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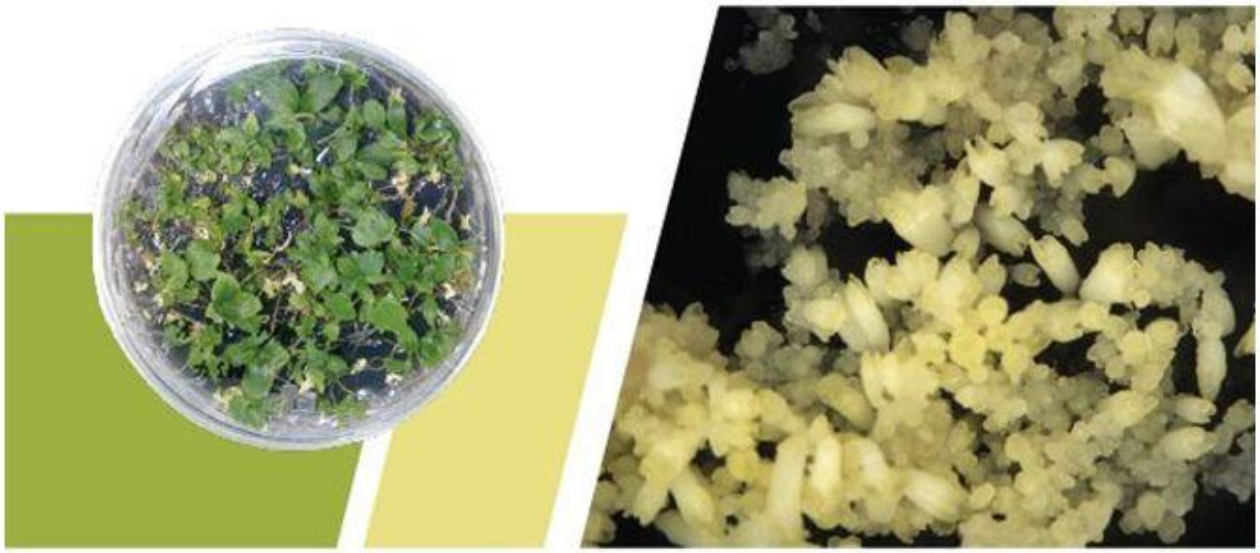
Vegetative Propagation of Forest Trees

Edited by
Yill-Sung Park, Jan M. Bonga and Heung-Kyu Moon



NIFoS

Vegetative Propagation of Forest Trees

Edited by

Yill-Sung Park
Jan Bonga
Hyeung-Kyu Moon

National Institute of Forest Science
2016

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Application of Vegetative Propagation of Forest Trees

Message from Director General of NIFoS

It has been 35 years since the beginning of active research in Forest Biotechnology at the National Institute of Forest Science (NIFoS), formerly Korea Forest Research Institute. Since forest trees are long-lived, it is difficult to develop tangible tree improvement technologies. Nevertheless, despite limited budgetary and human resources, persistent investments and dedicated efforts of researchers at NIFoS have led to the development of several applied forest biotechnologies, including somatic embryogenesis, tree improvement by genetic modification, and genomics.

Recently, in Korea and around the world, biotechnology has become a key strategy for future economic development, and the commercialization of forest tree biotechnology has begun. Accordingly in Korea, investments into the R&D of forest biotechnologies have increased steadily. Increasingly forest biotechnology is commercialized in tropical forestry and applied to species such as oil palm, Eucalyptus, coffee, etc. In temperate regions, increased forest production is achieved by integrating tree breeding and biotechnologies especially with several spruce and pine species. Among the new forest biotechnologies, somatic embryogenesis (SE) is an important vegetative propagation tool that, once refined, can produce superior tree varieties rapidly and effectively. Particularly, tree breeding integrated with SE and cryopreservation enables the delivery of a significantly high level of genetic gain in very short order.

Research in SE and other vegetative propagation technologies in woody plants is coordinated through the IUFRO Unit 2.09.02. The inaugural international conference of this IUFRO unit was held here in Suwon, Korea in 2010 and was hosted by the NIFoS. Subsequently, highly successful international conferences were held in Brno, Czech Republic in 2012 and Vitoria-Gasteiz, Spain in 2014. The main aim of these international gatherings is to promote communication and collaboration in the development and sustainable use of forest biotechnology.

Personally, if you are looking for an answer to problems, my favorite saying is "The answer can be found in the very field where you work." I also believe that, in order to achieve goals, it is very important to collaborate. This book is a collection of research results, reviews, and descriptions of practical experiences by leading

researchers in the field. This book not only deals with scientific development and trends but also with the practical application in tree breeding and commercialization of biotechnology. I am convinced that this book will be very useful to researchers in various disciplines and practitioners alike and will promote collaboration in future endeavors. Therefore, I would like to thank all the contributors.

In particular, I would like to thank Dr. Yill-Sung Park, Canadian Forest Service, for planning, coordinating with contributing authors, and editing; Dr. Jan M. Bonga, Canadian Forest Service, for providing detailed review and editing of all the contributions; and Dr. Heung-Kyu Moon, NIFoS, for conceiving, initiating and publishing this book.

Finally, I am hoping that this book will be a guide to current state of vegetative propagation in forest trees and to future development for students, researchers, academics, and forestry practitioners.

October, 2015.

Director General
National Institute of Forest Science
Seoul, Republic of Korea

NIFoS
NATIONAL INSTITUTE OF FOREST SCIENCE

FOREWORD

The IUFRO Unit 2.09.02 "Somatic Embryogenesis and other Vegetative Propagation Technologies" was founded in 2008 with an aim of fostering collaboration in the development and application of vegetative propagation technologies in woody plants. This unit has been active since then and has organized three well attended, friendly and lively conferences during that period. The first one was in Suwon, Korea in 2010, the second one was in Brno, Czech Republic in 2012 and the third in Vitoria-Gasteiz, Spain in 2014. Currently, one is being organized to be held in Bariloche, Argentina in 2016. During the last three conferences, while chatting during coffee and lunch breaks, the idea of putting a book together describing our experience with vegetative propagation technology and current trends in that field, slowly ripened among the meeting attendants. As a result of these deliberations, we decided to act upon it. We were very fortunate that Dr. H-K Moon was able to obtain strong support for this book project from the National Institute of Forest Science (NIFoS). We are very thankful that this project has come to fruition through NIFoS's dedicated efforts to promote forest science and through that of several members of our IUFRO unit. The book will appear in a printed version in a limited number but will also be freely available in an on-line edition. A few submissions that were not submitted in time for inclusion in the printed edition will appear in the online version. The online version will also be available for future additions/updates/corrections etc.

The book describes many different aspects of modern, vegetative propagation techniques of forest trees and is divided into two parts. Chapters in the first part deal with the current trends and status of research from a theoretical points of view. New technologies that are expected to have a major impact in future tree improvement programs, such as genomic selection and application of molecular analysis, are discussed in detail in this part. The second part is focused on a number of species of hardwoods and conifers and describes practical application of the various vegetative technologies including their use in industrial production.

In the first part of the book we find a general overview of current technologies by Bonga. This chapter describes various forms of clonal propagation, i.e., rooting of cuttings, organogenesis and somatic embryogenesis and deals with such issues as current problems with the various technologies, costs and deployment. It further describes how the various forms of propagation can be used together in tree im-

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provement efforts. Similar topics are also addressed by Monteuuis who stresses the complications still encountered by the various technologies. Furthermore he describes various pre-treatments that will rejuvenate plant material prior to explant excision. Current limitations in the application of somatic embryogenesis are also discussed by Ballester et al. who describe these problems for hardwood species. Maturation generally is a limiting factor in our ability to propagate plants vegetatively. The chapter by Diaz-Sala describes this problem in detail, outlining the involvement of cross talk between hormones and other signals, including molecular and genomic ones. Correia et al. describe in detail the molecular mechanisms that control somatic embryogenesis of hardwoods. Their review also includes a thorough description of epigenetic mechanisms involved in the process. Vondráková et al. present a detailed description of the role of plant growth regulators in somatic embryogenesis. They not only describe the function of the most common regulators, i.e., auxins, cytokinins and abscisic acid, but also that of less well known but yet very important ones like phenolics, jasmonic acid and brassinosteroids. Durzan gives an overview of how embryogenesis can be explained in terms of apomixes, meiosis, microsporogenesis, mitosporogenesis and totipotency. This chapter also describes how these processes relate to evolutionary trends. Klimaszewska and Rutledge deal with the potential of propagating mature spruce trees by somatic embryogenesis. Trontin et al. present the view that cloning of mature conifer trees is a cherished goal of many research institutions but that has proven to be a very difficult one to achieve. This chapter describes recent success and how genomic analysis has contributed to this achievement. Merkle provides insight in how the application of somatic embryogenesis and transgenic technology can help to conserve and restore forest tree species that may face extinction because of attacks by native and imported pests and pathogens. The use of genetic transformation in obtaining disease resistance is also the subject of the chapter by Corredoira et al. with emphasis on blight disease in European chestnut. Park et al. emphasize the importance of new forms of genetic analysis, in particular genomic selection, in programs aimed at genetic improvement of tree species, while Adams et al. present data that show how much genetic gain can be realised for use in an industrial multi-varietal forestry setting. Von Aderkas et al. describe conifer somatic embryogenesis in general terms and also deal with issues such as haploid embryogenesis and embryo rescue. The chapter by von Arnold et al. deals with pattern formation during embryogenesis and the processes associated with

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this, including stress and growth hormone effects, polarization, programmed cell death and gene expression, using Norway spruce as the model species. Miguel et al. reviewed the impact of already existing molecular studies that address (epi)genetic issues related to the embryogenecity of initiated lines. They also discuss the impact of (epi)somaclonal variation during the whole process and they assess the regenerative capacity (maturation ability) of propagated embryogenic lines.

In the second part of the book in vitro propagation of a number of hardwood and conifer species is described with emphasis on commercial application. The following hardwoods are included, Teak (Goh and Monteuuis); Eucalyptus (Pinto et al.); *Kalopanax septemlobus* (Moon et al.); yellow poplar (Kim et al.) and Ericaceae (Martins et al.); and the following conifers, Scots pine (Aronen); Nordmanns fir (Find); Norway spruce (Högberg and Varis); Larix (Lelu-Walter et al.); Maritime pine (Trontin et al.); radiata pine (Moncaleán et al.); Japanese black and red pines (Maruyama and Hosoi); Japanese red pine (Kim et al.); and pitch-loblolly hybrid pine (Kim et al.).

Overall, the chapters included give an overview of the rapid progress that has been made in the field of vegetative propagation and biotechnology of forest trees over the last few years. Our IUFRO unit has shown an excellent cooperative spirit in the past which has resulted in several joint efforts by various members. This has led to good progress and this, of course, is as we like it to be. It demonstrates the value of having an actively working group in which various international cooperative efforts have worked out very well. If this spirit is maintained in the future, good progress can be expected in the field that is dear to all of us. Happy networking!

October, 2015.

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Editors: Drs. Moon, Bonga, Park during Bruno2012 Conference

Part 1.

Development and Trends in Vegetative Propagation of Forest Trees

Conifer clonal propagation in tree improvement programs

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Abstract

Clonal propagation of conifers is achieved mostly by rooting of cuttings, organogenesis and somatic embryogenesis (SE). Of these, SE is the most powerful in obtaining genetic gain because SE cultures can be maintained in a juvenile state indefinitely by cryopreservation. This allows for long-term field testing of clonal lines while part of these lines are maintained in a juvenile physiological state until the field test has shown which are the best clonal lines for mass-production of propagules. This makes within family selection possible which is not the case with rooting of cuttings or organogenesis. However, as is explained in this review, one can expect that with advances in culture proceedings and in particular with increasing use of modern DNA analysis, within family selection may become possible for rooting of cuttings and organogenesis as well. Furthermore, issues such as deployment and field performance of clones and the cost of mass cloning are discussed.

Keywords: cost, cryopreservation, deployment, genetic gain, field tests, rooting of cuttings, organogenesis, somatic embryogenesis

1. Introduction

Forest plantations are increasingly needed to satisfy the growing demand for wood. Kirilenko and Sedjo reported in 2007 that only 3% of the world's forested land was plantation forest. However, despite this small percentage already more than one third of the industrial round wood production is provided by plantations and it is expected that by 2050 this production will rise to about 75%. Clearly, plantations are highly productive and with further improvement in genetic composition of planting stock and the application of biotechnology additional productivity increases can be envisioned (Fenning and Gershenzon 2002).

To operate plantations is expensive and requires a high productivity per hectare to make them economically viable. To achieve such productivity requires that good quality, i.e., genetically improved planting stock is used. Traditionally

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this was done by using high quality seed provided by seed orchards, either obtained by open pollination or more effectively by controlled pollination between superior parents. Although this has resulted in a marked improvement of planting stock much further improvement is potentially possible by combining traditional tree breeding with new biotechnology technologies. As summarized by Lelu-Walter et al. (2013) and El-Kassaby and Klápště (2014) seed orchards have limitations; not all parents contribute seed, some seed results from self-pollination thus producing poor progeny, poor quality pollen blows in from outside the orchard and there can be a negative correlation between fertility and vegetative growth. Therefore, many forest companies are currently considering clonal propagation in addition or in conjunction with their breeding programs.

2. Clonal propagation

2.1 Introduction

A comprehensive review of benefits, risks, risk management and technical problems of clonal propagation has been presented by Burdon and Aimers-Halliday (2003). The main benefits include capture of non-additive genetic gain, uniformity of the product and tailoring of the product to the growing site. The risks include genetic uniformity, biotic risks, inability to adapt to for example climate change and changing markets.

Clonal propagation can be achieved by various means, grafting, rooting of cuttings, coppicing or *in vitro* propagation. For many species clonal propagation occurs naturally often creating clonal populations that are very large. For example, root suckering has produced clonal populations of *Populus tremuloides* as large as 81 ha (Cook 1983) and one such clone is perhaps the largest living organism known (Mitton and Grant 1996). Clones can also be very old, e.g., it has been estimated that clones of the seagrass *Posidonia oceanica* have existed hundreds of thousands of years (Arnaud-Haond et al. 2012). Natural clones of conifers are rare; they occur occasionally in high stress environments, i.e., in arctic areas or at high altitudes near the tree line. Black spruce (*Picea mariana*), for example, will reproduce as a low bush in arctic northeastern Canada by rooting of low branches (air-layering) when environmental stress limits vertical growth (Pereg and Payette 1998). Similarly, clonal *Picea abies* is found at high altitudes in Sweden, these clones being thousands of years old and having living stems that show up to 600 growth rings (Öberg and Kullman 2011). One Gymnosperm is known that propagates vegetatively but lives in a temperate rather than severe climate environment, namely *Lagarostrobos franklinnii* (Huon pine) a native species in Tasmania (Shapcott 1997).

Many horticultural crops such as fruit trees have been cloned by grafting for centuries. For example, the *Vitis vinifera* cultivar Cabernet sauvignon has been propagated clonally since Roman times and since then has existed only in adult form. Interestingly, propagation *in vitro* of this cultivar has resulted in the reappearance of its juvenile form (Mullins et al. 1979). Some agricultural crops have been cloned for almost as long as the start of agricultural practices about

13.000 years ago (Allard 1999). Clonal forestry is not new either. In China *Cunninghamia lanceolata* (Chinese fir) has been mass propagated by rooting of stump sprout cuttings for at least 800 years (Minghe and Ritchie 1999).

2.2 Various methods of clonal propagation

Several methods of clonal propagation are being practiced with conifers. Of these the discussion in the following will be mainly focused on rooting of cuttings, organogenesis and somatic embryogenesis (SE). Grafting is not used for mass-propagation of conifers but is mainly restricted to the establishment of seed orchards. Coppicing, which is effective for many hardwood species (Wendling et al. 2014) is not an option for conifers because with the exception of a few species, i.e., *Sequoia sempervirens* (Bon et al. 1994), *Pinus serotina* (Bramlett 1990), *Taxodium distichum* (Wilhite and Toliver 1990) and *Cunninghamia lanceolata* (Minghe and Faxin 2001), conifers do not produce the juvenile stump or root sprouts needed for coppicing.

A comparative evaluation of rooting of cuttings, organogenesis and SE has recently been published (Bonga 2015). In this past review it is argued that in spite of the fact that with SE at present greater genetic gain is possible than with rooting of cuttings and organogenesis, the latter two still have a major function in conifer clonal propagation. It also points out that with expected future developments, especially by increasing use of genomic selection the advantage in genetic gain currently possible with SE may diminish. The objective of the current review is to elaborate on these subjects.

2.3 Rooting of cuttings

Rooting of cuttings has been highly effective for several hardwoods, in particular for several *Eucalyptus* species (Ondro et al. 1995). This success is based on the fact that rooted cuttings can easily be obtained from juvenile stump sprouts that are obtained from ortets (trees from which the cuttings were taken) that have shown superior characteristics. In Brazil clonal propagation of *Eucalyptus* has increased volume production from 33 m^3/ha/yr with unimproved seedlings to 70 m^3/ha/yr with rooted cuttings from selected trees (Zobel 1993). Unfortunately, for most conifers suitable cuttings are available only from plants that are too young to have demonstrated their growth potential. Nevertheless, rooting of cuttings can lead to genetic improvement. In breeding experiments improved seed is obtained but often in numbers far too low for planting. By producing seedlings from improved seed and by mass-propagating these by rooting of cuttings a large population is obtained with the same genetic makeup as the seed family. With this process selection within the family is not possible while, as explained later, with SE it is, making selection with the latter more powerful than with rooting of cuttings. Even though the period during which stock plants (ortets) can provide rootable cuttings can be extended by hedging, this extension is generally not long enough for proper long-term testing (Bonga 2015). Nevertheless, by rooting cuttings of plants obtained from seed that had been improved by breeding the gain

obtained can still be substantial. For example, a gain greater than 30% in height growth has been reported for *Picea glauca* when rooted cuttings of improved seed origin are compared with those obtained from non-improved seed (Weng et al.2010). *Pinus* species are sometimes mass-propagated by rooting of elongated fascicle buds instead of stem cuttings (e.g., *Pinus radiata,* South et al. 2005). How well rooted cuttings behave in comparison to seedlings depends on many factors, as described in a review by Ambebe et al.(2013).

2.4 Organogenesis

Because of age related maturation problems with rooted cuttings there is a tradition of trying to obtain clonal propagation by *in vitro* means. The first success in that respect was achieved with *Pinus palustris* by Sommer et al. (1975) who used excised zygotic embryos as explants. Adventitious shoots are formed on the zygotic embryo explant or on parts thereof or from meristematic nodules. These shoots are subsequently given a rooting treatment to form plantlets. However, for most conifer species this technology never reached a practical application stage primarily because of low plantlet formation rates and excessive handling and costs. For only a few species, most notably *Pinus radiata* (Aitken-Christie et al. 1988), have reasonable production levels been obtained. Instead of zygotic embryos another suitable type of explants are needle fascicles. For some *Pinus* species the use of needle fascicles has resulted in regeneration by organogenesis (e.g., *Pinus brutia ,* Jericó et al. 2012).

Organogenesis never became as popular as somatic embryogenesis because organogenic cultures can generally not be kept in a juvenile state by cryopreservation as is possible with SE cultures. Thus with organogenesis selection between but not within families is possible while with SE within family selection is possible (see below). Attempts have been made to prolong the juvenile state of organogenic cultures by low, non-freezing temperatures. Adventitious shoots of *Pinus radiata* have been stored for up to $5^1/_2$ years at 4^oC but rooting was restricted to shoots that had been stored for only 17 months (Aitken-Christie and Singh 1987) which is not long enough for a proper field test. However, since then it was discovered that partially desiccated cotyledons of *Pinus radiata* can be cryopreserved without killing them. After thawing the cryopreserved cotyledons produced the same number of adventitious shoots and plants as the non-cryopreserved control (Hargreaves et al. 2004). This procedure looks promising because it may allow long term testing in a fashion similar to that achieved with SE and cryopreservation.

2.5 Somatic embryogenesis (SE)

2.5.1 *Why SE?*

SE has become popular because SE cultures can be cryopreserved and retrieved in a viable state after cryopreservation. Cryopreservation of SE cultures makes it possible to select within the family rather than just between families.

Because of the large within family genetic variability such selection is more powerful than when selection only achieves the family average as is the case with other forms of vegetative propagation (see Park et al. in this volume). This makes SE attractive and generally the preferred method of clonal propagation. Unfortunately there are still many problems in its universal application. In the following part of the focus will be on such problems and on their resolution. Only a few technical aspects of SE will be discussed; for more detail several extensive reviews are available (Klimaszewska et al. 2007; Lelu-Walter et al. 2013).

2.5.2 *Initiation and maturation*

Initiation of SE in conifers is generally easier with immature than with mature zygotic embryos (Park et al. 1993; Miguel et al. 2004; Kvaalen et al. 2005). This creates problems because it limits collection of suitable material for SE initiation to a short period each year. Furthermore, zygotic embryos at an immature stage of development do not survive lengthy storage of cones and this limitation again restricts experimentation to short periods annually. It would be preferred to use cones with mature embryos that can be stored often for years in a viable state but for most conifer species such embryos either do not initiate SE or do so at reduced rates (Park and Bonga 2011).

For some gymnosperms initiation is restricted to a very early stage in the development of the zygotic embryo. For example, initiation of *Pinus banksiana* SE is possible only when the zygotic embryo is in the poly-cleavage stage and even then only leads to a low initiation rate. It is assumed that in that case initiation of SE is simply a continuation of the cleavage process for as long as the explant is maintained on a medium containing 2,4-D (Bonga 2012). Similarly, zygotic embryos of *Juniperus communis* can form embryogenic lines only when they are at the poly-cleavage stage (Helmersson and von Arnold 2009). *Pinus sylvestris* zygotic embryos produced somatic ones primarily when at the four-cell to the cleavage polyembryo stage whereas *Pinus pinaster* had the highest initiation rate at the stage just prior to elongation of the cotyledons (Lelu et al.1999). For some *Pinus* species initiation does not proceed directly from pro-embryogenic masses, as is usually the case, but from nodules (von Aderkas et al. 2005). SE via nodules has also been observed in cultures of *Picea abies* treated with histone deacetylase inhibitor (Uddenberg et al. 2011), in cultures of tissues from 10-year-old *Picea glauca* trees obtained by SE (Klimaszewska et al. 2011) and in cultures of adult *Larix decidua* and *L. x eurolepis* trees that formed embryo-like structures (Bonga 1996).

Once initiated, the culture consisting of masses of cloned embryos and suspensors grows rapidly. When large enough the masses are subdivided and are used to produce mature embryos that can be germinated and used for clonal field testing. Maturation generally requires transfer of the tissue to a culture medium free of auxin and containing abscisic acid (ABA) and an increased level of osmoticum to slow down growth. Mature embryos are germinated and the resulting plantlets are acclimatized and transferred to a greenhouse and eventually to the field (Klimaszewska et al. 2007; Celestino et al. 2013). Initiation and each

subsequent developmental stage of SE require a finely balanced application of various plant growth regulators that is specific for each developmental stage (for a review see Vondráková et al. in this volume).

2.5.3 *Cryopreservation*

After initiation has produced masses of embryos and suspensors large enough to be subdivided part of each mass is cryopreserved while the other part is used to produce clonal plants for field testing. Presumably, cryopreserved material can be maintained in a viable state for a very long time if not indefinitely. This allows for clones to be field tested for a time much longer than is possible with rooted cuttings, the ortets of which can, as already stated, be kept in a state capable of providing rootable cuttings for only a limited time. After the field test has determined which clones are superior, the best are then removed from cryostorage for mass production of germinating SEs ready for greenhouse and eventually field planting.

The suitability of cryopreservation depends on what effect, if any, it has on the genetic stability of the stored material. This is an important issue because if genetic stability cannot be assured the value of cropreservation is greatly diminished. For conifers this has been investigated extensively (Sutton and Polonenko 1999). Cryopreservation has to be capable of storing without ill effects a wide variety of genomes and families to be of value. These qualifications were met by Cyr et al. (1994) who obtained a 97% recovery rate of cryopreserved embryogenic cultures of 12 full-sib families of *Picea glauca engelmanni* using a large number (357) of genotypes. DNA fingerprinting showed no evidence of somaclonal variation resulting from the cryopreservation. Similar results have been obtained for other conifer species (Isabel et al. 1993; Cyr1999; Hazubska-Przybyl et al. 2013).

However, there are reports of abnormalities in tissues retrieved from cryopreservation, possibly in part due to the effect of the use of dimethylsulfoxide (DMSO) as cryoprotectant. Studies with SE cultures of some conifer species, for example *Abies cephalonica* (Aronen et al. 1999; Krajnakova 2011) and *Picea glauca* (De Verno et al. 1999), have indicated that this cryoprotectant can induce genetic and epigenetic changes. Consequently it has been attempted to achieve cryopreservation without cryoprotectant. This has been successful with cultures of *Picea glauca* and *Pseudotsuga menziessii* which could be cryopreserved without DMSO after a 4-8 week pretreatment at 5^{o}C (Kong and von Aderkas 2011). Similarly SE cultures of *Picea abies* exposed to desiccation in the absence of DMSO prior to cryo storage remained viable subsequently (Hazubska-Przybyl et al. 2013).

Regrowth after cryopreservation occurs for most clones. However, in some species, for example in an experiment with *Abies nordmanniana* regrowth depended on genotype since only one of five genotypes recovered from cryopreservation (Nørgaard et al. 1993). Cryopreserved embryo-suspensor masses are sometimes more productive embryo producers than their non-cryopreserved counterparts (Galerne et al. 1992). It appears that cryopreservation kills most

suspensor and non-embryogenic cells but not all embryogenic cells (Kristensen et al. 1994) thus presumably freeing the latter from competition.

2.5.4 *Abnormalities induced in vitro*

Conifers are generally considered to be genetically stable. Only a few cases of naturally occurring polyploids and aneuploids are known (Miksche and Hotta 1973; Saylor 1983). However, one has to consider the possibility that this natural genetic stability may not be strong enough to safeguard against changes induced by the *in vitro* culture environment. For example, Marum et al. (2009) found genetic variation at SSR loci in embryogenic cell lines of *Pinus pinaster* after prolonged proliferation and in some emblings recovered from these cell lines. Aronen et al. (2014) found that *in vitro* culture reduced telomere length of *Betula pendula*, a symptom of loss of regeneration capacity. Whether such reduction in telomere length occurs in conifer cultures is not known. Telomere length is of interest because it has been found that shortening of telomeres is associated in changes in gene expression during aging (Robin et al. 2014).

Genetic changes induced by *in vitro* culture do not necessarily show up in the trees regenerated from aberrant cultures. Many *Picea glauca* embryos regenerated after cryopreservation exhibited abnormal genetic patterns but these abnormal embryos did not form plantlets that survived transfer to soil while the embryos that were genetically normal did (DeVerno et al. 1999). Similarly, Harvengt et al. (2001) detected a high mutation rate in SE cultures of *Picea abies.* However, no allelic abnormalities were found in plants that originated from these SE cultures and the plants showed no abnormal growth behavior. These observations again suggest that there is an effective selection for normal genotypes when plants are formed by SE cultures.

2.5.5 *Problems with SE:*

Because SE is a very attractive technology a lot of research effort has gone into making it industrially applicable for most commercial conifer species. However, in spite of these efforts large scale industrial application has so far been restricted to a limited number of species. SE works well for several larches (Bonga et al. 1995), spruces and pines (Park et al. 2006; Park and Bonga 2011) but is difficult for the *Cupressaceae* (Helmersson and von Arnold 2009). For some pine species the initiation rates are still too low to be of practical value (Park and Bonga 2011). Low initiation rates are also common for some commercially important firs (Nørgaard and Krogstrup 1995; Vooková and Kormuták 2004). Furthermore, there often are considerable within species differences. Within 20 open pollinated families of *Pinus pinaster* initiation rates ranged from 35.8 to 2.0% (Miguel et al. 2004). Another experiment with that species also showed a wide variety in response depending on what parents were crossed (Lelu-Walter et al. 2006).

To be effective initiation rates should be greater than about 30%. Fortunately, initiation rate is a highly heritable trait and initiation rates can be improved if one parent capable of high initiation is included in each controlled

cross (Park et al. 1998). Furthermore, initiation rates are generally higher if immature rather than mature zygotic embryos are used as explants (Park et al. 1993; Klimaszewska et al. 2007). One consequence of low initiation rates is that only a few genotypes within the family are recovered resulting in a lack of genetic variation within the regenerated population (Högberg and Varis in this volume).

Recalcitrance in regeneration is a poorly understood problem. Several reviews have recently dealt with the subject (Zeng et al. 2007; Zavattieri et al. 2010; Bonga et al. 2010, 2012; Diaz-Sala in this volume) but the problem is persistent and difficult to solve. A variety of different potential explanations of what makes cells competent to initiate SE have been published, which suggests that several mechanisms may operate independently. For example, stress appears to reprogram deteriorating cells into a survival mode that stimulates SE (Dudits et al. 1995; Fehér et al. 2003) while Durzan (in this volume) noted that nutritional stress in embryonal initials initiates a meiotic process. On the other hand stress, even mild stress, often also results in abnormal phenotypes (Joyce et al. 2003). Zhang et al. (2010) identified four families of abiotic stress-induced miRNAs that are differentially expressed in embryogenic and non-embryogenic cultures of *Larix leptolepis*. An interesting novel approach has been proposed by Rutledge et al. (2013). They suggest that suppressing biotic defense mechanisms could perhaps initiate a physiological state that more readily initiates SE. Epigenetic factors also play a major regulatory role in SE initiation (Mahdavi-Darvari et al. 2015). Epigenetic factors are involved in developmental events such as phase change and rejuvenation and have to be manipulated to overcome recalcitrance (Us-Camas et al. 2014, Diaz-Sala this volume). Another factor that could perhaps influence initiation rates is the nutritional state of the explant. The amounts of various sugars, amino acids and soluble proteins in the megagametophyte and zygotic embryo can vary considerably depending on climate and seed source (Durzan and Chalupa 1968). Even when initiation rates are high the quality of the embryos can be low resulting in poor maturation, germination and formation of low quality plantlets (Thompson 2014; Monteuuis in this volume). Poor embryo quality was especially a problem shortly after SE was first developed. For example, Klimaszewska et al. (2007) list 15 *Pinus* species all of which produced SEs during the early years of SE experimentation. However, of these only three species produced plants that survived transfer to soil. In later years with improved protocols much better results were obtained, at least for some species. In an early experiment with *Picea glauca* (Park et al. 1998) nearly half the SEs were abnormal and showed low germination rates. Since then their culture protocol has improved and now most embryos are normal and show high germination and survival rates. Harrington (2003) reported an initiation rate of about 30% for *Picea sitchensis* but a loss of up to 50% of the cultures during proliferation. With an improved protocol for this species (Fenning and Park 2012) an initiation rate of about 70% was attained. This demonstrates that for some species unsatisfactory initial results can turn into much better ones later with improved culture procedures. However, there are still many species that in spite of extensive efforts at improvement are still intractable. An example of that is *Pinus banksiana* which has never initiated SE above rates of 3-4% in spite of years of research efforts (Park and Bonga 2011).

2.6 Combining the various propagation techniques

Combining SE with either rooting of cuttings or organogenesis can be useful. In that case SE together with cryopreservation is used to select superior genotypes that are subsequently mass-propagated by rooting of cuttings or organogenesis. This process is useful when SE maturation and germination rates are low and only a low number of field grown plants is produced for each clone. This is the case, for example, for *Pinus radiata* (Montalbán et al. 2010, 2011; Moncaleán et al. this volume). Harrington (2003) indicated that Coillte Teoranta in Ireland plans to produce up to 6 million rooted cuttings of *Picea sitchensis* using cuttings taken from 40.000 improved stock plants that had been produced by SE.

For many angiosperm species it is possible to combine rooting of cuttings with cryopreservation and organogenesis. Cryopreservation of shoot tips or dormant buds, and regeneration of plants thereof by organogenesis, is used mainly to preserve germplasm (Harding et al. 2009). Regeneration of plants from cryopreserved buds has been reported for a number of forest tree species for example, *Populus tremuloides* (Aronen and Ryyänen 2014) and *Melia azedarach* (Yang et al. 2011). With regard to gymnosperms, *Tetraclinis articulate* (Cupressaceae) (Serrano-Martinez and Casas 2011) and *Picea sitchensis* (Gale et al. 2003) shoot tips have been successfully cryopreserved.

In cases where regeneration of plants from cryopreserved shoot meristems or buds of conifers proves to be possible the following scenario can be envisioned. A few buds could be removed from seedlings and be cryopreserved while the seedling is subsequently assessed in a long-term field test. After the field test has determined which ortets are genetically superior, their cryopreserved buds could then be used for regeneration of offspring through organogenesis. This process could result in selection of superior clones similar to such selection after SE and cryopreservation and could be useful for species for which SE on a large scale is difficult.

Regeneration from shoot tips is difficult for most conifers and presumably would require that in most cases the buds are obtained from very young seedlings. Regeneration using shoots excised from buds of seedlings has been reported for some conifer species, e.g., *Pinus radiata* (Prehn et al. 2003), *Sequoia sempervirens* (Sul and Korban 2005) and *Pinus roxburghii* (Kalia et al. 2007). Whether regeneration from cryopreserved buds of these species is possible remains to be determined.

2.7 Attempts to clone mature conifers either by organogenesis or SE

Clonal propagation of adult conifers either by rooting of cuttings, organogenesis or SE is problematical. So far SE, the preferred method of propagation, has not been feasible on a commercial scale if at all (reviews see: Bonga et al. 2010, 2012; Diaz-Sala, this volume). However, propagating adult conifers by SE is still a cherished goal. It would be helpful if such propagation could be achieved at a practical level and with true-to-type offspring. In that case the period of field testing of clones, as is done when SE is obtained from zygotic

embryos, would not be necessary and much time would be gained. Furthermore, individuals could be selected for propagation that were not only superior for one trait but for many. When SEs are obtained from zygotic embryos, clones exhibiting all these good qualities combined may not appear and, furthermore, selection for traits that do not show until late in the life cycle would be difficult. Therefore, cloning of adult trees is still attractive and is being attempted in several laboratories.

There are means to return the ortet from a mature to an at least partially rejuvenated, more responsive state. These include forcing of pre-formed proventitious buds, serial grafting or micrografting and spraying with benzylaminopurine (Chang et al. 2010; Monteuuis et al. 2011and in this volume). In one case this has led to propagation of *Larix decidua* as old as 140 years (Kretzschmar and Ewald 1994). However, this technology is experimental and complex and has not yet lead to large scale application.

In an attempt to propagate adult *Larix decidua,* adventitious shoots were obtained *in vitro* but these rooted only rarely (Bonga and von Aderkas 1988). Attempts to obtain SE from tissues of adult conifers has been unsuccessful to date except in an experiment in which 10-year-old *Picea glauca* trees obtained by SE were used (Klimaszewska et al. 2011). A few instances of the appearance of embryo-like structures has been reported, i.e., *Pinus radiata* (Montalbán et al. 2010) and *Larix decidua* and *L. x eurolepis*. The *Larix* embryo-like structures arose from nodules, they germinated and formed elongating shoots but these lacked roots (Bonga 1996). Regeneration of rooted shoots from bud explants of adult trees by organogenesis has been reported for *Pinus pinaster* (De Diego et al. 2008) and *P. sylvestris* (De Diego et al. 2010).

Clonal propagation of adult conifers may lose some of its appeal as genomic selection technology progresses. It is conceivable that with expected future advances in that field it may eventually become possible to select clones with complex desired traits, quickly from among embryogenic cultures without the need to cryopreserve and long-term field test them.

3. Gains by breeding and SE

The ability to preserve SE clones over a long period of time in an unaltered state provides an effective way of improving the genetic makeup of planting stock. When SE is used for the clonal propagation of zygotic embryos in seed that was genetically improved by breeding, cryopreservation makes it possible to select and multiply the best clones within the breeding population, i.e., within family selection is possible. Thus a clonal population is obtained that on average will outperform the population obtained by breeding alone (Park 2002; Nehra et al. 2005; Lelu-Walter et al. 2013). This approach is highly effective because of the large degree of heterozygosity in most tree species, in particular in conifers (Ledig and Conkle 1983). Due to the long breeding cycle and self-incompatibility of most conifer species, little domestication has taken place. In fact, most conifers that have gone through a few breeding cycles are genetically still close to their wild populations and still are highly heterozygous and thus carry a lot of variation that one can potentially choose from (Libby 1987).

Breeding of *Pinus taeda* in the southeast US has resulted in the following gains in yield: 8% in the first generation by open pollination in a seed orchard; 11% by open pollination of the best mothers; 21% by full sib controlled pollination (Sedjo 2004). These percentages represent improvement in the average of performance of all individuals within the family. SE and cryopreservation can, as already explained, further improve these percentages. Sutton (2002) reports a 13% volume gain by using seed from open pollinated seed orchards of *Pinus taeda* while with the use of SE a gain in excess of 40% can be expected. Sorensson (2006) indicated that growth gains of 50% can be expected for *Pinus taeda* via SE. Superior genetic traits are often due to unique "non-additive" gene combinations that are difficult to capture by conventional tree breeding but that are captured by clonal propagation (Mullin and Park 1992; Bentzer 1993).

It is fortunate that for several conifers, including *Picea glauca* (Park et al 1993, 1998) and *Pinus taeda* (MacKay et al. 2006), SE initiation is under strong additive genetic control. Therefore, by having at least one parent with a high capacity for SE included in each breeding pair, families with a reasonably high SE initiation rate can be obtained. Thus some of the high qualities of the breeding partner with the low capacity for SE can still be captured by SE. Niskanen et al. (2004) found that the maternal effect was greater than the paternal effect on SE initiation. During prolonged maintenance of the cultures the effect of the mother's genotype diminished but had a significant effect during SE maturation. It has been suggested that the presence of the megagametophytes during initiation may prolong the maternal effect (MacKay et al. 2006).

A large degree of heterozygosity is not present in all conifer species. A few conifers lack diversity because their current population originated from a small remnant that survived after a catastrophe. Examples of this are *Pinus resinosa,* which appears to have originated from a small pocket of trees that survived the last ice age (Fowler and Morris 1977) and *Pinus torreyana* which became isolated otherwise (Ledig and Conkle 1983). Due to the lack of genetic variation in such species improvement by breeding and/or vegetative propagation cannot be expected and propagation by seed is the most economical and effective.

Epigenetics has attracted attention lately as a possible way of introducing beneficial traits through plant breeding (Mirouze and Paskowski 2011). In epigenesis environmental cues activate genes that initiate the formation of proteins that enable adaptation to environmental challenges. Sometimes the resulting phenotype is preserved through one or more sexual cycles which, for example, could quickly improve adaptability, especially in plants that possess limited genetic diversity. However, even though traits of interest may thus be acquired more or less permanently, the possibilities to acquire new traits presumably are far more limited via the epigenetic than via the Mendelian genetics route. A conifer example of traits that are epigenetically determined is presented by Kvaalen and Johnsen (2008). They observed that height growth and bud set in *Picea abies* are influenced by the temperature to which zygotic and somatic embryos were exposed and that the effect was still noticeable after two growing seasons. Exposure to high temperature (28^{o}C) during SE initiation of *Pinus radiata* resulted in increased drought stress tolerance one year later in the plants thus produced (Montalbán et al.

2014). In developing zygotic embryos of conifers the ability to initiate SE is reduced as the embryo matures and is rare after germination. However, it has been observed that once SE is induced in *Picea glauca* the ability to induce new SEs is maintained in some of the somatic trees that have developed from the SEs, even sometimes up to the point where these trees start to form sexual cones. It is assumed that this is due to an epigenetic fixation of the capacity to form SEs (Klimaszewska et al. 2011). Similarly, SE initiation continued in the shoots that developed from SEs obtained from shoot bud explants of adult *Larix decidua* (Bonga 1996). This repetitive SE initiation, although without the formation of proper root meristems, presumably also is epigenetic in nature. Durzan (in this Volume, Figure 7) noted that epigenetics silences embryonal initials that are not capable of SE.

4. Deployment

Clonal propagation is often seen in a negative light because of the perception that it promotes genetic uniformity in populations. However, when properly practiced uniformity problems can be limited to an acceptable level. Furthermore, plantations resulting from the application of modern biotechnology will take harvesting pressure off the natural forest (Sedjo 2005) and thus help to preserve these and their biodiversity. Because tree species have a long life cycle that requires a long-term investment, it is highly desired that clonal plantations remain risk free to the maximum possible extend.

In any plantation, clonally or sexually produced, there is a risk of insect or pathogen attack, sometimes of an unforeseen nature. For example, due to lack of diversity, problems have occurred in clonal poplar populations in several countries (reviewed by Stelzer and Goldfarb 1997; Bishir and Roberds 1999; Burdon and Aimers-Halliday 2003). Obviously, using only a few, highly productive clones may impose unacceptable risks (Burdon and Aimers-Halliday 2003). However, even though risks can decrease with a larger number of clones being deployed, too large a number can increase potential problems. It has been suggested that the risk level is unlikely to be reduced if the number of clones used exceeds 30 – 40 (Bishir and Roberds 1999). Another model suggests that approximately 18 genotypes are optimal under many conditions and that with regard to merchantable volume no more than 30 clones are needed for good risk protection and near-optimal timber yield (Yanchuck et al. 2006). This model also indicates that planting blocks with a mixture of clones has advantages over planting a mosaic of blocks with each block containing a different single superior clone. Another option is to mix clonal propagules with sexually produced seedlings and rogue these populations at regular intervals (Park et al. 1998). In general, the clones should be planted at highly productive sites and be well adapted to those sites, and a balance must be reached between genetic diversity and expected gain (Cyr and Klimaszewska 2002).

In several countries legislation has been enacted that regulates the deployment of clones. These regulations state what number and mixture of clones is to be deployed and what size of area can be planted with clones (Burdon and Aimers-Halliday 2003, Lelu-Walter et al. 2013, Högberg and Varis in this volume).

5. Field performance of clones

In a 12 year field test of *Pinus radiata,* obtained by organogenesis, clones were planted in both monoclonal blocks and blocks with mixtures of clones. Average performance was the same in both but variation was larger when clones were mixed. Uniformity is an advantage but risk is greater in monoclonal blocks (Sharma et al. 2008). Comparing SE emblings with seedlings from the same families showed that *Picea glauca x engelmannii* emblings had slower height, diameter and root growth rates than their sexually produced counterparts during the initial $2^1/_2$ months in the nursery but that growth of the former catches up after that (Grossnickle et al. 1994). Embling and seedling performance was similar over two years on a reforestation site (Grossnickle and Major 1994) and the emblings performed reasonably well under a variety of nutrient and stress conditions (Grossnickle and Folk 2007).

Field performance of somatic plants depends on the *in vitro* conditions under which SEs developed. For example, lengthy contact with ABA during maturation of *Picea abies* SEs and a non-optimal germination treatment reduced height growth during their first two growing seasons in the field (Högberg et al. 2001). By selecting SEs with lateral roots and epicotyls larger than 8mm, taller and more uniform plants were obtained (Högberg et al. 2003). In a 5 year field test of genetically matched Douglas-fir seedlings, rooted cuttings and plants produced by organogenesis, the plants obtained by organogenesis grew slower than seedlings and showed signs of early maturation (Ritchie et al. 1994). Early maturation (premature flowering) was also found in *Picea mariana* emblings (Colas and Lamhamedi 2014). On the other hand, Klimaszewska et al. (2011) observed that primordial shoots of some 10-year-old *Picea glauca* trees obtained by SE were still capable of SE initiation. Such initiation is considered to be a juvenile trait and for this species is normally restricted to zygotic embryos. These trees, therefore, expressed extended juvenility instead of early maturation. It has been suggested that this phenomenon could be due to a suppression of the biotic defense activation (Rutledge et al. 2013). Clones of *Picea glauca* obtained by SE showed, under the same growth conditions, greater variation in growth characteristics within the family than zygotic seedlings within that family (Lamhamedi et al. 2000). In 1999 a clonal test of 70 coastal Douglas fir SE clones was established from two full-sib families with the same female parent on five different sites. After $5^1/_2$ years of testing growth and survival were acceptable across the test sites and stable (Dean 2008). In a subsequent test with 37 SE clones from four full-sib families on five test sites, the SEs grew slower than their zygotic counterparts but 20% of the clones produced 25% greater stem volume after $7^1/_2$ years than the sexually produced seedlings. Clonal stability resulted in little variance due to clone x test interactions (Dean et al. 2009). Wahid et al. (2012) looked at the field performance of *Picea glauca* SE clones on two sites four years after out-planting. Selection of the top 38% of the clones provided a 4% genotypic gain in height and the genotype x site interaction was low. Since juvenile/mature height growth correlations are high for this species one may expect these clones to perform well at a later age (Wahid et al. 2013).

In all the above examples the performance of emblings was compared to that of seedlings of the same family, i.e., no comparison was made between performance of selected superior clones and the family average for seedlings. Data indicating what kind of gain is possible when clones are selected from within the family have, to my knowledge, not yet been published (Adams et al. in this volume). At higher selection intensities a higher genotypic gain can be expected. For example, Park (2002) estimated that a gain of 45% in height growth over that of the average of all clones can be expected at 5 years of age when the 10 best clones are selected from 300 *Picea glauca* SE cell lines.

6. Cost of clonal mass production

There are many potential technical problems involved in mass-clonal propagation that would affect costs of the operation. Aimers-Halliday and Burdon (2003) present a long list of them of which the following are just a few; problems with clonal storage, loss of genetic gain, potentially superior clones may be under-represented because of poor cloning ability of the genotype, somaclonal variation, systemic infections, epigenetic effects, inadequate testing and cultivar decline.

With regard to conifer SE, as was pointed out earlier, initiation rates for several species are low and embryo quality is often poor resulting in poor conversion to plantlet rates and operation costs that are prohibitive. SE requires a substantial input of labor and thus is expensive in comparison to seedling production. Because of the long rotation age of the product, costs must be carried for many years before they can be recovered (Lelu-Walter et al. 2013). This means that the savings obtained by improving productivity by SE should be higher than the extra cost incurred by using emblings instead of sexually produced seedlings. Furthermore, one has to consider whether the extra cost of SE outweighs the cost of gain in production attained by intensive sylvicultural management practices such as weed control, thinning and fertilizer application, inter-planting with nitrogen fixing species, all of which can significantly improve productivity (Binkley and Stape 2004; Sorennson 2006; Gyawali and Burkhart 2015).

An early cost analysis indicated that an increase in genetic gain can make clonal propagation cost effective. This study also suggested that because of the high cost of clonal propagation planting of superior clones should be restricted to high quality sites located close to the mill (Timmis 1985). High productivity on sites close to the mill presumably would result in considerable savings in road building, harvesting and transportation costs which could offset the initial high cost of clonal propagation, in particular of SE. This applies to all forms of intensive plantation management. With intensive management there is less of a need to harvest from less productive natural sites and costs associated with harvesting at these often poorly accessible sites need not to be undertaken. This takes harvesting pressure off these less productive natural sites (Sedjo 2005; Wahid et al. 2013). Other factors that reduce the impact of initial high costs are that with the faster growth obtained by using SE the percentage of logs suitable for saw-timber could rise between 35 to 80% (for *Pinus taeda)* (Sorensson 2006). This latter observation is important because saw logs are of greater value than pulp wood. The financial

gain possible by using selected clones of *Picea sitchensis* obtained by rooting of cuttings instead of sexually produced seedlings at harvest is substantial (Philips and Thompson 2010).

A factor in commercializing clonal propagation is seed productivity and phenology of the species. If seed production is high and occurs early in the life cycle, multiplying the seed in seed orchards for large scale planting is more cost effective than vegetative propagation unless the genetic gain by the latter is large enough to warrant the extra expense (Sutton and Polonenko 1999). For a few species the extra cost has been considered worth taking and large numbers of plantlets obtained either by organogenesis or SE are being produced. For example, Tasman Forestry Ltd by 1993 had developed the capacity to produce 3 million *Pinus radiata* plantlets annually by means of organogenesis from zygotic embryos from seed obtained by controlled pollination. This will result in an approximately 20% increase in yield over that provided by unimproved seed (Nairn 1993). JD Irving Ltd. planted 433,000 *Picea glauca* SE plantlets in 2012, 219,000 in 2013 and 212,000 in 2014. This company recently built a large new facility where they plan to produce 5 million SE plantlets annually (Andrew McCartney, Irving Ltd. Personal communication 2015). CellFor was producing about 2 million SEs annually by 2002 from control-pollinated families of loblolly pine and Douglas fir (Sutton 2002). Even higher numbers are reported for southern pines (Sorensson 2006).

To reduce the cost of producing SEs, efforts are being made to simplify the initiation, maturation, germination and planting protocols and to automate the process. For example, for the production and planting of *Pinus pinaster* SEs it has been possible to initiate them without subculture, to cryopreserve them without the need of a programmable freezer, to mature the embryos without subculture, to improve germination and to eliminate the need for a greenhouse in the acclimatization and planting process (Lelu-Walter et al. 2006).

For a number of non-coniferous species automation has reached a stage of considerable sophistication and effectiveness. For example, *Coffea* spp. SEs have been mass produced in mechanically agitated bioreactors up to the cotyledonary stage, matured in temporary immersion bioreactors and germinated in a raised CO_2 (photoautotrophic) environment. This system can proliferate embryogenic suspensions for about 6 months without causing excessive somaclonal variation (Ducos et al. 2007). To culture plantlets under photoautotrophic conditions and without sucrose in the culture medium was found to be beneficial, production and cost wise, for the conifer *Cunninghamia lanceolata* (Kozai and Xiao 2006). Bioreactors were initially developed for microbial culture and secondary metabolite production but more recently have also found application in plant cell cultures including of woody species (Yoeup and Chakrabarty 2003). An important aspect of automation is the ability to select and remove high quality SEs from a population containing both low and high quality embryos. This can possibly eventually be done with an image analysis sorting system (Ibaraki 1999). As discussed by Ingram and Mavituna (2000) conifer SE cell lines generally will proliferate in liquid medium but SE maturation in most cases requires a solid medium. They tested proliferation and maturation of *Picea sitchensis* in different

types of bioreactors and found bubble reactors to be the most satisfactory. Large scale SE production in liquid medium bioreactors has also been reported for *Pseudotsuga menziesii* (Gupta and Timmis 2005).

Mass production of SEs is only one stage in the commercialization process and perhaps not always the most severe bottleneck. For example, it has been stated that commercialization of SE is not primarily dependent on automation of SE production but on the current lack of reliable delivery systems, i.e., the lack of artificial seed (synseed; encapsulated SEs) that like natural seed can be stored for a long time, that will germinate at high rates and that are compatible with existing commercial propagation systems (Sutton and Polonenko 1999).Unfortunately, in spite of a great deal of effort to develop artificial seed technology into a commercially viable process, it often still does not work well due to often poor survival and germination rates and excessive dehydration of the capsules under field conditions (Sutton and Polonenko 1999; Roy and Tulsiram 2013). As stated by Onishi et al. (1994) the main requirement for synthetic seed to be effective in mass clonal propagation is a high and uniform conversion rate into viable plantlets under practical sowing situations. *Picea glauca engelmannii* somatic embryos require sucrose during germination until they have developed a functioning radicle (Roberts et al. 1995). Unfortunately, sucrose in synseed leads to microbial contamination and this and other problems have so far limited its practical use (Ara et al. 2000, Roy and Tulsiram 2013). The use of conifer synseed has so far been experimental and germination of synseed has been carried out aseptically *in vitro*, for example, with *Pinus radiate* (Aquea et al. 2008).

To improve maturation, germination and survival it has been attempted to load the embryos with storage nutrients to compensate for the absence of a nutritive megagametophyte. By using ABA and instead of sucrose a non-permeating osmoticum, Attree and Fowke (1993) and Attree et al. (1994) obtained *Picea glauca* SEs that contained greatly increased amounts of storage lipids. These SEs could be dehydrated to about 8% moisture content and stored for over one year at minus 20^{o}C, rehydrated and germinated. Embryos such as these are useful in attempts to achieve mechanization of the process (Sutton 2002).

7. DNA and other markers: marker assisted selection (MAS)

Conifers have large genomes and a long breeding cycle which causes problems in traditional breeding programs. These programs may become more effective with the aid of marker assisted selection (MAS) (Ritland et al. 2011, Chhatre et al. 2013). Early efforts in that direction involved the use of quantitative trait loci (QTLs). The use of such loci is based on the likelihood of a quantitative gene occurring near a marker in a particular linkage group. They are helpful in breeding but are not very effective in locating and identifying quantitative genes (van Buijtenen 2001). They are useful only for large families with known relatedness, i.e., full-sib families (Beaulieu et al. 2011, Thavamanikumar et al. 2013) and typically only explain a small proportion of phenotypic variation (Thavamanikumar et al. 2013). Nevertheless, marker assisted selection can be effective in tree breeding. For example, Beaulieu et al. (2011) identified single-

nucleotide polymorphism markers (SNPs) for several wood traits of *Picea glauca* that could be used to speed up future breeding schemes. Lately it was shown that some metabolites could serve as useful markers. For example, levels of inositol in the cambial area of *Pinus densiflora* during the middle of the growing season significantly correlate with stem growth (Kang et al. 2015).

Lately a procedure called genomic selection (GS) has become popular. It increases genetic gain per time unit while maintaining sufficient diversity in breeding schemes and clonal propagation and it predicts at an early age what phenotype will develop (Canales et al. 2013; El-Kassaby and Klápště 2014; Park et al. in this volume). GS predicts phenotype on the basis of the aggregate of whole-genome effects (Grattapaglia 2014). An example of how GS could be implemented in studies of SE or rooting of cuttings has been presented for *Pinus taeda* by Resende et al. (2012).

It is expected that GS will increasingly become more popular as the rapidity at which DNA is sequenced increases and its cost is lowered. As was already pointed out, this may eventually make it possible to optimize genetic gain by SE without the need for cryopreservation. Furthermore, one may expect that application of GS in rooting of cuttings and organogenesis will eventually result in obtaining a similar level of gain for these as is currently possible with SE.

8. Conclusion

Clonal propagation of conifers, primarily by rooting of cuttings, has long been practiced to maximize wood production. However, the development of SE technology, and its use in combination with cryopreservation, has led to genetic gain beyond that obtainable by rooting of cuttings. Because of the enormous genetic variation available in most conifer populations the possibilities of much further genetic gain by this procedure is far from exhausted. However, SE is still not practical for many conifer species. Presumably that problem will be solved for several commercial species eventually as culture protocols improve. In the meantime, rapid advances in genomic selection methods will add further possibilities in obtaining and mass-producing desired genotypes, not only by SE but also by rooted cuttings and organogenesis. Which of these will dominate the future scene will depend on species and local circumstances and combined use of these technologies can be expected to continue where appropriate. Cost of clonal propagation, especially by SE, is still an issue but will likely diminish with expected future automation.

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Micropropagation and production of forest trees

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Abstract

Forest tree species have been micropropagated *in vitro* for nearly 50 years by axillary budding first, then with increasing interest by *de novo* organogenesis, i.e., adventitious budding and somatic embryogenesis. The particularities of these three main techniques and more generally of *in vitro* micropropagation are reviewed, analyzing their respective pros and cons as well as their effectiveness and limitations for mass producing improved quality planting stock by comparison with more conventional propagation methods.

Keywords: Adventitious budding; Axillary budding; Field applications; Meristems; Nursery; Planting stock improvement; Rejuvenation; Somatic embryogenesis; Tissue culture; Vegetative propagation

1. Introduction

Plant micropropagation has been reviewed in the literature with special mention of its applications to forest trees (Bonga and Durzan 1982; 1987; Haines 1994) that were successfully cultured *in vitro* as early as the 1950's (Bonga and von Aderkas 1992). The purpose of the current paper is to reconsider this vegetative propagation technique from a broader point of view, highlighting its specificities and its usefulness for addressing issues related to the improvement and the production of forest tree planting stock.

2. Definition and expectations

Micropropagation literally refers to the propagation on a tiny scale of more or less differentiated cells that can be structured into organs, in order to produce, ultimately, complete plants. Micropropagation is a purely vegetative propagation technique, based on mitotic divisions that permit to replicate, theoretically unlimitedly, the original genotype while preserving all of its characteristics. The reality of cell totipotency as the conceptual basis of micropropagation (Durzan1984; Bonga and von Aderkas 1992) is strikingly demonstrated by single cell-derived

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somatic embryogenesis (Yeung 1995). The very small size of the vegetative organs or tissues that are being micropropagated requires highly controlled environmental conditions for manipulating these structures as well as for ensuring their further development (Bonga et al. 2010). Axenic *in vitro* culture conditions have been proven to be the most suitable to meet these requirements (Bonga and Durzan 1982; Bonga and von Aderkas 1992; George 1993).

The use of the term micropropagation should, therefore, be restricted to vegetative propagation under *in vitro* conditions. Irrespective of the environment, primary meristems remain the basic structures as the origin of shoots and roots, and as such of micropropagation. *De novo* micropropagation should, however, be distinguished from micropropagation by axillary budding, although for certain species like *Eucalyptus spp.* the two ways may coexist (Le Roux & van Staden 1991).

3. *De novo* micropropagation

3.1 Somatic embryogenesis

Somatic embryogenesis (SE) consists in producing embryos by mitotic divisions from somatic cells while preserving their original genetic make-up. It is, therefore, a cloning technique, as opposed to zygotic embryogenesis in which germinal cells give rise to seedlings that are all genetically different from each other. Apart from a very few cases of direct embryogenesis, for example genotype-dependent cleavage polyembryogenesis (Durzan and Gupta 1987; Sharma and Thorpe 1995; Durzan 2008), SE is mainly indirect. The somatic embryos are formed *de novo*, usually after callus formation artificially induced by the application of strong growth regulators that are assumed to be partly the cause of somaclonal variation (Jones 2002; Menzies and Aimers-Halliday 2004; Bairu et al. 2011). In the most favorable situations, some undifferentiated cells of these calli can gradually evolve into somatic embryos characterized, similarly to zygotic embryos, by a shoot–root bipolar structure (Yeung 1995). This basically distinguishes somatic embryos from adventitious and axillary budding-derived microcuttings that consist of a shoot from which adventitious roots must develop subsequently. By virtue of this analogy with zygotic embryos, SE remains the only way of achieving complete ontogenetic rejuvenation. It resets the ontogenetic program to zero through the formation of embryonic structures that characterize the very first stages of the ontogeny. The older the mother plant the greater the magnitude of this ontogenetic rejuvenation. In this respect, *Hevea brasiliensis* (Carron and Enjalric 1985), *Quercus robur* (Toribio et al. 2004; San–José et al. 2010; Ballester and Vieitez 2012), and more recently *Quercus ilex* (Barra-Jiménez et al. 2014) deserve special consideration as, contrary to most woody species,

somatic embryogenesis can be obtained from sporophytic tissues of mature genotypes. Notwithstanding genotypic and culture medium interference (von Aderkas and Bonga 2000; Bonga et al. 2010; Monteuuis et al. 2011), the physiological rejuvenation associated with this SE ontogenetical rejuvenation has been helpful for subsequent mass clonal propagation by rooted cuttings of mature selected genotypes of rubber trees (Masson et al. 2013), and of other tree species (Lelu-Walter et al. 2013).

3.2 Adventitious budding

Similarly to SE, micropropagation by adventitious budding or organogenesis (Bonga and von Aderkas 1992) depends on the *de novo* formation of new meristems or meristemoids from specialized cells. These need first to dedifferentiate with the possible formation of a transitory callus before reinitiating shoot development from a newly formed shoot apical meristem (SAM). This process is generally induced by the addition of high concentrations of growth regulators into the initiation culture medium. Apparently, this dedifferentiation capacity can be found mostly in superficial cells of vegetative structures like cotyledons or hypocotyls that characterize the early stage of the ontogeny. Contrary to SE, roots are developed also *de novo* subsequently and not concomitantly to shoot formation. Shoot elongation followed by root formation requires transfer to suitable media. Usually, a substantial proportion of the adventitious shoots fail to develop true-to-type when transferred to the field, which may be due to the growth regulators added to the initiation medium (Bonga 1991; Timmis et al. 1992). This and a too high production cost may account for a much more limited operational use of adventitious budding than initially expected (Timmis et al. 1987; Menzies and Aimers-Halliday 2004).

3.3 Micropropagation by axillary budding

Every part of a tree shoot system and all the vegetatively produced offspring derived from it arise from the organogenic activity of the initial SAM formed at the apical pole of the embryo. SAMs through intensive cell divisions produce leaf initia and primordia, which are going to develop into full leaves of limited growth, as well as newly formed axillary meristems, which are potential SAMs at the axil of each leaf. The secondary meristem located underneath the SAM in the main stem that is responsible for cambium formation arises also from SAM activity. Micropropagation by axillary budding stimulates the organogenic capacity of these preexisting axillary meristems that may remain quiescent under apical dominance for long time periods to become proventitious buds liable to produce epicormic shoots. *In vitro* culture boosts the potential of these axillary

buds to produce new shoots. This is, therefore, a much more natural process than the *de novo* micropropagation that occurs after cell dedifferentiation and callus formation, with the associated risks of unexpected occurrence of variants. Micropropagation by axillary budding is considered to be less powerful in terms of potential multiplication rates than *de novo* shoot formation (Haines 1994; Menzies and Aimers-Halliday 2004; Lelu-Walter et al. 2013). It has been proven, nevertheless, for different tree species to be more reliable and sustainable in the long term with a higher guarantee of phenotypic true-to-typeness (Goh and Monteuuis 2001; Monteuuis et al. 2008; Mankessi et al. 2009; Monteuuis et al. 2013). The shoots derived from axillary meristems are trimmed into microcuttings during each subculture transfer and need ultimately, like for *de novo*-derived shoots, to form adventitious roots in order to become independent and autotrophic plants.

4. Chronological steps

Except for SE, which must be considered as a special case (Bonga and von Aderkas 1992; Timmis 1998; Thompson 2014), micropropagation by adventitious and axillary budding involves different chronological steps which are: culture initiation, the stabilization phase, shoot production, rooting and acclimation to *ex-vitro* conditions.

4.1 Culture initiation

Primary culture or culture initiation is a crucial step of micropropagation as it is the starting point of the process. It consists in introducing primary explants, which can be of different types and sizes, to *in vitro* conditions. These primary explants must have at least one SAM for micropropagation by axillary budding, whereas *de novo* techniques are by definition more flexible. One has to apply disinfection protocols strong enough to destroy surface contaminants, while maintaining explant tissues alive.

The organogenic responsiveness of a primary explant is liable to vary tremendously according to its physiological status within the donor plant (Durzan 1984; Monteuuis 1989; Bonga et al. 2010). The stress caused by the excision itself, the smaller the quantity of tissues removed the stronger the impact, the storage conditions, the disinfection procedure before inoculation and the delays in placing the tissue onto proper *in vitro* culture medium can also interfere (Bonga and Durzan 1982; Bonga and von Aderkas 1992). The physiological status of the explants depends on metabolic activities under the influence of environmental conditions and of endogenous factors encompassing genotypic effects, ageing, short and long distance physiological correlations (Durzan 1984; Bonga et al. 2010). External as well as endogenous rhythms, too often neglected, can also

interact (Lüttge and Hertel 2009). Young tissues collected from actively elongating stems are usually less exposed to external contamination than older ones, which are less succulent and as such more resistant to strong disinfection procedures. Also, the smaller the explant, the lesser the surface exposed to contaminants, hence the contaminations risks, but also the higher the cut surface to volume ratio, thus the higher the degree of damage (Bonga and Durzan 1982). Tiny explants like SAMs are far more sensitive towards the composition of the culture medium than bigger ones like microcuttings (Durzan 1984; Monteuuis 1988, George 1993). This sensitiveness to medium composition increases also with the age of the donor plant (Monteuuis 1987). Although presenting, theoretically, the advantage of an higher effectiveness for initiating contamination-free cultures concurrent with the possibility of getting rid of endogenous contaminants (George 1993; Bonga et al. 2010), meristem culture remains in practice little used for forest tree species (Durzan 1984). SAM micrografting can be viewed as an elegant and useful alternative to meristem culture (Monteuuis 2012). An *in vitro* germinated seedling used as rootstock constitutes a more natural and suitable culture support for SAMs than synthetic culture media. In addition to their benefits for initiating healthy cultures, using SAMs as primary explants has been more efficient than using bigger explants for achieving the physiological rejuvenation needed for clonally multiplying true-to-type mature selected genotypes of several tree species (Bon and Monteuuis 1991; Monteuuis 1991; Monteuuis and Goh 2015). In spite of these arguments, meristem culture and micrografting remain in practice impeded by SAM size, which varies noticeably according to the species, its physiological stage and even to the plastochron (Romberger 1963; Mankessi et al. 2010). Personal dexterity for excising rapidly and without damage the SAMs used as primary explants also has a determining impact. For these reasons, shoot apices have replaced SAMs as primary explants for certain species (Monteuuis 1996). In practice 1cm long shoot tips and nodal explants are more widely used, the tissues beneath the organogenic meristems buffering the composition of the culture medium that is never optimal and usually enriched with growth regulators – auxins, cytokinins – for stimulating growth activity. The initiation phase ends with the first morphogenetic response from the contamination-free explants, at which time fungal contamination will be visible thus allowing removal of contaminated cultures. The use of transparent gelling agents like gelrite and phytagel permits better assessment of bacterial contamination diffusing into the culture medium than translucent agar (George 1993). In order to prevent the spread of contamination from one explant to others, especially for precious material and only partially effective disinfection protocols, it is safer to introduce only one primary explant per culture vessel, generally a test tube.

4.2 Stabilization phase

The stabilization phase involves explants that look contamination-free at the end of primary culture, although the risk that these explants may contain endogenous bacteria cannot be ruled out (George 1993). For higher efficiency, several explants can be cultivated in one flask or jar. The "memory" of their initial location within the original donor plant (Durzan 1984, von Aderkas and Bonga 2000) from which they have been collected disappears progressively under the effect of medium-added growth regulators resulting in a higher overall uniformity of the tissue cultured crop.

4.3 Production phase

The production phase corresponds to the sustainable propagation and development of shoots that can be rooted *in vitro* or in more natural *ex-vitro* conditions (Driver and Suttle 1987; Monteuuis and Bon 1987; Bonga and von Aderkas 1992). At regular time intervals the explants are transferred onto fresh culture media of well-defined and suitable composition in order to ensure, over time, sufficiently high multiplication rates, mortality and contamination losses included. This is the main requirement to ensure a sustainable production of microcuttings that can be used first for developing efficient rooting protocols. The production prospects of micropropagation are often overestimated in scientific publications: the size of the buds obtained is sometimes not even indicated, the multiplication rates are established over a too short a culture period and from a too limited sample size to be realistic and applicable on an industrial scale. The aim of such experimental studies seems to get the greatest attention in publications. The reported micropropagation results have usually been achieved by adding to the *in vitro* medium supraoptimal concentrations of growth regulators prone to be the cause of a rapid decline of the cultures which need then to be reinitiated with new explants (George 1993). A more sustainable, natural and thus preferable approach consists in adding to the culture media exogenous growth regulators at concentrations compatible with shoot elongation. On such media, multiplication by axillary budding is promoted by the suppression of apical dominance when the elongated shoots are trimmed into nodal explants at each subculture transfer. The multiplication rates X are lower, but they increase exponentially according to the number of successive subcultures n, resulting in an amount of X^n explants at the end of the process. Several tree species in various laboratories have been subcultured for many years and even decades using such practices, combining shoot elongation and multiplication by axillary budding (Bon et al. 1994; Dumas and Monteuuis 1995; Goh and Monteuuis 2001; Monteuuis et al. 2008; Mankessi et al. 2009; Monteuuis et al. 2013). On media with low cytokinin concentrations, microshoots can root spontaneously. Morpho-organogenic activities have been observed to vary significantly in the course of time according to species, clones and

steady culture conditions (Monteuuis 1988; Favre and Juncker 1989; Monteuuis 2004a). This is very likely due to the influence of endogeneous rhythms (Champagnat et al. 1986; Lüttge and Hertel 2009). Beside growth regulators, mineral components are also important: unsuitable salt compositions are liable to induce noticeable changes in the morphological and organogenic capacity of the explants, leading ultimately to culture failure (Monteuuis 1988).

4.4 Rooting and acclimation to *ex-vitro* conditions

The microshoots produced *in vitro de novo* or by axillary budding must ultimately be rooted to become a fully autonomous plant. There are several ways of producing adventitious roots from an *in vitro*-derived shoot (Monteuuis and Bon 1987). Basically, the process involves 3 successive phases: root induction, root initiation and root expression (Gaspar et al. 1994). Briefly, root induction corresponds to the biochemical/physiological signals sent to the target cells by the application of exogenous rooting substances or "auxins" at the base of the microcuttings as instant dips or during longer periods on an auxin-enriched *in vitro* rooting medium (George 1993). Consequently these target cells undergo concrete anatomical changes during the initiation phase to give rise to root primordia that elongate and become visible during the expression phase. For many tree species and conifers more specifically, root primordia require to be placed onto a specific auxin-free expression medium to elongate (Monteuuis and Bon 1986, Bon et al. 1994, Dumas and Monteuuis 1995). The whole process can be achieved entirely *in vitro*, or induced and initiated *in vitro* and then exposed to *ex-vitro* conditions for root elongation on more natural horticultural substrates (Driver and Suttle 1987; Monteuuis and Bon 1987; Bonga and von Aderkas 1992). The *in vitro* environment provides a better control of external parameters but is more costly, especially when specific media are required for root induction/initiation and expression. Moreover, microshoots *in vitro* are heterotrophic with limited capacity for photosynthesis which makes the transfer to *ex-vitro* conditions critical. Risk of hydric stress, especially for unrooted microshoots, must be prevented. Also, most of the time, the roots formed in gelled media differed anatomically and morphologically from roots adapted to a more natural environment (Monteuuis and Bon 1986; McClelland et al. 1990). According to species, these *in vitro* formed roots are often totally or partially replaced by more functional ones once transferred to *in vivo* conditions (Bonal and Monteuuis 1997). Most of the time, the new *ex-vitro* roots arise from the root structures developed *in vitro* which may justify, at least for certain species or for not fully rejuvenated material, to carry out the rooting process partially or completely *in vitro* (Hackett 1988; McCown 1988). However for cost, manipulation, time saving and greater efficiency reasons, it is usually preferable to

root directly the *in vitro* derived shoot in *ex vitro* conditions (McCown 1988; Bonal and Monteuuis 1997; Goh and Monteuuis 2001).

5. Usefulness

The advantages of using micropropagation to improve forest tree species planting stock have been discussed for several decades already. From a practical standpoint and with the benefits of hindsight, its main advantages seem to be:

5.1 Propagation efficiency

Providing suitable protocols can be developed, micropropagation permits to mass produce, theoretically, unlimited numbers of selected plants from a small group of cells that are more or less organized and that could not survive in *in vivo* conditions. This is particularly true for organs which, once removed from the donor plants, cannot be rooted *ex vitro* or grafted. Such rootless explants can be maintained and serially subcultured on proper culture medium during the time needed to ensure their mass multiplication or to restore their ability for adventitious rooting resulting from a sufficient degree of physiological rejuvenation (Bonga and Durzan 1982; Durzan 1984; Hackett 1988). Another main advantage of micropropagation is the possibility to mass produce in a restricted space, year around, regardless of the local outdoor conditions, enough material to make it more cost efficient than propagation under nursery conditions, especially when simple *in vitro* protocols are used (Monteuuis 2000).

5.2 Alternative to outdoor stock plants

Adapted micropropagation procedures permit to mass multiply sustainably by serial subcultures selected plant material without resorting to stock plants that need to be intensively managed to ensure the production of rooted cuttings in properly equipped facilities. The greater the production targets, the larger the required stock plant areas and rooting beds and the higher also the number of qualified staff that is needed to run all this efficiently. The overall cost of producing plants by rooted cuttings in nurseries together with the constraints this imposes increases dramatically with the quantity of planting stock needed. This should not be underestimated (Monteuuis 2000).

5.3 Establishment of contamination-free *ex-situ* gene banks

Tissue culture is by definition contamination-free, although endogenous contaminants like bacteria may exist surreptitiously for years to invade unpredictably the culture medium and thus affect all the explants of the same origin

after several subculture cycles. Shoot apical meristem culture has proven its efficiency for getting rid of such problems (George 1993). Cultures can be stored *in vitro* a long time at a temperature low enough to limit explant growth, reducing thereby the frequency of the subcultures. The most effective storage method is cryopreservation which requires special pre and post conditioning treatments (Bonga and von Aderkas 1992; George 1993; Jones 2002). Such an *ex situ* gene pool stored *in vitro* can be helpful for various species irrespective of the local natural conditions and can be used for different purposes, including DNA characterization in the absence of exogenous microbial contaminations.

5.4 International exchanges of vegetative material

Thanks to being contamination-free, tissue-culture remains to date the only way to introduce vegetative plant material to countries with very strict phytosanitation rules. Micropropagation is, therefore, essential for the international exchange and acquisition of germplasm for research as well as for operational and commercial purposes.

5.5 Requisite for GMO evaluation

Micropropagation of *in vitro* genetically transformed cells or group of cells to produce complete plants is also crucial for assessing the expected benefits resulting from genetic engineering experiments. Such assessment should be done *in vitro* first, and then ultimately outdoors (Bonga and von Aderkas 1992; Haines 1994; Timmis 1998).

5.6 Physiological rejuvenation

The possibility offered by tissue culture, in comparison with nursery techniques, to cultivate miniaturized organs, in particular SAMs that can be micrografted *in vitro,* is a real asset with regard to physiological rejuvenation prospects (Durzan 1984; Monteuuis 1989; Bonga and von Aderkas 1992). This is essential for successful true-to-type cloning of mature selected trees (Bonga 1991). In some cases, e.g., for clonal seed orchard establishment, it can be advantageous to rejuvenate the mature genotypes only to the degree needed to get rooted shoots, while avoiding too much vegetative vigor, delayed flowering and seed production that can result from a more advanced physiological rejuvenation.

5.7 Economics

Due to certain particularities developed previously, micropropagation can be economically more profitable than conventional propagation by rooted cuttings

from stock plants in the nursery. This mostly depends on the production scale and also on the capacity of the plants to be micropropagated using simple protocols. The coexistence of the two systems developed for teak within the same company in Sabah, East Malaysia established that if more than 100 000 teak plants are produced per annum, micropropagation was more cost effective than nursery techniques. This was mainly due to the savings made because the intensive and time consuming management of stock plants is not needed when propagating by tissue culture (Monteuuis 2000). *In vitro* culture cost can also be significantly reduced by micropropagating plants in countries where the financial investment needed for setting up and then running proper tissue culture facilities is lower, mainly because manpower in developing countries is far less than paid in developed ones.

6. Current limitations

It appears from the literature that micropropagation protocols have been successfully established for various forest tree species. This might be true at an experimental scale but not operationally where micropropagation development remains impeded by serious limitations.

6.1 Availability of responsive primary explants

Culture initiation success depends greatly on the type and the physiological condition of the explants inoculated (Durzan 1984; Bonga et al. 2010). Easy access to nearby donor plants to provide primary explants will definitely be beneficial. Also, resort to efficient nursery methods for preconditioning these explants prior to their introduction to tissue culture may greatly help, according to species and circumstances. These methods include grafting and optional use of BA sprays on successfully grafted scions, as well as keeping portions of branches or sticks under humid conditions in order to stimulate the production of young sprouting shoots to be utilized as responsive primary explants (Monteuuis et al. 2011).

6.2 Genotype responsiveness

The capacity for micropropagation often varies tremendously according to the genotype. For instance, at the genus level and notwithstanding a strong between and within species genotypic influence (Park et al. 1998), *Picea spp* demonstrate overall a higher capacity for somatic embryogenesis than pines or firs and douglas fir. For this latter species cleavage polyembryogenesis is strongly influenced by provenance (Durzan and Gupta 1987). Likewise, poplar (McCown et al. 1988) and

radiata pine (Aitken-Christie et al. 1988) have a higher predisposition for adventitious budding or meristematic nodule formation than other species. Also, marked differences of *in vitro* rooting capacity were observed between closely related *E. urophylla X grandis* hybrid clones derived from the same mother tree – half-sib genotypes (Nourissier and Monteuuis 2008, Mankessi et al. 2009).

6.3 Physiological ageing

The capacity for micropropagation decreases more or less rapidly according to species as genotypes physiologically age (Bonga and Durzan 1982, Durzan 1984, Hackett 1988). This is especially true for adventitious budding and SE which, except for a few exceptions like *Hevea brasiliensis* (Carron and Enjalric 1985), *Quercus spp* (San-José et al. 2010, Barra-Jiménez et al. 2014), remain restricted to very young individuals, mostly immature or mature embryos, too young for reliable selection (Bonga et al. 2010). When SE is successful part of the resulting emblings will be used for establishing clonal tests, while the others will be cryopreserved for as long as it takes to get results from the clonal tests, which allows a sounder selection (Park et al. 1998; Sutton 2002; Lelu-Walter 2013). Notwithstanding variations in the course of time, axillary budding multiplication rates are generally higher for physiologically juvenile explants than for more mature ones. These latter usually require higher concentrations of cytokinins in the nutrient medium, at least during the initiation and stabilization phases (Monteuuis 1988; 2004a). The negative influence of natural ageing on adventitious rooting ability and phenotypic true-to-typeness of the clonal offspring is well known (Bonga and Durzan 1982; Bonga 1991). More insidious is the *in vitro*-induced physiological ageing liable to affect prematurely soft and permeable cells exposed to non-optimal SE or adventitious budding culture media (McKeand 1985; Frampton and Isik 1987; von Aderkas and Bonga 2000).

6.4 Composition of the culture medium

In vitro culture media are usually synthetic, gelled or liquid, and consist of a combination of a restricted list of salts, vitamins, sucrose and growth regulators (Bonga and von Aderkas 1992; George 1993). The characteristics of these components as well as their interactions are likely to change uncontrollably during the autoclaving process, as well as during each subculture cycle due to nutrient uptake by the explants, evaporation, pH variation, and influence of temperature and light (George 1993). These unexpected changes are totally independent of the metabolic requirements associated to explant development in the course of time. Stress caused, for example by inappropriate medium components, unsuitable matrix strength, excessive concentrations of growth regulators and macro-salts,

ammonium especially, can affect the physiology and responsiveness of the explant (von Aderkas and Bonga 2000). *In vitro* micrografting can be an alternative solution to such limitations especially for tiny explants that are more sensitive (Monteuuis 2012).

6.5 Laboratory requirements

Micropropagation activities require proper facilities, equipment and human resources. These include a permanent supply of electricity and good quality water, as well as judiciously partitioned and equipped building facilities (George 1993). Location wise, the vicinity of a big city offers a lot of advantages like airport facilities, external services for easier maintenance and good delivery as well as more daily life convenience. Conversely, easy access to donor plants and suitable nursery facilities can help for initiating the *in vitro* cultures and for testing the post *in vitro* behavior of the tissue-cultured plants and adapting the protocols accordingly, bearing in mind the benefits of *ex vitro* rooting (Monteuuis et Bon 1987; McCown 1988; McClelland et al. 1990).

6.6 Ecomomics

Whatever the technique used, economics have a determining influence on the operational utilization of micropropagation and on how it benefits forest plantations. The main issues to be addressed should be: *i)* Is micropropagation the most suitable way of propagating the selected species taking into account its specificities? *ii)* For what end-use? *iii)* And what ultimate return on investment?

7. *In vitro*-induced effects

Contrary to more conventional vegetative propagation techniques, micropropagation can modify certain characteristics of the *in vitro* cultured plant material.

7.1 Rejuvenation

From an ontogenic standpoint, SE-derived offspring must be duly considered as completely rejuvenated, the more developed the initial donor plant, the greater the rejuvenation achieved. The maturation symptoms that can be observed within such ontogenetically-rejuvenated embling populations are likely due to non-optimal culture media (von Aderkas and Bonga 2000; Monteuuis et al. 2011). Serial micropropagation of microcuttings by axillary budding can also induce a certain degree of mature to juvenile reversion affecting traits like leaf

morphology, particularly visible in heteroblastic species like *Pinus sp* or *Acacia sp,* or in species with a conspicuous dimorphism between juvenile and mature foliage (Mullins et al. 1979; Hammatt and Grant 1993; Monteuuis et al. 2011). In the case of *Acacia mangium*, unpredictable morphological reversions of the mature phyllode type to the juvenile compound leaves at the SAM level during shoot elongation have been noticed only *in vitro* so far (Hatt et al. 2012). Higher capacities for growth, for adventitious rooting as well as for multiplication by adventitious and axillary budding have been noticed as the numbers of subcultures increased for various tree species (Fouret et al. 1986; Walker 1986; Monteuuis 1988; Dumas and Monteuuis 1991; Monteuuis 2004a and b). These changes must objectively be interpreted as physiological rejuvenation indicators influenced by the macro-salt composition of the culture medium, the addition of activated charcoal or exogenous cytokinins (Walker 1986; Monteuuis 1988; Dumas and Monteuuis 1991; 1995). Hence, micropropagation can be useful for at least partially physiologically rejuvenating *in vitro* of mature genotypes, even if most of these rejuvenations revert to the mature phase after acclimatization to *ex vitro* conditions (Mullins et al 1979; Fouret et al. 1986; Pierik1990).

The rare although demonstrative rejuvenation cases obtained from SAM cultures either directly on synthetic media or by micrografting may be due to the removal of potentially juvenile SAMs from ageing-induced inhibiting correlative systems to which they are exposed within the mature donor tree (Durzan 1984; Monteuuis 1989; Bonga et al. 2010; Monteuuis et al. 2011). It can be assumed that their inoculation on a suitable *in vitro* culture medium free of inhibitory ageing factors, while possibly benefitting from rejuvenating substances from the juvenile rootstock in the case of micrografts (Monteuuis 2012), will allow the expression of their juvenile characteristics. Explant miniaturization as well as the timing of SAM excision seems to have a determining influence on *in vitro* physiological rejuvenation, the juvenile "window" becoming more and more time and space restricted as the ortet develops (Monteuuis 1989; Bonga et al. 2010; Monteuuis et al. 2011), which is consistent with the cyclophysis concept (Schaffalitzky de Muckadell 1959; Olesen 1978). Conversely, the incomplete or transitory rejuvenation that is observed when bigger primary explants are used might be due to the persisting negative ageing influence by the mature tissues that are removed together with the meristems from the donor plant.

7.2 SAM characteristics

Cytohistological investigations of *Acacia mangium* have shown that SAMs of juvenile and mature origins displayed morphological and infrastructural similarities with SAMs of outdoor juvenile plants when micropropagated *in vitro*, even at the nucleus level (Hatt et al. 2012). *In vitro* culture of SAMs of *Eucalyptus*

urophylla x Eucalyptus grandis also resulted in a noticeable reduction of SAM size and cell numbers, depending on the plastochron (Mankessi et al. 2010; 2011a). This strengthens the assumption of a possible rejuvenating effect of tissue culture at the SAM level for different tree species (Fouret et al. 1986; Pierik 1990; Hammatt and Grant 1993). In contrast, cells of SAMs *in vitro* that had been excised from juvenile and mature ortets are characterized by a large vacuome which is more representative of the mature state. This reinforces the opinion that *in vitro* culture could also have a maturing effect on plant tissues (George 1993), which could account for the incomplete or transitory *in vitro* rejuvenation that has been reported (Pierik 1990; von Aderkas and Bonga 2000, Monteuuis et al. 2011).

7.3 DNA methylation

According to several reports, repeated *in vitro* subcultures of tree species could induce an overall increase of DNA global methylation as well as DNA methylation profiles that are different from those of outdoor growing plants (Li et al. 2002; Valledor et al. 2007; Monteuuis et al. 2008; 2009). A progressive re-methylation due to prolonged *in vitro* culture has been hypothesized (Lambé et al. 1997). However, reports are not consistent (Mankessi et al. 2011b) and are prone to vary according to species, *in vitro* culture conditions and duration (Lambé et al. 1997; Hasbun et al. 2005; Valledor et al. 2007).

8. Practical considerations

A lot of papers have been published on successful micropropagation of various forest trees, illustrating an intensive activity at the experimental level during the past 50 years. However, reliable reports on operational applications of these research activities are few (Lelu-Walter et al. 2013; Thompson 2014) and progress so far has been below expectations. This might be due to several reasons.

8.1 An increasing gap between research and short term applications

Research quality, especially in the public sector, is more and more evaluated with regard to the number of papers published in high ranking scientific journals. Consequently, research topics are getting more and more basic in nature, with far reaching and ambitious targets that are more and more disconnected from short term applications, and most of the time conducted by researchers who have not been exposed to the constraints associated with operational activities. As a matter of fact, the number of publications on micropropagation of forest trees has dramatically declined during the past decades, in spite of urgent needs to meet with the shortest delays a constantly increasing wood demand for various end-uses.

8.2 What species for what end-uses?

Forest tree species are highly diverse. Some have been selected, domesticated, genetically improved and planted for specific end-uses that encompass pulp and paper, particle boards, multipurpose lumber, veneer and slicing for the most precious timber ones. It can logically be assumed that the value of the final end product will have an impact on the selling price of the planting material: planters will be more eager to buy costly planting stock if the return on investment or the added value is higher. *Picea abies* is in this respect demonstrative: the mass production of selected clones by rooted cuttings initiated during the 1970-1980s in Germany (Kleinschmit 1974; Kleinschmit and Schmidt 1977) has progressively declined as this costly planting material failed to be economically profitable for chip or even lumber end-uses. Contrarily, producing *Abies nordmanniana* clones to be sold as Christmas trees (Nielsen et al. 2008), with a much higher added value and a better return on short term investment seems more justified (Lelu-Walter et al. 2013).

8.3 The best propagation strategy to meet the objectives: seed vs vegetatively produced plants.

In contrast with propagation by seeds, in which every seedling is genetically different from one another, asexual or vegetative propagation consists in duplicating, theoretically endlessly, genotypes while preserving through mitotic divisions the integrity of their original genetic make-up – and thus, consequently, all their individual characteristics. This is essential to ensure the transfer of phenotypic traits that are under non-additive control, especially for those that have a strong economic impact.

The choice of the propagation method remains highly dependent on species characteristics and more particularly on the range of variation of economically important traits among seedlings, especially for genetically related ones like half-siblings issued from the same mother tree. The greater this variability, the more justified the vegetative propagation option, at least theoretically (Bonga and Durzan 1982). Practically, how well plant material can be efficiently mass propagated vegetatively has a determining impact. Vegetatively produced plants are usually more expensive than seedlings. This is why the respective pros and cons of the sexual vs asexual propagation systems in relation to end-use targets and added value must be wisely pondered. For particle board and chip production, seedlings from good provenances are generally preferred for various reasons, cost especially, over vegetatively produced planting stock, as argued for *Acacia mangium* (Monteuuis et al. 2003). This basic question seems particularly relevant for *Picea spp* and *Pinus spp* considering the huge investment put into SE research

activities with these species during the past decades, unfortunately with concrete returns that are still far below expectations (Thompson 2014). Will all this pay off one day? The answer is obvious for teak due to the very cost efficient mass clonal micropropagation techniques that have been developed lately for producing, in short rotations, a high yield of premium quality timber of great market value (Goh and Monteuuis 2015 in this book).

8.4 Bulk vs clonal propagation

Bulk propagation consists in the vegetative propagation of a group of mixed genotypes without maintaining any individual identification. This can be useful for increasing the number of plants of presumably high genetic value but available in insufficient quantities, like for example those obtained by controlled pollinations. In clonal propagation, by contrast, genotypic identity is rigorously and individually preserved through successive propagation cycles, which may last several centuries in certain cases.

The main advantage of bulk propagation lies in the unnecessity to label and keep separated each genotype. This means that less handling and management is required than is needed for the clonal option, especially when large numbers of clones are concerned. Vegetatively propagating a mixture of unidentified genotypes will maintain a certain degree of genetic variability, depending on the number of genotypes involved, at least at the beginning. This may no longer be the case as the number of propagation cycles increases in the course of time as the genotypes with the higher multiplication and rooting rates are likely to supplant progressively the others. Clonal propagation while keeping each genotype separated, prevents such risks, in addition to a number of other advantages, including the possibility to mass produce superior planting material for establishing high yielding and uniform large-scale plantations of premium quality (Libby and Rauter 1984). Another issue to consider is that each seed-issued genotype is unique and there will always be a a “risk” that the time, energy, land and cost investments required by advanced tree breeding programs may not deliver, ultimately, genotypes that are as good as a particular outstanding one selected from the wild. This is partly due to biological processes like the DNA recombinations associated with chromosome crossing overs over which breeders have no control. Being able to mass clonally propagate true-to-type any selected individual regardless of its age is therefore of paramount importance. Practically, the success is highly dependent on the efficiency of the vegetative propagation methods used. In other words, special efforts must be devoted for adapting the cloning techniques to the particularities of the selected genotypes, rather than the other way around. As an illustration, clonal selection based on rooting capacity can be skewed by inefficient

rooting protocols and also by the lack of a strong positive correlation between rooting ability and other field traits of commercial importance.

8.5 Micropropagation vs more conventional vegetative propagation methods.

For micropropagation as for any other plant propagation technique, the simpler and therefore the cheaper to meet the ultimate objective the better. A wise approach of developing vegetative propagation protocols for new plant material should be to test first its responsiveness to conventional nursery techniques. Certain species like *Gmelina arborea*, *Populus spp* can be easily and advantageously mass clonally propagated from mature selected individuals by rooted cuttings in nursery facilities or even directly out planted. In case of nursery technique limitations and if the species is economically really worthwhile, then resort to tissue culture can be considered. In *Sequoiadendron giganteum* (Monteuuis 1988), the effectiveness of various conventional techniques like propagation by rooted cuttings, air layering, grafting for cloning mature selected individuals was first assessed and the information thus obtained warranted to work with smaller ramets (Monteuuis 1985). This was done by using *in vitro* techniques to miniaturize more and more the explants, starting with microcuttings (Monteuuis and Bon 1986), then shoot tips and finally SAM culture and micrografting (Monteuuis 1986, 1987).

Resorting to tissue culture must be warranted. There are too many examples of programs embarking on sophisticated *in vitro* programs without assessing first the capacities of simpler procedures to meet the actual needs. For *Eucalptus urophylla X E. grandis* hybrid clones for instance, the minicutting technique associated with intensively managed container-grown stock plants (Saya et al 2008) was found more efficient than micropropagation by axillary budding (Nourissier and Monteuuis 2008, Mankessi et al 2009).

9. Conclusion

Micropropagation is a remarkable tool for improving the quality of forest tree planting stock. Its usefulness has, however, to be seriously pondered according to the ultimate objectives and the particularities of the plant material to meet the desired goals. The advantages and limitations of vegetatively multiplying selected trees by tissue culture rather than in more natural and cheaper nursery facilities deserve special consideration. Practically, producing with the shortest delays and at the cheapest cost the needed quantity of improved quality planting stock to meet plantation requirements must remain the priority.

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Limitations of somatic embryogenesis in hardwood trees

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Abstract

Several limitations of somatic embryogenesis in mature and selected hardwood tree species hinder application of the process for mass clonal propagation. After many years of research, our understanding of the basic aspects of the process is still very limited. The objective of the present review is to highlight some difficulties in the different steps of somatic embryogenesis in adult hardwood trees. The experimental models, applied to species of the family *Fagaceae*, will be defined. These models could be used to increase the propagation capacity by applying both organogenesis and somatic embryogenesis together. Application of these experimental models to the study of basic aspects of embryogenesis will be highlighted. The potential use of somatic embryogenesis as a rejuvenation procedure for mature hardwood trees will also be discussed.

Keywords: developmental window; experimental models; explant source; germination; induction; molecular markers; organogenesis; rejuvenation;

1. Introduction

The application of somatic embryogenesis (SE) to multi-varietal forestry (MVF) (Park and Bonga 2011) is currently restricted to a few conifer species. This is mainly due to problems in the efficacy of the process, as deficiencies in the current protocols affect the induction of SE and also the maturation, germination and plantlet conversion steps (Thompson 2015). These problems affect both gymnosperms and angiosperms because initiation and, to a lesser extent, maturation and germination rates are under strong additive genetic control (Bonga et al. 2010). Successful SE may be induced in conifers when immature zygotic

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embryos are used as a source of explants. The 'developmental window' during which SE induction is possible is often very short but sufficient for application of the technique to enable selection of plant material derived from conventional breeding programs. Breeding of long-rotation hardwoods is less well developed than that of short-rotation species (poplars, eucalypts, and willows) and has always lagged behind that of conifers. The recently described genomic selection (genotype-dependent selection) (Park et al. 2015, El-Kassaby and Klápste 2015) would be of great interest for reducing the time required for genetic evaluation of economic traits in recalcitrant hardwoods. At present, the first approach for enhancing the productivity of hardwood plantations is the identification of mature trees with desired traits, followed by clonal propagation (SE), which has the advantage of capturing all the genetic superiority without involving gene segregation.

Despite major advances in forest biotechnology, regeneration through in vitro tissue culture techniques is still difficult, but feasible, in several mature hardwood trees. Although seedling explants are relatively easy to micropropagate, explants from mature trees tend to lose their regeneration potential. When cloning mature trees it is important to determine which part of the individual contains the most responsive cells, as some parts of the tree are morphogenically more competent than others. Responsive tissues in hardwoods are present at the root-shoot junction, in root or stump sprouts, sphaeroblasts and epicormic shoots (Bonga et al. 2010). Collection of these tissues during the most appropriate developmental period for culture is of great importance. In the absence of this type of material, mature reactive material may be produced by both hedging and stool bed methods, which enable the use of preformed dormant buds that remain quiescent after early initiation. Outgrowth of dormant buds often leads to the development of physiologically juvenile shoots, regardless of their position within the tree structure (Bonga et al. 2010, Monteuuis et al. 2011 and in this book, Vieitez et al. 2012). In addition, SE may also be induced in mature hardwoods by using tissues close to cells involved in the sexual process, in which the timing of explant isolation and the application of mild stress are extremely important. The possibility of using these types of tissues to induce SE in mature hardwood trees has proven quite efficient in certain cases, and this may be particularly important in relation to conifers, which (with the exception of a very few species) generally do not produce juvenile sprouts (Bonga 2013). However, only one study has reported successful induction of SE from mature material from a conifer (*Picea glauca*) (Klimaszewska et al. 2011).

In a pioneering review, Merkle (1995) clearly described the limitations of somatic embryogenesis in hardwood trees. Unfortunately, most of the limitations reported remain unresolved 20 years later, although significant advances have been made with some species. In hardwoods, induction of the embryogenic process from

juvenile material (immature or mature zygotic embryos) is generally not problematic. Several reviews (Merkle and Nairn 2005, Pijut et al. 2007) and books (Jain et al. 1995, Jain and Gupta 2005) provide information about the successful induction of SE in some species. However, most of those publications are based on single experiments, or experiments based on a single genotype, or do not provide sufficient data to allow replication of the work. Such incomplete information does not aid the development of experimental models that might assist in advancing knowledge of the basic processes underlying SE, which is fundamental for the practical application of the technology in MVF. Somatic embryogenesis has been induced in mature individuals in a small number of broad-leaved and deciduous species by using leaves in a suitable physiological condition and/or sporophytic tissues from the reproductive organs as primary explants, as demonstrated in various species of *Quercus* (Corredoira et al. 2014). Our experience accumulated during the last few decades of work with oaks and other species of the family *Fagaceae* has enabled us to define consistent and replicable protocols for the induction of SE in material from mature trees, and the experimental models proposed should be used to study fundamental aspects of the embryogenic process.

The objective of the present review is to describe the limitations of SE in adult hardwood trees. Specifically, the experimental model used to induce SE in mature *Fagaceae* species and the developmental aspects of the induction phase will be described and the key factors influencing the maturation and germination of somatic embryos will be defined. A procedure for improving the propagation of embryo-derived plants through organogenesis will also be proposed. The potential use of SE as a rejuvenation method for mature hardwood trees will also be discussed. For more exhaustive information on the subject, the reader should consult the chapter on chestnut (Corredoira et al.) in this book.

2. Searching for the explant source and the 'developmental window' for inducing SE in hardwoods

The presence of pluri- or toti-potent stem cells (the precursors of plant organs) is required for induction of SE. These stem cells are associated with shoot apices (Sablowski 2011) and exhibit a physiological juvenile condition confined to the shoot apical meristem (Monteuuis et al. 2011). In hardwoods, juvenile tissues found within the structure of mature trees can be used for induction of organogenesis and SE. In several hardwoods, tissues close to cells that are involved in the sexual process are also responsive (Corredoira et al. 2014).

Somatic embryogenesis has been induced from floral parts in several woody species, such as *Quercus bicolor* (Gingas 1991), *Q. petraea* (Jörgensen 1993) and horse chestnut (Capuana and Debergh 1997). To induce SE in holm oak, Blasco et al. (2013) used male catkins at different developmental stages collected

in late spring (May-June) as initial explants: floral buds up to 2-4 mm, catkins starting to develop, and elongated catkins (up to 2 cm in length). The embryogenic response was obtained in three of the five genotypes evaluated and restricted to the most advanced (catkin) developmental stage. Furthermore, SE has also been induced in developing ovules of several woody species such as *Carica papaya* (Litz and Conover 1982), *Hevea brasiliensis* (Carron and Enjalric 1985) and *Theobroma cacao* (Figueira and Janick 1993) as well as in hardwood species such as *Quercus ilex* (Barra-Jiménez et al. 2014). In the latter, the authors used female flowers before fertilization, flowers after fertilization, and immature acorns as initial sources of explants. The plant material was collected from mature trees of holm oak during June-July. Somatic embryogenesis was only successfully induced in ovules excised at an advanced stage of development and the somatic embryos regenerated arose from the integuments of fertilized ovules.

The information obtained using floral tissues for initiation of SE makes possible the identification of novel strategies and selective targets for improving the efficacy of the process. This would be an interesting experimental model for initiating basic studies of the embryogenic process. However, several aspects should be taken into consideration in determining whether or not floral tissues represent the most appropriate system for studying these aspects in mature hardwoods. Floral plant material can be collected only once a year, which constrains repetition of the results. In addition, plant material is influenced by environmental conditions and the time window for collecting the most appropriate plant material differs depending on the latitude. Taking all of these concerns into consideration, the development of a simpler, consistent and repetitive experimental method would be of interest.

As pointed out in the Introduction section, mature hardwood trees contain morphogenically responsive juvenile tissues that can be used to induce SE. Various pre-treatments have been proposed for the rejuvenation of mature material (Ballester et al. 2009, Bonga et al. 2010, Monteuuis et al. 2011 and in this book), however, in our opinion, the simplest and most efficient procedure is that based on forced flushing of branch segments. This method makes use of preformed dormant buds that remain quiescent after early initiation; outgrowth of these axillary buds (also called accessory buds by Evers et al., 1990 or proventitious buds by Monteuuis et al. 2011) often leads to the development of juvenile shoots. Flushing of these shoots is associated with branch scars and occurs 10-15 days after the beginning of the forcing period. Evers et al. (1990) distinguished between shoots associated with branch scars and shoots appearing elsewhere on trunk sections of an 8-year-old oak tree. The former were referred to as accessory, as they were considered to have once been associated with the terminal bud of the branch and then to have remained dormant, while the latter were referred to as epicormic as they emerged from a bud that was not formed in the last growing season. We

estimate that both accessory and epicormic shoots develop from preformed buds induced to flush when isolation of the branch segments broke apical dominance. The flushed shoots generally exhibit vigorous growth, long internodes and leaves resembling a more juvenile type. This material appears to be sufficiently rejuvenated to provide reactive explants. In practice, thick crown branches (3-5 cm diameter) are used to induce epicormic shoots. The plant material should be collected from the lower part of the crown during the resting period of the tree (between November and March in the northern hemisphere), cut into 25-30 cm segments, surface sterilized and placed on moist perlite beds in a growth cabinet (25ºC, 16 h light and 90% relative humidity) to flush new shoots. Expanding leaves excised from such shoots have been used as initial explants to induce SE in mature cork oak (Hernández et al. 2001) and in pedunculate oak trees (Toribio et al. 2004). Genotype and time of harvesting, as well as their interaction, were found to have a significantly influence on the rate of embryogenic induction in cork oak (Hernández et al. 2003). In pedunculate oak, 7 out of 30 trees tested showed an embryogenic response, which was also influenced by the collection date and the branch position in the mother tree (Toribio et al. 2004, Valladares et al. 2006). Genotype is clearly one of the limiting factors in the induction of SE. Attempts to induce SE from shoot tips, instead of expanding leaves, isolated from forced epicormic shoots of the corresponding trees were unsuccessful, suggesting that the sterilization process may have a negative effect on this type of explant. However, shoot tips excised from offshoots of adult date palm (Veramendi and Navarro 1997) and those excised from very juvenile seedlings of mahogany (Maruyama 2006) and peach palm (Steinmacher et al. 2007) have been used to induce SE. To circumvent the difficulties in inducing SE from shoot tips and leaf explants isolated from flushed epicormic shoots, we use a routine procedure by excising these type of explants from in vitro shoot proliferation cultures previously established from adult oak trees (see next section). A similar procedure has also been used to induce SE in a mature tree of *Cyphomandra betacea* (Correia et al., 2011). The results obtained to date confirm the suitability of the procedure for reproducible induction of SE in mature hardwood trees. A reliable and reproducible method for in vitro establishment and multiplication of shoot cultures is obviously required for application of the procedure already described. The use of in vitro shoot cultures as a source of explants for SE should be considered to enable better control of growing conditions of stock material and to prevent differences in the time of collection of plant material from trees growing in the field and possible differences in the physiological state of forced epicormic shoots. Shoot cultures produce uniform explants, thus preventing possible interactions between different types of plant material. Supply of an unlimited number of explants is guaranteed all year around, as flushing of branch segments is not required after in vitro establishment of the donor shoots.

This experimental model could be applied to other mature hardwood species to confirm its utility for studying why certain cells can become embryogenic and what factors trigger them to do so (Thompson, 2015), i.e. to understand the mechanisms underlying the process.

The experimental models developed for SE of oak species served to identify early markers of embryogenesis that can help to monitor the metabolic processes involved in SE induction, thus providing a better understanding of the physiology and mechanisms controlling plant cell reprogramming and acquisition of embryogenic competence. Although the molecular biology of somatic embryogenesis in hardwoods will be reviewed in another chapter of this book (see Coreira et al. in this book), we will summarize here the most recent findings achieved with the oak experimental models. Rodríguez-Sanz et al. (2014) studied the evolution of different markers during the induction of embryogenesis from both immature zygotic embryos and microspores (anther culture) of cork oak (Bueno et al. 2003). Specifically, these authors analyzed the changes in DNA methylation, sterification of pectins in cell walls and distribution of endogenous auxin (indole-acetic acid, IAA) during early embryogenesis as potential markers of the process. Interestingly, the response of the tissues in both embryogenic systems studied was very similar for all three markers considered. A significant decrease in global DNA methylation in early multicellular embryos was observed relative to that in immature zygotic embryos or microspores. Immunolocalization assays showed a decrease in 5-methyl-deoxy-cytidine as well as a change in its distribution pattern in small nuclear spots in contrast to that in large nuclear regions which corresponded to highly condensed chromatin masses of non-embryogenic cells. In addition, differences in the distribution pattern of highly-esterified pectins were observed; they were more abundant in early embryo cells than in non-embryogenic cells while they decreased with embryo development and differentiation. Finally, variable and significant increases in IAA endogenous levels were recorded in the cells of early multicellular embryos. Together with the fact that auxin transport inhibitors negatively affected the initiation of embryogenesis, this suggests the involvement of endogenous IAA biosynthesis and transport in switching the embryogenic program. These three markers are currently being analyzed in the embryogenic system of *Quercus alba* induced from leaf explants excised from shoot cultures (Corredoira et al. 2012). Preliminary results (Testillano, personal communication) indicate that the three markers already mentioned followed a similar pattern to that reported for the embryogenic processes of cork oak. Changes in various cell activities and in the structural organization of subcellular compartments were also reported during the in vitro microspore reprogramming to embryogenesis in olive (Solis et al. 2008). The availability of suitable molecular markers in different genotypes/species would be valuable for helping to understand the SE induction process in hardwoods.

3. Developmental aspects of somatic embryo induction, multiplication, maturation and germination steps

Several bottlenecks in the SE process require special attention in order to increase the efficiency of the procedure. Furthermore, there are not sufficient morphological markers available to identify competent cells for SE within a tissue/organ or to provide a good understanding of why certain cells become embryogenic. As previously mentioned and, to contribute to a better understanding of the embryo induction process, we have defined a procedure whereby SE was induced in shoot tips and leaf explants of pedunculate oak (San-José et al. 2010), white oak (Corredoira et al. 2012), swamp white oak (Mallón et al. 2013a), in different genotypes of red oak (Martínez et al. 2015a,b) and in mature genotypes of *Eucalyptus globulus* and the hybrid *E.saligna* x *E. maidenii* (Corredoira et al. 2015). In these species, a total of 14 genotypes were tested, 11 of which responded positively, indicating that the procedure is reproducible. We are currently applying this procedure to different genotypes of holm oak. Preliminary results show that induction rates of 4% are achieved in the 2 genotypes evaluated (Martínez et al., unpublished), confirming once again the suitability of the proposed procedure.

Shoot tip explants (2 mm long, comprising the apical meristem and 2-3 pairs of leaf primordia) were excised from proliferating shoot cultures (shoots, 3-4 cm long) at the end of the multiplication period and used as initial explants for SE induction. At the histological level, the response of the explants to the culture media started at the cut surface of the explants and consisted of cell proliferation derived from parenchymatic and vascular cylinder cells. Callus formation progressed from the basal part of the shoot apex but this cell proliferation did not affect the apical meristem itself, resulting in a senescent region that separated from the developing callus by 12-14 weeks of culture. The mass of callus increased by proliferation of parenchyma cells, and differentiation of cambium-like zones that generated vascular tissue. Some cells acquired an embryogenic character and they were interspersed with parenchyma cells in callus regions undergoing further vacuolization and degradation. The physical isolation of potential embryogenic cells from surrounding tissues seems to be a prerequisite for triggering the embryogenic program and expression of cell totipotency (Kurczynska et al. 2007). Embryogenic cells displayed a high nucleoplasmic ratio, a densely stained cytoplasm and small vacuoles, contained small starch grains and appeared to acquire an isodiametric form with a thick cell wall.

Miniaturization of shoot apical meristems to produce explants has been proposed for induction of SE in mature trees as such meristems are liable to contain cells that have remained juvenile and that would be responsive after excision and in vitro culture (Monteuuis et al. 2011). However, in our studies, the apical meristem itself generally senesced and died during culture, and the basal leaf primordia

attached to the axillary zone was the real source of calluses and, subsequently, embryogenic tissues (Corredoira et al. 2012). These results are consistent with those reported by Klimaszewska et al. (2011) on the induction of SE on needles of primordia shoot explants from mature white spruce trees, with the origin of somatic embryos associated with the formation of nodules or calluses generated at the base of elongated needle primordia.

In order to optimize the source of explants and their developmental stage, which are considered key elements in SE (Fehér 2006), an experiment was designed using leaf explants of shoot cultures derived from mature white oak trees (Corredoira et al. 2012). Leaf explants were taken from four successive nodes below the apex: node 1 was the apical most node, with a still-folded leaf, and nodes 2, 3, and 4 had expanding leaves. The rate at which explants produced SE was significantly affected by their position: the percentage of induction was highest in leaves from the node below the shoot apex (51%) and lowest in those from node 4 (4%). Interestingly, embryo induction rates were lower in the shoot tip explants than in leaf explants from nodes 1 and 2 of the same shoot cultures from which both were excised and cultured under the same conditions. The physiological condition and the 'developmental window' for selecting the most appropriate responsive explants for SE induction were again of prime importance even when in vitro shoots were used as a source of explants (there is 2-3 days of developmental delay from leaf of node 1 to leaf 4). Unfortunately, even using this system not all of the genotypes tested responded positively, confirming the well-known strong influence of the genotype on SE ability (Bonga et al. 2010). The histological analyses performed on leaf explants revealed that, leaves with the highest embryogenic capacity (nodes 1 and 2) contain not fully differentiated cells and tissues, including the presence of precursor guard cells of stomata, no accumulation of polyphenols in the epidermis, absence of intercellular spaces, and low starch content in the mesophyll. The higher level of differentiation in leaves at positions 3 and 4 included the development of vascular bundle sheaths enclosing smaller veins and of sclerenchymatic tissue formed by two or three layers of lignified cells surrounding the vascular system of the midvein (Corredoira et al. 2012). Asymmetric division of guard cells is of particular interest as these cells are highly differentiated but yet may be totipotent (Bonga 2013).

Few other factors can be tested in relation to improving SE induction, as the culture media and environmental conditions have not yet been optimized for many species/genotypes. The use of alternative or new chemicals may prove to be more effective than conventional culture media. In this respect, the positive effect of arabinogalactan from larch wood on SE induction in *Quercus bicolor* has been demonstrated (Mallón et al. 2013a). However, arabinogalactan proteins from *Larix* did not improve SE induction in holm oak (Barra-Jiménez et al. 2014) and those from Acacia (gum Arabic) inhibited the embryogenic response. Arabigalactan

proteins (AGPs) are a family of highly glycosylated hydroxyproline-rich proteins that are involved in several aspects of morphogenesis. The addition of exogenous AGPs to the culture medium has also been found to stimulate SE in other woody species (Ben Amar et al. 2007, Pereira-Netto et al. 2007). Although there is some evidence that oligosaccharides released from AGPs may act as signal molecules, their mechanism of action remains uncertain (Poon et al. 2012).

In most hardwood species, the induction rate, despite being low, is clearly not the most critical step of the embryogenic process. Once embryos are initiated, a large number of somatic embryos can be obtained by secondary embryogenesis. A recurrent embryogenic mechanism is triggered, thus enabling the formation of secondary embryos that give rise to clonal embryogenic lines. Production by scaling-up and long-term maintenance of SE in different species depends on the embryogenic capacity through secondary embryogenesis. The use of different components in embryo proliferation media may help to improve the multiplication response. In both white oak and red oak, the inclusion of silver thiosulphate, activated charcoal and 3% sucrose in the proliferation medium promoted development of well-formed and easily detachable cotyledonary-shaped embryos (Martínez et al. 2015b). High rates of maturation, germination, conversion and acclimatization is of prime importance as these are limiting factors for the practical application of SE in most hardwood species. The physiological quality of the somatic embryos being produced is critical for successful completion of these steps. At present, it is not clear which approach may yield positive results, and the design of a robust procedure covering most hardwood species is not yet possible. In many species, maturation has been hampered by repetitive embryogenesis, immaturity, embryo dormancy and precocious germination. The ultimate goal in this step is the production of high quality cotyledonary embryos ready to be subjected to the germination process. During the maturation stage, somatic embryos should accumulate specific storage products, and culture of embryos with abscisic acid and/or osmotic agents is recommended to induce desiccation tolerance. In addition, in some woody species, culture of SE in a maturation medium must be followed by a period of cold storage (2-month period in most *Fagaceae* species, Vieitez et al. 2012, Corredoira et al. 2014) to break embryo dormancy. Mature embryos are usually germinated in different media with or without different plant growth regulators.

In most species, including hardwood trees, the main bottleneck in the embryogenic process is the low production of viable plantlets from somatic embryos. Despite the numerous maturation and germination conditions tested and reported, plant conversion remains a limiting step for many hardwood species. In some cases, embryo germination with shoot-only or root-only elongation is the main response to germination treatments and very few embryos showing both shoot and root development (plantlet conversion) are obtained. An alternative

approach to the limited number of plants produced may be the stimulation of shoot development regardless of the presence or otherwise of the root. The shoot-promoting ability of thidiazuron (TDZ) may improve the overall efficiency of embryogenic lines exhibiting low conversion frequencies by inducing shoot development from the apical dome of somatic embryos, as demonstrated in embryogenic lines induced from mature *Quercus robur* trees (Martínez et al. 2008). In this species, the addition of TDZ to the germination medium for an initial period of 7 days induced multiple shoot formation in the epicotyl region of the germinating embryos, although root development may be restricted. Shoots excised from the partly germinated embryos can be elongated and rooted via axillary shoot proliferation (Martínez et al. 2008). This strategy could be taken into consideration for other recalcitrant hardwood species that display low plantlet conversion rates, in order to increase the total number of plants produced. To develop such a strategy, it is obvious that a method of axillary shoot development must be consolidated.

An alternative and complementary approach to increasing the efficiency of the maturation, germination and plantlet conversion steps is the use of bioreactors, initially seen as a rapid means of producing large volumes of embryogenic tissue; however, application of the technique is restricted by the high rates of hyperhydricity observed (Preil 2005). Bioreactors based on temporary immersion systems (TIS) have been shown to increase the number and quality of the somatic embryos produced. In several species, the morphology and physiology of TIS-derived plants resemble those of conventional propagated plants and yield higher survival rates than those cultured in semi-solid medium (Etienne and Berthouly 2002, Watt 2012). Most reports on the application of TIS to the multiplication of SE in woody species refer to subtropical species of commercial interest such as *Theobroma cacao* (Niemenak et al. 2008, Guillou et al. 2015), *Phoenix dactylifera* (Ibraheem et al. 2013) and *Coffea*. In the latter species, the potential for commercialization of the embryogenic process has already been demonstrated (Menéndez-Yuffá et al. 2010, Etienne et al. 2011). However, the application of TIS to temperate hardwood species has been investigated less frequently. In pedunculate oak, TIS promoted a significant increase in proliferated embryo biomass and had also a significant effect on somatic embryo synchronization, relative to culture in gelled medium, enabling higher production of cotyledonary embryos (Mallón et al. 2012). In addition, the use of suspension cultures including TIS increased the transformation frequency, relative to cultures on semi-solid medium, in genetic transformation experiments in different woody species such as sandalwood (Shekhawat et al. 2008), American chestnut (Andrade et al. 2009), yew (Zhang et al. 2011) and oak (Mallón et al. 2013b).

Combining the use of embryo-germinated plants and plants obtained by rooting axillary shoots derived from somatic emblings is a promising option for maximizing the number of plants yielded by embryogenic systems. In cocoa,

Guillou et al. (2015) found that only 7% of the somatic embryos convert into plants. These somatic plantlets are planted directly in the field or used to establish clonal gardens from which rooted cuttings are prepared to increase production of plants of selected genotypes. Unfortunately, it is usually difficult to root cuttings from recalcitrant hardwood trees, although the cocoa example should serve in the search for alternative strategies of enhancing the potential of SE in clonal propagation.

From the information available on the maturation, germination and plantlet conversion steps of the embryogenic process in hardwood species we can state that i) multiplication of somatic embryos is generally not a problem, regardless of whether the embryos are cultured in semi-solid or liquid medium; ii) the percentage of plantlet conversion is very low in most hardwood species, relative to the high proliferation capacity of the embryos; iii) basic research is required to understand the maturation step with special emphasis on finding appropriate markers to determine when the embryos are mature and ready for germination; iv) basic research is also required to understand why only a very small percentage of embryos convert into plants, while most only develop a root or a shoot or do not respond at all.

4. Rejuvenation through somatic embryogenesis

Recalcitrance is a common problem in the in vitro propagation of tree species, especially for micropropagation of mature genotypes. The phenomenon affects both organogenesis and somatic embryogenesis (in vitro micropropagation methods). Interestingly, often genotypes with a poor response to organogenesis may also respond poorly in the embryogenic process, as recently observed in three genotypes of adult eucalyptus (Corredoira et al. 2015). The search for alternative techniques is of interest in relation to mitigating the problems associated with recalcitrance in clonal propagation carried out by in vitro techniques. Many attempts have been made to rejuvenate mature material through both in vitro and ex vitro techniques, as juvenile stages of plants yield high morphogenetic responses. Recalcitrance in clonal propagation has been widely reviewed (Bonga et al. 2010, Bonga 2013). Furthermore, Monteuuis et al. (2011) described different approaches for overcoming recalcitrance, emphasizing that somatic embryogenesis is the only way of achieving complete ontogenetic rejuvenation as it resets the ontogenetic process to zero through the formation of embryos. However, examples demonstrating the re-acquisition of the micropropagation capacity in material from embryo-derived plants are, to our knowledge, limited to *Hevea brasiliensis* (Carron et al. 1995) and *Theobroma cacao* (Traore et al. 2003). Information on the subject in truly recalcitrant hardwoods is limited to *Fagaceae* species (Marínez et al. 2012, Ballester and Vieitez 2013).

We established shoot cultures from plant material simultaneously collected from juvenile shoots at the base of the trunk (BS) and branches from the crown of the same mature trees (three genotypes) of pedunculate oak (100-300-year-old trees). Genotypic differences in rooting capacity were evident; however, regardless of the genotype, rooting rates were significantly higher in BS shoots than in their C counterparts. These results were highly consistent over several years and allowed us to use leaf explants of shoot cultures of these materials to induce SE. Different embryogenic lines (BS-E and C-E) were induced and subjected to multiplication, maturation and germination steps. Shoot proliferation cultures were then established from somatic embryo-derived plants and, after stabilization of cultures, the multiplication and rooting rates were recorded and compared with those obtained with the BS and C counterparts. The rooting rates of BS-E and C-E were always significantly higher than those of BS and C shoots, confirming that some degree of rejuvenation took place through the embryogenic process (Martínez et al. 2012, Ballester and Vieitez 2013). Furthermore, plantlets of one genotype were rooted, acclimatized and grown in the greenhouse. After 12 months of growth, significant differences in plant growth, length of internodes and color of the leaves seem to confirm the juvenile character of the lines (BS-E and C-E) tested.

Estimation of the level of rejuvenation achieved in pedunculate oak should not be based only on micropropagation criteria, but also on biochemical, genetic and epigenetic markers. The results reported indicate that the proposed experimental model used in this research is valid not only for testing the rejuvenation hypothesis but also for increasing the rooting capacity of recalcitrant trees. The significant improvement of rooting rates obtained in stock shoot cultures of somatic plantlet origin, relative to those derived from mature trees could be applied in order to improve the micropropagation capacity of recalcitrant genotypes and should be tested in other hardwood species. The experimental model is very consistent and appears to be a suitable tool for studying the molecular aspects of rejuvenation in trees (Klimaszewska et al. 2009). The possibility of obtaining the juvenile phase in trees by overexpression of specific genes (Wang et al. 2011) opens up new possibilities for a better understanding of the phase change in trees.

5. Conclusions and future prospects

The SE process in mature, selected hardwood species has several limitations that hinder its application for mass propagation and, consequently, in multi-varietal forestry programs. The induction step is feasible but limited by the genotype, age of the mother tree, type of explants, composition of the culture medium and growth conditions. However, the main bottleneck in the process is the low rate of plantlet conversion, which limits the number of viable plants produced. This affects hardwood species as well as most woody plants. In this review, we

propose a combined procedure that uses both organogenesis and somatic embryogenesis to cover different aspects of the process: i) facilitation of SE induction by collection of initial explants at any time of the year, i.e. shoot apex and leaf explants from shoot multiplication cultures; ii) increased plant production by the multiplication and rooting of axillary shoot cultures established from plants derived from somatic embryos; iii) design of suitable and reproducible experimental models to facilitate the study of basic aspects of the embryogenic process. In addition, we propose a method for rejuvenating recalcitrant mature hardwood trees through SE, in order to enhance plant productivity and as a support for fundamental studies on rejuvenation.

Although a great deal of effort has gone into the development of SE methods in hardwood species, the results achieved are of academic rather than practical interest. This is mainly due to problems related to the efficacy of the process. The protocols used are adapted within species and within genotypes, and the embryogenic response is affected by the above-described factors. However after many years of research, understanding of the basic process remains limited. We still do not understand why certain cells can become embryogenic, what factors trigger them to do so, how to identify competent cells or how to identify which somatic embryos will go on to regenerate plants (Thompson 2015). Research in coming years should focus on these and similar topics, which represent the greatest challenges to large-scale propagation by SE. Suitable experimental models must be established to enable basic research in hardwood species. The knowledge gained in the different steps of SE by working with model plants should be adapted for application in hardwoods. The development of appropriate experimental models similar to those proposed in this review should be a priority in future studies.

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Physiological, cellular, molecular and genomic analysis of the effect of maturation on propagation capacity

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Abstract

The effect of maturation on the propagation capacity of trees is important for the propagation of selected adult trees. Approaches to understanding the recalcitrance of adult tissues to de novo regeneration have moved from the detection and quantification of plant growth regulators to the identification of specific genes through the application of molecular biology and genomics tools. Auxins and cytokinins are necessary, but not sufficient, signals for reprogramming adult cell specification. The crosstalk between hormones and other signals at various levels of regulation— molecular, cellular or tissue—is also important for de novo regeneration. This review describes the progress in understanding which signals and pathways contribute to maturation-related decline in propagation capacity.

Keywords: cell fate, dedifferentiation, pluripotency, stem cells, totipotency

1. Maturation, *de novo* regeneration and propagation capacity in forest tree species

It is difficult to overstate the importance of trees. They are essential components of the environment and play a crucial role in the equilibrium of global ecosystems. Trees also form the foundation of forest product industries, including the conversion of biomass to energy. Despite their importance from both environmental and economic perspectives, little is known about the mechanisms that underpin forest productivity. Because of the substantial lag time between seed germination and sexual maturity, trees have not been as amenable to the traditional breeding approaches that have been so useful in the improvement of short-lived

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crops. An alternative way to capture genetic gain more readily than by sexual reproduction is the clonal propagation of forest trees that have expressed desirable characteristics. In addition to conserving the genotype of the donor plant in the propagules, vegetative propagation also offers opportunities for selection and maintenance of both additive and non-additive gene effects (George et al. 2008). Non-additive gene effects can give rise to exceptional individuals, within superior families, and these genetic effects are best captured by vegetative propagation in most tree species. Therefore, vegetative propagation offers a way to increase the productivity of forest trees expressing yield traits of ecological or economic interest, such as wood quality or resistance to biotic or abiotic factors. In horticulture, the vegetative propagation of desired plant phenotypes has been successfully used for centuries. However, in forestry, aside from a few genera, vegetative propagation of "plus" trees has not been used extensively in most operational planting programs, despite the fact that many families have to be propagated by these procedures (Greenwood and Weir 1995). Woody species are generally more recalcitrant to regeneration and propagation than herbaceous plants, and gymnosperms are more recalcitrant than many angiosperm trees (McCown 2000; von Aderkas and Bonga 2000). Recalcitrance to organogenesis or somatic embryogenesis is a major limitation in the clonal propagation of many woody species, especially forest tree species such as conifers. Despite recent advances in our knowledge of the molecular basis of de novo regeneration, many aspects of the process and the causes of regeneration recalcitrance are still poorly understood. De novo regeneration depends on the plant species, varieties, or cultivars, and is highly variable and unpredictable. For a given species, endogenous and environmental factors such as genotype, tissue and timing of excision, explant position within the mother plant, phenology, tree maturation, light and temperature could be limiting for regeneration (Bonga et al. 2010; Rutledge et al. 2013).

The maturation and age of trees is a major limiting factor; it is well known that regeneration efficiency is much higher in tissues at the earliest stages of development (George et al. 2008). For many species, desirable yield traits are only adequately expressed at the phenotypic level after the tree has reached a certain degree of maturation. For example, wood specific gravity is only expressed at the phenotypic level after the tree has produced several growth rings, and early height growth is often a poor predictor of volume at the rotation age. Effects of maturation on stress responses have also been described for many forest species. However, after a critical age is reached, propagation capacity declines abruptly (Díaz-Sala et al. 1990, 1991). Propagation from juvenile material, although useful for certain studies, has the disadvantage that it is difficult to predict how a seedling will perform on reaching maturity. In addition, a certain degree of maturation sets in well before the flowering phase, which used to be considered diagnostic for the onset of maturation, and, therefore, a reduction in vegetative propagation ability

may occur very early for several species (even as young as one year old). Consequently, trees old enough for proper evaluation of their characteristics are generally difficult to propagate and clone, and individuals are often selected for clonal propagation at a stage too early for proper assessment. If age-to-age correlations for specific yield traits are weak, changes in the behavior of families during the transition from the juvenile to mature state can result in inappropriate selection. For many species the evaluation of their characteristics is, at present, not possible until the trees have reached about half their rotation age. Although many attempts have been made to overcome the inherent phase change associated with aging, and rejuvenation of adult trees has been described (Díaz-Sala et al. 1990, 1994, 1995; Monteuuis 1991; Sánchez et al. 1997; von Aderkas and Bonga 2000), barriers for the clonal propagation of adult selected trees are still present (Ricci et al. 2001, 2003; Selby et al. 2005; Prakash and Gurumurthi 2010; Klimaszewska et al. 2011; Vieitez et al. 2012; Xiao et al. 2014).

Maturation is an age-related developmental process in vascular plants that affects morphology, growth rate and other physiological and developmental traits, such as shoot height and diameter, foliar attributes, stomatal conductance, photosynthesis, respiration rates, rooting ability, and responses to abiotic and biotic stresses (Greenwood and Hutchison 1993; Day et al. 2002; Day and Greenwood 2011). Four phases of maturation have been recognized: (1) the embryonic phase, (2) the post-embryonic juvenile vegetative phase, (3) the adult vegetative phase, and (4) the adult reproductive phase (Poethig 2003). Although the most obvious phase change is the transition to reproductive development, trees exhibit a complex array of developmental phases, showing juvenile-adult transitions in numerous morphological and physiological traits that occur at very different times and continue for years. These juvenile–adult transitions are independently regulated to varying degrees (Poethig, 2003; Day et al. 2002; Brunner and Nilsson, 2004; Day and Greenwood 2011).

In forest tree species, a decline in the capacity to regenerate shoots, roots or embryos from somatic differentiated cells in an ectopic location is associated with tree age and maturation. The threshold age at which this decrease occurs, and the rate of decline may vary among species and even among clones within species (Díaz-Sala et al. 1996; Goldfarb et al. 1998). The rate and extent of reduction in rooting ability are species dependent. For example, in eastern larch the frequency of cuttings declines by 50% during the first 20 years (Greenwood et al. 1989). In contrast, loss of rooting ability occurs abruptly and early in loblolly pine (Díaz-Sala et al. 1996). The maturation-related decline of adventitious root formation is also very abrupt in many angiosperm tree species, such as chestnuts and oaks (Sánchez and Viéitez 1991; Sánchez et al. 1996). In addition, there is intraspecific and intraclonal variation in rooting capacity. This variation frequently dictates which trees will be available in a planting operation, limiting the use of clonal

propagation to capture the genetic superiority of selected individuals, even in the juvenile phase. Because of this, the benefit of clonal propagation cannot be maximized until appropriate procedures for the propagation of physiologically mature trees are developed (Day et al. 2002; Solé and Díaz-Sala 2003).

De novo regeneration can be achieved either directly from explants, or through an intervening callus stage. However, competent cells that can initiate organ primordia or embryos are required for regeneration to occur. The induction of meristematic or embryogenic cells from differentiated cells is the basis for de novo regeneration (Díaz-Sala 2014). Dedifferentiation, i.e., the loss of a specialized form or condition previously acquired during development that can be manifested by the loss of morphological cell identity or by re-entry into the cell cycle of non-dividing cells, is a central concept in plant de novo regeneration. Although apparent dedifferentiation and re-specification of cells seems to occur (Birnbaum and Sánchez Alvarado 2008), whether the competence to regenerate organs is acquired, as in animal cells, through dedifferentiation, via transdifferentiation or through pre-existing totipotent or pluripotent cells in adult tissues remains unknown (Stocum and Zupane 2008; Díaz-Sala 2014). The mechanisms behind the re-specification of a fully differentiated progenitor cell (switching its fate) into a pluripotent or totipotent cell that can develop a root, shoot or embryo in an ectopic location, especially in relation to the cell's developmental age, are also unknown (Abarca and Díaz-Sala 2009a,b). This raises the question of whether adult trees maintain certain cells, which have not been determined to develop an embryo or specific organ, outside the meristematic region in a specific differentiated state that can easily gain pluripotent or totipotent properties. Day and Greenwood (2011) suggest that there are two broad categories of mechanisms that regulate age- (or size-) related changes in tree growth habits and maturational traits: (i) physical constraints that occur through the interaction of the external environment with the physiological and morphological attributes of the tree, and (ii) the behavior of meristems and/or the resulting developing or differentiating cells that exhibit maturational properties. In general, most evidence indicates that the ease of rooting in the juvenile tissues of some trees is more a function of the ease of forming root initials than of physical restrictions for root emergence (Díaz-Sala et al. 1996; Goldfarb et al. 1998).

2. Physiological and cellular approaches to studying the maturation-related decline of propagation capacity

Classic regeneration assays demonstrated that de novo organogenesis and somatic embryogenesis requires elevated levels of both auxins and cytokinins, and that these hormones have antagonistic as well as synergistic roles. Although changes in the content and specific indices of several hormones during tree ageing

and maturation, as well as throughout organ maturation, have been demonstrated (Valdés et al. 2002, 2003, 2004a,b), and easy- and difficult-rooting lines from *Eucalyptus globulus* showed different levels of indole-3-acetic acid (Fett-Neto et al. 2001), differences in adventitious rooting capacity among individual cuttings of Scots pine and between competent hypocotyls and non-competent epicotyls from different families of loblolly pine were not related to the content, uptake, or metabolism of auxin; therefore, auxin does not account for all the developmental or genetic variation in rooting ability (Grönroos and Von Arnold 1988; Díaz-Sala et al. 1996). However, the effect of polar auxin transport inhibitors like 1-N-naphthylphthalamic acid (NPA) indicates polar auxin transport has an important role in this process (Greenwood and Weir 1995; Díaz-Sala et al. 1996). Polar auxin transport is required only during the first 2 d of the root induction process in hypocotyl cuttings from young loblolly pine seedlings, indicating that perhaps by 2–3 d cuttings are fully committed to root formation and are insensitive to NPA inhibition (Díaz-Sala et al. 1996; Hutchison et al. 1999). Although auxin transport appears necessary for adventitious root induction, the failure of epicotyl cuttings from young loblolly pine seedlings to root is not correlated with a decline in the intensity of polar auxin transport, and the inhibitory factors preventing the rooting response in loblolly pine epicotyls do not seem to be related to the lack of capacity to form roots (Díaz-Sala et al. 1996). The decline in rooting ability does not seem to be related to the lack of an initial auxin response either, but does seem to be associated with a loss of cells capable of fully responding to auxin for the induction of adventitious roots (Díaz-Sala et al. 1996). It is not known whether this is due to the loss of a specific cell type, the inability of individual cells to perceive auxin signals specific for root meristem organization, or the suppression of gene expression needed for cells to enter the root formation pathway (Hutchison et al. 1999). Greenwood et al. (2001) determined that the sequence and timing of cellular reorganization, the onset of cell division, and the mitotic frequency were the same in discs from rooting-competent hypocotyls and rooting-non-competent epicotyls; therefore, the overall hypothesis that epicotyl tissues respond slower to auxin than hypocotyl tissues in terms of cellular reorganization and cell division was rejected. Although other extrinsic factors may promote rooting, such as light, sucrose, or foliar-produced plant hormones other than auxin, a lack of these factors does not explain why epicotyls do not root (Díaz-Sala et al. 1996; Greenwood et al. 2001). Therefore, rooting ability is a result of the intrinsic capacity of cells to organize into a root meristem in response to auxin, perhaps due to the suppression of gene expression needed for cells to enter the root formation pathway (Hutchison et al. 1999). According to Greenwood et al. (2001) rooting competence is ultimately a function of differential expression of genes affecting all phases of root meristem formation. Given that no specific auxin signal-transduction pathways have been characterized in terms of rooting in conifers, elucidation of gene expression

programs affecting rooting should be a fruitful approach. Similar results have been described for recalcitrant angiosperm forest trees such as chestnut (Ballester et al. 1999; Vidal et al. 2003).

The regulated response of individual cells to a threshold concentration of hormone gradients is one way to translate general signals into cellular specific signals. Stable auxin response gradients have been shown to exist in many developmental processes (Bohn-Courseau 2010). It is becoming clear that auxin and other hormones are necessary, but not sufficient, to explain many specification events alone or to explain the switch of response competencies reflecting the developmental state of particular cells or the time window of the response. De novo regeneration and new cell specification are processes involving rearrangements of tissue polarity, with the temporal and spatial distribution of auxin being very important and contributing to tissue polarization and patterning (Xu et al. 2006). Although no differences in auxin uptake, accumulation or metabolism were found between rooting-competent and non-competent hypocotyls and epicotyls at the base of the pine cuttings (Díaz-Sala et al. 1996), an asymmetric auxin distribution was detected in rooting-competent tissues after excision, which was, at least, maintained during the initial 24 h of root induction (Abarca et al. 2014). An asymmetrical distribution was not observed in non-competent hypocotyls or epicotyls. Treatments with NPA, which inhibits rooting (Díaz-Sala et al. 1996) and does not change the number of cell layers in the vascular cylinder, cortex or pith, changed the auxin distribution pattern (Abarca et al. 2014), indicating that polar auxin transport resulted in auxin accumulation at the base of the cutting (Díaz-Sala et al. 1996), as well as auxin localization and distribution at the tissue or cellular levels. This result indicated that rooting-competent tissues could retain an intrinsic capacity to maintain or accumulate auxin after excision, which could be crucial for rooting. Auxin distribution largely depends on the dynamic expression and subcellular localization of the PIN-FORMED (PIN) auxin-carrier proteins (Friml 2010). However, PIN activity can be modulated by endogenous or exogenous signals, such as other hormones, stress or tissue-specific factors, to trigger developmental decisions that could initiate regeneration by triggering cell fates or other local changes (Grunewald and Friml 2010). The stress response associated with wounding has been related with de novo regeneration (Grafi et al. 2011; Da Costa et al. 2013). Additionally the somatic embryogenesis responsiveness may be antagonistic to biotic defense activation (Rutledge et al. 2013). No differences in the wounding stress response were observed between rooting competent and non-competent pine cuttings (Greenwood et al. 1997); therefore, other tissue-dependent signals could trigger re-patterning either by inducing cell-fate re-specification or by re-establishing the auxin distribution. Gibberellins perturb auxin transport affecting adventitious root formation in aspen (Mauriat et al. 2014) and nitric oxide influences rooting affecting auxin signaling in *Eucalyptus grandis* (Abu-Abied et al.

2012). Diphenylurea derivatives enhanced adventitious root formation of Monterey pine hypocotyls and in distantly related herbaceous and woody species in the presence of endogenous or exogenous auxin (Ricci et al. 2008; Brunoni et al. 2014). Diphenylurea derivatives modify the localization of the auxin response and auxin responsiveness as a function of exogenous auxin in DR5::GUS transgenic plants of *Arabidopsis*, indicating local changes in auxin gradients may enhance rooting. As DR5 is generally thought to be sensitive to auxin in a dosage-dependent manner, the results seem to indicate that diphenylurea derivatives affect auxin influx or transport along seedlings, or cell sensitivity to auxin, or both, enhancing adventitious rooting. Local changes of auxin maxima and enhancement of rooting were also detected in pine hypocotyls in the presence of diphenylurea derivatives.

Recently, remarkable progress has been made in understanding the mechanisms that control growth through the application of cutting-edge molecular biology and genome analysis tools, which provide a comprehensive picture of the genes and cellular processes involved in many aspects of plant growth and development, including de novo regeneration. The knowledge obtained in these studies points the way forward for strategies to enhance the quantity and quality of trees for desired end-uses.

3. Molecular and genomic approaches to studying the maturation-related decline of propagation capacity

Age and maturation-related trends are complex interactions between extrinsic factors, i.e., plastic reversible responses to external cues, and intrinsic factors, i.e., less plastic pre-programmed irreversible responses to ontogenetic cues (Day and Greenwood 2011). Cues internal to meristems and/or developing or differentiating cells would involve differential gene expression and epigenetic changes regulating genes needed for the expression of specific maturational traits. Molecular, genomic and epigenomic approaches are providing new insights into the developmental regulation of gene expression patterns associated with age- and maturation- related changes (Busov et al. 2004). Reprogramming of somatic cells towards embryogenesis or organogenesis has been recently analyzed in model plant species. The capacity to recruit meristem or embryonic programs in response to a specific stimulus, and the relevance of auxin and cytokinin signaling pathways in the regulation of key genes involved in the organization of stem cell niches, have a role in de novo regeneration (Yang et al. 2012; Feeney et al. 2013; Druege et al. 2014; Liu et al. 2014a). In addition, pre-existing stem-like xylem pericycle and pericycle-like cells, distributed throughout the entire body along the vasculature, directly originate different morphogenic programs for callus, roots or shoots, depending on the stimulus in the culture medium. De Almeida et al. (2012) suggested that pre-procambial cells can also act as niches for pluripotent and

totipotent stem-like cells that are responsive to the auxin/cytokinin ratio resulting in de novo organogenic or embryogenic programs in the shoot apex of peach palm.

The recruitment of meristematic programs or embryogenic-specific genes associated with the capacity for organogenesis and somatic embryogenesis has also been described in forest tree species (Legué et al. 2014). Putative embryogenesis-specific genes, such as *WOX2* (*WUSCHEL homeobox 2*) and a HEME-ACTIVATED protein 3, which is encoded by the *LEAFY COTYLEDON* (*LEC*) gene *HAP3A*, were analyzed in cultures of both shoot bud explants and zygotic embryos of *Pinus contorta* (Park et al. 2010). On the basis of these analyses, the authors postulated that *PcHAP3A* is expressed mainly in callus, and may be involved in cell division, but is unable to differentiate between embryogenic and non-embryogenic callus, whereas *WOX2* is expressed mainly in embryonal mass (EM)-like tissues and could be used as an early genetic marker to discriminate embryogenic cultures from non-embryogenic dividing callus. Similarly, *CHAP3A* and *WOX2* from *Picea glauca* were expressed exclusively in the early stages of somatic embryogenesis, and could potentially be used as markers of embryogenic capacity since they allow embryonal mass to be distinguished from callus and other types of tissue present in cultured shoot buds (Klimaszewska et al. 2010, 2011). These results corroborated those obtained for *PcWOX2* in lodgepole pine (Park et al. 2010). However, *CHAP3A* (a black spruce *LEC1* homolog) transcripts were high in white spruce EM but not detected in callus, and were also expressed in non-embryogenic calli of *P. contorta* (Park et al. 2010). Embryogenic and patterning genes, such as a Norway spruce *CUP-SHAPED COTYLEDON* orthologue to the *Arabidopsis* gene, and *WUSCHEL (WUS)-RELATED HOMEOBOX* genes, were also associated with somatic embryogenesis capacity in *Picea abies* (Larsson et al. 2012; Hedman et al. 2013).

Genes related to adventitious rooting have been identified by analyzing the expression of selected genes in rooting-competent and non-competent tissues, and by identifying QTLs controlling vegetative propagation using testcross strategies (Sánchez et al. 1995; Díaz-Sala et al. 1997; Greenwood et al. 1997; Hutchison et al. 1999; Buttler and Gallagher 2000; Ermell et al. 2000; Lindroth et al. 2001; Goldfarb et al. 2003; Gil et al. 2003). Embryonic or root patterning genes have been recently associated with adventitious root formation. Brinker et al. (2004) performed large-scale expression screening using a cDNA microarray for *Pinus taeda*, and identified a cell-fate meristem regulatory gene, *ZWILLE-LIKE*, related to the early stages of adventitious root formation. *WOX*-related genes have been associated with adventitious rooting in poplar (Liu et al. 2014b), and the *AINTEGUMENTA LIKE1* protein (Rigal et al. 2012), a homeotic transcription factor from the AP2/ERF family, is involved in the maintenance of cell meristematic competence during shoot organogenesis in *Arabidopsis* (Nole-Wilson et al. 2005). A gene encoding a transcription factor of the AP2/ERF family of

unknown function has a positive effect on adventitious and lateral root induction; its function has been linked to the auxin signaling pathway (Trupiano et al. 2013). Abarca et al. (2014) measured relatively high *GRAS* mRNA levels in non-differentiated proliferating embryogenic cultures and during somatic embryo development of *Pinus radiata*. The mRNA levels of putative GRAS family transcription factors, *SCARECROW (SCR)*, *PrSCR*, and *SCARECROW-LIKE (SCL) 6*, *PrSCL6*, were significantly reduced or non-existent in adult tissues that no longer had the capacity to form adventitious roots, but were maintained or induced after the reprogramming of adult cells in rooting-competent tissues. A subset of genes, *SHORT-ROOT (PrSHR)*, *PrSCL1*, *PrSCL2*, *PrSCL10* and *PrSCL12*, was also expressed in an auxin-, age- or developmental-dependent manner during adventitious root formation before the onset of cell division leading to the formation of a root meristem. The authors concluded that individual genes within each group have acquired different and specialized functions, some of which could be related to the competence and reprogramming of adult cells to form adventitious roots. Similarly, Vielba et al. (2011) described *GRAS* gene expression associated with the maturation-related decline of adventitious root formation in chestnut. In addition, asymmetrical increases of *PrSCL1* (Sánchez et al. 2007; Vielba et al. 2011) and *PrSHR* (Solé et al. 2008) transcript levels were described in the cambial region and rooting-competent cells were not detected in non-competent cuttings (Vielba et al. 2011; Abarca et al. 2014). The authors concluded that the asymmetrical increase in mRNA during the earliest stages of adventitious root formation in similar cell types at different developmental stages suggests the presence of specific cellular signaling pathways or specific factors in pine and chestnut. They also suggest that these pathways are perhaps distributed in cell type- and developmental-stage-specific contexts in the tissues involved in rooting, which could be crucial for rooting capacity, indicating a degree of evolutionary conservation of this response in distantly-related forest tree species.

The asymmetric auxin distribution detected in rooting-competent tissues after excision, and maintained during the initial 24 h of root induction (Abarca et al. 2014), matched the locations where *PrSHR* and *PrSCL1* are expressed (Solé et al. 2008). Transcription factors are the main players in regulatory modules controlling auxin gradients, positional information and the development of polarity fields, producing a cross regulatory network involved in organ formation (Feng et al. 2012). The differential expression of genes, such as *PrSCR* and *PrSCL6*, in rooting-competent and non-competent cuttings, as well as the differential responses of genes, such as *PrSCL1* or *PrSHR* (Solé et al. 2008; Abarca et al. 2014), to exogenous auxin during adventitious rooting may indicate the local involvement of specific GRAS transcription factors in rooting via auxin distribution, control of the division of certain cell types, or other mechanisms. The auxin-related increase of *PrSCL1* mRNA in competent tissues after 24 h of root induction (Solé et al. 2008)

could be associated with auxin localization in these tissues at the same time (Abarca et al. 2014). The overlap in the temporal and spatial distribution of auxin (Abarca et al. 2014) and the increase of the auxin-independent *PrSHR* mRNA (Solé et al. 2008) could indicate possible crosstalk between the signaling pathways, perhaps establishing response domains that activate a cascade of other *GRAS* genes or root-determining factors before the resumption of cell division.

PIN protein polarization is crucial for the generation of auxin gradients and localization. Fine-tuned crosstalk between microtubules (MTs) and the cell wall has been related with PIN protein polarization in adventitious rooting (Abu-Abied et al. 2015). Interactions among the cell wall, plasma membrane and cytoskeleton have been associated with the maturation-related decline of adventitious root formation in forest tree and model species. Díaz-Sala et al. (1997) and Hutchison et al. (1999) associated the expression of actin and expansin genes with adventitious root formation in pine, and Díaz-Sala et al. (2002) showed that rooting of hypocotyls from de-rooted adult *Arabidopsis* plants depended on the combined effect of auxin and peptides containing the RGD motif. The RGD peptide, which disrupts both the attachment between the extracellular matrix and the plasma membrane in animal cells (Ruoslahti 1996),and the adhesion between the cell wall and plasma membrane in plant cells (Canut et al. 1998), was biologically active in increasing the rooting capacity of hypocotyls from de-rooted adult plants. The effect of the RGD peptide was a necessary, but not sufficient, condition for rooting of hypocotyls from de-rooted adult plants. The authors proposed that these results support the hypothesis that cell wall–plasma membrane interactions of specific cells are involved in the loss of rooting capacity by hypocotyls from de-rooted adult plants of *Arabidopsis*. In addition, Abu-Abied et al. (2015) described perturbations in the adventitious rooting of *Arabidopsis* mutants impaired in MT-associated proteins and in mutants with altered cell walls. Recently, a comprehensive microarray analysis was performed to compare gene expression profiles in rooting-competent juvenile and rooting non-competent mature cuttings of *Eucalyptus grandis*, in the presence and absence of auxin. Among the functional groups of transcripts that differed between juvenile and mature cuttings were those coding for MT-associated proteins. The results suggested coordinated developmental and auxin-dependent regulation of several MT-related transcripts, annotated as coding for tubulin, MT-associated proteins and a kinesin motor, in these cuttings. To determine the relevance of MT remodeling to adventitious root formation, MTs were subjected to subtle perturbations by trifluralin, a MT disrupting drug, applied during auxin induction. Juvenile cuttings were not affected by the treatment, but mature cuttings showed increased rooting capacity in *E. grandis*. Thus, the authors provided evidence to suggest that MTs play a role in the shift from cell division to cell differentiation during adventitious root induction.

4. Epigenetics approaches to studying the maturation-related decline of propagation capacity

The restriction of reprogramming potential associated with the maturation-related decline in competence for de novo regeneration could be related to the presence of signals in tissues that retain a physiological or developmental memory. Chromatin status and epigenetic mechanisms resulting in a specific nuclear architecture could be involved in the control of cellular plasticity towards de novo regeneration (Díaz-Sala et al. 2014). Epigenetic regulation of the vegetative phase-change, dedifferentiation, and adventitious root formation has been described (Wang et al. 2011; Vining et al. 2013; You et al. 2014). In addition, epigenetically repressed embryonic programs could presumably be involved in callus repression and regeneration in postembryonic tissues (Chen et al. 2012; Chupeau et al. 2013; Ikeuchi et al. 2013). Epigenomic approaches are providing new insights into the developmental regulation of gene expression patterns associated with age- and maturation-related changes. DNA methylation and histone modifications are important epigenetic mechanisms for gene regulation in eukaryotes.

Aging and maturation are characterized by altered patterns of cell differentiation and organ formation processes, and the potential role of DNA methylation in maturation has been studied in some tree species (Fraga et al. 2002a; Valledor et al. 2007; Monteuuis et al. 2009; Santamaria et al. 2009). A clear relationship between DNA methylation levels and maturation has been established for some tissue types and species in woody plants; e.g. DNA methylation levels were higher in juvenile tissues than in adult ones in radiata pine (Fraga et al. 2002a; Valledor et al. 2007), and lower in adult tissues than in juvenile ones in *Acacia mangium* plants grown in vitro (Baurens et al. 2004; Monteuuis et al. 2009). Conversely, an increase in the degree of tree reinvigoration by serial grafting, measured by the recovery of morphogenic competence, was accompanied by a decrease in the global level of DNA methylation in meristematic tissue, thus pointing toward plasticity of DNA methylation marks during aging and maturation (Fraga et al. 2002b). Age-related changes in foliar traits were observed in *Larix laricina* whereas differences in DNA methylation levels between juvenile and mature scions could not be detected in DNA from whole needles (Greenwood et al. 1989).

Histone modifications are also important epigenetic mechanisms for gene regulation in eukaryotes. DNA methylation and histone modifications regulate de novo shoot regeneration by modulating *WUS* expression and auxin signaling in *Arabidopsis* (Li et al. 2011) and *KNOX* genes during in vitro regeneration of *Agave* spp. (De-la-Peña et al. 2012). Embryogenic potential and the expression of embryogenesis-related genes, such as *LEC*-type genes, in conifers are affected by treatment with a histone deacetylase inhibitor, suggesting a possible link between

chromatin structure and embryogenesis-related gene expression in conifers (Uddenberg et al. 2011). In addition, decreased levels of euchromatin-associated marks, such as histone 4 acetylation and specific histone methylation (trimethylation of histone 3 on lysine 4 or H3K4me3), have been observed in mature needles compared with juvenile ones (Valledor et al. 2010).

MicroRNAs (miRNAs) and snRNA play an essential role in regulating plant development by mediating target genes at the transcriptional and post-transcriptional levels, but the diversity of miRNAs and their potential roles in cell dedifferentiation and de novo regeneration capacity are poorly understood (Quiao et al. 2012; Liu et al. 2014c; Ohtani et al. 2015). Old *Arabidopsis* plants exhibit lower shoot regenerative capacity than young plants, which is largely due to a reduced cytokinin response (Zhang et al. 2015). An increased level of miR156-targeted SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors in old plants dampens shoot regeneration by interfering with the function of B-type ARRs, thus establishing a molecular link between developmental timing and cytokinin-mediated shoot regeneration responses (Zhang et al. 2015). In forest tree species, Levy et al. (2014) characterized the miRNA population of *E. grandis* and analyzed the relationship between the variation in the expression of miR156 and miR172 during development and the loss of rooting ability. While the expression levels of miR156 and miR172 were reversed in juvenile and mature tissues, no relationship was found between the high miR156 expression and the rooting ability in juvenile tissues, or the high miR172 expression and the loss of rooting ability in mature tissues. This was observed in both *E. grandis* and *Eucalyptus brachyphylla*, in which explants that underwent rejuvenation in tissue culture conditions were also examined. This suggests that in these *Eucalyptus* species, there is no correlation between the switching of miR156 and miR172 expression in stems and the loss of rooting ability. On the other hand, miR156 has been associated with aging and in vitro rejuvenation of apple trees (Xiao et al. 2014).

5. Conclusions

Recalcitrance to organogenesis or somatic embryogenesis, which intensifies in trees at the mature stage, is a major limitation for the clonal propagation of elite tree germplasm in many woody species, especially forest tree species. However, the mechanisms that enable a somatic differentiated cell to switch its fate into a pluripotent or totipotent cell that can develop a root, shoot or embryo, or repair damaged tissues are not well understood, especially in relation to the developmental age of the cell. The dynamic switching of cell fate during de novo regeneration results from regulatory interactions at various levels. The effect of spatio-temporal modifications of hormones, the crosstalk between hormones and

key transcriptional regulators involved in embryogenic and organ patterning and the contribution of cellular and tissue factors would result in reprogramming of gene expression patterns towards cell fate switching. Mechanical signals that could be related with modifications in the cell wall and cytoskeleton, and the role of epigenetic regulation at different levels activating or rechanneling a physiological or developmental cell memory could also be important mechanisms regulating de novo regeneration.

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Molecular biology of somatic embryogenesis in hardwoods

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Abstract

Somatic embryogenesis is one developmental pathway that has benefited from the integration of transcriptomics and proteomics data and has led to a better understanding of the molecular mechanisms that control embryogenic competence acquisition and somatic embryo development into viable plants. Nevertheless, most of the results obtained to date are based on traditional model systems that are often not easily applicable to somatic embryogenesis of economically relevant woody species. The aim of this report is to summarize the most recent understandings of particular aspects of the genetic and epigenetic regulation of the somatic embryogenesis process (in model and non-model systems) and how this applies to hardwood plants.

Keywords: Angiosperms, embryogenic competence, embryo maturation, epigenetic regulation, non-model systems, stress-related genes, totipotency.

1. Introduction

Somatic embryogenesis (SE) is a type of non-zygotic embryogenesis by which somatic cells, under suitable induction conditions, undergo a complete genome shift and embark into a new developmental pathway ending in the formation of asexual embryos that are morphologically identical to their zygotic counterparts (Radoeva and Weijers 2014; Smertenko and Bozhkov 2014). During this unique developmental process, cells have to dedifferentiate, activate cell division, and reprogram their physiology, metabolism and gene expression patterns

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(Yang and Zhang 2010; Elhiti et al. 2013; Fehér 2015). Thus, SE can be considered the most obvious demonstration of totipotency in plant cells, showing that somatic cells contain the essential genetic blueprint to complete plant development, and that embryogenesis is not an exclusive feature of the zygote and can proceed in the absence of fertilization (Fehér 2015). Since the first observations of somatic embryo formation in carrot cell suspension cultures (Reinert 1958; Steward 1958), the potential for SE has been shown to be characteristic of a wide range of tissue culture systems for both gymnosperms and angiosperms plants (Giri et al. 2004; Quiroz-Figueroa et al. 2006; Yang and Zhang 2010). Some of the angiosperms for which SE protocols have been established and applied are forest trees (Giri et al. 2004; Lelu-Walter et al. 2013), such as ash (*Fraxinus* spp.), cork oak (*Quercus suber*), English oak (*Quercus robur*), eucalyptus (*Eucalyptus* spp.), European beech (*Fagus sylvatica*), horse-chestnut (*Aesculus hippocastanum*), sweet-chestnut (*Castanea sativa*), poplar (*Populus* spp.) and walnut tree (*Juglans nigra*). Other economically relevant woody angiosperms have been propagated by SE as well (Giri et al. 2004), such as cacao tree (*Theobroma cacao*), pineapple guava (*Feijoa sellowiana*), grape wine (*Vitis vinifera*), rubber tree (*Hevea brasiliensis*), tamarillo (*Cyphomandra betacea*) or Valencia sweet orange (*Citrus sinensis*).

SE is not only an efficient system for in vitro clonal propagation, but also provides an outstanding model system that could lead to a better understanding of totipotency in higher plants as well as embryo development. This will overcome the difficulties that have been encountered when analyzing the early stages of zygotic embryogenesis during development of the embryo inside the ovular tissues (Smertenko and Bozhkov 2014). Analysis of proteomes and transcriptomes has led to the molecular identification and functional characterization of many genes involved in the initiation and development of somatic embryos. Nevertheless, most knowledge of the general principles underlining the SE regulatory pathways has been focused on traditional model organisms, such as *Arabidopsis thaliana* or *Medicago truncatula*, and has been intensively reviewed by several authors (Karami et al. 2010; Elhiti et al. 2013; Radoeva and Weijers 2014; Fehér 2015). There is no doubt that model organisms have many advantages and without them our understanding of the mechanisms underlying many developmental and physiological processes would have been much more limited. However, the discoveries based on model organisms such as *Arabidopsis* must be further tested in other species or systems to verify their effectiveness. Moreover, new model systems bringing new approaches that will broaden our scientific knowledge are welcome and, with the increase in the number of genome-sequencing projects carried out lately, the definition of model organisms has broadened (Hedges 2002). For example, since the *Arabidopsis* genome was sequenced, the genome of several important forestry woody species, including conifers such as *Picea abies* (Nystedt et al. 2013) or *Pinus taeda* (Zimin et al. 2014), and angiosperms such as *Populus*

trichocarpa (Tuskan et al. 2006), *Eucalyptus grandis* (Myburg et al. 2014) or *Quercus robur* (Plomion et al. 2015) were also deciphered and others are in the way to be published. Moreover, the genomic data for fruit trees such as *Citrus sinensis* (Xu et al. 2013) and *Malus domestica* (Velasco et al. 2010) also became available. Considering that there are approximately 350,000 botanically-described species of plants (www.theplantlist.org) and that model plants represent only a handful of species and families, even the arrival of these new model plants cannot reflect the biodiversity of the plant kingdom and all economic or agricultural interests (Castell and Ernest 2012). Some features and processes in a particular species are unique and cannot be approached via a model plant of a different species. Woody plants for example, are perennials with a long life cycle and special features to be analyzed, including those in their SE system. Thus, several approaches have been applied to study SE of several hardwood plant species such as cork oak (*Quercus suber*) (Gomez-Garay et al. 2013), Valencia sweet orange (*Citrus sinensis*) (Pan et al. 2009), grape wine (*Vitis vinifera*) (Marsoni et al. 2008), cacao tree (*Theobroma cacao*) (Noah et al. 2013), pineapple guava (*Feijoa sellowiana*) (Fraga et al. 2013) and tamarillo (*Cyphomandra betacea*) (Correia et al. 2012a). These reports included studies on gene/protein expression changes during SE and comparative studies of embryogenic and non-embryogenic cells as well as of zygotic and SE.

The main goal of this chapter is not to give a detailed review of all the molecular analysis studies carried out on SE of hardwood plant species, but to summarize some of the main achievements in that field and on their contribution to a better understanding of different aspects of the genetic and epigenetic regulation of the SE process in these plants (Figure1).

2. Genetic regulation of somatic embryogenesis in hardwood plants

Somatic embryogenesis induction proceeds by either direct somatic embryogenesis (DSE) or indirect (ISE) pathways (Williams and Maheswaran 1986). While in ISE an intermediate callus phase precedes embryo formation, in DSE somatic embryos form directly from the explant without a callus phase (Smertenko and Bozhkov 2014). Willemsen and Scheres (2004) proposed that while in DSE proembryogenic competent cells are already present, their embryogenic expression depends on desirable conditions and only minimal gene reprogramming is required. However, in ISE major gene reprogramming is essential for embryogenic induction and differentiation. Generally, in both types of SE, gene expression patterns change as some active genes in somatic cells are repressed and inactive embryogenic genes are expressed (Smertenko and Bozhkov 2014).

Somatic embryo models have been useful for studying cell differentiation

processes in plants and for increasing our understanding about the functional aspects of genes already implicated in SE (Quiroz-Figueroa et al. 2006; Fehér 2015). The development of somatic embryos closely resembles, both

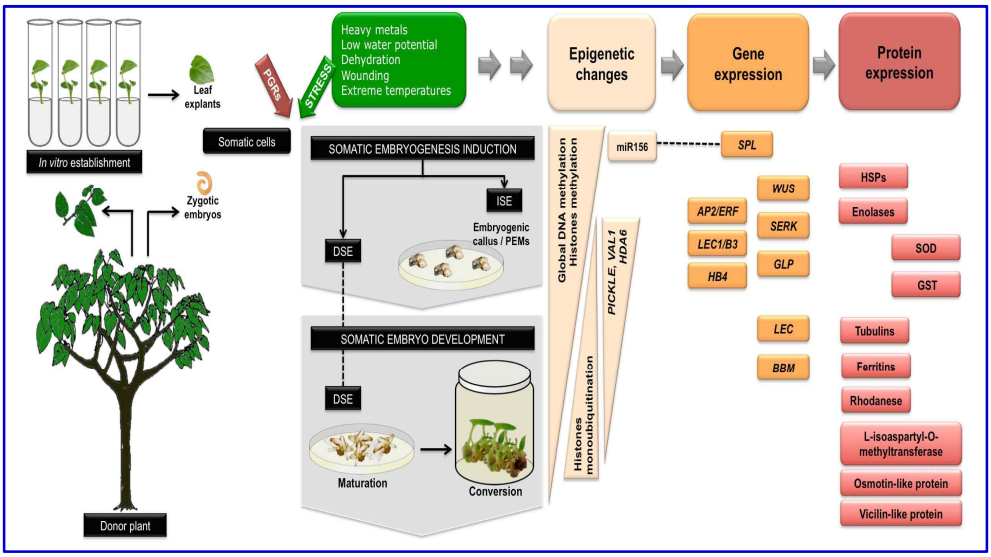

Figure 1. *Schematic diagram of somatic embryogenesis pathways in hardwoods with reference to identified epigenetic markers and genes/proteins that are potentially associated with key roles in somatic embryogenesis induction and embryo development. DSE – direct somatic embryogenesis; ISE – indirect somatic embryogenesis; PEMs – pro-embryogenic masses.*

morphologically and physiologically, the development of zygotic embryos, probably due to the conservation of the underpinning cellular and molecular mechanisms in both processes (Karami and Saidi 2010). Therefore, SE has been extensively used as an experimental system to investigate the morphological, biochemical and physiological events of embryogenesis (Radoeva and Weijers 2014). Nevertheless, SE induction in many woody species is often a recalcitrant process, strongly dependent upon culture conditions and on the genotype of the donor plants (Pinto et al. 2008). The difficulties in successfully establishing artificial conditions allowing continuous somatic embryo development from induction to maturity in hardwood species are to a large extent associated with the fragmented knowledge concerning the genetic programs that regulate embryogenesis. This in turn partly reflects the absence of genomic or transcriptomic data for these species. The species that have been studied are primarily from three families of angiosperms (Salicaceae, Myrtaceae and Fagaceae) — and four genera within these families (*Populus*, *Eucalyptus*, *Quercus* and

Castanea). There is no clear model species for all hardwood forest trees and this situation has surely hindered progress, but with the advent of next-generation sequencing (NGs) technologies, the large number of target species and the diversity among those species will become an asset for comparative genomic approaches (Neale and Kremer 2011). Also, proteomic approaches have shown great potential to study non-model species, because protein sequences have the advantage of being more conserved, making the high-throughput identification of non-model gene products quite effective by comparison to orthologous proteins (Liska and Shevchenko 2003).

In the next sections we summarize some of the main results obtained regarding the analysis of gene/protein expression changes during the stages of embryonic induction and embryo development in the SE process of hardwood species.

2.1 Embryonic induction

The switching of somatic into embryogenic cells involves a series of events associated with the molecular recognition of internal signals and external stimuli (Karami et al. 2010). The perception and response to these events triggers various signal cascades, and the downstream pathways followed during the transition of single cells to somatic embryos eventually result in specific gene expression and SE (Yang and Zhang 2010; Fehér 2015). Initiation of the embryogenic pathway seems to be restricted to certain responsive cells that have the potential to activate genes involved in generating embryogenic cells (Karami et al. 2010). Once these yet unknown genes are activated, an embryogenic gene expression program replaces the established gene expression pattern in the explant tissue (Quiroz-Figueroa et al. 2006).

Because SE induction in cultured tissues is a multi-factorial event, determining specific physical and chemical factors that switch on the embryogenic pathway of development is an important but also difficult step (Karami et al. 2009). It has been proposed that plant growth regulators (PGRs) and several stresses play a central role in mediating the signal transduction cascade leading to the reprogramming of gene expression, followed by a series of cell divisions that induce either unorganized callus growth or more organized growth leading to SE (Fehér 2015). Also, an increasing number of studies have indicated that the stress-response of cultured tissues, stimulated by heavy metal ions, high osmotic pressure, dehydration, explant wounding or high temperature, plays a major role in somatic embryo induction (Karami and Saidi 2010; Zavattieri et al. 2010).

The molecular pathways of the cells that undergo these drastic changes leading to embryogenic competence have been extensively studied in the model species *Arabidopsis thaliana*, in which 25 candidate genes were identified for their

effect in the acquisition of totipotency (Elhiti et al. 2013). Those candidate key genes, encoding DNA methyltransferases and enzymes of glutathione metabolism, as well as proteins that play integral roles in hormone perception and signaling, involved in the differential gene expression that affects the proteome and metabolome during in vitro embryogenesis, were identified by microarray technology (Elhiti et al. 2010).

Understanding the interactions among key factors initiating SE is a challenge in modern molecular biology. There are now many new molecular techniques that enable dissection of the molecular network at different developmental stages. Molecular techniques, such as differential display analysis, subtractive hybridization, and construction of cDNA libraries, have been crucial to identify genes that exhibit differential activity. These studies have resulted in the identification of several genes that are specifically activated or that exhibit differential expression during SE (Fehér et al. 2015), such as *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE* (*SERK*, Schmidt et al. 1997), *BABY BOOM* (*BBM*, Boutilier et al. 2002), *WUSCHEL* (*WUS*, Zuo et al. 2002) and *LEAFY COTYLEDON* (*LEC*, Curaba et al. 2004; Gazzarrini et al. 2004; Gaj 2004).

Nevertheless, the challenge is bigger when it is about the molecular analysis of SE competence acquisition in woody plants, for which most of the studies rely on the expression analysis and validation of the homologous of the genes previously identified to be expressed in *A. thaliana* or other model species. Therefore, not many comprehensive transcriptomic approaches were addressed until this moment, and several of these are based on proteomic analysis of the samples, which produces better results due to the conserved character of the proteins.

One important line of investigation, that has been used to analyze embryogenic competence acquisition in woody plants, is the comparison of responsive and non-responsive explants during the SE induction process (Zhang et al. 2009; a et al. 2012; Guzmán-Garcia et al. 2013). SE systems in which embryogenic (EC) and non-embryogenic (NEC) cell lines can be induced from the same cultured explant, like the ones of wine grape (*Vitis vinifera*) (Marsoni et al. 2008; Zhang et al. 2009) and tamarillo (*Cyphomandra betacea*) (Correia et al. 2012a), have been explored to obtain more information on important regulatory genes/proteins. Proteins, exclusively or predominantly expressed in EC, included iron-deficiency-responsive proteins, acidic ascorbate peroxidases and isoflavone reductase-like proteins (Zhang et al. 2009) and metabolism-related proteins, such as enolases and threonine synthases, and also heat-shock proteins (HSP) and ribosomal proteins (Correia et al. 2012a). Ascorbate peroxidases, catalases, calcineurin B-like proteins, 1,3-b-glucanases, cyclin-dependent kinases A1 (Zhang et al. 2009) and pathogenesis-related (PR) proteins were found mainly in NEC (Zhang et al. 2009; Correia et al. 2012a). The examination of differentially

expressed proteins between ECs and NECs suggests that the embryogenic status of EC cells could be related to a better ability to regulate the effects of stress conditions, namely through the controlling of oxidative stress by regulation of the reactive oxygen species (ROS) scavenging system (Marsoni et al. 2008), and by the action of HSP (Marsoni et al. 2008; Correia et al. 2012a). One hypothesis states that the expression of totipotency in cultured somatic cells is part of a general stress adaptative process that implies a fine regulation of auxin and stress signaling resulting in the restart of cell division and embryogenic competence acquisition. The observation that embryogenic tissues of different origins and obtained with the use of different auxins display similar protein profiles suggests a general behavior of cellular metabolism that can give important insights about the mechanisms triggering and controlling somatic embryo formation (Correia et al. 2012a).

Also for cork oak (Gomez-Garay et al. 2013), the role of ROS in the proliferative stages during SE and the up-regulation of proteins involved in cell division were reported. The comparison between somatic embryo cells type (SE-type) and pro-embryogenic masses type (PEM-type) of avocado (*Persea americana*) have confirmed the observations previously made in other systems (Guzmán-García et al. 2013). In this study the identification of high levels of HSP, glutathione S-transferases (GST), and superoxide dismutases (SOD) proteins in SE-type cells suggested that the generation of a significant amount of stress and ROS are prerequisites to induce SE, and that SE lines seem to be more efficient in coping with the necessary ROS and stress and, hence, have a higher regeneration capacity.

Of all the genes that have been isolated during SE, *SERK* has successfully been shown to be a specific marker distinguishing individual embryo-forming cells in carrot suspension cultures (Schmidt et al. 1997). Schmidt et al. (1997) suggested this gene as a suitable marker for SE as they found *DsSERK* expression only in SE, during proembryogenic mass formation and up to the globular stage, and in zygotic embryos but not in any other plant tissue. The role of *SERK* in the induction of SE has been demonstrated also in woody plants such as *Theobroma cacao* (De Oliveira Santos et al. 2005), *Citrus unshiu* (Shimada et al. 2005) or *Vitis vinifera* (Schellenbaum et al. 2008). Despite ample evidence that reveals the relation between early and ectopic expression of *SERK* and induction of SE (Hecht et al. 2001; Hu et al. 2005), other studies show a wider developmental role for *SERK*, for instance in the pathogen defense signaling pathway (Hu et al. 2005). Moreover, *SERK* genes have been detected in non-embryogenic calli (Zhang et al. 2011). Hence, while the *SERK* gene still must be considered as we study its specific role in SE, the search for gene markers for SE induction needs to look beyond this gene (Mahdavi-Darvari et al. 2015).

Another group of genes suggested as potential markers for the acquisition of SE competence are the germin and germin-like (*GLP*) encoding genes, whose

high expression at the earliest stages of SE has been demonstrated in *A. thaliana* (Membré et al. 1997) and also in *Vitis vinifera* embryogenic callus (Marsoni et al. 2008).

Using subtractive hybridization, during *Brassica napus* microspore embryogenesis, a differentially expressed cDNA was identified and named *BABY BOOM* (*BBM*, Boutilier et al. 2002). The *BBM* gene also encodes a transcription factor belonging to the *APETALA 2/ethylene-responsive element binding factor* (*AP2/ERF*) family. Although the negative effect of ethylene on somatic embryogenesis has been known for a long time (Benson 2000), a quantitative expression analysis of ethylene biosynthesis and ethylene responsive (*AP2/ERF*) genes identified 35 such genes with a unique expression pattern that may function as key elements during SE induction in *Hevea brasiliensis* (Piyatrakul et al. 2012). However, additional functional studies are needed to uncover the biological role of each of these genes to elucidate the underlying mechanism by which ethylene inhibits somatic embryogenesis (Elhiti et al. 2013). In *Coffea canephora WUSCHEL* (*WUS*) has been shown to cause dedifferentiation when expressed in somatic cells followed by the production of new stem cells that can lead to SE or organogenesis (Arroyo-Herrera 2008).

Moreover, transcriptomic studies were applied to investigate global gene expression in SE (Thibaud-Nissen et al. 2003; Stasolla et al. 2004). ESTs uniques to embryogenic cell clusters in *Coffea arabica* (Rojas-Herrera et al. 2002) have been detected. By means of subtractive hybridization and macroarrays, Zeng et al. (2006) isolated ESTs involved in SE, and then produced a draft molecular interaction network representing complex gene expression during SE (Zeng et al. 2007).

Three suppression subtractive hybridization (SSH) libraries were constructed using calluses of *Citrus sinensis* cv. 'Valencia' to explore the molecular mechanisms that underlie SE in citrus. A total of 880 unisequences were identified by microarray screening based on these three SSH libraries. Gene ontology analysis of the differentially expressed genes indicated that nucleolus associated regulation and biogenesis processes, hormone signal transduction, and stress factors might be involved in SE (Ge et al. 2012). Transcription factors might also play an important role. *LEC1/B3* domain regulatory network genes (*LEC1, LEAFY COTYLEDON1-LIKE, FUSCA3, ABSCISIC ACID INSENSITIVE 3*, and *ABSCISIC ACID INSENSITIVE 5*) were isolated in *Citrus* SE. Some new transcription factors associated with citrus SE, like a B3 domain containing gene and *HB4*, were identified. These genes could be necessary for SE initiation and might play a role in embryogenic competence maintenance in different cultivars (Ge et al. 2012).

Logan tree (*Dimocarpus longan*) is one recent example for which an embryogenic callus (EC) cDNA library was sequenced using an Illumina HiSeq

2000 system (Lai and Lin 2013), providing data on the types and quantities of genes expressed, as well as their functions, classification, and metabolic pathways. Twenty-three unigenes related to embryogenesis and reproductive and vegetative growth were differentially expressed in various embryogenic cultures, indicating their possible roles in SE.

2.2 Embryo maturation and conversion

Somatic embryo maturation and further germination (conversion) are two crucial steps to achieve plant formation through somatic embryogenesis. As expected, the genes controlling these two phases are different from those operating in the initial phases of totipotency acquisition and morphogenic embryo development. To develop into somatic embryos, somatic cells must regain their cell division activity. Thus, genes responsible for the control of cell cycle and cell wall synthesis and genes responsive to hormones and transcription process are associated with these stages of somatic embryogenesis (Yang and Zhang 2010).

Expression of genes responsible for the cell-cycle process is therefore important for the further development of the embryo. Hence, the division associated proteins, such as the proliferating cell nuclear antigen in grape wine (Marsoni et al. 2008) and the putative citrus DRT102 in Valencia sweet orange (Pan et al. 2009) are activated during embryogenesis. Besides, cytoskeletal proteins, such as tubulins associated to cell division, are also differentially regulated (Pan et al. 2009).

Regarding cell wall synthesis the role of genes are also important, as SE depends on proper formation of cell wall components. Among others, the following genes are included in the group responsible for cell-cycle and cell wall synthesis: *CYCLIN-DEPENDENT KINASES* such as *CDC2M*, *CEM6*, *SERPENTINE*, *ALPHA-1 ACID GLYCOPROTEIN*. The expression of these genes at the right time ensures proper construction of the embryo (Yang and Zhang 2010). Induction and growth of somatic cells can be stimulated by appropriate hormones that affect hormone-sensitive genes, including ABA (abscisic acid)-responsive genes such as *LATE EMBRYOGENESIS ABUNDANT* (*LEA*), but also genes responsive to auxins (indole-3-acetic acid – IAA and picloram - PIC) such as *GH3*, *PIN*, *ARF*, *SAUR*. The proper course of SE requires genes that regulate the individual stages and the entire process (Yang and Zhang 2010).

In several plant regeneration processes through SE, one of the major problems is an effective transition from the proembryogenic masses, forming the embryogenic tissue, toward embryo development, which is often impaired by the formation of abnormal embryos and precocious germination (Correia and Canhoto 2010; Vieitez et al. 2012). This situation may be due to an inadequate maturation of the embryos, an important phase of somatic and zygotic embryo development,

following the classic morphogenic phases from globular to cotyledonary embryos (Correia et al. 2012b). During maturation, embryo cells undergo various physiological changes, which become evident by the deposition of storage materials, repression of germination and acquisition of desiccation tolerance (Jiménez 2005; Vahdati et al. 2008). In cork oak, the activation of diverse ROS detoxification enzymes and the accumulation of reserve products (mostly starch and proteins) have been reported during the transition phase between morphogenesis and maturation, suggesting the requirement that cell division should be replaced with cell expansion for proper embryo differentiation (Gomez-Garay et al. 2013). In this system, energy requirements reached a maximum at the cotyledonary stage, suggesting the relevance of primary metabolite production, such as amino acids and fatty acids, whereas fermentation could constitute an alternative source of energy at the early steps of somatic embryo development (Gomez-Garay et al. 2013). Also, for Valencia sweet orange (Pan et al. 2009) several proteins involved in anti-oxidative stress response (GST), cell division (tubulins), photosynthesis (ferritins), and cyanide detoxification (rhodanese) exhibited different expression patterns and were likely to be associated with SE. Another species often used in studies aimed to detect and identify proteins expressed during the different stages of somatic embryo development is the myrtaceous tree pineapple guava (*Feijoa sellowiana*) (Cangahuala-Inocente et al. 2009; Fraga et al. 2013). The results obtained with this SE system indicate a high similarity in the profiles of the assayed somatic embryos at all developmental stages, suggesting that only a few specific genes are involved in the different developmental stages, and that gene expression changes occur prior to morphological changes. The hypothetical protein similar to L-isoaspartyl-O-methyltransferase active during the torpedo stage, and an osmotin-like protein active during the pre-cotyledonar stage of somatic embryos were suggested as embryonic markers for pineapple guava (Cangahuala-Inocente et al. 2009). The expression of the protein phenylalanine ammonialyase in all the assayed developmental stages confirmed the synthesis and accumulation of several phenolic compounds during the induction of pineapple guava embryogenic cultures and the development of somatic embryos. The presence of cytosolic glutamine synthetase and NmrA-like proteins revealed the activation of nitrogen metabolism, observed particularly in the later developmental stages in which the accumulation of storage compounds (mostly in the cotyledonary leaves) is enhanced (Cangahuala-Inocente et al. 2009). More recently, the comparison between “off-type” and normal phenotype proteomes of somatic plantlets of pineapple guava has brought new insights to the causes of abnormal somatic embryo development (Fraga et al. 2013). The presence of HSP was observed only during the formation of normal phenotype somatic plantlets, indicating that these proteins may be involved in the morphogenesis of normally developed plantlets. A vicilin-like storage protein was

only found in "off-types" at 20-day conversion, indicating that plantlets may present an abnormality in the mobilization of storage compounds, causing reduced vigor in the development of derived plantlets. HSP17 is a small heat shock protein that also transiently accumulates during somatic embryo maturation and germination in oak (Puigderrajols et al. 2002). These results indicate that peroxidase, RGP-1, and HSP17 may be involved in dedifferentiation of explant cells during SE (Elhiti et al. 2013).

3. Epigenetic regulation of somatic embryogenesis: an open gate

In recent years it has been found that epigenetic regulation plays a critical role in the regulation of multiple aspects of plant development through the modulation of gene expression in response to many environmental factors (Hauser et al. 2011). Epigenetics is defined as "heritable changes in gene expression that occur without a change in DNA sequence", and can be understood as a system to selectively regulate genome information through activating or inactivating gene expression (Valledor et al. 2007). At the molecular level, DNA methylation, posttranslational histone modifications, chromatin remodelling factors, transcriptional factors, and chromosomal proteins cooperate together (e.g. Hauser 2011, Brautigam et al. 2013). Epigenetic variation is likely to contribute to the phenotypic plasticity and adaptive capacity of plant species, and may be especially important for long-lived organisms with complex life cycles, including forest trees (Nicotra et al. 2010; Brautigam et al. 2013). Diverse environmental stress drivers can create reversible heritable epigenetic marks that can be transmitted to subsequent generations as a form of molecular "memory". Epigenetic changes might also contribute to the ability of plants to respond and adapt to different environmental challenges (Nicotra et al. 2010; Brautigam et al. 2013). Several authors consider the possible role of forest tree epigenetics as a new source of adaptive traits in plant breeding, biotechnology, and ecosystem conservation under rapid climate change (reviewed by Brautigam et al. 2013).

While epigenetic phenomena are clearly important for trees in a natural context (Grativol et al. 2012) they also could be of great consequence during specific tree production processes (Smulders and de Klerk 2011; Brautigam et al. 2013). Tissue culture can provide alternative means to keep desirable genotypes by vegetative propagation and to quickly produce commercial quantities of plants. During in vitro culture, plants have to cope with a specific microenvironment in which plant cells are exposed to a conjugation of stimuli such as exogenously applied growth regulators together with other artificially provided chemical and physical factors (Cassells and Curry 2001). In response to this cells acquire competence to switch fate in a clear demonstration of plant cell plasticity. Cellular proliferation, tissues and organ formation require the precise coordination of

genetic and epigenetic factors (Miguel and Marum 2011; Smulders and de Klerk 2011; Us-Camas et al. 2014). In fact, the variation that tissue culture can introduce in regenerated plants is an old issue (Cassells and Curry 2001). The intention of large scale clonal propagation is the production of phenotypically identical individuals but in practice this is not always achieved. Somaclonal variation can result in subtle to drastic phenotypic variation and has been found to be attributable to genetic or epigenetic variations (e.g., reviewed in Kaeppler et al. 2000; Miguel and Marum 2011). Besides, claims that in vitro culture procedures provide high genetic stability of in vitro regenerated plants are often not valid. A growing number of studies reveal high frequencies of epigenetic variation. This evidence, besides being unexpected, may reflect reflect the adaptation process of cells to different in vitro environmental stimuli (Miguel and Marum 2011, De-la-Peña et al 2012) similarly as occurs in nature. The best illustrative example is the case of somaclonal variants in somatic-embryo-derived oil palm (*Elaeis guineensis*) regenerated plants where phenotypic variation was associated to epigenetic mechanisms (Jaligot et al. 2011). However, the nature of the epigenetic changes occurring during *in vitro* regeneration is poorly understood. Most of the studies rely on the model plant *Arabidopsis* (Li et al. 2011) and too little information is available for woody plants. Exploring epigenetics, new insights might be of relevance for basic research and applications in plant propagation by providing a better understanding of the differentiation and dedifferentiation processes or for the selection of appropriate in vitro culture conditions (Kaeppler et al. 2000; Rodriguez Lopez et al. 2010). This idea is supported by a growing body of evidence that suggests that epigenetic mechanisms, such as DNA methylation and histone modifications, can be affected by typical in vitro conditions (De-la-Peña et al. 2012). Tissue-, organ-, and species-specific differences in DNA methylation levels are well known (Fraga et al. 2002; Valledor et al. 2007, 2010; Monteuuis et al. 2009; Santamaria et al. 2009; Rodriguez Lopez et al. 2010; Vining et al. 2012; Lafon-Placette et al. 2013). Changes in epigenetic marks were found to accompany morphological and physiological changes in trees in a wide variety of processes, including aging, phase change, organ maturation, and bud set or burst (Fraga et al. 2002; Santamaria et al. 2009; Valledor et al. 2010).

Epigenetic regulation of SE is a fascinating field of research itself but in the case of woody plants it is still in its infancy. In recent years (reviewed by De-la-Peña et al. 2015), epigenetic mechanisms have emerged as crucial factors during both somatic and zygotic embryogenesis (Nic-Can and De-la-Peña 2014, Nodine and Bartel 2010). Some early reports indicate that auxins and in vitro conditions modify the levels of DNA methylation in embryogenic cells. The changes in DNA methylation patterns are associated with the regulation of several genes involved in SE, such as *WUS*, *BBM1*, *LEC*, and several others (reviewed by De-la-Peña et al. 2015). Unfortunately most of the reports are related with non-woody plants even

though some recent publications have started to highlight these processes in woody plants. The evaluation of epigenetic modifications in plants cultured *in vitro* has been mostly focused on the analysis of DNA methylation (Miguel and Marum 2011) and this is also the case for woody plants. This is probably the case because it is one of the best described epigenetic mechanisms with several efficient tools for analysis of variation that has been optimized as well as tools for analysis of the specific sites of methylation (Valledor et al. 2007; Miguel and Marum 2011, Rodriguez Lopez et al. 2010). De-la-Peña et al. (2015) divided the methods for the determination of methylation levels in DNA into at least into six general groups: global DNA methylation, regional DNA methylation, genome-wide analysis, DNA methylation analysis by sequencing, detection of specific methylation patterns, and individual CpG analysis (see also Rodriguez et al. 2012; Us-Camas et al. 2014). Several of these methods were applied with success in SE cultures of woody species in order to elucidate the epigenetic role during the different steps of SE.

Rodriguez Lopez and co-workers (2010) reported a detailed analysis of genetic and epigenetic variation in relation to callus age in cocoa plants (*Theobroma cacao*) regenerated by somatic embryogenesis. Genetic variation was investigated using single sequence repeat (SSR) markers, and epigenetic variability was assessed by methylation-sensitive amplified polymorphism (MSAP), a method to detect genome-wide but anonymous DNA methylation patterns. Contrary to predictions, after an initial increase, a decrease in both genetic and epigenetic divergence between leaves of regenerants and the ortet plant was observed after the culture had reached an age of about 10 weeks (Rodriguez Lopez et al. 2010). One possible interpretation of the findings suggests a link between stable DNA methylation patterns and repression of *de novo* mutations during somatic embryogenesis (Rodriguez Lopez et al. 2010).

Another well-studied example of somaclonal variants and their relation to epigenetic marks in a tree species is the *mantled* phenotype in somatic-embryo-derived oil palm (*Elaeis guineensis*). This phenotypic variant, found in about 5% of regenerants, is characterized by abnormal inflorescence development and has been associated with global DNA hypomethylation, but not in changes in genomic structure or nucleotide sequence (Jaligot et al. 2000; Rival et al. 2008). This issue was recently revised by Jaligot et al. (2011) in so far that they suggest that apart from a historic perspective future efforts should also concentrate on the epigenetic regulation targeting of MADS-box genes and transposable elements of oil palm, since both types of sequences are most likely to be involved in the *mantled* variant phenotype.

With the aim to prove that both embryogenic suspensions and secondary embryogenesis provided reliable true-to-type propagation and large-scale conformity in commercial field plots of elite material of *Coffee arabica*, Bobadilla et al. (2013) quantified genetic and epigenetic modifications in the regenerated

plants through AFLP (Amplified Fragment Length Polymorphism) and MSAP molecular markers.They also cytologically characterized the karyotype of different phenotypic variants detected in the study. The results showed that genetic and epigenetic alterations were particularly limited during coffee SE and the main change in most of the rare phenotypic variants was aneuploidy. In order to deepen other biological questions related with the SE process Nic-Can et al. (2013) proposed that the embryogenic development of *Coffea canephora* proceeds through a crosstalk between DNA methylation and histone modifications during the earliest embryogenic stages of SE. They found that levels of DNA methylation, histone H3 lysine 9 dimethylation (H3K9me2) and H3K27me3 change according to embryo development. Moreover, the expression of *LEAFY COTYLEDON1* (*LEC1*) and *BABY BOOM1* (*BBM1)* were only observed after SE induction, whereas *WUSCHEL-RELATED HOMEOBOX4* (*WOX4*) decreased its expression during embryo maturation.

Beyond the previous study just a few others report histone modifications as a target for study apart from DNA methylation. The expression pattern of several genes related to chromatin modification and remodelling [two histone deacetylases (*HDACs*), *HDA6* and *HDA19*, two histone monoubiquitinases (*HUB1* and *HUB2*), a histone H3 kinase (*AUR3*), *PICKLE* and *VP1/ABSCISIC ACID INSNSITIVE 3-LIKE 1* (*VAL1*)], have been studied during the SE process of *Q. suber* (Pérez et al. 2015). It was found that *QsHDA19* decreases its expression as soon as the callus begins its differentiation, followed by a steady increase during the stage from immature cotyledonary embryo to an embryo with the cotyledons fully differentiated. On the other hand, a transient decrease in *QsHDA6*, *QsPICKLE*, and *QsVAL1* gene expression was observed in the transition from callus to the end of the mature embryo stage. *QsHUB1* and *QsHUB2* showed a transient increase in expression from white embryogenic structures and globular embryos to the immature cotyledonary embryo stage. The highest expression was observed in white opaque cotyledonary embryos, while *QsAUR3* was preferentially expressed in immature cotyledonary embryos. All of these results suggest that these epigenetic components play a key role during the development and maturation of *Q. suber* somatic embryos. Moreover, the change in the expression levels for all seven genes associated with epigenetic regulation showed that *QsHUB1* and *QsHUB2* may have a role in ABA signaling while *QsHDA6* and *QsHDA19* could act in different pathways in *Quercus* than in *Arabidopsis*. Furthermore, expression levels of *QsAUR3* indicated that histone phosphorylation is an early epigenetic mark in *Q. suber* somatic embryos while *QsPICKLE* and *QsVAL1* may be necessary for the correct development of cork oak somatic embryos (Pérez et al. 2015).

Global DNA methylation analyzed by HPLC is a suitable tool to check the involvement of epigenetic mechanisms in sexual embryogenesis and has been used as a marker of the somatic embryogenesis induction capability of *Castanea sativa*

(Viejo et al. 2010). The authors conclude that for this species a "developmental window" of SE exists and that a transient decrease in methylation is necessary after fertilization before SE can occur. Also in situ hybridization methods for studying the cytosine methylation status give accurate measures of the degree of total DNA methylation. But one of the most interesting aspects of these approaches is that they provide information regarding tissues specific methylation patterns. Data using immunolocalization coupled with confocal microscopy in order to localize 5-mdC have been used to resolve biological questions related with the SE process *in Q. suber* (Pérez et al. 2014). Immunohistochemical analyses showed that there was a specific spatial-temporal regulation during embryogenesis, particularly after the cold treatment. The acquisition of germination capacity concurred with a general low 5-mdC signal in the root meristem, while retention of the 5-mdC signal was mainly located in the shoot meristem and provascular tissues. These data where complemented with ABA immunolocalization and the results suggest that, in addition to ABA, epigenetic control appears to play an important role for the correct maturation and subsequent germination of cork oak somatic embryos (Pérez et al. 2014).

Recent studies have clarified the role of microRNAs in epigenetic regulation of some key transcriptional factors in early embryogenesis. For instance, in *Arabidopsis*, *LEC2* and *FUS3* have been found to be regulated by microRNAs (Willmann et al. 2011). Nodine and Bartel (2010) found that miR156 is activated throughout early somatic embryogenesis. Furthermore, miR156 showed a level of significant expression in differentiated embryogenic calli of Valencia sweet orange (Wu et al. 2011; Wu et al. 2015) and hybrid yellow poplar (Li et al. 2012). At this point, the main targets of miR156 identified through different methods, most recently through high throughput sequencing, are Squamosa Promoter Binding Protein-Like (SPL) families. More research is needed on other potential targets of miR156 related to somatic embryogenesis and other developmental processes. Thus, the presence of certain miRNAs in specific tissues seems to promote the silence of genes that are unnecessary to particular developmental stages (Us-Camas et al 2014). In sweet orange somatic embryogenesis, other several miRNAs were reported as being important during SE (Wu et al. 2011). For instance, miR156, miR168 and miR171 are involved during the somatic embryo induction process, miR159, miR164, miR390 and miR397 are dedicated to globular shaped embryo formation, and miR166, miR167 and miR398 are required for cotyledon-stage embryo formation.

Overall studies highlight the idea that epigenetic regulation plays an important role during somatic embryogenic development. As more accurate and powerful tools for epigenetic analysis become available for application in a broader range of plant species, analysis of the epigenetic landscape of plant cell cultures may be extended to more recalcitrant ones such as of woody plants.

4. Conclusions and future perspectives

The availability of model systems of plant somatic embryogenesis has created effective tools for examining the details of plant embryogenesis. However, the majority of mechanisms that regulate plant embryogenesis still remain to be clarified, and studies that used non-model plants for somatic embryogenesis are increasingly revealing their importance in the analysis of the molecular mechanisms in charge of gene expression during somatic embryogenesis. Thus, although few genes have been associated with somatic embryogenesis induction in hardwoods, it is today a major field of research with important fundamental and applied purposes. Numerous protocols for successful somatic embryogenesis induction and plant regeneration in different hardwood species have been published, suggesting that SE can perhaps be achieved for any plant, provided that the appropriate explant and culture treatment are employed. But a fragmented knowledge concerning the genetic programs that regulate embryogenesis in these species still remains as a bottleneck. The many technical challenges associated with accurate transcriptome profiling in tree species that lack reference sequences and have large genomes, contribute in large part to that problem. With the development of next generation sequencing (NGS) technologies and the concomitant availability of powerful bioinformatic tools to process and analyze large sequence data sets there should be a rapid advance in the depth and breadth of transcriptome profiling experiments in forest trees before long.

The results obtained over the last few decades strongly emphasize the role of stress pathways in somatic embryogenesis, revealing an intricate dynamism, variability and behavior of several regulatory proteins. Research in epigenetic regulation has also revealed a strong influence of epigenetic markers on SE.

The integration of the expressed protein data, together with transcriptome and even metabolome data, has the potential to provide the most comprehensive and informative clues for somatic embryogenesis in plants. Future research in this field should include new and/or complementary approaches, including more sensitive methods for protein detection and identification. These approaches are becoming more effective with the integration of new data from several genome-sequencing projects. Furthermore, efforts should be taken in the functional validation of the specific identified genes/proteins, in order to use them as markers for the SE process. The coordination of all this knowledge will give insight in future studies addressing the optimization of the somatic embryogenesis protocols for mass propagation and conservation strategies for several economically relevant woody species.

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Physiology and role of plant growth regulators in somatic embryogenesis

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Abstract

Somatic embryogenesis (SE) is the developmental process by which somatic cells, under suitable induction conditions, undergo restructuring through the embryogenic pathway to generate embryogenic cells and consequently the whole plant. Within the process of SE, a single cell or a group of cells with similar morphology and genetic background respond to external stimuli produced by the surrounding tissue, in the case of natural settings, or present in the tissue culture medium. These stimuli launch a genetic program that leads to the establishment of cell lineages with an altered gene transcription pattern, and a different morphology and developmental fate. The key substances controlling the whole process of SE are phytohormones. Generally they are characterized as natural substances with signalling ability important for connections between cells, tissues and/or organs in the plant. Their specific information depends on the chemical structure of the phytohormone and on the ability of plants to receive the signal and induce a physiological response in the target plant tissue. In the present review, we focussed on seven main groups of regulators that have a fundamental influence on different developmental stages of SE - auxins, cytokinins, abscisic acid, ethylene, jasmonic acid, polyamines and phenolic compounds. We provide an overview of current knowledge of phytohormonal regulation of embryo development including the effect of crosstalks between phytohormones and/or plant growth regulators in terms of highly coordinated interactions within phytohormones signalling pathways. We considered the main mechanism of regulation in plant/embryo development as revealed by studies from zygotic as well as somatic embryos using the modern approaches of molecular biology and advanced microscopic techniques. When possible, examples from SE of conifers will be given.

Keywords: phytohormones, tissue cultures, embryo, regulation, signalling, development

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Abbreviations

ABA - abscisic acid
ACC - 1-aminocyclopropane-1-carboxylic acid
BAP - N^6-benzylaminopurine
BR - brassinosteroids
CHS - chalcone synthase
CK - cytokinins
2,4-D - 2,4-dichlorophenoxyacetic acid
DHZR - dihydrozeatinriboside
ESM - embryogenic suspensor mass
GA - gibberellins
IAA - indole-3-acetic acid
IBA - indole-3-butyric acid
iP - isopentenyladenine
iPA - isopentenyladenosine
iPR - isopentenylriboside
JA - jasmonic acid
JAIle - jasmonoyl-L-isoleucine
kin - kinetin
MeJA - methyljasmonate
NAA - naphtylacetic acid
NPA - 1-N-naphtylphtalamic acid
OPDA - 12-oxo-phytodienoic acid
PAs - polyamines
PAA - phenylacetic acid
PAL - phenylalanine ammonia lyase
PCs - phenolic compounds
PCD - programmed cell death
PCIB - p-chlorophenoxyisobutyric acid
PGR - plant growth regulators
PhAs - phenolic acids
Put - putrescine
ROS - reactive oxygen species
SA - salicylic acid
SE - somatic embryogenesis
Spd - spermidine
Spm - spermine
TDZ - thidiazuron
TIBA - 2,3,5-triiodbenzoic acid
Z - zeatin
ZE - zygotic embryogenesis
ZR - zeatinriboside

1. Introduction

In 1902, the botanist Gottlieb Haberlandt theorized that, under the proper culture conditions, ‘‘one could successfully cultivate artificial embryos from vegetative cells’’ (Haberlandt, 1902; Krikorian and Berquam, 1969). Interestingly, effective plant regeneration techniques were established as soon as three decades later. In 1939, regeneration, using larger tissue explants from many species including woody ones, was successfully carried out in culture medium containing the critical phytohormone indole-3-acetic acid (IAA, auxin) (Gautheret 1940 a, b).

A further important advance in the study of plant regeneration was identification of the major effect of auxin/cytokinin ratios on regenerated tissue type. In 1957, Skoog and Miller found that treating tobacco pith with high auxin/cytokinin ratios led to root formation. In contrast, high cytokinin/auxin ratios induced shoot regeneration. When high concentrations of both hormones were added to cultivation media, a mass of growing cells known as a “callus” was induced on explants. This pioneering work provided the conceptual framework for the role of plant hormones and their interactions in establishing distinct regeneration paths in plant tissue cultures. Since that time in vitro culture has played a large role in the propagation of plants in large quantities and with desired characteristics. It has also been used as a tool for the conservation and rapid propagation of rare and endangered plants of economic importance. Based on differences in cell-fate transition, regeneration of higher plants can be classified into three main categories: tissue regeneration, *de novo* organogenesis, and somatic embryogenesis (Birnbaum and Alvarado 2008; Sugimoto et al. 2011; Xu and Huang 2014).

Somatic embryogenesis (SE) is the developmental process by which somatic cells, under suitable induction conditions, undergo restructuring through the embryogenic pathway to generate embryogenic cells. These cells then go through a series of morphological and biochemical changes that result in the formation of a somatic embryo and the generation of new plants (Yang and Zhang 2010, Smertenko and Bozhkov 2014). Somatic embryos resemble zygotic embryos and undergo almost the same developmental stages (Dodeman et al. 1997).

While zygotic embryogenesis (ZE) starts from a single cell followed by formation of a globular embryo containing a determined number of cells, SE starts from a single cell or a group of cells and attains a globular structure containing a variable number of cells. How a group of cells initiates embryo formation is not clear, but considering our knowledge about ZE, an asymmetric distribution of auxin must be established first (de Smet et al. 2010). Somatic embryo development encompasses key stages of ZE: the heart and torpedo stages in the case of dicotyledonous species; the globular, scutellar, and coleoptilar stages in the case of monocotyledonous species; early and late embryogenesis in the case of gymnosperm species (Zimmerman 1993; Singh 1978; von Arnold et al. 2002).

The mature somatic embryos resemble zygotic embryos morphologically and physiologically (Zimmerman 1993; von Arnold et al. 2002). They display apical–basal and radial polarity, possess a primary shoot and root meristem, and contain the typical embryonic organs radicle, hypocotyl, and cotyledons. The key

genes controlling ZE perform similar roles during SE (Mordhorst et al. 2002; Yang and Zhang 2010; Elhiti et al. 2013).

However, in contrast to the development of a zygotic embryo, the fact that it is possible to form embryos from a diverse set of tissues and that this can easily be observed has permitted somatic embryogenesis to serve as a model system for the study of morphological, physiological, molecular, and biochemical events occurring during the onset and development of embryogenesis in higher plants (Yang and Zhang 2010; Elhiti et al. 2013; Smertenko and Bozhkov 2014).

Since the first observations of somatic embryo formation in carrot cell suspension cultures by Steward et al. (1958) and Reinert (1959), somatic embryogenesis has been observed in a wide range of tissue culture systems of Gymnosperms (Park 2013) and Angiosperms, including dicot and monocot plants (Quiroz-Figueroa et al. 2006). The first reports on conifer SE from seed explants were published for Picea abies (L.) Karst, Norway spruce (Chalupa 1985; Hakman et al. 1985).

Propagation through SE allows formation of multiple, genetically identical embryos and avoids waiting for the following reproductive season. Cultivation of explants from microspores, ovules, embryos, and seedlings on media containing the appropriate balance of plant growth regulators (PGRs) can induce formation of embryos directly from the explanted tissue (direct SE) or induce proliferation of an embryo-forming (embryogenic) callus (indirect SE) (Quiroz-Figueroa et al. 2006, Pulianmackal et al. 2014).

SE starts from a single cell or a group of cells with a similar morphology and genetic background in response to external stimuli produced by the surrounding tissue, in the case of natural settings, or by stimuli present in the tissue culture medium. These stimuli launch a genetic program that leads to the establishment of cell lineages with an altered gene transcription pattern, and a different morphology and developmental fate. The first result of these multidimensional alterations is the formation of a morphologically asymmetric (polar) structure. Following the initiation of SE, the process becomes auto-regulatory and can sustain successive stages of the embryogenesis pathway without any or with minimal contributions from external signals. In spite of how complex the subsequent stages may seem, the original establishment and persistence of this polarity within a homogenous group of cells, including terminal differentiation and the demise of the embryo suspensor, represents the fundamental paradigm of plant developmental biology (Smertenko and Bozhkov 2014).

Phytohormones are key substances controlling plant growth and development. They are characterized as natural substances with a signalling ability that is important for connections between cells, tissues and/or organs in the plant. Their specific information depends on the chemical structure of the phytohormone and on the ability of plants to receive the signal and induce the appropriate physiological response in the target plant tissue. Many physiological effects, interrelated with phytohormones and other growth regulators (natural or synthetic), were investigated in various materials cultivated in vivo and in vitro. The role played by PGRs in SE has been examined widely but usually by using the traditional ‘one-factor-at-a-time’ and ‘trial-and error’ techniques. Hormonal requirements determined through such empirical methods were usually optimized

for particular cultivars or genotypes. Once identified, positively acting combinations of PGRs were usually used consistently for years with small modifications introduced in the case of less responsive genotypes.

Despite this fact, multiple problems still persist for several woody species. The main difficulties are a low yield of mature embryos, their low quality and low frequency of embryos able to convert into viable plantlets (Stasolla and Yeung 2003). To solve these problems it is important to focus our attention on obtaining an accurate understanding of the biosynthesis of phytohormones, their metabolism and transport as well as their mode of action. Recent research has uncovered coordinated interactions of phytohormones signalling pathways as the main mechanism of regulation in plant/embryo development. The crosstalk between the signalling pathways of growth regulators must be included into the investigation of the role of plant growth regulators in SE.

In our review we selected seven main groups of regulators that may fundamentally influence the process of SE: auxins, cytokinins, abscisic acid, ethylene, jasmonic acid, polyamines and phenolic compounds, especially during conifer SE. We are aware of the importance of other phytohormones and regulators in various developmental processes, but the data dealing with their effect in SE are not abundant.

2. Plant growth regulators and somatic embryogenesis

2.1 Auxins

The native auxin IAA (indole-3-acetic acid) has generally been accepted as the main regulator of plant morphogenesis since the thirties of the 20th century and discussion of its role still remains an important topic of contemporary plant science. Many other substances having auxin activity were detected - including the native auxins (e.g., IBA – indole-3-butyric acid; PAA – phenylacetic acid) or the synthetic ones (e.g., NAA – naphtylacetic acid; 2,4-D – 2,4-dichlorophenoxyacetic acid) that are effective in various developmental processes. The actual experiments are mainly focused on regulation of auxin biosynthesis, conjugation and degradation as well as their distribution in cells and tissues and on molecular mechanisms of auxin action in plant development.

The role of auxins in SE was studied using auxin application during the several developmental stages of SE. Classical experiments examined the morphological effects of auxins known from other developmental situations dealing with regulation of SE.

The induction of embryogenic cultures proceeds on standard mineral media supplemented with cytokinins; in many conifers the auxin (2,4-D) is also necessary. This auxin positively affected induction of larch and spruce embryogenic cultures while embryogenic cultures of firs are often initiated without auxin in the medium. Induction medium is often supplemented with 2,4-D in pine embryogenesis; another synthetic auxin NAA is also effective (summary in Jain et al. 1995). In other trees the induction process progresses in an analogical way, the mother explant – usually a zygotic embryo is cultivated on the induction medium supplemented with 2,4-D or eventually with NAA (Jain et al. 1995). During the

next step of SE the early embryos are multiplied while remaining in the developmental step of early embryos. This process occurs on proliferation medium (standard minerals) supplemented usually with cytokinins together with auxin. The 2,4-D application during proliferation is usual in embryogenic cultures of spruce, Douglas fir and larch (von Aderkas et al. 2015), the ratio between cytokinins and 2,4-D determines induction and proliferation of embryogenic culture of ginkgo (Laurain et al. 1996). Early somatic embryos proliferating on medium supplemented with auxin have a better chance to finish their development. Application of auxin later during maturation, instead of during proliferation, does not lead to successful development of embryos. This agrees with Stasolla and Yeung's (2003) argument that early events in embryogenesis are crucial for the successful development of somatic embryos. The auxin effect depends strictly on the species that is being investigated, e.g., the auxin effect in the regulation of fir somatic embryogenesis is not clear. According to the investigations carried out with several species and hybrids of fir, auxin is necessary during induction and proliferation of fir embryogenic cultures (Tautorus et al. 1991). On the other hand, Norgaard and Krogstrup (1991) described the inhibitory effect of auxin in the proliferation of early somatic embryos of *Abies nordmaniana*. Nevertheless, proliferation of the ESM (embryogenic suspensor mass) of *Abies balsamea* occurs on medium supplemented with NAA according to Guevin et al. (1994). During induction and proliferation of several Angiosperms the medium is supplemented with 2,4-D and cytokinins, e.g., in poplar (Michler and Bauer 1991), or eventually with NAA and cytokinins in oak (e.g., San-Jose et al. 2010) while proliferation medium was supplemented with IBA instead of 2,4-D in *Tilia* (Chalupa 2003) or in chestnut embryogenic cultures (Sezgin and Dumanoglu 2014).

Somatic embryo maturation occurs usually on medium without any auxins. Auxin (2,4-D) in maturation medium can actually inhibit the development of embryos, e.g., in the case of fir (Vondráková et al. 2011). The yield and quality of mature embryos can be enhanced by temporary cultivation (approximately 1 week before maturation) on phytohormone free medium (Bozhkov et al. 2002). Before maturation larch somatic embryos are usually cultivated on phytohormone free medium supplemented with activated charcoal (the effect of activated charcoal on plant tissue cultures was summarized by Thomas (2008)) and IBA together with ABA (abscisic acid) in the maturation medium (Lelu et al. 1994; von Aderkas et al. 2015). Auxin application is not necessary during the next steps of SE. During desiccation and germination embryos are cultivated without any medium or on germination medium without phytohormones.

Inhibitors of auxin transport and auxin action were used for the elucidation of auxin effects on somatic embryo development. Their effect was determined in early embryos as well as in embryos during maturation. An inhibitor of auxin transport TIBA (2,3,5-triiodbenzoic acid) was applied together with different auxins (2,4-D, IAA, NAA and picloram) during spruce ESM induction. The inhibition of ESM development occurs as the result of accumulation of auxin rather than the disruption of auxin polar transport (Ramarosandratana and van Staden 2004). According to Find et al. (2002) endogenously produced auxin decreases the yield of somatic embryos but may also play a critical role in proper development of cotyledons during later stages of embryo maturation. Exogenous treatment with the

auxin antagonist PCIB (p-chlorophenoxyisobutyric acid) reduced proliferation and promoted the development of high quality embryos in *Abies nordmaniana*. In contrast, the development of fir embryos treated by PCIB at the start of maturation was strongly inhibited (Vondráková et al. 2011). Larsson et al. (2008a) used another inhibitor of auxin transport – NPA (1-N-naphtylphtalamic acid) in a study of embryo differentiation, the role of PCD (programmed cell death) in this process and the effect of NPA application on endogenous auxin levels. They showed that auxin polar transport is essential for the correct patterning of both apical and basal parts of conifer embryos during maturation. The negative effect of NPA on the morphology of spruce somatic embryos was confirmed by Hakman et al. (2009) and demonstrated that early embryos are highly sensitive to this treatment. Immunolocalization of IAA manifested the highest level of IAA in early spruce embryos and continual IAA decrease during their maturation (Hakman et al. 2009). Palovaara et al. (2010) demonstrated the effect of NPA and auxin treatments on the PIN proteins (i.e.,, the family of auxin efflux transporters) and discussed the role of PaPIN1 not only in early steps of SE but also during apical and root meristem formation in spruce. Polar auxin transport was found to be the main regulator of the ratio between the volume of meristematic and suspensor cells in early embryos of pine (Abrahamsson et al. 2012).

The role of auxins in embryogenesis is linked especially with the polar auxin transport that is essential for the establishment of bilateral symmetry during plant embryogenesis (Liu et al. 1993). Wilson and Wilson (1993) showed that initiation of polarity in zygotic embryos is controlled by the direction of auxin movement in surrounding tissues. The role of auxin polar transport (and the changes in endogenous auxins) in plant embryogenesis (zygotic and somatic) was reviewed, e.g., by Liu et al. (1993) and Cooke et al. (1993).

Contemporary studies of the mechanisms of auxin polar transport and its role in plant embryogenesis continue at the biochemical, anatomical and molecular level and they are often linked with the investigation of the role of cytoskeleton in this process. Most information dealing with the role of auxins in embryogenesis regulation was obtained using the embryos of *Arabidopsis*. These data are used to establish a model of the regulation process of embryogenesis in plants. Briefly *Arabidopsis* embryo development proceeds as follows: The establishment of the auxin transport system is a prerequisite for patterning events in the apical region of the embryo and auxin signalling is required for the establishment of correct cell division patterns and for determining the cell fate of the suspensor (Souter and Lindsey 2000). Polar auxin transport controls differential growth, embryo and root patterning and vascular tissue differentiation (Friml and Palme 2002). Auxin transport is necessary for shaping the plant and auxin plays the role of hormone and morphogen in the process (Friml 2003). During early embryogenesis auxin regulates not only the apical-basal polarity of embryos (Friml 2003) but also the initiation of the root meristem (Geldner et al. 2000). During the last steps of embryogenesis and during seedling development it regulates the formation of lateral organs (Paquette and Benfey 2001). The auxins control the apical-basal polarity of the embryo axis, the initiation of the primary meristem and the phyllotaxis in the shoot apex (reviewed by Jurgens (2001) and Hamann (2001)). In addition, all findings show the connection between polarity of individual cells and

the establishment of polarity at the tissue, organ and whole plant level. Cell polarity depends on the subcellular polar targeting of PIN auxin transport components that determine the flow of auxin between the cells. The relationship between the establishment of cell polarity, auxin transport, cytoskeleton and cell division controls all developmental processes in the whole plant as it does in the embryo (reviewed by Dhonukshe et al. (2005)). Auxin biosynthesis as well as transport is linked with the process of bipolar structure formation; it seems that the tight control of distinct local auxin sources provides a non-cell-autonomous trigger for coordinated cell polarization and subsequent apical-basal orientation (Robert et al. 2013). A relatively recent insight into the molecular mechanism of auxin in the earliest stages of plant development – in plant embryogenesis – was provided by Jenik and Barton (2005). The role of auxin in somatic embryogenesis of *Arabidopsis* was investigated, e.g., during induction where the close link between auxin, LEC2 (LEAFY COTYLEDON2 transcription factor) activity and embryogenic capacity was confirmed (Ledwon and Gaj 2009) and the evidence that LEC2 controlled auxin biosynthesis that can trigger embryogenic development of somatic cells was demonstrated by Wojcikovska and Gaj (2015).

Larsson et al. (2008b) summarised that embryogenesis in conifers includes some steps that differ from those in Angiosperm embryogenesis. Nevertheless, the role of auxin polar transport in embryogenesis is crucial in embryo development of both *Arabidopsis* and conifers. It offers the possibility to compare the data obtained during embryogenesis of both plant materials. Furthermore we can (with care) use the data obtained during SE of conifers as a model for elucidation of zygotic embryogenesis of conifers as the several steps of somatic embryogenesis mimic the process of zygotic embryogenesis (Quiroz-Figueroa et al. 2006).

Exogenous auxin treatments used to regulate SE development induce changes of endogenous level of auxins – usually only the changes in IAA level are determined. The changes in IAA content in embryogenic cultures as well as in embryos during the whole process of SE can characterize the developmental processes necessary for embryo constitution. The link between 2,4-D application and endogenous IAA was described early by Michalczuk et al. (1992) in embryogenic cultures of *Daucus carota*. They demonstrated a positive effect of higher endogenous levels of IAA – on medium supplemented by 2,4-D at the start of SE – as a prerequisite for successful initiation of SE. A detailed analysis of the changes in auxin content in early somatic embryos of larch was demonstrated by Jourdain et al. (1997) and their investigation was complemented by von Aderkas et al. (2001) by their study of endogenous hormones during SE maturation. They detected a high level of 2,4-D and IAA-Asp (conjugate of IAA) in embryogenic cultures of larch during proliferation; during maturation the content of free IAA and IAA-Asp increased and the level of IAA became higher than the IAA-Asp level. These results concur with the data of Sandberg and Ernstsen (1987) obtained with germinated seeds of spruce and pine. Chiwocha and von Aderkas (2002) showed the continual increase of free IAA content and finally of the IAA-Asp content in Douglas fir zygotic embryos at the 14th week past pollination. Endogenous IAA was estimated also by Garcia-Martin et al. (2005) in oak somatic embryos. A higher level of IAA is typical for the early stage of oak embryo development while at the end of embryo development a higher IAA content

correlates with a better conversion capacity of mature embryos (Malá et al. 2000). Endogenous levels of IAA in mother explants (cotyledons) of *Prunus persica* influence the formation of embryogenic cultures, i.e.,, the embryogenic potential of cotyledons depends on their IAA level (Perez-Jimenez et al. 2013) and the differences in somatic embryogenesis capacity are determined by the content of endogenous IAA (and other hormones) (Perez-Jimenez et al. 2014).

Liao et al. (2008) connected the application of auxin inhibitors with the estimation of endogenous IAA levels and their data show that a higher level of IAA in spruce embryogenic cultures during proliferation correlates with a low yield of mature embryos; TIBA or PCIB application during proliferation decreases the IAA level and simultaneously can enhance the number of mature embryos. In contrast, application of auxin inhibitors has no effect on mature embryo yield in embryogenic cultures that have a low endogenous IAA level and a high yield of mature embryos without inhibitor treatment. This indicates that there is an optimal level of IAA during proliferation for optimal development of early embryos; an over-optimal level of IAA resulted in the inhibition of maturation.

A detailed analysis of phytohormones, including IAA, which was done by Vágner et al. (1998; 1999) on spruce embryogenic cultures cultivated in liquid as well as on agar medium demonstrated that precise phytohormones levels were essential during all phases of SE. During spruce SE the endogenous IAA level is quite low during proliferation but half way through maturation (approximately during the 3rd week of maturation) a rapid temporary increase of IAA content occurs. One week later the IAA content decreases again and the IAA level continues to decrease during maturation and desiccation. High peak of IAA content correlates with the process of embryo polarization. The high IAA content during apical and root meristem and vascular element formation agrees with the data obtained in studies of *Arabidopsis* embryo development. A slow continual decrease of IAA levels occurs during desiccation and at the start of germination. At the end of desiccation a small and short increase of the IAA level was observed in several embryogenic cultures. In embryos at the start of germination the IAA level is low, approximately the same as during proliferation. The next enhance in IAA content could, supposedly, occur during germination as auxin will regulate the development of seedlings (e.g., Berleth and Sachs 2001).

At present the study of auxins includes investigation of the effect of auxin on plant development (from embryos to the whole intact plants) through the study of auxin as a signal molecule with a specific mode of transport, that has a cell to cell function and has the ability to affect local metabolism (Petrášek and Friml 2009). The regulation of shape and pattern of plant development depends on mechanical and biochemical gradients (Hamant et al. 2010); the polarity of auxin transport through the plant/embryo plays a key role in this process. It depends on the coordinated polar localization of auxin efflux facilitators of the PIN family. PIN polarity is regulated by a feedback system, but the effect of auxin on PIN is linked with other auxin effects – like the change of cellular differentiation – that alter the polarity signal and the responsiveness to it (Geldner 2009). The specific regulation by auxin controls shoot apical meristem formation when auxin gradients activate PIN1 polar localization in embryogenic cultures. Polarized PIN1 is responsible for polar auxin transport and its accumulation in the shoot apical meristem (Su et al.

2009). Palovaara et al. (2010) suggest that WOX (the family of transcription factors essential for embryonic patterning) has a role in spruce somatic embryo development and confirmed the connection between polar auxin transport, PIN and WOX in the regulation of embryo patterning.

Vestman et al. (2011) recognized notable changes in the expression of genes involved in regulating auxin biosynthesis and auxin response. Somatic embryogenesis in *Picea abies* has become a model system for studying embryology in conifers (also see von Arnold et al. in this book). Somatic embryos exhibited gene expression patterns similar to the ones in zygotic embryos, although some differences were noted by Lara-Chavez et al. (2012). Cooke et al. (2002) declared that the mechanisms for mediating IAA responses probably represent modified pre-existing mechanisms operating in early land plants. Cairney and Pullmann (2007) provide the means to compare Gymnosperm and Angiosperm embryogenesis on the level of molecular biology. This will give us new possibilities to investigate the action of auxin in a different model of embryogenesis.

2.2 Cytokinins

Cytokinins (CKs) are low-mass molecules that represent an important group of phytohormones controlling many physiological and developmental processes in plants. They have been defined as factors capable of promoting growth of cultured plant cells (Skoog and Miller 1957) as they represent a group of growth regulators necessary for cell division. They are generally characterised as substances with a biological activity similar to that of zeatin. Zeatin (Z) was the first free CK identified from plants and was isolated from *Zea* endosperm. Chemically, CKs are classified as N^6-substituted derivatives of adenine with either isoprenoid or aromatic sidechains. The aromatic CK N^6-benzylaminopurine (BAP) is a highly active synthetic CK and its derivatives were also identified as natural CKs in several plant species. Other compounds with CK activity are synthetic derivatives of phenylurea (Spíchal 2012). Currently, more than 200 CKs or regulators with CK activity (natural and synthetic) have been identified.

The effects of CKs were tested by application of various CKs during various developmental processes; one main objective was to study CK regulation in plant tissue cultures by adding them to cultivation media. The role of CKs in the cultivation of explants is essential; CKs (and auxin) control organogenesis in vitro. The role of CKs in intact plants is large; CKs mediate bud growth, they influence transport mechanisms in plants and plant response to variable factors, e.g., light conditions in the shoots, they have a role in water uptake in the roots and in stress reactions etc. The mechanism of CK actions is often studied using CK antagonists – the substances that have structural similarities to CKs thus allowing them to compete for the same receptor as CKs (Spíchal 2008). CK action studies involve the determination of endogenous levels of CKs which are dependent on CK biosynthesis and uptake. CK-oxidase is the key enzyme in this process (Kamínek et al. 1997). How well changes in endogenous CKs could be determined depended on technological progress. New spectrophotometric methods provide large amounts of information about a large number of various CKs present in low concentrations in

plants and/or tissue cultures and their parts. This offers the possibility of determining the exact spectrum of CKs during different stages of plant/tissue culture development. Presently, the molecular basis of CK biosynthesis, metabolism, degradation, signalling and evolution are being investigated (Werner and Schmülling 2009).

CKs play an exceptional role in SE. They are required for induction of embryogenic cultures of various species of plants. In conifers induction medium is usually supplemented with BAP but a mixture of CKs can also be used – BAP+kin (kinetin) are often present in medium during induction of embryogenic cultures of trees (Jain et al. 1995); the synthetic CK TDZ (thidiazuron) is successful in induction of embryogenic cultures of *Abies fraseri* (Guevin and Kirby 1997). TDZ and iP (isopentelnyladenine) application during proliferation of spruce embryogenic culture affects the process of maturation depending on the embryogenic line used (Latkowska et al. 2001). Most of the protocols for SE of conifers recommend using CKs during induction of embryogenetic cultures and during early embryo development. Maturation, desiccation and germination are CK free stages of SE. Endogenous levels of CKs in both early and mature embryos are rather low. Jourdain et al. (1997) analysed endogenous levels of Z and iP, their metabolites and BAP. According to their results the low level of BAP and the high level of iPA (isopentenyladenosine) are characteristic in embryogenic larch cultures during proliferation. Von Aderkas et al. (2001) demonstrated an increase of iP level during maturation of larch embryos but all the CKs were present in very low quantities; nevertheless, iPA and iP were present in higher concentrations than Z or ZR (zeatinriboside). Endogenous Z, ZR, iP and iPA in spruce somatic embryos during the whole process of SE were measured by Vágner et al. (1998). The relatively high levels of CKs that were found in embryogenic cultures during proliferation decreased after transfer of early developing embryos onto maturation medium lacking CKs. The same CK pattern was detected by Březinová et al. (1996) in embryogenic cultures of oak. The higher CK content in oak mature embryos correlated with their better developmental capability (Malá et al. 2000). Endogenous levels of free and conjugated CKs (Z, ZR, iP and iPA) were measured during megagametophyte development in Douglas fir by Chiwocha and von Aderkas (2002). They demonstrated an increase of Z (and not ZR) level in the 13th week past pollination with a maximum iP level occurring between 10 and 13 weeks while the iPA level increased past the 13 weeks after pollination. Quesnelle and Emery (2007) studied early embryogenesis in *Pisum*. They suggested that it is possible that CKs regulate embryogenesis during seed development. They compared the role of different CKs during separate steps of embryogenesis and endorsed the idea that the main role of cis-Z is to promote embryo development. We expect that future investigations of the role of CKs in embryo development will focus on the elucidation of the specific roles of different CKs at separate stages of embryogenesis.

CK action is linked to their localization in plant tissues. Experiments were performed that dealt with immunolocalization of CKs at the cellular as well as plant tissue levels. Karkonen and Simola (1999) provided a detailed study of CK localization in developing embryos of *Tilia*. They showed that DHZR (dihydrozeatinriboside), ZR and iPR (isopentenylriboside) are concentrated in

highly cytoplasmic cells with meristematic characteristics. During embryo development CKs were located in meristematic areas (root and shoot apices). A strong CK signal was found in nucleoli and throughout the ground cytoplasm; occasionally it was associated with plastids and mitochondria. A large amount of CKs was located in the cell nucleus of *Actinidia* buds cultivated in vitro (Moncalean et al. 2001). Immunolocalization of Z and iP in spruce somatic embryos was performed by Vičánková et al. (2004). Both CKs were detected in meristematic embryonal heads of early embryos during proliferation. Localization of CKs changed during maturation – i.e., during embryo polarization. At that point both CKs were distributed unequally in fully developed embryos. During desiccation Z and iP were predominantly localized in the root pole and in the procambial part of somatic embryos.

Contemporary research focuses on the investigation of CK receptors and CK signalling. Experiments were carried out using primarily *Arabidopsis* and other model plants because the system of somatic/zygotic embryogenesis of trees is rather complicated and thus difficult to study. CK action is perceived via the molecular machinery of signal perception and transduction; the role of biosynthetic and metabolic enzymes in this regulation is crucial. The limiting step of the whole process is determined by the regulation of suitable CK concentration, correct place of their action and the right time for interaction with specific receptors (Frébort et al. 2011). A recent large scale analysis of the components of the CK signal transduction pathway revealed new CK receptors (Gruhn et al. 2015) and brings new information dealing with the reception of CKs. Kuderová et al. (2015) showed three CK receptors with distinct preferences for various CKs. Novák et al. (2015) demonstrated the interaction of signals involved in how root growth reacted to light exposition and they designated the CK receptor AHK3 as the major mediator of root signalling in response to illumination. It is expected that future CK research will focus on a better elucidation of CK receptor action, their characteristics and localization and on a search for CK receptor antagonists as well as CK transport mechanisms (Spíchal 2012).

2.3 Abscisic acid

Abscisic acid (ABA) is a sesquiterpenoid synthesized from xantophylls in all vascular plants but is also present in mosses and all algal classes (Rai et al. 2011). ABA represents a key signal that regulates plant development and growth as well as plant responses to various stresses. These diverse functions of ABA rely on complex regulatory mechanisms that control its production, degradation, signal perception, and transduction.

Genes of ABA biosynthetic enzymes are induced both under stress conditions (Tuteja 2007) and by sugars (Xiong and Zhu 2003), which allows plants to integrate signals from the outer and inner environment. ABA inactivation occurs either by oxidation, producing phaseic acid, dihydrophaseic acid and neophaseic acid, or conjugation with glucose, which produces ABA-glucose ester (Nambara and Marion-Poll 2005; Arc et al. 2013). Reverse hydrolysis of conjugates and release of active ABA takes place during dehydration stress conditions (Xu et al. 2012).

During seed development, ABA is known to initiate embryo maturation, synthesis of storage reserves and synthesis of seed proteins, such as the late embryogenesis abundant (LEA) class proteins, which function in desiccation tolerance of seeds (Chakrabortee et al. 2007). The induction of LEA protein synthesis allows embryos to survive in the extremely dry condition of desiccated seeds. This role of ABA is related to promotion of LEA-like proteins synthesis in vegetative tissues under dehydration stress (Xiong and Zhu 2003). In mature seeds, ABA stimulates dormancy and inhibits germination. In developing seeds ABA is either obtained from maternal tissues or is synthesized *de novo* in the embryo itself. Studies in *Arabidopsis thaliana* suggest that two peaks of ABA accumulation appear in the embryo during seed development (e.g., Finkelstein et al. 2002; Kanno et al. 2010). The first one is derived from maternal tissues and ABA promotes synthesis of storage proteins at this stage (Phillips et al. 1997). Maternal ABA can also stimulate its own biosynthesis in the embryo, as ABA is known to activate ABA biosynthetic genes (Xiong et al. 2001). This second peak of ABA accumulation induces the synthesis of LEA proteins and also initiates seed dormancy. The ABA level falls rapidly in the later stages of seed maturation and remains very low in dry *A. thaliana* seeds (Finkelstein et al. 2002). A similar pattern of endogenous ABA was described for coniferous seeds (Kong et al. 1997; Carrier et al. 1999).

In conifers, embryo maturation is initiated by arresting cell proliferation through the removal of auxins and cytokinins and by application of ABA (Stasolla et al. 2003). This external ABA is supposed to mimic the ABA signal coming from the maternal tissue during seed development. ABA is used not only to promote maturation of somatic embryos, but also to enhance somatic embryo quality by increasing desiccation tolerance and by preventing precocious germination (Rai et al. 2011). ABA is thus employed to induce somatic embryos to enter a quiescent state. Although ABA is commonly utilized to promote somatic embryo maturation in many coniferous genera, including *Picea, Larix*, and *Pinus*, the responsiveness of embryogenic cultures to ABA varies widely. Observed differences are not only species-specific, but also depend on the genotype (e.g., Stasolla et al. 2002). The level of endogenous ABA in somatic embryos is affected by its exogenous supply as demonstrated by Vágner et al. (1998) for *Picea abies* somatic embryos. The exogenous application of ABA in coniferous somatic embryogenesis is not limited to maturation; some researchers add ABA also into the induction medium. ABA alone is not able to induce the embryogenic culture, but in combination with auxins it can increase the number of successfully induced cultures (Pullman et al. 2003). Interestingly, in Angiosperms ABA can act also as a sole exogenous signal substance in SE induction, probably in cooperation with endogenous auxins (e.g., in stress-induced carrot seedling somatic embryogenesis) (Nishiwaki et al. 2000).

ABA influences the expression of a high number of genes, e.g., in developing *A. thaliana* seeds ABA induced about 10% of its genes (Nemhauser et al. 2006). The regulation of ABA signal transduction is realized mainly at the transcriptional level. There are three ABI (abscisic acid insensitive) transcriptional factors controlling ABA signal transduction in developing seeds – ABI3, ABI4 and ABI5, which were described for *A. thaliana* mutants germinating in the presence of ABA (Koornneef et al. 1984). Since then they were intensively studied, especially

in *A. thaliana* (e.g., Finkelstein et al. 2002; Lopez-Molina et al. 2002; Holdsworth et al. 2008; Reeves et al. 2011). It seems that these transcriptional factors act as nodes that interconnect ABA signal transduction with other phytohormones and with environmental signals (e.g., Brocard-Gifford et al. 2003; Chen et al. 2014a, b). In conifers ABI3 homologues were described for *Picea abies* (PaVP1; Footit et al. 2003) and *Callitropsis nootkatensis* (CnABI3; Lazarova et al. 2002). PaVP1 expression profiles in developing somatic embryos of *Picea abies* (Fischerová et al. 2008) were similar to those observed in developing *A. thaliana* seeds. Subsequent studies of CnABI3 revealed an ABI3 interacting protein (CnAIP2; Zeng et al. 2013a), the activity of which is under control of multiple hormonal signals (Zeng et al. 2013b). Future studies on the involvement of conifer ABI4 and ABI5 homologues in somatic embryo development will allow a better understanding of ABA signal transduction in this process.

2.4 Ethylene

Ethylene - the gaseous phytohormone is present in plant cells and tissues in low concentrations depending on its cytoplasm solubility. Most of the endogenous ethylene diffuses through the tissue and escapes into the atmosphere. The biosynthesis of ethylene starts by the conversion of L- methionine to S-adenozylmethionine and in the next step to the precursor of ethylene ACC (1-aminocyclopropane-1-carboxylic acid). This reaction is catalysed by ACC - synthase. Oxidation of ACC forms ethylene – the reaction is catalysed by ACC - oxidase. Both enzymes are encoded by a multigene family that has been investigated intensively – especially in *Arabidopsis* (Arc et al. 2013). The elucidation of the ethylene effect in developmental processes in plants was usually investigated using treatments with inhibitors of ethylene biosynthesis and with inhibitors of ethylene action. Kepczynska and Zielinska (2011) documented that in *Medicago* SE not only ethylene biosynthesis but also ethylene action is involved in the control of development. Most contemporary studies are focused on developing a better understanding of ethylene metabolism and signalling pathways in crosstalk with other phytohormones and/or other effectors.

Ethylene has a diverse effect on plant growth and development – it participates in stress responses, it is involved in ripening, flowering, aging and it regulates seed dormancy release and germination (Linkies and Leubner-Metzger 2012). The data available on zygotic embryogenesis suggest that there is an association of ethylene biosynthesis pathways and seed maturation (Matilla 2000). Corbineau et al. (2014) declared that ethylene is the key factor in the regulation of seed dormancy.

The impact of ethylene and ethylene inhibitors on conifer somatic embryogenesis was investigated during induction of embryogenic cultures as well as during maturation and germination. Kvaalen (1994) affirmed that ethylene and ACC are involved in the induction of spruce embryogenic culture and during the development of early somatic embryos. In experiments using *Pinus taeda* a difference between the effect of ethylene biosynthesis inhibitors and inhibitors of ethylene action was demonstrated.

Pullman et al. (2003) showed an increased initiation of embryogenic culture on medium supplemented with $AgNO_3$ (silver nitrate, an inhibitor of ethylene action). A negative effect of ethylene (or ACC) on secondary somatic embryogenesis was demonstrated by Saly et al. (2002) for hybrid larch. Investigation of ACC-synthase genes in embryogenic cultures of *Pinus sylvestris* (Lu et al. 2011) resulted in confirmation of the correlation between ethylene biosynthesis and embryo development. Expression of genes encoding ACC synthase corresponds with ethylene production and could serve as a genetic marker for early maturation.

Kong and Yeung (1994, 1995) demonstrated that there is a negative effect of ethylene during maturation of white spruce somatic embryos and simultaneously a stimulation of cotyledonary embryo formation after $AgNO_3$ treatment. Both effects were strictly dependent on endogenous ABA. El Meskaoui et al. (2000) confirmed these data. The ACC and ethylene enrichment inhibited embryo maturation and increased the browning of white spruce embryogenic cultures. They suggested that these effects are the result of interaction between ethylene and polyamines. Vágner et al. (1999) demonstrated that ethylene emanation decreased during maturation of spruce somatic embryos. A detailed proteomic study of oak somatic embryo development suggests that there are several steps of regulation by various substances – the increased level of ethylene in the advanced stages of oak somatic embryo development suggest that ethylene accumulation regulates embryo maturation (Gomez-Garay et al. 2013). The pool of ACC and ethylene production could be used as a marker of the embryogenic capacity of embryogenic cell lines of black spruce according to El Meskaoui and Tremblay (2001) since a higher embryogenic capacity correlates with lower ethylene production. On the contrary, in Brazilian pine higher values of ethylene production were observed in embryogenic cell lines that responded to maturation treatments than in lines in which embryo development was blocked (Jo et al. 2014).

The main attribute of ethylene action in plant development seems to be the crosstalk with other growth regulators. The complexity of its action was investigated for different developmental processes in various plants, from *Arabidopsis* to woody plants. The experiments were mostly focused on physiological processes that control seed dormancy and germination and coordinate regulation of embryo arrest or growth and maintenance or rupture of surrounding structures (Arc et al. 2013). The evidence of cross-talk regulation during dormancy breaking by ethylene and GA (gibberellins) was obtained by Calvo et al. (2004a, b) for beech seeds. According to their results the biosynthesis of ethylene is positively regulated by GA and cross-talk regulation by both phytohormones involved dormancy breaking and germination (Calvo et al. 2004a). The correlation between GA and ethylene biosynthesis was confirmed by Calvo et al. (2004b). The participation of ethylene, ABA and GA in regulation of endosperm weakening, which is at least partly based on evolutionary conserved mechanisms, was demonstrated by Linkies and Leubner-Metzger (2012). Linkies et al. (2009) found that in *Brassicaceae* endosperm cap weakening and rupture are promoted by ethylene and inhibited by ABA, but ethylene counteracts ABA-induced inhibition without affecting the ABA level in seeds. Ethylene promotes germination by acting as an antagonist of ABA. Experiments with *Arabidopsis*

plants provided information about the ethylene effect on ABA metabolism and signalling; simultaneously, ABA can inhibit the biosynthesis of ethylene (Arc et al. 2013). Ethylene and ABA signalling are also involved in defence against various pathogens (Chen et al. 2013).

During a response to stress and during other developmental processes ethylene can work in a synergistic or antagonistic way with JA (jasmonic acid) (Lorenzo et al. 2003; Lorenzo and Solano 2005). Staswick and Tyriaki (2004) found that ACC and JA form conjugates and they propose that synthesis of JA-ACC might provide the mechanism to regulate the conversion of both active phytohormones. On the contrary, an inhibitory effect of methyljasmonate on ACC conversion occurs before *Xanthium* seed germination (Nojavan-Asghari and Ishizawa, 1998). The direct ethylene - JA crosstalk was confirmed by Lorenzo et al. (2003) when they showed that ERF1 (ethylene response factor) is the key element in JA-ethylene interactions.

The crosstalk between ethylene and growth regulators of various characters (IAA, polyamines, brassinosteroides as well as glucose, oligosaccharides etc.) occurs in many developmental processes. It can be concluded that the simple chemical nature of ethylene contrasts strongly with its high regulatory complexity (Lin et al. 2009). The contemporary trends in ethylene studies focus mainly on the mode of the action of receptor systems in the control of nuclear transcription factors in ethylene signalling and on the molecular details of signalling convergence and synergism between ethylene and other phytohormones (Yoo et al. 2009).

2.5 Jasmonates

Jasmonic acid (JA) and its metabolites are lipid derived compounds acting as key signalling compounds in the response of plants to biotic and abiotic stresses (Linkies and Leubner-Metzger 2012) and generally in the developmental processes of plants (Wasternack and Kombrink, 2010; review Wasternack 2014). The jasmonate biosynthesis pathway was first described in *Vicia* by Vick and Zimmerman (1983). It is initiated in the chloroplasts with the conversion of α-linolenic acid to OPDA (12-oxo-phytodienoic acid) – the precursor of JA. JA can be converted into a variety of derivatives such as MeJA (methyljasmonate) or JA-Ile (jasmonoyl-L-isoleucine) (Browse 2009). Precursors as well as derivatives are biologically active (Wasternack 2007). JA, its metabolites MeJA, JA-Ile and its precursor OPDA are involved in counteracting biotic and abiotic stresses and in regulation of senescence, reproduction, pollen and embryo development etc. (Wasternack and Kombrink 2010; Linkies and Leubner-Metzger 2012). During plant development JA inhibits growth of roots, shoots and leaves. Seed germination and flower development are partially affected by its precursor OPDA (Wasternack 2014). Nevertheless, the role of jasmonates in seed germination seems to be crucial. JA and ABA together affected late stages of seed and embryo development (Hays et al. 1999). JA, ABA and SA (salicylic acid) are designated as stress hormones and are often used in plant tissue cultures, e.g., they promoted somatic embryogenesis and enhanced the quality of embryos in microspore embryogenic cultures of *Brassica* (Ahmadi et al. 2014).

JA and ABA exogenously applied exhibited a synergistic effect during embryo development and germination in *Brassica* and *Linum* cultures (Wilen et al. 1991). Exogenous jasmonate treatments regulated embryo development in various other plants, such as *Medicago* (Rudus et al. 2001), *Nicotiana* (Reinbothe et al. 1994). Madakadze and Senaratna (2000) investigated the effect of various phytohormones, including JA, on SE of geranium. JA and MeJA can prevent the precocious germination of *Brassica* somatic embryos (Wilen et al. 1991). JA and ABA levels increased during somatic embryo development in *Medicago* (Rudus et al. 2009). In developing embryos higher levels of JA, OPDA (and ABA) were detected than occurred in embryogenic tissue. Nevertheless, the effect of jasmonates in somatic embryogenesis of trees (conifers) has not yet been described.

The studies of the effects of jasmonates in trees and tree tissue cultures were focused rather on specific reactions linked with wounding, mycorrhizas and diseases and eventually with the special characteristics of the wood of *Quercus* (Moungsrimuangdee et al. 2011). JA was shown to be the important regulating molecule that appeared after wounding and pathogen or fungi attack, e.g., the first mycorrhizal contact of fungi with roots could be accelerated by JA treatment in spruce (Regvar et al. 1997). The data of Arnerup et al. (2013) suggest that JA-mediated signalling may be the prioritized module in the defence signalling network of spruce against fungi. The possible antagonism between JA and SA signalling was examined.

In most JA-regulated processes, the precise plant response is not activated by JA alone but is the result of a network of interactions between different signalling pathways. Different stimuli promote an asymmetric activation of these complex signalling networks, and the final balance of interactions determines the specific response to the initial stimulus, e.g., the developmental and stress responses of many plants require the coordinated interaction of the jasmonate and other signalling pathways - such as those for ethylene, SA and ABA (Lorenzo and Solano 2005). The complexity of jasmonate signalling and its cross-talk with other phytohormones provides a buffer system – crucial for plant development in a continuously fluctuating environment (Song et al. 2014).

In recent research the role of molecular mechanisms of GA and ABA in seed development, dormancy and germination has been studied intensively. The investigations are also focused on the effect of ethylene and ABA - ethylene interactions during germination as well as on the crosstalk between jasmonates and other phytohormones. Deeper understanding of the complex hormonal network and its interactions (including the effect of jasmonates) during seed (and embryo) development and dormancy is a prerequisite necessary for understanding successful regulation of plant seedling development (Linkies and Leubner-Metzger 2012). As ABA plays the key role in SE of many trees, including conifers, and since the influence of ethylene on the process of SE has been described, we should also consider the role of jasmonates and SA in this process. The hormone signalling pathways are not isolated but connected in complex regulatory systems (Lorenzo and Solano 2005) and thus it is reasonable to suggest that the interactions of signalling pathways of all these phytohormones – regulators of seed and embryo development as well as the regulators of stress reactions – are involved in the SE of trees.

2.6 Other plant growth regulators

The function of other plant growth regulators in SE has not been completely established yet. The role of gibberellins during SE was reviewed by Jimenez (2005), Moshkov et al. (2008) and Rose et al. (2010). The effect of exogenously applied gibberellins (GAs) on induction of SE is highly variable and depends on the species, tissues or endogenous levels of GAs (Jimenez and Bangerth 2001). SE was stimulated by exogenous gibberellic acid (GA3), for example in tissue cultures of *Medicago sativa* L. (Rudus et al. 2002), several rose cultivars (Kintzios et al. 1999, Li et al. 2002), *Gossypium* species (Sun et al. 2006), *Cocos nucifera* (L.) (Montero-Cortes et al. 2010) and *Magnolia obovata* Thunb. (Park et al. 2012). A promoting effect of GA was observed during in vitro germination of coconut embryos (Ake et al. 2007) and *Tylophora indica* (Thomas 2006). However, it appears that, for many species, SE is inhibited by exogenous GAs, for example, in *Arabidopsis* (Ezura and Harberd 1995), linseed (da Cunha and Ferreira 1997), *Geranium* (Hutchinson et al. 1997), *Centaurium erythraea* Gillib. (Subotic et al. 2009) and wheat (Miroshnichenko et al. 2009).

For coniferous SE, Pullman et al. (2005) found an improvement in the initiation of SE for several conifers by using paclobutrazol (an inhibitor of gibberellin synthesis) which indicates a negative effect of endogenous GA in embryogenic culture initiation. Krajňáková et al. (2013) demonstrated the importance of the timing of exogenous GA_3 application during the maturation phase of *Abies alba*. The addition of 10 μM GA3 at the beginning of the maturation phase had a negative effect on the development of somatic embryos and reduced the number of cotyledonary embryos. However, when GA3 was added later, for the last 6 weeks of maturation, the number of cotyledonary somatic embryos increased.

The role of GA (and ABA) during seed dormancy has often been investigated. GA releases dormancy, promotes germination and counteracts the ABA effect during dormancy (Kucera et al. 2005). The synergism between GA and ABA was demonstrated by Nolan et al. (2014) in *Medicago* SE. Detailed studies of the role of various GAs during the different steps of SE have not yet been published.

A possible effect of brassinosteroids (BR) in SE initiation of conifers was reported by Pullman and Bucalo (2011). The effect of BR during different steps of microspore embryogenesis of *Brassica* was investigated by Ferrie et al. (2005). They confirmed a positive effect of BR during early stages of embryogenesis, not during conversion of embryos into plantlets. However, Jiang and Lin (2013) reviewed the role of BR in seed development of *Arabidopsis* and concluded that there probably will be a considerable role of BR applications in future agricultural production. BR as well as strigolactones belong to the "new" phytohormones that have been discovered recently. Strigolactones control root formation in rice and their action is connected with auxins (Sun et al. 2015) and/or with ABA. The relation between stress conditions, ABA and strigolactones regulate root development in *Lotus* (Liu et al. 2015). Based on these results we can speculate that there is crosstalk between strigolactones, ABA and other phytohormones during SE but currently appropriate data dealing with the effect of strigolactones in SE are not yet available. Other regulators with specific effects on plant

development are also used during in vitro cultivation. For example, the application of salicylic acid as a stress related phytohormone was tested, e.g., during SE of *Arabidopsis* by Wojczikowska and Gaj (2015); during induction of microspore embryogenesis in *Brassica* (Ahmadi et al. 2014) and/or during SE of cotton (Kouakou et al. 2007). In suspension cultures of cotton the effect of various phenolic compounds, including salicylic acid and benzoic acid, was demonstrated. The effect of other substances on in vitro cell cultivation could be mentioned, e.g., arabinogalactan and haemoglobin proteins can enhance cell viability and proliferation in pepper cultures (Kaparakis and Alderson 2003). Haemoglobins are ubiquitous proteins present in plants and animals. They participate in regulation of induction and the first steps of somatic embryo development of chicory (Smagghe et al. 2007). Their positive effect on the SE process was also demonstrated, e.g., in cotton (Ganesan and Jayabalan 2004) or in peanut (Jayabalan et al 2004). The haemoglobin control of SE was often linked with the regulation of PCD (Hill et al. 2013; Huang et al. 2014).

To develop initiation and multiplication media that are suitable for conifer SE requires the optimizing of the redox state during early embryo development (analogous to during zygotic embryo development). The addition of special redox agents improved embryogenic culture initiation as well as the next stages of SE (Pullman et al. 2015).

The analysis of plant hormone action is progressing rapidly as illustrated, e.g., by the recent discovery of several plant hormone receptors. We can expect that important, as yet unknown growth regulators will be isolated in the future (such as the new phytohormones BR and strigolactones several years ago) (Caboche 2010). The cross-talks between known and as yet unknown phytohormones during regulation of developmental processes, including SE, remain to be elucidated. A successful step in that direction was initiated, e.g., by the presentation of a broader view of auxin/cytokinin cross-talk by Xu et al. (2013). They analysed the molecular mechanism of cotton SE regulated by auxin and cytokinin. They connected the primary role of phytohormones in SE regulation to other factors that influence the interactions between phytohormones during specific steps of embryo development. The development of protocols that will increase SE initiation and subsequent growth should as much as possible try to duplicate the environment found inside the seed (Pullman and Bucalo 2014).

3. Other substances that regulate somatic embryogenesis

3.1 Polyamines

Polyamines (PAs) are small aliphatic amines found in all living organisms. Their synthesis starts from two amino acid precursor molecules: L-arginine and L-methionine (Kusano et al. 2008). Three commonly occurring PAs in plants are diamine putrescine (Put), triamine spermidine (Spd) and tetramine spermine (Spm). All these compounds are present in the free form or as conjugates with other low molecular substances (e.g., phenolic acids) or macromolecules (proteins, nucleoproteins). They are found in cell walls, vacuoles, mitochondria, chloroplasts and cytoplasm (Kaur-Sawhney et al. 2003). Put, Spd and Spm are involved in

fundamental living processes such as gene expression, translation, cell proliferation and differentiation, membrane stabilization and modulation of cell signalling. A better understanding of the roles of PAs in various plant developmental processes can be achieved by precise investigations of biosynthetic pathways using biosynthesis inhibitors, PA mutants and/or various transgenic strategies (Bagni and Tassoni 2001; Kakkar and Sawhney 2002).

PAs play an important role in stress responses and diseases of plants indicating their importance for plant survival (Kusano et al. 2007). High endogenous PAs correlated with a higher tolerance against biotic and abiotic stresses (Kakkar and Sawhney 2002). Bouchereau et al. (1999) demonstrated changes in PA metabolism caused by environmental challenges; they concluded that stress tolerance can be associated with the production of conjugated and bound PAs and with stimulation of PA oxidation. Plants with a high content of PAs are considered to have higher nutritious value which is important for mammals; PAs were also specified as important regulators of developmental processes including floral and fruit development, senescence, organogenesis and embryogenesis.

PAs play a fundamental role in the regulation of somatic and zygotic embryogenesis (Kong et al. 1998; Silveira et al. 2004). The accumulation of high levels of PAs in somatic embryos contributes to their reserves of proteins and triglycerides, which are utilized during embryo germination. Minocha et al. (1999) demonstrated parallel morphological and anatomical patterning in the development of somatic and zygotic embryos and they established that the ratio Spd/Put determines embryo germination. The changes in PA level and/or the ratio of Spd, Put and Spm correlate with defined stages of embryogenesis in *Brassica* suggesting that PAs could perhaps serve as markers of embryogenesis (Puga-Hermida et al. 2003). A relationship between total content of free PAs and embryogenic potential was shown by Noceda et al. (2009) - a high content of free Put and Spd was found in non-embryogenic lines of *Pinus nigra*. A review dealing with the role of PAs during in vivo and in vitro development, including somatic embryogenesis, was published by Baron and Stasolla (2008).

The elucidation of the role of PAs in embryo development was investigated by using PA application, followed by evaluation of the changes in PA content and their link with PAs metabolism. These studies were done mostly during maturation and germination of embryos. Nevertheless, Vuosku et al. (2012) investigated PA metabolism in liquid pine embryogenic cultures and they focused their experiments on the effect of specific stressors acting in the course of early embryo development. They compared the role of Put, Spd and Spm during the interphase between initiation of embryogenic culture and proliferation. Their results suggest that Put participates in proliferation and that Spd plays a dual role as a protector against stress and is a suppressor of cell growth in proliferation. A link between the accumulation of Put and biosynthesis of Spd (and Spm) was demonstrated. The association of a high Put level with low cellular growth in embryogenic suspension cultures of *Pinus taeda* was shown by Silveira et al. (2004). Santanen and Simola (1992) compared the total PA level in embryogenic and non-embryogenic tissues of *Picea abies*. The total Spd level was higher in embryogenic cultures but the decrease of Spd after the application of an inhibitor of Spd biosynthesis did not affect the development of embryos.

The evaluation of the effect of PAs in maturation of embryos is not definite. According to Nakagawa et al. (2011) PAs can promote the development of early embryos of *Picea glehnii* and their maturation. Put and Spd application increased the maturation capacity and decreased the time required for somatic embryo formation. A positive effect of Spd on maturation of pine embryogenic cultures was shown by Niemi et al. (2007) and was confirmed by Santa-Catarina et al. (2007) who demonstrated that Spd and Spm promote somatic embryo maturation of *Ocotea catharinensis*. Nevertheless, PA application did not promote maturation of *Cryptomeria* embryos but L-ornithin (the precursor of PA biosynthesis) treatment had a stimulatory effect on their development (Nakagawa et al. 2006). Vondráková et al. (2015) treated the embryogenic cultures of *Picea abies* with Put during proliferation and maturation. They demonstrated an increase of free and conjugated PAs after Put application and stimulation of cell division in embryonal meristems. However the treated embryos were not able to release from polyembryogenic centres and in consequence the yield of malformed embryos was high. The results of El Meskaoui and Tremblay (2009) support the idea that PAs are essential for maturation of spruce somatic embryos. Spd and Spm application improved somatic embryo production and helped to synchronize maturation. They were also effective in reducing necrosis in white spruce embryogenic cultures. A positive effect of PAs often occurred during germination of embryos. Put promotes germination and the recovery to plantlets in cotton (Sakhanokho et al. 2005). Kevers et al. (2002) used Spm treatment for harmonious development of plantlets of *Panax*. Pieruzzi et al. (2011) showed that the ratio between Spm + Spd/Put is a marker for completion of germination. These results coincide with those of Shoeb et al. (2001) that show that PAs could serve as biomarkers for plant regeneration.

Changes in endogenous PA concentration during zygotic embryo development of *Ocotea catharinensis* were described by Santa-Catarina et al. (2006). Free Put concentration decreased and free Spm increased during embryo development suggesting that Put has a role in the initial phases of embryogenesis while the role of Spm is essential later. The differences in PA content in somatic/zygotic embryos of *Pinus* were measured by Minocha et al. (1999). During both somatic and zygotic embryo development the Spd content increased leading to a big increase of the Spd/Put ratio. Mature somatic embryos capable of germination have a higher Spd/Put ratio than abnormal embryos incapable of forming plants. The stage of embryo development can be characterized by changes in PAs and their biosynthetic enzymes rather than by the period of growth on maturation medium (Minocha et al. 2004). The PA content and activities of PA biosynthetic enzymes in Norway spruce somatic and zygotic embryos were studied in relation to anatomical changes during embryo development by Gemperlová et al. (2009). They found that the activities of PA biosynthetic enzymes steadily increase during the development of somatic embryos, from formation of the embryogenic suspensor mass until that of early cotyledonary embryos. In these stages the Spd level was significantly higher than Put. The biosynthetic enzyme activity subsequently declined in mature cotyledonary embryos, accompanied by sharp reductions in PA content. The start of germination was associated with a rise in PA biosynthetic activity which was accompanied by a marked increase in Put content. The accumulation of high levels of PAs in somatic embryos may be causally linked

to their germination ability. Higher concentrations of free PAs were commonly observed in somatic embryos than in zygotic ones. The link between a high level of ABA, a high PA content and low germination ability of somatic embryos was discussed by Gemperlová et al. (2009).

The role of PAs in plant development (including SE and ZE) was often connected with the actions of other phytohormones. Stasolla and Yeung (2003) demonstrated that ABA and physiological changes induced by ABA involves changes in PA biosynthesis in maturing somatic embryos of conifers. The interactions between ethylene and PAs were often examined but the results are not entirely conclusive; a relationship between ethylene and PA application was demonstrated by Roustan et al. (1994) in different steps of carrot SE and its importance during white spruce SE maturation was discussed later by El Meskaoui et al. (2000). Nevertheless, Quan et al. (2002) found no competition between ethylene and PA pathways in poplar. Mauri and Manzanera (2011) measured the production of ethylene in correlation with endogenous levels of PAs during SE of oak but they found no interference between the ethylene and PA content. A close ethylene and PA participation in another developmental process was demonstrated by Parra-Lobato and Gomez-Jimenez (2011), i.e.,, fruit abscission in olive. The interaction between PAs and auxins was shown, e.g., during the initiation of embryogenesis in *Panax* as the embryogenic process started on medium supplemented with auxin, a process that can be stimulated by Spd (Monteiro et al. 2002). The participation of PAs in vein definition in *Arabidopsis,* managed by polar auxin transport, was confirmed by Clay and Nelson (2005). A more complex study was done by Steiner et al. (2007) with *Araucaria* embryogenic cultures. They determined the effect of PA treatments on endogenous levels of phytohormones and demonstrated the occurrence of an increase in IAA and ABA content in embryogenic cultures just after the PA application and discussed the direct PA influence on ABA accumulation. The relationship between the CK content and PA biosynthesis was examined, e.g., by Danin et al. (1993) in celery embryogenesis.
PAs are essential for the growth and function of normal cells and their interaction with various macromolecules is having a variety of cellular effects that seem to be a characteristic feature of these PGRs. They are also implicated in the regulation of PCD. The direct effect consists in their association with particular biological processes, including a direct contribution to the molecular regulation of PCD. Indirectly PAs regulate PCD through their metabolic derivatives and catabolic products (Moschou and Roubelakis-Angelakis 2014). Recent data indicate that PA biosynthesis, conjugation, catabolism and transport modulates the homeostasis of PAs in plants due to regulation of endogenous PA levels and that they simultaneously actively participate in, e.g., the action of PA transport in stress tolerance, PA-dependent transcriptional and translational modulation of genes and transcripts etc. (Tiburcio et al 2014).

The regulation of PA content within cells occurs at several levels including transcription and translation (Wallace et al. 2003). Elucidation of crosstalk of PAs with other growth regulators as well as the investigation of molecular aspects of PA metabolism and action in plants serves to advance the understanding of in vivo and in vitro plant growth and development, including embryogenesis. It is expected that this will result in future large scale biotechnological applications that are of

agronomic/economic importance (Baron and Stasolla 2008). Currently a broad perspective of the value of PA investigations is presented in several reviews; the basic contributions to the elucidation of PA action during essential biological processes were summarized by Alcázar and Tiburcio (2014).

3.2 Phenolic compounds

Phenolic compounds (PCs) represent a huge group of secondary metabolites broadly distributed in the plant kingdom. The chemical structure of PCs is based on an aromatic ring that bonds one or more hydroxyl groups. Phenolics are classified as simple phenols or polyphenols according to the number of phenol units in the molecule. Therefore phenolics show large diversity of structures and their classes consist of thousands of unique compounds fulfilling specific roles in the course of plant life (Khoddami et al. 2013). PCs are derived from the combination of the shikimate pathway with the phenylpropanoid pathway and the acetate/malonate derived "polyketide" pathway which represents the flavonoid biosynthetic pathway (for details see Quideau et al. 2011 and Cheynier et al. 2013). Accumulation of phenolics in plant tissues as a response to plant stresses is due to the increased activity of phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS), key enzymes of these pathways.

The family of PCs abounds with members; the three most important groups will be discussed: phenolic acids, flavonoids and tannins. Phenolic acids (PhAs) represent one of the main groups of phenols occurring in the form of esters, glycosides or amides. They differ in the number and position of hydroxyl groups on the aromatic ring. PhAs are derived from hydroxycinnamic acid (e.g., ferulic, caffeic, coumaric, and sinapic acids) or from hydroxybenzoic acid (e.g., gallic, vanillic, syringic and protocatechuic acids) (Dai and Mumper 2010). Salicylic acid takes a special position and can be formed either from cinnamic acid or from benzoic acid depending on the plant species or on instant needs of the plant caused by pathogen attack (An and Mou 2011). Hydroxycinnamic acid derivatives can be reduced and thus provide aldehydes. Their further reduction gives monolignols, including coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. These monomers are polymerized to generate various forms of lignans, lignin and suberin, important components of plant cell walls (Quideau et al. 2011).

The largest phenolic class consists of flavonoids which are responsible alongside carotenoids and chlorophylls for many colours of plant tissues, particularly of flowers. Flavonoids thus play a significant role as attractants of the pollinators (Harborne and Williams 2000). Their basic structure is the flavone nucleus containing 15 carbon atoms arranged in three rings, which are called A, B, C. Flavonoids are divided into six subgroups according to the oxidation state of the central ring C: flavones (e.g., luteolin), flavonols (e.g., quercetin, kaempferol), flavanols (e.g., (epi)-catechin, epigallocatechin), flavanones (e.g., naringenin), isoflavones (e.g., genistein) and anthocyanins (Dai and Mumper 2010).

Tannins are polyphenols, which can form complexes with proteins, metal ions and polysaccharides (Schofield et al. 2001). Tannins can be divided into three groups: hydrolysable tannins, condensed tannins also called proanthocyanidins and phlorotannins. Hydrolysable tannins are compounds containing sugar (typically a

glucose unit) in the central core, to which motifs derived from gallic acid or ellagic acid are esterified. Hydrolysable tannins have a very restricted taxonomic distribution. Condensed tannins are oligomers or polymers of flavonoid units (flavan-3-ol) linked by carbon bonds. They are abundant in woody plants, but less common in herbaceous plants. Phlorotannins are derived from phloroglucinol and were found in red-brown algae (Haslam 2007; Quideau et al. 2011).

Plants as sessile organisms are exposed to manifold biotic and abiotic stresses which they have to overcome. Since defence costs plants a large quantity of energy, only some defence strategies are expressed constitutively under normal plant growth. These defences are present in the plant without any challenge, while inducible defence mechanisms are generated upon stress conditions (Franceschi et al. 2005). Plants synthesize PCs partly constitutively and partially as a response to stress. Phenolics (especially flavonoids and proanthocyanidins or salicylic acid) play a key role in plant photoprotection and in defence against microbial infection and attacks of herbivores.

The role of flavonoids in the plant's defence against injuries by light, especially by UV-B radiation is widely accepted. Flavonoids are mostly located in the cell walls and vacuoles of epidermal cells and in trichomes but they also occur in various compartments of leaf mesophyll cells, such as the vacuole, chloroplast and nucleus. Rather than being UV - absorbers (for this role hydroxycinnamates located in vacuoles or cell walls have better UV-B screening capacities) they serve as reactive oxygen species (ROS) scavengers (Agati et al. 2012; Agati et al. 2013).

Flavonoids fulfil numerous biological roles other than those attributed to their potential cytotoxicity and their ability to scavenge free radicals. They are known as signalling molecules which participate in allelopathic plant-plant interactions. Root exudates can be phytotoxic to other plants and inhibit their growth and seed germination (Treutter 2006). However, flavonoids in roots are important for *Rhizobium*/legume symbiosis, since they stimulate the synthesis of Nod factors in the infection tube. Flavonoids also serve as signalling molecules between plants and arbuscular mycorrhizal fungi and are known to stimulate mycorrhization (Cheynier et al. 2013). Flavonoids can modulate essential physiological processes. Interestingly, they also can affect the metabolism of auxin. Monohydroxy B-ring flavonoids are suggested as cofactors of IAA oxidase, contrary to the dihydroxy B-ring flavonoids which inhibit the degradation of IAA (Mathesius 2001). The flavonoids quercetin, apigenin and kaempferol can modulate polar auxin transport as they bind to a plasma membrane protein known as NPA receptor (Peer and Murphy 2007).

Flavonoid oxidation is related to plant defence mechanisms and is accompanied by browning of plant tissues (Pourcel et al. 2007). In these processes two major groups of enzymes are involved: the diphenol oxidases (catecholases and laccases) catalysing the oxidation of PCs to the corresponding quinones in the presence of molecular oxygen and the peroxidases catalysing the oxidation of phenols in the presence of hydrogen peroxide. Nevertheless, the presence of these three types of enzyme in plants can be species specific. For example, *Arabidopsis* possesses only laccase (Pourcel et al. 2005). Quinones are highly reactive and undergo further non-enzymatic reactions. They polymerize with themselves or with amino acids or proteins, yielding complex heterogeneous structures of brown,

black, red or even blue colour (Walker and Ferrar 1998; Pourcel et al. 2007). Plant tissue browning is a well- known problem of fruit and vegetable food producers, appearing during storage or after tissues disintegration and is one of the main causes of quality loss (Cheynier 2012).

Nevertheless, flavonoid oxidation performs physiological roles in the course of plant development and growth. During the desiccation phase of pea or cotton seeds flavonoids that had accumulated in the seed coat are oxidized; brown complexes of polymerized quinones harden the seed coat and increase its water-impermeability (Werker et al. 1979; Halloin 1982). Seed coat browning caused by the laccase oxidation of epicatechin and proanthocyanidins was also studied in *Arabidopsis* during the desiccation phase (Debeaujon et al. 2003; Pourcel et al. 2005). The role of quercetin as an antimicrobial agent in the outer dried scales of onion bulbs is discussed by Takahama (2004). Yellow onion bulbs are known to be resistant to pathogens, contrary to the white bulbs. The dried scales of yellow bulbs contain 3,4-dihydroxybenzoic acid, an oxidative product of quercetin (Takahama and Hirota 2000; Takahama et al. 2001).

Normally, in healthy cells or tissues, enzymes and their substrates are usually separated in different compartments. Because of this separation the enzyme is inactive, the substrate is in precursor form or co-substrates are not present. Catecholases are enzymes localized in plastids (Chevalier et al. 1999); laccases are probably secreted to the apoplast (McCaig et al. 2005); peroxidases are classified according to their localization. Class-I peroxidases using ascorbate as the electron donor are localized in chloroplasts, cytosol or peroxisomes. These peroxidases are the scavengers of H_2O_2 produced in these organelles. Class III peroxidases, using phenolics as preferential electron donors, are localized in vacuoles and in the cell wall. These peroxidases participate in the modification of the cell wall, since they are implicated in the formation of lignin and suberin, cross-linking of cell wall components and cell elongation; these peroxidases also can participate in the process of tissue browning (Takahama 2004; Passardi et al. 2004). PCs (like anthocyanins, flavan-3-ol monomers, condensed tannins and glycosylated flavonols) are usually sequestered in the vacuole (Walker and Ferrar 1998; Pourcel et al. 2007) or in the cell wall (Takahama 2004).

Aging of the tissues or wounding or attack of pathogens can cause disorganization of tissue or cell structures when enzymes, substrates (phenolics) and co-substrates (O_2 or H_2O_2) meet in the same space. This destruction of the natural biological barriers between enzymes and substrates lead to non-enzymatic autooxidation or enzymatic oxidation of phenolics by molecular oxygen, diphenol oxidases or peroxidases (Pourcel et al. 2007). Tissue browning can then occur. This process may in fact be a part of the plant´s first line of defence, since quinones, as the products of oxidation of constitutively produced PCs, are potentially toxic for insects or pathogens. For instance, catecholase released from one type of trichomes of *Solanum berthaultii* catalyses the oxidative polymerization of secreted components on the mouthparts and tarsi of the insect, thus leading to its death (Kowalski et al. 1993). Products of the polymerization of quinones at the site of infection or wounding can also form a physical barrier to further infection (Walker and Ferrar 1998).

Salicylic acid (SA) plays an important role in many physiological processes, among them the plant immune response is of outstanding significance. When plants are subjected to attack by a microbial pathogen, they are able to recognize signals from injured cells and activate the immune response through a SA signalling cascade. SA interacts with other phytohormones, such as JA, ABA, ethylene, auxin, GA, CK and others (for details see An and Mou 2011).

A high content of PCs and their exudation into the cultivation medium is considered to be one of main limits of in vitro propagation of woody plants (Winkelmann 2013). Browning of tissue cultures is caused by oxidation of phenolics after disorganisation of the cells of primary explants which are rich in tannins (like those of *Quercus* or *Pinus* species) or it can be caused by culture aging when cells are dying and their compartments disintegrate. Callus cultures derived from shoot tips of mature Scots pine (*Pinus sylvestris*) grow very slowly and are turning brown within 2 or 3 weeks of culture. This browning interrelates with increasing activity of peroxidase in callus tissue. Nevertheless, browning is also a typical feature of embryogenic and non-embryogenic cultures of Scots pine, initiated from immature zygotic embryos (Laukkanen et al. 1999). Microscopical observations showed the occurrence of alterations in the cellular structure of browning callus tissue, such as accumulation of PCs and lignification of cells. Cell death is a consequence of these processes and creates a serious hurdle for Scots pine propagation by tissue culture (Laukkanen et al. 2000). Tissue browning is considered as a major factor in the recalcitrance of date palm (*Phoenix dactylifera*) to tissue cultures. Enhanced tissue browning is caused by increasing phenolic contents and peroxidase activity during long subculture intervals. Shortening of these intervals together with a lower concentration of the cytokinin BAP in the cultivation medium helped to reduce the tissue browning and thus overcome the impediment to the proliferation of embryogenic cells (Abohatem et al. 2011). Schnablová et al. (2006) have shown that overproduction of endogenous cytokinins in transgenic tobacco caused over-accumulation of phenolics and an increase in peroxidase activity and lignin content.

Micropropagation of woody plants can be limited not only by oxidized polyphenolic compounds, but also by phenolic acids present in the tissue. Cvikrová et al. (1998) studied two types of sessile oak (*Quercus petraea*) somatic embryos with a different ability to convert into plantlets. Besides determining the content of endogenous ABA, polyamines and aromatic monoamines the authors also analysed the content of PhAs, lignin and peroxidase activity. Embryos that were able to convert to plantlets possessed a lower content of total PhAs (the sum of free, soluble ester– and glycoside-, and insoluble cell wall-bound PhAs) than non-converting embryos. Interestingly, differences have been found in the spectrum of the PhAs that were found. Whereas ferulic acid, together with *p*-coumaric and vanillic acids dominated in cell walls of converting embryos, the contents of sinapic acid and its esters and glycosides were higher in non-converting embryos. In these embryos a higher content of lignin and an increased activity of peroxidase were observed as well. Ferulic acid in cell walls of converting embryos may participate in the restriction of cell elongation, which is important for cell division, while sinapic acid as a precursor of monolignol sinapyl alcohol may promote lignification. Composition and content of PhAs also played a significant role in the

propagation of elm (*Ulmus glabra*) (Malá et al. 2006). Primary elm cultures were formed by excised apical meristems of dormant buds. Multiplied shoots obtained during organogenesis were excised, cut into two parts – apical and basal – and cultivated separately either on shoot multiplication medium or used for rooting. The shoot–forming capacity was higher in apical than in basal parts, while root formation was delayed in the apical part contrary to its occurrence in the basal parts. These effects were connected not only with higher amounts of the polyamines Put and Spd in the apical parts, where tissues are generally less differentiated, but also with higher contents of endogenous auxin IAA and glycoside-bound *p*-coumaric acid, ferulic and sinapic acids in the basal parts. Caffeic acid was the predominant PhA in both types of explants. The authors discussed the role of phenolics in the catabolism of auxin. According to Volpert et al. (1995) caffeic, ferulic and sinapic acids exhibit in vitro IAA protection against peroxidase oxidation; these PhAs may thus positively influence the hormonal balance required for root formation. These PhAs are precursors for lignin biosynthesis as well. Initiation of lignification occurs during root induction in micropropagated walnut shoots (Bisbis et al. 2004). These findings indicate that phenolic compounds present in the primary explants or in the medium are not always deleterious compounds for in vitro propagation of woody plants. Reis et al. (2008) studied the effect of caffeic acid and phloroglucinol added to the cultivation media during induction of somatic embryos of pineapple guava (*Feijoa sellowiana*). These phenolics at low concentrations significantly increased the rates of embryo induction and of embryo germination. The effect of phloroglucinol was more obvious than that of caffeic acid. Analyses of the contents of endogenous PC showed that gallic acid derivatives and flavan-3-ols were produced in cultures cultivated on medium supplemented with phloroglucinol, while flavones and dihydroflavonols were present in embryos induced on caffeic acid-containing medium. Flavones were the main phenols detected in the control. Histological and ultrastructural studies showed the presence of cells with vacuoles filled with PCs during formation of somatic embryos. Later on during embryo development these cells form a barrier zone between the maternal tissue and the embryos. Ultrastructural changes in the phenolic-rich cells showed signs of degeneration. The authors proposed that these cells might undergo the process of programmed cell death (PCD) to separate the embryo proper from the maternal tissue. Association of early stages of somatic embryo differentiation with phenolic-rich cells was shown in other woody plants, such as myrtle (Canhoto et al. 1999a), bay laurel (Canhoto et al. 1999b) and carob (Canhoto et al. 2006).

Accumulation of phenolics in cells somehow associated with developing somatic embryos seems to be a feature not only of Angiosperm species but of Gymnosperm species as well. Gutman et al. (1996) reported the presence of polyphenolic compounds, particularly monomeric flavonols, catechins and polymeric proanthocyanidins in the proximal suspensor cells and basal cells of the embryo proper in all stages of larch somatic embryo development. Later on polyphenols were detected in the cells of the root cap of matured embryos. Similarly, PCs were observed in the same regions of spruce somatic embryos (Eliášová et al., non-published data). The question is if these phenolic-rich cells are of similar nature as the ones separating the embryos from explants in feijoa (Reis et al. 2008) and if they have a role in the separation of conifer somatic embryos from

the rests of the embryogenic suspensor mass. It remains also unclear if we can relate the presence of phenolic-rich cells to the gradual process of PCD in suspensor cells which was well described for somatic embryogenesis of conifers (Smertenko et al. 2003; Bozhkov et al. 2005; Smertenko and Bozhkov 2014). Accumulation of PCs in the cells on the embryo surface seems to be a feature of embryos growing under inappropriate conditions, such as a lack of ABA in the maturation medium (Gutman et al. 1996; von Aderkas et al. 2002) or when maturation is extended too long and malformation of embryos occurs (Svobodová et al. 1999). Accumulation of PCs in larch somatic and zygotic embryos is also affected by light and partially by ABA (von Aderkas et al. 2015). Somatic embryo development can proceed in either light or dark conditions. Nevertheless, mature light-treated embryos produced a higher amount of phenolics than embryos developing in the dark. Light conditions induced red colouring of somatic embryos, especially in part of the root cap. The flavonoid quercetrin has been identified as the dominant phenolic compound in these embryos. Embryos cultivated in the dark did not possess the red colour. Zygotic embryos that develop in cones were devoid of phenolics. However, when cultivated in light on medium with ABA (which prevents germination) the zygotic embryo root caps turned slight red and cotyledons with hypocotyls turned green within several days. Interestingly, zygotic embryos placed on ABA-free medium in the light germinated quickly and began to accumulate phenolics within a day in both hypocotyls and root caps. The phenomenon of red colouring of spruce somatic embryo root caps also occurred during desiccation without medium in the light (Eliášová et al. non-published data). Gutman et al. (1996) and von Aderkas et al. (2002) also studied the histology of larch somatic embryos. They mentioned the presence of long idioblastic cells in the subepidermal and pith region or in cotyledons in which phenolics can eventually accumulate. Similar cells were observed in somatic and zygotic embryos of Norway spruce as well (Eliášová et al. non-published data). Woodenberg et al. (2014) reported the existence of idioblasts with tannin content called tannin channels in the periphery of zygotic embryos of the cycad *Encephalartos natalensis*. These findings could indicate a role of flavonoids and proanthocyanidins in plant photoprotection and defence against pathogens or insects.

The importance of the role of JA in plant defence has already been mentioned above. Nevertheless, jasmonates are also known as elicitors of plant secondary metabolites. Many transcription factors (TFs) have a role in the JA-modulated regulation of metabolism. Members of the MYB TFs family are involved in the regulation of metabolic pathways of many phenolics, such as anthocyanins, proanthocyanidins, flavonols and lignins (De Geyter et al. 2012).

4. Concluding remarks and perspectives

The similarities between the developmental stages of zygotic and somatic embryogenesis make somatic embryogenesis an attractive system for the study of embryogenesis. In future, the importance of model species like *Arabidopsis thaliana*, its mutants, and *Picea abies* will increase with a mounting understanding of the mechanisms that accompany formation of their SEs.

In the future, molecular and genetic approaches will be employed to analyse gene regulatory mechanisms involved in the cellular origin of regenerated organs or SEs when these are under hormonal regulation. In addition, high-resolution data sets from live imaging and histological analysis will be used to validate the cytological basis of specific cells during organ regeneration while this regeneration is being regulated by distinct hormonal interactions. The principles revealed by such approaches may be critical to an understanding of the hormone-regulated plant regeneration processes. This would include the complex network of interactions and mechanisms that facilitate crosstalk between ROS and hormone signalling. They should also assist in the study of in vivo plant development (Yang and Zhang 2010, Sugimoto et al. 2011, Xia et al. 2015).

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Totipotency as a form of diversification in a gymnosperm artificial sporangium

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Abstract

Embryonal initials of conifers in an aqueous artificial sporangium initiate apomixis and discharge "spores" that develop into somatic embryos. Other initials revert to meiosis (automixis) and discharge microspores in a display of heteromorphic asexual heterospory. Initials failing these forms of diversification are silenced and dispersed as micronucleated cells or undergo apoptosis and provide a source of necrohormones and renewed substrates. Multi-nucleated eggs of *Taxus brevifolia* and *Ephedra californica* are transdifferentiated into oögonial tubes forming cells that were discharged as mitospores. The formation of cells within a cell represents a symbiotic form of diversification in the model for plant cell totipotency. The bringing forward of apomixis, microsporogenesis and mitosporogenesis to an earlier stage in a life history is known in plant evolution as progenesis. Diversified products in an artificial sporangium responded in a fundamental way like ancestral unicellular Algae to environmental conditions. Symbiotic expressions of cell totipotency may be used to diversify and capture genetic gains for silvicultural practices in boreal forests faced with climate change.

Keywords: totipotency, artificial sporangium, apomixis, meiosis, apoptosis, mitosporogenesis, somatic embryogenesis, genome silencing, symbiosis, progenesis, evolution

1. Introduction

It was known that the line of gravity had no influence on the developmental process of a pine embryo collected from tree seed (Wakayama 1929). The orientation of the egg was not even disturbed by inverting the position of the cone when compared to a control. After fertilization, four free nuclei passed

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to the base of the egg without regard to their geotropic response. The elongation of the suspensor was not affected with regard to the action of gravity.

Unit gravitational forces acting on eggs and single suspended cells in an aqueous culture medium are significantly reduced. FC Steward et al (1964) demonstrated that large populations of phloem cells of the wild carrot suspended in an aqueous medium developed into somatic embryos. When embryos were removed from nippled flask they developed into plants in soil where they flowered, produced seeds and completed their life cycles.

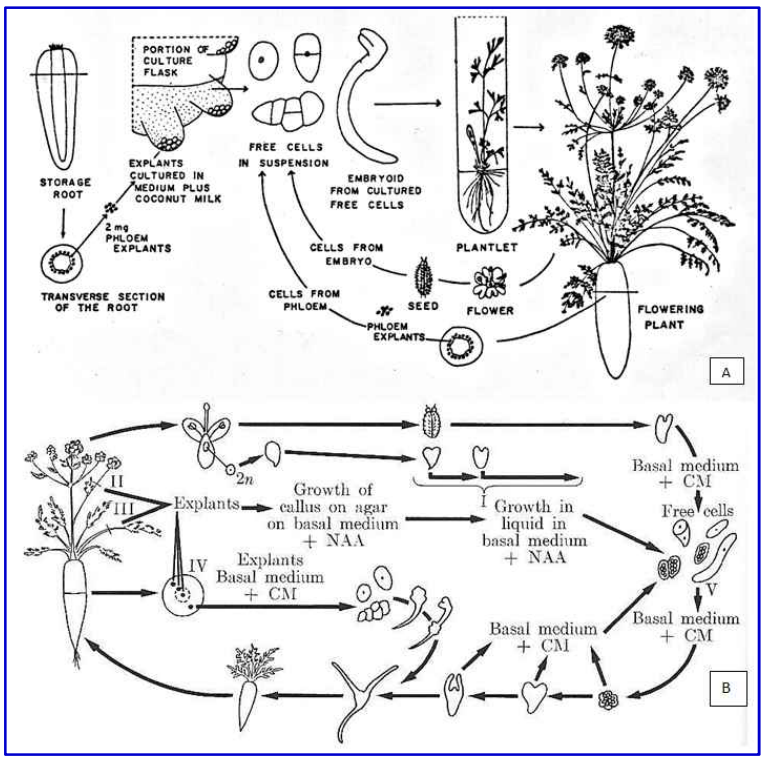

Figure 1. ***A*** *Experimental proof of plant cell totipotency (Steward et al.1964).* ***B*** *Diversified expressions of cell totipotency in carrot cells included "unusually large uninucleate cells with very conspicuous cytoplasmic strands"... They "were among the first to be observed in this freely suspended state". Their development "may continue until a densely packed mass of small cells has been formed which fills the original lumen of the large cell..." (Mitra et al. 1960). The culture medium contained coconut milk (CM) and naphthalene acetic acid (NAA). The cyclic production of small cells within the lumen of a large cell (bottom right) represents a latent form of "diversification" in totipotent cells (Steward 1968, pp 478, 485).*

Tissues from the flower, seed and phloem of the root of the cloned plants were again used to re-establish new cell suspensions. These cells formed somatic

embryos and again regenerated flowering plants that completed their life cycles in soil. Repeated iterations of this sequence demonstrated that plant cells are "totipotent" (Figure 1 A). Without expressions of totipotency, somatic embryos currently used to capture genetic gains would not be able to complete their life histories.

The living together of a cell or group of cells within a single cell becomes "symbiotic" when such a condition is of mutual advantage (Figure 1 B). Diversifications, arising from symbiotic cells, are now recognized as an untapped source of evolutionary innovation (Kliers and West 2015, Zehr 2015) and are addressed in this review.

Apomixis is known in conifers (Dogra 1967, 1984, 1978, Orr-Ewing 1957a, b). It is defined as the replacement of sexual reproduction by various types of asexual reproduction which does not result in fusion of gametes (Rieger et al. 1976). This review summarizes how symbiotic expressions contribute to apomixis, androsporogenesis and genome silencing in embryonal initials from Norway spruce (Finland), Douglas-fir (USA) and *Araucaria angustifolia* (Brazil). These forms of asexual reproduction and sporogenesis led to the designation of nippled flasks and a Multigen bioreactor as an "artificial sporangium" (Durzan 1996a, 2011, 2012, 2013).

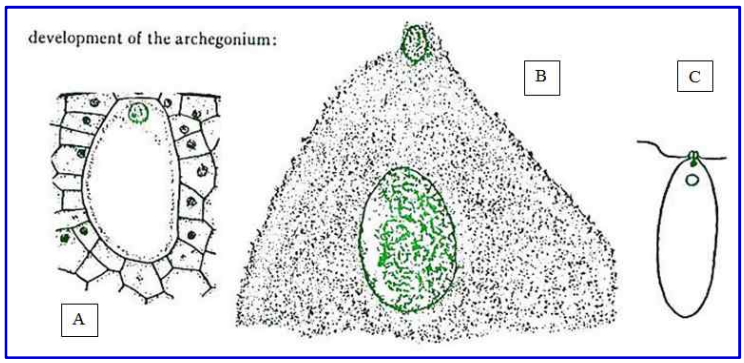

Figure 2. *Development of the archegonium in a Norway spruce tree (Håkansson 1956).* ***A*** *A large central cell is close to the neck of the archegonium.* ***B*** *Failure of the first or second meiotic division of the central cell nucleus results in the formation of a large restitution nucleus and small ventral canal nucleus (Andersson 1947). The ventral canal nucleus undergoes programmed cell death (apoptosis).* ***C****. Avoidance of meiosis under field conditions is called "asyndesis." Apomixis involving diploid parthenogenesis was regarded as absent from the group (Mogie 1952).*

Embryonal initials bypassing embryogenesis and expressing apomixis requires that they be neo-functionalized and transdifferentiated into archegonial tubes with a diploid egg-equivalent nucleus and an apoptotic ventral canal nucleus.

The egg-equivalent nucleus forms a neocytoplasm and a thin cell wall forming a symbiotic cell within a tubular archegonium (Figure 2). Diploid egg-equivalent cell were discharged into the culture medium where they rapidly developed into free-nuclear proembryos and in most cases early embryos (Durzan et al 1994, Durzan 2011, 2012).

When food supplies declined in the culture medium, other embryonal initials reverted to meiosis (automixis) in neo-functionalized and transdifferentiated androsporangial tubes. Androsporogenesis is another term for microsporogenesis. Products of meiosis were discharged as monads, dyads, triads, and pentads as "androspores" into the culture medium in a display of heteromorphic asexual heterospory. Sterling (1963) suggested that the microspore be called an embryonal cell but his view was strongly disputed. We now know embryonal initials of conifers are capable of expressing both apomixis and automixis in a display of heteromorphic asexual reproduction.

Embryonal cells failing heterospory developed bouquets of DNA on nuclear membranes and dispersed micronucleated cells in the culture medium. Cytomixis is the extrusion or passage of chromatin and DNA from one cell into the cytoplasm of another cell. It would represent an example of terminal diversification in Steward's concept of totipotency. Cytomixis is known in meristematic, tapetal, nuclear, ovary and in pollen mother cells (Rieger et al 1976).

The life cycles of gymnosperms have a haploid and diploid phase. Eggs of *Taxus brevifolia* and *Ephedra californica*, when cultured in nippled flasks, were rapidly neo-functionalized and transdifferentiated into oögonial tubes. Their free nuclei were replicated as mitospores with cell walls "within the lumen of a large cell" before being discharged into the aqueous culture medium (mitosporogenesis) (Durzan 2012, 2013). A mitospore is any haploid or diploid cell resulting from mitotic divisions that undergoes mitotic growth to produce the kind of organism from which it arose (Rieger et al 1976).

Stebbins (1950) pointed out that "the rates of evolution are very diverse, not only in between different groups of organisms living in different environments or even in the same environment but also between the same line at different periods in its evolutionary history and in between different parts of the same organism. Evolutionary plasticity depends on the presence in the population of genetic variance in adaptive traits that are available for natural and artificial selection to act upon".

All diversifications in an artificial sporangium were classified according to reproduction in Algae (Maggs and Callow 1992). The fact that latent expressions of heteromorphic asexual reproduction and mitosporogenesis were brought forward and expressed in embryonal initials and eggs in an aqueous environment when model-referenced to Algae addressed an old but fundamental question on the

evolution and the alternation of haploid and diploid generations in plant life cycles. "When the higher plant in which a regular alternation of generations was established returned to the unicellular condition (spore, zygote) how far did it become capable of responding in a fundamental way like its remote ancestors the unicellular Algae to environmental influence?" (Bower1908).

In plant evolution, the bringing forward of plant reproduction as a form of diversification to an earlier stage of development is called "progenesis". Progenesis is most notable during the appearance of the seed habit in flowering plants (Mogie 1992). Its interpolation is postulated as accounting for the origin of the land plant sporophyte (Hemsley1994). It is also evident in diversified expressions of totipotency in an artificial sporangium.

2. Parthenogenetic apomixis in Norway spruce

Parthenogenesis is the production of an embryo from a female gamete without the assistance of a male gamete, with or without development into an adult. One of several conditions leading to non-recurrent expressions of parthenogenesis involves the propensity of the genotype to show polyembryony (Darlington 1958). The term "nonrecurrent" signifies that the expression of parthenogenetic apomixis was latent and not a fixed feature in development. It is classified according to the mode of reproduction, the mechanism of sex determination and on cytological data.

Four conditions have to be met for expressions of parthenogenesis in plants (Mogie 1992). First, the capacity for parthenogenesis must be present. Second, this capacity must be allowed expression by preventing male gametes from fertilizing the eggs. Third, the eggs must exhibit the same ploidy level as the mother, so meiotic reduction during egg production must be avoided. Fourth, the first three conditions must be met almost simultaneously.

Each of the four conditions is deleterious when expressed in the absence of the others. If more than a single mutation is required for the first three conditions, the mutations must become phenotypically expressed simultaneously, or at the very most within a few mitotic generations of each other.

All four conditions for the development of an archegonium were met in suspension cultures of Norway spruce embryonal initials expressing latent diploid parthenogenesis and monozygotic cleavage polyembryony (MCP) (Durzan et al 1994). Observations were model-referenced to archegonial development in Norway spruce (Håkansson 1956) (Figure 2). MCP is not commonly observed in Norway spruce (Dogra 1967) and Douglas-fir seeds (Orr-Ewing 1957a, b). Apomixis was expressed in embryonal initials at 22 to 25 ^{o}C in one-liter nippled flasks and in a 1.4 L Multigen bioreactor (Figure 3A). The mean temperature for apomixis in Norway spruce in Finland was 16 ^{o}C (range 5 to 30 ^{o}C). Experiments relating to apomixis in my laboratory were repeated at 14^{o}, 23^{o}, and 25^{o} ± 2^{o}C. The lowest

temperature represented a rough estimate of the mean global temperature of earth in Mesozoic times (D. I. Axelrod, personal communication). The highest temperature is an estimation of the tropical mean annual temperature in the Cretaceous (Stewart and Rothwell 1993).

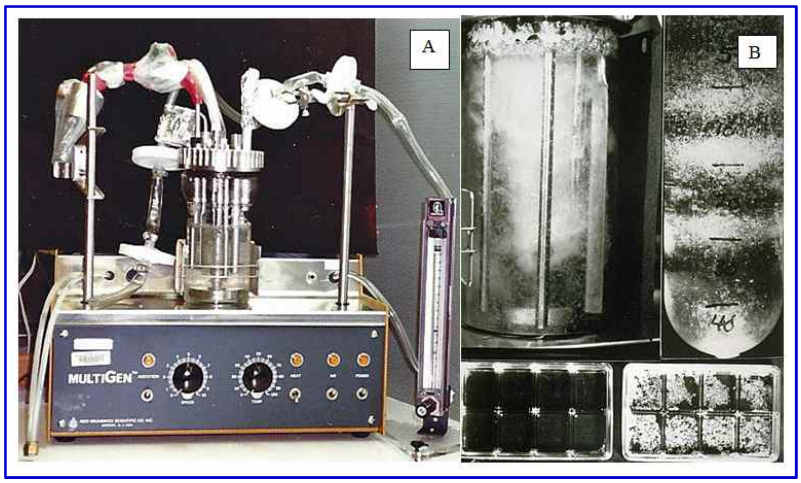

Figure 3. ***A*** *Multigen 1.4 L bioreactor enabled the scale-up embryonal suspensor masses undergoing cleavage polyembryony under prescribed, controlled environmental and nutritional conditions. Levels of 2,4-D, myo-inositol, casein hydrolysate (low sodium) and abscisic acid in the culture medium were optimized (Durzan et al 1994). After two weeks, rapidly growing and cleaving proembryos were wound around a propeller (1 rpm).* ***B*** *Products of apomixis, androsporogenesis, proembryogenesis, genomic silencing and apoptosis (cell death) were fractionated and collected according to their buoyant densities. Products were cytologically examined and evaluated for their developmental expressions in multiwell plates.*

Embryonal initials and their products in nippled flasks designed by Steward and in a Multigen bioreactor (Figure 3A) were fractionated by their buoyant density into size classes and collected for cytological examination in multiwell plates (Figure 3B). The developmental fates of embryonal initials were model-referenced to archegonium development and reproduction in Norway spruce (Håkansson 1956).

Assays revealed that many embryonal initials neo-functionalized into central cells with a large nucleus that divided and formed an egg-equivalent nucleus and a small ventral canal nucleus in a tubular cell. These cells were designated as tubular proembryonal megakaryocytes (TPMs) (Durzan et al 1994) (Figure 4).

Bell (1994) commented that in Norway spruce “Compared with the elegant apomictic systems which have been evolved in the angiosperms, asexual reproduction in the gymnosperms is poorly developed and rarely extend beyond

polyembryony. That the potential to deviate from the normal sexual cycle is nevertheless present in conifers has now been ably demonstrated by Durzan et al. in their experimental work with Norway spruce".

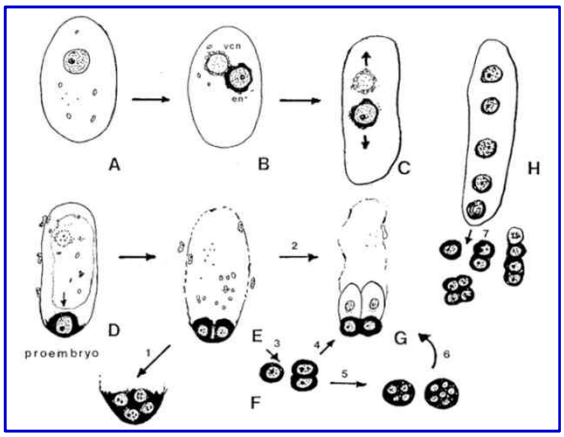

Figure 4. *Diploid parthenogenesis and automixis in embryonal initials of Norway spruce (Durzan et al 1994).* ***A*** *Embryonal initials developed into central cells with a large nucleus in a large cell designated as a TPM.* ***B*** *An amitotic nuclear division produced a small ventral nucleus (vcn) and an egg-equivalent nucleus (en).* ***C*** *The vcn migrated to one end of the tube and the en formed a neocytoplasm rich in mitochondria.* ***D*** *A thin cell wall formed around the en. Osmotic contraction of cytoplasm led to the separation and discharge of the new cell (parthenospore) into the culture medium. (****E, F, G****) The spore rapidly underwent free nuclear divisions and developed into early embryos wound around the rotating spindle of the bioreactor (Figure 3 B).* ***H*** *Neo-functionalized narrow tubes contained cells that were responsible for the formation and discharge of monads, dyads and tetrads. These tubes were later identified as androsporangial tubes. A linear tetrad of four megaspores was observed (far right) but its development into a female gametophyte was not explored.*

"One of the many interesting points raised is whether the behavior of the embryonal cells was a consequence of extreme cultural conditions (involving high temperatures, low organic nitrogen and probably high interstitial levels of CO_2) were without significance for the normal cycle. These conditions may have reproduced those to which the gymnosperms were subjected in the late Mesozoic times and the reproductive lability revealed may have played a part in their evolution… the discovery of that manipulation of environmental conditions alone can induce a diploid somatic cell to behave as a central cell in an archegonium of the same species is altogether fascinating…. the parthenogenetic behavior of the product of the division of the pseudo-central cell disclosed that it was not a true egg cell, but a totipotent cell equivalent to those which initiate embryogenesis." "The pycnotic condition of nuclei is an indicator of apoptosis (programmed cell death) and is under genetic control."

Spores produced by apomixis were discharged into the culture medium and formed proembryonal coenocytes (Figure 5 A to D) that developed into cleaving

early embryos with an axial tier (Figure 5 E). Colchicine added to the culture medium produced multi-nucleated and polyploid early embryos (Figure 5 F). They

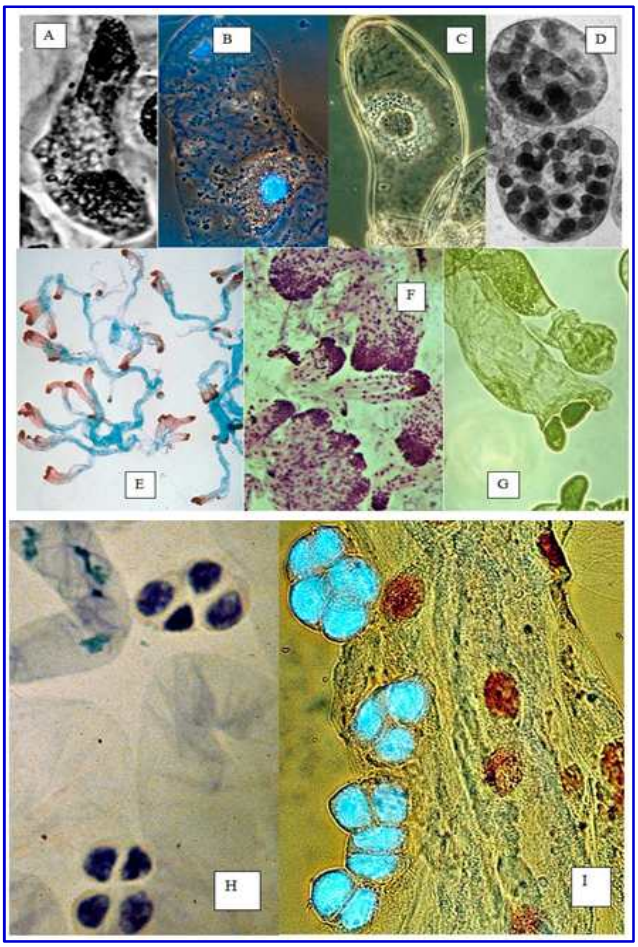

Figure 5. *Norway spruce. Apomixis and microsporogenesis.* ***A*** *Amitosis.* ***B*** *An egg-equivalent and a ventral canal nucleus (vcn) is produced by amitosis in a neo-functionalized archegonial tube (DAPI fluorescence). The small vcn is apoptotic. The egg-equivalent nucleus develops a neo-cytoplasm.* ***C*** *Neo-cytoplasm becomes enriched with mitochondria and a thin cell wall is formed. Degradation of the vcn and osmotic changes contribute to the discharge of the egg-equivalent cell as a spore into the culture medium.* ***D*** *Spores rapidly became free-nuclear and developed into proembryos (parthenogenetic apomixis).* ***E*** *Cleavage polyembryogenesis (acetocarmine) occurs in somatic embryos produced by apomixis but not in zygotic embryos. Embryonal suspensors along the axial tier become enucleated and stain with Evans blue.* ***F*** *Colchicine (5 x 10^{-4} M) added to the culture medium multiplied nuclei in early embryos produced by apomixis (Feulgen).* ***G*** *Androspores produced by meiosis were rapidly discharged from a transdifferentiated androsporangial tube (silver nitrate).* ***H*** *Tetrads and spent androsporangial tubes in the culture medium (Feulgen).* ***I*** *Nuclei in microspores fluoresced blue when stained with DAPI. Nuclei, failing meiosis and staining red (TUNEL) were retained within androsporangial tubes.*

were not examined for their ability to develop into somatic embryos. As substrates in the culture medium changed the remaining embryonal initials reverted to meiosis (Durzan 2011, 2012). (Figures 5 G, H, I). Another term for meiosis and its variant expressions is "automixis" (Suomalainen et al 1987).

The four conditions specified by Mogie (1992) required for expressions of apomixis were met and repeated over a 5-year period. Reproductive development could now be brought to an earlier developmental phase in the life cycle of conifers. No longer would growth and development of parthenogenesis and microsporogenesis be masked by colonies of cells and somatic embryogenesis in culture plates.

3. Apoptosis, necrohormones and cell-cycle regulation

Haberlandt (1938) observed that wounded cells released a substance or substances that stimulated cell division in adjacent intact cells. He drew attention to the fact that dying cells were often a feature of sites of apomictic reproduction and that they might bring about the development of unfertilized eggs.

Genetically programmed cell death (apoptosis) was first demonstrated in the early developmental plan for somatic embryogenesis in Norway spruce (Havel and Durzan 1996a). It was identified and recognized as an important factor in plant life histories (Havel and Durzan 1996b). Cytochemical stains were developed to identify the early signs of apoptosis in somatic embryos and to demonstrate how it contributed to the differentiation and elongation of embryonal suspensors (Durzan 2010) (Figure 6 A to E). Based on these observations apoptosis could now be considered as an intrinsic part of somatic embryogenesis in conifers and not accidental or sporadic phenomenon.Proteasomes in cells are a supramolecular assembly of enzymatic complexes that are highly conserved and critical for the regulation of many cellular processes along the axial tiers of somatic embryos (Durzan 2008). They function in the degradation of damaged or unneeded proteins during the development and contribute to the metabolic turnover of cell regulatory proteins. This provides a metabolic energy source for embryonic development and renews the availability of amino acids for protein and nucleic acid biosynthesis (Durzan 1996b, 2010).

Ubiquitin is a small highly conserved protein that targets cell regulatory proteins for degradation by proteasomes. In the late 1960s there were no records in the plant literature showing evidence for the role of ubiquitin in plants. The metabolic turnover of cell regulatory proteins in an early somatic embryo was shown to be mediated by ubiquitination (Durzan 1996b, 2008). It functions in the biosynthesis of organelles, plant hormone regulation, cell cycle and division, differentiation, morphogenesis and development, responses to stress, apoptosis (programmed cell death), somatic embryogenesis, apomixis, androsporogenesis, parthenogamy and genome silencing. When first reported in plants the turnover of cell regulatory proteins was referred to as ubiquitation" (Durzan 1996b). Ubiquitination in embryonal initials is now recognized as being involved in cell-cycle regulation, DNA repair, protein turnover, hormone-mediated signaling pathways and apoptosis (Durzan 2010).

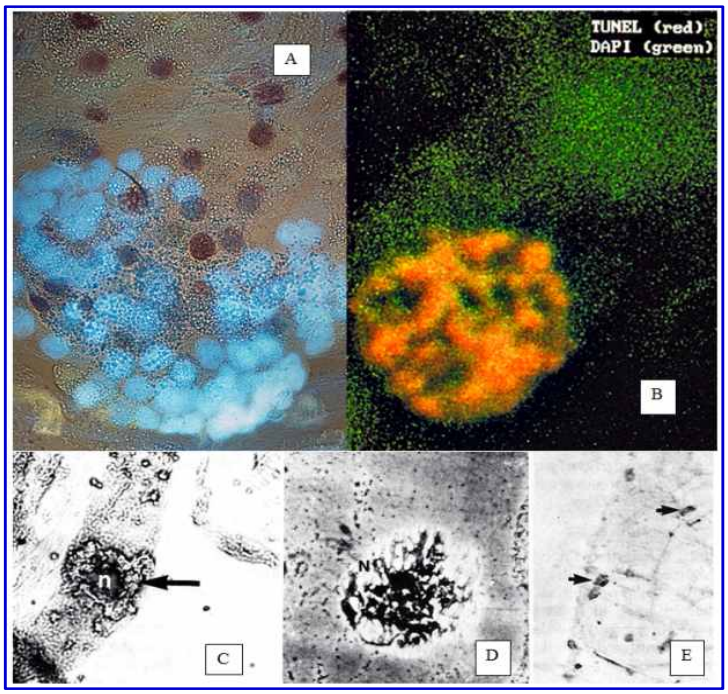

Figure 6. *Apoptosis (programmed cell death) occurs along the axial tier of an early Norway spruce early somatic embryo (Havel and Durzan 1996a, b).* ***A*** *Nuclei in newly formed proembryonal cells reacted with DAPI (blue fluorescence). Nuclei in cells forming suspensors undergo apoptosis (red fluorescence, TUNEL reaction).* ***B*** *Chromosomal degradation products (green fluorescence) are released into the cytoplasm of elongated cells.* ***C*** *An apoptotic nucleus (n) becomes surrounded by a condensed cytoplasm (arrow) that becomes fragmented.* ***D*** *Residual nuclear chromatin (N) (double-stained, silver and Giemsa).* ***E*** *Enucleated embryonal suspensors have little residual protein and DNA around proteasomes (arrows) (Durzan 2010).*

Bell (1996a) pointed out that investigations of somatic embryogenesis in *Picea abies* "have demonstrated that the regular apoptotic removal of a proportion of the nuclei identified by the TUNEL assay … and the ubiquitin mediated digestion of the nuclear and cytoplasmic proteins… in which nuclei and protoplasts are removed in ordered fashion, can now be accepted as established". It remained unclear why and how some embryonal initials were silenced in an artificial sporangium.

4. Genome silencing, gene imprinting and cytomixis

Genomes of embryonal initials failing apomixis and automixis (meiosis) were silenced by DNA methylation and cytomixis (Figure 7). Methylated DNA sequences in plants represent a "memory trace" and an imprint of past genomic events. Gene imprinting evolved from the targeted methylation of transposable element insertions followed by a positive selection when expression change was advantageous (Gehring et al 2009).

Silencing in embryonal initials in plants was characterized by the formation of a Rabl bouquet on the nuclear membrane and by the extrusion of methylated heterochromatin bodies from nuclei (Giorgetti et al 1995, 2007). It also avoids the risk of unequal pairing and crossing over with single copy DNA.

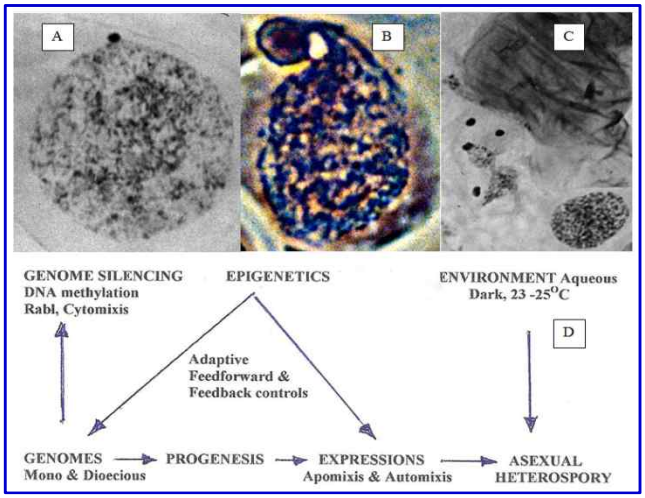

Figure 7. *Genomes of embryonal initials not capable of developing into embryos or expressing asexual heterospory were silenced epigenetically.* ***A*** *Cells and parthenospores stuck in incompletely reprogrammed states formed small Rabl chromatin configurations (bouquets) at telomere sites on the nuclear envelope (Feulgen-Giemsa).* ***B*** *Pycnotic chromosome ends became clustered on the nuclear envelope before being separated from the nucleus.* ***C*** *Their separation accounted for the formation of micronucleated cells (cytomixis).* ***D*** *Genome silencing and expressions of heteromorphic asexual heterospory (apomixis and androsporogenesis) were mediated and controlled by epigenetic factors within a narrow temperature range, in darkness and in an aqueous culture medium. The self-destruction of meiotic cells (automixis) may provide an embryonic stimulus (Mogie 1992).*

Norway spruce cells that were not able to express apomixis or androsporogenesis (meiosis, automixis) silenced their genomes by forming Rabl bouquets on nuclear membranes. Bouquets were separated from the nucleus and discharged into the culture medium as micro-nucleated cells (cytomixis) (Figure 7).

Epigenetic expressions are bidirectional and a function of past and new external conditions. Genome silencing of DNA is unidirectional and terminal. Genome silencing and cytomixis accounted for the dispersion of pycnotic micro-nucleated cells in Norway spruce, Douglas-fir and *Araucaria angustifolia* in an artificial sporangium (Durzan 2010, 2011, 2013).

5. Apomixis and androsporogenesis in Douglas-fir

Apomixis occurs in Douglas-fir seed orchards (Orr-Ewing 1957 a, b). Apomixis is expressed in embryonal initial in nippled flasks on a clinostat apomixis. This process was designated as female parthenogenetic apomixis (fPA).

Endomitosis led to the formation of a binucleate cell with a diploid egg-equivalent and an apoptotic ventral canal nucleus (vcn). In this process embryonal initials were neo-functionalized and transdifferentiated in archegonial tubes (Figures 8, 9). The egg-equivalent nucleus with a surrounding and developing neocytoplasm formed a thin cell wall. It migrated to the distal end of the tube before it was discharged like a spore into the culture medium (Figure 8 a to i). The cytoplasm retained the vcn and contracted as the cell was discharged into the culture medium. These thin-walled cells rapidly became multinuclear before developing into proembryos showing cleavage polyembryogenesis (Figure 8 j to m). Cleavage polyembryony is not observed in Douglas-fir seeds.

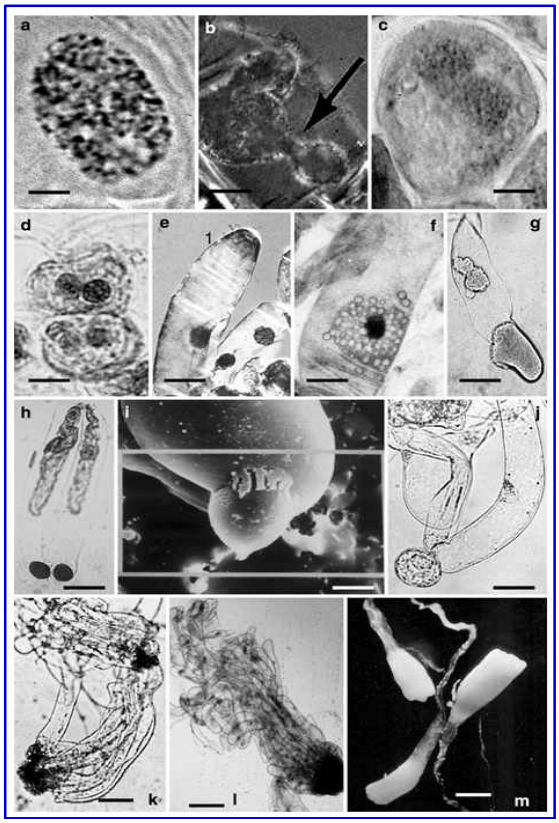

Figure 8. *Cytology of female parthenogenetic embryogenesis in Douglas-fir embryonal initials.* ***a*** *Prophase nucleus in neo-functionalized archegonial tube, Acetocarmine.* ***b*** *Amitotic nuclear constriction (arrow). Orcein, polarized light.* ***c*** *Egg-equivalent nucleus and vcn.* ***d*** *Twin binucleate cells elongate will form twin archegonial tubes.* ***e*** *Nuclei in archegonial tubes migrate to opposite poles. Vcn (**1**) at top of the tube has undergone autophagy. Orcein, polarized light.* ***f*** *Dark-stained egg-equivalent nucleus is surrounded by a neocytoplasm rich in amyloplasts and mitochondria.* ***g*** *Cytoplasm fluorescence hides an apoptotic vcn (top). A neocytoplasm develops around a basally migrating parthenote. Osmotic forces lead to the discharge of parthenotes.* ***h*** *Twin archegonial tubes each having cells within cells. The contracted cytoplasm retains the apoptotic vcn. A parthenote with a thin cell wall is at the base of each tube.* ***i*** *The*

parthenote breaks through the archegonial tube into the culture medium. ***j*** *Parthenote with a large nucleus next to a spent archegonial tube. Unstained.* ***k*** *Early embryos with elongated suspensors show cleavage polyembryony not commonly found in seed embryos.* ***l*** *Tubular cells, sloughing off flanks of the axial tier become binucleate and reinitiate fPA, Acetocarmine.* ***m*** *Early embryos with suspensors.*

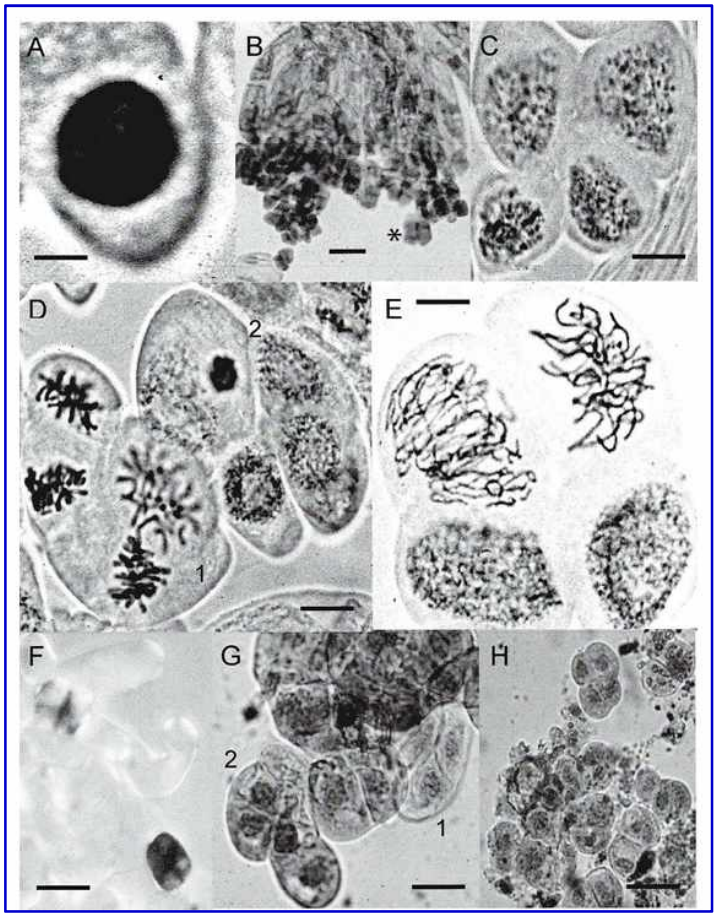

Figure 9. *Androsporogenesis in Douglas-fir was aberrant and asynchronous. Normal meiosis produces spores having 13 chromosomes.* ***A*** *Androspore (monad) in androsporangial tube just before its discharge, Acetocarmine.* ***B*** *Clusters of elongated and poorly developed androsporangial tubes release androspores into the culture medium. Tetrad (asterisk). Dyad (lower left), Acetocarmine.* ***C*** *Asynchronous tetrad.* ***D*** *Tetrad (**1**) with reduced diploid chromosome numbers in one or more cells. Tetrad (**2**) with asynchronous nuclear cycles and delayed wall formation, Acetocarmine.* ***E*** *Asynchronous tetrad with sticky chromosomes. Centric rings arise from two breaks in a chromosome followed by a reunion of the chromosomal fragments.* ***F*** *Total RNA in two tetrads.* ***G*** *Rescued and cultured microsporangium from a tree shows dyad (**1**) and an aberrant tetrad (**2**).* ***H*** *Microspores released by an immature Douglas-fir microsporangium. Microspores were exposed to the same temperatures promoting mPA in suspension cultures.*

Darlington (1958) in his "Evolution of Genetic Systems" pointed out that one of several conditions leading to non-recurrent parthenogenesis in plants includes the propensity of a genotype to express polyembryony. Proembryos removed from nippled flasks and cultured separately showed cleavage polyembryony. Cleavage in somatic embryos was inhibited by adding abscisic acid (Hong et al 1991, Durzan and Gupta 1987). Embryos with cotyledons were converted to plants in soil (Durzan and Gupta 1987, 1998).

Apomixis was accompanied by androsporogenesis (mPA) (Durzan 2011). Cytokinins maintained DNA replication and cell cycle progression. 2 4-D having auxin and cytokinin activity contributed to the triggering of fPA and mPA. Male and female gender expressions were separated over one year (dichogamy). The formation and release of dyads, triads and tetrads (mPA) occurred between January and April. Female PA was prevalent from July to November.

Thermostat functions in plants are located on nucleosomes containing the H2A.Z protein (Deal and Henikoff 2010). This protein was claimed to be conserved throughout evolution. Depletion or modifications of H2A.Z-carrying nucleosomes due under constant temperature may have stabilized gene-specific transcriptional responses that enabled sequential expressions of fPA and mAP.

Mitochondria with DNA of maternal origin when apportioned to daughter cells would have provided the metabolic energy for expressions and timing of fPA and mPA. The ratio of fPA or mPA to the number of all possible cytological outcomes and transcription factors could be used to provide a rough estimate or index of reproductive adaptation in each experimental run. Defining the molecular mechanism of this provision should bring us closer to a better understanding genomic diversification in expressions of totipotency.

6. Apomixis and androsporogenesis in *Araucaria angustifolia*

The evolution of Araucariaceae trace back to the Triassic and Jurassic in North and South Hemispheres when climates were warmer and well before angiosperms evolved (Sporne 1965). There remain just two genera restricted to the southern hemisphere. Araucariaceae were dominant in the Triassic when ocean temperatures ranged between 21^{o}C to 36 ^{o}C. Due intensive exploitation of this conifer's valuable wood, only 2% of the original population now remains.

Proembryo development in Araucariaceae is considered one of the most primitive and evolutionary divergent stage unrelated to the evolutionary trends of other conifers (Johansen 1950). Their seeds evolved from megaspores inside the parent and carried out all the steps through embryo formation.

An "early embryo" culture of an elite genotype of *Araucaria angustifolia* was obtained from Miguel Guerra at the University of Santa Catarina in Brazil. This genotype was established in the warm and humid climate of Santa Catarina, Brazil. Its expressions of fPA and mAP provided comparisons with similar observations in Norway spruce (Durzan et al 1994) and Douglas-fir (Durzan 2011) from cold climates in the Northern hemisphere.

The cell suspension medium was comprised of inorganic macro and trace nutrients, vitamins, sucrose, *myo*-inositol, a supply of amino acids (casein

hydrolysate) and amides at pH 5.8 (23 ± 2 °C). It was the same formulation that was used to demonstrate fPA and mAP in Norway spruce and supported apomictic and automictic cell cycles in runs lasting two weeks in darkness at 23 ± 2 °C. Six runs were repeated between March to September in the first year and two separate runs were repeated in the second year (July and September).

Products were harvested after 14 to 16 d to track the fates of embryonal initials in darkness at 23 ± 2 °C. Somatic embryogenesis was model-referenced to the literature on proembryogenesis in Araucariaceae (Haines and Prakash 1980, Johansen 1950). Androsporogenesis was model-referenced to the limited literature for microsporogenesis in Araucariaceae (Eames 1913, Bhatnager and Moitra 1996, Johansen 1959, Singh 1978).

Cytological characteristics, which distinguish the proembryonal stages of an Araucarian proembryo from those of other Coniferophyta involve are: the retention of free nuclei in the approximate center of the archegonium. Daughter nuclei divide simultaneously producing a centrally located proembryo with four free nuclei (Johansen 1950). The concentric arrangement of free nuclei at the time of wall formation, complete encirclement of the embryonal initials by the cap and suspensor cells, and the formation of a massive secondary suspensor system was observed.

7. Parthenogenetic apomixis in *Araucaria*

In neo-functionalized and transdifferentiated embryonal initials, the egg-equivalent nucleus migrated to the basal end of the archegonial tube where it was discharged and dispersed into the culture medium as a female parthenospore (Figure 10). A prolonged free-nuclear phase in proembryo development is a primitive feature of uncertain origin in Araucariaceae (Sporne 1965). Most parthenospores displayed some of the hierarchical and plesiomorphic features of free-nuclear replications typical of proembryogenesis in Araucariaceaen seeds. But the development of early embryos failed.

8. Automixis and androsporogenesis in *Araucaria*

Parasexual meiotic recombinations (automixis) led to the neo-functionalization and transdifferentiation of embryonal cells into narrow androsporangial tubes that discharged monads, dyads, tetrads and polyads into the culture medium.

The recombination activating enzyme (RAG-1) is one of two proteins required for joining DNA segments in recombinant nodules (RNs). RNs normally occur on the central element of the synaptonemal complex (SC) during zygotene and pachytene of meiosis. They were localized at sites of reciprocal recombination (Figure 11 A) and are known to contribute to homologous chromosome synapsis, crossing over, and cross-over interference.

Polyploidy is not observed in Araucariaceae (Delevoryas 1980). In a separate set of nippled flasks, colchicine (2.5×10^{-4}M) was added a culture medium to induce C-meiosis (colchicine meiosis). Colchicine increased polyploidy but

androspore formation was aberrant and fragmented (Figure 11 D, E).

9. Heterokaryotic protoplasts discharge parthenospores

Inbreeding depression in conifers is high (Zobel and Talbert 1984). Plating eggs and selecting for their protoplast fusion products would remove the heavy load of recessive lethal genes in genotypes of interest to tree breeding and improvement programs. Protoplast fusions were used to create heterokaryotic coeno-variants in Araucaria (Durzan 2011).

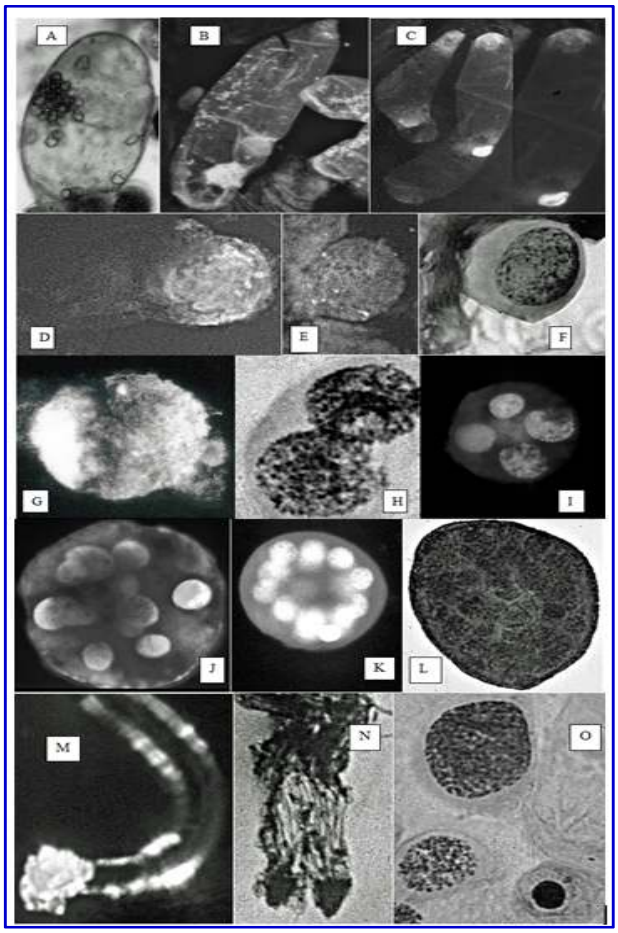

Figure 10. *Female parthenogenetic apomixis in embryonal initials of Araucaria.* ***A*** *Transdifferentiated archegonial tube with an egg-equivalent nucleus surrounded by amyloplasts and mitochondria. Amyloplast and mitochondrial DNA are derived from the male gamete after fertilization, Feulgen-Giemsa.* ***B*** *Migrating free nuclei in a binucleate archegonial tube, Acridine orange.* ***C*** *Apoptotic vcn at top of archegonial tubes. Radially elongated nuclei are common in Araucaria and Agathis, DAPI, UV fluorescence.* ***D*** *PCNA on nuclear chromosomes before the free-nuclear stage. Streak lines at the left show the inertial forces that accompanied the discharge of a thin-*

walled spore into the culture medium. ***E*** *The discharged spore with a large nucleus has now separated from its archegonial tube. PCNA (proliferating cell nuclear antigen) is essential for DNA replication and repair.* ***F*** *Discharged parthenote stained with Feulgen-Giemsa.* ***G*** *PCNA in dividing nuclei at telophase, FITC fluorescence.* ***H*** *Binucleate parthenote with replicating chromosomes, Feulgen-Giemsa. (2n = 24).* ***I*** *Four-nucleate stage, DAPI.* ***J*** *Free nuclear "jacket stage" in proembryogenesis, DAPI.* ***K*** *Nuclei become arranged for internal cell wall formation. In seeds, the peripheral nuclei are more numerous on the lower side than on the upper side. Cell elongation starts in upper and lower groups of cells after wall formation, Acetocarmine.* ***L*** *Nuclei in position for internal cell wall formation. The lower group develops the "cap". Upper group forms the suspensors. The "central cells" are embryonal initials, Cell elongation starts in the upper and lower groups to form the suspensors. Embryonal cells react strongly with acetocarmine.* ***M*** *Aberrant pro-parthenote (proembryo) with a single elongated suspensor after 30 d, (polarized light).* ***N*** *Two proembryos rescued from a seed.* ***O*** *Endomitosis in embryonal initial (top right). Monad released from an androsporangial tub (lower right).*

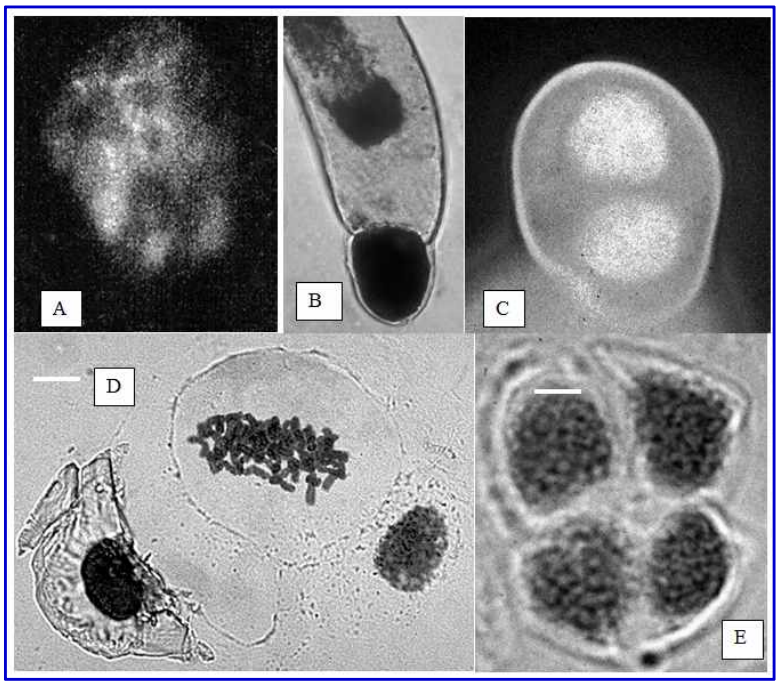

Figure 11. *Androsporogenesis in Araucaria.* ***A*** *Anti-RAG-1 probe localizes highly fluorescent recombinant DNA nodules in an embryonal initial nucleus undergoing automixis (meiosis), Rhodamine, FITC.* ***B*** *Monad in prophase with compacted chromatin at the base of an androsporangial tube, Feulgen-Giemsa.* ***C*** *Binucleate androspore with callose wall, DAPI fluorescence, Aniline blue.* ***D*** *Fragmentation of a colchicine-induced C-meiotic decussate triad (2 nuclei in prophase, one with paired metaphase chromosomes), Feulgen-Giemsa.* ***E*** *Isobilateral (tetragonial) androspore with a thick cell wall, Feulgen-Giemsa.*

A single heterokaryotic protoplast has nuclei with more than one genetic type. Coeno-variant fusions led to the neo-functionalization of sporangial tubes that replicated free nuclei with a thin surrounding cytoplasm and discharged them as "spores" into the culture medium (Figure 12). Heterokaryotic reconstructions through protoplast fusions could be used to introduce and explore chimeric competency.

When cell walls are laid down, multicellular continuity in fusion products would be maintained by epigenetic factors passing through plasmodesmata. Recovery of "coeno-variant" genotypes has utility in exploring how variations in free-nucear replications contribute to embryo development, survival under field conditions and how different coeno-variations contribute to adaptive responses to factors responsible for climate change.

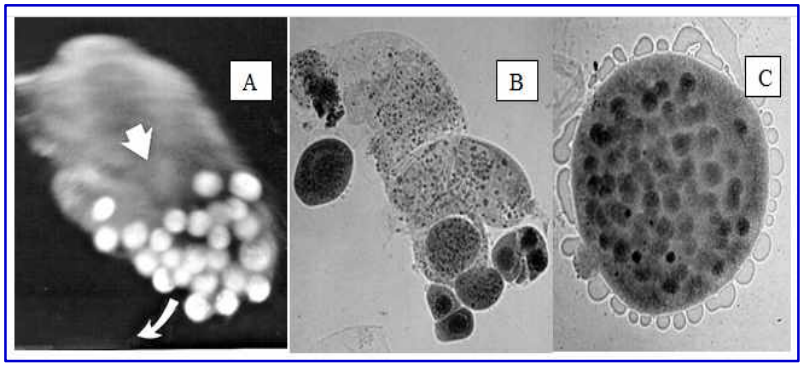

Figure 12. *Araucaria protoplast cultures discharge products of apomixis and androspores in multiwell plates.* ***A*** *Neo-functionalized embryonal initials protoplasts discharge spores into the culture medium, DAPI fluorescence.* ***B*** *Androspores were discharged from androsporangial protoplast, Acetocarmine.* ***C*** *Free-nuclear proparthenote (proembryo) regenerated an aberrant new cell wall in the presence of cell-wall digesting enzymes, Acetocarmine (Havel and Durzan, unpublished).*

Coeno-variant genotypes in conifers would be classified according to their genetic origins, chimeric diversity, properties, spatially defined growth responses and adaptive plasticity under varying laboratory and field conditions. This information would enrich our fundamental understanding of pluripotency in evolution and expand the repertoire of cellular tools that can be used to explore chimeric diversity in expressions cell totipotency. A direct test of the degree of the evolutionary divergence of two compatible genomes would be the relative variability of their F2 filial generations (offspring).

10. Mitosporogenesis in eggs of *Taxus brevifolia* and *Ephedra californica*

Egg suspension cultures of dioecious females *Taxus brevifolia (*Taxaceae, Taxales*)* and *Ephedra californica S. Wats* (Ephedraceae, Gnetales) were established in late summer from specimens in the University of California Arboretum. Eggs were no longer a parasite within the megasporangium of the plant.

The culture medium had the same formulation as the one used for *Araucaria*. Parthenosporulation (mitosporogenesis) is classified according to the mode of reproduction and by the mechanism of sex determination (Rieger et al. 1976).

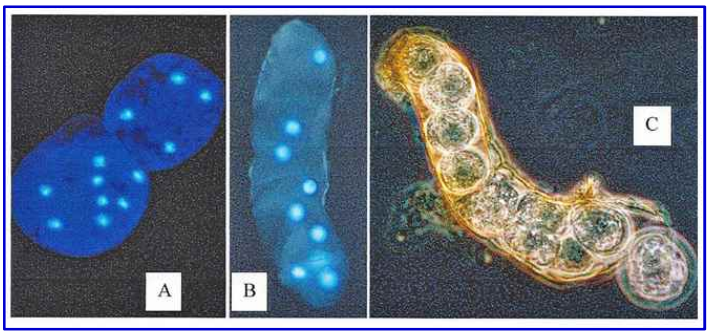

Figure 13. *A Four free nuclei in a rescued egg of a Pacific yew (Taxus brevifolia) produced eight free nuclei (DAPI fluorescence). B Neo-functionalized and transdifferentiated oðgenic tube with eight free nuclei. Free nuclei developed a neocytoplasm and a thin cell wall. C Mitosis produced at least ten mitospores that were discharged into the culture medium (phase contrast) (Durzan 2012).*

In *Taxus* the central cell nucleus functions directly as an egg (Sporne 1965). A vcn is not produced. Replication of the egg nucleus produced multiple female parthenospores (mitospores) with cell walls, one of which is shown being discharged into the culture medium (Figure 13) (Durzan 2012). Complete meiotic apomixis (gametic genome doubling and diploidy as described by de Meeûs et al (2007) was not ruled out. Spent tubes floated to the air-water interface in the rotating nippled flasks.

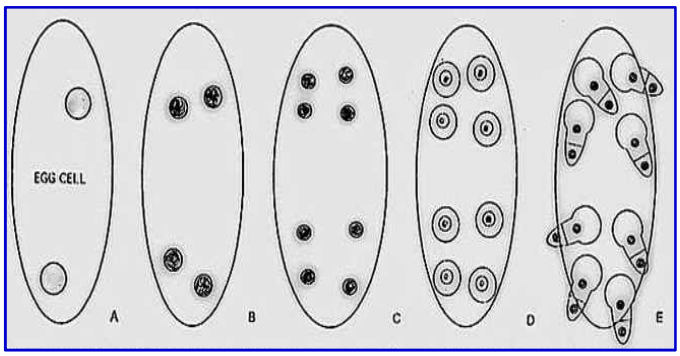

Figure 14. *Embryogenesis in Ephedra (**A** to **E**). With the completion of fertilization, the zygotic nucleus undergoes three relatively synchronous free-nuclear divisions within the former egg. Four of eight free nuclei in the lower half of the archegonium are produced from the zygote (**A** to **C**). D. The remaining four nuclei in the upper half are derived from the fusion product of the ventral canal nucleus and a second sperm nucleus (second fertilization). **E**. After mitotically derived diploid nuclei are established as spherical proembryos, filamentous growth is initiated by each proembryo. The growing tip of each proembryo penetrates into tissues of the female gametophyte.*

Ephedra is considered unique among gymnosperms (Gnetales) and in evolution by having a distinct proembryogeny (Figure 14). Evolutionary botanists believed the Gnetales to be the ancestors of flowering plants (Sporne 1965).

In the absence of fertilization five mitospores were discharged from eggs into the culture medium instead of the eight found in seeds (Figure 15 A, B). Spent tubes floated and accumulated at the air-water interface. The reduced number of discharged mitospores provides an example of reproductive diversification in Gnetales (Durzan 2010) .

Adaptive changes in reproduction occur over relatively short periods of evolutionary time (Sporne 1965). The expressions of single-celled symbioses offer insights into how gymnosperms might have evolved and how native species might become genetically diversified and adapted to local climate change.

11. Conclusion

Diversification in Steward's model for totipotency (1968) is extended to include apomixis in embryonal initials of conifers and mitosporogenesis in eggs of *Taxus brevifolia* and *Ephedra californica.* In the apomictic process, recessive combinations are not exposed and short-term selection acts on the total genetic and additive variance (Crow 1994). Multi-nucleated eggs of *Taxus brevifolia* and *Ephedra californica* under the same conditions formed cells within cells that were discharged as mitospores. Products of mitosporogenesis are genetically identical to the parent.

Embryonal initials, bypassing somatic embryogenesis and expressing apomixis, were neo-functionalized and transdifferentiated into archegonial tubes with central cells forming a diploid egg-equivalent nucleus and an apoptotic ventral canal nucleus. Products of apoptosis stimulated the development of a diploid egg-equivalent nucleus with a neocytoplasm and thin cell wall within a neo-functionalized and transdifferentiated archegonial tube. Osmotic contraction of the cytoplasm in the tube discharged these diploid cells as parthenospores into the aqueous culture medium.

In Norway spruce and Douglas-fir these parthenospores rapidly underwent free-nuclear replications and formed somatic embryos that matured and were planted in soil. In *Araucaria angustifolia*, the free-nuclear plesiomorphic features of typical of proembryogenesis in Araucariaceae were retained but the development of somatic embryos was not explored.

As food supplies declined and apoptosis increased, embryonal initials reverted to automixis (meiosis) in neo-functionalized and transdifferentiated androsporangial tubes. Monads, dyads, triads, tetrads and pentads were discharged in a display of heteromorphic asexual heterospory in an aqueous environment. Embryonal initials, failing diversification, silenced their genomes and dispersed micronucleated cells that no longer developed.

Features of asexual reproduction in embryonal initials and eggs in an aqueous artificial sporangium, when classified, emulated the reproduction of Algae (Durzan 2013). Diversified expressions of asexual heterospory displayed cenogenesis and palingenesis. Cenogenesis is the introduction during embryonic

development of characters and diversified structures not thought to be present in the earlier evolutionary history of the species. Palingenesis is the recapitulation of development that might have undergone in the evolution of the species (Mogie 1992). These observations extend the model for totipotency and provide new experimental insights into how gymnosperms might have evolved in the deep past.

Macro-evolutionary changes are best viewed over the perspective of geological time (Sporne 1965). Rhode (1923) postulated that a multi-nucleate plasmodium (coenocyte) was responsible for the phylogenetic evolution of cellular tissues. Although his views remain controversial, it is remarkable that protoplast fusions of embryonal initials led to the neo-functionalization of sporangial tubes that discharged proembryonal protoplasts. Through protoplast fusions, novel "coeno-variant" somatic proembryos could be used to explore and enhance genomic plasticity.

Could the first diploids have arisen via rare endomitotic errors as proposed by Wilkins and Holliday (2009)? Expressions of fPA in Norway spruce, Douglas-fir and *Araucaria* employed endomitosis, amitosis, genome silencing and meiosis in expressions of parthenosporulation and asexual heterospory. Could meiosis have originated from mitosis as postulated by Wilkins and Holliday (2009)? Embryonal initials undergoing mitoses reverted to meiosis as food supplies declined and hormones were altered within narrow temperature ranges.

Signatures of acidification and climate change are found in the Permian extinctions 250 million years ago when gymnosperms were evolving (Hand 2015). Diversified expressions of totipotency may have value in exploring how investments in clonal somatic embryogenesis may someday deliver tangible genetic gains in boreal forests faced with global warming. Advances in DNA sequencing, genome-wide association studies and epigenome-editing tools offer new ways to identify specific gene combinations, effects of introgression, conditions that enhance carbon storage, survive acidification and contribute to primary production in coniferous forests.

12. Acknowledgements

Professor Ledyard Stebbins at the University of California defined much of what was worth doing in plant biosystematics, evolution and biological conservation. He advised that observations with Norway spruce (Durzan et al 1994) be published as soon as possible. Without collaborations with Michael Mogie (Bath UK) and support from Scott Russell (Editor of Sexual Plant Reproduction) it would have been difficult to address the significance of observations in an artificial sporangium. Peter Bell at the University of London offered comments on the significance of apoptosis in expressions of latent diploid parthenogenesis. Manfred Eigen, while visiting Cornell in 1965, encouraged my interests in self-organization in prebiotic systems in an aqueous environment. Without experiences gained in FC Steward's laboratory it would not have been possible to relate diverse expressions of cell totipotency to opportunities in experimental evolution. The author is indebted to Jan Bonga for his editing and corrections made to this review.

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Is there potential for propagation of adult spruce trees through somatic embryogenesis?

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Abstract

Although large-scale cloning of elite adult conifer trees via somatic embryogenesis (SE) has long been a holy grail for many tree improvement programs, attempts by a number of research groups to induce embryonal tissues from vegetative explants has, to date, produced very limited success. It has been speculated that this recalcitrance is related to (potentially reversible) epigenetic suppression of the embryogenicity that underpins SE induction, for example, within zygotic embryos. However, our limited understanding of the molecular mechanisms contributing to SE induction, particularly within conifers, provides little support for such a supposition. Exacerbated by a paucity of responsive vegetative explants, the discovery of a clonal line (G6) of somatic embryo-derived white spruce (*Picea glauca*) trees whose primordial shoots have remained responsive to SE induction for well over a decade, presented an outstanding opportunity to explore SE-induction at a genomic level via transcriptome analysis. Subsequent screening further revealed that some of these G6 trees had lost responsiveness, further presenting an opportunity to conduct gene expression analysis under an epigenetic context. That was done in the absence of genotype-specific factors which, as described here, confound identification of genes directly involved in SE-induction responsiveness. This chapter describes the origin of these somatic trees and of the extensive SE induction experimentation conducted over the last five years, including microscopic analysis of the tissues generated. In addition we describe absolute qPCR analysis comparing gene expression within responsive and nonresponsive G6 explants, and ending with a brief overview of recent efforts to apply RNA-seq analysis.

Keywords: vegetative buds, *Picea glauca*, primordial shoots, somatic trees, stress response, gene expression

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1. Introduction

Modern forest management relies extensively on breeding and reforestation programs to support both the sustainability of forest productivity and conservation of natural forests, with the expectation that plantation forestry will play a major role (Fenning et al. 2008). As such, vegetative propagation has become an integral part of many tree improvement programs, primarily due to the ability to clonally propagate elite genotypes. This is indicative of the widely held belief that cloning of individual trees allows large genetic gains to be achieved within a single selection cycle. There is great interest in developing the capability to clone adult trees because many elite characteristics only become evident after sexual maturation (Park 2002; Nehra et al. 2005; Bonga et al. 2008; Bonga et al. 2010).

Somatic embryogenesis (SE) provides an alternative approach that in many ways is analogous to large-scale seedling production from seed (Park 2002; Klimaszewska et al. 2015). In addition to allowing exploitation of existing reforestation infrastructure, SE further provides the capability for large-scale clonal propagation, in that somatic embryos produced from an individual embryonal cell line are genetically identical. Combined with the ability to cryopreserve large numbers of embryonal cell lines (genotypes), SE has the potential to generate an unlimited number of somatic seedlings, albeit with one major limitation.

Notwithstanding the potential of SE for clonal propagation, the recalcitrance of adult conifer tree explants to respond to SE induction has precluded the ability to clone individual adult trees. While young needles from one- and three-year-old Norway spruce trees have been found to have limited responsiveness to SE induction (Ruaud et al. 1992; Harvengt et al. 2001), this responsiveness is lost as donor trees age (von Aderkas and Bonga 2000; Bonga et al. 2008; Bonga et al. 2010).

2. Ontogenic aging, maturation and phase change in conifers

In conifers, a shoot apex undergoes three phases of growth during its post-embryonic development: a juvenile vegetative phase (seedling), an adult vegetative phase, and an adult reproductive phase (Poethig 1990; 2010). Depending on the species, this adult vegetative phase may last for many years with the appearance of reproductive organs signaling sexual maturation (Poethig 2010). This maturation process is further characterized by a reduction in the growth rate, loss of rooting propensity of cuttings, and changes in morphological parameters (Haffner et al. 1991). Maturation is thus composed of two different components: physiological aging, which corresponds to the increase in size and/or structural complexity (Borchert 1976) and ontogenic aging, which is localized in the meristem, at the

level of the individual cell or of the entire meristem (Hackett 1985). Although juvenile (a few years old) conifer trees can be cloned via rooted cuttings, as they mature they become increasingly unresponsive, which is particularly difficult to reverse in conifers (Bonga et al. 2010; Díaz-Sala 2014).

It has been suggested by some authors that epigenetic control, which affects qualitative and quantitative gene expression, is strongly implicated in tree-specific growth phases (Greenwood 1995; von Aderkas and Bonga 2000; Miguel and Marum 2011). Valledor et al. (2010) suggested that epigenetic marks also define a unique molecular signature for morphogenetic competence such as embryogenicity in conifers. Epigenetics entails three major groups of molecular mechanisms affecting gene expression under phase-change or environmental constraints (Grant-Downton and Dickinson 2005; 2006): 1) cytosine methylation of DNA; 2) post-translational modifications of histones (methylation, acetylation) and associated euchromatin/heterochromatin transitions; and 3) small RNA (sRNA) signatures involved in both transcriptional (siRNA: small interfering RNA) and/or post-transcriptional silencing (miRNA: micro RNA). For example, in *Picea abies* sRNA (particularly miRNA) was involved in the temperature-induced memory of embryogenic responsiveness, including somatic embryogenesis initiation frequency from zygotic embryos (Kvaalen and Johnsen 2008; Yakovlev et al. 2010).

3. Identification of *Picea glauca* somatic trees responsive to SE induction

A major impediment to studying the mechanisms of SE induction in adult conifers has been the paucity of responsive explants. Explants other than zygotic embryos, which of course are not genetically identical to the mother tree from which they are collected, are generally not responsive. Additionally, it was unknown which medium formulation would be optimal in promoting SE induction within vegetative tissues versus those known to be effective for zygotic embryos. To address both issues simultaneously, the responsiveness of vegetative bud explants taken from 38 genotypes of 2 to 3.5 years old white spruce somatic trees grown in a greenhouse, were determined for 13 medium formulations (K. Klimaszewska, unpublished). Clonal trees of four additional genotypes of 3-year-old somatic trees (893-1, 893-2, 893-6 and 83-12 abbreviated as G1, G2, G6 and G12) were also established in a plantation at Valcartier, Quebec, Canada in 2003 (Klimaszewska et al. 2011) (Figure 1). The rationale for targeting somatic trees was based on the premise that trees generated from somatic embryos could have a higher propensity for SE induction (Ruaud et al. 1992).

The induction of SE was carried out within longitudinally cut sections of primordial shoots (PS) that were excised from pre-flush vegetative buds in early spring, or from the developing buds in early autumn (Klimaszewska et al. 2011,

Figure. 2 a, b, c). Lateral as well as apical and subapical buds were collected for comparison. High contamination frequency of explants, both fungal and bacterial,

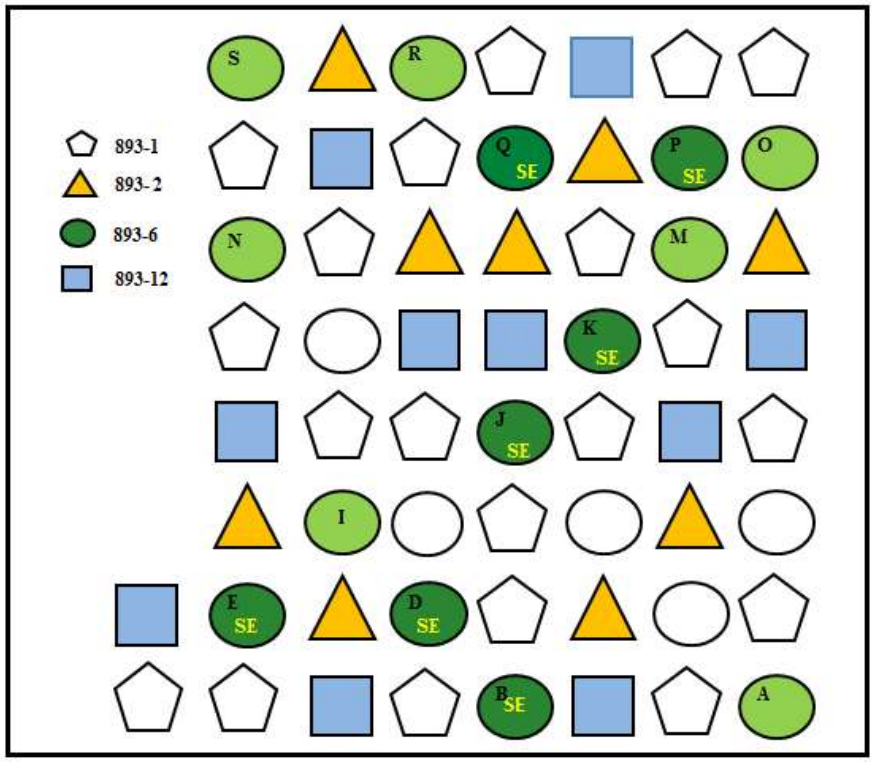

Figure 1. *Plantation layout of 58 white spruce somatic trees established at Valcartier, Quebec, Canada in 2003 using 3-year old somatic trees generated from four genotypes (G1, G2, G6 and G12). The trees were fertilized every June/July with 11.2 N, 8.1 P, 14.1 K and 2.9 Mg slow release fertilizer (SynAgri, Canada) and maintained according to standard silvicultural practices. SE: designates the seven responsive G6-derived trees.*

was a major problem initially, but an effective disinfection protocol was subsequently developed that resulted in 0 to a maximum of 10% contaminated explants. After collection, the shoot buds were prepared for disinfection by removing the basal scales (Figure 2 d), and disinfection was carried out in either 15 ml or 50 ml centrifuge tubes, depending on the number of buds. The buds were then shaken in 95% ethanol for 2 min to dissolve traces of resin, washed (by shaking) in de-ionized tap water containing a drop of Tween-20 for 6 min, rinsed three to four times in de-ionized tap water and then shaken in 75% ethanol for 2 min. The ethanol was then replaced by a solution of 10% (v/v) H_2O_2 containing a drop of Tween-20, and the buds shaken for 8 min. The next step was performed in the active laminar flow unit where the buds were collected in a sterile tea strainer

and rinsed several times with sterile distilled water. The last rinse was done using a 1% (w/v) solution of polyvinylpyrrolidone (PVP) and the wet buds placed in a sterile Petri dish on a moist sterile filter paper to prevent drying. If necessary, the buds can be stored at 4°C in a sealed Petri dish for up to 2 days, although buds stored for up to 1 week have been found to remain responsive. Under a stereomicroscope the buds were sectioned longitudinally generating two sections of PS, with the option of sectioning again depending on the size (Figure 2 e, f). The explants were then placed on a medium with cut surfaces down. Sections of four PS were cultured per Petri dish (1 x 9 cm).

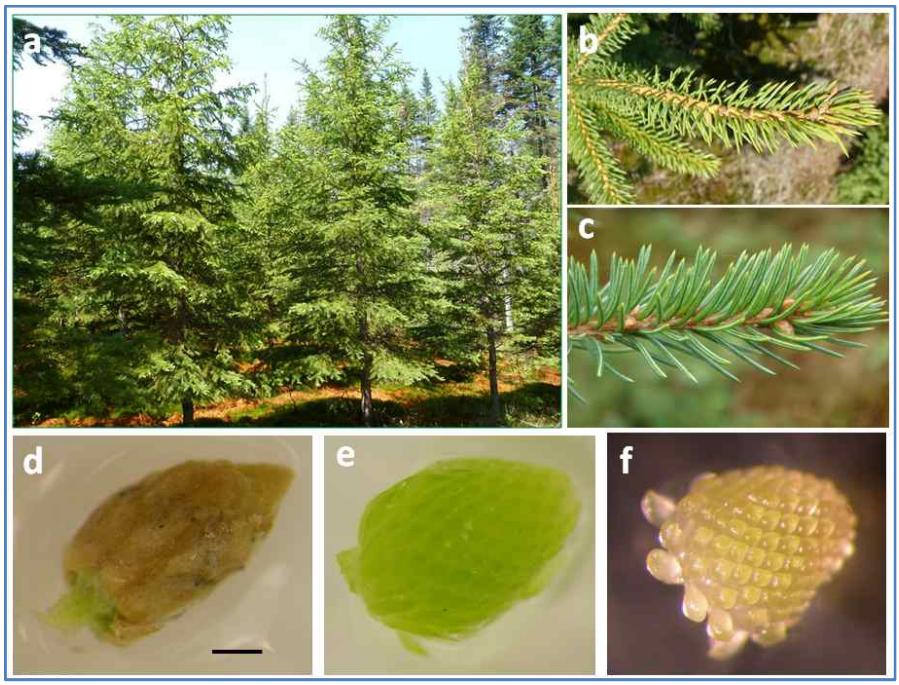

Figure 2. *Vegetative bud collection from somatic white spruce trees.* ***a*** *15 year-old somatic white spruce trees (7 to 8 m high) in June 2015.* ***b*** *a twig with the pre-flush spring vegetative buds (3 to 4 mm long) at collection time.* ***c)*** *a twig with forming buds in September (2 to 3 mm long).* ***d*** *a spring bud with base scales removed and ready for disinfection.* ***e*** *a spring primordial shoot. Bar = 0.06 mm.* ***f*** *a forming primordial shoot from a bud collected in September. Bar = 0.5 mm.*

Of the first 38 genotypes tested, five genotypes responded to induction of SE following culture on five media formulations: MLV or MSG + 13.5 µM 2,4-D + 4.5 µM BA; MLV or MSG + 9.0 µM 2,4-D + 4.5 µM BA + 0.4 µM Ancymidol; and MLV + 1.0 µM BSSA + 4.5 µM BA) (Klimaszewska et al. unpublished). MLV is a modified Litvay medium (Litvay et al. 1985; Klimaszewska et al. 2001) and

MSG is a modified Murashige and Skoog (1962) medium (Becwar et al. 1990). Ancymidol was used in one of the media to inhibit gibberellin synthesis in order to verify whether gibberellin might act as a suppressor of SE as observed in *Arabidopsis* pickle (pkl) mutant (Ogas et al. 1997). BSSA (3-(benzo (B) selenyl) acetic acid was used to replace 2,4-D. Based on the above results, the medium formulation chosen for all subsequent SE induction experiments was MLV with 9.0 µM 2,4-D and 4.5 µM BA, which in fact is the standard medium for induction of SE in zygotic embryos of spruce and other species in the Pinaceae family.

4. SE induction within shoot primordium explants

Of the four white spruce genotypes (G1, G2, G6 and G12, total of 58 trees) grown within a plantation, explants of G1 and G6 initially responded, but only explants from the G6 trees have retained responsiveness to present (2015, 15 years old). All explants enlarged during the first few days of culture, with most generating calli from the cut surfaces within 15 days of culture (Klimaszewska et al. 2011, Figure 3 a, b, c). Needle primordia elongate, often with calli forming at their

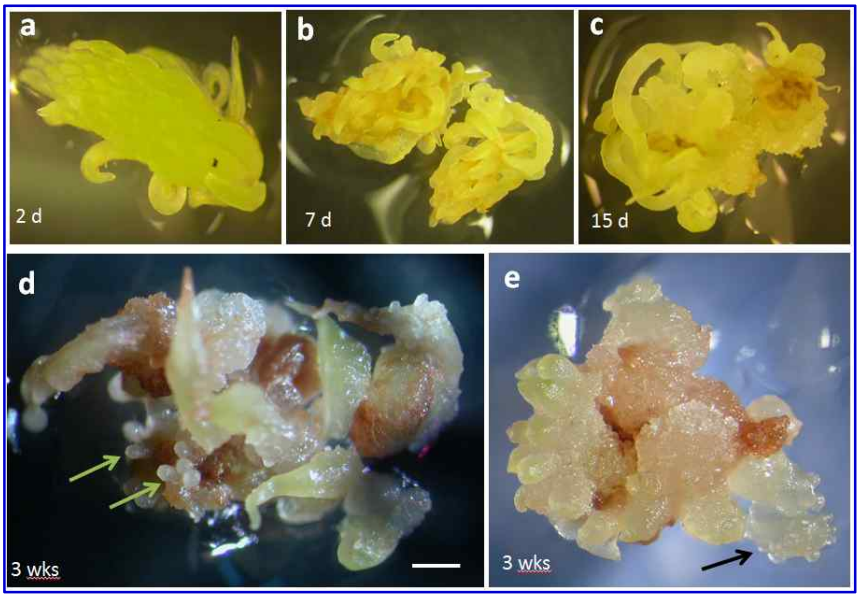

Figure 3. *Primordial shoot explants in culture after 2 to 21 days.* ***a- c*** *the needle primordia elongate and the explants produce a small amount of calli. Bar = 0.8, 1.2 and 1 mm, respectively,* ***d, e*** *between 15 and 21 days, round protuberances (nodules) appear on different parts of explants preceding, in most instances, the initiation of somatic embryogenesis. Bar = 0.04 and 1.25 mm, respectively* ***d*** *explant from spring bud and* ***e*** *explant from fall bud.*

bases. During the same time period, many explants of G6 produced round protuberances (nodules) most visible along the needle primordia, although some formed within calli, albeit infrequently (Figure 3 d, e and Figure 4 a). During the third and fourth week of culture these round protuberances became more prominent with some displaying cell elongation at the site of attachment to the explant, mimicking the suspensor and embryonal mass of an early-stage somatic embryo (Figure 4 b, c, d, e). However, microscopic examination failed to confirm these structures as true early-stage somatic embryos, although, importantly, many embryonal masses were found to develop from the cells of these structures. That is,

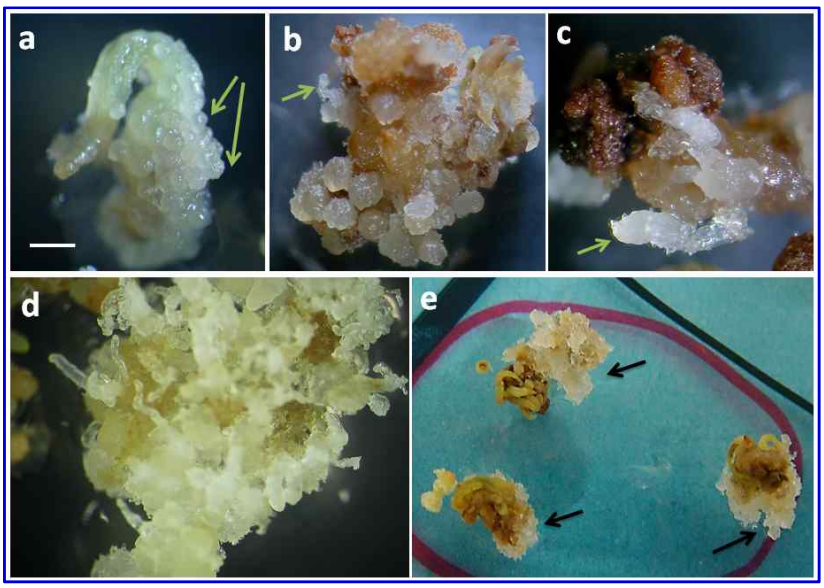

Figure 4. *Primordial shoot explants with protuberances at different stages of growth/development.* ***a*** *an explant with protuberances formed on a needle primordium cultured for 5 wks. Bar = 1.5 mm.* ***b*** *an explant with large protuberances and an early somatic embryo cultured for 5 wks. Bar = 1.6 mm.* ***c*** *an explant with elongated protuberances mimicking early somatic embryos with large, opaque dome subtended by a tail of elongated cells cultured for 7 wks. Bar = 0.67 mm.* ***d, e*** *mix of elongated protuberances and early somatic embryos on explants after 12 weeks of culture. Bar = 1.3 and 1.88 mm, respectively.*

there were clear differences between these elongated protuberances versus early-stage somatic embryos (Figure 5a, b, c, d, e). The most obvious differences were the large size of the former, consisting of densely packed small cells within the white, opaque dome and the tail of long cells, and dissimilar to suspensor cells, subtending the dome. Once embryonal masses differentiated from these elongated

protuberances, over time they became identical, both macro- and microscopically, to those initiated from zygotic embryos.

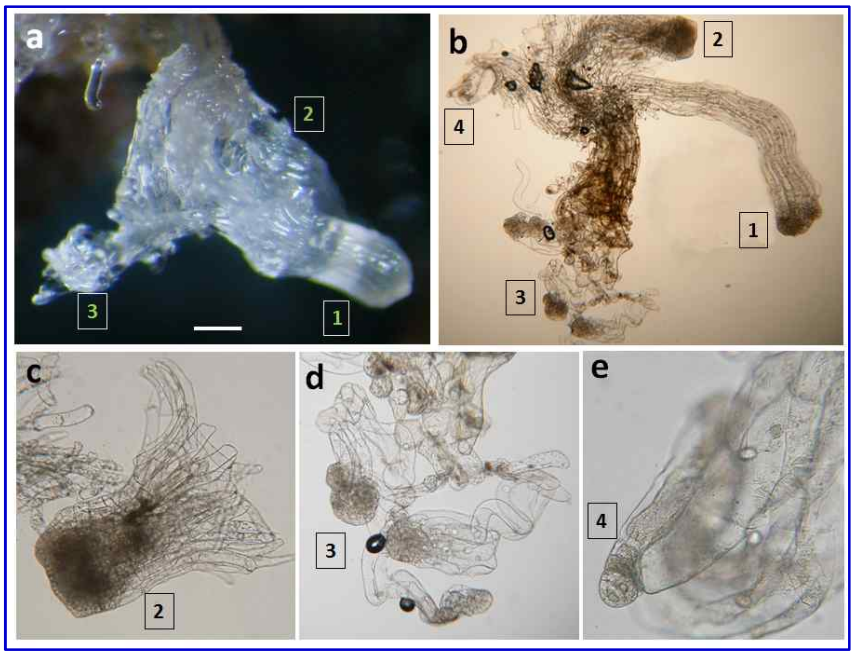

Figure 5. *Composition of an elongated protuberance (nodule) at the time of somatic embryogenesis induction.* ***a*** *an elongated protuberance viewed in a stereomicroscope showing a smooth, opaque dome subtended by a tail of elongated cells. Bar = 0.29 mm.* ***b*** *The same but slightly squashed and viewed under bright-field microscope. Note two elongated protuberances and early somatic embryos. Bar = 0.24 mm.* ***c, d, e*** *the same as on* ***b*** *under larger magnification. Note the clear difference in the phenotypes of elongated protuberance versus early somatic embryos.* ***e*** *initial stages in early somatic embryo formation. Numbers in squares are labels of the same parts of the elongated protuberance. Bar = 80, 80 and 50 µm, respectively.*

To further explore the nature of these induced embryonal cultures, expression profiles of 11 genes known to be involved in embryogenesis and meristem development were analyzed. This encompassed PS explants before culture, 3 and 6 days in culture, separated needles with the protuberances (rp) described above, callus with rp, embryonal masses and nonembryogenic calli (Klimaszewska et al. 2011). The analysis revealed that four genes known to be associated with embryogenesis (VP1, SAP2C, a spruce LEC1 homolog (CHAP3A) and WOX2) were exclusively expressed in embryonal masses, while IAA2, SKN1, SKN2 and SERK1 were expressed in both embryonal masses and vegetative

tissues, albeit at different levels.

Embryonal masses initiated from explants of somatic trees of increasing ages (2 to 11 years old) were subsequently cultured on medium of the same composition used for SE induction, and mature somatic embryos generated using a standard spruce maturation protocol (Klimaszewska et al. 2011) (Figure 6 a).

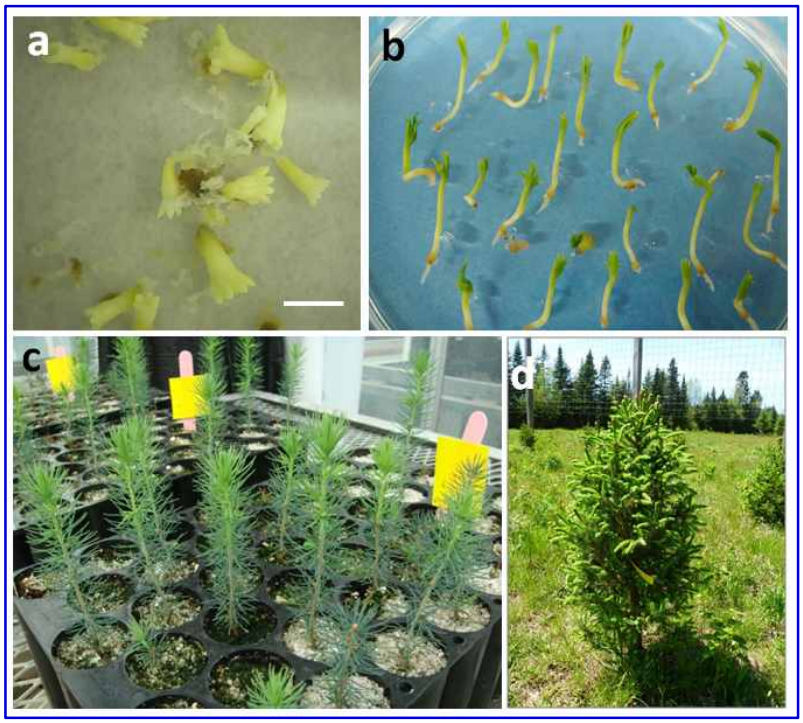

Fig. 6 *Stages in somatic tree production from PS explants.* ***a, b*** *maturation and germination of somatic embryos. Bar = 1.88 and 5 mm, respectively.* ***c*** *first growth from flushed buds in a greenhouse. Bar = 3.7 cm.* ***d*** *4-year-old somatic white spruce grown from somatic embryo initiated in the explants of 8-year-old somatic white spruce G6. Photo taken in June 2015 at Valcartier, Quebec, Canada. Bar = 19.5 cm.*

These were germinated (Figure 6 b), transferred to a greenhouse (Figure 6 c) and afterwards to a nursery at Valcartier, Quebec with the primary goal of continued observation of morphology and growth patterns. Subsequently, in 2013, 38 bud-derived trees from 8-year old G6 donor somatic trees were planted in the field (Figure 6 d). The trees have demonstrated growth and vigour similar to that of seed-derived seedlings, indicative of lacking mature traits, an undesired phenomenon that can arise when using other vegetative propagation methods (Greenwood 1995).

5. Gene expression profiling within explants of responding (G6) and non-responsive (G12) genotypes (age 9 years)

In 2009, pooled primordial shoot explants taken from clonal G6 and G12 trees were used to conduct microarray-based transcriptome analysis (Rutledge et al. 2013). Explants used for the microarray analysis were taken at the point of collection in the field (day 0) and after 1 week of SE induction treatment (day 7). An inter-genotype comparison revealed many small differences, with 167 targets differing significantly at day 7 compared with 27 targets at day 0. Overall, the induction treatment generated a largely shared response with regards to the genes involved in both genotypes. However, this did not take into account quantitative differences in expression levels. A two-way ANOVA analysis of interaction between genotypes and the induction treatment revealed 8,433 targets differentially expressed with about 90% responding solely in relation to the SE induction treatment.

To identify candidate genes for the absolute qPCR analysis (Rutledge and Stewart 2008; 2010), the microarray data were sorted based on the largest fold differences relative to the other genotypes at day 7 of induction. Subsequently, four candidate genes that most greatly differentiated each genotype at day 7 were examined in detail. In the responsive genotype, G6, the two most highly differentially expressed genes were dehydrin (DHN1), consisting of a small conifer-specific gene family that have been previously identified in Norway spruce (Yakovlev et al. 2008), in addition to a putative apoplastic class III peroxidase that is most similar to *Arabidopsis* AtPrx52. The last two candidates encode for unusual proteins that appear to be conifer-specific, containing repetitive segments rich in threonine-glutamine and proline, respectively. In the non-responsive genotype, G12, putative homologues were found for all four candidates. The two most differentially expressed genes were found to encode for closely related proteins with high levels of sequence similarity to an unusual class of serine protease inhibitor (PI). The third candidate encodes for an apoplastic class III peroxidase that is most similar to *Arabidopsis* AtPrx21, with the fourth candidate encoding for a cell wall invertase most similar to the *Arabidopsis* AtcwINV1.

To profile the dynamics of these candidate genes in greater detail, absolute qPCR was used to expand the analysis by inclusion of three additional time points, extending the analysis to day 21 of the induction. In addition to confirming differential expression at day 7 as predicted by the microarray analysis, all of the candidate genes maintained differential expression within their originating genotype throughout the entire induction treatment, although to varying degrees. In particular, all four G12 candidate genes maintained high levels of expression beyond day 7 within the G12 explants, which averaged 9-fold higher than that of the four G6 candidate genes within the G6 explants. This suggested that a major

distinguishing characteristic of the nonresponsive explants was an intense physiological response to the SE induction treatment. Furthermore, all four of the G12 candidate genes encode for proteins with similarity to proteins known to play prominent roles in biotic defense in angiosperms, such that their induction is likely indicative of an intense defense response, which in turn could be antagonistic to SE induction. On the other hand, a weaker induction response within the G6 explants, including induction of a conifer-specific dehydrin, is more consistent with elicitation of an adaptive stress response.

6. SE induction responsiveness of adult G6 vegetative and reproductive phase trees from 2010 to 2015

In 2010, instead of pooling buds as had been done for previous SE induction experiments, buds were collected separately from each of the G6-derived somatic trees in order to determine the response of individual trees (Figure 1). Initially, this included 19 clonal trees (labeled A to S); however, five trees were subsequently excluded following micro-satellite analysis that revealed that they were not clones of G6, likely due to an error in labeling. Of the remaining trees, seven have shown responsiveness, albeit of variable frequency, while the remaining seven trees were found to be completely unresponsive (Figure 1, Table 1). Importantly, this presented an unprecedented opportunity to examine differential gene expression in the absence of genotype-specific factors, potentially allowing the exploration of SE responsiveness in relation to that governed by epigenetic factors.

7. Gene expression profiling of responsive and nonresponsive PS within individual G6 trees

In 2014, pooled PS of six responding G6 trees were compared with those taken from seven nonresponsive G6 trees (Table 1) using absolute qPCR to quantify expression of candidate genes previously identified from microarray analysis conducted in 2009 (Rutledge et al. 2013). In addition to G12, this analysis included G2, another genotype that has never responded to SE induction. Expression profiles of the G12 inducible candidate genes, PI20a, PI20b, and Prx21were not only similar for the two nonresponsive genotypes (Figure 7 a, Rutledge et al. 2013), but were also remarkably similar to that observed for G12 during the spring 2009 induction experiment. In addition to demonstrating an exceptional repeatability of the qPCR-based gene expression profiling, this also revealed a low to absent induction within both responsive and nonresponsive G6 PS, suggesting that the differential gene expressions observed during the 2009 inductions are primarily reflective of genotype-specific factors, bringing into doubt

Table 1. *Response of individual G6-derived clonal trees to SE-induction treatments conducted from 2010 to 2015. Number of primordial shoots that generated embryonal masses with the total number of shoots presented in brackets. Note that some of the inductions (2014 and 2015) were conducted using pooled primordial shoots. nt: not tested.*

G6 clone	2010		2011		2012		2013	2014			2015
	spring	autumn	spring	autumn	spring	autumn	spring	spring	Sept	Oct	spring
B	nt	4 (30)	40 (58)	20 (54)	58 (67)	6 (28)	8 (40)		8 (44)		
D	26 (48)	0 (18)	36 (46)	6 (40)	25 (31)	3 (28)	11 (23)		5 (26)		
E	9 (77)	0 (32)	3 (57)	0 (56)	2 (69)	nt	10 (35)		0 (36)		
J	6 (34)	1 (26)	6 (51)	4 (57)	9 (64)	nt	6 (38)		2 (52)		
K	4 (54)	0 (24)	1 (52)	3 (62)	0 (63)	nt	0 (35)		0 (28)		
P	36 (44)	0 (27)	44 (58)	4 (62)	42 (64)	nt	5 (18)		2 (26)		
Q	0 (98)	0 (28)	1 (48)	0 (54)	1 (62)	nt	nt	nt	0 (19)	nt	nt
							Pooled:	14 (111)		17 (120)	20 (80)
R	nt	0 (22)	0 (14)	0 (58)	0 (48)	nt	nt		0 (16)		nt
S	0 (30)	0 (31)	0 (26)	0 (71)	0 (63)	nt	nt		0 (24)		nt
A	0 (93)	0 (28)	0 (40)	0 (56)	0 (66)	0 (28)	nt		0 (29)		
I	0 (64)	0 (30)	0 (27)	0 (61)	0 (64)	nt	nt		0 (24)		
M	0 (86)	0 (20)	0 (18)	0 (67)	0 (63)	nt	nt		0 (44)		
N	0 (66)	0 (29)	0 (28)	0 (60)	0 (64)	0 (32)	nt		0 (23)		
O	0 (62)	0 (28)	0 (25)	0 (42)	0 (65)	nt	nt		0 (23)		
							Pooled:	0 (42)		0 (152)	0 (60)

a direct role of these genes in SE induction responsiveness.

This was further supported by the expression profiles of the G6 candidate genes, although to a lesser degree. For example, induction of Prx52 was observed within both responsive and nonresponsive G6 PS, indicative of a genotype-specific response rather than being related to SE induction responsiveness, as was previously proposed (Rutledge et al. 2013). However, the expression profiles of the other two G6 candidate genes were less definitive (Figure 7 b).

Despite the revelation that genotype-specific factors can significantly confound identification of genes directly involved in SE induction responsiveness, analysis of PS from responsive and nonresponsive G6 trees (Table 1) does provide an unprecedented opportunity to eliminate genotype-specific factors. This in turn would allow direct analysis of the differential gene expression under an epigenetic context, which would supposedly be more effective in identifying genes related to SE induction responsiveness within PS of G6. Indeed, this was instrumental in initiating RNA-seq transcriptome analysis within responsive and nonresponsive G6 PS sampled at days 3, 7, 15 and 21 of induction in the fall of 2014. Although the analysis is still ongoing, preliminary results have indicated that the differential expression is complex, with substantial differences evident at each time point of the induction treatment, which is not unexpected based on the extensive morphological differences that are observed at the different stages of SE

development (Figs. 3 and 4). Subsequently, this has led to the identification of over 2,000 genes differentially expressed at a level greater than 2-fold, combined over the four time points of induction.

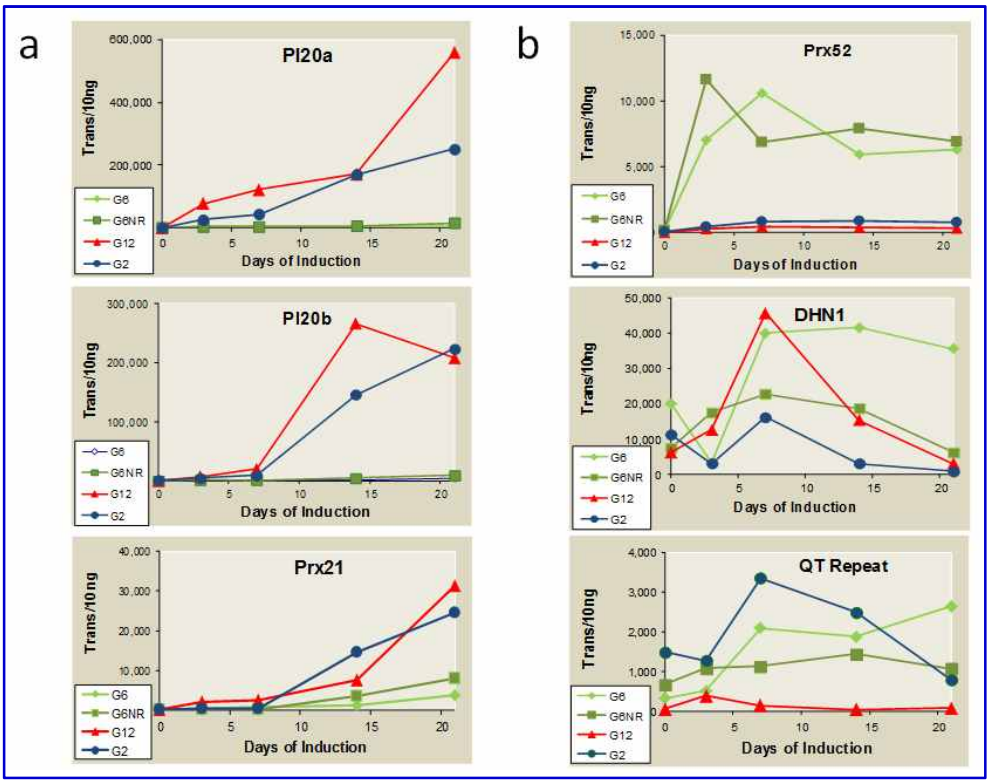

Figure 7. *Absolute quantification of six candidate genes during SE inductions conducted in the spring of 2014. Single replicate samples of PS collected from two nonresponsive genotypes (G2 and G12) and from responsive and nonresponsive (NR) clonal trees of G6 were used to quantify the expression of six candidate genes identified from microarray analysis of an induction experiment conducted in the spring of 2009 (Rutledge et al. 2013).* ***a*** *G12-inducible genes.* ***b*** *G6-inducible genes.*

8. Conclusion / Future research

Identification of clonal adult trees differing in their SE responsiveness has provided an unprecedented opportunity to examine factors influencing SE induction under an epigenetic context. This has particular significance in that epigenetics has been proposed to have a major role in determining SE responsiveness, likely related to the role of epigenetics in ontogeny (Poethig 2010);

however, as described here, the application of even the most advanced gene expression analysis technologies can be confounded by genotype-specific factors. Therefore, it was with great anticipation that transcriptome analysis via RNA-seq was initiated, particularly in view of the fact that it is unknown how long these trees will remain responsive.

Although this analysis is still in a preliminary stage, a MADS-box transcription factor has been found to be induced within responsive PS during the first days of induction, which is within a gene family known to include developmental regulators. Indeed, this is consistent with a general presumption that master regulator genes are most likely involved in generating the developmental events required for the formation of embryonal tissues in culture, as exemplified by the nodular structures that have been found to be so closely associated with G6 SE induction (Figure 3). Among many expectations is the possibility that if genes that directly influence SE responsiveness could be identified, they could provide effective targets for assessing the efficacy of applying small molecules known to modify the epigenome (Grant-Downton and Dickinson 2005), as part of an attempt to reverse the epigenetic suppression of SE responsiveness. Such an approach is founded on the general presumption that genome-wide epigenetic changes underpin the formation of stem cells (Birnbaum and Sánchez Alvarado 2008), which in turn could be essential for the formation of embryonal tissues.

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International effort to induce somatic embryogenesis in adult pine trees

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Abstract

The genus *Pinus* includes several species that are economically important and planted outside their natural ranges as plantation species. Somatic embryogenesis (SE), a biotechnological tool for mass propagation of pines, has been reported for many species but only from seed embryos. Cloning the individual adult trees, through SE from vegetative explants, could potentially benefit the forest industry in that only trees with elite characteristics would be planted commercially. The attributes of conifer trees may only be evaluated after many years of growth and often not until the reproductive growth phase. This chapter describes a concerted effort by several research teams in five countries to initiate SE in primordial shoot explants of six pine species, each commercially important in its respective country. In spite of the multi-year experiments, SE was induced in only one species (*Pinus sylvestris*), but embryogenic lines showed some instability at microsatellite loci and the somatic embryos did not germinate. Some cell lines

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initiated in different species showed embryogenic-like characteristics at the microscopic level. Expression of embryogenesis specific genes (*LEC1*/*CHAP3A*, *WOX2*, *VP1*) was detected in such calli/cell aggregates of all three tested pine species (including those with embryogenic-like characteristics) even when the presence of early somatic embryos could not be confirmed. Overall, the results presented in this chapter are indicative of the existing challenges in propagation of adult pines as in other conifers.

Keywords: callus, embryonal mass-like, explant pre-treatment, gene expression, needle fascicles, *Pinus* spp., primordial shoot explants, vegetative shoot buds

1. Introduction

The ability to vegetatively propagate adult conifer trees as opposed to zygotic embryos or seedlings, or juvenile trees, all of which are of unproven genetic potential, would be more advantageous in that only field-proven trees would be propagated for commercial plantings. Although rooted plants have been produced from axillary buds of adult trees of some pine species, such as *P. pinea, P. radiata, P. pinaster* and *P. sylvestris* on a laboratory scale, the method has many limitations including the lack of potential for scale-up as only a limited number of plants could be established (Cortizo et al. 2009; De Diego et al. 2008, 2010; Montalbán et al. 2013, respectively). Somatic embryogenesis (SE) of adult trees would provide not only a potential for scale-up production of clonal trees but coupled with the ease of cryopreservation of embryonal masses (EMs) would ensure a continuous supply of elite genotypes for plantings (*see also the chapter in this book "**Is there potential for propagation of adult spruce trees through somatic embryogenesis?**" by Klimaszewska and Rutledge*). Moreover conventional breeding is likely to benefit in the near future from the synergistic application of both genome-wide (genomic) selection and vegetative propagation (cloning) through SE of elite varieties to implement multivarietal forestry (Klimaszewska et al. 2007, El-Kassaby and Klápště 2015).

Starting in 2003 and onwards, a number of publications by Malabadi and collaborators claimed that SE could be initiated from adult trees (10-20 years old) of different tropical and subtropical pine species of high economic interest in South Africa (*Pinus patula,* Malabadi and van Staden 2003, 2005a) and northeastern India (*P. kesiya*, *P. roxburghii*, *P. wallichiana*, Malabadi et al. 2004, Malabadi and Nataraja 2006, 2007a, b). The authors described relatively similar tissue culture procedures for all the tested species to achieve the initiation of SE from shoot apical domes (primordial shoot, PS) and, in one case, from secondary needles (Malabadi and Nataraja 2007a). Positive results were presented for all three

genotypes tested in each species suggesting that the method is sufficiently refined to be considered generic, i.e., most genotypes would be responsive within a species. The SE initiation protocol was based on a modified DCR (Gupta and Durzan 1985) medium formulation (mDCR) and involved three main steps taking place in the dark (Table 1): (1) cold pre-treatment of explants for 3 days on a pre-treatment medium (PM) including activated charcoal but no plant growth regulators (PGR), (2) culture of explants on an initiation medium (IM) with high PGR content until white mucilaginous embryogenic tissue (ET) was detected (28-42 days) and (3) ET proliferation on maintenance medium (MM) with reduced PGR content.

Table 1. *Description of cold pre-treatment (PM), initiation (IM) and maintenance (MM) steps and media for inducing SE in explants of adult trees in different Pinus spp. (according to Malabadi et al.). Note: "?" indicates lack of information in the publication.*

Step - Medium	1- PM					2 - IM					3 - MM				
Pinus species[a]	Pp	Pk	Pr	Pr	Pw	Pp	Pk	Pr	Pr	Pw	Pp	Pk	Pr	Pr	Pw
Reference[b]	[1-3]	[4]	[5]	[6]	[7]	[1-3]	[4]	[5]	[6]	[7]	[1-3]	[4]	[5]	[6]	[7]
Explants source[c]	AD	AD	AD	ND	AD	AD	AD	AD	ND	AD	TS	TS	TS	LS	TS
Explants type[d]	TS	TS	TS	LS	TS	TS	TS	TS	LS	TS	EM	EM	EM	EM	EM
Ø 25 glass tube size (mm)	75	145	145	145	145	?	?	?	?	?	?	?	?	?	?
Medium volume/tube (ml)	10	15	15	15	15	?	?	?	?	?	?	?	?	?	?
Temperature (°C)	2	4	4	4	2	25	25	25	25	25	25	?	?	?	?
Incubation time (days)	3	3	3	3	3	28	28	42	?	?	30	?	28	30	30
Number of subculture	0	0	0	0	0	3	?	?	?	?	2	?	?	2	2
DCR macronutrients[e]	1X	0.5X	1X	1X	1X	1X	0.5X	1X	1X	1X	1X	0.5X	1X	1X	1X
DCR micronutrients[e]	1X	0.5X	1X	1X	1X	1X	0.5X	1X	1X	1X	1X	0.5X	1X	1X	1X
DCR vitamins[ef]	1X	?	?	?	?	1X	?	1X	1X	1X	1X	?	1X	1X	1X
Myo-inositol (g l^{-1})	?	?	?	?	?	1	1	1	1	1	?	?	?	?	?
PVP-40 (g l^{-1})	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	/	/	/	/	/
Activated charcoal (g l^{-1})	3	3	3	3	3	/	/	/	/	/	/	/	/	/	/
Maltose (mM)	90	83	83	83	83	90	/	/	/	/	120	111	167	83	167
2,4-D (µM)	/	/	/	/	/	20	22.6	22.6	22.6	22.6	2	2.26	2.26	2.26	2.26
NAA (µM)	/	/	/	/	/	25	26.8	26.8	26.8	26.8	2.5	2.68	2.68	2.68	2.68
BA (µM)	/	/	/	/	/	9	8.9	8.9	/	8.9	1	0.88	0.88	/	0.88
Triacontanol (µM)	/	/	/	/	/	/	/	/	5	/	/	/	/	2	/
pH[g]	?	?	?	?	?	5.8	5.8	5.8	5.8	5.8	?	?	?	?	?
Phytagel (g l^{-1})	1.5	/	/	2	2	1.5	2	2	2	2	2	4	4	1.8	4
Bacto-Agar (g l^{-1})	/	7	7	/	/	/	/	/	/	/	/	/	/	/	/
Casein hydrolysate (g l^{-1})[h]	/	?	?	?	?	1	1	1	1	1	/	/	/	/	/
L-Glutamine (g l^{-1})[h]	/	?	?	?	?	1	1	1	1	1	/	/	/	/	/

[a]Pp: *P. patula*; Pk: *P. kesiya*; Pr: *P. roxburghii*; Pw: *P. wallichiana*

[b][1] Malabadi and van Staden (2003); [2] Malabadi and van Staden (2005a); [3] Malabadi and van Staden (2005b); [4] Malabadi *et al.* (2004); [5] Malabadi and Nataraja (2006); [6] Malabadi and Nataraja (2007a); [7] Malabadi and Nataraja (2007b).

[c]AD: apical domes from shoot apices; ND: secondary needles; [d]TS: transverse sections (0.5-1 mm thick); LS: longitudinal sections of basal ND part (1-1.5 cm long); [e]Gupta and Durzan (1985); [f]Original vitamins excluding myo-inositol; [g]Medium pH was adjusted to 5.8 before gelling agent was added and autoclaving (121°C, 15 min, 103 kPa). [h]Filter-sterilized and added after autoclaving to the warm cooled medium (50°C).

Mean initiation rates of SE obtained by using this basic procedure (Table 2) were in the range of 2-7% (standard treatment) or 4-8% (treatment with dithiothreitol, DTT), in *P. patula*, 3-6% (standard) or 13-27% (treatment with smoke-saturated water, SSW) in *P. wallichiana*, 34-63% (apical domes of PS) or 46-65% (secondary needles) in *P. roxburghii,* and as high as 86% in *P. kesiya*. It was therefore conceivable that such a simple procedure could be successfully

applied, with potentially minor modifications, to other pine species of commercial interest. Based on the published studies, there were two key complementary factors to obtain initiation of SE within explants of PS of mature pines: (1) cold pre-treatment for 3 days at 2°C (*P. patula*, *P. wallichiana*) or 4°C (*P. kesiya*, *P. roxburghii*) on mDCR supplemented with 0.3% (w/v) activated charcoal and (2)

Table 2. *The best initiation rates of SE obtained from mature pine genotypes (Malabadi et al., procedure and media described in Table 1). Note: "?" indicates lack of information in the publication.*

Pinus spp.	Genotype	Age (years)	Explant source (type)[a]	Collection date (developmental stage)	Mean initiation rate ± standard error (%)	Ref.[a]
patula	PP3	15	AD (TS)	May (?)	4.5 ± 1.2 6.2 ± 0.5 (DTT)[b]	[1-2] [3]
	PP13	15	AD (TS)	March (?)	7.0 ± 1.2 8.2 ± 0.5 (DTT)[b]	[1-2] [3]
	PP18	15	AD (TS)	June (?)	2.0 ± 0.6 4.2 ± 0.2 (DTT)[b]	[1-2] [3]
kesiya	PK08	15-20	AD (TS)	April-July (?)	86.3 ± 7.4	[4]
	PK351	15-20	AD (TS)	April-July (?)	86.3 ± 7.4	[4]
	PK934	15-20	AD (TS)	April-July (?)	86.3 ± 7.4	[4]
roxburghii	PR11	14	AD (TS)	April (bud break)	57.0 ± 1.3	[5]
	PR105	14	AD (TS)	April (bud break)	63.0 ± 5.6	[5]
	PR521	14	AD (TS)	April (bud break)	34.5 ± 1.2	[5]
	PR17	10	ND (LS)	May (needle sprouting)	51.5 ± 4.1[c]	[6]
	PR100	10	ND (LS)	May (needle sprouting)	46.2 ± 3.2[c]	[6]
	PR321	10	ND (LS)	May (needle sprouting)	65.0 ± 1.9[c]	[6]
wallichiana	PW10	13	AD (TS)	May (bud break)	3.0 ± 0.2 13.0 ± 1.2 (SSW)[d]	[7]
	PW39	13	AD (TS)	May (bud break)	4.0 ± 0.6 21.0 ± 1.4 (SSW)[d]	[7]
	PW120	13	AD (TS)	May (bud break)	6.0 ± 0.3 27.0 ± 2.1 (SSW)[d]	[7]

[a]See Table 1. [b]Explants were submerged in 0.1% (w/v) dithiothreitol (DTT) for 10 min before cold pre-treatment (PM) and initiation (IM). [c]BA replaced by 5 µM triacontanol (TRIA) in IM medium. [d]10% (v/v) smoke-saturated water (SSW) in cold pre-treatment (PM) and initiation (IM) media.

the developmental stage of PS or secondary needles. Very low initiation rates or only hard non-embryogenic calli (NEC) were obtained if shoot explants were not exposed to cold pre-treatment (Malabadi and van Staden 2003, Malabadi et al. 2004, Malabadi and Nataraja 2006, Malabadi and van Staden 2005a), or if incubated at temperatures above 2-4°C (Malabadi et al. 2004, Malabadi and van Staden 2005a), or if cultured on medium with activated charcoal at a lower or higher concentration than 0.3% (Malabadi and van Staden 2005a, Malabadi and

Nataraja 2006, Malabadi et al. 2004, Malabadi and Nataraja 2007b) or if the explants were cultured on charcoal medium for longer than 3 days (Malabadi and van Staden 2005a, Malabadi and Nataraja 2006). Cold pre-treatment for only 1 or 2 days resulted in very low initiation rate of SE (Malabadi and van Staden 2005a). The optimized cold pre-treatment conditions, i.e., cold incubation for 3 days on half-strength (*P. kesiya*) or full-strength (other pine species) DCR supplemented with 0.3% activated charcoal, 0.2 g l^{-1} PVP-40, 83-90 mM maltose and 1.5-2.0 g l^{-1} gellan gum (Phytagel™) or 7 g l^{-1} agar (Difco-bacto), apparently fulfilled the requirement for SE initiation in all four species tested. Only the optimal cold incubation temperature appeared to be slightly variable among the species (2 to 4°C). It was found that PS explants produced ET only at specific collection dates from March to July depending on the pine species, the genotype, and the explant source (Table 2). Only white, hard NEC was obtained on other collection dates. It was noteworthy that all tested genotypes were able to produce ET during a relatively short period of competence of 1 (*P. patula*, *P. roxburghii* and *P. wallichiana*) to 3 months (*P. kesiya*). The "right" developmental stage of the shoot buds was apparently determined as being the early stages of elongation, i.e., immediately after bud break (Malabadi and Nataraja 2007b) or at the stage of needle fascicle sprouting (Malabadi and Nataraja 2007a).

Notwithstanding, none of the publications presented sufficient and unambiguous photographic evidence to illustrate progression of the SE initiation within the pine explants nor did the authors present regenerated plants from the somatic embryos established *ex vitro* except apparently in *P. kesiya* (Malabadi et al. 2004). It is also unclear whether the experiments were repeated in the consecutive years and if the same genotypes responded or whether the described responses were single events. However, owing to the importance of the subject matter for commercially important conifers, the above published results triggered the commencement of an international project, initiated in 2006 and coordinated by K. Klimaszewska, on SE induction in adult pine trees in two laboratories in Canada, two laboratories in France, and one each in Finland, Spain and New Zealand. Each laboratory worked on locally important pine species with specific protocols that were adapted following common discussion of the protocols published by Malabadi and collaborators from 2003 to 2007.

In this chapter, we present the results obtained in the above-listed laboratories for *P. pinaster, P. sylvestris, P. radiata, P. patula, P. strobus* and *P. contorta,* followed by conclusions. Most of these results have either not been published at all or have been included in conference abstracts and proceedings. Only the results on *P. contorta* and *P. radiata* were published by Park et al. (2010) and Garcia-Mendiguren et al. (2015), respectively and are also included in this chapter.

2. *Pinus pinaster* Ait. (Maritime pine) - Trontin J-F, Quoniou S, Lelu-Walter M-A

At FCBA and INRA, the research mainly focused on the determination of the responsive developmental stage of the shoot buds, applying the cold pre-treatment to the explants for 3 days at 2 or 4°C on mDCR supplemented with 0.3% (w/v) activated charcoal. Two genotypes of 5-year-old trees were used at INRA and four genotypes of 11- to 34-year-old trees at FCBA. We report here only the more advanced results obtained at FCBA from cultured explants. The shoot buds were collected in 2007 (10 experiments), 2008 (7 experiments) and 2009 (3 experiments).

2.1 Donor trees and PS explant sources

Three F1 genotypes of adult trees selected from elite G0 families (FCBA breeding populations) were compared in the SE initiation experiments: 1443 (0041 x 0022), 2599 (4301 x 3110) and 2849 (1337 x 0243). One genotype of a somatic tree (0136) was also introduced in several experiments as a tissue culture derived material. In the microscopic analyses, an embryogenic line initiated in 1999 (PN519) from an immature zygotic embryo (4304 x 4301) was included as the reference for embryogenic culture characteristics.

In March 2007, genotypes 1443, 2599 and 2849 were available as dozens of 4-year-old grafts; potted plants in a greenhouse or plants established outdoors in soil containers. The ortet age was 34 ± 1 year. This grafted mature material (producing female cones) has also been involved in rejuvenation experiments using micrografting techniques (Trontin et al. 2005). Significant knowledge has thus been gained about meristem activity and shoot growth during several growing seasons. The somatic genotype 0136 was acclimatized in 1996, grown outside since 1997 and planted in the field in the spring of 1999. This tree was 11 years old at the time of the first experiment and had just started its reproductive phase (first female cones were observed in spring 2007).

2.2 Preparation of PS explants

The grafts and somatic plants were treated monthly with fungicides by alternating aluminum ethylphosphite and thirame treatment, and weekly application with iprodione or vinchlozoline. Branch tips with shoot buds (about 20 cm long) were collected from mother plants 1-2 days after the foliar fungicide treatment. Unless specified otherwise, the shoots were randomly sampled within clone and individual plants. Secondary needles were removed from the subapical zone and the shoot bud (about 5-10 cm long) was excised, washed with a home

detergent solution and thoroughly rinsed with running tap water. Shoot buds were

Table 3. *Sampling, tested factors and success in decontaminating PS slices (SAS) during initiation experiments from four genotypes of mature P. pinaster (1443, 2599, 2849, 0136) launched at FCBA in 2007-2009.*

Exp.	Collection date	Plant setting[a]	Nb of genotypes	Buds Nb / genotype	Tested factors[b]	SAS (Nb)[c]	Decontaminated SAS (%)
1	09/03/07	G	3	2	Bud type	121	34.7
2	27/03/07	G	1	15	Decontamination + subculture	150	36.0
3	17/04/07	G	3	6	Bud type + decontamination	180	41.7
4	15/05/07	O	1	15	Decontamination	150	59.3
5	12/06/07	O	1	15	Decontamination	150	32.7
6	29/06/07	O	1	10	Decontamination	100	56.0
7	17/07/07	O	3	5-10	Decontamination	200	65.0
8	27/08/07	O	3	5	SAS position	295	39.3
9	02/10/07	O	3	5	SAS thickness	270	38.5
10	06/11/07	O	3	5	/	157	31.8
11	22/01/08	G	3	2	Bud stage + basal	177	95.5
		O	4	2-4	medium	266	80.8
12	04/02/08	G	3	4-8	Bud stage + basal	235	67.2
		O	4	4	medium + SAS thickness	264	59.1
13	19/02/08	G	3	4-6	Bud stage + basal	255	80.4
		O	4	4-6	medium	327	53.8
14	31/03/08	G	3	4	Bud stage + basal	230	90.9
		O	4	4	medium	312	56.7
15	12/05/08	G	3	7	Bud stage + initiation	144	100
		O	4	7	method	192	77.1
16	06/06/08	G/O	4	4	Medium composition (IM)	164	96.9
17	04/07/08	G/O	4	4	Medium composition (IM)	160	98.7
18	17/03/09	G	2	8	SAS pretreatment	166	100
		O	2	13-16		283	73.1
19	20/04/09	O	4	8	SAS pretreatment	320	98.1
20	11/05/09	O	4	4	Bud and SAS pretreatment	163	97.5
		O 4°C	4	9		370	98.1
		O Cryo	4	3		120	94.2
1-20	Total	/	4	495	/	5921	70.3

[a]*As genotypes 1443, 2599 and 2849 were available as dozens of potted plants (clones), some were established outdoors (O) in a nursery in soil containers during May 2007, whereas a second plant lot was put back in the greenhouse (G) in November 2007. Genotype 0136 was available as one tree planted outdoors (O), i.e., without any soil containers. O 4°C: buds collected at the time of experiment 18 (17/03/09) and stored at 4°C in dark (50 cm-long twigs in water + aluminum ethylphosphite; weekly sprayed with iprodione/vinchlozoline). O Cryo: buds collected in April (08/04/09) and immediately cryopreserved.*
[b]*In addition to genotype;* [c]*Sample size = total number of SAS investigated (nb of SAS x buds x genotypes).*

then surface-sterilized. Four methods were tested: (1) calcium hypochlorite: 90 g l^{-1} $Ca(OCl)_2$ with 70% active chlorine + 0.01% (v/v) wetting agent (home detergent) for 20 min (with stirring). Shoot buds were rinsed three times in large volumes of sterile water (exp. 1-7, 16, Table 3). (2) Hydrogen peroxide: H_2O_2 30% for 20 min

(with stirring). Shoot buds were rinsed three times in large volumes of sterile water (experiments 5-15, 17-18). In experiments 19-20, the H_2O_2 treatment lasted 60 min and was immediately followed by 70% (v/v) ethanol for 15 min before the three final rinses in sterile water. (3) Bleach/ethanol/$HgCl_2$: NaOCl containing 2.6% active chlorine + 0.01% (v/v) wetting agent for 5 min. Shoot apices were rinsed three times in a large volumes of sterile water, then immersed in 70% (v/v) ethanol for 5 min followed by 0.2% (w/v) $HgCl_2$ for 2 min. Shoot buds were finally rinsed four times in large volume of sterile water (experiment 2). (4) Sodium dichloro (iso) cyanurate: 5 g l^{-1} NaDCC + 0.01% (v/v) wetting agent for 2 min (with stirring). Shoot buds were rinsed two times in a large volume of sterile water. Bud scales were removed and shoot buds were further soaked for 1 min in 0.167 g l^{-1} NaDCC without rinsing (experiments 4-5).

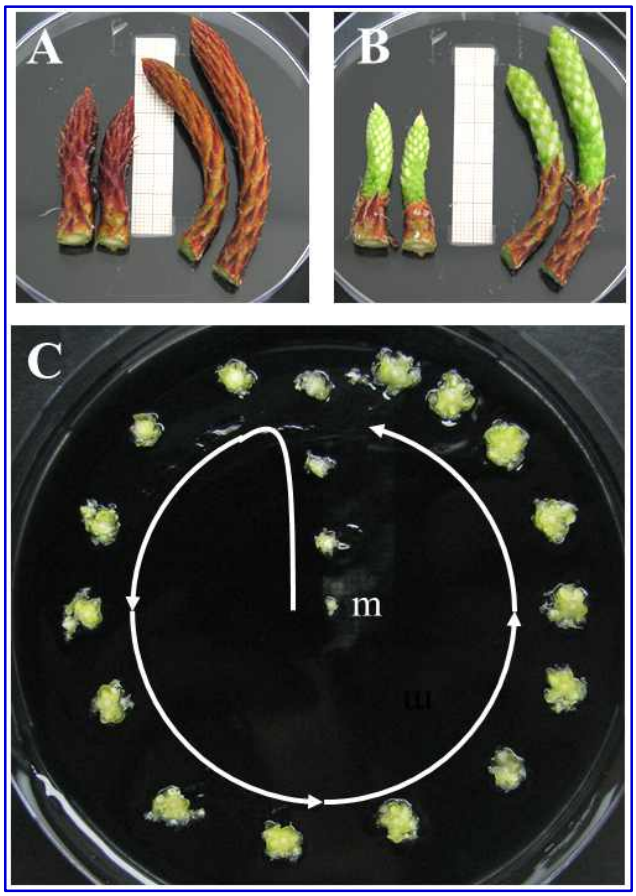

Figure 1. *Preparation of PS explants for initiation experiments in P. pinaster. Shoot buds were surface sterilized (**A**) and the bud scales were aseptically removed (**B**). Transverse PS apex slices (10-20 per shoot, 0.5-1 mm thick) were arranged in Petri dishes (**C**) from the top to the bottom of the PS apex (arrows). The first slice including the apical meristem is indicated (m).*

Transverse PS apex slices (SAS) were the final explants used in the initiation experiments with different medium formulations (Table 4). After removing the shoot bud sheath, the PS apex was transversely cut into slices (SAS) using sterile surgical blades #11 (slice thickness: 0.5-1 mm, exp. 1-8, 13-20) or razor blades (slice thickness ≤ 0.5 mm, experiments 9-12). An effort was made to cut thin slices (≤ 1 mm) because thick slices (1-2 mm) were reported to have a reduced SE initiation rate (Malabadi et al. 2004). A maximum of 10 (up to 20 in some experiments) transverse slices per PS, beginning from the top of the shoot bud (one slice including the apical meristem) to the subapical region (1-2 cm below the former) were cut. Slices were arranged on a culture medium according to their original position in the shoot bud (Figure 1). Overall (in 20 experiments, Table 3) and on average, we sampled about four shoot buds (up to 20) and 36 SAS per treatment (up to 164) from different shoot developmental stages (Figure 2), types and positions (Table 5).

Figure 2. *Developmental stages of one PS apex in P. pinaster monitored during 6 months from autumn 2006 to spring 2007.* ***D****: Dormant buds (arrested shoot growth); apical bud length ~ 10 cm.* ***S****: Bud swelling; terminal shoot length ~ 11 cm.* ***E****: elongating bud; terminal shoot length ~ 16 cm.* ***EF****: elongating shoots with female cones emergence (****EF1****, red arrows) and growth (****EF2****); terminal shoot length ~ 18 cm (EF1) or 27 cm (EF2).* ***NS****: secondary needles sprouting from elongated shoots; terminal shoot length ~ 34 cm.*

Of the different methods for disinfection of shoot buds, the H_2O_2 method consistently yielded the best results (68% of non-contaminated SAS) followed by NaOCl/ethanol/$HgCl_2$ (44%), $Ca(OCl)_2$ (41%) and NaDCC (34%). Removing the scales prior to disinfection did not improve the sterility of SAS, while explant viability was significantly reduced. In individual shoot buds from the same clone, the sterility varied from 0 to 100% suggesting that most (if not all) contaminants were endophytes. Subsequently, the H_2O_2 method was used in experiments 8 to 18 (excluding experiment 16) and the overall sterility of SAS reached 68% (N = 3733). Significant differences were found among genotypes 2599 (51%, N = 910), 2849 (60%, N = 1250), 1443 (80%, N = 1148) and 0136 (97%, N = 425). The sterility of the cultures was also very good in experiments 19-20 using a H_2O_2 modified protocol (97%, N = 973). Large fluctuations were observed among collection dates (Table 3), from 31% in experiment 10 (06/11/07), 86% in experiments 11 (22/01/08) and 15 (12/05/08), and 98% in experiments 17 (04/07/08) or 19 (20/04/09), suggesting that the endophytes within a plant are highly variable and poorly controllable by pesticides. It was clear however from experiments 11 to 15 and 18 (clones 1443, 2599 and 2849) that potted plants in the greenhouse yielded more sterile explants (87%, N = 1207) than the plants established outdoors in soil containers (only 56%, N = 1259).

2.3 Initiation of cultures from PS explants

The PS explants (SAS) were subjected to two initiation procedures: IP1 (experiments 1-18) and IP2 (experiments 19-20). In both IP1 and IP2 the standard cold pre-treatment of SAS was for 3 days in the dark at 2°C or at about 4°C (experiments 18-20) on PM medium. In one IP1 experiment we also tested 4°C for 17 days, 23°C for 3 days or no pre-treatment (experiment 18). In two IP2 experiments we investigated whether high *versus* low temperature pre-treatment could be beneficial for maritime pine using the following temperature sequences: 4°C for 1 day, 40-53°C (heat shock) for 4 hours, 4°C for 2 days (experiments 19-20), 23°C for 1 day, 40°C for 4 hours, 23°C for 2 days (experiment 19) or 40°C for 3 days (experiment 20). After pre-treatment, the PS sections were incubated on the initiation medium (IM) at 25 ± 2°C in the dark for 4 weeks or until soft, translucent to whitish mucilaginous tissue was detected (no subcultures were carried out). This potentially EM was then separated from the surrounding tissue and subcultured biweekly onto maintenance medium (MM) at 25 ± 2°C in the dark. PS explants were cultured in Petri dishes (9 x 1.5 cm) containing 23.5 ml medium and sealed with two layers (experiments 1-12) or only one layer (experiments 13-20) of cling film.

The recipes for pre-treatment (PM), initiation (IM) and maintenance media (MM) used in IP1 and IP2 are listed in Table 4. In IP1, the formulation is mDCR

including full-strength macronutrients, micronutrients and "vitamins" (meso-inositol, nicotinic acid, pyridoxine and thiamine hydrochloride) from Gupta and Durzan (1985) with the modifications proposed by Malabadi and van Staden (2005a). We also tested a modified Litvay (mLV) medium (Litvay *et al.* 1985) with modifications as above. Compared with DCR, Litvay-based media were found best-suited for initiation of SE from immature zygotic embryos in maritime pine (Park et al. 2006, Trontin et al. 2009, see also Trontin et al., Chapter on Maritime pine in this book). Unless specified otherwise, in the text and in Table 5 (mDCR2,3,4), all media were supplemented with vitamins Gupta and Durzan (1985) or Litvay et al. (1985) to obtain mDCR1 or mLV1 recipes, respectively (Table 4). In IP2, substantial modifications of the original mDCR1 recipe were made to obtain mDCR5 (Table 4). The main change was the high calcium content in mDCR5 (2.5 g l^{-1} $CaCl_2$ x 6 H_20) compared with mDCR1 (0.085 g l^{-1}). High calcium was found to mediate cold-enhanced SE in *P. patula* (Malabadi and van Staden 2006).

Table 4. *Composition of the pre-treatment (PM), initiation (IM) and maintenance media (MM) for SE initiation in P. pinaster (standard IP1 and IP2 procedures, this study).*

Basal medium	mDCR[a]						mLV[b]		
Basic procedure (medium)	IP1 (mDCR1)			IP2 (mDCR5)			IP1 (mLV1)		
Medium	PM	IM	MM	PM	IM	MM	PM	IM	MM
Macronutrients	DCR 1X			DCR 1X with 2.5 g l^{-1} $CaCl_2$, 2 H_20			LV 0.5X		
Micronutrients	DCR 1X			DCR 1X without $NiCl_2$, 6 H_20			LV 1X		
Vitamins[c]	DCR 1X			DCR 1X			LV 10X		
Meso-inositol (g l^{-1})	0.2	1	1	/	1	1	0.2	1	1
PVP-40 (g l^{-1})	0.2	0.2	/	0.2	0.2	0.2	0.2	0.2	/
Activated charcoal (g l^{-1})[d]	3.0	/	/	3.0	/	/	3.0	/	/
Casein hydrolysate (g l^{-1})	/	1	1	1	1	1	/	1	1
Maltose (mM)	90	90	120	83.3	83.3	83.3	90	90	120
2,4-D (µM)	/	20	2	/	22.6	2.3	/	20	2
NAA (µM)	/	25	2.5	/	26.8	2.7	/	25	2.5
BA (µM)	/	9	1	/	8.9	0.9	/	9	1
pH[e]	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.8
Phytagel (g l^{-1})	1.5	1.5	2	2	2	2	1.5	1.5	2
L-Glutamine (g l^{-1})[f]	/	1	1	1	1	1	/	1	1
Gel strength (N)[g]	0.20	0.29	0.34	0.12	0.09	0.08	0.17	0.22	0.26

[a]Gupta and Durzan (1985); [b]Litvay *et al.* (1985); [c]Original vitamins excluding myo-inositol; [d]Purchased from Sigma Ca. N°C6289; [e]Medium pH was adjusted to 5.8 before gellan gum was added and autoclaving (121°C, 15 min, 1.05 kg cm^{-2}). [f]pH adjusted to 5.8, filter-sterilized and added after autoclaving to the cooled medium (50°C for IP1, 60-65°C for IP2). [g]Mean gel strength (in Newton) measured 24 h after autoclaving and pouring into Petri dishes.

Other major changes included nickel deprivation in all media, no myo-inositol in PM, PVP-40 added to MM, PM supplemented with casein hydrolysate and glutamine, higher maltose content in MM, higher gellan gum in PM and IM.

The gel strength of media was much lower in this IP2 protocol, around 0.10 Newton (N) versus 0.20-0.34 N, owing to the 29-fold higher calcium concentration.

2.4 Generalized morphogenetic response of SAS to the initiation protocols (IP1 and IP2).

Table 5. *Mean number of white tissues (WT) produced per SAS explant of P. pinaster after 4-week culture on IM medium.*

Exp.	Shoot stage[a]	Shoot type[b]	SAS position[c]	Mineral base[d]	SAS pretreatment on PM (temperature, duration)	N[e]	Necrotic SAS (%)[f]	Reactive SAS (%)[f]	WT
1	E	S	A+SA	mDCR1	2°C 3 d	22	0.0	95.5	0.45
		L				20	0.0	95.0	0.60
2	EF2	L	A	mDCR1	2°C 3 d	54	27.8	96.3	0.52
3	EF2	LT	A	mDCR1	2°C 3 d	43	0.0	100	0.84
		LA				32	0.0	93.7	0.87
4	NS	L	A	mDCR1	2°C 3 d	89	0.0	100	0.73
5	D	L	A	mDCR1	2°C 3 d	49	6.1	93.9	0.94
6	S	L	A	mDCR1	2°C 3 d	56	0.0	100	1.00
7	E	L	A	mDCR1	2°C 3 d	130	0.0	100	0.91
8	NS	L	A	mDCR1	2°C 3 d	67	3.0	97.0	0.82
			SA			49	32.7	81.6	0.29
9	D	L	A	mDCR1	2°C 3 d	104	1.0	91.3	0.81
10	D	L	A	mDCR1	2°C 3 d	50	10.0	76.0	0.58
11	S	L	A+SA	mDCR1	2°C 3 d	86	3.5	96.5	0.72
				mLV1		83	22.9	69.9	0.58
	D			mDCR1		98	8.2	89.8	0.57
				mLV1		117	73.5	18.8	0.13
12	S	L	A	mDCR1	2°C 3 d	94	8.5	83.0	0.64
				mLV1		64	29.7	57.8	0.48
	D			mDCR1		75	0.0	92.0	0.55
				mLV1		81	70.4	23.5	0.10
13	E	L	A+SA	mDCR1	2°C 3 d	111	10.8	85.6	0.59
				mLV1		94	24.5	78.7	0.63
	D			mDCR1		104	5.8	94.2	0.80
				mLV1		82	29.3	73.2	0.61
14	EF2	L	A+SA	mDCR1	2°C 3 d	116	0.0	100	0.33
				mLV1		93	25.8	69.9	0.26
	S			mDCR1		90	3.3	96.7	0.92
				mLV1		86	60.5	34.9	0.23
15	NS	L	A	mDCR1	2°C 3 d	72	1.4	98.6	0.28
				mDCR2		72	87.5	1.4	0.00
	EF2			mDCR1		83	1.2	98.8	0.30
				mDCR2		65	92.3	0.0	0.00
16	NS	S	A	mDCR1	2°C 3 d	81	1.2	96.3	1.14
				mDCR3		78	85.9	10.3	0.00
17	D	S	A	mDCR1		80	0.0	93.7	0.89
				mDCR4	2°C 3 d	79	0.0	91.1	1.03
18	E	S	A	mLV1	2-4°C 3 d	84	34.5	61.9	0.54
					2-4°C 17 d	82	84.7	13.4	0.01
	S	S	A	mLV1	2-4°C 3 d	100	53.0	51.0	0.26
					23°C 3 d	86	61.6	54.7	0.14
					No pre-treatment	21	71.4	38.1	0.29

19	E/EF1	S	A	mDCR5	2-4°C 3 d	79	84.8	11.4	0.00
					4°C 1 d, 40°C 4 h, 4°C 2 d	158	75.9	11.4	0.00
					23°C 1 d, 40°C 4 h, 23°C 2 d	77	83.1	16.9	0.00
20	EF2	S	A	mDCR5	4°C 1 d, 53°C 4 h, 4°C 2 d	84	100	0.00	0.00
					4°C 3 d	75	4.0	6.7	0.00
	E cryo	S	A	mDCR5	4°C 1 d, 53°C 4 h, 4°C 2 d	80	100	0.00	0.00
					4°C 3 d	33	66.7	0.00	0.00
	S 4°C	S	A	mDCR5	4°C 1 d, 53°C 4 h, 4°C 2 d	122	100	3.3	0.00
					4°C 3 d	118	5.1	5.1	0.00
					40°C 3 d	123	100	0.00	0.00
1-20	/	/	/	/	/	4171	35.7	57.9	0.41

[a]Shoot developmental stages defined in **Fig. 2**.
[b]S = Short shoot (≤ 5 cm in length); L = Long shoot (≥ 10 cm in length); T = Terminal shoot; A = Axillary shoot.
[c]A: apical zone (< 10 mm from meristem); SA: subapical zone (10-20 mm from meristem).
[d]mDCR1 standard procedure and media (IP1, **Table 4)** ; mDCR2: explants were individually cultivated in glass tubes (25 x 200 mm; medium volume = 20 ml) instead of Petri dishes with some modification of PM (no vitamins and meso-inositol; gel strength = 0.02 N) and IM media (no maltose; casein hydrolysate was filter-sterilized and added after autoclaving; gel strength = 0.17 N); mDCR3: mDCR standard procedure but without maltose added to IM ; mDCR4: mDCR standard procedure with 10 g l^{-1} maltose added to IM; mDCR5: mDCR1 modified procedure (IP2, **Table 4**).
[e]N: sample size (decontaminated SAS, see **Table 3**).
[f]Evaluated after 2 weeks on IM medium. Necrotic SAS: black localized areas or affecting the whole explant are observed. SAS are reactive when swelling, healthy tissues are observed at least in some parts of the explant (**Fig. 3B, 3D**). Note that reactive SAS could also be classified as necrotic in the case of localized necrosis.
Notes: SAS thickness is 0.5-1 mm except during experiments 9-12 (< 0.5 mm); Petri dishes were sealed with 2 (experiment 1-12) or only 1 ring (experiment 13-20) of cling film.

In the case of IP1 (mDCR1 or mLV1 media), the SAS integrity appeared to be largely preserved after cold pre-treatment on PM medium (Table 4) for 3 days at 2°C and incubation for 1-5 days on IM medium (Figure 3A). The whole structural organization of the sections remained recognizable with an epidermis, a broad cortex parenchyma containing resin ducts and a ring of yellowish vascular bundles surrounding the whitish pith. Some enlargement and elongation of young wounded needle fascicles or primordia surrounding the SAS usually started after 6-9 days on IM medium. We also observed some translucent tissues mainly located in the cortex but not in the central pith (Figure 3D). At the same time, some SAS became yellowish to brownish in colour, which started in the central parenchyma pith zone (Figure 3B) eventually affecting other tissues within only 1 week (Figure 3C). In other cases, a white, often translucent and soft tissue (WT) morphologically resembling EM was growing out from both SAS edges (needle fascicles) and cortical zone. The WT could rapidly, within 1-2 weeks, cover the entire SAS surface (Figure 3E) and/or more frequently underwent localized profusion, especially at the contact site between the explant and the gelled IM medium (Figure 3F, arrows). Some signs of likely oxidative stress (browning) were already detectable at this step and WT usually turned hard and entirely brown within 4 weeks after the transfer to MM medium (Figure 3G). Some WT reappearance (usually hard tissue) was consistently observed after biweekly subculture of

browning calli for 6 weeks (Figure 3F) but, again, sustained proliferation of these cells could not be achieved. In IP2, most explants turned brown (Figure 3B, 3C) and finally died within 2-3 weeks following either low or high temperature pre-treatment. None or very discrete WT production could be observed. The high calcium content of mDCR5 (2.5 g l^{-1} $CaCl_2$, $2xH_2O$) was most likely toxic to maritime pine.

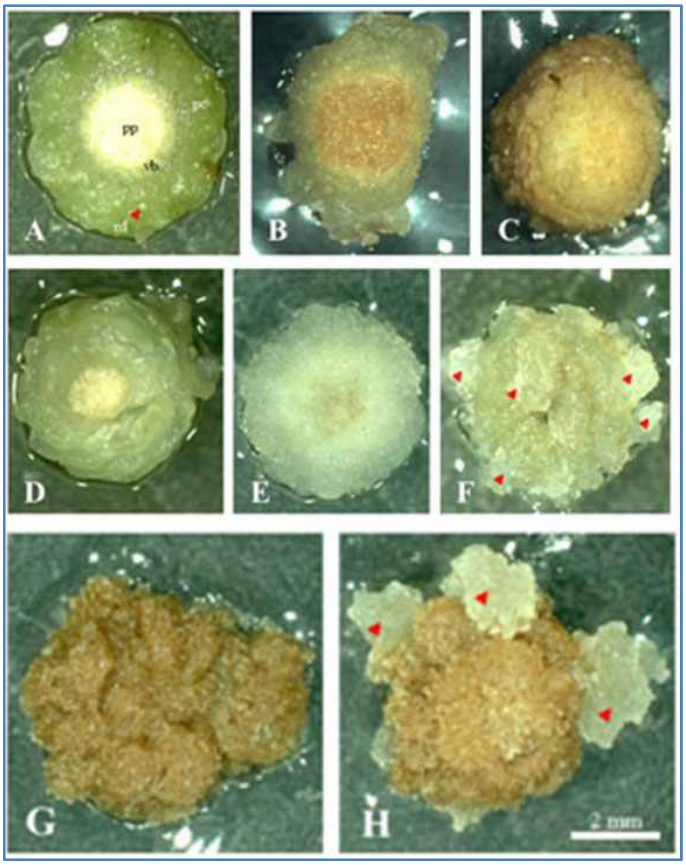

Figure 3. *General morphogenetic response of SAS from P. pinaster subjected to the initiation procedure (see Table 1 and Table 4) after 1-5 (**A**), 6-9 (**B, D**) or 10-14 days (**C, E**) on initiation medium (IM), and 1-2 (**F,** note the localized profusion of whitish soft tissue, arrows), 3-4 (**G**) or 5-6 more weeks (**H,** note the resurgence of white hard tissue, arrows) on maintenance medium (MM). **e**: epidermis; **pc**: broad parenchymatous cortex; **pp**: parenchymatous pith; **rd**: resin ducts (arranged in a ring in the cortex, arrow); **vb**: vascular bundles arranged in a ring around the pith.*

2.5 Factors affecting production of white tissue (WT) in IP1

Culture medium - The occurrence of WT was scored 4 weeks after SAS transfer onto IM (Table 5). The medium (mDCR, mLV) strongly affected SAS

response and necrosis, and ultimately WT production. Medium mDCR1 (N = 774) consistently supported a high SAS response (92.2%), low necrosis (5.2%) and a high WT production rate per SAS (0.63). By comparison, only 52.1% SAS were reactive using mLV1 (N = 700), with 43.4% explants showing large necrotic areas and a low mean of 0.36 WT per SAS. This medium interacted with the genotype. On mDCR1, a similar SAS response (91.4-94.6%) and necrosis (3.8-5.9%) were obtained and quite low differences in WT production rate per SAS (0.57-0.76) were detected among genotypes (Table 6). Conversely, higher variability was observed using mLV1. Genotypes 2599 and 0136 were poorly responsive (15.9-36.7%), highly necrotic (51.1-79.9%) and sporadic WT producers (0.07-0.09). Genotype 1443 was moderately responsive (51.2% response; 46.8 % necrosis) with low WT (0.36) produced per SAS (as compared with 0.74 on mDCR1). Only genotype 2849 showed a similar and quite high WT production rate on both mLV1 (0.64) and mDCR1 (0.67) but with slightly decreased responsiveness (81.7 vs. 94.5%) and increased necrosis (16.5 vs. 5.9%).

In experiment 15, we compared mDCR1 medium and procedure (Table 4) with a new DCR method (mDCR2, see Table 5) considered to be experimentally closer to the procedure described by Malabadi and collaborators (Table 1). Surprisingly, most decontaminated explants (89.8%) died within 2 weeks on IM

Table 6. *Mean number of white tissues (WT) produced per SAS explant in P. pinaster as influenced by the medium (mLV1, mDCR1) and genotype.*

Genotype	1443		2599		2849		0136	
Basal medium	mDCR1	mLV1	mDCR1	mLV1	mDCR1	mLV1	mDCR1	mLV1
N*	755	346	387	90	492	284	221	164
Necrotic SAS (%)	3.8	46.8	4.6	51.1	5.9	16.5	4.1	79.9
Reactive SAS (%)	94.2	51.2	94.6	36.7	94.5	81.7	91.4	15.9
WT	0.74	0.36	0.57	0.07	0.67	0.64	0.76	0.09

*N: sample size (decontaminated SAS, see **Table 3**), experiments 1-18, cold pre-treatment 2-4°C 3 days.

medium (experiment 15, Table 5). As IM was not supplemented with maltose in mDCR2 (in contrast with PM medium), we hypothesized that the use of such a carbon source during both pre-treatment and initiation was critical for SAS responsiveness. General SAS necrosis could also result from the very low gel strength of mDCR2 PM medium in glass tubes (0.02 N) compared with mDCR1 PM medium in Petri dishes (0.20 N). In experiment 16, we thus tested the standard mDCR1 in Petri dishes (Table 1) with (mDCR1) or without maltose (mDCR3) added to IM (Table 5). The very low survival rate of SAS on IM medium, when deprived of maltose, was confirmed (85.9% vs. 1.2% necrosis). SAS from the top position were much more affected by necrosis than SAS from a more basal position. SAS response was also significantly reduced (10.3% vs. 96.3%) and no WT

production was observed on mDCR3, as compared with 1.14 for mDCR1 (data not shown). We thus concluded that IM must be supplemented with maltose (32.4 g l^{-1}) to prevent necrosis and to achieve a high SAS response and WT production. By reducing the maltose concentration from 32.4 g l^{-1} (mDCR1) to 10 g l^{-1} (mDCR4) in IM medium (experiment 17, Table 5) we obtained a very similar SAS response (93.7 vs. 91.1% with no necrosis) and WT production rate (0.89 vs. 1.03) after 4 weeks induction. However WT induction and growth was much more discrete on mDCR4 than on mDCR1 and sometimes originated from the central part of the SAS (vascular bundle and pith zones) instead of on the cortex parenchyma (data not shown). This is an interesting point because WT production in *P. patula*, *P. roxburghii* and *P. wallichiana* seems to exclusively arise from the vascular bundle/cambial cells of SAS (Malabadi and van Staden 2005a, Malabadi and Nataraja 2006, 2007b).

There are thus some troubling similarities between the SAS reaction figures obtained in *P. pinaster* with reduced maltose concentration and the SE initiation figures reported by Malabadi and collaborators. As already mentioned, there are some inconsistencies in IM medium formulation proposed by these authors, especially in maltose concentration (0-90 mM, Table 1). We concluded that a more detailed spatiotemporal study of WT induction from SAS as a function of maltose concentration in IM medium is required in maritime pine.

SAS position - In addition to the medium type and genotype, SAS position relative to the meristem was identified as another factor affecting WT production in *P. pinaster*. We consistently observed that the 4-5 first slices (including the meristem) were more prone to WT production. This was specifically tested during experiment 8 (Table 5) by comparing SAS located in the apical (< 10 mm) and subapical zones (10-20 mm below the meristem). Apical SAS produced more WT (0.82 per SAS) than the subapical slices (0.29), with the latter ones being also more necrotic in this experiment (32.7 vs. 3.0%) and less reactive (81.1 vs. 97.0%). Pooling the data from all the experiments (mDCR1 basal medium only) we calculated that SAS restricted to the apical region of the shoot (N = 1159) produced more WT (0.74) than SAS originating from both apical and subapical zones (0.61 N = 696).

SAS thickness - Although not specifically tested in this work, our results suggested that slice thickness affected WT production. Considering only the data collected under the most favourable conditions (mDCR1, SAS belonging to the apical zone), we found that very thin slices (< 0.5 mm thick, N = 323, experiments 9-12, Table 5) were less reactive (86.7 vs. 97.7%), slightly more necrotic (4.3 vs. 2.8%) and produced less WT (0.66 vs. 0.77) than slices 0.5-1 mm thick (N = 836).

Shoot developmental stage - The effect of shoots developmental stages (D, S, E, EF, NS Figure 2) on SAS responsiveness and necrosis and incidence of WT production were studied over the 18 IP1 collection dates from 9/03/2007 to

17/03/2009 (mDCR1 or mLV1 media, cold pre-treatment for 3 days at 2-4°C). Using the mDCR1 standard procedure (all genotypes) we obtained low necrosis (4.1-5.6%) and a high response (90.9-98.5%) of SAS (Figure 4A). We observed high WT occurrence (mean of 0.69/SAS) but with significant variation as a function of the shoot developmental stage (0.47-0.80, Figure 4A). Similar results (0.69-0.80 WT/SAS) were observed at the D (dormant buds), S (swelled buds), E (elongating buds) and NS (needle sprouting) stages with a maximum at the S stage (0.80). In contrast a strong decrease was observed at the time of elongating shoots with female cone growth (EF2, 0.47 WT/SAS), but without any clear correlation with SAS necrosis and response. Similar conclusions were drawn by analyzing the data from independent genotypes, especially 1443 (Figure 4A) and 2599 (data not shown). A slow decrease in WT production from stage D (0.76) to NS (0.60, Figure 4A) was however observed in the case of genotype 2849.

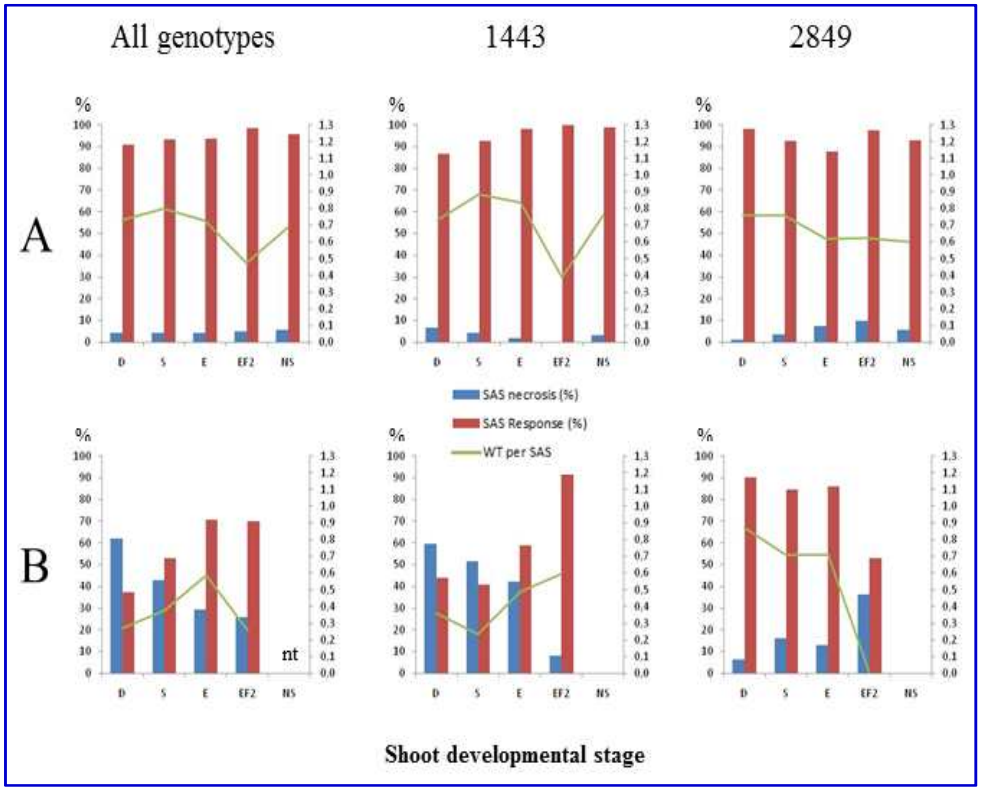

Figure 4*. SAS necrosis and responses (%, left vertical axis) and WT occurrence per explant (WT index, right vertical axis) in P. pinaster as a function of shoot developmental stage and culture medium.*
A. *mDCR1 medium;* ***B.*** *mLV1 medium. See* ***Table 4*** *for medium composition. Shoot developmental stages (D, S, E, EF2 and NS) are defined and illustrated in* ***Figure 2****. Data were computed from all genotypes or from the highly responsive genotypes 1443 and 2849. Nt: not tested.*

Different results were obtained using mLV1 (data from all genotypes, Figure 4B). The high necrosis rate of SAS observed on this medium decreased with advanced developmental stages, from 61.9% at D stage to 25.8% at the EF2 stage. The SAS response concomitantly increased from 37.4 to a mean of 70.5% at the E and EF2 stages. Interestingly, the WT occurrence increased with SAS response from the D (0.27) to E stages (0.58) before decreasing at the EF2 stage (0.26). This general trend was however not representative of individual genotypes (Figure 4B), especially responsive genotypes on mLV1 (1443, 2849). WT production was found to increase from D (0.36) and S stages (0.23) to EF2 (0.59) in genotype 1443 but a strong decrease was observed for genotype 2849 (from 0.87 to 0). In both cases a negative correlation with SAS necrosis and positive correlation with SAS response was observed. We finally concluded that WT occurrence was affected by the shoot developmental stage on both mDCR1 and mLV1 media. Early stages (D, S, E) produced better results in most genotypes in the case of mDCR1. In contrast, the SAS response to developmental stages on mLV1 appeared much more genotype-dependent with the optimal stages being D, S, E in 2849 but EF2 in 1443.

2.6 Microscopic observations

Potential EMs collected from explants on IM and/or MM media were stained with acetocarmine (1.5% w/v) directly on the glass slides for 3-4 min. Stained samples were gently rinsed with water and mounted (with cover slide) in a fructose syrup (150 g fructose + 100 ml water). Squashes were then incubated overnight at 37°C and sealed with two layers of nail varnish. A light microscope (Optiphot, Nikon, Kogaku, Japan) equipped with a digital camera (DX20N, Kappa opto-electronics, Gleichen, Germany) was used for observations and picture taking.

For cellular organization (CO) analysis of squashes, we used the nomenclature (five classes CO1 to CO5) defined by Breton et al. (2005) for maritime pine. Classes CO1 and CO2 refer to numerous, loosely aggregated, small and cytoplasmically dense cells with rare (CO1) or some occurrence (CO2) of clustering (less than 50 µm cluster size) and/or organized cell divisions. Class CO3 is characterized by large clusters of cytoplasmically dense cells (up to 100-200 µm in size) forming early EM (usually shapeless) with differential growth (somatic polyembryony). In this CO3 class, EMs were sometimes attached to unequally elongating, loosely aggregated vacuolated cells forming early secondary suspensors (embryonal tube). Such early embryogenic structure with a clear bipolarity, i.e., apically situated EM sustained by a well-organized (compact) secondary suspensor, are found in class CO4 (small embryoids with EM of about 100 µm in size) and in CO5 (well-developed embryoids with EM up to about 500 µm in size). In both CO4 and CO5 (late stages of early embryogeny) differential growth of regions of the embryoid EM is possibly observed (delayed cleavage

polyembryony). CO1 and CO2 cells and structures are frequently observed in CO3, CO4 and CO5. The occurrence of each CO class, early embryogenic structures and somatic embryos is largely affected by culture aging (Breton et al. 2005, 2006).

We were unable to promote sustained growth of soft WT resembling EM on either mDCR- or mLV-based IM or MM media (IP1 protocol) using various subculture methods and frequencies. Similar results were obtained with adult *P. pinaster* genotypes growing in Spain (Humánez et al. 2012). WT usually became yellowish to brownish shortly after initiation, i.e., within a few days regardless whether the tissue was kept attached (see Figure 3F-G) or removed from the explant. In the latter case, browning could sometimes be observed within a few minutes after excision suggesting a severe wounding stress response. As a result, soft WT rapidly ceased to grow and reversed towards hard, yellowish callus later producing hard WT with similar morphological progression (data not shown). No more than a few mm^3 of apparent volume growth could be obtained for most sampled soft WT. The non-destructive morphological observation of such a tiny amount of WT was ambiguous (Figure 5A) and micromorphological analysis was required to differentiate potential embryogenic from non-embryogenic tissue (Figure 5B). WT were thus usually sacrificed shortly after initiation (usually at the time of collection) to prepare squashes stained with acetocarmine. A total of 187 WT were analyzed from experiments 7 to 18 (IP1, cold pre-treatment for 3 days, mLV1 or mDCR1 media). As expected from the response and WT production data (Table 6, Figure 4) most squashed WT were from genotypes 1443 (40.1%, $8.7*10^{-2}$

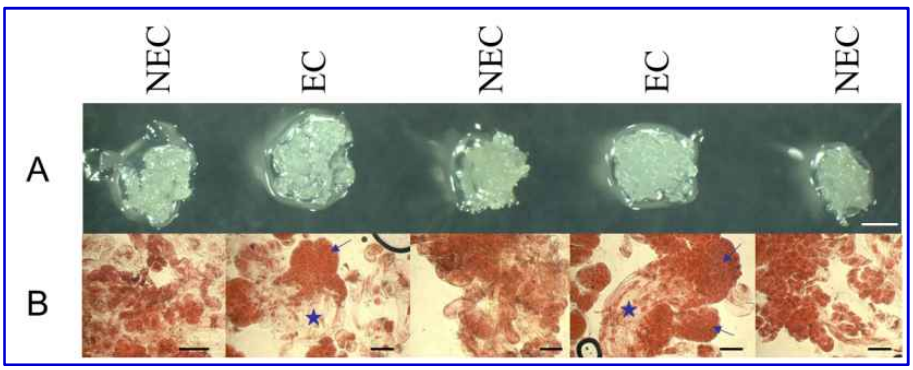

Figure 5. *Macro- (**A,** bar = 1 mm) and micromorphological (**B,** bar = 100 µm) observations of small amount of soft WT (a few mm^3, less than 10 mg) collected from embryogenic-like (EC) or non-embryogenic (NEC) cultures in P. pinaster.*
The macromorphological observation of small, soft WT initiated from SAS on IM medium was unsuitable to distinguish EC vs. NEC. Microscopic observations were required to detect EM (blue arrows) and secondary suspensor cells (embryonal tube, blue stars) forming early embryogenic structures in EC.

squashes/SAS) and 2849 (38.5%, $10.8*10^{-2}$ squashes/SAS). Genotypes 2599 and 0136 accounted for only 17.6% ($7.1*10^{-2}$ squashes/SAS) and 3.7% ($1.8*10^{-2}$ squashes/SAS) of slides, respectively. Most squashes (88.2%, 165 WT) only revealed quite hard, highly difficult to disaggregate clusters of cells. These compact tissues were usually accompanied by numerous loosely aggregated, cytoplasmically dense spherical (Figure 6, WT3) or elongated cells (Figure 6, WT4). We therefore concluded that the sampled tissue did not exhibit any advanced cellular organization of EM (CO3 to CO5 classes, Breton et al. 2005) and was best classified in this work as NEC. Interestingly, the cellular organization consisting of loosely aggregated (unorganized divisions), oval cells illustrated in Figure 6 (WT3) was similar to the CO1 class usually found largely interspersed with the CO3, CO4 and CO5 in EC. The frequency of CO1 increased with culture aging or in response to an unsuitable treatment (Breton et al. 2005).

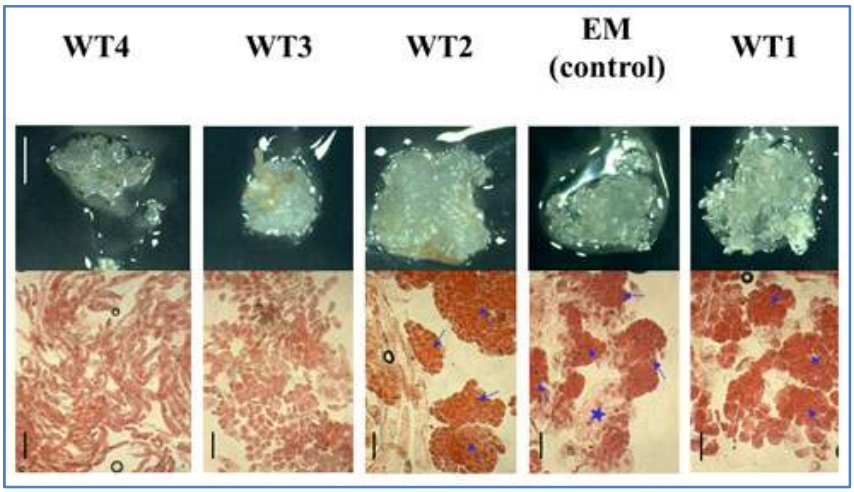

Figure 6. *Micromorphological observation (lower panel, bar = 100 µm) of several WT (upper panel, bar = 1 mm) initiated on SAS explants collected from mature P. pinaster trees compared with control EM. Compact aggregates of cytoplasmically dense cells (blue arrows) detected in some WT are very similar to early EM forming suspensor cells (blue stars) in embryogenic culture.*

In a significant number of squashes (11.8%, 22 WT), we detected (after about 4 weeks on IM medium) more advanced and/or intriguing structures with cellular organizations resembling the CO3, CO4 or CO5 classes found in embryogenic culture. Large clusters of cytoplasmically dense cells actively dividing in a cohesive way and forming compact cell aggregates (Figure 6, WT1, WT2) were observed. Usually shapeless but with apparent differential growth (e.g. WT2), those cell aggregates 100-500 µm in size were found very similar to early

EM undergoing somatic polyembryony in SE cultures from seed embryo (Figure 6) with CO3 cellular organization. Very similar figures were obtained from mature trees in *P. contorta* (Park et al. 2010, see *Figure 3c, d* in that paper) and *P. pinaster* (Humánez et al. 2012, see *Figure 8a, b* in that paper). Interestingly, elongating cells were usually (86.4%, 19 WT) found in the vicinity of such EM-like tissues, especially big clusters, and often (68.2%, 15 WT) in direct connection suggesting early embryonal tube formation. This hypothesis was further supported in a few squashes (22.7%, 5 WT) where more advanced and quite well-organized embryonal tubes were found connected to potential EM (Figure 7A, B, D). The

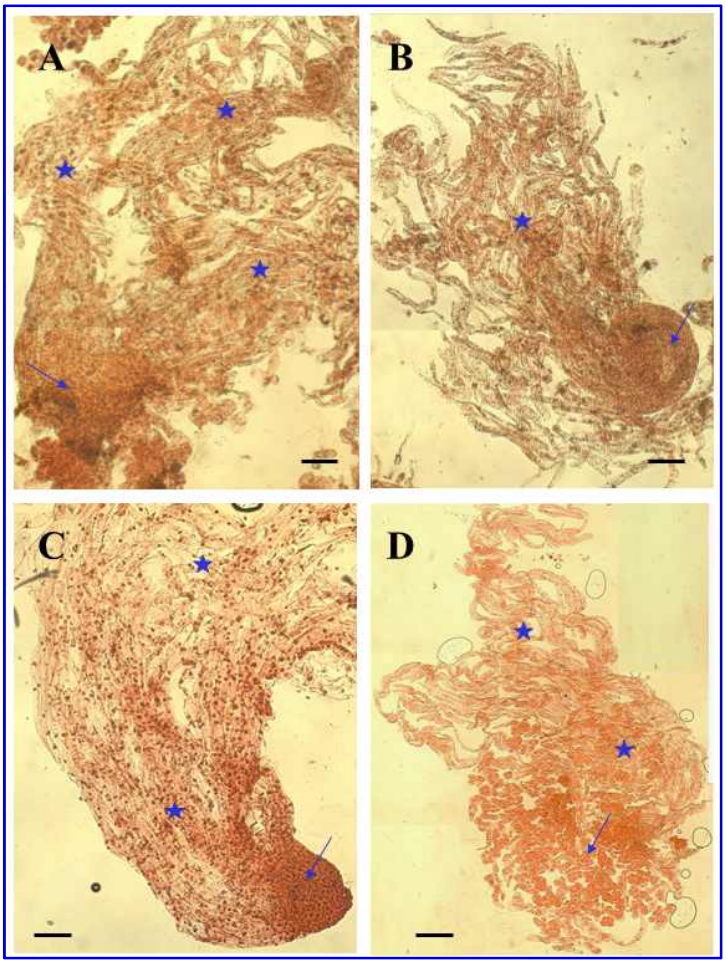

Figure 7. *Examples of EM-like structures (**A, B, D**) obtained from SAS of mature tree of P. pinaster (clone 1443, experiment 11, see Table 5). Typical well-developed early immature embryo in proliferating control embryogenic culture (clone PN519) is shown for comparison of both size and structure (**C**). Putative embryonal tubes cells (blue stars) were found connected to potential EM (blue arrows). Scale bars = 200 µm.*

resulting bipolar structures are similar to the early embryogenic structures observed in the CO4 and/or CO5 cellular organization of embryogenic culture both in size (200-500 μM) and micromorphology (Figure 7C). No differential growth of the putative EM region of such embryoids (delayed cleavage polyembryony) was observed in contrast to what is usually the case during the late development stages of early embryogenic structures. This is in close agreement with the very low growth ability of WT. We thus concluded that under our experimental conditions EMs were capable of some development towards the late stages of early embryogeny but were apparently unable to propagate through cleavage polyembryony. In contrast, some proliferation of early somatic embryos was obtained from mature trees of *P. sylvestris* (Aronen et al. 2009), as described below in this chapter.

The majority of EM-like structures were obtained from genotypes 1443 (68.2%, 15 WT) and 2849 (27.3%, 6 WT). In contrast, genotype 0136 did not produce any promising WT whereas only one was observed in 2599 (4.5%). EM-like tissues were initiated on both mLV1 (59.1%, 15 WT) and mDCR1 (40.9%, 9 WT) media but with apparently some interaction with genotype, with 2849 being more "productive" on mLV1 (5 out of 6 squashes) compared with 1443 (8 out of 15 squashes). Considering the developmental stage, no conclusions could be drawn because of the small sample size (N = 22). Most potential ET (77.3%, 17 WT) were initiated from SAS collected at the early stages (D/S/E, N = 862 SAS) rather than later stages (EF2/NS, N = 303 SAS). However, estimation of EM-like tissue occurrence per SAS (thus correcting for sample size) was similar at early ($1.9*10^{-2}$) and late developmental stages ($1.6*10^{-2}$).

3. *Pinus sylvestris* L. (Scots pine) - Aronen T, Ryynänen L

All the experiments described below were performed in 2008, 2009 and 2010 at the Finnish Forest Research Institute (METLA), Punkaharju Unit. On 01 January 2015, METLA became a part of the Natural Resources Institute Finland (LUKE).

3.1 Plant material

Two progeny trials of Scots pine (*Pinus sylvestris* L.) located at Punkaharju, Finland (61° 49' N; 29° 19' E) were used as a source of donor trees. Trial 1323/3 was planted in 1991 using 1-year-old seedlings, and trial 1801/7 in 1995 using 3-year-old seedlings, both with F1 progenies of selected plus trees. From the trial 1323/3 families 22/G01-86-0178 (trees A-F) and 36/G01-84-0071(trees A-F), and from the trial 1801/7 families 123/G04-86-0474 (trees 25-30), 136/G04-85-0392 (trees 31-36), and 141/G01-87-0418 (trees 37-42) were used for

SE initiations in 2008 when the donor trees were 18 or 16 years of age. In 2009, the same trees from trial 1801/7, now 17-year-old, were used. In 2010, only nine trees from trial 1801/7 (26, 28, 29, 34, 35, 36, 37, 38, and 39), now 18-year-old, were used.

In addition to the field-grown trees, somatic trees regenerated from the embryogenic lines 13, 51 and 76, all originating from open-pollinated seed embryos of donor K374, were used as explant source in 2009 and 2010. The somatic trees were 2 year-old in 2009, 3 year-old in 2010, and grew in pots in a nursery.

3.2 Shoot bud collection and surface sterilization

Tips of the branches with pre-flush shoot buds (Figure 8a) or flushing shoot buds (Figure 8b) were collected from the donor trees in the spring, when the temperature sum was between 35-180 d.d. (day degrees, i.e., the sum of daily average temperatures with a threshold of 5°C). Collections were performed once or twice a week, 5-10 collections per year (Table 7). Branch tips were brought immediately to the laboratory, where long needles were carefully removed. Then the shoot buds attached to a few centimeters long stem were immersed into 10% H_2O_2 overnight (approximately 16 h). Next morning the shoot tips were immersed in sterile water until being used for explant excision.

Surface-sterilization of the explants using H_2O_2 proved successful and explant contamination rates varied from 3.6 to 9.0% in different experiments.

3.3 Culture initiation

Bud scales of the surface-sterilized buds were first peeled aseptically. Then thin (approximately 1 mm) cross-sectioned slices (Figure 8c), beginning at approximately 1-2 mm below the bud tip, were cut and placed on the medium with the upper surface upwards. Each slice was considered as a separate explant, and numbered. A subgroup of the explants was subjected to heat shock at +37°C on different pre-treatment media for 3 days prior to placement onto initiation medium (Table 7). In 2008-2009, the explants were subcultured once from the initiation medium onto the same, fresh medium within 2 weeks of culture. In 2010, the explants were kept on the original initiation medium without subculturing. No cold pre-treatment was tested in Scots pine, since the temperature in Finland drops close to 0°C during the bud break period. Instead a heat shock of +37°C for 3 days was tested.

3.4 Subculture and proliferation of the induced tissues

Only the tissues considered potentially embryogenic, i.e., whitish or light in colour, translucent and soft, were separated from the explants and subcultured. In 2008-2009, the separated tissues were first placed on initiation medium and cultured on that for 4-8 weeks, with 2-week subculturing intervals.

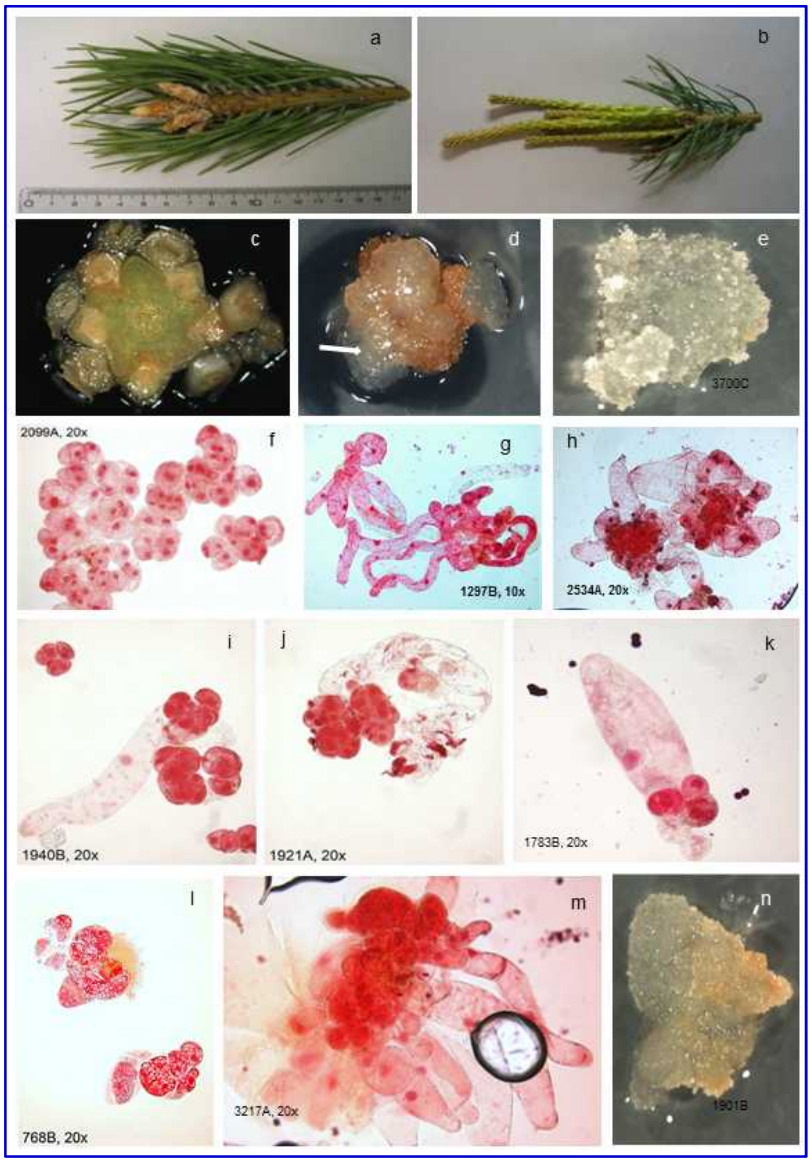

Figure 8. *Initiation of the cultures from PS explants in P. sylvestris. Developing buds (**a**) or flushing buds (**b**) collected between 35-180 d.d. were used as source of explants. Following surface-sterilization, thin cross-sectioned PS slices (**c**) were placed on an initiation medium, on which proliferation of both callus and potentially embryogenic tissue (arrow) was induced (**d**). Acetocarmine staining and microscopic examination of induced tissues that have been isolated as potentially embryogenic i.e., being soft,*

*white, and/or translucent (**e**) revealed ovoid cells (**f**), elongated cells (**g**), or cell clusters consisting of both small cells with dense cytoplasm and bigger cells having distinguishable nucleus and lightly stained cytoplasm (**h**). Often EM-like structures (**i, j, k**) are found in the same samples with callus cells. Presence of endophytes in the induced tissues is common and can be observed as brownish or orange growth (**l, m**), often resulting later in visible browning (**n**) and loss of the cultures.*

***Table 7.** Initiation treatments and responses of PS explants of P. sylvestris.*

Explants					**Initiation treatment**			**Response of explants**		
Year	Nb of families	Nb of genotypes	Age & type of donors	Nb and timing of collections	Nb of explants	Pretreatment [1]	Medium [1]	Number of isolated tissues (translucent, whitish, soft)	Potentially embryogenic, microscopy	Remaining cultures after 6 months (genotypes)
2008	5	30	15/17-year-old F1 progenies	10x, 50-178 dd	4500	none	MB2+Ca	253	56 / 9[2]	0
	5	30	15/17-year-old F1 progenies	10x, 50-178 dd	4500	3d +37°C, MB1	MB2+Ca	3185	1000 / 11[2]	5 (2)
					total 9000			**total 3438**	**20 (0.6 %[3])**	**0.1 %[3] 5 (2)**
2009	3	18	16-year-old F1 progenies	10x, 35-180 dd	900	none	MB2+Ca	900	16	2 (1)
	3	18	16-year-old F1 progenies	10x, 35-180 dd	900	3d +37°C, MB1	MB2+Ca	777	11	0
	3	18	16-year-old F1 progenies	10x, 35-180 dd	900	3d +37°C, MB1+Ca	MB2+Ca	744	13	4 (3)
	3	18	16-year-old F1 progenies	10x, 35-180 dd	900	none	mLV+Ca	695	2	1 (1)
	1	3	2-year-old emblings	10x, 35-180 dd	150	none	MB2+Ca	98	2	0
	1	3	2-year-old emblings	10x, 35-180 dd	150	3d +37°C, MB1	MB2+Ca	82	0	0
	1	3	2-year-old emblings	10x, 35-180 dd	150	3d +37°C, MB1+Ca	MB2+Ca	80	1	0
	1	3	2-year-old emblings	10x, 35-180 dd	150	none	mLV+Ca	115	0	0
					total 4200			**total 3491**	**45 (1.3 %[3])**	**0.2 %[3] 7 (4)**
2010	3	9	17-year-old F1 progenies	5x, 50-170 dd	450	none	MB2+Ca	267	15	1 (1)
	3	9	17-year-old F1 progenies	5x, 50-170 dd	450	3d +37°C, MB1+Ca	MB2+Ca	268	11	3 (2)
	3	9	17-year-old F1 progenies	5x, 50-170 dd	450	none	DCR	64	6	0
	3	9	17-year-old F1 progenies	5x, 50-170 dd	225	none	DCR+Ca	46	1	0
	1	3	3-year-old emblings	5x, 50-170 dd	150	none	MB2+Ca	99	1	1 (1)
	1	3	3-year-old emblings	5x, 50-170 dd	150	3d +37°C, MB1+Ca	MB2+Ca	77	14	1 (1)
	1	3	3-year-old emblings	5x, 50-170 dd	150	none	DCR	12	0	0
	1	3	3-year-old emblings	5x, 50-170 dd	75	none	DCR+Ca	19	0	0
					total 2100			**total 852**	**48 (5.6 %[3])**	**0.7 %[3] 6 (3)**

[1] Media used during pretreatment and initiation:

MB1 = Original DCR macro- and micronutrients and vitamins (Gupta and Durzan 1985), except myo-inositol 1 g l^{-1}; with added L-glutamine 1g l^{-1}, casein hydrolysate 1g l^{-1}, PVP 200 mg l^{-1}, activated charcoal 3 g l^{-1}, 90 mM maltose, no PGR; 2g l^{-1} gellan gum (Phytagel™) (Malabadi and van Staden 2003, 2005a,b)

MB1+Ca = MB1 with addition of extra 2.5g l^{-1} of $CaCl_2 \times H_2O$

MB2+Ca = Original DCR macro- and micronutrients and vitamins (Gupta and Durzan 1985), except myo-inositol 1 g l^{-1} with added L-glutamine 1g l^{-1}, casein hydrolysate 1g l^{-1}, PVP 200 mg l^{-1}, 90 mM maltose, 9µM BA, 20 µM 2,4-D, 25 µM NAA; 2g l^{-1} gellan gum (Malabadi and van Staden 2003, 2005a,b); with addition of extra 2.5g l^{-1} of CaCl2 x H2O

mLV+Ca = Litvay's medium (Litvay et al. 1985) modified according to Lelu-Walter et al. (2008) i.e. containing half-strength macroelements, 90 mM sucrose, 2.2 µM 2,4-D and 2.3 µM BA; 4g l^{-1} gellan gum with addition of extra 2.5g l^{-1} of CaCl2 x H2O

DCR = Original DCR macro- and micronutrients and vitamins (Gupta and Durzan 1985); with added L-glutamine 250 mg l^{-1}, casein hydrolysate 500 mg l^{-1}, 90 mM sucrose, 13.5 µM 2,4-D and 2.2 µM BA; 2.5 g l^{-1} gellan gum

DCR+Ca = DCR as above; with addition of extra 2.5g l^{-1} of CaCl2 x H2O

[2] Microscopic examination repeated; first sampling within a month from separation, second one 1-2 months later. Results from 1st / 2nd observations shown.

[3] Percent of initial number of explants

Afterwards and if still growing and considered potentially embryogenic, based on the acetocarmine staining and microscopic examination (Latutrie and Aronen (2013)), the cultures were transferred onto proliferation medium. In 2010, the separated tissues were placed directly onto proliferation medium. From the same explant, tissue could be isolated several times, and the cultures originating from the same explant were distinguished by adding the same letter to the line number. The first separations were done approximately 2-3 weeks from the onset of initiation, and the last ones over 3 months later.

Different proliferation media were used. In 2008, we used MB3 containing original DCR macro- and micronutrients and vitamins (Gupta and Durzan 1985), with added L-glutamine 250 mg l^{-1}, casein hydrolysate 500 mg l^{-1}, PVP 200 mg l^{-1}, 70 mM maltose, 1 µM BA, 2 µM 2,4-D, 2.5 µM NAA, and gelled with 2g l^{-1} of gellan gum (Phytagel™). MB3 was based on Malabadi and van Staden (2003, 2005 a,b) formulations but with various modifications (see Table 1). In 2009, the initiated tissues were subcultured onto MB2 medium (Table 7) and were proliferated on MB3 with the addition of extra 2.5g l^{-1} of $CaCl_2$ x H_2O for the first 4 weeks. The tissues initiated on mLV+Ca medium (see Table 7) were isolated and proliferated on mLV (Lelu-Walter et al. 2008) containing half-strength macrosalts, 90 mM sucrose, 2.2 µM 2,4-D and 2.3 µM BA, and gelled with 4g l^{-1} of gellan gum. Likewise, in 2010, MB3 was used as proliferation medium for tissues initiated on MB2+Ca. In the case of tissue initiated on DCR (see Table 7), proliferation medium was a DCR formulation (Gupta and Durzan 1985) supplemented with L-glutamine 250 mg l^{-1}, casein hydrolysate 500 mg l^{-1}, 90 mM sucrose, 9.1 µM 2,4-D and 2.2 µM BA and gelled with 2.5g l^{-1} of gellan gum.

The cultures remained on proliferation medium for over 6 months (Table 7); afterwards they were cryopreserved using slow-cooling in the PGDI (aqueous solution of 10% polyethylene glycol 6000, 10% glucose and 10% dimethylsulfoxide) cryoprotectant mixture (Latutrie and Aronen 2013), using MB3 medium supplemented with sucrose instead of maltose.

The results of the SE initiation experiments performed at the Finnish Forest Research Institute in 2008-2010 with bud explants is summarized in Table 7. Based on the visual examination, a huge number of EM-like tissues i.e., whitish, translucent and soft (Figure 8d), were separated and subcultured (Figure 8e). Visual discrimination of EM-like tissue from non-embryogenic tissue parts proved impossible, and most of the isolated tissues were found non-embryogenic (Figure 8f) when acetocarmine stained samples were examined under a microscope. There were, however, also tissues (a tiny percent of the examined ones, see Table 7) containing early EM-like structures with densely stained small, dividing cells and longer suspensor-like cells (Figure 8g-k).

The majority of the tissues considered potentially embryogenic degenerated during the proliferation stage. Most of them were from the start a

mixture of both potentially embryogenic cells and callus cells, and the latter contributed to the change in the morphology of the tissues to yellowish and hard. This took place also in old cultures in which predominantly callus cells were found in microscopic samples (Figure 9a, e.g. lines 1901B, 2382C). Another big problem was caused by endophytic contaminants, observed as orange-stained growth or film in the acetocarmine-stained samples (Figure 8 l, m). The cultures suffering severely

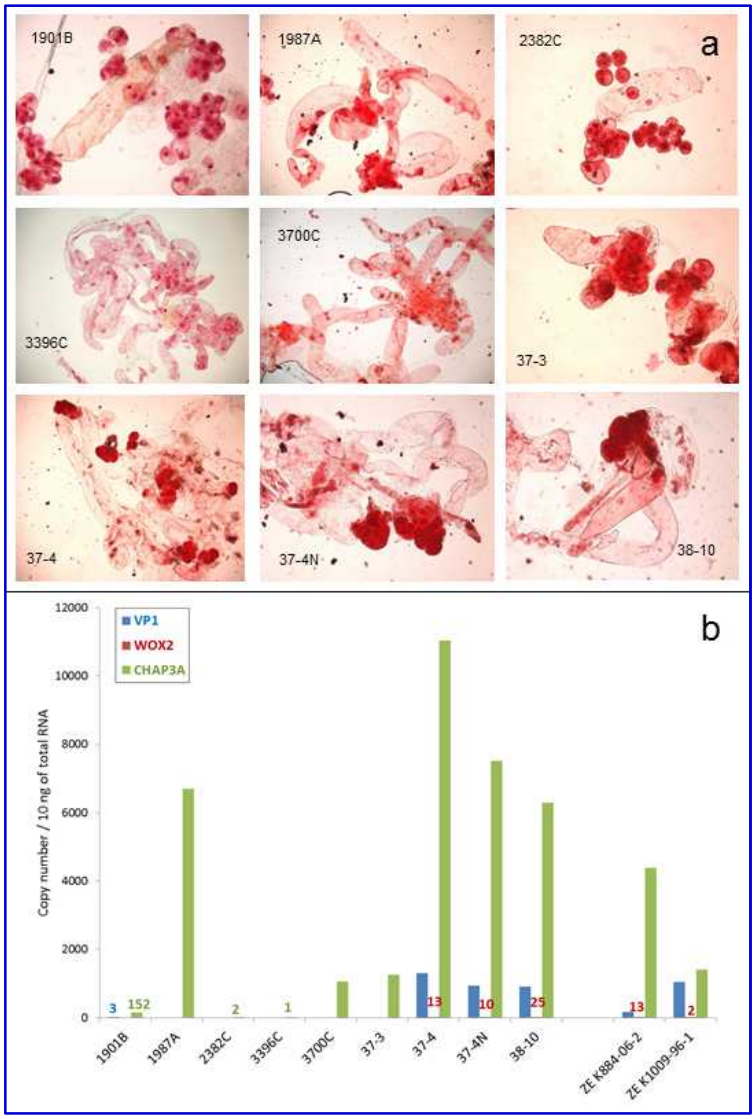

Figure 9. *Microscopic observations of acetocarmine stained samples (**a**) versus expression of the VP1, WOX2, and CHAP3A genes (**b**) in P. sylvestris lines of PS explant origin: 1901B, 1987A, 2382C, 3396C, 3700C, 37-3, 37-4, 37-4N, and 38-10 remained under long-term culture. For gene expression, results from the control lines of ZE origin, K884-06-2 and K1009-96-1, are shown.*

from endophytes remained soft in structure but gradually turned brown (Figure 8n) and ceased growing. However, in total 18 lines (Table 7) appeared embryogenic and these were subjected to the maturation treatment (see below).

The pre-treatment at +37°C, or the initiation media tested did not affect number of isolated tissue pieces (Table 7). However, when the remaining cultures after 6 months were examined, most of them were initiated from the pretreatment and MB2 medium supplemented with extra calcium. The majority of the remaining cultures originated from the PS belonging to one family, 141/G01-87-0418. The collection time (developmental stage of the shoot bud) did not affect the number of isolated tissue pieces, and the remaining cultures originated from the explants collected at various times, from 52 to 176 d.d.

3.5 Maturation and germination results

Maturation of the Scots pine lines initiated within the PS explants was started with methods developed in collaboration with INRA, and the procedure was based on Lelu-Walter et al. (2008). Prior to maturation, the cultures were proliferated on mLV as described above, with 200 mg l^{-1} PVP to prevent browning of the tissues. Tissue was then suspended in liquid maturation medium containing 10 g l^{-1} of activated charcoal but no PGR, and spread on a Whatman #2 filter paper placed on mLV maturation medium containing L-glutamine 500 mg l^{-1}, casein hydrolysate 1 g l^{-1}, myo-inositol 100 mg l^{-1}, 0.2 M sucrose, 80 µM ABA with 10 g l^{-1} of gellan gum or 120 µM ABA with 12 g l^{-1} of gellan gum. No subculturing took place during the 12-week maturation. Filter maturation on MB4 medium [original DCR macro-, micronutrients and vitamins, from Gupta and Durzan (1985), with added L-glutamine 250 mg l^{-1}, casein hydrolysate 500 mg l^{-1}, 0.18 M maltose, 80 µM ABA, gellan gum 9 g l^{-1}] was also tested with subcultures onto fresh medium every 2 weeks. Germination of the mature somatic embryos was performed on MB5 medium according to Aronen et al. (2009).

In the maturation tests, only two lines, 37-4N and 38-10, showed some kind of somatic embryo development **(**Figure 10**)** on mLV medium. The number of normal-looking somatic embryos was small; most of them turned brown before reaching the cotyledonary stage. On the germination medium, none of these somatic embryos developed a root, and only a few developed cotyledons. None of the produced somatic embryos survived.

3.6 Analysis of microsatellite loci in initiated lines

To study genetic fidelity of the induced lines, genomic DNA was extracted from the tissue cultures and from the buds of the corresponding donor trees using the modified method of Lodhi et al. (1994), as described in Valjakka et al. (2000), and the microsatellite loci were analyzed as described by Varis et al. (2008).

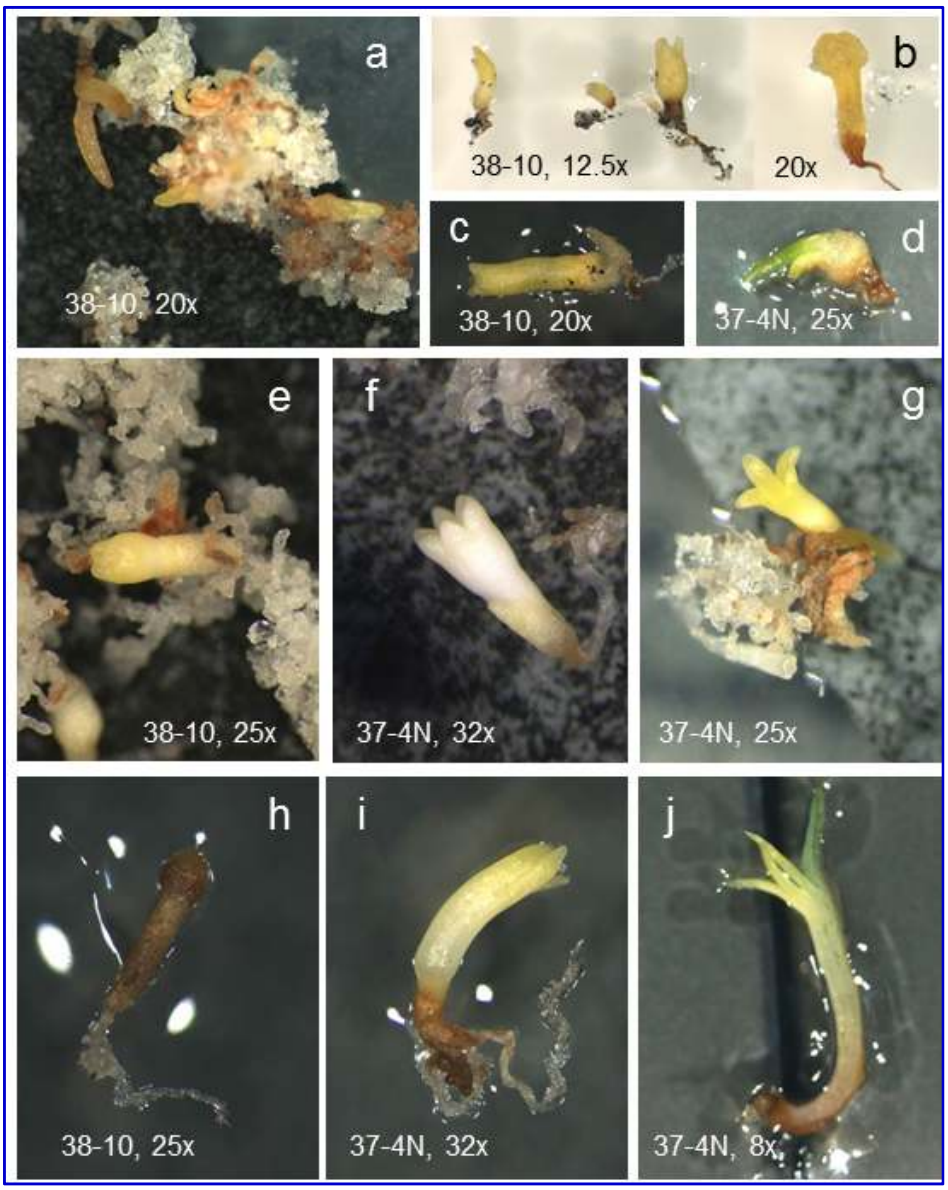

Figure 10. *Somatic embryo maturation in P. sylvestris lines originating from PS explants.* ***a)*** *Tissue spread on maturation medium often continued proliferation while development of somatic embryos was blocked and necrosis ensued. Photo taken after 11 weeks on maturation medium. Most of the developed somatic embryos were abnormal (**b, c, d**). Occasionally development of normal-looking somatic embryos was observed: photos taken after 8 weeks (**e, f**) or 11 weeks (**g**) on maturation medium. All the embryos, however, died following transfer onto germination medium: either without showing any further development (**h**), or after developing cotyledons but no root (**i, j**). Photos **h** and **i** taken after 3 weeks, and **j** after 1 month on germination medium.*

Table 8. *Alleles detected at three microsatellites loci in PS derived cell lines of P. sylvestris in comparison with the corresponding donor trees.*

	Microsatellite locus		
Genotype	S125	S714	P2146
Donor 29	174/197	204/204	181/220
Line 3217A	174/197	204/204	181/220
Line 3700C	174/197	204/204	181/220
Donor 36	163/163	184/214	166/220
Line 1901B	163/163	184/214	166/220
Line 2382C	163/163	184/214	166/220
Donor 37	147/166	202/228	209/235
Line 37-3	147/166	202/228	209/235
Line 37-4	147/166	202/228	209/235
Line 37-4N	**166**/166	202/**202**	**189**/235
Donor 38	147/147	190/228	195/220
Line 38-10	**166/166**	**205/205**	**189**/220
Line 3396C	147/147	190/228	**193**/220
Donor 40	147/147	204/229	203/220
Line 1987A	147/147	204/229	203/**203**
Line 2958A	147/147	204/229	**220**/220

Note: differences in allele size between initiated lines and donor tree are highlighted in bold.

Examination of three microsatellite loci in lines originating from PS explants showed some deviance from the donor trees (Table 8), especially in the lines that had shown some somatic embryo production ability (37-4N, 38-10), but also in other lines (3396C, 1987A, 2958A). In an earlier study, Burg et al. (2007) had shown variations of microsatellite markers (four loci) taking place both during zygotic and somatic embryogenesis of Scots pine, and found some families having higher mutation rates in tissue culture than in seed embryos. Interestingly, families with low genetic stability during establishment of embryogenic culture had higher maturation ability than those that were genetically more stable. In the present study, the pretreatment of the explants at +37°C, and a high concentration of calcium in the medium during initiation of the cultures could have imposed stress, hence potentially increasing cellular and genetic instability. This result may indicate some genetic plasticity of tested genotypes to cope with stressful culture condition.

3.7 Expression of embryogenesis-related genes in initiated lines

Expression of *VP1*, *WOX2*, and *CHAP3A* genes in nine proliferating cultures of PS origin and in two embryogenic lines of zygotic embryo origin was analyzed as described in Klimaszewska et al. (2011). These genes were chosen for their potential to be markers of embryogenicity (Klimaszewska et al. 2011, Park et al. 2010, Uddenberg et al. 2011). A variable level of *CHAP3A* expression was revealed in all the studied lines. The expression of both the *VP1* and *WOX2* genes, on the other hand, was observed not only in the lines of zygotic embryo origin, but also in three lines of PS origin (37-4, 37-4N, 38-10, Figure 9b). When samples of these three lines were observed under a microscope, the presence of early stage somatic embryos, although not well-structured, were detected (Figure 9a). Two of these three lines (37-4N, 38-10) also showed some kind of embryo maturation (Figure 10).

4. *Pinus sylvestris* L. (Scots pine) - Supplementary short note *(*Lelu-Walter MA)

4.1 Plant material

In 2007, PS buds were collected from one adult 17-year-old tree (818, INRA, Orleans, France) known to be responsive to SE induction from seed embryos in previous years (25% initiation rate from control pollinated seeds, the highest initiation frequency ever obtained for Scots pine). Pre-flush shoot buds were 3-5 cm in length and both apical and lateral shoot buds (16 in total) were pooled for the experiment.

4.2 Shoot bud disinfection and PS explant excision and culture

PS buds covered with scales were disinfected for 20 min in 0.5% $HgCl_2$ solution followed by stirring in $CaCl_2$ solution twice to neutralize the residual $HgCl_2$. Each $CaCl_2$ treatment lasted 10 min and then PS buds were rinsed three times, each for 10 min in sterile water. The scales were kept in order to minimize the effect of the sterilization agent on the shoot tissue (we assumed that these pre-flush elongated PS were aseptic). The scales and basal parts of the shoot buds were removed and transverse slices were cut from the entire PS under the binocular. The slices were positioned with the basal part on the medium, and the slices were marked from the base to the apex of a PS. The disinfection method proved very effective and no explant contamination was observed.

Two media were used for both pre-treatment and initiation: mDCR (Malabadi and van Staden 2005a, Table 1) and mLV (Lelu-Walter et al. 2008). The explants were pre-treated at 2 or 4°C for 3 days on the two pre-treatment media

(Table 1) and then cultured for 12 weeks on the initiation medium (Table 1) in darkness at 24°C.

4.3 PS explant responses

After the first 2 weeks, explants produced translucent calli appearing to arise from the cambium. There was also some cell proliferation at the base of the needle fascicles. After 1 month, the calli were still white but after two months they turned brown on mDCR, whereas on mLV the calli remained white. However, in spite of the explant necrosis on mDCR medium, the growth of white callus was observed, which upon microscopic observation did not display any EM characteristics. Callus growth was greater on mLV than on DCR. After 12 weeks of culture a total of 590 PS explants produced calli and remained alive without any noticeable trend regarding the length of the shoot buds. In order to promote proliferation of the white-translucent EM-like tissue, tiny pieces were isolated from the surrounding callus as soon as they were identified, and transferred onto nylon mesh placed over the maintenance medium (Table 1), a procedure developed to rescue EM of Scots pine (Lelu-Walter et al. 2008). However, the white calli that grew on the nylon mesh did not produce EM.

5. *Pinus radiata* D. Don (Monterey pine, radiata pine) and *Pinus patula* Schiede ex Schltdl. & Cham. (patula pine) - Hargreaves C, Reeves C

5.1 Plant material

Five shoot bud collections were made in 2007 at weekly intervals between early May and mid-June, from one *Pinus patula* tree and 20 *Pinus radiata* trees from the Long Mile Archive, at Scion in Rotorua, New Zealand. The *P. radiata* buds were from 10-20-year-old grafted clones and these buds had whorls of small, developing female cones. The buds were on average 5 cm long. The *P. patula* buds were from the lower crown of the approximately 15-year-old tree. The *P. patula* buds were approximately 3 cm long and with a smaller diameter than those of *P. radiata*. At collection time, the buds were near their strongest period of winter dormancy and the buds were enclosed in tightly packed brown scales. A further three collections were made of a subset of the *P. radiata* genotypes in August-September and this is detailed in the results and discussion section (see below).

5.2 Shoot bud disinfection, treatments and media

The buds were disinfected in commercial bleach 50:50 v/v (Chlorodux 5% sodium hypochlorite) plus surfactant (0.1 ml Silwet L-77.L–1), for 10-15 minutes.

The buds were then rinsed three times in sterile water, and the brown scales were peeled off aseptically, which was more difficult with the smaller *P. patula* shoot buds. Contamination was very low for these five collections (< 5%) and was likely to have been positively influenced by the tight sheath of scales on the bud material.

Transverse slices of the PS buds were then made and were about 1 to 2 mm thick. These PS explants were divided between a 2°C pre-treatment, a 4°C pre-treatment and a control pre-treatment (22°C), with larger numbers exposed to cold pre-treatments. The medium used for these pre-treatments was a modified Quoirin and Lepoivre medium (Quoirin and Lepoivre 1977; modification of Aitken-Christie et al. 1988) and included 5 g l^{-1} activated charcoal (Merck). Care was taken to ensure the PS slices were orientated in an upright position (small needle fascicle primordia can be seen on the bud slices and this also helps with regard to correct positioning on the medium). Following incubation, all explants were transferred to three initiation media: DCR (Gupta and Durzan 1985), EDM (Smith 1996) and Glitz (Litvay et al. 1985). The modifications to both EDM and Glitz are detailed in Hargreaves et al (2009). Cultures were incubated in the dark at 22°C and then assessed for callus formation.

In total there were 350, 680, and 301 *P. radiata* PS explants, whereas in *P. patula* there were 320, 160 and 170 explants cultured on DCR, EDM and Glitz media, respectively.

5.3 PS explant responses

Calli were obtained on all explants irrespective of species, genotype, pre-treatment and medium. It should be noted that both EDM and Glitz are extremely good media for SE in *P. radiata* (Hargreaves et al. 2009, 2011). The callus was generally glassy and friable and sometimes with a yellowish colouration. In the case of the smaller *P. patula* explants the calli formed all over the explant but with the *P. radiata* there were often two distinct areas of growth, one from around the outer edges of the explant, where the needle primordia were, and from the centre of the slice, in what is the early cambial tissue. We also observed translucent callus being produced from the edges of explants (needle fascicle primordial regions) that contain long "stringy" cells, visible after staining with acetocarmine. These cells looked like elongating epidermal cells, which of course may be exactly what they were. The bulk of the callus tissue tended to be a mixture of highly vacuolated cells, some quite elongated and a little like suspensor cells with some darker staining nucleic material and starch granules scattered through the cells. Isolated callus tissue grew with variable success on the three media irrespective of medium formulation but subsequent staining of this showed it all to be non-embryogenic. Further collections (3) were made at 3-week intervals with 10 genotypes rather than 20 of *P. radiata* starting six weeks after the shortest day in 2007 (August-

September). The elongation of the PS buds was rapid and the contamination of the explants increased from 13.4% to 52.6% for the last collection and probably can be attributed to the open nature of the buds at this stage (very elongated and only a few sparse brown scales at the very tip of the bud). Only two pre-treatments (2 ^{o}C and 4 ^{o}C) were tested with this material due to the results observed in the samples taken earlier in the year. The same initiation media were used (DCR, EDM and Glitz). From each genotype, 15 to 50 transverse PS slices were placed on each of the test media for each of the pre-treatments. Care was taken to observe the tissue every few days in case EM was appearing and being overgrown by the proliferating callus cells. As with the earlier work, callus growth was in general prolific and WT was easily isolated and in most cases continued to proliferate. However, in all cases microscopic examination of the WT revealed regions of large vacuolated cells resembling suspensor cells with some densely staining cells, perhaps meristematic in origin, but nothing that was convincingly embryogenic, or that on isolation and subsequent proliferation retained an 'interesting' morphology. We have to conclude from this work, somewhat surprisingly, that both our *P. patula* and *P. radiata* genotypes were not as responsive to similar treatments tested for *P. patula* by Malabadi and co-workers as previously discussed in this chapter.

6. *Pinus radiata* D. Don (Monterey pine, radiata pine) - Moncaleán P, Montalbán IA, Garcia-Mendiguren O

6. 1 Experiment 1

In 2008, seven 19-year-old trees were selected in the seed orchard established by Neiker-Tecnalia in Deba, Spain. Shoot buds (3–5 cm long) were taken from the mid-basal part of the trees. The buds were collected fortnightly from February 18 (Figure 11a) to April 29 (Figure 11b), wrapped in moist paper to prevent dehydration and stored in polyethylene bags at 4ºC for a maximum of a week.

Buds were sprayed with 70% (v/v) ethanol, and then rinsed with sterile distilled H_2O. Afterwards, the buds were submerged in 50% (v/v) commercial bleach (active chloride >5%) plus two drops of Tween 20® and agitated for 10 min. Finally, they were rinsed three times in sterile distilled H_2O in aseptic conditions. When possible bud scales were removed, explants were cut transversely into 1–1.5 mm thick slices with a surgical scalpel blade and were laid on the culture medium (Figure 11c).

On the first and the second collection dates (February 18 and March 3) bud slices were cultured on two initiation media. The first medium was embryo development medium (EDM) (Walter et al. 1994) with 30 g l^{-1} sucrose, 1 g l^{-1} inositol and a combination of 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and

2.7 µM BA. Before autoclaving, the pH of the medium was adjusted to 5.7 and then 3 g l^{-1} gellan gum (Gelrite®) was added. Medium was autoclaved at 121ºC for 20 min. After autoclaving, filter-sterilized solutions (pH = 5.7) of 550 mg l^{-1} L-glutamine, 525 mg l^{-1} asparagine, 175 mg l^{-1} arginine, 19.75 mg l^{-1} L-citrulline, 19 mg l^{-1} L-ornithine, 13.75 mg l^{-1} L-lysine, 10 mg l^{-1} L-alanine and 8.75 mg l^{-1} L-proline were added to the cooled medium.

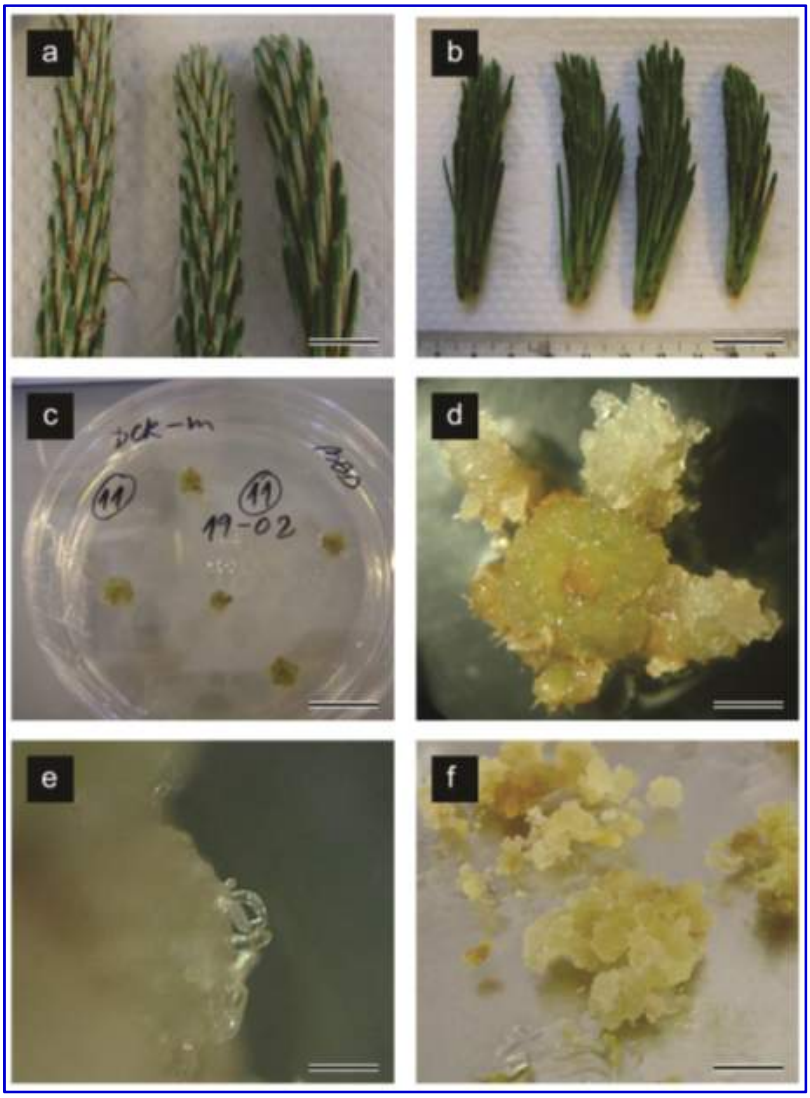

Figure 11. *Pinus radiata cultures initiated from PS explants (Experiment 1).* ***a*** *Shoot buds collected at the end of February (bar=12 mm).* ***b*** *Shoot buds collected at the beginning of April (bar=19 mm).* ***c*** *PS explants cultured on DCRI (February collection) (bar=14 mm).* ***d*** *PS explant cultured on DCRI for 3 weeks (bar=4 mm).* ***e*** *Elongated cells in the proliferating tissue (bar=1 mm).* ***f*** *Tissue proliferating on DCRM (bar=9 mm).*

The second medium was full-strength DCR medium (DCRI, Gupta and Durzan (1985) modified by Malabadi and Van Staden (2005a), see Table 1) containing 0.2 g l^{-1} polyvinylpyrrolidone-40 (PVP-40), 3.24 % (w/v) maltose, 1 g l^{-1} inositol and supplemented with 20 µM 2,4-D, 25 µM 1-naphthaleneacetic acid (NAA) and 9 µM BA. Before autoclaving, the pH of the medium was adjusted to 5.7 and then 1.5 g l^{-1} gellan gum was added. After autoclaving, filter-sterilized solutions (pH= 5.7) of 1 g l^{-1} casein hydrolysate and 1 g l^{-1} L-glutamine were added to the cooled medium prior to dispensing into gamma-irradiated Petri dishes (90 x 20 mm).

From the third to the sixth collection date (from March 17 to April 29) half of the PS explants were cultured on DCRI, and the other half were subjected to cold pre-treatment. This pre-treatment (see Table 1) consisted of culturing the explants at 4ºC on full-strength DCR medium containing 0.2 g l^{-1} PVP-40, 3.24 % (w/v) maltose, 0.3% (w/v) activated charcoal (AC), and 1.5 g l^{-1} gellan gum (Phytagel); after 3 days, these explants were subcultured on DCRI.

At each collection date, five PS slices per Petri dish were cultured; seven Petri dishes per treatment were laid out randomly on the shelves of the growth chamber. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks. Then the explants and/or the proliferating tissues were transferred to maintenance medium. Maintenance medium for explants cultured on EDM was the same as for initiation. Maintenance medium for explants cultured on DCR (DCRM) had the same basal composition but contained 4.32 % (w/v) maltose, 1 g l^{-1} inositol and was supplemented with 2 µM 2,4-D, 2.5 µM NAA and 1 µM BA. This formulation is similar to that of Malabadi et al. (2005a) (see Table 1). The amino acid mixture was the same as for initiation. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks on maintenance medium.

On the first and second collection dates and in all the genotypes tested, the PS slices cultured on EDM produced tissue from the margins (Figure 11d). These cell proliferations were formed by white-translucent tissue formed by elongated cells (embryogenic-like cells, Figure 11e) and a small population of round cells (callus cells). The explants cultured on DCRI showed the same growth of tissue but it grew less and more slowly than on slices cultured on EDM. When transferred to maintenance medium, the tissues continued proliferating rapidly but the population of round cells increased and the tissue became yellow-brown (Figure 11f).

In all the genotypes from the third to the sixth collection dates, the growing tissues on DCRI showed the same trend as described above for the initiation and maintenance. The cold pre-treatment did not have any effect on the responses of the PS slices and half of these slices had no cell proliferation and necrotized. The other explants, when transferred to DCRI, had cell proliferation in the needle primordia areas. Although this tissue was white-translucent and EM-like cells were

identified (Figure 11e), when proliferated on DCRM, the tissue became yellowish and after 1 month was predominantly composed of round cells (Figure 11f).

6.2 Experiment 2

Ten trees over 20 years old were selected from a seed orchard established by Neiker-Tecnalia in Amurrio, Spain. Apical shoot buds (3–5 cm long) were taken from the mid-basal part of the trees (Figure 12a). The shoot buds were collected fortnightly from December 2009 to January 2010. The buds were stored

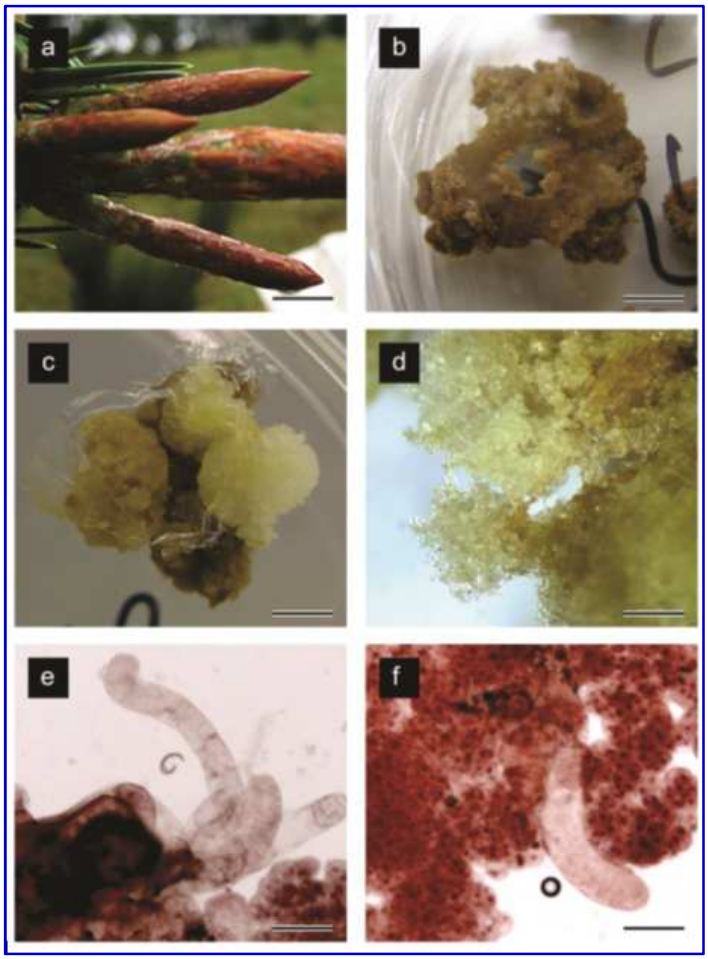

Figure 12. *Pinus radiata cultures initiated from PS explants (Experiment 2).* ***a*** *Shoot buds collected at the end of December (bar=17 mm).* ***b*** *PS slice (1.5 cm thick) cultured on EDM (bar=4 mm).* ***c*** *Tissue growing on 7 mm PS slices cultured on LPI medium (bar=4 mm).* ***d*** *Elongated and round cells in the proliferating tissue (bar=3 mm).* ***e*** *Micro-morphology of elongated cells in the proliferating tissue (bar=0.15 mm).* ***f*** *Micro-morphology of elongated and round cells in the proliferating tissue (bar=0.2 mm).*

and disinfected as described in *Experiment 1*. PS were cut transversely into 1-1.5 mm thick slices and 6-7 mm thick slices. These slices were cultured on EDM and on modified LP basal medium (LPI, Quoirin and Lepoivre (1977), modified by Aitken-Christie et al. (1988)). This LPI medium was supplemented with 30 g l^{-1} sucrose, 1 g l^{-1} inositol, 20 µM 2,4-D, 25 µM NAA and 9 µM BA; before autoclaving the pH of the medium was adjusted to 5.8 and 3 g l^{-1} gellan gum (Gelrite®) were added. After autoclaving the same amino acid mixture as used in EDM medium was added. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks.

The PS slices cultured on EDM were transferred to the same medium. The slices cultured on LPI were subcultured either on LPI or on EDM. The slices cultured on LPO (the same as LPI without PGRs) were subcultured to LPI.

The 1-1.5 mm thick slices necrotized rapidly and did not produce any tissue (Figure 12b). When the initial explants were 6-7 mm thick slices and cultured on EDM, a developing white-translucent tissue was observed with embryogenic-like cells (Figure 12e); this tissue was subcultured onto the same medium and became yellowish with a higher proportion of round cells.

On explants cultured and subcultured on LPI, the tissue grew slower than on those subcultured on EDM or cultured from the beginning of the experiment on EDM. But after 4 to 8 weeks, the tissue growing on LPI showed the same macro- and micro-morphological features as the ones growing on EDM (Figure 12f). When cultured on LPO most explants displayed cell proliferation in the brachyblast meristems surrounding the PS slices; when transferred to LPI, 50% of them necrotized; the others developed globular structures (Figure 12c) that proliferated into the tissue type previously observed on the other culture media (Figure 12d).

6.3 Experiment 3

In 2010, the explants were from *in vitro* adventitious shoot buds. These were obtained from shoot buds of 10 trees that were over 20 years old and from the same seed orchard mentioned in *Experiment 2* (Figure 13a). These explants were obtained by culturing the shoot buds collected from the field on LPO for 4 weeks to induce axillary shoots and adventitious shoots and then transferring the explants to LP medium lacking PGRs and supplemented with 0.2% (w/v) AC (LPAC). The explants were subcultured every month. After 1 year, *in vitro* buds were cut into halves, quarters or slices (Figure 13b) and cultured on EDM or on EDM supplemented with 20 µM 2,4-D, 25 µM NAA and 9 µM BA (EDM2) (Figure 13). After 4 to 8 weeks, when proliferation of tissue was observed, the explants were transferred to maintenance medium. The maintenance media were those used for initiation. Cultures were maintained in the dark at 21±1ºC.

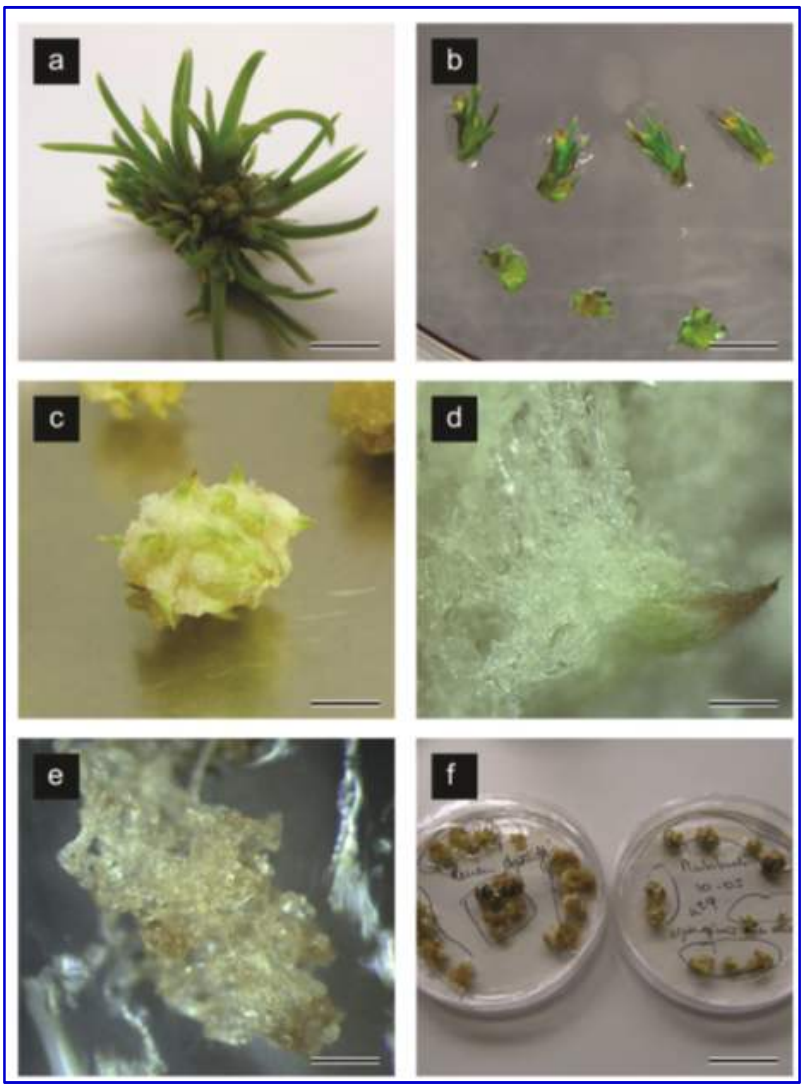

Figure 13. *Pinus radiata cultures initiated from sections of adventitious shoots (Experiment 3).* ***a*** *Adventitious shoots and shoot buds regenerated in vitro (bar=7 mm).* ***b*** *Shoots cut into quarters, halves and small sections (bar=5 mm).* ***c*** *Shoot explants after culture on EDM for 4 weeks (bar=6 mm).* ***d*** *Long cells within proliferating tissue cultured on EDM for 4 weeks (bar=0.8 mm).* ***e*** *A tissue piece with EM-like morphology cultured on EDM for 6 weeks (bar=0.7 mm).* ***f*** *Explants cultured on EDM on the left, and on EDM2 on the right (bar=30 mm).*

Small pieces of proliferating tissue were stained with acetocarmine (2% w/v) directly on glass slides for 4 min. Then, the samples were rinsed with water and mounted with a cover slide. Samples were observed with an inverted microscope (Leica DM4500) using a 40-fold magnification.

Tissue proliferation was observed in the three types of explants tested (halves, quarters and slices) and this tissue growth was most abundant in shoot buds cut into quarters (Figure 13c). In the explants cultured on EDM, the tissue grew at a higher rate than in the explants cultured on EDM2 (Fig 13d). As observed in previous experiments, when transferred to maintenance medium the tissue became yellowish (Figure 13f) and a large population of cells was round despite that initially, the proliferating tissue was white-translucent and that EM-like cells were observed (Figure 13 d, e).

6.4 Gene expression profiling of callus lines

Through the collaboration with NRCan-CFS, Canada, a project was undertaken to analyze gene expression profiles in six callus lines: two derived from shoot buds (PS) of somatic trees and three derived from axillary shoots generated *in vitro* from shoots collected from four seed-derived trees (Garcia-Mendiguren et al. 2015). In addition, expression profiles of three embryogenic lines derived from seed embryos were also generated for comparison. The explants were cultured on media with four different PGR compositions. The analysis revealed that culture medium had no significant impact on gene expression. High level expression of two *Knotted1*-like genes further reflected the vegetative character of these callus lines along with the expression of *WOX4*, a marker of vascular procambium tissue. Most notable was expression of embryogenesis-related gene *LEC1* in all five callus lines, although expression of two other embryogenic markers (*ABI3* and *WOX2*) was undetectable. Whether *LEC1* expression could be reflective of some level of embryogenic character, which might have progressed in the presence of additional embryo inducing factors, remains to be studied.

7. *Pinus strobus* L. (eastern white pine) - Klimaszewska K, Overton C

At the Laurentian Forestry Centre of the Canadian Forest Service, the SE induction experiments were conducted with clones of four genotypes (1053-8, 1053-12, 1073-12 and 1145-23) of somatic trees planted in Valcartier, Quebec in 2003. The trees were regenerated through SE induced in seed embryos (Klimaszewska et al. 2001). The first experiments started in November 2007 with dormant shoot buds, and in the spring of 2008, 2009, 2011 and 2014 with pre-flush buds when the trees were 7, 8, 9, 11 and 14 years old, respectively. Apical and subapical buds were pooled for the experiments.

7.1 PS bud disinfection and explant excision

The shoot bud disinfection protocol was the same as described for white spruce (Klimaszewska and Rutledge 2015 in this volume). Prior to disinfection, the

majority (not all) of the scales were removed together with the young needle fascicles, which invariably left small wounds on the stem. This protocol resulted in 70 to 80% explant sterility. In November 2007, PS was transversely sliced or cut longitudinally into four pieces and cultured (Figure 14 a, b, c). At least 100 transverse 1 to 2 mm thick PS slices, including PS apices, were cultured per genotype and pre-treatment. In 2008, shoot buds were collected in May (Figure15 a, b) and in June (Figure 15 g). In May, the PS were transversely sliced as described above (Figure 15 d); small PS were also cut longitudinally in four parts (Figure 15 c). In June the buds were longer and the primordia of needle fascicles were also elongated (Figure 15 g). These needle fascicles were separated from the buds, the basal scales were removed (Figure 15 h) and 30 were cultured per Petri dish. In 2009, 2011 and 2014, shoot buds were collected in May.

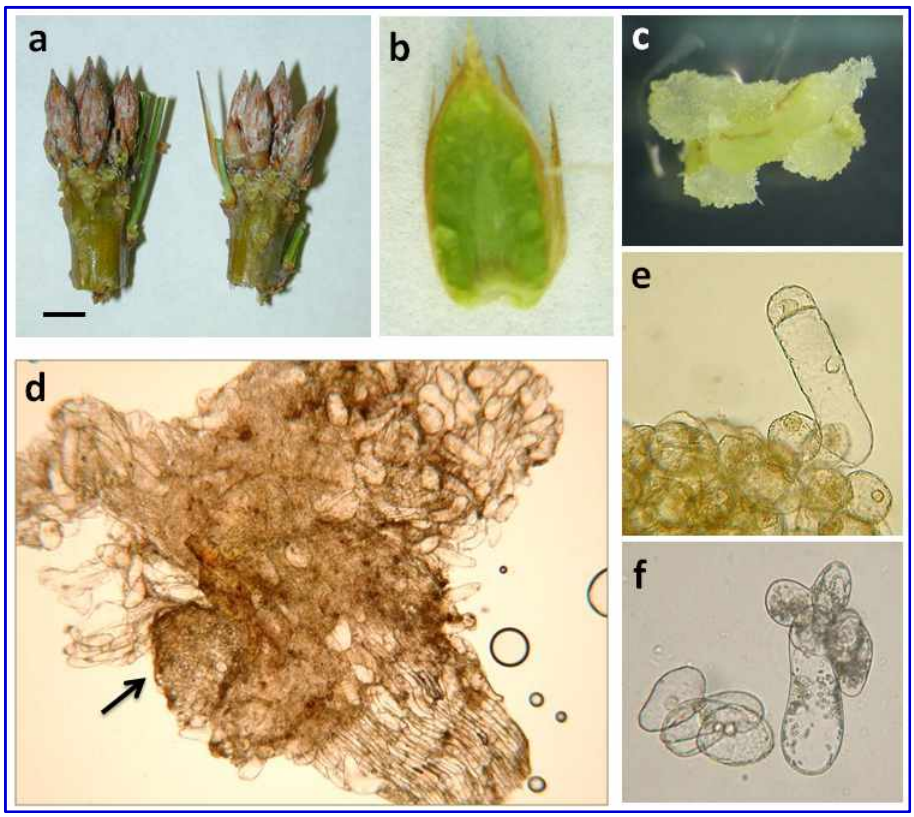

Figure 14. *Pinus strobus cultures initiated from PS longitudinal and transverse sections collected in November 2007 from 7-year-old somatic trees.* ***a*** *Dormant shoot buds prior to excision and disinfection (bar=1cm).* ***b*** *Longitudinal section through the disinfected shoot bud (bar=0.36cm).* ***c*** *White, partially translucent callus growing from the longitudinal section of entire PS after 2 weeks of culture (bar=037cm).* ***d*** *Slight squash of the PS explant showing callus cells and a small protuberance (arrow) (bar=60µm).* ***e, f*** *Asymmetrically dividing cells identified within callus cells (bar=30 µm).*

7.2 Culture medium and pre-treatments

In 2007, the PS explants were cultured according to the experimental design listed in Table 9, whereas in the following years only MLV-S medium was used. Explants were cultured in 90 x 10 mm Petri dishes, in darkness at 24°C.

Table 9. *Pre-treatment temperatures and culture media for PS explants of P. strobus in 2007.*

Pre-treatment conditions (medium composition)	Initiation medium (composition)
None	MLV-S (MLV + 3% sucrose, 0.5 gl^{-1} glutamine, 1 gl^{-1} CH, 100 mgl^{-1} inositol, 9.5 µm 2,4-D + 4.5 µM BA)
None	MLV-HPGR (MLV + 3% sucrose, 0.5 gl^{-1} glutamine, 1 gl^{-1} CH, 100 mg l^{-1} inositol, 20 µM 2,4-D, 25 µM NAA, 9 µM BA)
2/4°C, 3 days, dark (MLV (no N, no sucrose) + 100 mg l^{-1} inositol, 0.3% charcoal, 0.2 % gellan gum, no PGR)	MLV-S
2/4°C, 3 days, dark (same medium as above)	MLV-HPGR
32°C, 2 days, dark (same medium as above)	MLV-S
32°C, 2 days, dark (same medium as above)	MLV-HPGR

Note: Explants were split in equal numbers between 2 and 4°C pre-treatment. In the following year's experiments, only MLV-S was tested as a culture medium.

Explant responses- Regardless of the experimental design, within the first 2 weeks of culture, the majority of PS slices produced some calli from the cambial region (Figure 15 e) and also from the edges (Figure 15 f). The calli arising from the edges of explants had initially a white/translucent phenotype and might have originated from the wound areas / needle primordia. However, invariably after 4-6 weeks of culture and regardless of the subcultures on the "SE maintenance medium", the calli changed the phenotype and while still white they became a typical callus composed of spherical loose cells without any degree of organization. No discernible embryo structures were detected upon microscopic examination except sporadic cells undergoing asymmetric division (Figure 14 e) and a few cell aggregates composed of small cells and a large, elongate cell (Figure14 f). After

each subculture the callus pieces grew more slowly, became hard in texture and finally necrotized after several weeks of culture. Occasionally, the PS slices became necrotic with one or two distinct areas of white tissue growth, which, when isolated and subcultured, did not survive.

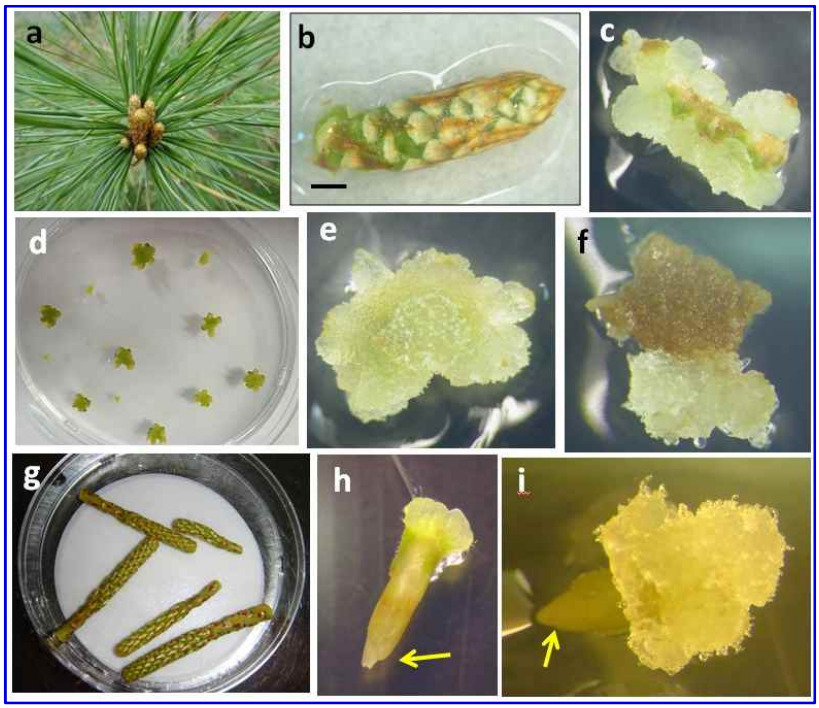

Figure 15. *Pinus strobus cultures initiated from spring PS explants collected in May (a – f) and June (g – i) 2008, 7.5- year-old trees.* ***a*** *Pre-flush shoot buds on a tree prior to collection (bar=2.5cm).* ***b*** *Disinfected pre-flush shoot bud (bar=3.25cm).* ***c*** *Callus growing from a longitudinal section of PS (bar=1cm).* ***d*** *Transverse slices of PS at the onset of cultures (bar=1.2cm).* ***e, f*** *Callus growing on the transverse slice of PS after 2 weeks (**e**) and on another explant after 6 weeks (**f**) (bar=2.4cm).* ***g*** *Disinfected shoot buds with developing needle fascicles (bar=1.38cm).* ***h, i*** *Isolated needle fascicle after 1 week (**h**) (bar=2mm) and 3 weeks of culture (**i**) (bar=1mm). Arrows indicate the tips of the needle fascicles. Note the calli growing from the bases of the fascicles.*

8. *Pinus contorta* Doug. ex Loud. (lodgepole pine)*:* Brief description of the published results (Park et al. 2010)

8.1 Plant material

Branches of fifteen 20-year-old lodgepole pine (*P. contorta* Dougl. ex Loud. Engelm.) genotypes, which had previously been selected based on the level of resistance to mountain pine beetle (MPB) attack, from the British Columbia

Ministry of Forests and Range seed orchard 307 (British Columbia, Canada), were collected bi-weekly from February to July in 2008 and 2009 (Park et al. 2010).

8.2 Explant preparation

The shoot bud disinfection and pre-treatment of explants were as described in Malabadi and van Staden (2005a) with some modifications (Park et al. 2010). Five transverse slices (0.5–1 mm thick) were taken sequentially from the tip of each PS. Approximately 25 slices were placed in Petri dishes (90 × 15 mm) containing a Gupta and Durzan (DCR) pre-treatment medium (Gupta and Durzan 1985) supplemented with 3 g l^{-1} activated charcoal (AC) and 0.2 g l^{-1} polyvinylpyrrolidone (PVP) for pre-culture at 38°C for 4 h followed by 4°C for 3 days in the dark (Park et al. 2010). The control PS explants had no pre-treatment and were cultured on DCR induction medium with high PGR concentrations (Table 1).

After 8 weeks of initial culture, calli originating from the cambial region of a PS slice were recorded. The calli were then proliferated and cultured on maintenance medium with reduced PGR concentrations (Table 1) and subcultured every 2 or 4 weeks for further development. The putative embryogenic cultures were preliminarily identified by microscopic observation.

8.3 Explant responses

In both 2008 and 2009, the highest number of explants producing EM-like white tissue was obtained between 27 March and 2 April. The survival rate of explants from later collections decreased and EM-like tissue could not be recovered. Genotypic specificity was also observed, as genotypes 1506 and 1537 produced EM-like tissue from more than 10% of all explants with pre-culture treatment. The same two genotypes consistently showed a similar response throughout the experiment. In contrast, two additional genotypes (1520 and 1530) were strongly recalcitrant and did not produce EM-like tissue at any sampling time. Shoot bud growth ceased in the middle of May, and needles flushed. More than 50% of the explants taken from apical PS collected from these latter time points became necrotic, whereas the remainder of the explants produced only brownish callus. Throughout the entire collection series, less than 2% of the PS explants produced callus from the cambial region, regardless of genotype. The calli that did develop grew slowly compared with those originating from tissues other than the cambium and displayed a characteristic transparent and white morphology. Staining the isolated calli with 2% aceto-carmine clearly showed the presence of EM-like cells that were small and dense with cytoplasm. These calli initially grew quickly, but after 6 months began to grow slowly and eventually turned brown and subsequently necrotized.

The calli originating from the cambial region were carefully separated from the explants after 2 months of culture and were transferred to fresh initiation medium. After 2 months, the proliferating calli were transferred to maintenance medium for further development and proliferation, and were sub-cultured every 2–4 weeks depending on their growth. When the calli were substantially proliferated, sub-samples were observed under the microscope to investigate the developmental stage of the EM-like aggregates. Some calli clearly revealed the presence of EM-like aggregates, which consisted of dividing cells and elongated suspensor-like cells. Despite the abundance of EM-like cells, the cultures were developmentally arrested even after culturing on spent medium or on the embryo development medium, which contained half the amount of PGRs compared with maintenance medium. These cultures also failed to produce somatic embryos on maturation medium with abscisic acid (ABA). Expression of embryogenesis-related genes *WOX2* and *LEC1/HAP3A* was studied in a line derived from PS explants as well as in a control EM and NEC. *WOX2* was expressed in all lines although at low levels compared with EM. Very low *WOX2* expression was detected in NEC. Similarly *LEC1/HAP3A* was expressed in all lines but at variable levels similar to either NEC or EM. Therefore, the cell lines derived from PS buds could be EM (Park et al. 2010), similarly to those in *P. sylvestris* described above.

9. Conclusions

The experiments carried out in different labs with six pine species (both somatic and zygotic embryo-derived trees) failed to produce SE from PS slices except for *P. sylvestris* in which two cell lines were embryogenic, but maturation yield was very low and the resulting somatic embryos were mostly abnormal and failed to grow into plants. Such behaviour of embryogenic line is often seen in pine EM of seed origin after continuous, prolonged subcultures. Because genetic variation was observed at several microsatellite loci for these two lines and others, follow up experiments with the same trees will be required to confirm these positive results and to establish a final protocol.

Otherwise, all authors observed very similar explant responses in culture, which initially produced translucent/white and embryogenic-like in appearance proliferating cell aggregates during the first 2-3 weeks and later, after a few subcultures, displayed reduced growth rate, changed appearance with respect to colour and consistency and eventually necrosis ensued. These changes were reflected in the cell shapes, the majority of which became round and formed aggregates of various sizes. On some necrotic explants, outgrowth of white tissue was observed later in culture. It was technically very difficult to separate and culture tiny pieces of embryogenic-like tissue that could only be identified through

staining and microscopic viewing, thus inadvertently rendering them non-viable for culture. Based on the micromorphology, seemingly EM-like cell aggregates were observed in *P. sylvestris*, *P. contorta*, *P. pinaster* and *P. radiata*. Interestingly, *P. radiata*, *P. contorta* and *P. sylvestris* cell lines obtained from PS explants expressed the embryogenesis-related gene *LEC1* at various levels. Similarly, *WOX2* expression was detected in *P. contorta* and *P. sylvestris* whereas *ABI3/VP1* was detected in *P. sylvestris*. These results suggest that activation of some prerequisite stages for SE might have occurred but did not progress further.

We conclude that the positive results on SE induction in adult pines attributed to the methods of Malabadi et al. (see the references) have largely proven unrepeatable with other pine species, particularly regarding the ease and high frequency inductions of SE. Furthermore, the insufficient details documenting the initiation and progression of SE from PS domes and slices, missing or confusing information detailing medium composition (see Table 1**,** e.g. maltose concentration in IM) and no proof, in most species, of normal germination and of established somatic embryo-derived plants bring to doubt the authors' claims that SE was achieved in explants of adult pines. Various media modifications were cited by the same authors as beneficial for particular pine species. Unfortunately, no comparative work was done simultaneously between species. These modifications included calcium, antioxidants, triacontanol and the addition of extracts from smoke-saturated water. In all cases, the discussion cited very solid research with other plant species by other researchers. However, the links of the effect observed in the *Pinus* spp. SE with the wider reported literature were often tenuous.

Certainly, the work by this chapter's international group of scientists negates the general applicability of the published methods together with the claim that SE in adult pine explants is feasible at high efficiency. Indeed, the challenge still exists in pines and other conifers as exemplified by the multiyear study on induction of SE in adult *Picea glauca* somatic trees (Klimaszewska et al. 2011, see also Klimaszewska and Rutledge 2015 in this volume).

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Application of somatic embryogenesis and transgenic technology to conserve and restore threatened forest tree species

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Abstract

North American forest trees are under increasing pressure from exotic pests and pathogens accidently introduced from overseas, some of which have the potential to completely eliminate these trees from their native ranges. While conventional selection and breeding may help restore these threatened forest trees, these approaches could be greatly enhanced or complemented by applying biotechnological tools. Somatic embryogenesis (SE) has the potential to make major contributions to conservation and restoration of trees threatened by forest health problems. Here, I describe three cases involving foundational forest species in the U.S. that are threatened with extinction by exotic pests and pathogens, and how we have developed embryogenic culture systems and applied them to aid with germplasm conservation and restoration. Embryogenic cultures have been developed for eastern and Carolina hemlocks, important coniferous species native to the Appalachian Mountains that have been devastated by hemlock woolly adelgid (HWA). These cultures can be cryostored to facilitate hemlock germplasm conservation. Embryogenic cultures have also been initiated from seeds collected from putatively HWA–tolerant individuals and hybrids with HWA-tolerant Asian hemlocks to produce somatic seedlings for HWA tolerance screening and potential use in restoration plantings. Similarly, white and green ash trees, widely used for wood products, are under attack from emerald ash borer (EAB). After developing an embryogenic cultures system for green ash, we applied it to produce cultures and somatic seedlings from putatively EAB-resistant "lingering" white ash trees. The first of these somatic seedlings should reach stem diameters large enough for testing for resistance to EAB within a few years. The loss of American chestnut to

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the chestnut blight fungus is arguably the most destructive forest tree disease in recorded history. A decades-long effort to develop and apply SE technology to address chestnut blight in the U.S. is finally culminating in multiple applications that may help restore the tree to the forests it once dominated. SE technology and cryostorage are being applied to enhance The American Chestnut Foundation's hybrid backcross breeding program by facilitating clonal testing of advanced generation hybrid backcross genotypes, and could eventually be used for scaled-up production of elite hybrid backcross material. American chestnuts engineered with different candidate genes for resistance to blight and Phytophthora root rot, produced by transforming embryogenic cultures, are now being deployed in field tests, and at least one candidate gene for blight resistance is showing very promising results in the field. While SE technology is just one tool to be deployed in addressing forest health problems, the ability to manipulate embryogenic cultures for propagation, cryopreservation and gene transfer applications makes it a key technology for conservation and restoration of threatened forest trees.

Keywords: eastern hemlock, Carolina hemlock, *Tsuga canadensis*, *Tsuga caroliniana*, green ash, white ash, *Fraxinus pennsylvanica*, *Fraxinus americana*, American chestnut, *Castanea dentata*, germplasm conservation, species restoration, cryopreservation, gene transfer

1. Introduction

Several North American forest tree species have suffered dramatic declines over the past decades due to the accidental introduction of forest pathogens or insect pests from other regions of the world. While resources have been directed at finding resistance to these pests or pathogens, progress has been slow and successes few. Conventional selection and breeding approaches that have allowed development of disease- and pest-resistant crop species are difficult to apply to forest trees, most of which have long juvenile periods before they can be bred or selected for resistance. Because forest trees are undomesticated, out-crossing organisms, homozygous pure lines, the basis of hybrid breeding in crop plants, are not available for them. Selection and breeding programs for a few forest tree species under attack from devastating fungal pathogens have been undertaken with some promising results, but only after decades of difficult work. In addition, given the experience with crops bred for disease-resistance, the ability of plant pathogens to overcome resistance means that new genetic material must continually be selected for integration into these programs. Meanwhile, new forest health threats continue to arise, almost on an annual basis. Thus, there is a need to explore

supplemental or alternative approaches for dealing with these forest health threats, and hopefully, to accelerate our response times.

Several biotechnological tools have become available that may augment or even substitute for conventional breeding approaches for conservation and restoration of threatened forest trees, including marker-assisted selection, in vitro propagation and transgenics. Somatic embryogenesis (SE), in particular, has multiple applications that could be useful for research and perhaps eventual operational deployment of pathogen- or pest-resistant genotypes. In collaboration with other scientists and groups, we are already making use of SE technology in research projects that we believe will contribute to restoration of some foundational North American species that have been devastated by exotic pests and pathogens—some that have been under attack for over a century and some struck more recently. Here, I will describe embryogenic culture systems we developed for three of these threatened foundational forest species and how SE technology, in combination with conventional breeding approaches, cryopreservation and/or transgenics, may make major contributions toward their conservation and restoration.

2. Somatic Embryogenesis in eastern hemlock and Carolina hemlock

Two species of hemlocks, which are members of the Pinaceae, are native to the eastern United States, eastern hemlock (*Tsuga canadensis*) and Carolina hemlock (*Tsuga caroliniana*). Eastern hemlock is a major component of the Northern Hardwood Forest climax forest in the U.S. and is distributed from New England south through the Appalachian Mountains into north Georgia and Alabama. The tree is considered to be a foundational forest species (Ellison et al. 2005) of particular importance to the ecosystem in that its shade helps maintain stable temperatures in Appalachian streams. Carolina hemlock is a relatively rare species found in scattered populations, mainly on rocky outcrops and dry ridges in the southern Appalachians. Stands of both species are currently under attack from the hemlock woolly adelgid (*Adelges tsugae*; HWA). The insect, native to Japan, was first seen in Virginia in the 1950s, but was reported in western North America 30 years earlier. It feeds on xylem ray parenchyma at the base of needles, leading to desiccation of the needles and death of buds. While western North American species of hemlock are relatively resistant, both the eastern species are highly susceptible. The adelgid has already caused extensive damage and mortality of these species in the mid-Atlantic region (Small et al. 2005) and infestation by the adelgid threatens to greatly reduce or even eliminate the two hemlock species from eastern North American forests in the coming decades (Ellison et al. 2005). Impact of the adelgid on populations in the southern Appalachians has been particularly severe (Vose et al. 2013). The loss of eastern hemlock and the dense shade it produces is likely to have severe ecological consequences on forest composition,

nutrient cycles, hydrologic processes, wildlife and aquatic life in mountain streams (Potter et al. 2008). Current management approaches include spraying or injecting insecticides to protect individual trees and applying predator beetles as biocontrol agents (McClure et al. 2001). However, neither of these management approaches will make a significant contribution to conserving or restoring the hemlock trees. A system for long-term preservation of eastern and Carolina hemlock germplasm is needed to ensure that the genetic diversity of these species can be maintained for restoration purposes. Some work to generate germplasm banks via seed storage and establishment of seedlings or rooted cuttings outside the range of the pest is underway (Jetton et al. 2013). However, both of these approaches have potential drawbacks. The viability of seeds of eastern and Carolina hemlocks in cold storage appears to be limited to a few years (Robert Jetton, personal communication). Plantings of hemlocks outside their natural ranges may expose them to new pests, pathogens or other stresses that they do not face in their natural range, leading to loss of the populations or sub-optimal growth and reproduction.

We developed SE technology for eastern and Carolina hemlocks not only for germplasm conservation purposes, but as a possible tool for restoration of these species, working with geneticists and breeders who are identifying potentially HWA-tolerant native trees and breeding the native species with HWA-tolerant Asian hemlock species to generate HWA-tolerant hybrids. We began research to establish embryogenic cultures of both eastern and Carolina hemlocks by applying SE induction protocols that had been effective with other Pinaceae members, in particular southern U.S. pines (Merkle et al. 2005). We cultured seeds collected on different dates during May - August from four eastern hemlock and four Carolina hemlock source trees in Georgia and North Carolina, on three different induction media (Merkle et al. 2014). Medium type and cone collection affected embryogenesis induction frequency, with an induction rates as high as 52% for eastern hemlock seeds collected in mid-July in Georgia and 17% for Carolina hemlock seeds collected in late July in North Carolina. Smith's (1996) EDM6 medium was the best overall for embryogenesis induction for both species. Embryogenic hemlock cultures were maintained by monthly transfer to fresh EDM6 medium. Experiments using a modified Litvay's medium (Litvay et al. 1985) improved production of coytledonary-stage embryos over Smith's (1996) EMM2 medium (Merkle et al. 2014).

We wanted to develop cryostorage of embryogenic hemlock cultures as an alternative to storing seeds or installation of plantings outside the range of HWA, for conserving genetic diversity of the hemlock species. We tested a cryopreservation protocol that previously had been applied to cryostore and recover embryogenic cultures of different hardwood forest tree species (Holliday and Merkle 2000, Vendrame et al. 2001). We found that embryogenic eastern and Carolina hemlock cultures pre-treated in liquid EDM6 (Smith 1996) supplemented

with 0.4 M sorbitol and cryostored in the same medium supplemented with 5% dimethylsulfoxide (DMSO) as cryoprotectant could be thawed and regrown with 100% efficiency, even after more than seven months in cryostorage, for four of five tested genotypes (Merkle et al. 2014).

In addition to germplasm conservation, the ability to produce embryogenic hemlock cultures and their amenability to cryostorage has important implications for producing and testing material that may be tolerant to HWA infestation. The clonal multiplying power of the embryogenic cultures can greatly enhance the products of conventional intra-species and hybrid hemlock breeding programs by facilitating "clonal testing" of genotypes for HWA tolerance and, eventually, by providing a means for scaled-up production of the best hybrid clones. Seeds resulting from crosses between surviving native hemlocks that appear to possess resistance/tolerance to HWA (Caswell et al. 2008) can be used as explants to start embryogenic cultures. Once established, these cultures can be used to generate populations of trees that will provide "clonal repeatability" data from screening trials to determine if there is a genetic basis for the HWA tolerance of the parent trees. Similarly, hybrid breeding between susceptible native hemlocks and resistant Asian species such as Chinese hemlock (*Tsuga chinensis*) and southern Japanese hemlock (*Tsuga sieboldii*) can be combined with SE to generate clones of hybrid trees for tolerance screening. Embryogenic cultures of *T. caroliniana* x *T. chinensis* and *T. caroliniana* x *T. sieboldii* have already been generated and recently, the first putative hybrid somatic seedlings were produced (Figure 1A, 1B; Ahn et al., in press).

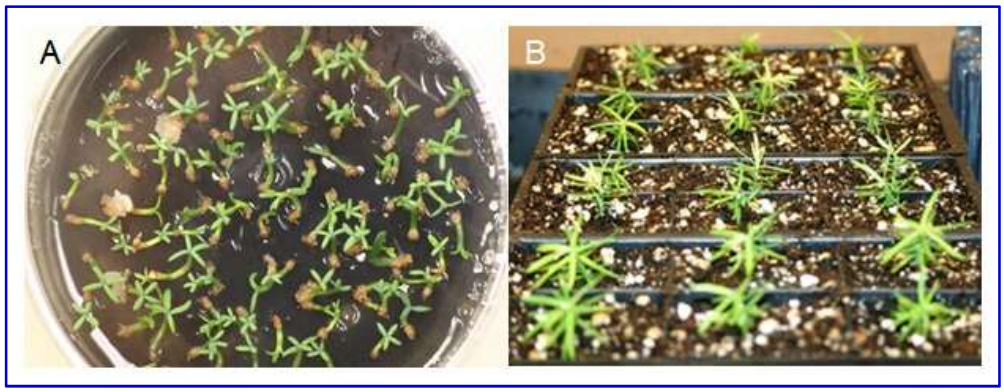

Figure 1. *Hemlock somatic embryogenesis.* ***A.*** *Germinating hybrid (Tsuga caroliniana x Tsuga sieboldii) hemlock somatic embryos.* ***B.*** *Hybrid (Tsuga caroliniana x Tsuga sieboldii) hemlock somatic seedlings continued growth in the hardening off chamber following potting.*

Finally, we may be able to take advantage of the relatively high level of self-compatibility that has been reported for both eastern and Carolina hemlocks (Bentz et al. 2002). Seeds collected from surviving, putatively HWA-tolerant hemlocks may include a high percentage of selfed seeds. Thus embryogenic cultures initiated from these seeds could provide a collection of clones with HWA tolerance genes in various heterozygous and homozygous combinations, partially overcoming the inability to directly clone the HWA-tolerant parents via somatic embryogenesis. In all of these scenarios, the fact that hemlock embryogenic cultures can be recovered following cryostorage means that they can be held indefinitely while somatic seedlings derived from them are screened for HWA resistance or tolerance. Then, if screening results indicate that any of the clones are especially promising, the cultures from which they were derived can be thawed, regrown and scaled-up to make somatic seedlings for restoration purposes.

3. Somatic embryogenesis in green ash and white ash

Ash trees, in particular white ash (*Fraxinus americana*) and green ash (*Fraxinus pennsylvanica*), are among the most abundant hardwood species in the eastern U.S. and are integral to the ecology of many ecosystems in the region. Not only are ash trees valued as urban tree and landscape species, but ash wood, which is strong, straight-grained and dense, is used for a variety of products, including tool handles, baseball bats, furniture, flooring and cabinets. While white ash is the most common species (Schlesinger 1990), green ash is the most widely-distributed. White ash commonly occurs on deep, moist, fertile upland soils, where it can grow to over 25 m, while green ash is most abundant along rivers and streams and in bottomland forests. Green ash is highly stress-tolerant and has been used successfully in shelterbelts (Hardin et al. 2001). Both ash species are valued as urban and landscape species. All North American ash species are under threat of extirpation from their native ranges by the emerald ash borer (EAB; *Agrilus planipennis)*, an exotic wood-boring beetle introduced from Asia, first discovered in Michigan in 2002. The larvae feed on the inner bark of ash trees, disrupting the tree's ability to transport water and nutrients. Since its discovery, the insect has killed millions of ash trees in 15 U.S. states and Canada (Poland and McCullough 2006). Some parasitoids of the borer have been found in China that may be useful biocontrol agents in North America (Zhang et al. 2005), but few control measures have been implemented in the U.S. other than restrictions on interstate movement of firewood.

The development of EAB- tolerant ash trees will be critical for ash reforestation in both urban and natural forests. Research is already underway to develop EAB-resistant ash trees via hybrid breeding with EAB-resistant Asian ash species, such as Manchurian ash (*F. mandshurica*; Rebek et al. 2008). In addition,

individual native white ash and green ash trees have been identified as potentially EAB-resistant by their persistence in populations where EAB-induced mortality exceeds 99% (Knight et al. 2010). These trees, commonly referred to as "lingering ash" by those researching the EAB infestation, constitute potentially valuable sources of resistance genes that could be used in a breeding program. A more direct approach to rapidly provide EAB-tolerant varietal planting stock would be to simply clonally propagate "lingering ash" individuals to generate varietals. A similar approach has been successful with generating Dutch elm-disease resistant American elm (*Ulmus americana*) trees (Shukla et al. 2012). Thus, while conventional selection and breeding approaches are promising for ash forest restoration, they could be greatly enhanced by the availability of a system for mass clonal propagation of the best EAB-resistant material, such as somatic embryogenesis. Somatic embryogenesis has been reported for multiple ash species, including white ash (Preece et al. 1989, Bates et al. 1992), narrow-leafed ash (*F. angustifolia*; Tonon et al. 2001), common ash (*F. excelsior*; Capuana et al. 2007) and Manchurian ash (*F. mandshurica*; Kong et al. 2012), but not for green ash. The SE system described by Tonon et al. (2001) demonstrated the potential for scalable ash somatic embryo production using suspension culture. Therefore, we undertook a study aimed at establishing scalable systems for in vitro propagation of green ash and white ash via somatic embryogenesis.

As detailed in Li et al. (2013), we collected immature seeds from three local Athens, GA green ash trees in late August 2012, divided them into three embryo developmental classes [< 1 mm long (EM1), 1-3 mm long (EM2) and >3 mm long (EM3)], and cultured embryos of each stage on semisolid woody plant medium (WPM; Lloyd and McCown 1980) or yellow-poplar induction medium (YP; Merkle et al. 1990) with different combinations of 2,4-D and BA. We individually transferred cultures producing proembryogenic masses (PEMs) to plastic Petri plates with induction-maintenance medium (IMM; Andrade and Merkle 2005), which was semisolid WPM supplemented with 2 mg/l 2,4-D and 1 g/l filter-sterilized L-glutamine, and transferred them to fresh medium every 3 weeks. After three months, proembryogenic masses (PEMs) were produced by embryo explants from all three source trees. Embryogenesis induction appeared to be affected by the developmental stage of the zygotic embryo in the explant, since five of the seven embryogenic cultures were derived from stage EM2 explants. Basal medium and plant growth regulator treatment were also important variables, since all the embryogenic cultures except one were produced on WPM with 2 mg/l 2,4-D and 0.5 mg/l BA and the other was produced on YP medium with the same PGR levels.

To produce somatic embryos, we transferred PEMs to liquid IMM and grew them for 3 weeks on a gyratory shaker at 100 rpm, then size-fractionated them on Cellector® stainless steel sieves (Bellco Glass). We collected the PEM

fraction between 38 µm and 140 µm on filter paper using a Büchner funnel and then incubated the filter with PEMs on semisolid embryo development medium (EDM; Andrade and Merkle 2005), which was WPM with 1 g/l L-glutamine, in the dark at 25° C to allow somatic embryos to develop. PEMs proliferated rapidly following transfer to liquid IMM and approximately 6 weeks following size fractionation and plating on basal medium, they produced highly dense masses of somatic embryos (Figure 2A).

When somatic embryos reached at least 3 mm in length with visible cotyledons, we picked them from the masses of developing embryos, transferred

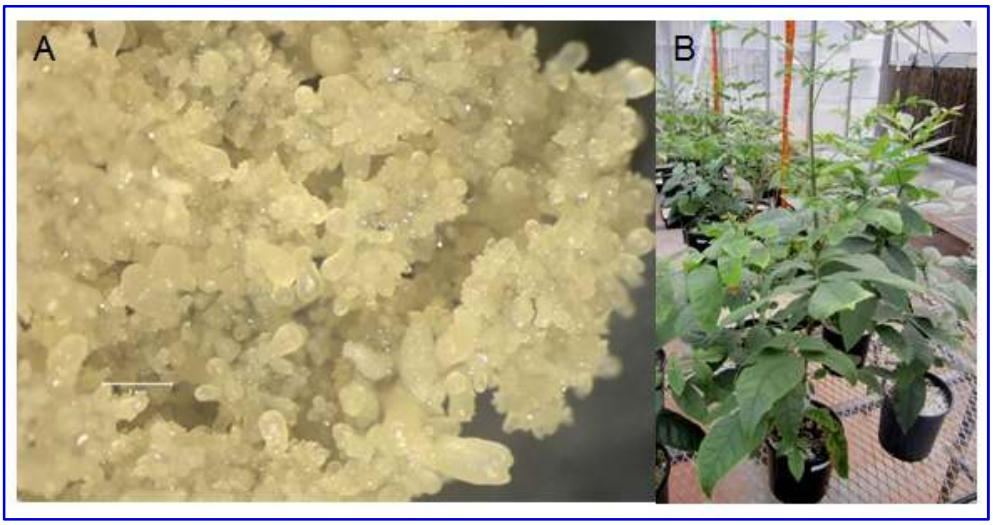

Figure 2. *Ash somatic embryogenesis.* ***A.*** *Proliferating green ash embryogenic culture. Bar = 1 mm.* ***B.*** *"Lingering" white ash somatic seedlings in the greenhouse.*

them individually to fresh plates of EDM and incubated them for another 3-4 weeks in the dark at 25° C to mature. Then, we moved them to a lighted incubator under cool white fluorescent lights (100 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) with 16 h day lengths at 25° C to encourage germination. Embryos of only two of the three tested genotypes greened and converted, while embryos from the third line remained white and failed to germinate. We removed germinating embryos with roots from *in vitro* conditions and potted them in moistened peat:perlite:vermiculite (1:1:1) mix in plastic pots, which we placed on top of water-saturated perlite in a clear plastic dome-covered tray under cool white fluorescent lights (80 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) and 16 h day lengths. The germinated embryos grew into vigorous somatic seedlings that continued growth following transfer to the greenhouse, eventually producing the characteristic compound leaves.

In summer 2013, we applied the same embryogenesis induction protocol to seeds collected by Ohio State University collaborators from four "lingering" female white ash trees in Michigan. We reasoned that even though the seeds collected from these surviving trees were open-pollinated, it was very likely that the pollen parents were also putatively EAB-tolerant "lingering" white ash trees, since EAB had eliminated all other white ash trees in the area. We tested the effects of collection date, auxin treatment and explant treatment (whole seeds or excised zygotic embryos) on embryogenesis induction. Excised zygotic embryos had higher induction rates than whole seeds, and embryos from seeds collected in mid-August had the highest embryogenesis induction (Mitchell and Merkle, in press). Unlike the green ash cultures, most white ash embryogenic cultures tended to produce somatic embryos while still on medium with 2,4-D, rather than PEMs. Embryos picked from these cultures and transferred to basal medium often continued to produce repetitive embryos, instead of enlarging or germinating, unless they were first shaken overnight in liquid basal medium to remove residual 2,4-D. A preliminary experiment indicated that, following a 15 week pre-germination cold treatment, the highest conversion rates were achieved by incorporating 0.5 g/L activated charcoal and 0.01 mg/L gibberellic acid into the germination medium (Mitchell and Merkle, in press). Several white ash somatic seedlings derived from the "lingering ash" explants were acclimatized and are continuing to grow in the greenhouse (Figure 2B). It will be at least a few years before their stems reach a diameter where they can be tested for resistance to EAB. In the meantime, we have cryostored copies of the "lingering ash" cultures so they will be available for scaled-up production should any of the clones demonstrate durable EAB resistance.

4. Somatic embryogenesis in American chestnut and hybrid chestnuts

American chestnut (*Castanea dentata*) once dominated the forests of the Appalachian Mountains in the Eastern United States, where it was a major timber and nut-producing tree. Its durable wood was used for poles, pilings, posts, shingles and railroad ties and furniture and the bark was an important source of tannins for the leather industry. The large, reliable annual nut crop provided nutrition for wildlife as well as people (Anagnostakis 1987). The chestnut blight fungus (*Cryphonectria parasitica*), accidentally introduced from Asia on Japanese chestnut trees, began killing American chestnut trees around 1900. Within 40 years, the fungus had killed millions of chestnuts throughout the tree's natural range. Today, the tree occurs mainly as an understory shrub, due to its ability to re-sprout from stumps and the fact that the root systems remain uninfected by the blight fungus (Burnham 1988). Attempts to restore the species to the forest have included (1) searching for natural blight resistance in surviving American chestnut

trees, (2) hybridizing American chestnut with blight-resistant Asian chestnuts, (3) induction of mutations using gamma irradiation and (4) the use of hypovirulent strains of the blight fungus as biocontrol agents (Griffin 2000). In the 1980s, the American Chestnut Foundation (TACF) began a hybrid backcross breeding program, based on hybrids between American and blight-resistant Chinese chestnut (*Castanea mollissima*), that is currently producing BC_3F_3 seedlings that were intended to resemble American chestnut in form and other aspects, while possessing levels of blight resistance that approach that of Chinese chestnut (Hebard 2005). However, preliminary results from field tests of these seedlings indicate that many trees were lost to Phytophthora root rot, another devastating disease of American chestnut and other trees caused by the exotic Oomycete *Phytophthora cinnamomi* (Clarke et al. 2014). Unfortunately, TACF's breeding program failed to breed for resistance to this second pathogen. Thus, the BC_3F_3 trees would not survive if planted in much American chestnut's original southern range, since the soils in the region are infested with *P. cinnamomi*.

It is with American chestnut that SE technology may ultimately have the largest impact on conservation and restoration of any North American tree, because there are multiple levels where this technology can make contributions. Not only can SE technology help conserve remaining American chestnut germplasm before it is lost, but it has the potential to greatly enhance TACF's hybrid backcross breeding program. Perhaps most importantly, there now appears to be evidence that some transgenic American chestnuts, the production of which was facilitated by somatic embryogenesis, are displaying high levels of blight resistance. Thus SE-derived chestnuts may become a major component of a restoration program for the tree.

The first *in vitro* propagation work and first successful transformation of *Castanea* were accomplished in Europe. Viéitez (1995) regenerated several plantlets of *C. sativa* x *C. crenata* hybrids via somatic embryogenesis using zygotic embryos as explants. Saur and Wilhelm (2005) regenerated plantlets from embryogenic cultures of pure *C. sativa*, initiated from ovaries, ovules and immature zygotic embryos. Corredoira et al. (2004) achieved a transformation frequency of 25% and regenerated stably transformed European chestnut trees by co-cultivation of these leaf-derived embryogenic cultures with *Agrobacterium*, and more recently produced transgenic chestnuts expressing a thaumatin-like protein gene that may confer resistance to fungal pathogens (Corredoira et al. 2012).

Somatic embryogenesis research with American chestnut has been underway for the past 25 years (Merkle et al. 1991; Carraway et al. 1997; Xing et al. 1999, Robichaud et al. 2004), but has only made significant progress for propagation and gene transfer applications in the last ten. We developed a system for manipulating embryogenic chestnut cultures in suspension that allows us to produce hundreds of somatic seedlings per gram of PEMs (Andrade and Merkle

2005). We also now routinely cryostore copies of all chestnut cultures once they are established, and can reliably recover them from cryostorage and regrow them when needed using the protocol reported by Holliday and Merkle (2000). The ability to cryostore and recover embryogenic cultures means that for any American chestnut tree that survives long enough to produce seeds from which we can initiate embryogenic cultures, we can conserve its germplasm indefinitely. We have several cultures in cryostorage that were derived from seeds of American chestnut trees that have died from blight or other causes since we initiated the cultures.

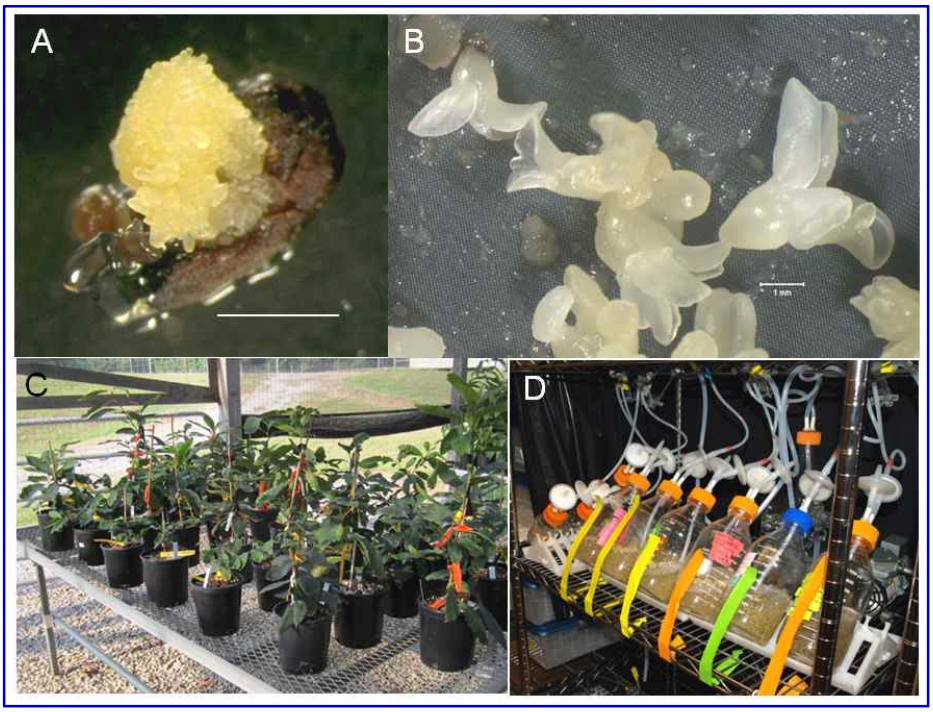

Figure 3. *Chestnut somatic embryogenesis.* ***A.*** *Newly-initiated embryogenic culture from BC_3F_3 hybrid backcross seed explant. Bar = 1 mm.* ***B.*** *BC_3F_3 somatic embryos produced from embryogenenic suspension culture that was size-fractionated and plated on nylon mesh overlaid ion basal medium. Bar = 1 mm.* ***C.*** *BC_3F_3 somatic seedlings in lath house.* ***D.*** *Embryogenic chestnut suspension cultures designated for gene transfer in air-lift bioreactors.*

For the past five years, our lab has been part of the Forest Health Initiative (FHI), a multi-institution research project, the mission of which is to demonstrate the application of biotechnological tools to address forest health threats in the U.S. We have been employing embryogenic cultures to address multiple FHI objectives focused on restoration of the American chestnut. One particular goal of the FHI

was to establish embryogenic cultures of TACF BC_3F_3 material to facilitate clonal testing. Prior to the beginning of the FHI project, only pure American chestnut material had been propagated via SE, so the potential to propagate advanced generation hybrid material using this approach was unknown. Open-pollinated BC_3F_3 seeds representing two lines of blight-resistance were collected from BC_3F_2 seed orchard parents by TACF cooperators and used to initiate cultures in 2010 and 2011 (Figure 3A). Average embryogenesis induction percentages were 0.85% for nine open pollinated (OP) BC_3F_3 families in 2010 and 1.63% for 11 OP BC_3F_3 families in 2011. These "capture" percentages were not significantly different from those for American chestnut cultures initiated in those years, and BC_3F_3 somatic embryos and somatic seedlings were produced from the cultures (Figure 3B, 3C; Holtz et al. 2013). In order to take full advantage of SE for production of elite chestnut varieties, it needs to be combined with full-sib breeding. Selected BC_3F_2 parents were crossed to for this purpose in 2012. While the average embryogenesis induction percentage for the full-sib material (0.5%) was lower than for OP seeds, at least one embryogenic culture was produced for eight of the nine crosses (Holtz et al. 2013). Now that we have confirmed that SE can be applied to propagate BC_3F_3 material, all the pieces are in place to apply the varietal forestry approach to blight-resistant chestnuts. Somatic seedlings derived from BC_3F_3 cultures initiated from crosses between the best BC_3F_2 parents can be field tested while the cultures from which they were produced are held in cryostorage. Once the best varieties are identified, those cultures can be recovered from cryostorage and scaled-up for mass somatic seedling production of elite planting stock.

Another FHI objective was to develop a "pipeline" for scaled-up production of transgenic American chestnut trees engineered with different candidate genes (CGs) for blight resistance and *Phytophthora* root rot resistance. My lab at UGA and the Powell/Maynard Lab at SUNY-ESF had already developed transformation and regeneration systems for American chestnut using embryogenic cultures as target material (Polin et al. 2006, Andrade et al. 2009). In my lab, we used selection in suspension culture to produce over one hundred transgenic American chestnut somatic seedlings from multiple genotypes following *Agrobacterium*-mediated transformation of embryogenic cultures (Andrade et al. 2009). To meet the FHI objective of testing dozens of CGs, we collaborated with scientists from multiple universities (SUNY-ESF, Pennsylvania State University, Clemson University), TACF and the USDA Forest Service. CGs were identified by the Genomics/Gene Discovery group (U.S. Forest Service, Penn State University, Clemson University) working from the compared transcriptomes of chestnut blight canker tissue from blight-resistant Chinese chestnut and blight-susceptible American chestnut. Once identified, the CGs were cloned from Chinese chestnut libraries by Dr. Bill Powell (SUNY-ESF) and then cloned into transformation vectors by Dr. Joe Nairn (UGA). Both our lab at UGA and the Powell Lab at

SUNY-ESF then worked to transform dozens of CGs into American chestnut "workhorse" lines that were chosen for high transformation and somatic seedling production efficiencies. In addition to CGs from Chinese chestnut, we also transformed a number of anti-fungal CGs from heterologous sources into American chestnut. Transformation of the "workhorse" lines with CG and reporter gene constructs began in my lab at UGA in 2010, using the transformation protocol detailed in Andrade and Merkle (2009). Our adoption of airlift bioreactors for growing embryogenic suspension cultures (Figure. 3D), rather than shaken flasks, greatly accelerated production of embryogenic material for both somatic embryo production and *Agrobacterium*-mediated genetic transformation. Sufficient new target material for transformation experiments could be produced every two weeks, and transformation frequencies for some workhorse lines grown in the airlift bioreactors were very high, producing almost 700 putative transformation events per 50 mg of inoculated tissue of one line (Kong et al. 2014). To date, we have transformed over 30 constructs with CGs and marker genes into different American chestnut backgrounds, and have transgenic trees in the ground or in pots representing over 120 transgenic events. Field plantings of the trees for blight resistance screening and evaluation of growth have been established at three locations in Georgia and Virginia. In addition, chestnuts engineered with candidate genes for resistance Phytophthora root rot from Chinese chestnut and other sources are being screened in large containers of *P. cinnamomi*-infested potting mix by a collaborator in South Carolina.

While the FHI "pipeline" has been very successful at producing transgenic chestnuts for screening candidate genes for pathogen resistance, these trees are only now reaching a size where they can be screened for blight resistance. However, American chestnut trees engineered with a wheat oxalate oxidase (*OxO*) had already been produced by our collaborators in the Powell/Maynard Lab at SUNY-ESF prior to the inception of the FHI project (Polin *et al*. 2006). The oxalate oxidase enzyme encoded by the *OxO* gene breaks down oxalic acid, and since *C. parasitica* infection involves the killing of tissue with oxalic acid, the overexpression of this gene in chestnut stem tissues was expected to confer resistance to the blight fungus. Recent results indicate that this gene may offer very effective resistance to the blight fungus (Newhouse et al. 2014).

5. Conclusions

With the incidence of forest health problems expected to accelerate due to factors such as global trade and global climate change, forest scientists will need every tool at their disposal to mitigate the effects of pests and pathogens that threaten to eliminate forest species. The abilities to mass propagate resistant or tolerant genotypes for testing or operational deployment, as well as the ability to

test the function of genes that may confer pest/pathogen resistance or tolerance, will become more and more critical to managing these threats. Forest tree SE technology is already making useful contributions as a component of strategies to address the conservation and restoration of some forest species threatened by exotic pests and pathogens, such as eastern North American hemlocks, North American ash species and American chestnut. As I hope the examples I have used here clearly demonstrate, the mass propagation and long-term storage potential of embryogenic cultures make a very powerful tools for conservation and restoration when combined with conventional selection, hybrid breeding or transgenics. I believe that very soon, approaches incorporating SE technology will begin to make a real impact on programs focused on addressing forest health threats in North America and other regions of the world.

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Advances in somatic embryogenesis and genetic transformation of European chestnut: Development of transgenic resistance to ink and blight disease

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Abstract

Somatic embryogenesis (SE), which is considered the most efficient in vitro procedure for mass propagation of plants, shows great potential for use in forest tree improvement programs. This chapter presents a summary of recent advances made in the development of SE systems for European chestnut and hybrid chestnuts. As in most other woody species, immature zygotic embryos constitute the most suitable material for induction of SE in European chestnut. However, somatic embryogenesis has also been induced in leaf and shoot apex explants derived from axillary shoot cultures. Although the initial rate of induction of SE is low, a large number of somatic embryos can be obtained by secondary embryogenesis. An efficient protocol for the production of transgenic somatic embryos mediated by *Agrobacterium* co-culture with marker genes has been described for European chestnut. A number of parameters were evaluated with a view to maximizing the transformation efficiency. The transformation efficiency was not significantly affected by wounding, co-culture temperature or bacterial growth phase, but it was significantly influenced by other parameters such as strain/plasmid combination, co-cultivation time, selective agent, genotype and developmental stage of the somatic embryos. Genetic transformation experiments aimed at inducing tolerance to ink disease and blight disease have been performed with the thaumatin-like protein (*CsTL1*) and chitinase protein (*CsCh3*) genes, respectively. The presence of transgenes was confirmed by histochemical GUS assay, GFP, PCR and Southern blot analysis. The chestnut plants obtained are not transgenic *sensu stricto*, because the overexpressed genes are isolated in chestnut, and they could be considered cisgenic plants. Vitrification-based cryopreservation procedures have been successfully used with zygotic embryos and with

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untransformed and transformed somatic embryos.

Keywords: *Castanea sativa*, Chitinase, Cisgenic, Cryopreservation, Forest biotechnology, Genetic transformation, Micropropagation, Pathogenesis related proteins, Thaumatin-like protein, Tree breeding.

1. Introduction

The genus *Castanea* (family *Fagaceae*) comprises 13 species of chestnut trees and chinkapins native to the Northern Hemisphere. Among the chestnut species, *C. sativa* Mill. (sweet chestnut, European chestnut) is considered the only native species of the genus in Europe. It is a widely distributed tree species of economic importance in Europe where it covers an area of more than 2 million hectares (Conedera et al. 2004). The nutritional value of the chestnut fruit has long been recognised, and some ancient Greek and pre-Roman tribes even considered chestnuts to be superior to almonds and walnuts. In around 1570, the writer and philosopher de Montaigne wrote (in his *Journal du Voyage*) that Roman legions subsisted on chestnuts during the Gallic Wars, considering them "bread from the forest" and denominating the tree *Arbus panis* (Vieitez and Merkle 2005). The Romans also used the timber from chestnut trees to make baskets and produce stakes and posts for protecting and training vines. Chestnuts have thus been cultivated for centuries and have become a very important part of the cultural heritage in many rural areas of Europe. Chestnut timber is also used as a construction material, for making furniture and flooring, for extraction of tannins and as a source of renewable energy. In addition to their original productive role, European chestnut stands may also play an important role as wildlife habitats, in rural tourism, as recreational areas, for landscaping and also for protection of land from erosion.

Root-rot or ink disease (caused by *Phytophthora cinnamomi* Rand and *P. cambivora* (Petri) Buis) and chestnut blight (caused by *Cryphonectria parasitica* (Murrill) Barr) are the most important diseases that affect European chestnut. The economic importance of chestnut and the severity of the diseases that threaten the species justify the implementation of genetic improvement programs. The first breeding program aimed at developing tolerant/resistant Euro-Japanese hybrids with *Castanea crenata* (Japanese chestnut), which shows natural resistance to both ink and blight diseases, was initiated in Spain as early as 1921 (Vieitez et al. 1996). Conventional tree breeding programs are very slow, and large backcross breeding programs have not been (and probably will never be) carried out in Europe to produce disease-resistant chestnut trees. However, only first-generation hybrids have been distributed throughout different European countries by means of conventional breeding programs, indicating that a number of the specific

characteristics of *C. sativa* may have been lost in many chestnut stands, as undesirable traits may have been introduced into the native species.

Somatic embryogenesis (SE) is a potentially powerful biotechnological tool for overcoming some of the difficulties inherent in forest tree breeding programs. SE is the primary enabling tool for many tree biotechnology procedures, including genetic transformation and subsequent mass propagation (Park and Bonga 2010). One of the greatest advances in plant breeding has been the generation of modified plants through genetic transformation, which involves inserting specific genes into the plant genome. A fundamental premise for success in the production of transgenic plants is the availability of an in vitro regeneration system that can support the regeneration of plants from cells, organs or tissues (target cells) that are susceptible to infection by *Agrobacterium tumefaciens*. SE is the procedure of choice for transformation protocols as regeneration via this micropropagation method offers the advantage of a single cell origin, whereas plant regeneration from transformed tissues via organogenesis can often give rise to chimeras (Giri et al. 2004). In research involving European chestnut, the first objective was the micropropagation by axillary shoot cultures of ink resistant hybrids (*Castanea sativa x C. crenata*) obtained by conventional breeding (Vieitez et al. 1986). The method thus developed has been transferred to private companies for large scale propagation of ink-resistant genotypes (Vieitez et al. 2007). Interest subsequently arose in the induction of consistent somatic embryogenic methods for use in genetic transformation experiments, with the aim of obtaining disease resistant trees.

This chapter presents a review of all relevant findings related to somatic embryogenesis in European chestnut and its applications in propagation, genetic transformation and cryopreservation.

2. Culture initiation

Chestnut species are considered recalcitrant to clonal propagation. Selection of an appropriate initial explant, excised at defined developmental and/or physiological stages, is a crucial factor for propagation by axillary shoot proliferation and also for clonal propagation by SE (Vieitez et al. 2012). Most embryogenic systems obtained in European chestnut and their hybrids have used zygotic embryos or parts of them as initial explants. Thus the material being propagated is of unproven genetic value. Induction of somatic embryogenesis in explants other than immature or mature zygotic embryos is very difficult. This chapter, although describing SE from zygotic embryos, will mainly focus on embryogenic systems developed from plant material other than zygotic embryos.

2.1 Somatic embryogenesis from zygotic embryos

Indent reports described the induction from cotyledonary explants of organized structures resembling somatic embryos, but which did not develop into plantlets (González et al. 1985, Piagnani and Eccher 1990). The first report clearly describing the induction of true somatic embryos and plant regeneration from immature zygotic embryos of *Castanea sativa* x *C. crenata* trees was published by Vieitez et al. (1990). Subsequent reports also described the induction of somatic embryos from zygotic embryos of *Castanea sativa* x *C. crenata* (Vieitez 1995, Corredoira et al. 2006) and *C. sativa* (Sauer and Wilhelm 2005, Corredoira et al. 2006, Sezgin and Dumanoğlu 2014) trees.

The most important factors determining the induction of somatic embryogenesis in chestnut zygotic embryos are the genotype, the developmental stage of the zygotic embryos and the type of growth regulators used. Initiation of embryogenic cultures is generally achieved in a two-step culture procedure, in which the explants are successively cultured in induction medium and expression medium with or without auxin (Vieitez et al. 1990, Vieitez 1995, Sauer and Wilhelm 2005, Corredoira et al. 2006, Sezgin and Dumanoğlu 2014). Inclusion of an exogenous auxin, usually 2,4-dichlorophenoxy acetic acid (2,4-D), is an essential pre-requisite to the initiation of chestnut embryogenic cultures.With the exception of Sezgin and Dumanoğlu (2014), who used indole-3-butyric acid (IBA) in combination with thidiazuron to initiate somatic embryos, researchers have generally initiated embryogenic cultures by using 2,4-D. A cytokinin, such as benzyadenine (BA), kinetin or zeatin (Z), is also added to the induction medium at a lower concentration than the auxins.

The induction rate is clearly influenced by the developmental stage of the zygotic embryo and the time of collection, defined by weeks post-anthesis (Vieitez 1995, Sauer and Wilhelm 2005, Corredoira et al. 2006). Immature zygotic embryos harvested at 6-12 weeks post-anthesis (approximately end of July to first week of September in NW Spain) were found to be the most suitable explants for induction of European chestnut somatic embryos. Whole zygotic embryos (Vieitez et al. 1990, Vieitez 1995, Sauer and Wilhelm 2005, Corredoira et al. 2006) and pieces of cotyledon (Corredoira et al. 2006, Sezgin and Dumanoğlu 2014) have been used as initial explants. In an evaluation of the embryogenic capacity of the embryonic axis and the cotyledonary pieces, the induction efficiency was found to be twice as high in the former as in the latter (Corredoira et al. 2006).

Somatic embryos formed on the surface of nodular friable cell masses that developed from the initial explants (Figure 1A). Depending on the genotype, the time required from initiation of the experiment until the appearance of the first somatic embryos generally ranged from 3 to 5 months. The induction frequency of somatic embryos is generally low, less than 11% (Vieitez et al. 1990, Vieitez 1995, Corredoira et al. 2006, Sezgin and Dumanoğlu 2014), although an exceptional induction rate of 57.1% has been reported (Sauer and Wilhelm 2005).

2.2 Somatic embryogenesis from leaf and shoot apex explants

Induction of somatic embryos from material other than zygotic embryos in the genus *Castanea* has to date only been achieved by our research group (Corredoira et al. 2003, 2006). We have initiated embryogenesis in leaf and apex explants excised from stock shoot multiplication cultures established from *C. sativa* plantlets (2 to 3 months-old) derived from the germination of zygotic embryos. Use of this type of explant to initiate embryogenic systems offers advantages over the use of zygotic embryo tissues, as no sterilization procedure is required and experiments can be programmed all year round (San José et al. 2010).

In the first experiments, 1-3 of the uppermost unfurled expanding leaves were excised from 4-week-old shoot cultures from the Clone 12 of *C. sativa* and were cut transversally across the midvein. Proximal (basal) halves of leaves were subjected to a three-step culture method. This procedure involved successive culture of explants on induction medium (M1) consisting of MS (Murashige and Skoog 1962) mineral salts and vitamins, 500 mg/L casein hydrolysate, and different combinations of naphthaleneacetic acid (NAA), IBA, 2,4-D and BA. After 8 weeks in dark conditions, the explants were transferred to fresh medium of the same composition, except that the concentrations of auxin and BA were reduced to 0.54 μM and 0.44 μM respectively (M2 medium), for a further 4 weeks, and finally to plant growth regulator-free medium (expression medium M3) for another 8 weeks (i.e. the explants were cultured for a total of 20 weeks after the start of culture). With this procedure somatic embryos generally appeared on the surface of a callus 10-20 months after culture initiation, a period that was longer than that observed for induction from zygotic embryo explants. The best results were obtained when leaf explants were initially cultured with 5.4 NAA plus 4.44 μM BA or 21.48 μM NAA plus 2.22 μM BA, with an induction frequency of 1% (Corredoira et al. 2006). Unlike in induction of SE from zygotic embryos, 2,4-D and IBA were ineffective when they were applied to leaf sections. The combination of NAA and BA was also used to induce SE in *Quercus* species (Corredoira et al. 2014).

Experiments were carried out to test the effect of arabinogalactan proteins (AGPs) from arabic gum (AG) and arabinogalactan from larch wood (LW) on the initiation of somatic embryos in leaves and shoot tip explants, to further define the requirements for SE induction in European chestnut (unpublished results). AGPs are a family of proteins in which the core protein is linked to arabinogalactan residues (van Hengel et al. 2002). These proteins play an important role in plant development and somatic embryogenesis (Majewska-Sawka and Nothnagel 2000). The addition of exogenous LW or AGPs to the culture medium has been found to stimulate embryogenic development in several species (Ben-Amar et al. 2007, Pereira-Netto et al. 2007).

The shoot apex and the most apical expanding leaf isolated from the first node in the apical region of the actively growing shoots were excised from shoot proliferating cultures of Clone 12 and Clone 818 and used as initial explants. Somatic embryos were induced following the above procedure but with an induction medium (M1) consisting of 21.48 µM NAA and 2.22 µM BA. Arabinogalactan from larch wood (0, 2 or 4 mg/L) or gum arabic (40 mg/L) were filter sterilized and added to the autoclaved culture medium (M1, M2 or M3) to evaluate the effect on initiation of somatic embryos in European chestnut.

Our studies on European chestnut indicate that the addition of larch arabinogalactan to the culture medium enhances the embryogenic response in both explant types. Somatic embryos were obtained from leaf and shoot apex explants (Figure 1B, C)in both of the genotypes evaluated, although the best results were obtained with leaf explants (5.3%) of Clone 12 cultured on medium supplemented with 2 mg/L LW. Similarly, the presence of LW improved SE in leaf and shoot apex explants excised from the three genotypes of swamp white oak (Mallón et al. 2013). The stimulatory effect of this complex polysaccharide arabinogalactan was demonstrated in the present study and the data obtained on its positive effect on the embryogenic system of *C. sativa* represent the first report of such a finding in the genus *Castanea*.

The influence of genotype during the initiation of somatic embryos from leaf and shoot tip explants was also observed in European chestnut. The most responsive material was Clone 12, in which both types of explant showed a higher embryogenic capacity than Clone 818. Genotype specificity for induction of SE has also been reported in other woody species, including *Q. suber* (Hernández et al. 2011), *Q. alba* (Corredoira et al. 2012a) and *Eucalyptus* species (Corredoira et al. 2015a).

Findings regarding the ability of exogenous application of AG 40 mg/L to induce somatic embryos in leaf and shoot apex explants of European chestnut are not conclusive, although addition of this compound to induction medium enhanced SE rates in several oak species (Corredoira et al. 2014).

3. Maintenance of embryogenic cultures

The maintenance of embryogenic capacity by repetitive embryogenesis makes the continuous supply of somatic embryos possible, as embryogenic cultures can be efficiently multiplied by both secondary embryogenesis and subculture of nodular embryogenic masses and proembryogenic masses (PEMs). In European chestnut, the multiplication and maintenance of embryogenic capacity can be carried out by both of these methods.

Repetitive somatic embryogenesis frequently leads to the formation of many secondary somatic embryos, although the genotype has been found to affect

maintenance of embryogenic cultures. Thus, we have observed that the competence of different embryogenic lines originated from zygotic embryos of *C. sativa* and hybrid material for repetitive embryogenesis varies from line to line, highlighting the effect of the genotype on embryo proliferation (Vieitez 1995, Corredoira et al. 2003, 2006). In addition to the genotype, the composition medium also affects the embryo proliferation rates and the quality of the secondary embryos. For proliferation, clumps of somatic embryos at globular to early-cotyledonary stages are subcultured on semi-solid medium of the same mineral composition as the induction medium (Sauer and Wilhelm 2005) or at levels less than full-strength (Vieitez 1995, Corredoira et al. 2003 and 2006, Sezgin and Dumanoğlu 2014). Low levels of BA (0.44 or 0.89 μM) with or without 0.54 NAA are used for secondary embryo proliferation (Sauer and Wilhelm 2005, Corredoira et al. 2003, 2006). The type and concentration of carbon source was investigated for maintenance of hybrid embryogenic cultures, and 3 % sucrose was found to be superior to fructose, glucose and maltose, with the latter being the least effective (Vieitez 1999).

Although somatic embryos can be developed on the cotyledons of primary embryos, secondary embryos mainly form on the hypocotyl or root zone of primary somatic embryos (Figure 1D). The embryonic cells in the hypocotyl-root zone of primary embryos of chestnut are probably embryogenically determined, and a single stimulus for cell division may be sufficient for the formation of secondary embryos (Figure 1E).

In European chestnut, the maintenance of embryogenic capacity by subculture of nodular callus (Corredoira et al. 2003) and PEMs (Vieitez 1995, 1999) has also been described (Figure 1F, G). Basically, embryogenic lines of *Castanea sativa* x *C. crenata* have been maintained by subculture of PEMs on half-strength macronutrients MS (½MS) semi-solid medium containing 0.91 μM Z and 0.25 μM IBA or on liquid suspension medium (Vieitez 1995). Suspension cultures were established by transferring PEMs to liquid suspension medium consisting of ½MS supplemented with 1.13 μM 2,4-D and 0.45 μM BA. Every 8 days, the suspension cultures were filtered through 104 μm size nylon screens and the filtrate, which contains only floating free cells and smaller cell aggregates, was subcultured by 1:1-5 dilution with fresh liquid suspension medium, making a final liquid volume of 60 ml. The culture fraction retained on the 104 screens was collected and transferred to semi-solid medium supplemented with 0.46 μM Z, where embryos at all stages of development were observed after 3-4 weeks of culture.

In addition to secondary embryos, the subcultured primary embryos of *C. sativa* also begin to develop nodular masses from their cotyledons as a form of repetitive embryogenesis (Corredoira et al. 2003). The nodular clumps produced somatic embryos with the same PGR combination as used for secondary embryogenesis, although fewer somatic embryos were obtained from nodular

masses than in subculture of somatic embryos or PEMs. In the case of embryogenic

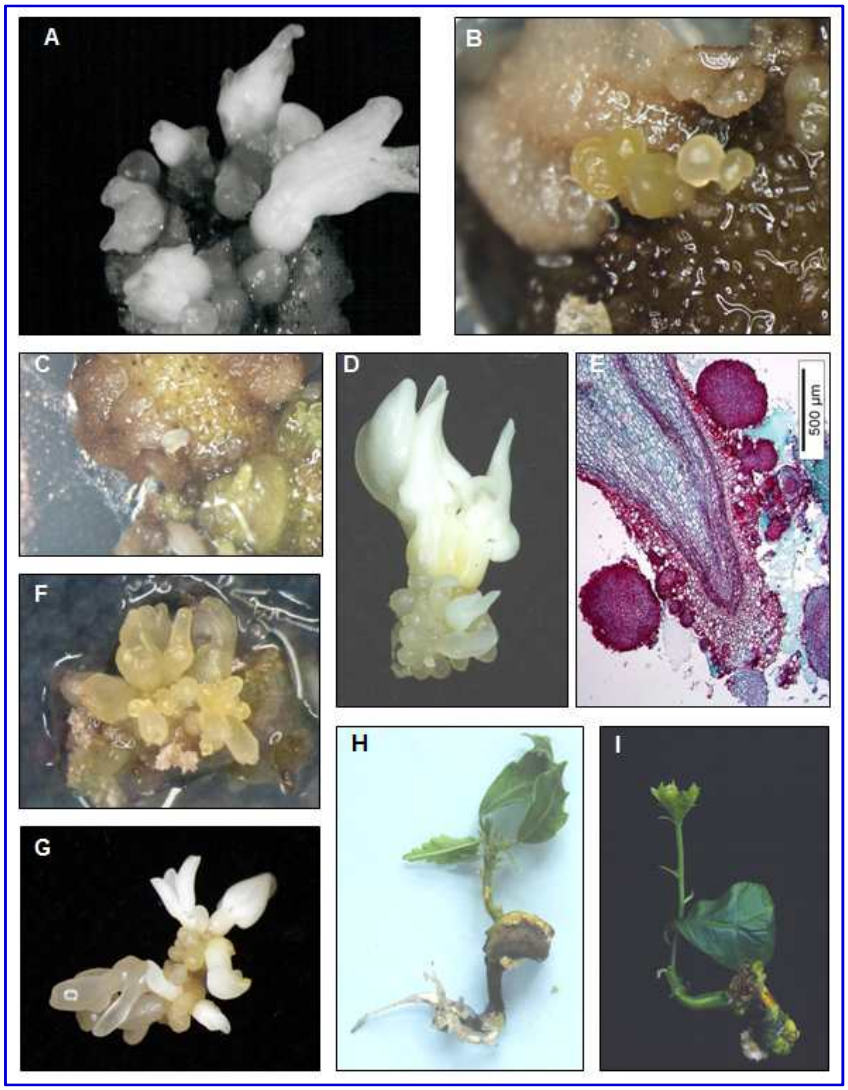

Figure 1. *Somatic embryogenesis in European chestnut.* ***A*** *Somatic embryos generated from an immature zygotic embryo collected from a Castanea sativa x C. crenata tree.* ***B, C*** *Somatic embryos initiated from a leaf* ***(B)*** *and a shoot apex* ***(C)*** *explants derived from axillary shoot cultures of C. sativa.* ***D*** *Secondary embryos produced in the root region of a primary somatic embryo.* ***E*** *Longitudinal section of an embryo showing meristematic cellular areas on the surface layers in the root region of a primary embryo.* ***F, G*** *Secondary embryos developing from a nodular callus* ***(F)*** *and a PEM* ***(G)*** *cultured on semi-solid proliferation medium.* ***H, I*** *Germinated somatic embryos exhibiting shoot plus root development* ***(H)*** *and only shoot development* ***(I)****, after 8 weeks of culture in germination medium.*

nodular masses that had originated from cotyledonary cells (which are more differentiated), a certain number of mitotic divisions seem to be necessary to produce the masses prior to somatic embryo development (Corredoira et al. 2006). Therefore, direct secondary embryogenesis and indirect proliferation through proembryogenic masses or nodular calli can be considered as two extremes of a continuum (Merkle 1995).

4. Germination and plantlet conversion of somatic embryos

In addition to the low rates of embryogenic induction, another difficulty in chestnut somatic embryogenic systems is the relatively poor germination of somatic embryos and the low rate of development into functional plants. This is a common difficulty in many other woody species, including American chestnut. In zygotic embryogenesis, maturation occurs after cell division, differentiation and embryo elongation (Dodeman et al. 1997). During this stage, reserve substances accumulate, metabolic activity decreases and drying takes place, thus preparing the embryo for germination and development of the plants (Kermode 1990). In embryogenic systems, the procedure generally used to obtain plants from somatic embryos has been to mimic the conditions undergone by zygotic embryos prior to germination (Merkle 1995). Although in some species this can be done by use of a single medium, the most common procedure is to apply different approaches for maturation and then for germination. Addition of certain PGRs such as abscisic acid (ABA) or gibberellic acid (GA_3) to the culture medium, as well as the use of different osmotic agents, desiccation and cold storage may favor maturation of the embryos (Merkle 1995, Jiménez 2005, Pinto et al. 2013).

4.1 Maturation treatments

Somatic embryos usually mature in the presence of osmotic agents (sugars, alcohol-sugars or inert compounds such as polyethyleneglycol) added to decrease the osmotic potential of the medium in order to reduce water adsorption (Klimaszewska et al. 2000). In addition to causing loss of water due to hydric stress, osmotic agents also induce synthesis of reserve products (Yeung 1995). The addition of sucrose or other sources of carbon produces osmotic stress when used at concentrations higher than those required to sustain embryo growth (Lipavská and Konrádová 2004). In this case, on being hydrolyzed in the cell, the osmotic potential increases, leading to accumulation of the reducing sugars and sucrose that are used to produce reserve substances during maturation (Thompson and Thorpe 1987).

In European chestnut, the source of carbon and the concentration at which it is used had significant effects on the maturation and subsequent germination and conversion ability (Corredoira et al. 2003). Plantlet conversion was achieved in

embryos matured with 6% sucrose or 3-6% maltose, whereas mean shoot length, root length and leaf number in the plants produced were not significantly affected by these maturation media. Overall, the best results were obtained with 3% maltose-treated embryos, which produced 6% plant recovery in addition to 33% of embryos exhibiting partial germination with only shoot development (Figure 1H, I). In our embryogenic systems, addition of activated charcoal or sorbitol did not yield any improvement in the germination and plantlet conversion.

In European chestnut, osmotic stress was also induced by increasing the concentration of agar to 1.1% in the maturation medium. This treatment stimulated the maturation and plantlet conversion rates (10-25%) in *C. sativa* embryogenic lines of zygotic origin (Sauer and Wilhelm 2005).

ABA regulates the synthesis of storage proteins and lipids in zygotic embryos, thus promoting seed dormancy and tolerance to desiccation (Rohde et al. 2000). ABA is widely used for the maturation of somatic embryos of gymnosperms and, to a lesser extent, angiosperms. In chestnut, the culture of isolated embryos of hybrid material on media supplemented with ABA (0.38-7.45 μM) alone or in combination with different gelling agents was very poor in relation to supporting embryo maturation and had no effect on the subsequent conversion (Vieitez 1995, 1999).

4.2 Pre-germination treatments

In most embryogenic systems developed for European chestnut, direct transfer of somatic embryos from maturation to germination medium results in poor germination and abnormal development of plantlets. This has led to the need for the application of pre-germination treatments (prior to transfer of embryos to the germination medium) such as cold storage, drying and addition of gibberellic acid (GA_3). The aim of such treatments is to break the dormancy imposed by ABA or by osmotic agents, to stimulate germination and to synchronize root and shoot development.

Chilling has been associated with an increase in GA_3 and with a reduction in endogenous ABA (Corredoira et al. 2014). Of the known reports concerning SE in chestnut, only one reported that chilling did not influence the conversion frequency (Sezgin and Dumanoğlu 2014). In the embryogenic systems developed by our research team, inclusion of a period of cold storage considerably improved the plantlet conversion rates and proved essential for some embryogenic lines (Corredoira et al. 2006). For hybrid material, the rate of conversion of cold-treated somatic embryos into plantlets (12-16 weeks at 4ºC) was 29-32% (Vieitez 1995, 1999). For *C. sativa*, and after cold storage for 2 months at 4ºC (Corredoira et al. 2003), the rate of plantlet production was 38.9%, which included both the percentage of embryos directly developing into plants and the percentage of

embryos that only developed shoots that had to be subsequently rooted. These conversion rates were improved when somatic embryos were stored for 2 months at 4ºC in empty plates (unpublished results). The positive effect of cold storage (6-12 weeks at 4ºC) as a post-maturation treatment has also been reported for American chestnut somatic embryos (Andrade and Merkle 2005). Zygotic embryos of chestnut species require cold stratification for optimum germination, and it is, therefore, not surprising that conversion of chestnut somatic embryos may benefit from a similar treatment.

Desiccation is a natural step in seed development and is necessary to initiate the change from embryo maturation to germination. Inclusion of a desiccation step consistently improved the conversion response in almost all European chestnut embryogenic cultures evaluated. The combination of cold storage and desiccation was found to be effective in enhancing conversion in somatic embryos derived from leaves (Corredoira et al. 2003, 2008) and from zygotic embryos (Corredoira et al. 2008). Two or three weeks of slow desiccation in sealed empty plates yielded a slight reduction in water content that nevertheless increased the total potential plant recovery, shoot length and the number of leaves per plantlet. However, the best results were achieved by rapid drying (2 hours) in a laminar flow hood, which reduced the embryo moisture content to 57-58% and enhanced the potential plant recovery and quality of regenerated plantlets (Corredoira et al. 2008). Similarly, plantlet regeneration of somatic embryos initiated from zygotic embryos of *C. sativa* was stimulated by desiccation in a Nalgene® desiccator for 4 days (Sezgin and Dumanoğlu 2014). However, these authors did not observe any significant effect on the germination of somatic embryos when a combination of desiccation and cold storage and/or GA_3 was used.

4.3 Germination treatments

After the maturation and pre-germination treatments, chestnut somatic embryos are usually germinated on the same mineral medium used for somatic embryo proliferation. Addition to the germination medium of a low concentration of BA or Z, with or without IBA or NAA, has been used to promote germination and plantlet conversion. Thus, somatic embryos from hybrid material were germinated on MS medium containing 0.92 μM Z (Vieitez 1995). In another study, we also observed that incorporation of PGRs in the germination medium affected the conversion ability of somatic embryos derived from zygotic embryos of *C. sativa* and *C. sativa* x *C. crenata* (Corredoira et al. 2008). The best results (highest percentage of plantlet conversion and percentage of embryos forming only shoots) were obtained with treatments including 0.44 μM BA with or without auxin (0.54 μM NAA or 0.49 μM IBA), although shoot length, root length and leaf number were enhanced in both PGR-free medium and BA plus IBA supplemented medium.

Likewise, Sezgin and Dumanoğlu (2014) obtained the highest conversion rates with germination medium supplemented with 0.44 μM BA and 0.54 μM NAA.On the other hand, plant yield was also promoted by the addition to the germination medium containing 0.92 μM Z plus 150 μM Fe-Na-EDTA (Vieitez 1995) or 0.44 μM BA plus 200-438 mg/l of glutamine (Corredoira et al. 2008).

As an alternative method of improving the overall plantlet production, only shoots derived from germinating somatic embryos were used to establish axillary shoot culture lines, which were then multiplied by axillary branching and rooted following the micropropagation techniques previously developed for chestnut multiplication (Vieitez et al. 2007). Although this procedure provides an opportunity to obtain an unlimited number of European chestnut plants, efforts should obviously be made to increase plantlet conversion from somatic embryos.

As noted in previous sections, the rate of conversion of chestnut somatic embryos into plantlets greatly depends on the genotype (Vieitez 1995, 1999, Sauer and Wilhelm 2005, Corredoira et al. 2008). Therefore, further efforts should be made to optimize maturation and germination protocols, for application to a wide range of genotypes.

5. Genetic transformation

One of the greatest advances made in plant breeding has been the genetic modification through genetic transformation by insertion of specific genes into the plant genome. As mentioned above, a fundamental premise for success in the production of transgenic plants is the availability of an in vitro regeneration system that can support the production of plants from cells, organs or tissues susceptible to infection by *Agrobacterium tumefaciens*.

Our research group developed the first protocols for the production of transgenic somatic embryos in European chestnut and for establishment of plants in the greenhouse prior to testing the stability of the inserted genes (Corredoira et al. 2004a). As part of this research, we evaluated the effect of a bacterial strain/plasmid combination and the length of co-cultivation period (3 or 4 days). Somatic embryos were initiated from zygotic embryos and leaf explants obtained from shoot multiplication cultures of *C. sativa* were used as target material. Initially, we used only marker genes such as neomycin phosphotransferase (*nptII*) and β-glucuronidase (*uidA*) genes, which enabled selection of the transgene in media containing kanamycin and identification of transformed embryos by the GUS reporter system (Figure 2A). Culture of the initial 624 explants for 12 weeks on selective medium yielded 112 resistant embryogenic explants. Control cultures of somatic embryo clumps on selective medium in the absence of *Agrobacterium* did not grow and finally died. The combination of strain/plasmid and the period of

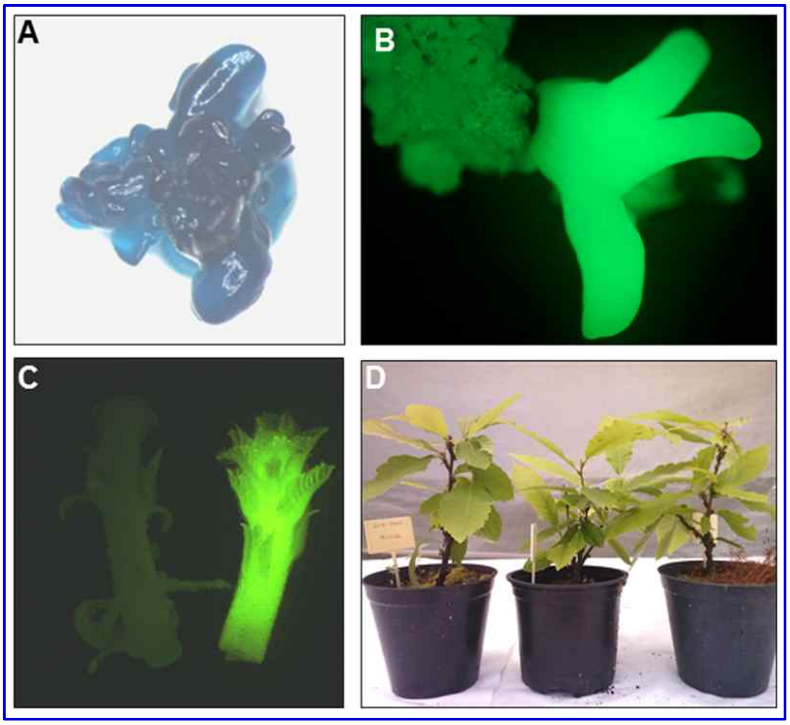

Figure 2. *Genetic transformation of chestnut somatic embryos.* ***A*** *Histochemical GUS analysis of transgenic somatic embryos obtained with EHA105pUbiGUSINT.* ***B*** *GFP expression in a cotyledonary embryo obtained with EHA105pK7WG2D-CsTL1.* ***C*** *GFP expression on the apex of a transgenic plant (right) and an untransformed plant (left) visualized with an epi-fluorescence stereomicroscope. D Transgenic plants obtained with EHA105pK7WG2D-CsTL1after one year of acclimatization in the greenhouse.*

co-culture clearly influenced the number of Kan-resistant explants and also the transformation efficiency. Transformation was defined as the percentage of initial explants that developed GUS-positive embryogenic cultures. The highest transformation efficiency (25%) was achieved after 4 days of co-culture with strain EHA105 and plasmid pUbiGUSINT. Subsequent experiments were therefore carried out using this strain/plasmid combination and 4 days of co-culture. The selection efficiency (defined as the percentage of kanamycin-resistant explants producing embryos that developed GUS-positive embryogenic cultures) was relatively high in all treatments assayed and ranged from 55 to 100%.

For optimization of a genetic transformation protocol with marker genes, factors such as the explant type, wounding, selective agent and temperature of co-cultivation were evaluated (Corredoira et al. 2005, 2007). Explant type had an important influence on the transformation efficiency; isolated globular embryos and embryo clumps consisting of 2-3 somatic embryos at globular and early-

torpedo stage (30% in both explants types) yielded a significantly higher transformation response than obtained with cotyledonary embryos (6.7%). The low transformation frequency of cotyledonary explants may be due to the lower proliferation capacity of this type of explant (3 SE per explant) than that of embryo clumps and globular embryos (9.2 and 9.0 SE per explant, respectively). The transformation efficiency was clearly genotype dependent, as two of the seven lines evaluated yielded higher transformation frequencies (21.7 and 33.8%) than the other five lines (3.3, 5.0, 1.7, 1.7 and 10%). Nevertheless, neither changing the temperature of the co-cultivation period, nor bacterial growth phase, nor wounding the embryos improved the transformation efficiency. Inclusion of the antibiotic kanamycin in the selection medium was more effective than inclusion of paramomycin. As a result of this intensive research, a total of 288 embryogenic lines with marker genes were obtained (Corredoira et al. 2004a, 2005, 2007).

Specific genes for resistance to ink and blight diseases have not yet been identified; however, it is suspected that ink disease resistance is conferred by a single gene in some populations while blight resistance is conferred by more than one gene (Nelson et al. 2014). An alternative to obtain resistant trees would be the over-expression of genes that codify pathogenesis-related (PR) proteins, which play a major role in natural defence against pests and pathogens. Among these, the PR-5 family of proteins (thaumatin-like proteins) are generally of low molecular weight (below 35 kDa) and cause transmembrane pores on fungal plasma membranes promoting osmotic rupture (Roberts and Selitrennikoff 1990). Chitinases, belonging to the PR-3 family of proteins, hydrolyze the β-1, 4 glycosidic bonds that link the *N*-acetylglucosamine residues of chitin and play a direct role in plant defense by hydrolyzing chitin (Veluthakkal and Dasgupta 2010). Hence, we are using a thaumatin-like protein gene (*CsTL1*) and a chitinase-like protein gene (*CsCh3*) to induce resistance to ink disease and blight disease, respectively. As these genes were isolated from European chestnut seeds (Collada et al. 1992, García-Casado et al. 2000), we are transforming chestnut somatic embryos with native genes.

For transformation with these genes, explants consisting of small clumps of 2 to 3 chestnut somatic embryos at globular or early-torpedo stages were co-cultured for 5 days with *Agrobacterium tumefaciens* strain EHA105 harbouring pK7WG2D-CsCh3 or pK7WG2D-CsTL1 binary vectors. Both plasmids also contain the *nptII* as a selective gene and the green fluorescent protein (*egfp*) as a reporter gene. The fluorescent protein simplified and improved the evaluation of transformation events relative to the GUS assay used in previous protocols in chestnut transformation. Selection of whole fluorescent embryos facilitates the proliferation of transgenic embryos (Figure 2B), limiting subculture of escape tissues. As in the transformation experiments with marker genes, the transformation efficiency, determined on the basis of the fluorescence of surviving

explants, was clearly genotype-dependent. The rates of transformation of the *CsTL1* gene ranged from 32.5% to 7.1% (Corredoira et al. 2012b), whereas for the *CsCh3* gene, the values ranged from 20% to 4.2% (unpublished results). A total of 126 and 175 independent transformed lines were obtained with the thaumatin and chitinase genes, respectively. The selection efficiency, defined as the percentage of GFP-positive kan-resistant explants, was higher (80-100%) in all experiments. This indicates that double selection with kanamycin and GFP was stringent, thus minimizing escapes and avoiding loss of samples, as when the GUS histochemical assay is applied. The presence and integration of chestnut *CsTL1* and *CsCh3* genes in genomic DNA was confirmed by PCR and Southern blot analyses. The qRT-PCR analysis revealed that *CsTL1* expression was up to 13.5 times higher in transgenic lines than in the corresponding untransformed line. Finally, chestnut chitinase and thaumatin-like protein genes were simultaneously introduced into chestnut somatic embryos of two embryogenic lines (Corredoira et al. 2015b). The transformation frequency was 1% in line CI-3 and 33.3% in line CI-9, and a total of 34 independent transformed lines were obtained.

The transgenic embryogenic cultures obtained in different transformation experiments, either with marker genes or with genes of interest, were routinely maintained by secondary embryogenesis with sequential subculture at 6-week intervals according to the previously defined conditions (Corredoira et al. 2003). Transformed plantlets were obtained after the transgenic somatic embryos were subjected to maturation and germination treatments following previously reported methods (Vieitez 1995, Corredoira et al. 2003, 2008); however, the plantlet regeneration frequencies were low. As mentioned above, to increase the conversion rates, plantlets derived from germinating somatic embryos of transgenic lines were used to establish axillary shoot culture lines that were multiplied by axillary branching. The presence of trans-genes in leaves of plants obtained after germination of transformed somatic embryos was confirmed by GFP expression (Figure 2C), PCR and Southern blot analysis (Corredoira et al. 2004a, 2007, 2012b). The transgenic plants obtained by germination of somatic embryos with higher levels of *CsTL1* gene expression are growing in the greenhouse (Figure 1D) and their resistance to fungi is being evaluated.

The regenerated chestnut plants are not transgenic (we are over-expressing native genes and foreign genes are not introduced), and the new term “cisgenic” plants seems more appropriate (Joshi et al. 2011, Vanblaere et al. 2011). This is an important difference fromthe research carried out by the American groups: American chestnut plantlets are engineered with a candidate anti-fungal oxalate oxidase (*OxO*) gene cloned from wheat (Newhouse et al. 2014) and laccase-like gene cloned from *C. mollissima* (Nelson et al. 2014). Our interest in the production of cisgenic plants is linked to the regulatory systems designed to manage the risks associated with genetically modified organisms and to respond to public concern in

this regard. However, at present, the same regulatory systems are used for both cisgenic and transgenic plants.

The experience gained in European chestnut during recent years has also been applied to oak species. Although oaks are also affected by fungal attack (e.g. *Phytophthora cinnamomi, P. ramorum* or *P. querciana*), the diseases caused are not as severe as in chestnuts. Chestnuts and oaks belong to the same family and we therefore expect similar responses regarding application of biotechnological tools. We also have wide experience in carrying out in vitro tissue culture of oak species including *Q. robur*, *Q. rubra*, *Q. alba* and *Q. bicolor*, and we have successfully induced somatic embryos in different explants (leaves, shoot apex) obtained from axillary shoot cultures (Vieitez et al. 2012, Corredoira et al. 2014). We have also successfully carried out *Agrobacterium*-mediated genetic transformation of somatic embryos from *Q. robur*, and transgenic plants including the thaumatin gene are currently being grown in the greenhouse (Mallón et al. 2014).

6. Cryopreservation

Cryopreservation of zygotic embryos or the embryonic axes is one way of preserving the genetic diversity of species with recalcitrant seeds, which cannot be stored for long periods. Chestnut seeds, which belong to this group, can only be stored for 30-40 months at temperatures of between -2°C and 5°C (they can germinate at 2°C) under stratification conditions (Vieitez et al. 1996). In addition, long-term maintenance of embryogenic lines obtained from selected individuals by subculture involves the concomitant risk of contamination, somaclonal variation and possible loss of embryogenic capability (Corredoira et al. 2014). Cryopreservation or freeze-preservation at ultra-low temperature (-196°C, i.e. the boiling point of liquid nitrogen (LN)) is a good option for the long-term conservation of plant genetic resources, as under such conditions, biochemical and most physical processes are completely arrested (Panis and Lambardi 2005). Cryopreservation may also be a reliable option for facilitating management of transgenic embryogenic lines, limiting the risk of contamination and may also reduce labour and supply costs. Both untransformed and transformed plant material can be stored indefinitely.

Early attempts to cryopreserve chestnut zygotic embryos were reported by Pence (1990, 1992). Although embryonic axes survived after LN immersion, the embryos did not develop sufficiently to regenerate plants. The potential of embryonic axes in the long-term cryopreservation of European chestnut was outlined in the study of Corredoira et al. (2004b). Zygotic embryonic axes of European chestnut have been cryopreserved using a simple desiccation-based protocol. Prior to storage in LN, embryonic axes were desiccated under sterile conditions in a laminar airflow bench for different periods of time (1-7 h). Between

93% and 100% of excised embryonic axes of chestnut seeds survived storage in liquid nitrogen (LN) following desiccation to moisture contents of 20-24% (on a fresh weight basis), and 63% of these axes subsequently developed into whole plants.

Cryopreservation of European chestnut somatic embryogenic cultures has been successfully achieved after desiccation or application of PVS-based vitrification procedures, the latter of which was most successful (Corredoira et al. 2004b, Vieitez et al. 2011). Vitrification is defined as a physical process whereby a concentrated aqueous solution solidifies into a stable amorphous glass without the formation of ice crystals as the temperature decreases (Sakai 2004). The formation of ice in cells is prevented by the use of highly concentrated cryoprotectants that increase the viscosity in target cells, to a point where formation of glass is induced and crystallization of water is bypassed (Pegg 2010). One of the most common cryoprotectant solutions used is plant vitrification solution nº 2 (PVS2), which comprises 30% w/v glycerol, 15% w/v DMSO and 15% w/v ethylene glycol in MS medium containing 0.4 M sucrose (Sakai et al. 1990). In European chestnut, embryogenesis resumption rates of 68% were achieved by preculture of embryo clumps at globular or heart-stage for 3 days on solid medium containing 0.3 M sucrose, incubation in PVS2 vitrification solution for 60 min at 0°C, and direct immersion in liquid nitrogen. This procedure was successfully used in the cryopreservation of chestnut embryogenic lines transformed with marker genes (Corredoira et al. 2007), a thaumatin-like protein gene (Corredoira et al. 2012b), and a chitinase gene (unpublished results). The embryogenic resumption rates of three lines transformed with marker genes, ranging from 52 to 65%, were found to be similar to those achieved with cryopreserved somatic embryos derived from the wild-type line (66%) (Corredoira et al. 2007). Following a similar procedure, embryo recovery rates of between 56 and 84% were achieved for cryopreservation of somatic embryos isolated from embryogenic lines obtained after transformation with the thaumatin-like protein gene (Corredoira et al. 2012b). Cryopreservation of transgenic embryogenic lines enables long-term storage of transgenic lines while the same, non-cryopreserved, lines are evaluated for disease resistance.

As the result of research carried out in recent years, a cryopreservation bank has been established for European chestnut by application of a simple vitrification protocol to embryogenic lines derived from zygotic embryos, leaf explants and apex explants and to embryogenic lines established during different transformation experiments.

7. Conclusion and future prospects

Somatic embryogenesis is very important for improvement of European chestnut, including its use for mass propagation, genetic engineering and

germplasm conservation. Although European chestnut is a recalcitrant species, significant progress has been made regarding its propagation by somatic embryogenesis since the first report (Vieitez et al. 1990). Induction of SE in explant tissues other than zygotic embryos has been achieved in recent years, unlike in American chestnut. However, the low rates of somatic embryo initiation and plantlet conversion remain problematical. Additional research is needed to refine the process of somatic embryo initiation, which will enable somatic embryos to be obtained from other genotypes. Establishment of embryogenic cultures from explants derived from mature trees is one of the major research goals that should be achieved in the near future. It is necessary to optimize the mineral and PGRs composition of the induction and expression media, but above all a careful selection of the initial explant is needed. We must increase efforts to study the physiological condition/development of the initial explant to determine which is the most suitable state to obtain a good embryogenic response in a majority of genotypes. Information obtained from molecular and anatomical studies that identify the "stem cells" that control the process of acquisition of embryogenic competence will be very useful for advancing chestnut somatic embryogenesis.

Combining genetic studies with somatic embryogenesis and genetic transformation is probably the most appropriate way to accelerate the improvement processes in this species.Considerable progress has been made in transforming somatic embryos of European chestnut and producing transgenic plants. Although specific genes involved in chestnut resistance to ink and blight diseases are not yet identified, an integrated web-based resource for members of the *Fagaceae* family, including *Castanea* (http://www.fagaceae.org), disseminates genomic data which are being posted as they become available and thousands of genes have been isolated. It is expected that in the coming years genes directly related to chestnut diseases would be identified to facilitate the production of resistant trees.

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Multi-varietal forestry integrating genomic selection and somatic embryogenesis

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Abstract

Multi-Varietal Forestry (MVF) is defined as the deployment of a range of genetically tested tree varieties in commercial plantation forestry. Somatic embryogenesis (SE) and cryopreservation are the enabling technologies for the implementation of MVF. Recently, it has been shown that genomic selection (GS) has a great potential to be incorporated with MVF. MVF is well suited for intensively managed, high-productivity sites. MVF offers a much greater genetic gain than conventional tree breeding because it captures both additive and non-additive variations. Furthermore, MVF integrated with forward GS and SE eliminate the time required for producing seeds and, thus, gain per unit time is notably increased. In white spruce breeding in eastern Canada, for example, the gain is delivered 15 years sooner than by conventional seed orchard breeding. Moreover, GS will make the testing and selection efforts more efficient and streamlined through pre-screening. Sufficiently refined and efficient SE protocols for commercial MVF are available for a number of conifers, primarily the spruces, some pines, and a few larches, but more refinements are required for several economically important conifers. The main challenge for implementing industrial MVF, however, is the relatively high cost of SE seedling production due to manual handling of embryos, both pre- and post-germination. In order to be cost effective, it requires the development of a mechanized embryo handling system for transplanting into mini-plugs for greenhouse culture, which is under development. However, with the current lack of an automated transplanting system, complementary serial rooting of cuttings may be used as a mass propagation tool

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once the suitable varieties are developed from the SE-based system. In addition to obtaining a large genetic gain at a significantly reduced time, MVF offers flexibility to adapt to changing breeding goals, environment, disease and insect conditions, and this flexibility is provided by up-to-date information obtained from varietal field testing. Furthermore, in MVF, plantation diversity is dynamically managed over time by carefully balancing genetic gain and diversity based on the best available varietal field test data.

Keywords: Cryopreservation, deployment strategy, varietal field testing

1. Introduction

Somatic Embryogenesis (SE) is an important biotechnology in conifers for the development and production of tree varieties with desirable traits. The plant production by the SE process in conifers involves the initiation, proliferation, maturation, germination, and greenhouse culture steps with varying degrees of success at each step. Nonetheless, the SE system for several conifers has advanced to the stage where it can be implemented in a large-scale commercial production.

SE is not a tree breeding method, but it is a required biotechnology aiming at the development of tree varieties and their deployment in conjunction with tree breeding. The most important advantage of conifer production by SE is that the embryogenic cell lines can be cryogenically stored in a juvenile state indefinitely, which was not possible with other vegetative propagation techniques for trees. This allows for the long-term field testing and for the subsequent selection and retrieval of tested cell lines for mass propagation. This led to the operational implementation of Multi-Varietal Forestry (MVF) in eastern Canada by JD Irving Limited and the Quebec Ministry of Forests, Wildlife, and Parks.

The purpose of this paper is to review the successes and issues of implementing SE and how novel biotechnologies such as genomic selection can be integrated with SE into modern tree breeding systems.

2. Somatic embryogenesis

Since the first successful report of SE in conifers (Nagamani and Bonga 1985, Hakmann et al. 1985, Chalupa 1985), rapid progress has been made, particularly for most spruce species and some pine species. In these species, SE is initiated efficiently from immature zygotic embryos. In spruce species, SE can be obtained from mature zygotic embryos, but, in pines, SE from mature seed has met limited success. Several media formulations were successfully used including mLV (Litvay et al. 1985), DCR (Gupta and Durzan 1985), MSG (Becwar et al. 1990),

and these formula typically contain auxin and cytokinin, such as 2,4-D and BAP. The proliferation of embryogenic tissue is usually accomplished either on solid or in liquid medium of the same formulation. The initiation of SE in many conifers is influenced by additive genetic variation offering a possibility of breeding for increased SE initiation (Park 2002).

Maturation of somatic embryos is achieved by removing auxin and cytokinin and supplying ABA. In addition to the use of ABA, it was discovered that a critical factor for developing a large numbers of somatic embryos was the restriction of water availability either by physical, or osmotic, or both means. The most commonly used methods are the use of high molecular weight PEG and increased gel strength. The quality of mature somatic embryo is very important as it affects germination rates and somatic seedling quality. This is the most important but challenging step, because maturation success is widely variable from total recalcitrance to abundance.

Germination of somatic embryos is usually carried out on a semi-solid medium without growth regulators. Normal germination and zygotic-like development are common provided that mature somatic embryos are well formed and vigorous. This is the step linking the automated transplanting and greenhouse culture. With a lack of an automated system, the current transplanting process is a manual process, consequently time consuming and expensive. Thus, in order to be cost-effective, the development of a mechanized somatic seedling transplant system, or direct germination of somatic embryos into micro-plugs, or their incorporation into artificial seed is highly desirable. Despite these challenges, SE of many conifers, most of spruce and some pine species, is sufficiently refined to the point that it can be used in industrial production.

3. Cryopreservation

Cryopreservation is the key element of conifer SE programs that makes long-term storage of embryogenic tissue at an ultra-low temperature possible while lengthy field testing of cell line is being carried out. For most species, cryopreservation is a routine with an excellent recovery rate, using rather simple procedures. The current protocol entails incubating EM with sorbitol in liquid maintenance medium. Then, the cooled cell suspension, with added DMSO, is dispensed into cryo-vials, which are placed in an alcohol-insulated freezing container (Nalgene®). The freezing containers are pre-cooled and placed at -80 ºC for 1-2 hours, where slow cooling takes place. Subsequently the vials are immersed into liquid nitrogen (-140 ºC to -196 ºC). The recovery of EM involves a rapid thawing in water at 37 C for 1-2 minutes, then the EM suspension is poured over a filter-paper disk allowing the drainage of storage solutions, and placing of the disk with EM onto the semi-solid proliferation medium for regrowth.

The genetic stability of cryopreserved cell lines has been studied in various species (Cyr et al. 1994; DeVerno et al. 1999; Sutton and Plonenko 1999), showing no evidence of somaclonal variation. Harvengt et al. (2001) found no allelic difference, nor abnormal growth behavior, among *Picea abies* plants raised from somatic embryos obtained from up to 3-year-old plants and their ortets. However, a high mutation rate was detected during the *in vitro* phase. Nonetheless, owing to an effective selection for normally formed somatic embryos, the resulting plantlets were all normal. Cryopreservation of conifer cell lines is already used commercially (Cyr 1999).

Given the success of cryogenic storage for conifers, the production of identical genotypes consistently over time without somaclonal variation or loss of juvenility is now possible, which is analogous to the development of agronomic and horticultural varieties. Somatic embryogenesis in combination with cryopreservation offers the means for forward selection and mass producing tree varieties after the varietal field testing of an appropriate length has determined which cell lines have the desirable attributes. The development of tree varieties in conifers was not possible previously.

4. Conventional tree breeding

Conventional tree breeding typically employs a form of recurrent selection, and the production of genetically improved material is accomplished by wind pollinated seed orchards (White 1987). This procedure, for each generation, involves the formation of multiple breeding populations, controlled pollinations among parents within the breeding population, establishing, maintaining, and evaluating the progeny test at multiple sites, and the establishment of clonal seed orchards for the production improved seed, while the selected parents form a new breeding population for the next cycle of breeding. Therefore, tree breeding programs require extensive resources and an extended period of time. Also, the establishment of land-based seed orchards is expensive and remains fixed and inflexible until the establishment of the new next generation orchards. However, these orchards will deliver substantially increased productivity.

A typical breeding cycle using a subset of a breeding population for white spruce (*Picea glauca*) is illustrated in Figure 1 and takes about 15 years to complete. This is primarily due to time required to attain the flowering maturity needed to allow breeding. The time can be shortened by the use of stimulants but this has limitations. This seed orchard-based tree breeding scheme typically produces about 10% volume increase per generation (Fullarton 2015).

The most commonly used conventional genetic evaluation is based on the mixed linear model using pedigree information:

$$\mathbf{y} = \boldsymbol{Xb} + \boldsymbol{Zu} + \boldsymbol{e} \qquad (1)$$

where **y** is the vector of observed phenotype (trait); *X* and *Z* are known design matrices of fixed and random effects, respectively; ***b*** is an unknown parameter of

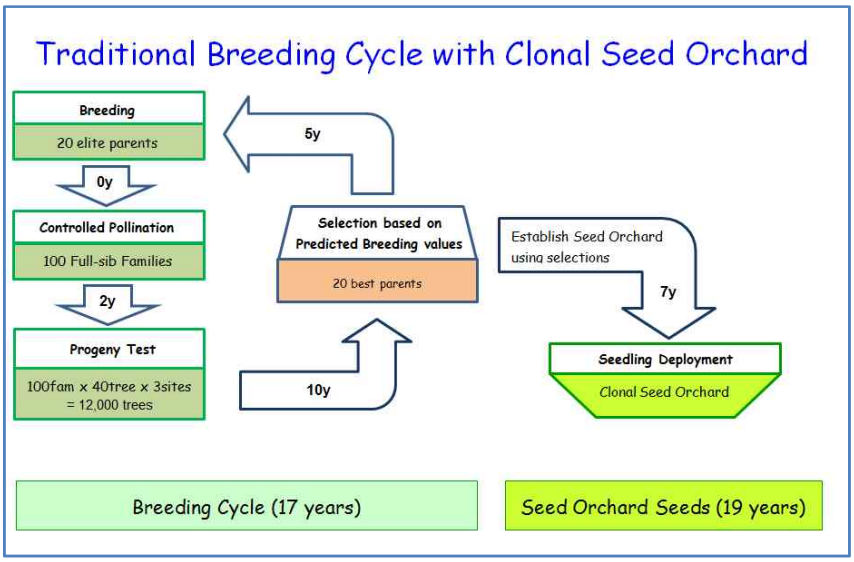

Figure 1. *Schematic presentation of white spruce breeding in New Brunswick, Canada: A white spruce breeding population is divided into 20-tree sub-populations and this example uses an elite sub-population thereof. Controlled-pollinations are performed within this elite sub-population to produce full-sib families (typically 100 families) that are planted in the field tests at multiple locations. When the test is about 10 years old, growth is assessed and the 20 best parents are selected to form the next generation breeding population. Since the flowering maturity for white spruce is about 15 years, it takes 17 years to begin the next generation breeding cycle. The genetic gain from this breeding cycle is delivered through the establishment of a clonal seed orchard using grafts of selected parents. Due to flowering constraints it takes 19 years after the beginning of the breeding cycle before appreciable amounts of seeds are produced.*

fixed values to be estimated; and ***u*** and ***e*** are vectors of breeding values (random) to be predicted and residuals, respectively, such that E(***u***)= E(***e***)=0, Var (***u***)=$\boldsymbol{A}\sigma^2_A$ and Var (***e***)= $\boldsymbol{I}\sigma^2_e$, where ***A*** is the numerator relationship matrix based on genes identical by descent, σ^2_A is additive genetic variance and σ^2_e error variance. The Best Linear Unbiased Prediction of ***u***, known as A-BLUP, is obtained by replacing the variance-covariance matrix of the individual trees in the mixed linear model by the ***A*** matrix (Henderson 1975). These breeding values are the genetic merits of the individuals, and thus the highest values should be used for selection. Alternatively,

the breeding values (BV) of an individual *i* in a population based on the narrow-sense heritability (h^2) is defined as:

$$\mathbf{BV_i = m_0 + h^2 (y_i - m_0) = m_0 + V_A/V_P (y_i - m_0)} \quad (2)$$

where y_i is the phenotypic value of individual *i*, m_0 is the population mean. The estimated narrow-sense heritability (h^2) is computed as the ratio of the estimates of additive variance (V_A) to total phenotypic variance (V_P) from the analysis model. The BV predicted in this manner is referred to as estimated BV (EBV), while the BV predicted by the genome-wide markers will be referred to genomic EBV (GEBV) (see below).

5. Molecular markers, marker-aided selection, and genomic selection

In the past 20 years, there has been a rapid development in marker technologies, and the availability of inexpensive molecular markers offers a possibility of using them to improve the efficiency of tree breeding. Various classes of DNA markers, such as Simple Sequence Repeats (microsatellites), Single Nucleotide Polymorphisms (SNP), Diversity Arrays Technology (DArT), Genotyping-By-Sequencing (GBS), and Restriction site associated DNA (RAD) have been developed for commercially important species such as spruces and pines (Pavy et al. 2013a; Liu et al. 2014; Neves et al. 2014), eucalypts (Sansaloni et al. 2010; Silva-Junior et al. 2015), and poplars (Schilling et al. 2014), among others. High-throughput genotyping technologies were also developed by companies such as Sequenom Inc. (San Diego, Ca, USA), Illumina Inc. (San Diego, Ca, USA) and Affymetrix (Santa Clara, Ca, USA). Thus, depending on availability of markers for a given species, a large number of individuals can be genotyped for a few dozen of DNA markers to many thousands of them. For species like eucalypts, a flexible multi-species genome-wide 60K SNP genotyping chip is available (Silva-Junior et al. 2015) for any genotyping purpose, while for other species, custom DNA chips must still be designed and built for specific needs using the DNA marker information that is available on public domain databases (Pavy et al. 2013b; Pavy et al. 2015).

Application of DNA marker technology in breeding covers two main areas: population management and selection. A wise use of molecular markers in the context of population management is in the pedigree reconstruction proposed by El Kassaby and Listiburek (2009), where they could reconstruct a full pedigree from the open-pollinated seed of a lodgepole pine seed orchard through genotyping using DNA microsatellite markers. When such pedigree reconstruction is implemented in breeding populations, it will circumvent the expensive controlled pollination step and the resulting pedigree can more inclusively of all available

cross combinations. The genetic evaluation of the progeny from the reconstructed pedigree can be carried out in the usual manner.

The use of molecular markers for selection in breeding was initially focused on marker-aided selection (MAS). The mapping of quantitative trait loci (QTL) and candidate gene association approaches have been explored to relate gene architecture and trait expressions, i.e., based on the presumption that causative mutations underlying genetic variation can be localized with DNA markers. The concept of MAS entails that if the QTL associated to a given trait is identified with corresponding molecular markers, they could be used to select superior genotypes in the breeding population. The general process of MAS consists of two phases, training and selection phases. In the training phase, phenotypes in the mapping population are investigated to identify significant associations of a phenotype with marker genotypes using statistical procedures and identify MAS markers for use in the selection phase. In the selection phase, genotyping is necessary for the targeted region of the quantitative trait of interest to screen for MAS markers and selection. However, QTL mapping and candidate gene association approaches in forest trees have not been used widely, primarily due to the fact that the most important traits are controlled by many QTLs, each with only a small effect, and because only a limited portion of the existing variation in a given trait can be explained by the several associations or QTLs detected (Beaulieu et al. 2011, Pelgas et al. 2011).

Genomic selection (GS) or genome-wide selection is a form of MAS; however, it is distinctly different from the traditional MAS based on QTLs. Indeed, GS aims to trace all the QTLs controlling an individual's phenotype and simultaneously estimate all marker effects across the entire genome to calculate its genomic estimated breeding value (GEBV). If the marker coverage is sufficiently dense, all the QTLs controlling the phenotype should theoretically be in linkage disequilibrium (LD) with at least one marker, and unlike the QTL-based MAS, prior information on the association between the phenotype and markers, and on the effects of QTLs is not necessary. However, GS also consists of two phases. A model to predict GEBV is first developed with a training population using both genotypic and phenotypic data. In the ensuing selection phase, only genome-wide genotypic data are needed to obtain GEBVs using the prediction model developed in the training phase. The selection is then based on the GEBVs. The stages of genomic selection are illustrated in Figure 2.

Various statistical methods have been developed for GS and they can be classified in two main groups (de los Campos et al. 2013). The first is based on the idea of Meuwissen et al. (2001) that it is possible to predict the genetic value of individuals by regressing phenotypes on all available markers using a regression model. However, because the number of available markers generally exceeds the number of individuals of the training population, variable selection or shrinkage

estimation procedures are required. Since then, several shrinkage estimation methods, using Bayesian estimation procedures, have been proposed to address this issue, such as ridge regression (RR) (Hoerl and Kennard 1970) and the least absolute angle and selection operator (LASSO) (Tibshirani 1996). The second group uses genomic relationships derived from markers in a mixed model framework to predict the genomic breeding values of individuals. Thus, contrary to the methods of the first group, the effects of individual markers are not estimated,

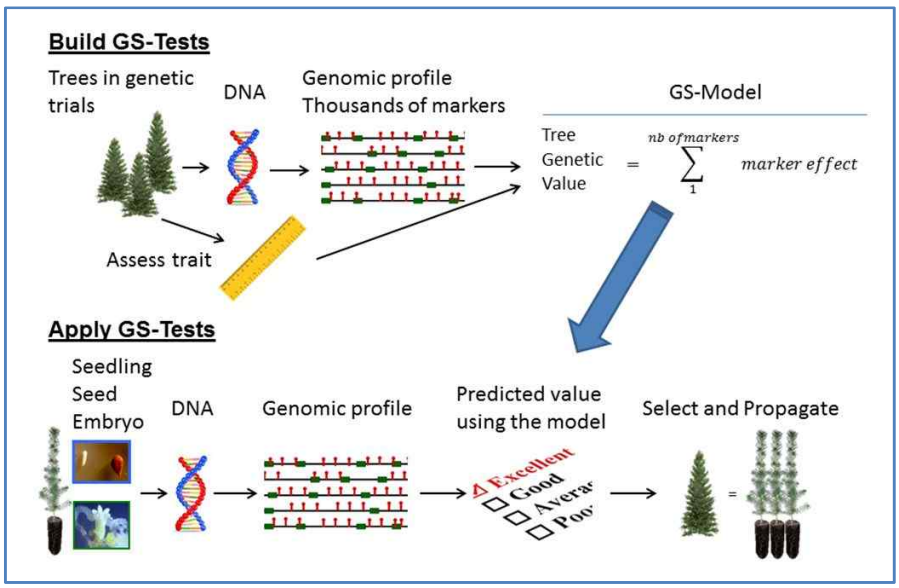

Figure 2. *The process of genomic selection and application in tree breeding: Genomic selection (GS) involves two phases, the development of a GS model in the training population and the application of a validated GS model to the breeding population. In forestry, training populations can be a well-established existing genetic test plantation or can be taken from a selection plantation. In the training population, both phenotyping (traits) and genotyping (e.g., SNP makers) are required to build a GS model. In the GS model, phenotype is typically considered as the sum of all marker effects and is validated by using a subset of the training population. The breeding population (BP) is an offspring population of the training population and the selection is to be made from this BP. In the BP, only genotype data are required to calculate the genomic estimated breeding value (GEBV) using the GS model developed from the training population. The best GEBV individuals are selected to form the next generation breeding population and are used to establish a seed orchard; however, in "Forward GS", the selections are mass propagated using SE or rooting of cuttings for immediate deployment. Thus, vegetative propagation techniques such as SE and/or rooting of cuttings are required to mass produce selections without sexual recombination.*

although they can be obtained with extra calculation. This method is usually referred to as Genomic Best Linear Unbiased Prediction or simply G-BLUP, and can be used in the context of an additive infinitesimal model in which the standard pedigree-based numerator relationship matrix is replaced with a marker-based estimate of additive relationships (Van Raden 2008, de los Campos et al. 2013).

In multiple-marker regression, many markers are simultaneously estimated as random effects in an individual tree model:

$$\mathbf{y} = \boldsymbol{Xb} + \boldsymbol{Za} + \boldsymbol{e} \qquad (3)$$

y is the vector of observed phenotype (trait); *X* and *Z* are known design matrices of fixed and random effects, respectively; $\boldsymbol{b}$ is an unknown parameter of fixed values to be estimated (including an overall mean and population structure); and $\boldsymbol{a}$ is a vector of random marker effects with the *n* x *m* incidence matrix containing marker covariates coded as Z_{ik} = (0, 1, 2) so that the sum of marker effects approximates the individual (additive) genomic estimated breeding value (GEBV) $\hat{g}_i = \sum_{k=1}^{m} z_k \, a_k$, and e_i is the vector of residual error effects. It is assumed that E($\boldsymbol{u}$)=0, E($\boldsymbol{e}$), and that m follows a normal distribution (~ N (0, $I\sigma_a^2$)), and *I* is an identity matrix. Such a model with a normal distribution of marker effects is often called ridge regression best linear unbiased prediction or RR-BLUP (Meuwissen et al. 2001, Van Raden 2008). Under a Bayesian approach, all SNP effects are assumed to have a common variance by assigning a Gaussian prior as $a_{RR,k} \sim N(0, \sigma_a^2)$, and all markers are shrunk to the same extent toward the mean and the degree of shrinkage is controlled by the prior variance. This method appeared most appropriate when a quantitative trait is controlled by many QTLs, each with a small effect. Several GS studies following this approach have been recently published for conifers (Beaulieu et al. 2014a,b, Resende et al. 2012a,b, Zapata-Valenzuela et al. 2012). The LASSO is an alternate shrinkage method that minimizes the residual sum of squares constraining the sum of absolute values of the regression coefficients if the predictors are standardized. Some estimated regression coefficients can be zero, contrary to ridge regression.

The genomic-estimated breeding value (G-BLUP) can be obtained by using the same mixed linear model that is used to obtain A-BLUP, and by replacing the numerator relationship matrix ($\boldsymbol{A}$) with the realized genomic relationship matrix ($\boldsymbol{G}$) derived from the markers (Van Raden 2008, Legarra et al. 2009, Zapata-Valezuela et al. 2013). The $\boldsymbol{G}$ matrix can be computed as:

$$\boldsymbol{G} = \frac{\boldsymbol{ZZ'}}{2\sum_{j=1}^{m} \boldsymbol{p_i}(\boldsymbol{1}-\boldsymbol{p_i})} \qquad (4)$$

where $\boldsymbol{Z} = \boldsymbol{M} - \boldsymbol{P}$, $\boldsymbol{M}$ is a matrix which elements are set to 1, 0 and -1, i.e. the number of minor alleles minus 1, and $\boldsymbol{P}$ is a matrix that contains allele frequencies

as $\boldsymbol{P_i = 2(p_i - 0.5)}$ where $\boldsymbol{p_i}$ is the minor allele frequency of the marker i. The denominator of the formula scales the $\boldsymbol{G}$ matrix to be similar to the $\boldsymbol{A}$ matrix (Van Raden 2008). Computation could pose some challenges if GS involves tens of thousands SNPs, but the BLUP computations can be accomplished by using statistical software that fits linear mixed models using Residual Maximum Likelihood (REML) such as ASReml (Gilmour et al. 2009).

Several factors can affect prediction accuracy. Presence of linkage disequilibrium between markers and QTLs controlling the trait of interest (Habier et al. 2007) is of course crucial to maintain a high level of accuracy over the generations. The number of markers used for estimating the GEBVs can also have an important influence (Schaeffer 2006, Poland and Rife 2012). Grattapaglia and Resende (2011) for instance showed that a high accuracy level can be obtained even at a marker density of 2 markers per centiMorgan (cM) when the effective training population size (Ne) is as small as about 30. However, for larger effective population sizes, marker density must be considerably increased to obtain high prediction accuracy (Daetwyler et al. 2008, Jannink et al. 2010). Trait heritability also influences the prediction accuracy (Heffner et al. 2009), but its impact is less important than marker density and the effective population size (Grattapaglia and Resende 2011). The existence of relationships between training and testing sets has also been shown to be essential (Albrecht et al. 2011; Beaulieu et al. 2014, Zapata-Valenzuela et al. 2012) unless the marker density is very high.

6. Multi-varietal forestry (MVF)

Multi-Varietal Forestry (MVF) can be defined as the deployment of a range of genetically tested tree varieties in plantation forestry. It is also known as clonal forestry; however, with advances in conifer SE and cryopreservation, the term MVF is more descriptive when applied to commercial plantation forestry (Park 2004). In general, a clone refers to any genotype with its genetic copies or ramets, whereas a variety refers to a clone that is selected or bred for certain attributes (and has test data to show to what extent these attributes have been achieved). In the past, the MVF concept in conifers was not realistic because of our inability to produce the same genotype over time. With the use of SE and cryopreservation combined with varietal testing, it is now possible to produce the same test-proven genotypes consistently over time, similar to the production of agronomic and horticultural varieties.

For several conifers, particularly for spruce and several pine species, the SE process is sufficiently refined to the stage that it can be implemented in industrial production. In New Brunswick, Canada, MVF is being practiced with spruce species, e.g., *Picea glauca* and *P. abies*, by JD Irving Limited since the late 1990s. A schematic representation of MVF by JD Irving Limited is shown in

Figure 3. Briefly, the MVF process takes the following steps: it begins with controlled crossing of superior parents selected from the breeding population; the resulting seeds are subjected to somatic embryogenesis; once embryogenic cell lines are proliferated, they are cryopreserved; once a number of lines to test is determined, a portion of each line is thawed and propagated; using the plants from the thawed lines, varietal testing is conducted at multiple locations; based on the periodic evaluation of varietal test, superior varietal lines are identified and retrieved from cryogenic storage; a selected number of superior varietal lines are mass vegetatively propagated; and the varietal lines are deployed in commercial plantations using appropriate numbers of varietal line mixtures.

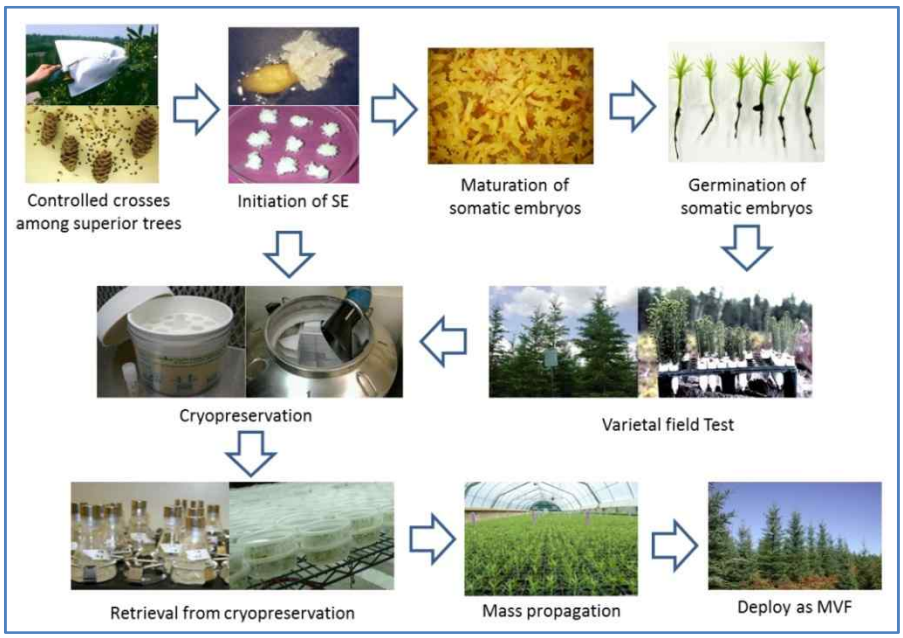

Figure 3. *The current MVF implementation using somatic embryogenesis at JD Irving Limited in eastern Canada. Selected parents from a long-term breeding program are controlled crossed and the resulting seeds are subjected to somatic embryogenesis for development of clonal lines. Embryogenic lines are cryopreserved and then a portion of each line is thawed and propagated to produce plants for varietal field testing. Once field testing shows which are the best lines, the corresponding embryogenic tissue are retrieved from cryopreservation, mass propagated, and deployed in the plantations.*

The field testing is an important phase of MVF because it is a critical part of selecting cell lines with desired attributes and of developing varietal lines. It is also the most time-consuming and expensive part of the process because trees are long-lived and, unlike agricultural crops, they grow slowly. Field tests are

evaluated at regular intervals, and the most current genetic information is used to amend the composition of multi-varietal mixtures thus offering the flexibility to adapt to changing conditions.

7. MVF incorporating forward genomic selection (GS)

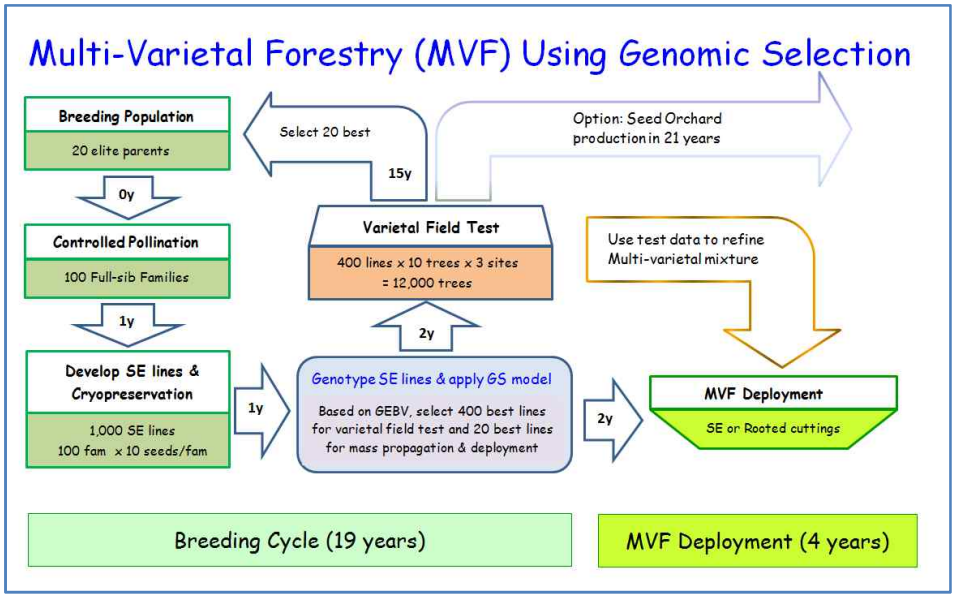

Figure 4. *Multi-varietal forestry integrating somatic embryogenesis and genomic selection: Similar to traditional breeding, controlled pollinations are performed to produce, e.g., 100 families from a subset of a breeding population. From each full-sib family, 10 embryogenic lines are developed (a total of 1,000 embryogenic lines) and cryopreserved. Genotyping of SE lines can be performed using embryogenic tissue, mature somatic embryos, or plantlets. The genomic data of SE lines are then applied to the GS model developed from the training population of the previous generation to obtain their GEBV. Based on best GEBV, about 400 SE lines, for example, may be selected to establish a varietal field test (VT), while the 20 lines that have the best GEBV may be selected for immediate deployment. In the VT, when trees begin to flower, the 20 best individuals are selected to form a new breeding sub-population. Thus, the breeding cycle is prolonged by 2 years due to the SE and cryopreservation steps that are required, thus resulting in a 19 year cycle. However, high GEBV SE lines are available for immediate mass propagation and deployment in MVF. This results in the deployment of genetically improved material 15 years sooner than via traditional seed orchard breeding by skipping one sexual reproduction cycle. Since the seed orchard is dependent on the time it takes to reach flowering maturity, GS has a limited advantage when production is based on sexual reproduction.*

With the availability of relatively inexpensive genotyping costs, GS is becoming attractive in tree breeding. In eastern Canada, integrated MVF using GS and SE in white spruce is based on forward GS and vegetative deployment. In this scheme, the GS model is developed in the mature genetic test plantations (training population) and the GS model is applied to the offspring population of the training population, hence forward GS as is illustrated in Figure 4: the elite individuals in a subset of breeding population are controlled-crossed to produce full-sib families; from these families, SE lines are developed and cryopreserved; after several months of cryo-storage, these lines are thawed, genotyped, and GEBVs are calculated; based on the GEBV, desirable individuals are mass propagated by SE or rooted cuttings from SE plants as proposed by Park et al. (1998) and deployed in the MVF. Thus, SE combined with GS can deliver genomically tested varietal mixtures for MVF in 4 years. This is a huge time saving when compared to delivery of genetic improvement by seed orchard, which may take 19 years even with GS.

Even though GS can identify superior genotypes at a very early stage without phenotyping, "varietal field testing (VT)" is necessary as it will verify the performance of the selections based on the GS model. Also, since GS can provide genetic information of the individuals at a very early stage, it can be used to pre-select genotypes to be included in VT. For example, instead of testing all available embryogenic lines obtained from seed produced by breeding, a breeder can select an upper 20-25% of high GEBV lines based on the genomic prediction, and propagate them to establish VT. This will reduce test establishment and maintenance cost drastically. VT is an important component of this MVF scheme because it provides continuously updated performance data that can be used to revise or modify varietal mixtures for deployment in the plantations, offering flexibility to adapt to changing conditions. Also, VT offers opportunities to capture non-additive variability as well as non-targeted traits (trait stacking) when observed during testing. Finally, the best selected trees in the VT will be used as the parents of the next cycle of breeding when they produce flowers and commence the next cycle of breeding.

8. Benefits of multi-varietal forestry

There are many benefits of MVF, but a few of the more important ones are:

1. Much greater genetic gain is possible than is obtained by using seed orchard seed. This is due to the capture of both additive and non-additive genetic variance.

2. MVF integrated with GS enables fast delivery of genetic gain and improves cost and efficiency of varietal testing. In turn, this will result in drastically higher genetic gain per unit time.
3. MVF can deliver trees with superior wood quality and uniformity
4. MVF offers flexibility to rapidly adapt to changing breeding goals, insect and disease conditions, and climate change through the use of continuously updated VT data.

9. Deployment strategies for MVF

The diversity of multi-varietal plantations is of concern, because there is a perception that narrow genetic variation may make MVF plantation more vulnerable to disease and insects than seedling-based plantations, and may results in plantation failure. However, for known diseases and insects, MVF has an advantage because more resistant varieties may be developed while simultaneously improving economic traits. But, for unknown or introduced pests, the protection is rather limited regardless of genetic variability existing within the species. It is difficult, if not impossible, to design protection against unknown diseases and insects. Nevertheless, it is generally assumed that, the more varieties in the MVF mixture, the lower the risk. However, the use of an increased number of varieties will reduce the genetic gain, Therefore, it is necessary to balance genetic gain and diversity, and this leads to a question of what is an appropriate number of varieties in a plantation.

Based on various assumptions, scientists generally agree that 10-20 varietal mixtures are sufficient for protection while providing benefits of MVF (Huhn 1987; Libby 1982; Zobel 1993; Roberds and Bishir 1997, Namroud et al. 2012). Such a threshold assures that alleles with population frequency of 10% or more are generally conserved, which are responsible for most of genetic variance in quantitative traits. Lindgren (1993) suggested some basic considerations: (1) if the species is used for short rotation, a lower number of varieties may be used because the exposures to the potential risk is short; (2) a lower number of varieties is acceptable if plantation management is intense and includes pest management; (3) the more well-known a variety, the more acceptable is its extensive use. Planting of varieties can be in varietal blocks or random mixtures, notwithstanding that they could also be used in mixed-species plantation schemes. In general, a random mixture is appropriate when varieties are not well-known or the future pest situation is uncertain (Lindgren 1993).

In eastern Canada, an approach called “Desired gain and diversity” is used to determine the number of varieties in a mixture. In this approach, the number is dynamically decided by selecting a desired or predetermined level of genetic gain and diversity based on the VT data (Figure 5). For example, a larger number of

varieties are included in the mixture at an early stage of VT in favor of diversity; however, at a later stage VT when the data are more reliable and varietal characterization is complete, a smaller number of varieties are used in the mixture in favor of larger genetic gain. This strategy is also combined with the previously proposed "Mixture of varieties and seedlings," which is mixture of selected varieties and seed orchard seeds (Park et al. 1998). This strategy will increase initial plantation diversity and reduce the stock cost as the seed orchard seeds are cheaper. Typically, in eastern Canada, about 40% of a plantation's basal area is commercially thinned at half-rotation age leaving superior quality trees for the final harvesting regardless of genetic origin. Thus, it is expected that the majority of trees are varietal trees with some exceptional trees of seedling origin. Therefore, the diversity of plantations is dynamically managed over time, where selection of varieties will be continuously revised based on the current VT data throughout the rotation age.

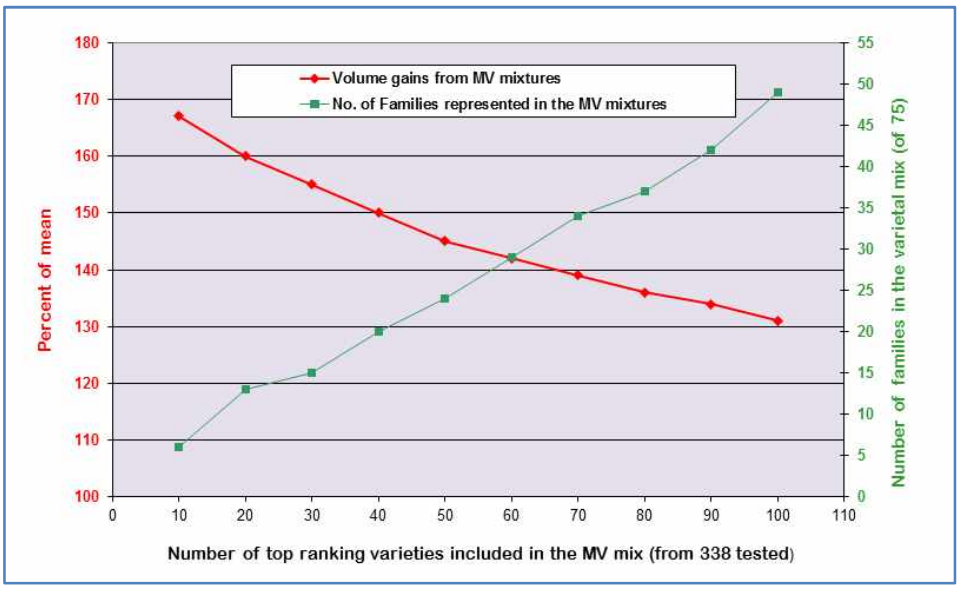

Figure 5. *Available genetic gains and diversity from MVF from a clonally replicated genetic test of white spruce assessed at age 14. The test contained 338 candidate varietal lines developed from 75 full-sib families. Since the parents of the crosses are the same parents that were used in the seed orchard, the overall mean (100%) represents the theoretical output of the orchard. If we take the 10 best clones in the varietal mix, the volume gain is 68% better than provided by the seed orchard but it contains only 5 families. If we take the 100 best clones, genetic gain is 30% better than the seed orchard output but it includes much greater genetic variation, i.e., 50 of 75 families. Thus, a breeder can set a desired level of genetic gain at given level of diversity.*

10. Hybrid varieties

Hybridization is a useful and widely used breeding approach in crop improvement, e.g., hydrid corn, through the crossing of usually different homozygous lines. In forestry breeding, hybridization usually refers to inter-specific or, sometimes, inter-provenance crosses. The main objectives of hybridization are to capture hybrid vigor and a combination of desirable characters. An example of hybrid vigor is demonstrated by the interspecific cross between Japanese (*Larix kaemferi*) and European (*L. decidua*) larch where certain lines are outperforming either parental species. In Korea, the pitch (*Pinus rigida*) and loblolly (*P. taeda*) pine hybrid was successfully used in reforestation to take advantages of the trait combination of the fast growth of loblolly pine and cold tolerance of pitch pine. However, despite the large potential, hybridization in conifers has rarely been used in modern tree breeding due to the labor intensiveness of hybrid seed production through mass controlled pollination and/or inefficiencies of bi-species seed orchards. SE appears as the ideal technology for developing hybrid varieties in conifers, because it can mass produce hybrid seedlings from a small number of seed obtained by interspecific controlled crosses. Moreover, with cryopreservation and VT, it offers further improvement through selection of the best individuals within the interspecific crosses. The development and deployment of hybrid varieties may be carried out similarly to the MVF as described here.

11. Commercial implementation of MVF

The industrial implementation of MVF is at an early stage. Many forestry companies and organizations are known to produce somatic seedlings from SE, including Arborgen (USA), Weyerhaeuser (USA), JD Irving Limited (Canada), FCBA (France), Arauco (Chile), Scion (New Zealand), Coillte (Ireland), Forestry Commission (UK), Government of Quebec (Canada) and others; however, their production rate is generally unknown but it seems relatively small in most cases. With the exception of JD Irving Limited and the Province of Quebec, the current SE production is mostly a laboratory-based system with *in vitro* germination, which is suitable for establishing varietal tests or small-scale commercial production but not for a large-scale production.

The primary challenge for MVF is the efficiency of the SE process from initiation to somatic seedling production. For many economically important species, SE initiation and maturation rates are too low; however, for most spruce and several pine species, the SE process is sufficiently refined to be used in the industrial MVF. For example, in white spruce, initiation of SE is at about 70%, proliferation in both liquid and semi-solid media generally works well. Usually, a

gram of embryonal mass produces on average about 500 mature embryos, and germination on appropriate culture media works well. However, there is a large variability in proliferation and maturation rates among embryogenic lines, and it is well-known that the SE process is affected by genetic background and culture conditions, offering a possibility of further refinements.

Cryopreservation of embryogenic lines using previously mentioned "Freezing Containers" is relatively simple. The recovery of cryopreserved lines is also satisfactory. For example, the recovery rate of 234 cell lines that were cryopreserved for 22 years was 95% (Park, unpublished data). The presence of contaminating microbes was also observed in the thawed cultures but the loss due to contamination was only about 1 percent of the total sample.

Perhaps, the most important challenge is the relatively higher cost of producing trees by somatic embryogenesis when compared to the seedling production using seed. In eastern Canada, it is estimated that SE trees cost more than 1.5 times the cost of seedlings, which is a net improvement compared to a generation ago, but still slightly too high even when the higher genetic gains are considered. Based on a series of crude assumptions, it was estimated that the SE production cost should not exceed 1.3 times the cost of seedlings in order to be profitable. Currently, the most expensive part of SE-derived trees is the manual transplanting of germinated embryos (*in vitro* state) into a commercial container system in the greenhouse. Therefore, it is critically important to develop either a semi-automated transplanting system or the means for direct germination into a growth substrate (micro plug) system; these options are currently being explored experimentally.

In the absence of a fully operational mechanized SE transplanting system, an alternative path to implementing MVF is the use of serial rooted cuttings from juvenile donor plants. Once superior embryogenic varieties are identified and thawed from cryopreservation, a small number of donor plants are propagated by SE, forming "stock" hedge plants (Park et al. 1998). Subsequently, mass propagation from the hedge stock can be accomplished by rooting of cuttings, which can be relatively inexpensive and automatable. These hedges can be used as stock hedges for about 5 years. The mass production of stecklings by rooting of cuttings from juvenile plants has been accomplished in several conifers (Park and Fowler 1987; Mullin et al. 1992; Kleinschimit et al. 1993; Russell 1993).

Finally, preliminary cost-benefit assessments of integrating SE with forward GS indicate that MVF will deliver unprecedented economic returns, much higher than achievable by any tree breeding effort (Beaulieu & Bousquet, unpublished data). This is the case because MVF can deliver much greater genetic gain than seed orchard breeding by capturing both additive and non-additive genetic variation without recombination through sexual reproduction. Furthermore, forward GS and SE eliminate the time required to produce seeds and, thus, gain per

unit time is notably increased. Therefore, SE becomes a key enabling technology for delivering the forward GS strategy.

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An industrial perspective on the use of advanced reforestation stock technologies

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Abstract

J.D. Irving, Limited is a forest products company with large forest land holdings in Eastern North America. The company has been active in tree improvement programs for many years for a number of conifer species. As well as using traditional seed orchards to produce improved seed, the company has also integrated vegetative propagation initially through rooted cuttings and then via somatic embryogenesis (SE), primarily of spruces. The objective of the SE program is to obtain tested varietal lines to be deployed in multi-varietal forestry plantations. Data are presented that show the genetic gain in height, diameter, and volume over that of the varietal test average at various selection intensities after a decade in a field test.

Keywords: clonal propagation, rooted cuttings, somatic embryogenesis, multi-varietal forestry, genetic gain, weevil resistance

1. Introduction

Forest geneticists have identified the potential incremental gain for a range of traits afforded by alternative, advanced reforestation stock strategies over that provided by traditional seed orchards. These strategies include rooted cutting propagation of tested full-sib families, mass control-pollinated production of full-sib families and propagation of tested varieties capturing additive and non-additive genetic variation. The latter strategy has primarily been employed in reforestation

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with hardwood species such as poplar and eucalyptus species, where vegetative propagation by coppice production of cuttings is biologically feasible (Zobel 1993, Ondro et al. 1995). Varietal production options in conifers have, in general, received less attention due to the relatively higher production costs of clonal plants and the poor success with non-juvenile cuttings.

The development and rationale behind industrial application of advanced reforestation stock technologies is described from the perspective of a forest products company, J.D. Irving, Limited (JDI), in North-Eastern North America. The companyis a privately owned diversified forest products company managing 1.3 million hectares of Freehold land in the Provinces of New Brunswick and Nova Scotia, Canada and the State of Maine, United States. The company land holdings cross a broad diversity of forest types, from high quality tolerant hardwood through to lowland softwood. Products produced include softwood and hardwood pulp, white pine and eastern white cedar lumber, Kraft pulp, tissue, corrugated medium, light-weight coated paper and consumer products (JDI Sustainability Report 2013). The company has maintained a long-standing commitment to land stewardship and as part of that commitment has planted over 620 million conifer seedlings since 1957on its privately owned land, while hardwood forests are managed using natural regeneration. The company initiated a tree improvement program in 1979 and has been extensively involved since then, both independently as well as in collaboration with two regional cooperatives (New Brunswick Tree Improvement Council and Nova Scotia Tree Improvement Working Group). The primary species of interest are white spruce, red spruce, black spruce, Norway spruce, white pine and jack pine. All seedlings produced originate from seed orchards and to dateover 320 million seedlings have been produced with improved orchard seed. The company became interested in vegetative propagation based on its widely understood potential genetic gain (Park et al. 2015, in this volume) and advances that have been made are described below.

The purpose of this chapter is to describe the development of advanced seedling production strategies in an industrial context including gains observed in recent multi-varietal testing (MVF) using somatic embryogenesis.

2. Multiplication of elite families by rooted cuttings

While methods related to rooting juvenile cuttings of spruce were relatively well known at the time when the company's tree improvement program was initiated, successful propagation of rooted cuttings required specialized expertise and resulted in higher production costs than in seed-based production. Seed orchards were coming into production by the late 1980's and progeny testing had not yet provided sufficient genetic data to be used to identify specific combinations of parents that could yield sufficient genetic gain to warrant the

additional production costs. By the early to mid-1990's estimates of parental breeding values became available based on open-pollinated or polycrossgenetic test data. This made it possible to produce control-pollinated crosses among high value parents followed by hedging of potted seedlings and multiplication with rooted cuttings (Adams and Tosh 1998). By the end of the 1990's rooted cutting production was semi-operational with annual production in some years in the hundreds of thousands before the program evolved towards the somatic embryogenesis approach.Nevertheless, the rooted cutting approach is often practical when seed orchard seed is not available due to poor seed production or timing (Ahuja and Libby 1993).

3. Varietal testing and production of rooted cutting with serial propagation

In species where seed orchard production is abundant and predictable, the incremental production cost of rooted cuttings based purely on making crosses among high value parents is a significant obstacle, especially when making use of such advances as flower induction using gibberellic acids (Greenwood et al. 1991) and supplemental mass pollination or controlled mass pollination. It was well understood that there is a substantial amount of non-additive genetic variation in spruce species (Mullin and Park 1992, 1994). Utilizing this variation to increase incremental gain is complicated by the poor rooting and plagiotrophic growth of non-juvenile spruce cuttings,i.e., typically around age 4 juvenility wanes (Bentzer 1993). By the time varietal field testing is accomplished, the donor plants are no longer viable for cutting production. Partial circumvention of this issue was proposed by Kleinschmitt (1993) who described a technique whereby juvenility could be maintained through serial propagation. By this method, hedge plants are re-propagated on a four-year cycle. In order to test the potential gain through this approach, JDI produced 32 control pollinated full-sib families among 34 selected black spruce parents. From each family, eight varieties were developed and hedged for a total of 256 varieties. In 1996, varietal tests from rooted cuttings were established at two sites in New Brunswick with 10 replicates at each, consisting of a single tree plot for each variety. Tests were evaluated for height, breast height diameter and individual calculated tree volume (Horner et al. 1983) at ages 5, 10 and 15. Based on varietal means and selection of the top 20% (51 of 256 varieties),gains of 8.5% and 29.2% for height and volume growth respectively were observed compared to the height and volume growth of all varieties at age 15 (6.41 m height, 0.027 m^3 volume). This test series also illustrated important genetic diversity management implications of varietal selection. For instance, the top 20% of varieties selected, based on height, were distributed across 19 of 32 families and 28 of 34 parents. If selection had been based on family means alone, selection at the same intensity would have included only 6 of 32 families and 10 of 34 parents.

At the same time, gain based on family selection would have been lower at 6.1% for height and 18% for volume. While advantages of varietal selection with rooted cutting compared to multiplication of elite families was demonstrated to be significant, there is little information in the literature regarding serial propagation of the species that JDI is concerned with. Furthermore, serial propagation in a nursery setting during the lengthy field testing phase involves significant logistical management and costs.

4. Integration of somatic embryogenesis into advanced seedling production

The potential for advanced seedling production was improved with the discovery of somatic embryogenesis (SE) in conifers in the mid-1980's. The use of cryo-storage of SE cell lines presented a solution to the problem of not being able to root cuttings from trees which were no longer juvenile. Storing cell lines in liquid nitrogen during the lengthy field testing phase to identify superior varieties has become essential to progress in this area. Immediate recognition of the enabling potential of cryogenic storage drove initial efforts by the company to evaluate somatic embryogenesis. Tree improvement programs at the time were most advanced for black spruce and on this basis, this species was chosen for initial focus (Adams et al., 1994). Initial efforts demonstrated the responsiveness of spruce species to all phases of somatic embryogenesis, including initiation of SE callus from zygotic embryos using modified Litvay (mLV) (Park et al.1993), maturation of somatic embryos, germination of mature embryos and transplanting of germinantsinto *ex-vitro* conditions in the greenhouse (Park et al. 1993; Adams et al. 1994.).

Currently, JDI's SE-based advanced seedling production system, a.k.a. Multi-Varietal Forestry (MVF), is following the process presented by Park et al. 2015 (in this book, Figure 3). Briefly, it involves: Selected parents from a long-term breeding program are controlled crossed and the resulting seeds are subjected to somatic embryogenesis for development of clonal lines;embryogenic lines are cryopreserved and then a portion of each line is thawed and propagated to produce plants for varietal field testing; once field testing shows which are the best lines, the corresponding embryogenic lines are retrieved from cryopreservation, mass propagated, and deployed in the plantations.

White spruce is most suited for planting on highly productive sites. Yields fromsilvicultural investments will be greater than on lower productivity sites, in contrast to species such as black spruce which are ecologically adapted to poorerlocal conditions. By the mid-1990's, early progeny testing results in white spruce enabled identification of first-generation white spruce selections with high breeding values, which were then used as the parents of controlled crosses. The seeds from these crosses were subjected to SE for the development of candidate

varietal lines. As a result of the company's changing focus on higher value species, white spruce varietal tests were initiated beginning in 1999 using candidate varieties derivedfrom the full-sib crosses. From 1999-2004, 1367varieties of white spruce from 82families and 58parents were established at multiple sites across the region. Varietal testing was also initiated with full-sib crosses among Norway spruce parents with demonstrated resistance to white pine weevil (Lavallee et al. 1999; Figure 1). This species has been widely planted in North-Eastern North America for over 100 years and in many cases it out-performs local spruce species (Nova Scotia DNR report). However, Norway spruce is highly susceptible to white pine weevil damage, as are a number of native spruce species such as Sitka spruce and interior spruce in British Columbia (Alfaro and Ying 1990). White pine weevil kills current year leaders of trees and does not result in mortality but seriously impacts quality of stems. While work continued on improving SE productivity and greenhouse culture, emphasis was mainly on producing a small number of somatic seedlings from as many varieties as possible for establishment of field tests across the region. Varietal tests are typically established at 3-4 sites with the test design being ten replications with single-tree plots for each variety randomly planted in each replication.

Figure 1.*Norway spruce varietal test at age 15. Embryogenic varietal lines are developed from controlled crosses among weevil resistant parents.*

5. Observations from varietal tests

Of the many series of SE-based varietal tests, we present data from two tests; however, we observed similar trends from other series. Evaluation of varietal tests began after 5 growing seasons with subsequent remeasurement at ages 10 and 15. Individual tree volume is calculated using metric volume equations (Honer et al. 1983) when height and diameter measurements are available. At an early age, e.g., age 10, the calculated individual volume may not have practical use but it can be used as an index value combining height and diameter. Significant variation in average varietal height and diameter is observed and results are summarized in Tables 1 and 2 at age 10 for two separate varietal test series planted in 2000 and 2002 with 224 and 315 varietal lines included,respectively. From a quality perspective, the ratio of branch and stem diameter at breast height is also measured.

In Table 1, the 10 year performances of series 1 test of the varietal mixture consisting of 10, 20 , .., 100 top ranking varieties based on the volume indexis tabulated and compared to the overall test mean. Incidentally, the overall test mean represents expected seed orchard output, without inefficiencies of the seed orchard, because the parents used in producing embryogenic lines are the same asthose used for the seed orchard clones. The deployment of the top 10 varieties in the test would result in a realized gain of 18 and 27% for height and diameter, respectively, over the seed orchard gain at age 10. In general, at approximately 20% selection intensity, results are very similar across the two test series at 10-12% gain over the varietal means for height and approximately 17% for diameter (Table 2).

Table 1*.Varietal test of white spruce (Series #1) at age 10. The test was established in 2003at 3 locations in New Brunswick, Canada using plants produced by somatic embryogenesis while maintaining corresponding embryogenic tissue in cryo-storage.*

No. of top ranking varieties	Proportion selected	Height (cm)		Diameter (mm)		Volume (m^3)		Diversity	
		Mean	% of overall mean	Mean	% of overall mean	Mean	% of overall mean	No. of Families	No. of parents
10	4.4%	452.9	18%	70.8	27%	0.009724	78%	7	10
20	8.9%	441.7	15%	69.0	23%	0.008950	63%	11	14
30	13.4%	435.9	14%	67.5	21%	0.008476	55%	13	17
40	17.9%	432.6	13%	66.3	19%	0.008139	49%	16	19
50	22.3%	429.6	12%	65.4	17%	0.007889	44%	17	19
60	26.8%	425.4	11%	64.8	16%	0.007688	40%	18	20
70	31.2%	422.3	10%	64.3	15%	0.007509	37%	18	20
80	35.7%	419.0	9%	63.7	14%	0.007324	34%	19	23
90	40.2%	416.2	9%	63.1	13%	0.007167	31%	21	23
100	44.6%	413.5	8%	62.6	12%	0.007014	28%	21	23

Varietal test summary	
Number of varieties tested	224
Number of parents	27
Number of Families	27
Mean Height	383.1
Mean Diameter	55.91
Volume	0.0054

Table 2.*Varietal test of white spruce (Series #2) at age 10. The test was established in 2002at 2 locations in New Brunswick, Canada using plants produced by somatic embryogenesis while maintaining corresponding embryogenic tissue in cryo-storage.*

No. of top ranking varieties	Proportion selected	Height (cm)		Diameter (mm)		Volume (m^3)		Diversity	
		Mean	% of overall mean	Mean	% of overall mean	Mean	% of overall mean	No. of Families	No. of parents
10	3.2%	494.1	16%	83.76	27%	0.014176	75%	6	11
20	6.3%	483.9	14%	81.49	24%	0.013228	63%	7	13
30	9.5%	479.5	13%	79.87	21%	0.012652	56%	12	17
40	12.7%	473.5	11%	79.09	20%	0.012272	51%	14	18
50	15.9%	472.0	11%	78.18	19%	0.011956	47%	15	18
60	19.0%	470.8	11%	77.40	17%	0.011701	44%	15	18
70	22.2%	469.0	10%	76.83	16%	0.011485	41%	15	18
80	25.4%	467.9	10%	76.21	16%	0.011286	39%	16	20
90	28.6%	465.2	9%	75.75	15%	0.011128	37%	16	20
100	31.7%	462.9	9%	75.26	14%	0.010944	35%	17	21

Varietal test summary	
Number of varieties tested	315
Number of parents	23
Number of Families	21
Mean Height	425.4
Mean Diameter	66.0
Volume	0.0081

Genotype by environment interactions are observed but the varietal rank changes were small at the upper and lower ranges of overall performance. From a selection standpoint, varietal rank shifts across sites are evaluated based on consistency across the environmental gradient tested.

6. Scaling up production of somatic embryos

While incremental gain added through varietal production has clearly been demonstrated to be substantial, benefits of using SE and realizing return for the investment in technology development and varietal testing relies on producing SE seedlings for operational reforestation. The key factors are the cost of production compared to the value of additional volume produced, sawlog versus pulp production ratios and/or reduction in rotation length. Since 2008, JDI has focusedon improving all aspects of producing high quality SE seedlings. Important indicators include number of mature embryos per gram of embryogenic suspensor mass (ESM)matured, conversion of mature embryos to acceptable germinants and successful transition through transplanting into the greenhouse. Current laboratoryprotocols include proliferation of thawed embryogenic lines in suspension culture (Figure 2 A) and/or on semi-solid media,maturation of embryos on filter paper over semi-solid growth media in petri dishes, separating mature embryos through several cleaning steps (Figure 2 B), drying embryos and dispensing them onto semi-solid germination media in petri dishes or trays (Figure 2 C). An optimized timing has been developed for the germination period prior to

greenhouse transplanting. Germinants are transplanted manually into pre-slit miniplugscontaining polymerized peatmoss(e.g., Jiffy Preforma mixture manufactured by Jiffy Products of America Inc., or Grow-Tech FlexiPlugs manufactured by Grow-Tech LLC). Greenhouse culturehas also been optimized and after several months, miniplugs are transplanted into Multipot 67 cavities (currently done manually,Figure 2 D). Success rate through to transplanting is typically 80-90% for white spruce and 70-80% for Norway spruce. Total production targets have been in the 300,000 – 400,000 range annually in the last several years with production cost and greenhouse recovery being the determining factors for future production increases.

Figure 2. ***A)*** *Proliferation of thawed embryogenic lines in liquid culture;* ***B)*** *Mature somatic embryos ready for germination;* ***C)*** *in vitro germination of somatic embryos;* ***D)*** *Operational production of somatic seedlings at J.D. Irving's greenhouse.*

7. Challenges for cost effective production of SE seedlings

Significant challenges remain to be addressed before the cost of SE seedlings will allow for step changes in production. In the laboratory production phase, the greatest costs occur after the maturation stage. Multiple steps are required to separate mature embryos from ESMmaterial and increased handling and drying introduces the potential to damage embryos. Uniform distribution of embryos on germination containers has an important impact on germinated to transplanted conversion rates as well as on transplanting productivity. Technology

development is currently underway to automate these steps which are intended to improve both productivity and quality.

The most critical step from a cost standpoint is transplanting of germinated SE seedlings from sterile germination media to miniplugs in the greenhouse. This step typically involves individual handling of the fragile germinants with forceps and placing them in the peatmoss plug, whichis a significant hurdle from an economic production perspective. This remains the largest challenge to JDI implementing varietal production on a larger scale.Transplanting of miniplugs following greenhouse growth to larger seedling containers is currently done manually,however, horticultural automation systems are well developed to handle the transfer of miniplugs to larger containers which should reduce a significant portion of the overall cost of SE production.

7. Long-term opportunities for advanced seedling production

Traditional tree improvement by field testing and more recently, varietal testing, have demonstrated the broad genetic variability of conifer species which is important worldwidefor providing traits related to economic value and adaptiveness. Plantation establishment is a long-term investment in our region with rotations in the 35-45 year time window. Traditional seed orchard approaches, while effective, are not very flexible to respond to changing values and conditions. As well as providing incremental gains to growth rate through accessing additive and non-additive genetic variance, varietal production through SE offers significant gain through trait stacking. An important example is selection for white pine weevil resistance in Norway spruce. Increased resistance could be achieved through traditional seed orchards; however, the timeframe for having resistant seed would be approximately 15 years because of the long time period required to establish and wait for a seed orchard with resistant parents to come into production. In contrast, varietal testing and evaluation has made the production of weevil resistant seedlings possible in a much shorter timeframe, while at the same time increasing the number of resistant individuals deployed to plantations by selecting and propagating highly resistant individuals. Genetic gain for individual traits is often compromised by addition of other traits due to negative or even neutral genetic correlationsamong traits (Novaes et al. 2010). Varietal selection often offers the potential to mitigate this issue (Park et al. 2012). While growth rate is always important, value of plantations is also influenced by branching, stem form and wood quality. Varietal selection is a more efficient method for incorporating these traits and this helps to produce seedlings with greater value. Another long-term opportunity is related to integrating new technologies such as genomic selection. Progress in genomic selection may make it feasible to evaluate individual varieties for a range of traits much earlier than can be quantitatively measured in

the field. Traits that can thus be tested include growth rate and wood properties such as density and microfibril angle (Park et al. 2012). Once this technology is proven, it could be incorporated much earlier in the selection cycle than is currently possible (one year versus 10-20 years).

Adaptation to changing climatic conditions is a concern thathas been looming for over a decade. Tree improvement field testing, as well as varietal testing has been conducted across a climate gradient that exceeds projected climate changes over the next 50 years. While breeding zones were established based on adaptive potential within the region, this aspect will need to be re-examined constantly in response to projected climate change. While forest tree genetics testing will provide intelligence regarding the adaptive potential of parents within regional breeding programs, varietal production strategies will provide the best means to respond from the standpoint of providing the best adapted genotypes for operational reforestation stock in a changing environment.

8. Deployment strategies

Most discussion around deployment strategies of varietal production has focused on pure versus mixed varietal planting (Park et al. 1998). These discussions weigh factors such as risk and advantages based on uniformity. The JDI approach has been mainly one of varietal mixture deployment. To determine the number of varieties in a mixture, an approach called“desired genetic gain and diversity” is used. In this approach, plantation diversity is managed dynamically based on the most up-to-date varietal test data. For instance, when the test is young we can include more varieties in the mixture with a reduced genetic gain. When the test is mature and varietal lines are well characterized, we can use a smaller number of varieties in the mixture while optimally increasing genetic gain (Park et al 2015, in this volume).

9. Conclusions

Advanced Reforestation Stock Technologies (ARST) of J.D. Irving, Limited is based on a long-term tree improvement program and incorporates the latest technological advances. Multi-varietal forestry based on somatic embryogenesis at JDI has demonstrated a substantially higher range of genetic gain than can be obtained with conventional seed orchard breeding. The main challenge for implementing industrial MVF, however, is the relatively high cost of SE seedling production due to manual handling of embryos, both pre- and post-germination. Thus, the development of an automated embryo handling system is required. J.D. Irving’s ARST program will be ideally suited to incorporate genomic selection with vegetative deployment as outlined in Park et al. (2015, in this volume).

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In vitro techniques for conifer embryogenesis

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Abstract

Somatic embryogenesis in conifers requires stage-specific manipulation. This review focuses on the technical aspects required for successful in vitro culture. The review covers induction of embryos, and embryo-like structures and organs, secondary embryogenesis, production of embryos from haploid tissues and some of the techniques used to optimize these processes. Induction of embryos is achieved by using zygotic embryo explants at various stages of development, i.e., from early embryos through to mature embryos, depending on species. Further multiplication of in vitro embryos is commonly done on semi-solid media supplemented with organic nitrogen sources and appropriate combinations of auxins and cytokinins. Bulking up these repeatedly cleaving embryos is optimized by using liquid cultures, in particular, bioreactors. This particular stage suffers from aging effects, which result in a diminution and eventual loss of embryo yield and quality. To avoid aging effects, young high-yielding good quality stock cultures are processed for cryopreservation. They are stored until needed. Loss of embryogenicity is a bottleneck to experimental physiology. One way to circumvent this bottleneck is to select immortal cultures, i.e. cultures that do not lose their embryogenicity over decades of culture. Techniques and /or factors promoting embryo maturation and embryo conversion to somatic seedlings are also reviewed.

Keywords: somatic embryogenesis, haploid embryogenesis, embryo rescue, organ formation, secondary embryogenesis

1. Introduction

Vegetative propagation methods have been widely used to add value. The value may be economic in nature, e.g. capture of genetic gain in density, form, yield or resistance to diseases, pests or environmental factors. A species'

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conservation status, and even, in some cases, its cultural place in society, e.g.Golden Spruce in British Columbia, may provide reason enough to warrant special attention. Most commonly, breeding programs and seed production programs use a variety of vegetative propagation techniques ranging from rooted cuttings, grafts, to *in vitro* embryogenesis (see several other chapters in this book).

Multiplication of desirable genotypes by somatic embryogenesis can spare a tree improvement program years compared to waiting for planted seed to grow into a plant that delivers seed. Furthermore, in vitro haploid embryogenesis (of megagametophyte origin) captures unique genotypes that could otherwise only arise after years, or in the case of trees, decades, of inbreeding (Nagmani and Bonga 1985; Simola and Santanen 1990; von Aderkas and Bonga 1993). Embryo rescue, another technique, is used to capture crosses that might otherwise abort. As with any type of propagation, somatic embryogenesis is only attempted when the monetary gain justifies the investment in infrastructure and cost of highly specialized personnel. *In vitro* embryogenesis is generally used sparingly, because of high cost.

Given the expense of *in vitro* embryogenesis, it is used to solve some problems associated with other methods of vegetative propagation, or to attack problems for which other methods are unsuited. In the case of the former, somatic embryos can be cryopreserved at early developmental stages and used as a perpetual un-aging stock for bulk multiplication of embryos whenever desired. This spares the expense of maintaining nursery stocks, such as hedges for cuttings, which will eventually become increasingly difficult to propagate as they age. The number of propagules that somatic embryogenesis can produce is virtually unlimited. Furthermore, large numbers can be produced in short order. *In vitro* embryogenesis also provides completely unique ways to create genetically desirable materials, be they haploid embryos, or embryos rescued from crosses that normally abort. In addition, *in vitro* embryogenesis is a platform within biotechnology that provides aseptic material for transformation technologies.

In this paper we will discuss methods that optimize *in vitro* embryogenesis. There are a variety of induction techniques that have met with varying degrees of success. Once induced, there are methods that optimize handling, maintenance and maturation, some of which are more suited to small scale laboratories, others to industrial-level production. Cryopreservation methods are relatively straightforward, but the types of chemicals used may affect propagule growth. Acclimatization of young plants to greenhouse or phytotron conditions is easily achieved in some species, but difficult for others.

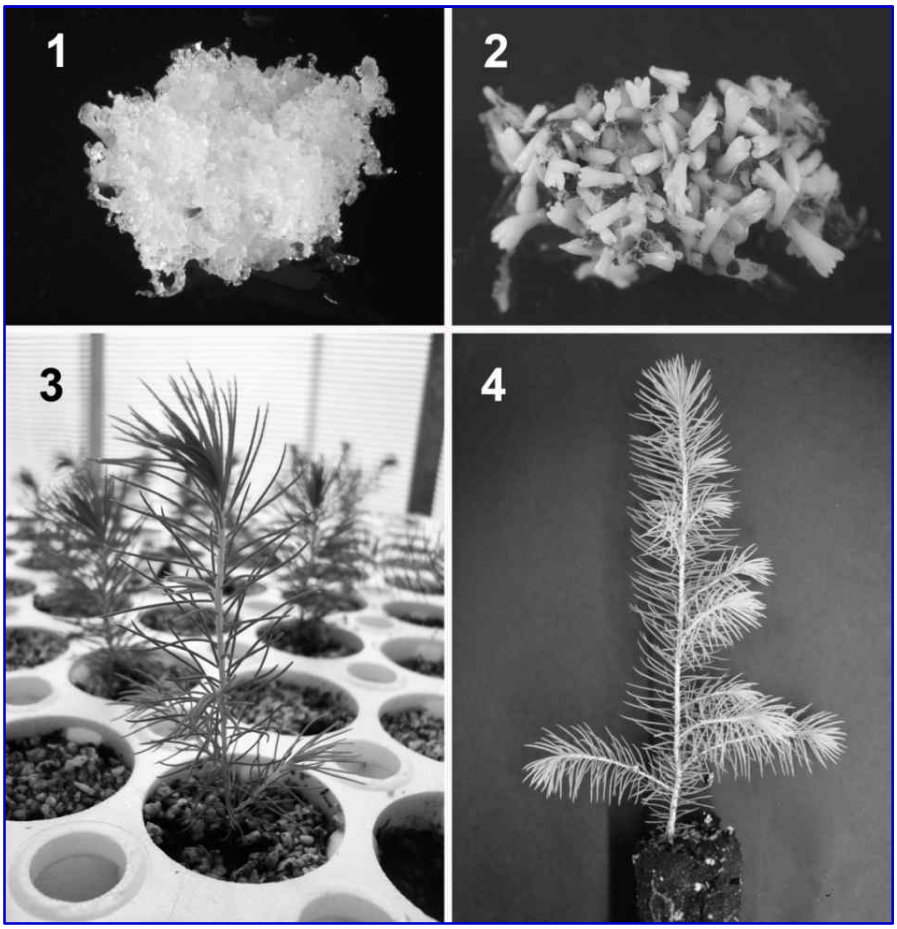
2. Somatic embryogenesis

Somatic embryogenesis is an advanced propagation technology for higher plants. Somatic embryos possess both shoot and root meristems, unlike organogenesis-derived plants, which most commonly have only a shoot that must later be rooted. The first success in conifer somatic embryogenesis was achieved in 1985 (reviewed by Stasolla et al. 2002). During the last three decades, success in inducing somatic embryogenesis was reported in many coniferous species with the result that this technology has been developed for clonal forests of elite trees (Stasolla et al. 2002; Park 2002). Currently in North America, millions of somatic embryo-derived seedlings and trees of species such as loblolly pine, Douglas-fir, interior spruce are growing. The major steps in obtaining clonal trees through somatic embryogenesis include: 1) induction of embryogenic tissue; 2) maintenance of embryogenic tissue, including cryo-preservation and regular maintenance; 3) bulking up of embryogenic tissue; 4) maturation of somatic embryos; 5) germination and conversion of mature embryos; 6) transition of plantlets from *in vitro* to *ex vitro* conditions, and establishment of plants in soil.

In most coniferous species, embryogenic tissue is induced from immature zygotic embryos of developmental stages ranging from proembryo to pre-cotyledonary. Developmentally advanced zygotic embryos are usually dissected from megagametophytes after the seed coats are removed. Both the embryo and megagametophyte are placed onto the surface of an induction medium with the suspensor that links the embryo to the megagametophyte. If the embryo is too small to be dissected out, the entire megagametophyte can be used as the explant once the seed coat has been removed. Embryogenic tissue can also be induced from mature embryos in certain species, such as white spruce (Tremblay 1990), larch (Lelu et al. 1993), Sitka spruce (Figure 1). It is more difficult to induce embryogenic tissue from vegetative tissue and organs. Some success has been reported in a few species, such as larch (Lelu et al. 1993; Bonga 2004). There is strong interest in induction of somatic embryogenesis from mature trees using vegetative tissue since this technique can propagate elite trees in true-to-type clones without a long period of time for clone test and selection. If induction is to be successful, it is critical to select suitable explants due to phase changes during plant development (von Aderkas and Bonga 2000).

Generally, for initiation of somatic embryogenesis, high concentrations of plant growth regulators (PGRs), usually 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzyl-aminopurine (6-BA), are used in induction medium and lower concentrations of the same PGRs in maintenance media. Pullman and his colleagues reported that addition of supplements to induction medium such as abscisic acid (ABA), silver nitrate, brassinolide and/or gibberellin inhibitors enhanced embryogenic tissue initiation in coniferous species (Pullman et al. 2003a, 2003b, 2005). In addition, liquid culture or liquid overlay on the surface of semi-solid media improved embryogenic tissue induction (Pullmanand Skryabina 2007).

In most cases, long-term maintenance in either liquid or semi-solid cultures results in a decline or even arrest in embryogenic capability. In order to avoid qualitative and quantitative decline, embryogenic tissue needs to beand Skryabina 2007).



Figures 1-4. *Somatic embryogenesis in Sitka spruce (Picea sitchensis). 1. embryonal masses/suspensor cultures 2. mature embryos 3. young somatic seedling in greenhouse 4. Somatic seedling prior to field planting.*

Inorder to avoid qualitative and quantitative decline, embryogenic tissue needs to be cryopreserved in liquid nitrogen for long term storage.In conventional cryopreservation methods, dimethyl sulfoxide (DMSO) is used as a major component of cryoprotectant for pre-treatment before the tissue is ready for storage in liquid nitrogen. However, DMSO causes genetic variations in embryogenic tissue (Aronen et al. 1999) and even later in plants that were derived from DMSO-treated tissue (DeVerno et al. 1999). We have eliminated DMSO during cryopreservation; instead, we plate embryogenic tissue on maturation medium and

culture under low temperature (4 °C) prior to cryopreservation (Kong and von Aderkas 2011). With this technique, cryopreservation of embryogenic tissue, or immature embryos, was successful in both interior spruce and Douglas-fir (Kong and von Aderkas 2011). This treatment not only induced cryotolerance of the tissue, but embryos later produced from once cryopreserved cultures were normal in form and maintained their embryogenic yield (von Aderkas et al. 2007; Kong and von Aderkas 2011).

Efficiency of bulking up is generally higher in liquid cultures of embryogenic tissue than semi-solidified cultures. At CellFor Inc. (1999-2011, Victoria, BC Canada), airlift bioreactors were built and used to bulk up embryogenic tissue of Douglas-fir and loblolly pine in large qualities (results not published) for production of mature somatic embryos subsequently. This type of bioreactor was also used to bulk up embryogenic tissue in other woody species, such as American chestnut (Kong et al., 2013). High quality embryogenic tissue after bulking up is essential for the subsequent steps if embryo production is to succeed. Important factors include not only the quality of initial tissue but also the conditions in the bioreactor, such as tissue inoculation density, airflow rate, ratio of fresh to conditioned media, etc. In either semi-solid or liquid cultures, pre-treatment using no PGR-containing medium for a few days prior to embryo maturation culture was beneficial to embryo maturation. In addition, activated charcoal can be added into pretreatment medium to absorb PGR residues from the tissue (reviewed by Thomas 2008). Reduction or elimination of PGRs in the pretreatment may reduce unwanted tissue proliferation, thereby enhancing embryo development and maturation.

Successful embryo maturation (Figure 2) depends on tissue plating, abscisic acid treatment, and water relations (reviewed by Stasolla et al. 2002; Stasolla and Yeung 2003). Plating embryogenic tissue on to maturation medium requires some care. It is difficult to get mature embryos of high quality in coniferous species if the tissue is immersed in liquid cultures. The process of embryo development and maturation may require gradients of plant hormones and/or other medium elements. For mass production, a stationary bioreactor (or diffusion bioreactor) was used successfully to mature somatic embryos of white spruce (Attree et al. 1994) and Douglas-fir (results not published). In order to obtain high quality mature embryos, embryogenic tissue must be plated evenly onto a surface of the nutrient substrate. Optimum tissue plating density depends on species and genotypes. To achieve an even plating, the tissue was suspended in liquid medium and poured into a funnel with a piece of mesh or filter paper (Klimaszweska and Smith 1997). While stirring, liquid is sucked down by vacuum. The tissue is left on the mesh or paper in the funnel. When this mesh or paper is placed on medium, it should, in an ideal culture, suppress tissue proliferation. Embryos mature readily on such filters. We have also used another method, which

is more primitive and brutal, but has met with great success in our hands. Half-a-gram of embryonal masses and suspensors is taken from a culture and placed on a polypropylene mesh ranging in mesh size from 710 μm to 1025 μm. The embryonal masses are flattened using the back of a spoon. The number of mature embryos was significantly higher than controls of sub-cultured, divided lumps of embryonal masses and suspensors (Pond 1999). This method had another benefit – it was easier to harvest mature embryos spread evenly across a surface than in clusters or clumps. A third benefit was in handling. For example, in desiccation tolerance experiments conducted on early stage white spruce embryos, embryos flattened into 3 cm^2 discs of polypropylene mesh (980 μm) were exposed uniformly to desiccation and temperature treatments (Pond et al. 2002). Later, these same discs could easily be transferred for final maturation of embryos. Since the advantages in handling, treatment and maturation are significant, no matter how embryos are subcultured, they should be uniformly spread prior to maturation treatments. Avoiding culturing somatic embryos on lumps or piles of embryonal masses prevents ethylene accumulation, which reduces embryo yield (Kong andYeung 1994). In order to suppress unwanted tissue proliferation, an auxin inhibitor, 2-(*p*-chlorophenoxy)2-methylpropionic acid (PCIB), can be applied during maturation (Find et al. 2002).

The second factor that must be controlled during maturation is abscisic acid, a hormone that occurs in both conifer megagametophtyes and embryos (Kong et al. 1997). Exogenously supplied ABA is essential for promoting embryo maturation. Of the various types of ABA available commercially (Kong and von Aderkas 2007) the most effective one in the cultures of embryogenic tissue in spruces is (+) *cis, trans* -ABA, or s-ABA (Dunstan et al. 1992; Kong and von Aderkas 2007). However, under particular light or heat conditions during medium preparation and storage part of *cis, trans* -ABA is altered to *trans, trans*-ABA, which is not bioactive (unpublished results).

The third major factor influencing embryo maturation is water availability, which is determined by both gel strength and the osmotic balance of the medium. High concentrations of gellan gum in medium improved the number and quality of mature embryos (Klimaszewska and Smith 1997). Significant increase in the yield of mature embryos was also achieved when polyethylene glycol (PEG) was added into the media (Attree et al. 1991; Kong and Yeung 1995; Kong et al. 1998). PEG is regarded as a non-plasmolysing agent that affects gene expression during embryo maturation (Stasolla et al. 2003) and increases accumulation of storage substances and desiccation tolerance in maturing embryos (Attree et al. 1991). In previous reports, different carbon sources could also affect embryo maturation (Tremblay and Tremblay 1991). In Douglas-fir (Kong and von Aderkas 2011) and hemlock (Merkle et al. 2014), maltose, instead of sucrose, reduced tissue proliferation and enhanced embryo maturation.

The quality of morphologically matured embryos contributes substantially to the ability of embryos to germinate and convert. Lowered moisture content in mature embryos enhanced their germination and conversion (Roberts et al. 1989; Kong and Yeung 1992). High quality embryos can tolerate desiccation after either a fast or slow desiccation treatment (Attree et al. 1991, 1994; Pond et al 2002). Desiccated embryos can be stored in a freezer for a few years. In mature somatic embryos, less structural abnormality was found in root meristems than in shoot meristems in white spruce (Kong 1994). This is likely due to the location of these meristems. The root meristem is enclosed deeply within the root cap, but the shoot meristem is exposed (Kong et al. 1999). As a result, intercellular spaces were frequently observed in shoot meristem tissue (Kong and Yeung 1992, 1994). During germination/conversion, a functioning shoot meristem could be established from only a part of the cells in the meristemic region of the somatic embryo (Kong and Yeung 1992), whereas in zygotic embryos, cells of shoot meristem were packed tightly and all functioned together during germination (Kong 1994; Yeung et al. 1998). Addition to maturation media of ethylene inhibitors (Kong and Yeung 1994), ascorbic acid (Stasolla and Yeung 1999), or glutathione (Stasolla et al. 2004; Belmonte et al. 2005) improved shoot meristem formation and embryo conversion. Although PEG enhances embryo maturation, it may result in a low conversion rate with PEG-matured embryos. Negative effects of PEG on embryo conversion were reported in white spruce (Kong and Yeung 1995) and Norway spruce (Bozhkov and von Arnold 1998) when PEG was added into embryo maturation media. A longer process of embryo imbibition under lower temperatures (4°C - 15°C) before germination may allow PEG to diffuse slowly out of embryos, which could enhance embryo conversion (unpublished results).

Care must be taken at the stage of transition from *in vitro* to *ex vitro* conditions for obtaining somatic seedlings (Figures 3, 4). In order to reduce germination costs in mass production systems, mature embryos are rehydrated and then primed in liquid media (Rise et al. 2011) with temporary immersion bioreactors, or airlift bioreactors. Subsequently, germinants are planted into mini plugs, the holes of which are filled with a carbohydrate-free medium that contains nutrients and mineral salts. Large losses often occur during the transfer of *in vitro*-produced plantlets into a greenhouse. High humidity in the greenhouse at the time of the transfer is important for plantlet survival in soil, but a regulated and gradual decrease in humidity is necessary to harden plants. The root system will then be able to develop functioning lateral roots. However, providing high humidity for too long not only causes roots to rot, but encourages moss growth and/or insect attack. Plants developed new roots and needles. Mini plugs with established plantlets can be removed from the block and transferred either to larger containers or machine-planted in the field.

3. Haploid embryogenesis

Induction of haploid embryogenic cultures in conifers is difficult and labour-intensive due to starting materials. Conifer researchers have a unique problem. Unlike angiosperm species that can be induced quite readily from microspores, which can be isolated by the hundreds of thousands for culturing, gymnosperm microspores cannot be induced to form haploid embryos. Perhaps it is because of the developmental complexity of conifer microgametophyte, e.g. pinaceous pollen is generally five-celled at time of anthesis, as opposed to the simplicity of angiosperm pollen, which is often one- or two-celled. Attempts by us to culture *Chamaecyparis nootkatensis* pollen, a simpler conifer pollen with only two cells, resulted in callus cultures; however, these could not be further induced to produce embryos (Hay 1997).

Fortunately, megagametophytes have proven easier to culture than microgametophytes or microspores. The stages required for induction of haploid embryogenesis are late in a megagametophyte's development, i.e. around the time of normal fertilization. This necessitates individual dissection of megagametophytes during a rather narrow window of their development (Baldursson et al. 1993). A few labs have been able to induce embryonal masses (Nagmani and Bonga 1985; Krutovsky et al. 2014), as well as mature embryos (Simola and Santanen 1990) and trees (von Aderkas and Bonga 1993). Megagametophyte induction to produce haploid cultures and trees is also characteristic of other gymnosperms, e.g. *Gingko* (Laurain et al. 1993), *Ephedra* (Singh et al. 1981) and cycads (Chavez et al. 1992). A general drawback in haploid conifer cultures is the lack of genetic stability (Pattinavibool et al. 1995; von Aderkas et al. 2003; Tretyakova and Voroshilova 2014). This brings into question the starting material itself. Reports have varied by species. Ball (1987) reported that cells in megagametophytes of *Sequoia* range in ploidy level from haploid to 16-ploid (Ball 1987). In contrast, a study of maritime pine using microsatellites showed a generally haploid composition in derived cell lines (Arrillaga et al. 2014).

Megagametophyte induction in conifers is generally simple. Megagametophytes are removed and directly plated on to medium. They need to be cut in half: only the chalazal end is retained for culture. Throwing out the micropylar half, which, at this time, contains the zygotic embryo, ensures that the experiment is only inducing haploid embryos and not propagating already formed zygotic embryos. In some species, such as larch and Douglas-fir, megagametophyte development proceeds to egg formation in the absence of pollination. In this case halving megagametophytes is unnecessary. Bagging the female cones at anthesis to prevent pollination is sufficient. Megagametophytes that would otherwise abort, will, if selected prior to the beginning of abortion, readily take to culture. Abortion is arrested, and proliferation may begin, resulting

in either a callus or in embryonal masses. Any medium that is good for somatic embryogenesis is suitable for haploid embryogenesis. There is no consensus on hormone treatments during multiplication. To date there are no reports of abscisic acid supplementation of maturation medium; this lack of ABA supplementation either represents an oversight in experimentation, or a fundamental difference between haploid and somatic embryogenesis. Given how similar embryogenesis following induction is between the two types, it is far more likely that it is an oversight.

4. Embryo rescue

Embryo rescue is an in vitro tool for breeders. Seeds from some crosses may fail due to an inability to germinate, or due to incompatibility resulting from either inbreeding or interspecific crossing (Reed 2005). In the case of conifers, embryo rescue represents a possible solution to such problems, but, in fact, has been more written about than performed. The reason that embryo rescue has been so little employed is that most breeding programs for conifers, unlike those for many agricultural crops, have had little or no occasion to use this technique. For nearly a hundred years, conifer researchers have known that removing embryos, particularly mature embryos, from their surrounding megagametophytes, will result in a germinated plant (Schmidt 1924). The proviso is that this is done before either the embryo or the megagametophyte has shown signs of degeneration.

5. Organ formation and organ failure

Generally, organogenesis is an undesired outcome in any somatic embryogenic protocol. We will not discuss organogenesis arising from somatic embryogenic cultures that have begun to produce callus. Normally, such cultures would be thrown out. However, there may be occasions where organogenesis is useful, as will be discussed below. Induction of somatic embryogenesis is successful if embryonal masses and suspensors, as opposed to callus, appear en masse. The proof that cultures are embryogenic resides in their ability to produce mature embryos capable of germination. Morphological criteria on their own can be deceptive, because there are many developmental possibilities in the formation of somatic embryos, as has been shown in studies of larch (Hay 1997) and spruce (von Arnold et al. 2002) in which individual embryos were traced from their origins. One of the alternative outcomes includes organogenesis. Bonga (1996) described structures that resembled mature embryos. These developed directly from embryonal masses with suspensors and to all intents and purposes appeared normal. However, only shoots developed: these “embryos” were missing roots. This is not the only case of organogenesis from somatic embryogenic cultures. *Pinus strobus* and *P. banksiana* are capable of producing somatic embryos from

nodules, but in the case of *P. banksiana* many somatic embryos were developmentally abnormal and lacked root meristems (von Aderkas et al. 2005).

6. Secondary embryogenesis (immortal lines)

Secondary embryogenesis occurs from somatic embryos that are placed on initiation medium and allowed to proliferate new embryos. It may also occur in maturing embryos that have not been well spread, resulting in mature somatic embryos that have embryonal masses and suspensors pushing out from their base, making harvesting difficult and even pointless. However, secondary embryogenesis may be desirable. For example, if the failed embryos of *Pinus banksiana* mentioned above that developed from nodules and which lacked root meristems were placed back on induction medium, cells at the surface of the cotyledons and hypocotyls proliferated, creating embryonal masses with suspensors. The resulting somatic embryos matured normally and were fully capable of germination (von Aderkas et al. 2005). This points to one of the latent capacities of somatic embryos, which is that they can be used to re-initiate lines that may have begun to lose their embryogenicity. In fact, one virtually immortal line of *Larix* x *eurolepis* (no. 69) was created in exactly this manner (von Aderkas et al. 2015).

The usefulness of immortal lines is for experimental embryogenesis. Somatic embryos represent a golden opportunity to study embryo development free from the constraints of the tree. However, there are drawbacks to such experiments. Physiological and molecular biological studies require repetition, which, in rapidly aging somatic embryogenic cultures forces a researcher to either re-initiate the lines from seed from the same crosses or from cryopreserved stocks. Naturally, this raises the question of whether experimental materials are, at the outset of an experiment, physiologically equivalent. This may appear to be a subtle problem in experimental design, particularly experiments that are only concerned with some feature of mature embryos or germinated seedlings. However, for any experiment that tracks the development of physiological and molecular events during embryo development, aging cultures are a big problem. Immortal lines get around this problem, as they are stable in yield and performance over many decades that we have cultured them. Such lines, particularly of larch, have been used extensively to study embryo physiology. Some examples are studies of hormones during embryogenesis (Gutmann et al. 1996; Jourdain et al. 1997; von Aderkas et al. 2002a, 2002b; von Aderkas et al. 2015), effects of gelling agents on protein expression (Teyssier et al. 2011), phenotypic variation in cotyledon morphogenesis (Harrison and von Aderkas 2004; Nagata et al. 2013), mycorrhizal associations with seedlings (Piola et al. 1995) to name but a few of the applications.

7. Conclusions

Somatic embryogenesis is a powerful method to create aseptic cultures that are useful in breeding and biotechnology. Many bottlenecks to production have been eliminated with technical and methodological improvements. However, some tissue culture methods, e.g. haploid cultures from microspores, have proven intractable. The limitations on somatic embryogenesis methodologies can be overcome with more experimentation in embryo physiology and molecular biology.

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Norway spruce as a model for studying regulation of somatic embryo development in conifers

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Abstract

Somatic embryogenesis in Norway spruce has been used as a model for studying embryo development in conifers. This model represents an excellent system for studying development in an evolutionary perspective and for the production of protocols for mass propagation. Somatic embryogenesis includes a well-characterized array of developmental stages, which can be synchronized by specific treatments, making it possible to collect a large number of somatic embryos at specific developmental stages. To improve the robustness of somatic embryogenesis, it is important to identify and eliminate *in vitro* treatments that impair the quality of somatic embryos. Therefore, the pathways controlling the successive developmental stages leading to cotyledonary somatic embryos must be fundamentally understood in order to obtain optimal management of somatic plant regeneration systems. In this review, we summarize the up-to-date knowledge about the regulation of somatic embryo development in Norway spruce (*Piceaabies*).

Keywords Apical-basal polarization · Cotyledon organogenesis · Embryonal shoot apical meristem · Nurse cells · Polar auxin transport · Programmed cell death · Protoderm · Radial patterning

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1. Introduction

Somatic embryogenesis is a valuable method to propagate conifers vegetatively both in breeding programs, and in clonal forestry. The whole procedure of conifer plant regeneration through somatic embryogenesis is comprised of several steps including initiation and proliferation of embryogenic tissue, differentiation of early embryos and development of late and cotyledonary embryos (von Arnold and Clapham 2008). Efficient deployment of somatic embryos requires a number of critical physical and chemical treatments with proper timing. A deeper understanding of the genetic regulation of embryo development may help to identify what goes wrong during somatic embryo development in recalcitrant genotypes of Norway spruce and other conifers. This can also provide clues on how to improve the culture conditions in order to propagate economically important conifers via somatic embryos.

Most morphogenic events in plants occur in the sporophyte after seed germination. However the embryonic phase is crucial as it is then that the embryo polarity and the primary meristems are established. Knowledge about the molecular regulation of embryonic pattern formation in plants has to a large extent been derived from studies of embryo-defective mutants in the angiosperm model plant Arabidopsis (*Arabidopsis thaliana*). By contrast, our knowledge about molecular regulation of zygotic embryo development in conifers is scarce, partly owing to the lack of characterized embryo-defective mutants. Thus somatic embryogenesis coupled with reverse genetics has become a promising alternative system for studying regulation of embryo development in conifers.

In this review we will summarize the up-to-date knowledge about the regulation of somatic embryo development in Norway spruce (*Picea abies*).

2. Pattern formation during somatic embryo development in Norway spruce

Embryogenic cell lines of Norway spruce are routinely established from mature, non-desiccated zygotic embryos, so that each cell line represents one genotype (von Arnold and Clapham 2008). In the presence of the plant growth regulators (PGRs) auxin and cytokinin, the embryogenic cultures proliferate as proembryogenic masses (PEMs). The PEMs are composed of two cell types: small meristematic cells and elongated, highly vacuolated cells (Figure 1a). To stimulate differentiation of somatic embryos, the cultures are transferred to medium lacking PGRs. These early embryos have a polar structure with a compact, globular embryonal mass in the apical part, and an elongated suspensor in the basal part (Figure 1b). The embryonal mass and the suspensor are separated by a layer of gymnosperm-specific cells called tube cells. Further development and maturation of somatic embryos is stimulated by transferring the cultures to medium

supplemented with abscisic acid (ABA). Late embryos develop after one to two

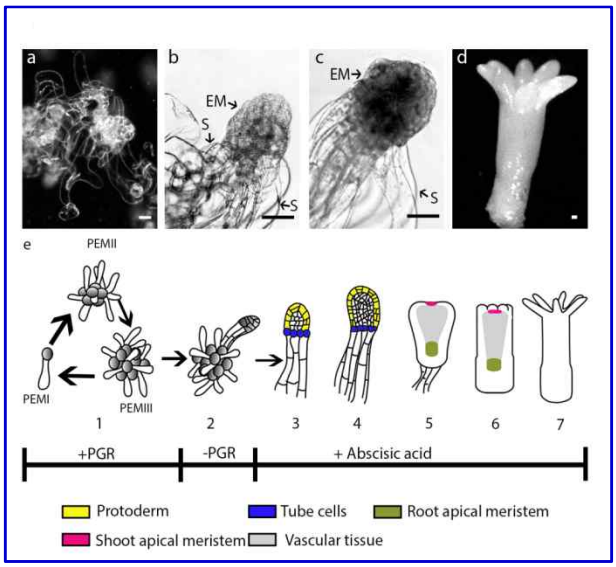

Figure 1. *Somatic embryo development in Norway spruce: Developmental stages during somatic embryo development. (a) Proliferating proembryogenic masses (PEM) in the presence of the plant growth regulators (PGRs) auxin and cytokinin. (b) Early embryo (EE) after one week on pre-maturation medium lacking PGRs. (c) Late embryo (LE) after one week on maturation medium containing ABA. (d) Mature embryo (ME) after five to eight weeks on maturation medium containing abscisic acid (ABA). The time it takes for development of EEs and LEs varies among cell lines. In some cell lines EEs develop after one week on maturation medium and LEs after two weeks on maturation medium. EM, embryonal mass; S, suspensor. Scale bar=100 µm. (e) Schematic representation of the developmental pathway of somatic embryos in Norway spruce. The process of somatic embryogenesis involves two broad phases. The first phase, which occurs in the presence of auxin and cytokinin (+PGR), is represented by proliferating PEMs, cell aggregates which can pass through a series of three characteristic stages distinguished by cellular organization and cell number (1, PEM I, II and III). The second phase encompasses differentiation, development and maturation of somatic embryos. (2) Differentiation of somatic embryos from PEM III is triggered by withdrawal of auxin and cytokinin (-PGR). When embryos have differentiated, their further development into (3) EE, (4) LE, (5) maturing embryos, characterized by the initiation of cotyledons, (6) almost fully matured embryos and (7) fully mature, cotyledonary embryos is stimulated by abscisic acid. The developmental pathway of somatic embryos in Norway spruce is based on the original publication by Filonova et al. (2000a).*

weeks on maturation medium (Figure 1c). The terminally differentiated suspensor cells are successively eliminated by programmed cell death (PCD), a process starting already during differentiation of early embryos (Filonovaet al. 2000b; Bozhkov et al. 2004).The root and shoot apical meristems are delineated. Mature embryos develop after five to eight weeks on maturation medium (Figure 1d). Finally, after partial desiccation, the embryos are germinated on medium lacking PGRs. The developmental pathway of somatic embryos of Norway spruce is schematically presented in figure 1e.

3. Phase change during embryo development

Embryo development can be divided into two distinct phases, the early morphogenic phase and the late maturation phase (Goldberg et al. 1994). During the morphogenic phase, most cell divisions and differentiation processes occur and the basic body plan of the embryo is established. During the maturation phase, embryo morphogenesis is arrested and the embryo increases in size by cell expansion. Seed germination marks the end of the embryonal development and rapid repression of embryonic genes is observed with seed imbibition (Tai et al. 2005). Histone deacetylases (HDACs) are involved in the repression of embryonic properties upon germination (Tanaka et al. 2008). During early embryogenesis in Arabidopsis the *LEAFY COTYLEDON* (*LEC*) genes are required to maintain the embryonic cell fate and to specify cotyledon identity (Santos-Mendoza et al. 2008). The activity of *LEC* genes must be repressed post-embryonically to allow vegetative development to proceed (Braybrook and Harada 2008). *ABI3,* and its ortholog*Viviparous-1* (*VP1*) in maize (*Zea mays*) is another master regulator that together with the *LEC* genes acts to promote embryo maturation (To et al. 2006).

Two *LEC1-like* genes (*PaHAP3A* and *PaHAP3B*) and one *VP1 gene(PaVP1*) have been characterized in Norway spruce (Footitt et al. 2003; Uddenberg et al. 2011). A phylogenetic analysis of the *LEC1*-type genes revealed a conifer-specific subclade, and the expression of *PaHAP3A* is high in PEMs, in early and late embryos, but low in mature embryos (Uddenberg et al. 2011). In contrast, the expression of *PaVP1* is low in PEMs and early embryos and high in late and mature embryos. When the embryogenic cultures are treated with the HDAC inhibitor trichostatin A (TSA) during maturation the process is arrested and the expression level of *PaHAP3A* remains high while the expression of *PaVP1* remains low.

Taken together, these results suggest that the transition from the early morphogenic phase to the late maturation phase is accompanied by shifts in the expression levels of *PaHAP3A* and *PaVP1* and provides a possible link between chromatin structure and expression of embryogenesis-related genes. Furthermore, the results show a divergent evolutionary history of the conifer and angiosperm

LEC1-type genes, indicative of either neo-or subfunctionalization. In contrast, the conifer *ABI3/VP1* homologs are closer to their angiosperm homologs, both when it comes to gene expression patterns and sequence analysis (Uddenberg et al. 2011).

4. Processes associated with pattern formation during early development of somatic embryos

Putative processes associated with early development of Norway spruce somatic embryos have been identified by studying changes in global gene expression using microarray analyses (Vestman et al. 2011).

4.1 Stress-related processes

Genes involved in defense and oxidative stress are over-represented among differentially expressed genes during both differentiation of early embryos and development of late embryos. Oxidative stress and the production of reactive oxygen species have been associated with the activation of PCD (Swidzinski et al. 2002). In Norway spruce, PCD is responsible for the degradation of PEMs, when early somatic embryos differentiate, and for eliminating terminally differentiated suspensor cells during early embryo development (Filonova et al. 2000b). The importance of PCD for embryo development will be discussed later.

During embryo maturation several genes coding for heat-shock proteins (HSPs), which protect cellular components from severe dehydration, are up-regulated (Stasolla et al. 2004).

4.2 Auxin -mediated processes

The array data indicate that auxin biosynthesis increases during early embryogeny and that the auxin-responsive machinery is up-regulated in the beginning of late embryogeny. In addition, polar auxin transport starts early during embryo differentiation (see below under “Polar auxin transport”).

4.3 Nurse cell functioning

It has long been known that conditioned medium (spent medium harvested from cultured cells) from embryogenic cultures can promote embryogenesis. For example, conditioned growth medium from highly embryogenic cultures can induce embryogenesis in non-embryogenic cultures (Hari 1980). This ability of conditioned medium to sustain or stimulate somatic embryogenesis implies that secreted soluble signal molecules play an important role for differentiation and development of somatic embryos. Several proteins identified in conditioned medium are seed-specific, therefore it has been assumed that some cells in cell

cultures have endosperm-like properties. Interestingly, three putative homologues of *MATERNAL EFFECT EMBRYO ARREST* (*MEE)* in Arabidopsis were found to be differentially expressed during early and late embryo development in Norway spruce (Vestman et al. 2011). In Arabidopsis several female gametophyte-expressed *MEE* genes are essential for embryo development, and mutations in these genes lead to arrested embryogenesis (Pagnussat et al. 2005).

In embryogenic cultures of Norway spruce "nurse cells" positioned close to the embryo, but not cells in the embryo itself, express the chitinase 4 encoding gene *Chia4-Pa* (Wiweger et al. 2003). This gene is also expressed in the single cell-layered zone surrounding the corrosion cavity of the megagametophyte in Norway spruce seeds. Furthermore, an endochitinase from sugar beet (*Beta vulgaris*) stimulates early embryo development in Norway spruce (Egertsdotter and von Arnold 1998), and one of the secreted proteins, which has been shown to promote somatic embryogenesis in carrot (*Daucuscarrota*), is a glycosylated acidic endochitinase (De Jong et al. 1992). Since chitin is not present in plants, the mechanistic role of chitinases in stimulating embryo development is not clear. However, they might be involved in the cleavage of compounds such as lipo-chitooligosaccarides (LCOs) (Dyachok et al. 2002) and arabinogalactan proteins (AGPs) (van Hengel et al. 2001), thereby releasing signaling molecules that in turn stimulate embryo development and growth. LCOs have been isolated from conditioned medium of Norway spruce (Dyachok et al. 2002), and a partially purified fraction of these LCOs stimulates development of somatic embryos. In addition, AGPs isolated from seeds of Norway spruce can stimulate development of somatic embryos (Egertsdotter and von Arnold 1995). Furthermore, a strong coincidence in the temporal and spatial presence of both chitinases and AGPs that contain a chitinase cleavage site has been shown in carrot, which is interesting since chitinase-mediated processing generates AGPs with an increased capacity to promote somatic embryogenesis (van Hengel et al. 2002).

Altogether, it seems that extracellular compounds in conditioned medium can substitute for the female gametophyte and promote development of somatic embryos. However, more research is required for understanding the interaction between the secreted signaling molecules and somatic embryo development. Such knowledge will be of great importance for improving the culture conditions in order to increase the yield and quality of somatic embryos.

5. Apical-basal polarization

Early embryogenesis is a critical developmental phase when the apical-basal polarity is established through directional auxin transport and through specification of distinct expression domains of transcription factors. Early somatic embryos of Norway spruce are polar structures consisting of three major cell types:

the meristematic cells of the embryonal mass, the embryonal tube cells and the terminally differentiated vacuolated, expanding suspensor cells.

5.1 Polar auxin transport

Polar auxin transport (PAT) is of major importance for the correct patterning of the embryonal shoot and root meristems. Already after the first asymmetric cell division in Arabidopsis, auxin is transported polarly from the larger basal cell to the smaller apical cell (Friml et al. 2003). This has been suggested to trigger the initiation of transcription programs leading to contrasting developmental fates, where the apical cell gives rise to the embryo proper and the basal cell divides transversely to form one file of suspensor cells (Robert et al. 2013).

The role of PAT during somatic embryo development in Norway spruce has been studied by treating embryogenic cultures and developing embryos with the well-established PAT inhibitor 1-N-naphtylphthalamic acid (NPA) (Larsson et al. 2008a; Palovaara et al. 2010). During early embryo development, NPA-treatment leads to an increased amount of endogenous indole-3-acetic acid (IAA), suppression of PCD and abnormal differentiation of the suspensor. Mature embryos that have been treated with NPA show both apical and basal abnormalities. Typically the embryos have fused cotyledons, lack an organized SAM and have irregular cell divisions in the area of the root meristem. This shows that PAT is essential for the correct patterning of both the apical and the basal parts of Norway spruce embryos throughout the whole developmental process, and indicates that the role of PAT during embryogenesis is conserved in higher plants.

In order to monitor auxin responses during somatic embryo development in Norway spruce, the auxin responsive promoter of*GRETCHEN HAGEN3* (*GH3*) from soybean (*Glycine max*) fused to the GUS reporter gene was introduced into embryogenic cultures. According to the GUS assay results, auxin responses in PEMs one week after subculture in proliferation medium are patchy (Larsson 2011; Vestman 2012). At this time point, supplemented 2,4-D has been gradually depleted, and differential auxin levels between adjacent cells might be important for the outgrowth of early somatic embryos. During early embryogeny, an auxin response maximum is generated in the basal part of the embryonal mass. However, early embryos treated with NPA show no GUS activity in the embryonal mass, but increased GUS staining in the embryonal tube cells and suspensor cells, suggesting that polar transport of auxin from the suspensor to the embryonal mass is blocked. At late embryogeny, GUS activity is restricted to the basal part of the embryonal mass, embryonal tube cells and uppermost suspensor cells. Following NPA treatment, GUS activity is only detected in the upper part of the embryonal mass (Vestman 2012). This suggests that auxin during late embryogeny is transported

from the apical to the basal part of the embryo, which is in accordance to what has been shown in Arabidopsis (Friml et al. 2003; Robert et al. 2013).

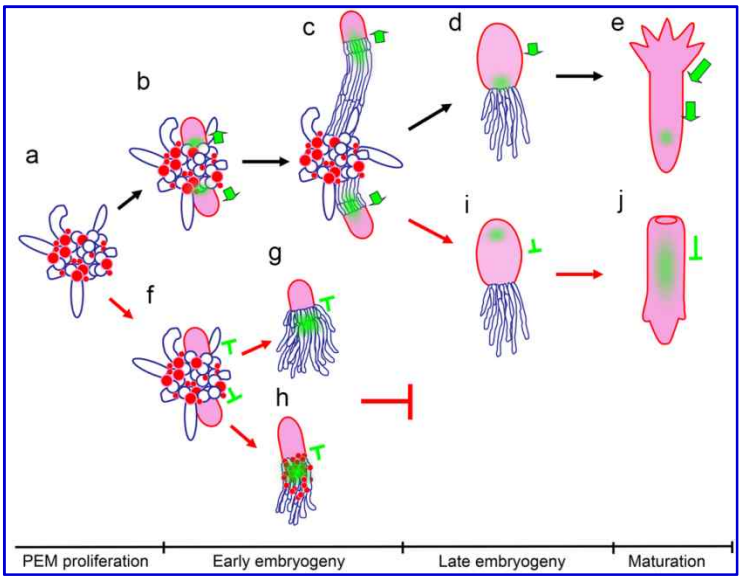

Figure 2. *Model for how polar auxin transport controls somatic embryo patterning in Norway spruce: (a) Proliferating proembryonic masses (PEMs) in the presence of the plant growth regulators (PGRs) auxin and cytokinin. (b) As early embryos start to differentiate from PEMs after withdrawal of PGRs, endogenous auxin is transported towards the newly formed embryonal mass. (c) During early embryo development auxin is transported from the suspensor and the tube cells to the embryonal mass. (d) During late embryogeny the auxin transport changes direction so that auxin is transported from the apical to the basal part of the embryonal mass. (e) During maturation, auxin is presumably transported from the developing cotyledons through the procambium and down to the root pole, thereby stimulating the outgrowth of separated cotyledons and the establishment of an organized root meristem. (f) Treatment with NPA blocks the polar auxin transport to the embryonal mass, leading to an auxin accumulation in the suspensor cells, tube cells and perhaps also in the cells of the embryonal mass most adjacent to the tube cells. (g) Embryos with supernumerary suspensor cells are formed if polar auxin transport is inhibited only during the earliest stages of suspensor differentiation. (h) Embryos with meristematic cells in the suspensor are formed if polar auxin transport is inhibited during both differentiation and elongation of the suspensor. We assume that these abnormalities abort further development and maturation of viable embryos. (i) Treatment with NPA during late embryogeny blocks the polar auxin transport from the shoot to the root pole, which leads to the development of mature embryos with fused cotyledons and*

unorganized root meristem (j). Green arrows indicate polar auxin transport, red arrows indicate NPA-treatment, green T indicates blocked polar auxin transport, green shadings indicate auxin accumulation. The model is adapted from Larsson et al. 2008b and extended according to new data (Larsson et al., unpublished).

The observations of the effects of blocked PAT on auxin response and embryo development enables the prediction of a model for auxin regulated embryo development in Norway spruce (Figure 2). In this model auxin is initially transported from the suspensor cells to the embryonal mass during early embryo development. This transport is essential for the developmental decisions of the embryonal tube cells and the suspensor thus affecting embryo patterning. During late embryogeny, the auxin transport direction is shifted so that auxin is transported from the apical part of the embryonal mass towards the basal part, where it presumably is involved in the specification of the future root meristem (Vestman 2012). Furthermore, auxin drainage from the apical part through gradually established pre-procambial cell files is most likely required for the outgrowth of separated cotyledons, as the cotyledons grow out like a doughnut-shaped ring if PAT is blocked (Larsson et al. 2008a).

5.2 Establishment of the primary body axis

Embryonic pattern formation requires highly regulated spatio-temporal cell division to set up the organ plan and the overall shape of the embryo. The orientation of the cell division plane is critical as it not only determines the position but also the fate of the daughter cells (van den Berg et al. 1995). The existence of stem cells at the basal part of the embryonal mass in Norway spruce embryos is so far conjectural, largely owing to the difficulties to distinguish these cells under microscope as they are anatomically very similar to the rest of the cells in the embryonal mass. We assume that the basal cells in the embryonal mass are distal stem cells, which after asymmetric divisions give rise to two daughter cell types: apical meristematic cells in the embryonal mass and basal vacuolated suspensor cells.

The *WUSCHEL-RELATED HOMEOBOX* (*WOX*) genes encode a family of plant-specific transcription factors. Members of the *WOX* gene family are characterized by the presence of a highly conserved DNA binding homeodomain. All *WOX* genes examined show very specific expression patterns, both spatially and temporally, which seems to be important for their molecular functions (Ueada et al. 2011). Phylogenetic analyses have divided the *WOX* gene family into three major clades (van der Graaff et al. 2009). The Norway spruce *WOX* genes are represented in all three clades (Hedman et al. 2013).

The *WOX* gene family members, *AtWOX2*, *AtWOX8* and *AtWOX9* regulate

early embryonic patterning in Arabidopsis. *AtWOX2* is expressed in the apical daughter cell of the zygote and *AtWOX8* and *AtWOX9* are expressed in the basal cell and its descendants (Haecker et al. 2004; Breuninger et al. 2008; Ueda et al. 2011). The Arabidopsis *wox8wox9* double mutants show aberrant cell division orientations both in the embryo proper and in the suspensor (Breuninger et al. 2008). The Norway spruce *PaWOX8/9* is most similar in sequence to *AtWOX8* and *AtWOX9*, and it is preferentially expressed during embryo development (Palovaara et al. 2010; Hedman et al. 2013; Zhu et al. 2014). The expression level of *PaWOX8/9* is high during early and late embryogeny and decreases with the onset of the embryo maturation phase. Down-regulation of *PaWOX8/9* results in embryos having an aberrant morphology caused by disturbed orientation of the cell division plane in the basal part of the embryonal mass during early and late embryogeny (Zhu et al. 2014). In addition to the normal anticlinal division, the stem cells also divide periclinally and incline, which retains both daughter cells in the basal part of the embryonal mass and results in a radial growth of the embryonal mass. As a consequence, a high frequency of the late embryos are cone-shaped and they develop into mature embryos having a heart-shaped morphology. Furthermore, the expression of several key cell-cycle-regulating genes are affected when *PaWOX8/9* is down-regulated.

These results suggest that *PaWOX8/9* acts as an important regulator for establishing the apical-basal embryo pattern in Norway spruce and that its function is evolutionarily conserved between angiosperms and gymnosperms. This function is accomplished by controlling the orientation of the cell division plane and cell fate determination during early embryonic pattern formation.

5.3 Programmed cell death

The dynamics of embryo growth require the removal of cells by PCD once their functions are no longer needed. The development of somatic embryos in Norway spruce is dependent on two successive waves of PCD (Filonova et al. 2000b). The first wave is responsible for the degradation of PEM cells when early embryos differentiate in response to withdrawal of PGRs, and the second wave eliminates the terminally differentiated suspensor cells during early embryogeny.

The establishment of the apical-basal polarity in early embryos is dependent on a gradient of cells at different stages of PCD along its apical-basal axis, starting with living meristematic cells in the embryonal mass, via the embryonal tube cells that are committed to death, to the cell corpses at the basal end of the suspensor (Bozhkov et al. 2005a). Thus, cells at all successive stages of PCD can be observed simultaneously in the same embryo along its apical-basal axis. During execution of this PCD, the cytoplasm and organelles are gradually removed by growing lytic vacuoles (Filonova et al. 2000b), which are the

morphological hallmark of the vacuolar type of cell death that is common for most examples of developmentally regulated PCD in plants (van Doorn et al. 2011). Re-organization of actin filaments into thick cables distributed parallel to the apical-basal axis of the suspensor cells is essential for their disassembly, which also involves gradually dismantling of cortical microtubules (Smertenko et al. 2003). Execution of vacuolar cell death in the suspensor is a slow energy-demanding process that takes several days. Noteworthy, the plasma membrane remains intact until the culminate stage of PCD when the tonoplast collapses and the release of hydrolytic enzymes lead to rapid digestion of the remaining cytoplasm leaving behind only the cell wall (Bozhkov et al. 2005a).

Vacuolar cell death is associated with and critically requires enhanced autophagic activity. Knockdown of autophagy-related genes *ATG5* and *ATG6* in Norway spruce embryogenic cultures does not prevent cell death but induces a switch from vacuolar cell death to necrosis, thereby abolishing suspensor differentiation and apical-basal patterning (Minina et al. 2013). In contrast to the completely cleared contents of cell corpses produced by vacuolar cell death, necrotic cell corpses remain largely unprocessed owing to mitochondrial dysfunction and energetic catastrophe. A series of reverse genetics experiments have further revealed that activation of autophagy during vacuolar PCD in the embryo-suspensor is downstream of a type II metacaspasemcII-Pa (Minina et al. 2013), whose activity is likewise instrumental for both PCD and embryogenesis in Norway spruce (Suarez et al. 2004; Bozhkov et al. 2005b). Since suppression of *mcII-Pa* expression by RNAi inhibits autophagic flux and led to a similar switch from vacuolar to necrotic cell death due to autophagy deficiency (Minina et al. 2013), we postulate that type II metacaspase and autophagy play dual roles in cell death regulation: they execute vacuolar cell death and at the same time protect differentiated cells from necrosis (Minina et al. 2014a; Minina et al. 2014b).

Molecular mechanism of mcII-Pa-dependent activation of autophagy remains elusive. The only so far known substrate cleaved *in vivo* by mcII-Pa is an evolutionarily conserved multifunctional regulator of gene expression, Tudor staphylococcal nuclease (TSN) (Sundström et al. 2009). In a recent study using Arabidopsis we demonstrated a critical requirement of TSN in the formation and function of the cytoplasmic messenger ribonucleoprotein complexes, stress granules and processing bodies, the major sites of translational repression during stress (Gutierrez-Beltran et al. 2015). Since it has also been shown that autophagy is required for both degradation and assembly of stress granules (Buchan et al. 2013; Seguin et al. 2014) it is tempting to speculate that mcII-Pa-mediated processes of TSN cleavage and activation of autophagy are parts of the same signaling nexus that governs execution of vacuolar PCD in the Norway spruce embryo-suspensor. More information about PCD in somatic embryogenesis can be found in a recent review by Smertenko and Bozhkov (2014).

6. Radial patterning

In addition to the apical-basal polarity along the shoot-root axis the basic body plan of plant embryos also shows a radial organization of primary tissue layers: epidermis (derived from the protoderm), cortex and endodermis (derived from the ground tissue) and pericycle and vascular tissues (derived from the procambium). The epidermal cells secrete lipids and waxes to their outer cell wall. This results in the formation of a cuticle layer early during embryo development, after differentiation of the protoderm (Yeats and Rose 2013).

Differentiation of the protoderm is the earliest event of radial pattern formation. In angiosperms, the protoderm formation is promoted by periclinal divisions at the octant stage and later maintained by strict anticlinal cell divisions in the growing embryo (Jürgens et al. 1994). In conifers, cells in the outer layer of the embryonal mass divide mainly anticlinally, but also periclinally giving rise to additional internal layers (Singh 1978). Consequently, the strict division pattern for the protodermal and epidermal cells is less pronounced in conifers.

The *ARABIDOPSIS THALIANA MERISTEM L1* (*AtML1*) gene, belonging to the homeodomain GLABRA2 (HD-GL2) family also named the HD-Zip IV family, is first expressed in the apical cell after the first asymmetric division of the zygote (Lu et al.1996). At the dermatogen stage the expression becomes restricted to the protoderm. Similar to its angiosperm homologues, the expression of the *Piceaabies Homeobox1* (*PaHB1*) gene switches from a ubiquitous expression in PEMs to a protoderm-specific expression in developing embryos (Ingouff et al. 2001). Furthermore, ectopic expression of *PaHB1* in the inner cell layers of the early embryo leads to an early developmental arrest caused by a lack of protoderm. This shows that the specific expression pattern of *PaHB1* is important for protoderm specification. In accordance, a phylogenetic analysis of the HD-GL2 family has revealed that *PaHB1* is strongly associated with a subclass consisting of protoderm/epidermis-specific genes (Ingouff et al. 2001). Another member of the HD-GL2 family in Norway spruce is *PaHB2* (Ingouff et al. 2003). *PaHB2* is a homolog to the *ANTHOCYANINLESS2* (*ANL2*) gene in Arabidopsis, which is involved in maintaining the subepidermal-layer identity (Kubo et al. 1999). *PaHB2* is uniformly expressed in PEMs and early embryos. Later during embryo development *PaHB2* is expressed in the outermost layer of the cortex and the root cap. However, it is presently not clear if *PaHB2* is involved in the specification and/or maintenance of the cortex identity. Together, these resuts suggest a conserved protodermal/epidermal and subepidermal expression of HD-GL2 in seed plants.

In Arabidopsis *AtWOX2* is regulating the differentiation of the protoderm. Embryos from the Arabidopsis *wox2* mutant fail to correctly form the protodermal layer by periclinal divisions in one or more cells in the upper part of the embryo

(Breuninger et al. 2008). The expression of *PaWOX2*, the corresponding homolog of *AtWOX2* in Norway spruce, is transiently up-regulated during early and late embryogenesis (Zhu et al. under preparation) and it is expressed both in the embryonal mass and the suspensor (Palovaaraet a. 2010). Down-regulation of *PaWOX2* results in aberrant early embryos which fail to form a protoderm, instead vacuolated cells ectopically develop on the surface of the embryonal mass. In addition, the aberrant embryos lack a cuticularized layer covering the embryonal mass. Embryos lacking a functional protoderm cannot develop into normal mature embryos. This shows that *PaWOX2* is important for specification of a protoderm and suggests that *WOX2* exerts a conserved role in protoderm development in both gymnosperms and angiosperms.

The functions of lipid transfer proteins (LTPs) have been widely discussed. One suggestion has been that LTPs are involved in secretion or deposition of extracellular lipophilic material, such as cutin monomers, the main components of the cuticle layer (Thoma et al. 1994). The Arabidopsis *LTP* gene (*AtLTP*) is expressed in the protoderm at the dermatogen stage (Vroemen et al. 1996). Furthermore, the carrot *LTP* gene, *EP2*, is expressed in precursor cell clusters from which somatic embryos develop (Sterk et al. 1991). Expression of the gene is then restricted to protodermal cells. In the temperature sensitive (ts) carrot mutant *ts11,* the embryos are develomentally arrested at the late globular stage. The mutant embryos lack a fully formed protoderm, and the *LTP* gene is expressed in the subepidermal cell layer rather than in the protoderm (Sterk et al. 1991). The *Pa18* gene, encoding a putative Norway spruce LTP, is expressed during somatic embryogenesis in Norway spruce (Sabala et al. 2000). The *Pa18* gene is expressed in all cells in PEMs, however, the expression is restricted to the protoderm in developing embryos. Ectopic expression of *Pa18* causes aberrant embryo development, which coincides with the maintenance of *Pa18* expression in the inner cell layers of the embryo. In the aberrant embryos, the outer cells in the maturing embryos frequently become elongated and vacuolated instead of remaining small and uniform. This shows that a correct expression pattern of the *LTP* gene *Pa18* is crucial for development of a functional protoderm, which is in accordance to what has been shown for other *LTP* genes in angiosperms.

Taken together, expression of *PaWOX2* is required for definition of the protoderm. Furthermore the inner cell layers in the early embryo must be devoid of *PaHB1* and *Pa18* to proceed through embryogenesis.

7. Differentiation of shoot meristem and separated cotyledons

In seed plants major patterning events, such as the establishment of stem cell niches in shoot and root meristems take place during embryogenesis. The establishment of the embryonal SAM in Arabidopsis is dependent on the

expression of the homeodomain containing transcription factor *SHOOT MERISTEMLESS* (*STM*). *STM* is one of four class 1 *KNOTTED-like homeobox* (*KNOX1*) genes in Arabidopsis. The three other *KNOX1* genes act redundantly with *STM* to maintain SAM characteristics. *STM* is first expressed in a single cell in the apical part at the dermatogen stage (Long et al. 1996). Later *STM* is expressed in the incipient SAM between the emerging cotyledons. Strong *stm-1* homozygous mutants do not form an embryonal SAM (Barton and Poethig 1993). Four *KNOX1* genes have been identified in Norway spruce, *HBK1*, *HBK2*, *HBK3* and *HBK4* (Sundås-Larsson et al. 1998; Hjortswang et al. 2002; Gulliet-Claude et al. 2004). Phylogenetic analyses have shown that the four Norway spruce *KNOX1* genes form a monophyletic group suggesting that they have diversified after the split between angiosperms and gymnosperms (Guillet-Claude et al. 2004). *HBK1* and *HBK3* are expressed in all tested embryogenic cell lines of Norway spruce, including cell lines which are developmentally arrested, while *HBK2* and *HBK4* are only expressed in cell lines that are competent to form mature embryos (Hjortswang et al. 2002; Larsson et al. 2012a). Overexpression of *HBK3* leads to enlarged SAM in somatic embryos and an accelerated differentiation of early embryos from PEMs, while down-regulation of *HBK3* precludes embryo differentiation (Belmonte et al. 2007). During embryo development the *HBK2* and *HBK4* genes are significantly up-regulated concomitantly with the formation of an embryonic SAM, while the up-regulation is delayed in somatic embryos with aberrant SAM formation after NPA treatment (Larsson et al. 2012a). In contrast, *HBK1* and *HBK3* are up-regulated prior to SAM formation, and their temporal expression is not affected by NPA treatment. This suggests that the function of *HBK2* and *HBK4* are connected to the establishment of SAM, while *HBK1* and *HBK3* have more general functions during embryo development.

In Norway spruce, the cotyledons develop as a crown surrounding the incipient SAM and the radial symmetry is retained in the seedling. In contrast, in most angiosperms, cotyledon organogenesis breaks the radial symmetry in the apical embryo domain and marks the transition to bilateral symmetry. In Arabidopsis, the formation of cotyledon boundaries and the establishment of the embryonal SAM are dependent on the redundant function of three *CUP-SHAPED COTYLEDON* (*CUC*) genes (Aida et al.1997; Takada et al. 2001; Vroeman et al. 2003). The *CUC* genes belong to the large plant-specific *NAC* gene family (from petunia *NAM*, Arabidopsis *ATAF1* and *ATAF2*, and *CUC2*) (Aida et al. 1997). Two *CUC* orthologues have been described in Norway spruce (*PaNAC01* and *PaNAC02*; Larsson et al. 2012b). Based on phylogenetic analysis together with motif analysis it seems that *PaNAC01* is most similar to *CUC1* and *CUC2,* and *PaNAC01* can functionally substitute for *CUC2* in the *cuc1cuc2* mutant. The expression of *PaNAC01* increases as early embryos differentiate, and remains at a steady state until the separated cotyledons are clearly visible. However, the up-regulation of

PaNAC01 is reduced in embryos that form fused cotyledons and lack a functional SAM after being treated with NPA. This expression profile of *PaNAC01* shows that the gene is PAT-regulated and indicates that it is associated with SAM differentiation and cotyledon formation. In accordance, the Arabidopsis *cuc1cuc2* double mutant lacks a functional SAM and is therefore seedling lethal (Aida et al. 1997).

These results suggest that differentiation of a functional SAM and separated cotyledons are regulated by *PaNAC01* and *HBK2/HBK4* and that the expression of these genes are PAT-dependent, indicating that central parts of the regulatory network for SAM and cotyledon formation are conserved between angiosperms and gymnosperms.

8. Conclusion

By using somatic embryos and reverse genetics it has been possible to identify and characterize important processes during early embryo development in Norway spruce, which have been summarized in Table 1.

Table 1. *Key processes during development of somatic embryos in Norway spruce.*

Process	Developmental stage						Essential genes
	PEMs to EEs[a]	EEs	EEs to LEs[b]	LEs	Early MEs[c]	MEs[d]	
Signaling between the embryo and nurse cells	+	+	+ +	+			*MEE* genes *Chia4-Pa*
PCD	+	+	+				*ATG5*, *ATG6*, *mcII-Pa*
Apical–basal polarization		+	+	+			*PaWOX8/9*
Transition from morphogenic to maturation phase	+	+	+	+			*PaHAP3A*
				+	+	+	*PaVP1*
Radial patterning		+					*PaHB1*, *PaWOX2*, *Pa18*
				+			*PaHB2*
Differentiation of SAM and separation of cotyledons				+			*PaNAC01*
					+		*HBK2*, *HBK4*

[a] Transition from proembryogenic masses (PEMs) to early embryos (EEs).
[b] Transition from EEs to late embryos (LEs).
[c] Early mature embryos (MEs).
[d] Fully mature embryos.

Taken together, the regulation of embryo development in Norway spruce has many similarities to what has been reported from angiosperms. Although it has to be kept in mind that we have had to focus on genes that have been characterized in angiosperms, mainly owing to the lack of gymnosperm reference genomes and technical limitations. Lately there has been a rapid emergence of technological advances in plant molecular biology and recently the first draft of genome sequences of three conifer species, including Norway spruce (Birol et al. 2013; Nystedt et al. 2013; Zimin et al. 2014), was presented. Together these new tool-kits will facilitate functional analyses of genes important for unique conifer traits; genes that in previous microarray and RNA-seq experiments have been annotated to angiosperm genes without known function or that completely have lacked an angiosperm homologue. The knowledge gained from genes and processes that take place during differentiation and development of somatic embryos can now serve as a basis for further studies of conifer specific processes. The successive developmental stages leading to cotyledonary somatic embryos must be understood fundamentally for optimal management of somatic plant regeneration systems.

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Impact of molecular studies on somatic embryogenesis development for implementation in conifer multi-varietal forestry

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Abstract

Somatic embryogenesis coupled with cryoprerservation has clear implications for the implementation of multi-varietal or clonal plantation forestry in conifers. To achieve this, there are strong requirements for high performance of the process from initiation of embryogenic tissue to the production of somatic embryo and plants with the quality that is standard for seedlings. The tedious trial and error strategy currently in use to develop somatic embryogenesis in conifers could greatly benefit from targeted molecular studies of embryogenesis-related candidate genes and from the more recent development of genome-wide "omics" studies. The question is: has the complex and still fragmented knowledge of this subject already delivered accurate molecular markers or new tools to refine somatic embryogenesis protocols? We review the impact of already existing molecular studies that address (epi)genetic issues to demonstrate the embryogenic state (embryogenecity) of initiated lines, to check for (epi)somaclonal variation during

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the whole process and to assess the regenerative capacity (maturation ability) of propagated embryogenic lines. We further highlight a few studies with some practical outcomes to check or stimulate the embryogenecity and maturation ability in conifers.

Keywords: Embryogenic potential; Embryo development; Embryo quality; (Epi)Genetics, Gene functional studies; Metabolomics; Molecular markers; Proteomics; Maturation ability; Transcriptomics

1. Introduction

A long life cycle, an unreliable seed production, a very limited capability for conventional vegetative propagation (i.e., through cuttings) and impeded crossing procedures predestine most conifers for vegetative multiplication by somatic embryogenesis. This process has worldwide been investigated for economically important conifer species and an increasing number of secondary species since the early 1980s (Klimaszewska et al. 2015). In the meantime advantages of the somatic system have taken effect and the possible integration of somatic embryogenesis in the chain of seed propagation and clonal plantation (multi-varietal) forestry has become tangible (Klimaszewska et al. 2007; Lelu-Walter et al. 2013; Colas and Lamhamedi 2014). Somatic embryogenesis offers new tree improvement strategies in favour of productivity, genotypic diversity and stability of improved varieties, particularly in regard to future socio-economical demands in the context of climate change. The ongoing development of genome-wide methods in conifers has huge perspectives for breeding (reviewed in Plomion et al. 2015), particularly for the accurate, early identification of elite genotypes (varieties) in breeding populations (e.g., genomic selection). Scaling-up the production of these selected genotypes will ultimately require powerful technologies such as somatic embryogenesis. Therefore, it is likely that synergies will be gained from conventional breeding, genomic selection and somatic embryogenesis to implement innovative, multi-varietal or clonal plantation forestry in conifers (El-Kassaby and Klápště 2015).

A strong requirement for the application of somatic embryogenesis is the availability of highly effective protocols for initiation and subsequent culture of embryogenic tissue (ET) up to the maturation and germination phases of fully mature cotyledonary somatic embryos (SEs). The resulting somatic seedlings (emblings) must be of high quality, similar to that of conventional seedlings, to be widely adopted at operational costs in both breeding programmes and plantation forestry. Klimaszewska et al. (2015) recently gave a clear picture about the tedious

trial and error strategies traditionally used to refine protocols in conifers. Such strategies are still in use due to the poor understanding of the biological, physiological, cellular and molecular mechanisms regulating ET initiation and subsequent embryo patterning and development. Most successful efforts in developing somatic embryogenesis were achieved from very juvenile material, i.e., zygotic embryos (ZEs) from immature to mature seeds. Recalcitrance in initiating somatic embryogenesis is high in older trees up to the adult vegetative and reproductive phases (Bonga et al. 2010). Long-term cryopreservation of initiated ET from ZEs propagated in the form of embryonal masses (EMs) or proembryogenic masses (PEMs) is thus required to preserve juvenility while emblings are field-tested for traits of interest. Although cryopreservation has clear advantages for the management of genetic resources, it is adding complexity to the somatic embryogenesis process. Ineffective methodological transfer between species within the highly diversified conifer group further illustrated real needs for a better knowledge of the somatic embryogenesis process at the molecular level and for marker-assisted development.

Classical genetic approaches used to elucidate embryo development in herbaceous angiosperms are largely not practical for trees owing to their delayed adult reproductive phase, large physical size and, in the case of conifers, very large genome. Targeted molecular expression studies, and more recently the development of genome-wide profiling of genes (transcriptomics), proteins (proteomics) and metabolites (metabolomics) provided increasing opportunities in conifers to unravel critical processes and gene cohorts involved at specific embryo developmental stages and transitions (Vestman et al. 2011; de Vega-Bartol et al. 2013; Businge et al. 2012, 2013; Morel et al. 2014a,b; reviewed in Trontin et al. 2015). Furthermore, the power of conifer "omics" is becoming even more evident as a means to investigate both genetic and epigenetic issues as large genome sequence resources or full genome sequences are now available for a number of pine and spruce species (Plomion et al. 2015). These studies also clearly benefit from reference data in the model plant *Arabidopsis*. Reverse genetics, through genetic transformation of ET, is also useful for some conifers to test the function of embryogenesis-related candidate genes (Belmonte et al. 2007; Trontin et al. 2013; Zhu et al. 2014).

Molecular aspects of developmental pathways from early SE differentiation and patterning to late embryo development and embling growth in conifers have now been investigated for more than 20 years. Progress achieved up to now has resulted mostly from targeted gene expression studies and, more recently, from genome-wide approaches (since Cairney et al. 2000). Comprehensive reviews of this complex and still fragmented knowledge with regard to conifers can be found in Dong and Dunstan (2000), von Arnold et al.

(2002), Stasolla et al. (2003a), Cairney and Pullman (2007), and more recently Trontin et al. (2015). Furthermore, Elhiti et al. (2013) and Mahdavi-Darvari et al. (2015) reviewed this topic for plants in general with a focus on the initial step of embryogenic induction. Most inputs in conifers deal with *Picea abies* as the species most used for modelling SE development (reviewed in von Arnold et al. 2015, in this book).

Besides this fundamental knowledge, molecular studies have the potential to deliver accurate molecular markers or tools in order (1) to demonstrate embryogenecity (i.e., the embryogenic state/potential) in competent cells from explants and in initiated tissues, (2) to check for (epi)somaclonal variation and, in a broader sense, to establish clonal identity during the whole somatic embryogenesis process including in enabling technologies such as cryopreservation, (3) to assess the regenerative capacity of propagated ET, i.e., to test their ability to regenerate high-quality cotyledonary SE after the maturation and germination steps, and ultimately, (4) to stimulate the embryogenic potential as well as to improve the regenerative capacity of embryogenic lines. Such practical outcomes could be of significant interest to help in refining somatic embryogenesis of conifers and to achieve an operational level of performance in plantation forestry.

Our objective for this chapter was specifically to review some current perspectives gained from conifer molecular studies in regard to the four above listed practical considerations with a focus on molecular markers of putative interest from genes or from other DNA regions in the genome. Such markers should be sensitive enough, easily detectable and characteristic of the somatic embryogenesis step and/or of the SE developmental stage (Lara-Chavez et al. 2012). For embryogenecity we primarily reviewed studies aimed at a comparison between ET and non-embryogenic callus (NEC) or non-embryogenic vegetative tissue. For regenerative capacity, we mainly considered studies that compare productive (plant-forming) and non-productive embryogenic lines with regard to both maturation yield and SE quality.

2. Is this tissue embryogenic?

Embryogenic induction is the first stage of somatic embryogenesis, during which specific genes are activated by either application of exogenous plant growth regulators (PGR) or stresses to change somatic cells into embryogenic cells (reviewed in Elhiti et al. 2013). This early stage is difficult to analyze since there is no clear cytological or molecular marker that allows distinguishing and separating embryogenic cells from non-embryogenic ones.

Adult conifers are commonly accepted as highly recalcitrant to vegetative propagation (reviewed in Bonga et al. 2010). Therefore, immature or mature seed has routinely been used to initiate somatic embryogenesis for the production of

clonal trees (reviewed in Klimaszewska et al. 2015). The potential to initiate embryogenic cultures decreases as the zygotic embryos develop (e.g., *Pinus* spp.) or later during germination (e.g., *Picea* spp., Uddenberg et al. 2011). ET often becomes non-embryogenic during sub-culture (i.e., non-plant forming) in some conifers. Interestingly, somatic embryogenesis-derived plant material may have a higher embryogenic potential than seed-derived material (Klimaszewska et al. 2011).

Therefore, the practical application of somatic embryogenesis as a micropropagation tool in clonal forestry can highly benefit from a more consolidated knowledge of gene markers associated with the embryogenic potential of tissues. Embryogenic induction from somatic cells includes cell dedifferentiation, expression of totipotency and commitment to somatic embryogenesis (Elhiti et al. 2013; Mahdavi-Darvari et al. 2015). Elhiti et al. (2013) provided a list of twelve genes likely to be involved in the different steps of embryogenic induction in plants: *Auxin Response Factor 19* (*ARF19*); *Polycomb Repressive Complexes 1* (*PRC1*); *WUSCHEL* (*WUS*), a member of the *WOX* gene family; *Shoot Meristemless* (*STM*), a member of the class-1 KNOX homeodomain-containing proteins; *Leafy Cotyledon 1* (*LEC1*); *Cyclin-dependent Kinase A;1* (*CDKA;1*); *Somatic Embryogenesis Receptor-like Kinase 1* (*SERK1*); *PROPORZ1* (*PRZ1*); *Curly Leaf* (*CLF*); *type-1 hemoglobin* (*GLB1*); *Heat Shock Protein 17* (*HSP17*); and *Reverse Glycosylating Protein* (*RGP-1*). Furthermore, Elhiti et al. (2013) identified a set of genes encoding 51 proteins that may be functionally associated to embryogenic induction. Mahdavi-Darvari et al. (2015) similarly reported a short list of 10 “clue” genes for embryogenic induction in plants. Three are common genes and part of the list established by Elhiti et al. (2013), i.e. *SERK*, *LEC*, and *WUS*. Two genes (*AGL15* and *PKL*) have been described by Elhiti et al. (2013) as functionally associated with genes involved in the acquisition of totipotency. *AGL15* (*Agamous-like 15*) is involved in repression of gibberellic acid (GA) biosynthesis and is reported to be directly induced by *LEC* genes. *PKL* (*PICKLE*) encodes a putative chromodomain helicase DNA-binding protein 3 involved in chromatin remodeling that may activate some genes such as *SERK* (Elhiti et al. 2013). Five additional genes were also highlighted by Mahdavi-Darvari et al. (2015): *Glutathione-S-transferase* (*GST*); *Germin-like Protein* (*GLP*); *Baby Boom* (*BBM*); *Ethylene Response Factor* (S*ERF1*); and *Arabinogalactan Protein1* (*AGP1*). These authors focused on *SERK*, *LEC* and *WUS* genes which have been widely considered to be markers of cell totipotency in plant species (Elhiti et al. 2013). Mahdavi-Darvari et al. (2015) gathered evidence suggesting that the *SERK* gene may be a less reliable marker of early somatic embryogenesis in plants because it can be detected in all stages of somatic embryogenesis and in NEC as well. On the other hand, *LEC* and *WUS* genes seem to be reliable because their high expression primarily occurs in

the early stage of cell differentiation and because of its embryo-specific expression pattern. Moreover, *GLP*-encoding genes (and their encoded proteins) are proposed to be useful markers of embryogenic potential because of their high expression, specifically at the earliest stages.

Conifers apparently express homologs of some of these 19 plant genes involved in embryogenic potential (Table 1). For most genes it remains to be established whether they share similar expression patterns and functions (Cairney and Pullman 2007). To date there is no conifer study reporting on *PRC-1* (dedifferentiation), *AGL15, GLB1, PKL* (acquisition of totipotency), *PRZ1, STM* and *SERF1* (commitment to embryogenesis). Only a few have been properly

Table 1. *Putative key plant genes involved in embryogenic induction steps which have been identified in conifer species during somatic embryogenesis.*

Induction step	Key genes[a]		Conifer related gene	Species investigated	Reference
	Elhiti et al. (2013)	Mahdavi-Darvari et al. (2015)			
Dedifferentiation	*ARF19*		*ARF19*	*Picea glauca*	Rutledge et al. (2013)
	RGP-1		*RGP-1*	*Picea glauca*	Lippert et al. (2005)
			AaRGP	*Araucaria angustifolia*	Schlögl et al. (2012)
	HSP17		*HSP17*	*Picea glauca*	Rutledge et al. (2013)
Totipotency	*SERK1*	*SERK*	*AaSERK1*	*Araucaria angustifolia*	Steiner et al. (2012)
			SERK	*Picea balfouriana*	Li et al. (2014a)
			SERK1-like	*Picea glauca*	Klimaszewska et al. (2011)
	LEC1	*LEC*	*AaLEC*	*Araucaria angustifolia*	Schlögl et al. (2012)
			PaHAP3A	*Picea abies*	Uddenberg et al. 2011
			CHAP3A	*Picea glauca*	Klimaszewska et al. (2010, 2011)
			PcHAP3A	*Pinus contorta*	Park et al. (2010)
			PsHAP3A	*Pinus sylvestris*	Uddenberg et al. (2011)
			LEC1	*Pinus radiata*	Garcia-Mendiguren et al. (2015)
	WUS	*WUS*	*AaWOX*	*Araucaria angustifolia*	Schlögl et al. (2012)
			PaWOX2, 3, 4, 5, 8A, 8B, 8/9, 13	*Picea abies*	Palovaara et al. (2010), Palovaara and Hakman (2008), Hedman et al. (2013)
			WOX2, WUS (ectopic)	*Picea glauca*	Klimaszewska et al. (2010, 2011)
			WOX9, 12	*Picea balfouriana*	Li et al. (2014a)
			PcWOX2	*Pinus contorta*	Park et al. (2010)
			WOX2	*Pinus radiata*	Garcia-Mendiguren et al. (2015)
	CLF		*CLF*	*Pinus pinaster*[b]	de Vega-Bartol et al. (2013)
Commitment	*CDKA;1*		*cdc2Pa*	*Picea abies*	Footitt et al. (2003)
			Various CDK	*Picea glauca*	Rutledge et al. (2013)
		GST	*Glutathione-S-transferase*	*Picea glauca*	Stasolla et al. (2003b)
		GLP	*LmGER1*	*Larix x marschlinsii*	Mathieu et al. (2006)
			GLP-like	*Pinus radiata*	Bishop-Hurley et al. (2003)
		BBM	*SAP2C*	*Picea glauca*	Klimaszewska et al. (2011)
		AGP1	*AGP*	*Picea balfouriana*	Li et al. (2014a)

[a]Additional key genes (see Elhiti et al. 2013, Mahdavi-Darvari et al. 2015) not identified in conifers to date: *AGL15, GLB-1, PKL* (acquisition of totipotency), *PRZ1, STM, SERF1* (commitment to somatic embryogenesis). [b]*CLF* was found expressed during ZE development.

investigated to date, i.e., *LEC, SERK* and *WOX* genes (Table 1). In the following we review conifer molecular studies reporting on expression of these key (and some other) genes during the embryogenic induction step and in initiated tissue obtained from explants. Our attempt was mainly to consider studies that compared ET and NEC or other non-embryogenic vegetative tissue.

2.1 Tissue from explants subjected to induction treatment

It is noteworthy that there are very few molecular studies dedicated to the early steps of induction of somatic embryogenesis in juvenile explants of conifers

(Uddenberg et al. 2011, Trontin et al. 2015). Significant insights in this matter came from studies of shoot bud explants of SE-derived clonal white spruce (*Picea glauca*) trees that have been responsive to induction treatment (Klimaszewska et al. 2011, Rutledge et al. 2013). With the aim of identifying marker genes to discriminate between NEC and EM, Klimaszewska et al. (2011) followed the expression pattern of eleven transcription factor (TF) genes before and during *in vitro* culture of primordial shoot explants. The selected genes included *Apetala2-like2* (*AP2-L2*), *LEC1-like* (*CHAP3A*, heme activated protein 3), *IAA2-like* (*IAA2*), *BabyBoom* (*SAP2C*), *SERK1-like* (*SERK1*), *KNOX* (*SKN1,2,3,4*), *ABI3/Viviparous* (*VP1*) and *WOX2-like* (*WOX2*). Several of these genes were differentially expressed in bud explants as early as 3-6 days after induction (i.e., *AP2-L2*, *SERK1*, *SKN1-4*, *IAA2* and *SAP2C*). However, only *CHAP3A*, *VP1*, *WOX2* and to a lesser extent *SAP2C* came out as potential markers of embryogenecity because they were found to be expressed almost exclusively in the early stages of somatic embryogenesis and thus allow EM to be distinguished from NEC and other types of tissue present before and during culture of the primordial shoot buds. *WOX2*, *VP1* and *CHAP3A* but not *SAP2C* were expressed exclusively in EM (Klimaszewska et al. 2010) and, therefore, they seem to be good markers of the embryogenic state. Although not clearly demonstrated, because expression levels were under reliable detection limits, a similar function was suggested to exist for *CHAP3A* and *VP1* by Uddenberg et al. (2011) during induction of secondary somatic embryogenesis from cotyledonary and germinating SE of *P. abies*. It was shown that expression of both genes is affected by the histone deacetylase inhibitor trichostatin A (TSA) which has a demonstrated impact on embryogenecity in *Arabidopsis* through activation of *LEC* and *ABI3* genes. A possible link between these genes and the epigenetic regulation of the chromatin structure is suggested.

Rutledge et al. (2013) combined microarray analysis with absolute quantitative PCR (qPCR) to investigate differentially expressed genes after somatic embryogenesis induction between a responsive and a non-responsive genotype of *P. glauca*, the same ones investigated by Klimaszewska et al. (2011). Eight of the most differentially expressed candidate genes remained differentially expressed until the end of the induction treatment, although to differing degrees. The four candidate genes activated in the responsive genotype were a *conifer-specific dehydrin* called *DHN1*, a homolog of *apoplastic class III peroxidase AtPrx52* (*PgPrx52*), and two putative conifer-specific protein coding genes (QT-repeat and proline-rich). This set of genes was associated to an adaptive stress response. In contrast activated genes in the non-responsive genotype were two *protease inhibitors* (*PgPI20a* and *PgPI20b*), a homolog of *apoplastic class III peroxidase AtPrx21* (*PgPrx21*) and a *cell wall invertase* similar to *AtcwINV1* (*PgcwINV1*). These genes were strongly indicative of a biotic defense response. However, it

remains unknown if there is a direct association between biotic defense elicitation and suppression of somatic embryogenesis induction in *P. glauca* (Klimaszewska et al. 2011). More genotypes need to be tested. Interestingly, several members belonging to the gene families described by Elhiti et al. (2013) were also detected in this study, namely *ARF19, HSP17* and *CDK* genes; however their fold-changes were less drastic than those of the chosen candidates. Table 2 lists the currently known embryogenesis-related genes which were found deregulated in conifer explants and tissue upon somatic embryogenesis induction.

Table 2. *Deregulated genes identified during somatic embryogenesis induction in conifers. Potential marker genes of embryogenecity highlighted by authors are in bold.*

Species	Gene differentially expressed	Compared material[a]	Reference
Picea glauca	Apetala2-like2 (*AP2-L2*), LEC1-like (***CHAP3A***), IAA2-like (*IAA2*), BabyBoom (***SAP2C***), SERK1-like (*SERK1*), Knotted1-like (*SKN1, SKN3/HBK2, SKN4*), Knotted2-like (*SKN2*), Viviparous (***VP1***), WOX2-like (***WOX2***)	1 responsive G 1 responsive G. Shoot buds/explants (3-6 d induction), nodules (needle, callus), ET, NET (later in induction)	Klimaszewska et al (2011)
	Dehydrin (*DHN1*), apoplastic class III peroxidase (*PgPrx52*); QT-repeat protein coding gene, Proline-rich protein coding gene, serine protease inhibitor 20a (*PgPI20a*) and 20b (*PgPI20b*); apoplastic class III peroxidase (*PgPrx21*); Cell Wall Invertase (*PgcwINV1*), *ARF19, HSP17, CDK*	1 responsive G producing (EM, NET) vs. 1 non-responsive G (NET) Shoot buds (0, 3, 7, 15, 21 days induction)	Rutledge et al (2013)
Picea abies	ABI3/Viviparous (***PaVP1***), LEC1-like (***PaHAP3A***)	4 SE dev. st.	Uddenberg et al. (2011)
Pinus sylvestris	ABI3/Viviparous (***PsVP1***), LEC1-like (***PsHAP3A***)	8 SE dev. st.	Uddenberg et al. (2011)

[a]dev. st.: development stage; G: genotype; ET: embryogenic tissue; EM: embryonal mass; NET: non-embryogenic callus or vegetative tissues; SE: somatic embryo

2.2 Initiated tissue from explants

More studies have reported on conifer embryogenesis-related gene expression in initiated ET because an unlimited amount of tissue can be obtained during proliferation. There are studies of targeted expression of a few genes and also of genome-wide analysis of expressed sequences that include small non-coding RNA (sRNA).

The *WUSCHEL (WUS)*-related homeobox (*WOX*) homologous genes have been the focus of several studies in conifer species. For *P. abies*, *PaWOX2* and *PaWOX8/9* were suggested as possible markers of embryogenic potential (Palovaara and Hackman 2008; Palovaara et al. 2010). Both genes shared a similar expression pattern during the entire embryo development, but their expression levels were highest at the early stages. Moreover, no *PaWOX2* expression was detected in NEC (Palovaara and Hackman 2008). Hedman et al. (2013) performed an extended study of the *WOX* gene family in *P. abies* and they were able to clone eleven *WOX* genes: *PaWUS*, *PaWOX2,3,4,5, PaWOX8/9, PaWOX8A,B,C,D a*nd

PaWOX13. Expression analysis of these genes confirmed previous observations of their role as possible markers for embryogenic potential. *PaWOX2* and *PaWOX8A* expression patterns were very specific as they could only be detected in PEMs proliferated in the presence of PGR and in late embryos, i.e., two weeks after transfer to maturation medium containing abscisic acid (ABA), but not in early (one week after withdrawal of PGRs) and mature embryos (5 weeks after transfer to ABA-containing medium). It was still possible to detect *PaWOX8A* in young needles but at very low levels. *PaWOX8/9* profile of expression agreed with the one that had been previously presented (Palovaara et al. 2010), namely a decrease in expression along with the progression of SE development, although it could still be detected in post-embryo development tissues but at very low expression levels (Hedman et al. 2013). Interestingly, *PaWOX8B* expression was only detected in PEMs, and *PaWOX5* was the only gene not expressed in PEMs (Hedman et al. 2013). Also in *P. glauca*, *WOX2* was found to be expressed exclusively in ET while remaining undetected in NEC (Klimaszewska et al. 2011) which was also observed in *Pinus radiata* (Garcia-Mendiguren et al. 2015). Overexpression in *P. glauca* of the related *WUS Arabidopsis* gene did not affect EM growth and pattern but affected SE maturation which suggests a role for *WUS/WOX* genes during early embryogenesis (Klimaszewska *et al.* 2010). Similarly, in *Pinus contorta*, *PcWOX2* was found to be expressed mainly in EM-like tissues initiated from mature trees whereas no expression was detected in the NEC derived from a seedling needle (Park et al. 2010). A *WOX* gene (*AaWOX*) was also upregulated during early somatic embryogenesis in *Araucaria angustifolia* (Schlögl et al. 2012). Overall, *WOX2* appeared to be a marker that can discriminate ET from NEC among conifer tissues.

For *P. contorta*, Park et al. (2010) found that a *LEC1*-like gene (*PcHAP3A*) was mainly expressed in NEC although it was also present in EM. Similarly, Garcia-Mendiguren et al. (2015) showed that *LEC1* is expressed in NEC lines initiated from shoot explants from adult *P. radiata* but at lower rates than in control EM initiated from ZEs. In contrast *LEC1/CHAP3A* was expressed almost exclusively in EM initiated from shoot bud explants of *P. glauca* (Klimaszewska et al. 2010, 2011) whereas a *LEC1* gene (*AaLEC*) was expressed at a low level from early to late SE development in *A. angustifolia* (Schlögl et al. 2012). Expression of this gene (*HAP3A*) is also high during early embryogenesis in *P. abies* and *P. sylvestris* (Uddenberg et al. 2011). It has been suggested that *LEC1*-like genes may have different, as yet unclear functions in conifers. *LEC* genes including *LEC1* are thought to act as an inductive signal for transition from GA to auxin biosynthesis (Elhiti et al. 2013). However ectopic expression of *CHAP3A* during early and late stages of SE development in *P. glauca* (Klimaszewska et al. 2010) had no phenotypic effect and did not affect expression of other TFs studied by

Klimaszewska et al. (2011). It was proposed that in *P. contorta* the *PcHAP3A* gene may be involved in cell division, which may be the reason why it is expressed differently in EM and NEC, as both are actively dividing tissues (Park et al. 2010). It has also been postulated that *LEC1* expression in NEC could reveal some relic, non-functional embryogenic potential, especially when EM and NEC are obtained from the same explant or when EM converts into NEC during prolonged subculture (Park et al. 2010; Garcia-Mendiguren et al. 2015).

Several studies in different species of angiosperms have associated *SERK1* with somatic embryogenesis potential of cell cultures (Steiner et al. 2012; Elhiti et al. 2013; Mahdavi-Darvari et al. 2015). However there are only two reports that describe putative *SERK* homologs in the literature for conifers, one for *P. glauca* (Klimaszewska et al. 2011) and the other one for *A. angustifolia* (Steiner et al. 2012). *P. glauca* putative *SERK* (*SERK1-like*) was ubiquitously expressed in EM and NEC although with expression values that were lower than the ones observed for non-cultured fresh explants (Klimaszewska et al. 2011). In *A. angustifolia,* Steiner et al. (2012) found that *AaSERK1* is transcriptionally active in embryogenic cell clusters but not in non-embryogenic cell aggregates. As observed for the *LEC1-like* gene and as mentioned by Mahdavi-Darvari et al. (2015), conifer *SERK* homologs may have different functions in conifers and may be a less reliable marker of early SE than genes such as *WOX2*.

Other transcripts related to key genes were found to be differentially expressed during early embryogenesis in conifers, either in zygotic (*CLF*) and/or in somatic embryogenesis (*RGP*, *CDK*, *GST*, *BBM*, *GLP* and *AGP*, Table 1) but it is still unclear if they can serve as gene markers of embryogenecity. Outside the key gene lists (Elhiti et al. 2013, Mahdavi-Darvari et al. 2015), only *ABI3/VP1* is a good candidate marker of embryogenecity in conifers (see Table 2). This gene is specifically expressed in emerging tissue during embryogenic induction (see above) in *P. glauca* (Klimaszewska et al. 2011), *P. abies* and *P. sylvestris* (Uddenberg et al. 2011). *VP1* was previously shown to be expressed in *P. abies* (Footitt et al. 2003; Vestman et al. 2011) and is still expressed at a high level in productive embryogenic lines until the cotyledonary stage (Fischerova et al. 2008). Furthermore *ABI3* expression remained undetectable in NEC of *P. radiata* while it is expressed in embryogenic lines (Garcia-Mendiguren et al. 2015).

Transcriptomic studies represent an additional and valuable source of new potential markers of embryogenecity as they give more information about clusters of genes that share similar patterns of expression. Bishop-Hurley et al. (2003) performed an initial genetic characterization of early embryo development in *P. radiata*, starting with a cDNA library built from cultured tissue that formed stage 1 embryos. Six gene families were found to be highly expressed during embryogenesis, in comparison to in NEC obtained from needles and to vegetative

tissues (roots, shoots and needles), namely four putative extracellular proteins: germin-like protein (*GLP*, in accordance with the proposed list by Mahdavi-Darvari et al. 2015), *β-expansin*, *21 kDa protein precursor, cellulase*, a *cytochrome P450 enzyme* and a gene with unknown function (*PRE87*). All these genes except the *cellulase* gene were shown to be expressed in both plant-forming and non-plant forming sublines of the same genotype (two genotypes tested), i.e., in both the juvenile and aged version of embryogenic lines. The authors proposed that these genes could be used as markers of ET but not as markers for both embryogenic and plant-forming potential (regenerative capacity).

For the same species, Aquea and Arce-Johnson (2008) performed an analysis of cDNA-AFLPs (amplified fragment length polymorphism) using three different stages of embryo development (stage 1-7 days in proliferation medium; stage 2-14 days in maturation medium; stage 3-30 days in maturation medium) and NEC induced from needles. The authors identified transcript-derived fragments up-regulated (50) or down-regulated (32) in PEM compared to in NEC. The up-regulated genes were similar to genes involved in cellular metabolism and in the stress response and the down-regulated genes were similar to genes involved in proteolysis, cell wall modification and signaling pathways. Five genes were proposed as marker of embryogeneicity: β-*expansin, enolase, sugar transport (STP1), metacaspase type II, SPRY protein, and uridilate kinase* (Aquea and Arce-Johnson 2008).

In *P. abies*, van Zyl et al. (2003) reported a cDNA array analysis of expression patterns of 373 genes at the beginning of embryo development. The authors reported an "up, down and up again" global gene expression signature as embryos developed through PEM-to-embryo transition (up), early embryogeny (down) and late embryogeny (up). Such a signature was not observed in a developmentally arrested "embryogenic" line incapable to form embryos. Among 35 cDNA clones differentially expressed between normal and blocked lines, 22 could be associated with early embryo pattern formation (mainly from the "cellular process category") and could be considered as marker genes of early embryogenesis in *P. abies*. However these markers are not ideal in practice as most appeared as downregulated genes in PEM. Only ten cDNA clones were found differentially expressed in the blocked cell line, half of them represented the functional category "Metabolism". Another microarray study in *P. abies* conducted by Stasolla et al. (2004) on differentially expressed genes between two normal and one blocked cell lines (including lines studied by van Zyl et al. 2003), further extended the list of potential marker genes of early embryogenesis. Again, the 52 genes identified by these authors are mostly repressed genes in PEM (Table 3).

More recently, high-throughput RNA-seq technology was applied to investigate the transcriptomes of ET and NEC from the same *Picea balfouriana*

genotypes (three genotypes were investigated, Li et al. 2014a). A set of 1,418 differentially expressed genes were identified in the ETs relative to the NEC, including 431 significantly up-regulated and 987 significantly down-regulated genes. The most significantly altered genes were involved in plant hormone signal transduction, metabolic pathways (starch and sucrose metabolism) and plant-pathogen interaction and phenylalanine metabolism (Li et al. 2014a).Among these genes Li et al. (2014a) highlighted *SERK*, *AGP* genes and *WOX* genes (*WOX9, WOX12*) as putative molecular markers of the early stages of somatic embryogenesis.

Few proteomic studies on ET induction and proliferation have been conducted in conifer species (Lippert el al. 2005, Zhao et al. 2015 and references herein). To date, only Zhao et al. (2015) compared ET with NEC derived from the same explant of the conifer *Larix principis-rupprechtii* Mayr using isobaric tags for relative and absolute quantitation (iTRAQ) combined with LC–MS/MS. From the 503 proteins detected, 71 were differentially expressed between ET and NEC with a high prevalence of proteins involved in metabolic processes and development, with also high prevalence of stress-related proteins (Zhao et al. 2015). A total of 12 proteins most upregulated in ET were proposed as marker of embryogenic potential (see Table 3).

Several recent studies in angiosperms have evidenced the involvement of epigenetic processes and particularly of sRNA such as microRNAs (miRNAs) in the regulation of somatic embryogenesis, both in the induction phase and in development, through repression of specific genes (Mahdavi-Darvari et al. 2015). By contrast, few reports have been released focusing on conifer species. Zhang et al. (2010a) hypothesized that aberrant expression of miRNAs could cause the loss of embryogenic ability in Japanese Larch (*Larix leptolepis = L. kaempferi*). The authors analyzed 3 genotypes for a comparison of miRNA expression patterns between ET and NEC derived from the same culture (i.e., NEC was obtained after prolonged subculture of ET), at 3 and 14 days after subculture. Four abiotic stress-induced miRNA families dominated the 165 differentially expressed miRNAs found in the comparison: *miR159*, *miR169*, *miR171* and *miR172*. Interestingly, *miR171* was up-regulated in the ET, whereas *miR159*, *miR169* and *miR172* were down-regulated (also partially supported by Zhang et al. 2012b). All four miRNA families target TFs that regulate a group of genes important for cell differentiation and development (Zhang et al. 2010a). In another study, the same authors confirmed the *MYB* homolog in *L. kaempferi*, *LaMYB33*, to be a target gene of *miR159* (Li et al. 2013), providing new evidence for miRNA-mediated ABA regulation during somatic embryogenesis, especially maintenance of the embryogenic/non-embryogenic potential. Similarly, Li et al. (2014b) showed that

Table 3. *Some deregulated genes identified from initiated tissues after somatic embryogenesis induction in conifers. Potential marker genes of embryogenecity highlighted by authors are in bold.*

Species	Gene/protein differentially expressed	Compared materiala	Reference
(Epi)Genetics			
Larix leptolepis	***miR159*** targeting *MYB101, MYB33 TFs*	3 G, ET vs. NET	Zhang et al. (2010a), Li et al. (2013)
	miR169 targeting *NF-YA* TFs (*LoNFYA1,2,3,4*)	3 G, ET vs. NET	Zhang et al. (2010a, 2015)
	miR171 targeting *Scarecrow-like6* TF (*SCL6*)	3 G, ET vs. NET	Zhang et al. (2010a), Li et al. (2014b)
	miR172 targeting *Apetala2* TF	3 G, ET vs. NET	Zhang et al. (2010a)
Targeted gene expression			
Picea abies	*WOX* (***PaWOX2***,*3,4,5,****8/9,8A***,*8B,13*)	Several G, various SE dev. st., ET, NET	Palovaara and Hackman (2008), Palovaara et al. (2010), Hedman et al. (2013)
	LEC1/HAP3; ***ABI3/VP1***	2 G, 4 SE dev. st.	Uddenberg et al. (2011)
	ABI3/VP1	1 G, 2 SE dev. st.	Footitt et al. (2003), Fischerova et al. (2008)
Picea glauca	***CHAP3A***, ***VP1***, ***WOX2***, ***SAP2C***, *AP2-L2, IAA2, SERK1, SKN1, SKN2, SKN3* and *SKN4*	1 responsive G. Shoot buds/explants (3-6 d induction), nodules (needle, callus), ET, NET	Klimaszewska et al. (2010, 2011)
Araucaria angustifolia	*AGO, CUC, WOX, LecK, SCR, VIC, LEC, RGP,* ***SERK1***	ET, 2 SE dev. st., NET	Schlögl et al. (2012), Steiner et al. (2012)
Pinus radiata	*LEC1,* ***ABI3***, ***WOX2***, *WOX4, SKN1, SKN2, SKN3, SKN4, histone 4, PCNA* and *YLS8*	ET (3 G), NET (5 G)	Garcia-Mendiguren et al. (2015)
Pinus sylvestris	***LEC1/HAP3***; ***ABI3/VP1***	1 G, 8 SE dev. st.	Uddenberg et al. (2011)
Pinus contorta	***PcWOX2***, *PcHAP3A*	EM-like (5 G), EM (1 G), NET (1 G)	Park et al. (2010)
Larix x marschlinsii	*LmGER1*	1 G, ET, NET	Mathieu et al. (2006)
Transcriptomics			
Pinus radiata	***Germin-like protein***, ***ß-expansin***, ***21 kDa protein precursor***, *cellulase*, ***cytochrome P450 enzyme*** and ***PRE87*** (unknown function).	2 G (ET with or lost maturation ability), NET	Bishop-Hurley et al. (2003)
	50 genes upregulated; 32 genes downregulated. ***-expansin, enolase, sugar transport (STP1), metacaspase type II, SPRY protein, uridilate kinase***	3 SE dev. st., NET	Aquea and Arce-Johnson (2008)
Picea abies	***Serine/threonine protein phosphatase***; *L-ascorbate peroxidase* (2); ***HSP70***; ***RNA binding protein***; ***Histone H3.3***; *SAM synthetase*; ***Cyclophilin*** (2); ***Ubiquitin-conjugating enzyme 9 and10***; ***Proline-rich protein***; ***Cyclophilin***; ***Glutaredoxin*** (2); LEA/***Dehydrin***; *Calmodulin*; ***Ribosomal S29-like protein***; *Tubulin beta-3 chain*; No hit (***drought-stress responsive***) (2); ***EF1α***; *K+channel protein*; ***10 kDa chaperonin***; *Thioredoxin H-type*; *HSP80*; *PCEBR*; ***SOD***; ***PS II 44 kDa*** *protein*; *Putative arabinogalactan protein*; *SAMDC proenzyme*; ***uncharacterized protein***; *Laccase*; ***Drought-induced protein***; ***Polyubiquitin***;	2 lines (normal vs. blocked)	Van Zyl et al. (2003)
	Ribosomal proteins (25); hypothetical and unknown proteins (13); *initiation factor 4A-15*; *flavanone 3-hydroxylase* (*FH3*); *-fructofuranosidase 1*; *UDP-galactose 4-epimerase like*; *glyceraldehyde 3-P precursor*; *CONSTANS-like*; *thioredoxin H*, *cytosolic ascorbate peroxidase*; *HSP*; *expansin*; *ubiquitin-conjugating enzyme*; *auxin-inducible protein 11* (*IAA11*); *casein kinase I*; *high mobility group protein-2 like*	3 lines (2 normal vs. 1 blocked)	Stasolla et al. (2004)
Picea balfouriana	987 downregulated and 431 upregulated genes. Most differentially expressed genes: *germin-like proteins* (*GLPs*); *ABA-receptor*; *cytochrome P450*; *chitinase*; *auxin-induced protein*; ***SERK***; ***AGPs***; ***WOX9***; ***WOX12***	3 G (ET vs. NET)	Li et al. (2014a)

Proteomics			
Larix principis-rupprechtii	32 proteins upregulated and 39 proteins downregulated (see Table 1 in Zhao et al. 2015). *Most downregulated proteins* in ET: oxidoreductases; catalases; 6-phosphogluconate dehydrogenase; ATP synthase; flavonoid 3' hydroxylase; profiling. *Most upregulated proteins in ET*: **triosephosphate isomerase**; **citrate synthase**; **aldose 1-epimerase**; **ARFs-GAPs**; **tubulin alpha-1**; **actin isoforms**; **alpha-1,4-glucan protein synthase**	1 G (ET vs. NEC)s	Zhao et al. (2015)

[a]dev. st.: development stage; G: genotype; ET: embryogenic tissue; EM: embryonal mass; NET: non-embryogenic callus (NEC) or vegetative tissues; SE: somatic embryo

miR171 is targeting the *SCARECROW-LIKE* 6 gene in *L. kaempferi* (*LaSCL6*), and concluded that posttranscriptional regulation of *LaSCL6* might participate in the maintenance of embryogenic potential.

An increasing number of new potential markers for embryogenecity of established cell lines can be expected thanks to genome-wide transcriptomics, epigenetic (sRNA/miRNA) and proteomics approaches. Table 3 provides a tentative summary of putative gene markers of embryogenic potential in conifers.

3. Is this somatic material true-to-type? Did (epi)somaclonal variation occur during *in vitro* culture?

3.1 After propagation and/or SE recovery in different conditions

After successful induction of embryogenesis, the ETs need to be propagated by regular subculture to fresh medium in order to obtain adequate amounts of tissue for subsequent recovery of mature SE or for cryopreservation. At this stage, the high rate of cell division represents an increased risk of accumulation of mutations in the tissues, which can be deleterious for SE developmental progression, and thus compromise mature SE recovery. Whenever SE can be recovered and converted to emblings these should be uniform and somaclonal variation, defined as a phenotypic variation, either genetic or epigenetic in origin, displayed among somaclones (Schaffer 1990), should be absent.

Normal morphology does not provide in all cases a reliable indicator of genetic and/or epigenetic stability (von Aderkas et al. 2003). Although *in vitro* cultures of conifers were originally considered to be genetically stable (Mo et al. 1989; Eastman et al. 1991; Isabel et al. 1993; Roth 1997), a number of approaches beyond morphological analyses have been used as diagnostic tools to investigate and monitor the true-to-typeness of either ETs or SEs. These approaches include karyology, flow cytometry, and molecular biology techniques to analyze DNA alterations and gene expression profiles. As a result of such studies, the occurrence of somaclonal variation in *in vitro* cultures of conifers has been reported for several species.

At the chromosomic level, the occurrence of alterations such as polyploidy and aneuploidy, have been found in *P. abies* (Fourré et al. 1997; Lelu 1988), *Picea mariana* (Tremblay et al. 1999), *Larix decidua* (Pattanavibool et al. 1995; von Aderkas and Anderson 1993; von Aderkas et al. 2003), *Abies alba* (Roth et al. 1997), *P. radiata* (O'Brien et al. 1996) and *Pinus nigra* (Salajova and Salaj 1992). In *A. alba* Roth et al. (1997) found that 3 years after addition of organic nitrogen to the medium, all cells in a 6-year-old embryogenic cell line were trisomic contrasting with the subclone maintained on medium without organic nitrogen. In addition, malformation of the suspensor cells and a loss of maturation capacity occurred. *L. decidua* embryogenic cultures maintained for 17 years showed duplication of DNA content and chromosome number several years after initiation, stabilizing around 24 chromosomes for most cultures (von Aderkas et al. 2003). Embryogenecity was lost completely in some lines, while in others the loss was only temporary suggesting physiological and or epigenetic effects. By contrast, the combination of morphological profiling and flow cytometric analysis to assess genetic stability during the several steps of *Pinus pinaster* somatic embryogenesis pointed to the absence of major changes in ploidy level even though abnormal phenotype embryos were included in the analyses (Marum et al. 2009a). Another type of DNA alterations was investigated in Norway spruce embryogenic cultures by measuring the sites of apurinic/apyrimidinic (AP) which are considered major lesions in DNA formed during base excision and repair of oxidized bases (Boiteux and Guillet 2004). Using this approach it was found that cultures grown on fructose displayed elevated levels of DNA damage when compared to cultures that had grown on sucrose or glucose (Businge and Egertsdotter 2014).

At the DNA sequence level, polymorphisms in specific DNA sequences have been observed which may result from point mutations of the DNA or from slipped-strand mispairing during the replication process (Kunkel and Benek 2000). Sequences such as simple sequence repeats (SSRs) are among the most variable sequences in genomes and have been particularly targeted in the investigation of genetic variation studies during the somatic embryogenesis and cryopreservation of conifers (Burg et al. 1999; Helmersson et al. 2004, 2008; Burg et al. 2007). SSR markers have yielded different results in several conifer species, and sometimes within the same species. Harvengt et al. (2001) found no variation in Norway spruce plants regenerated from SEs derived from the same clone as analyzed by six nuclear SSRs, or between these and the mother plants. Genetic stability of Norway spruce SEs was also unaffected by the induction of programmed cell death (PCD) caused by withdrawal of auxin and cytokinin, using three SSR markers at successive stages of somatic embryogenesis in two cell lines (Helmersson et al. 2004). By contrast, when analyzing allele frequencies at four variable nuclear microsatellite loci in 314 plants regenerated from SE from six families of *P. abies*,

significant variation was found (Helmersson et al. 2008). However, the authors concluded that the procedure for somatic embryogenesis does not produce any large changes in allele frequency based on a comparative analysis with seedlings from half-sib families. In *P. sylvestris* a significant difference in genetic stability among families was found by comparing the stability of four variable nuclear microsatellite loci in embryogenic cultures and ZEs (Burg et al. 2007). Interestingly, families showing a low genetic stability during establishment of embryogenic cultures had a higher embryogenic potential than those which were genetically more stable although embryo development was suppressed in such families. The higher mutation rate during somatic embryogenesis was related to the plasticity of the families to adapt to stress. Genetic variation was also detected at seven SSR loci in *P. pinaster* embryogenic cell lines under proliferation conditions for 6, 14 or 22 months, and in 5 out of 52 emblings recovered from SEs, of which three showed an abnormal phenotype (Marum et al. 2009b). However, no correlation was established between genetic stability at the analyzed loci and abnormal embling phenotype.

When using another type of molecular markers, the RAPD (Random Amplified Polymorphic DNA) markers, to analyze tissues from 57 lines of *A. alba* obtained by somatic embryogenesis from six genotypes, somaclonal variation was detected in the lines of 4 out of 6 genotypes (Nawrot-Chorabik 2009). However, structural rearrangements in DNA caused by different types of DNA damage might not be readily detected by RAPDs (Danylchenko and Sorochinsky 2005) and, therefore, this approach has been less used in recent studies.

Epigenetic mechanisms, including DNA methylation, histone modifications and RNA interference (RNAi) mediated by miRNAs are highly dynamic and can be affected by the conditions imposed during *in vitro* culture (Miguel and Marum 2011). As a result, access to the genetic information can be altered leading to modified gene transcriptional profiles (epitypes), ultimately affecting phenotypes. Therefore the epigenetic regulatory layer should also be taken into account in the analysis of true-to-typeness. A convincing example came from the study by Zhang et al. (2010a) who compared miRNA expression profiles of EM and NEC of 3 Japanese Larch genotypes. Interestingly NEC was originating from the same initial ET, i.e., from ET turned into NEC after (prolonged) subculture. Four main miRNA families known to be activated by abiotic stress (environment) were differentially expressed and associated to either embryogenecity (*miR171*, *miR159*, see part 2 in this chapter) or in early determination and maturation of SE in response to ABA signaling (*miR169* targeting *NF-YA* genes, Zhang et al. 2015, see part 4 in this chapter). These data evidenced the variability of expression patterns (epitypes) exhibited by the same genotype.

Another clear example of epigenetic phenomena has been reported for *P. abies* by Yakovlev et al. (2014). When analyzing embryogenic cultures (PEM) from two phenotypically well-characterized full-sib genotypes, the authors found that temperature conditions (18 vs. 30 °C) significantly alter transcriptional profiles and resulted in different temperature-induced epitypes. Differentially expressed genes included numerous orthologs of transcriptional regulators, epigenetic-related genes, and large sets of unknown and uncharacterized transcripts, as revealed by the use of Illumina-based MACE analysis. Among these, thirty-five highly expressed transcripts orthologous to epigenetic-related genes showed consistent transcript patterns differing between the two temperatures for both genotypes.

3.2 After cryopreservation

Cryopreservation of ETs can contribute to preserve juvenility and minimize (epi)genetic change by shortening the duration of *in vitro* culture maintenance, thus avoiding repeated cell division over prolonged time periods and thus reducing mutation risk.

The cryopreservation protocol most widely-used for conifer ETs is the slow-cooling and fast-thawing method. This protocol usually involves the use of cryoprotectants such as DMSO (dimethyl sulfoxide) which possesses mutagenic potential at the employed concentrations because of its direct interaction with chromatin.

A few studies in conifers have addressed putative correlations between the cryopreservation process and the occurrence of somaclonal variation during somatic embryogenesis. Using RAPD markers for monitoring the genetic stability of cryopreserved *Abies cephalonica* embryogenic cultures, Aronen et al. (1999) found genetic variation in DMSO-treated, but non-frozen samples, at a higher level than the background variation seen in the controls. The use of mixtures containing polyethylene glycol (PEG) and glucose seemed less detrimental than DMSO alone. This variation was not reflected in any morphological changes following the cryoprotectant treatments and cryostorage. A more recent study with a subset of these embryogenic cultures that have been under cryostorage for 6 years (Krajnakova et al. 2011) indicated some changes in one of the lines when comparing the profiles prior and after long-term cryopreservation, but the embryogenic cultures were able to proliferate and to produce SEs with normal morphology. In contrast, De Verno et al. (1999) reported that trees regenerated from white spruce SEs that matured or germinated abnormally exhibited altered RAPD fragment patterns. Somaclonal variation was also detected in embryogenic cultures 2 and 12 months after re-establishment following cryopreservation for 3 and 4 years (De Verno et al. 1999), when compared to freshly thawed embryogenic cultures. However, the tested genomic regions were genetically stable in the

corresponding regenerated trees (De Verno et al. 1999) leading the authors to suggest that variation observed due to the *in vitro* culture process rarely affects trees regenerated from normally maturing and germinating SEs. In *P. nigra* no genetic variation was observed in cryopreserved tissues using a RAPD approach (Salaj et al. 2011). However, intraclonal variation in the RAPD profiles was detected in the case of two cell lines that were pretreated with DMSO and sorbitol but were not frozen. This observation further supports the idea that pretreatment with cryoprotectants, and not the freezing, thawing and regrowth processes, might be the most relevant factor to consider concerning genetic fidelity of cryopreserved ETs of conifers.

The genetic stability of ETs of Norway spruce cryopreserved by a different method was also analyzed. In such a study a droplet vitrification-based procedure was used involving pregrowth-dehydration consisting of a preculture on media that only contained sucrose at increasing concentrations either alone or supplemented with ABA, followed by desiccation of the plant material prior to cryopreservation. Also in this case, no genetic variation was detected in five SSR loci in the ETs or SEs derived from them (Hazubska-Przybył et al. 2013). As far as genetic fidelity is concerned this method has the advantage of avoiding the use of toxic and/or mutagenic cryoprotectants but the recovery rates might be lower.

4. What is the regenerative capacity of the propagated embryogenic tissue?

The developmental switch from early to mature embryo results from a highly complex and impressive cascade of molecular events involving multiple structural, TFs and epigenetic-related genes (Vestman et al. 2011; de Vega-Bartol et al. 2013). There are large data sets accumulating for conifers from transcriptomic and targeted expression studies, and to a lesser extent from proteomics and metabolomics of embryo development (reviewed in Trontin et al. 2015) showing significant homology with model angiosperms, particularly in apical-basal embryo patterning driven by polar auxin transport and in radial patterning resulting from activation of the auxin-mediated, ABA-dependent response machinery during late embryogenesis. Differences in the molecular regulation of embryogenesis may arise mainly from spatiotemporal variations in gene expression with central roles of TFs with synergistic effects (e.g. *LEC* genes, *ABI3* and *FUS3*) on both auxin- and GA-mediated responses during early embryogenesis and induction of ABA-dependent response during late embryogenesis. In combination with other well-known triggers (e.g., sucrose, PEG, gellan gum), ABA signaling may modify ET responsiveness to auxin and GA as well as to other signaling (ethylene, polyamine) and regulating molecules, particularly sRNA/miRNA and other epigenetic regulators (DNA methylation, histone modification/chromatin remodelling)

involved in temporal and organ-specific expression of homeotic genes. Major processes involved in the correct embryo development include (Trontin et al. 2015),

Table 4. *Some outputs from recent molecular studies of regenerative capacity of embryogenic cultures in conifers.*

Species	Compared material*	Factors investigated / →Main conclusion*	Reference
Epigenetics			
Larix × eurolepis	1 SE line (undifferentiated, immature and mature)	Global DNA methylation during maturation – Treatment with hypomethylating (5-azaC) or hypermethylating (HU) drugs → Each developmental step associated with different levels of global DNA methylation → 5-azaC and HU affect morphogenesis (potential tools to improve morphogenesis)	Teyssier et al. (2014)
Larix leptolepis	8 SE dev. st.	Small RNA library construction + RNA-seq (Illumina) 11 conserved miRNA families involved in SE development →PEM to SE transition: miR397, miR398 →Transition from early to late SE: miR162, miR168 →Cotyledonary SE: miR156, miR159, miR160, miR166, miR167, miR390	Zhang et al. (2012b)
	ET matured with/without ABA (synchronous and asynchronous SE maturation)	Small RNA library construction – Quantification of redundant sRNAs → *miR156* as regulator of embryo synchronism → miRNAs expression differed from that of target gene in asynchronous cultures	Zhang et al. (2014)
Species	**Compared material***	**Factors investigated / →Main conclusion***	**Reference**
Epigenetics			
Larix × eurolepis	1 SE line (undifferentiated, immature and mature)	Global DNA methylation during maturation – Treatment with hypomethylating (5-azaC) or hypermethylating (HU) drugs → Each developmental step associated with different levels of global DNA methylation → 5-azaC and HU affect morphogenesis (potential tools to improve morphogenesis)	Teyssier et al. (2014)
Larix leptolepis	8 SE dev. st.	Small RNA library construction + RNA-seq (Illumina) 11 conserved miRNA families involved in SE development →PEM to SE transition: miR397, miR398 →Transition from early to late SE: miR162, miR168 →Cotyledonary SE: miR156, miR159, miR160, miR166, miR167, miR390	Zhang et al. (2012b)
	ET matured with/without ABA (synchronous and asynchronous SE maturation)	Small RNA library construction – Quantification of redundant sRNAs → *miR156* as regulator of embryo synchronism → miRNAs expression differed from that of target gene in asynchronous cultures → miRNAs and target genes coexpressed in synchronous cultures: *miR159a (MYB33)*, *miR160a (ARF)*, *miR162 (DCL1)*, *miR166a (HD-ZIPIII)*, *miR397 (laccase)*, *miR398a (plastocyanin)* → Synchronous SEs: 29 miRNAs up-regulated, e.g. *lleniR-13*, *miR535b*, *miR535c*, *lleniR-11*. Majority of redundant sRNA are 24 nt long. More non-redundant unknown sRNAs. → Asynchronous SE: 59 miRNAs up-regulated, e.g. *miR166b*, *miR4414b*, *lleniR-160*, *miR4414e*. Majority of redundant sRNAs are 21 nt long. High proportion of sRNA matching repeat regions.	Zhang et al. (2014)
Picea omorika	1 SE line (long-term proliferation) Various auxin/cytokinin ratios	Methylation level established with RAPD and DNA digestion: → DNA methylation decreased with 2,4-D reduction or application of 5-azaC → 2,4-D reduction improved regenerative capacity	Leljak-Levanic et al. (2009)
Picea abies and Pinus sylvestris	1-2 SE lines matured with TSA	Treatment with 10 µM TSA - *PaHAP3A* and *PaVP1* expression → TSA treatment: proliferation is enhanced; the maturation process is arrested. *PaHAP3A* and *PaVP1* expression are abnormally maintained to high level.	Uddenberg et al. (2011)
Pinus nigra	3 SE lines (high, medium, no maturation)	Quantification of global genomic DNA methylation → More methylation associated with less regenerative capacity	Noceda et al. (2009)
Pinus pinaster	1 aged SE line (no maturation) 2 SE lines (young vs. aged)	Global DNA/site specific methylation (MSAP/HPCE) → Global DNA methylation levels similar in all tested samples → EM aging was associated with net DNA demethylation at 5'-CCGG-3' sites Analysis of hypomethylating drug (5-azaC; 5-40 µM) on aged line → Alteration of methylation profiles (largest alterations with 15 µM 5-azaC)	Klimaszewska et al. (2009)
Transcriptomics			
Araucaria angustifolia	3 ZE stages 2 SE lines (responsive vs. blocked)	Illumina platform; paired end protocol → ABA-responsive lines (SE1): mainly differential expression of defence, cell wall and secondary metabolite genes → Non-responsive lines (SE6): mainly differential expression of genes involved in DNA replication, transcription, translation and cell division → Various TFs differentially expressed (SE1/SE6), mainly *NAC*, *WRKY*, *ERF*, *MYB*, *HD-ZIP*, *bZIP*. For full list of function associated unigenes, see Figure 5a, Elbl et al. (2014) → Regeneration impairment results from auxin signalling failure	Elbl et al. (2015)
Picea balfouriana	1 SE line. Prolonged subcultures with BAP (2.5-5.0 µM)	Illumina platform → BAP may regulate transcripts involved in specific enrichments of genes associated with ribosomes, glutathione metabolism and plant hormone signal transduction → Two putative *Wuschel homeobox proteins* (*WOX8/9*) and 30 putative nucleotide binding site-leucine-rich repeats (NBS-LRR) proteins genes were upregulated. Differentially expressed unigenes: see Table 1, Li et al. 2015) → Best maturation and germination results obtained with 3.6 µM BAP in proliferation medium	Li et al. (2015)

(Table 4 to continue)

Table 4. Contunued

Species	Compared material[a]	Factors investigated /→Main conclusions[ab]	Reference
Proteomics (and biochemistry)			
Araucaria angustifolia	2 SE lines (responsive vs. blocked)	2-DE and MALDI-TOF/TOF MS →11 differentially expressed proteins → Responsive cell line: SAMet synthase; higher values of ethylene and ROS → Blocked line: protein linked to oxidative stress, subunit F of NADH dehydrogenase; higher levels of diamine putrescine and lower levels of ethylene (for full list see Jo et al. 2014, Table 2).	Jo et al. (2014)
Larix × eurolepis	1 SE line (immature, mature SEs)	2D-PAGE+LC-MS/MS analysis → Revealed proteins are associated with functional classes: Metabolism (70), Genetic Information processing (43), Environmental processes (14), Cellular processes (19), Organismal system (10), Others (16), see full list of identified proteins (Teyssier et al. 2014; Table 3) → Most proteins found in mature SEs. Storage proteins of the legumin- and vicilin-like families → Differentially expressed proteins in mature SEs mainly involved in primary metabolism	Teyssier et al. (2014)
Picea abies	2 SE lines matured in maltose/PEG vs. sucrose	SDS-PAGE+GC/MS → Maltose/PEG treatment: mainly Dehydrin 2, HSP, LEA, LEA-like proteins, sHSP. Improved maturation ability but low germination rate → Sucrose treatment: mainly strorage proteins. Improved germination ability	Businge et al. (2013)
	1 SE line, 10-500 µM putrescine (proliferation/maturation)	Quantification of polyamines (putrescine, spermidine, spermine) with HPLC → Effects on polyamine levels were observed in subsequent steps following putrescine treatment → Exogenous putrescine had no effect on subsequent embryogenesis	Vondráková et al. (2015)
Picea balfouriana	Mature SEs from 1 line. Prolonged subcultures with BAP (2.5-5.0 µM)	HPLC and LC-MS/MS proteomic analyses of iTRAQ labeled peptides →Most frequent functional groups: binding (44.63%), catalytic activity (41.26%), 'cellular processes' (17.98%) and 'metabolic processes' (17.58%). See list of differentially expressed proteins in Table 2; Li et al. 2015). → Best maturation and germination with 3.6 µM BAP in proliferation	Li et al. (2015)
Pinus nigra	3 SE lines (high, medium or no maturation)	Quantification of polyamines (putrescine, spermidine, spermine and 1,7-diamino heptane) with HPLC → More free polyamines was associated with less maturation ability	Noceda et al. (2009)
Pinus pinaster	2 SE lines. Young (productive) vs. aged (non productive).	Hormones and polyamine analyses (HPLC) Acetic acid, indole-3 aspartate, zeatin, zeatin riboside, isopentenyladenine, isopentenyl-adenosine, ABA and abscisic acid glucose ester. →Inconsistent profiles of endogenous hormones between genotypes. → Higher IAA concentration in young tissue Polyamines (putrescine, spermidine, spermine) - HPLC → Polyamine profiles are inconsistent. In 1 line (MM25) no significant differences in specific polyamines. Another line (NM18) had higher spermidine and spermine contents. → Different IAA-sp and ZR profiles in studied genotypes could have affected maturation ability and different polyamine profiles.	Klimaszewska et al. (2009)
	2 SE lines (cotyledonary SEs) vs. cotyledonary ZEs	2D-PAGE + LC-MS/MS analysis →CotyledonarySEs similar to fresh cotyledonary ZEs →23 protein markers of the cotyledonary stage: 5 HSPs (2 isoforms of class II HSP 17.6, HSP18.2, HSP60, HSP70-4); 2 other stress-related proteins (aldose reductase, 6-phosphogluconate dehydrogenase family protein); 4 LEAs (embryonic proteins DC-8 and 63, LEA-like, responsive to ABA 28); 5 energy storage proteins (2 cupin domain-containing proteins, legumin-like, 2 vicilin-like); 2 proteins involved in purine metabolism (adenosine kinase 2, SAMet synthase); 5 proteins of unknown function	Morel et al. 2014b
Pinus strobus	Cotyledonary SEs (2 lines) and mature ZEs (1 tree, 3 collection dates); 6-16 weeks maturation; 3 or 6% sucrose	Proteins identified by SDS-PAGE, amino acid sequencing and MS/MS → SEs accumulated most storage proteins after 9 weeks maturation (60-65%), declining afterwards (16-40%) → Identical storage proteins (SE/ZE) - Most abundant proteins are 11S-globulins, 7S vicilin-like proteins (in temporal fashion) → Sucrose (6%) resulted in increase of three sets of storage peptides	Klimaszewska et al. (2004)
Metabolomics			
Picea abies	3 SE lines (normal, aberrant, blocked SE development). 4 developmental stages.	Metabolic profiling with GC/MS analysis Amino acis and derivates, carbohydrates, sugar alcohols, organic acids and other metabolites (full list in Businge et al. 2012, see Table 1) → Significant levels of sucrose in proliferation, and maltose during late embryogenesis → Pinitol only present in normal cell line → stress-linked mechanism – due to malnutrition or environmental pressure; caused by impaired nutrient uptake – cellular stress	Businge et al. (2012)
Picea abies	2 SE lines matured in maltose/PEG vs. sucrose	GC/MS-based metabolomics →SE line with normal development: high sucrose in proliferating SEs, high maltose in late SEs → Pinitol only present in normal SEs → Presence of tryptophan only in cell lines that formed mature SEs – link to auxin, as essential factor for proliferation and embryo development → Desiccation tolerance was associated with high levels of sucrose, raffinose and LEA proteins	Businge et al. (2013)
Pinus taeda	5 SE lines assessed for their maturation ability	GCMS-based metabolomics → A subset of metabolites correlated with maturation ability: sucrose, threonine, glutamic acid, carbohydrate, glucopyranose, 4-amino-butric acid (for full list, see Robinson et al. 2009, Table 1)	Robinson et al. (2009)

[a]ZE : zygotic embryo ; SE: somatic embryo

[b]5-azaC: 5-azacytidine; 2-DE: two-dimensional electrophoresis; 2D-PAGE: two-dimensional polyacrylamide gel electrophoresis; GC/MS: gas chromatography coupled with MS; HU: hydroxyurea; iTRAQ: isobaric tags for relative and absolute quantitation; LC/MS: liquid chromatography coupled with MS; MSAP: methylation-sensitive amplification polymorphism; nt: nucleotide; MS: mass spectrometry; RAPD: random amplified polymorphic DNA; SDS: sodium dodecyl sulfate; sRNA: small RNA; TSA: trichostatin A.

1) developmentally regulated PCD, 2) genes with a function in megagametophyte development and/or signaling, 3) genes related to cell wall modifications, 4) auxin response machinery and other important regulators of embryo patterning including epigenetic regulation, 5) an ABA-mediated developmental switch, 6) changes in metabolisms (carbohydrates, proteins, energy) and 7) stress-related genes and the maintenance of redox homeostasis. Practical outcomes to refine somatic embryogenesis in conifers may primarily result from an opportunity to modulate any of these pathways. Early marker-assisted screening of embryogenic cultures can help preventing unnecessary expenses resulting from the use of proliferation, maturation and germination conditions unfavourable for the conversion of immature embryos into high quality mature embryos and somatic seedlings.

There is a large inventory of active genes and associated processes at each developmental stage and transition from early to late embryogenesis (Trontin et al. 2015). However, the current knowledge is highly fragmented and substantial support for robust markers of correct embryo development (maturation yield, synchronism and quality) would require confirmation studies of candidate genes or proteins in different species through various molecular approaches. Nevertheless, we review below evidence for genes that may affect maturation ability by focusing on comparative studies of productive and non-productive embryogenic lines of different genetic origin, physiological/ontogenetic ages, or cultures conditions. We also considered comparative studies of SE and ZE as a way to determine SE quality and produce refined protocols. Following this strategy we highlighted below a few candidate genes (mainly regulators) and/or important processes (Table 4) mainly involved in epigenetic regulation (DNA methylation, chromatin structure, sRNA/miRNA), auxin-mediated and ABA-dependent embryo patterning, various metabolisms (polyamines, ethylene, storage proteins, amino-acids), desiccation tolerance and response to stresses, particularly maintenance of redox homeostasis, but also abiotic stress.

4.1 DNA methylation

Noceda et al. (2009) analyzed ET from 3 genotypes of *P. nigra* exhibiting different regenerative capacities (from non-productive to productive) and found that low global DNA methylation is associated with higher maturation ability, i.e., yield in cotyledonary SE. The withdrawal of auxin/cytokinin prior to an ABA application was found to affect the global DNA methylation status of ET which was ultimately associated with differences in maturation ability. Leljak-Levanić et al. (2009) studied the effect of precultures (one to four weeks; different auxin/cytokinin ratios and PGR-free medium) on the extent of global DNA methylation and regenerative capacity of ET of *Picea omorika*. They found that a reduction of the 2,4-dichlorophenoxyacetic acid (2,4-D) level or its omission

(PGR-free medium supplemented with activated charcoal) resulted in a decreased methylation level and subsequently in improved embryo maturation on ABA containing medium.

A further phenomenon, which is frequently observed in ET is the loss of the ability to produce cotyledonary SEs after several subcultures. Klimaszewska et al. (2009) investigated such a discrepancy in *P. pinaster* by comparing in 2 genotypes i) young EM cultures (3-month-old lines since the first subculture) that produced cotyledonary SEs, ii) tissue of the same lines of significantly increased age (18-month-old lines, aged EM) with an impaired maturation capacity, and iii) secondary "reinvigorated" EMs induced from mature SEs that have improved maturation ability compared with aged cultures. In all culture types they analyzed the global DNA methylation and also targeted methylation patterns as detected by MSAP (methylation-sensitive amplification polymorphism). Even though global DNA methylation patterns did not significantly change among tested cultures, MSAP revealed that ageing is associated with net DNA demethylation or methylation at specific target sequences.

Of further interest is the possible alteration of the epigenetic state in embryonic cultures which offers the opportunity to analyze and possibly to improve the regenerative capacity with hypo- or hypermethylating agents as well as with histone deacetylase inhibitors. To determine if it is possible to restore losses caused by line ageing, Klimaszewska et al. (2009) analyzed the effect of the DNA hypomethylating drug 5-azacytidine (5-azaC) on embryo maturation in *P. pinaster* and on the viability of an aged embryogenic line. After nine days of treatment (>5 µM 5-azaC), the mean fresh mass increase of the maturing EM was significantly reduced. However, and interestingly, EM treatment with 10-15 µM 5-azaC led to a slight increase of the number of maturing embryos. MSAP revealed that high 5-azaC concentrations (15 µM) noticeably altered the DNA methylation pattern. Surprisingly, the culture showed maximal hypermethylation after nine days of treatment, which changed to maximal hypomethylation after 14 days. As a consequence, considerable variation in the EM growth was detected in presence of 5-azaC, but no consistent conclusions could be drawn from the resulting culture behaviour, probably because of the cytotoxicity of this drug (Klimaszewska et al. 2009). Leljak-Levanić et al. (2009) similarly observed a reduction in global DNA methylation of EMs after a one-week exposure to 5-azaC in the presence of 2,4-D and 6-benzylaminopurine (BAP). However, the subsequent embryo development during maturation was not affected. Teyssier et al. (2014) also analyzed the influence of 5-azaC and also the hypermethylating drug hydroxyurea (HU) during SE maturation in *Larix* × *eurolepis*. Without treatment, global DNA methylation (methylated cytosine) varied from 45.8±3.8% in proliferating EMs to 61.5±3.1% in SEs that had matured for 1 week. Later during maturation at the SE cotyledonary

stage (8 weeks) the global DNA methylation decreased (53.4±7.8%). The presence of 100 µM 5-azaC or HU in the maturation medium, respectively, affected global DNA methylation levels, growth and EM maturation ability. Although toxic effects of these drugs could not be excluded, these results indicate an important role for DNA methylation in embryogenesis and probably in the regenerative potential as well.

4.2 Chromatin structure

In the epigenetic complex of regulation of gene expression a possible link between chromatin structure and expression of embryogenesis-related genes at different somatic embryogenesis steps, including maturation, has been suggested for *P. abies*. Uddenberg et al. (2011) took advantage of the use of TSA with the aim to cause de-repression of master regulators like *LEC1/PaHAP3A* or *ABI3*/Pa*VP1* during maturation. *LEC* genes including *LEC1* are part of a complex regulatory network with *ABI3* and other genes (e.g. *FUS3*) resulting in ABA-dependent gene regulation in plants (Vestman et al. 2011; Cairney and Pulman 2007). *LEC1* induces homeotic genes expression such as the coordinated *AGAMOUS* and *APETALA2* that have direct implication on embryo patterning. *LEC1* is expressed during early embryogenesis and then is significantly downregulated at the onset of late embryo development promoted by exogenous ABA (Vestman et al. 2011; Uddenberg et al. 2011). The resulting putative spatio-temporal modulation of both auxin- and GA-mediated signaling pathways could be involved in the developmental switch from embryonic to vegetative growth. The expression of *PaHAP3A* decreases during SE maturation in *P. abies* whereas *PaVP1* expression increases (Footitt et al. 2003; Fischerova et al. 2008; Uddenberg et al. 2011). TSA treatment during maturation resulted in continuous ET proliferation and arrested SE development whereas normal expression levels of *PaHAP3A* and *PaVP1* were precluded. Various histone deacetylase genes (*HD2C, HDA8, HDA9*) were regulated from early to late embryogenesis in *P. pinaster* (de Vega-Bartol et al. 2013) as were genes involved in histone H4 acetylation, methylation of H3K9 (*SUVH1*) and H3K27 (*CLF* a member of the polycomb group Pc-G) or genes encoding chromatin-remodeling ATPases (*CHC1, RAD5, BSH*). In the same species, many *ubiquitin-protein ligase* transcripts and *ubiquitin-/small ubiquitin-related modifier (SUMO)-conjugating* genes were detected during early SE development (Morel et al. 2014a). Ubiquitin-protein ligases are associated to SUMO activation (specific post-transcriptional modification of chromatin), suggesting that early maturing EMs were subjected to large-scale reorganization of gene expression towards embryo development. Ubiquitin-protein ligases are also activators of the PGR-regulated ubiquitin/26S proteasome pathway resulting in controlled proteolysis with increased supply in amino acids. Morel et al. (2014a)

proposed that ubiquitin-protein ligases can be used as predictive markers of SE development.

Micro RNAs and other small RNAs have crucial roles in regulating embryo development in conifers. RNA-seq analysis of various SE developmental stages in *L. leptolepis* (Zhang et al. 2012b) revealed 83 conserved miRNA from 35 families. Expression of 11 conserved miRNA families and putative targets was stage-specific suggesting their possible modulation during initiation (*miR159, miR171*, see part 2 in this chapter) and SE maturation from the transition of PEM to early embryo (*miR397,398*), early to late embryo development (*miR162,168*) and production of cotyledonary SE (*miR156,159,160,166,167,390*). Target genes of miRNAs include i) genes of the auxin-response machinery such as *ARF* genes (*miR160, miR167, siRNA TAS3/miR390*), *SPL3* involved in phase change (*miR156*), *class III HD-ZIP* involved in regulation of abaxial pattern formation (*miR166*), ii) other regulators of embryo body plan, i.e., *AGO* (*ARGONAUTE*) genes from the RNA-induced silencing complex (*miR168*) and *SCR* genes involved in radial patterning and delineation of embryonic root (*miR171*), iii) positive regulators of ABA (*miR159*) and iv) antioxidant enzyme genes such as *Cu/Zn SOD/plastocyanin* (*miR398*). These miRNAs may provide markers of full SE maturation and new tools to modulate SE maturation yield and quality in conifers. In the same species, the *miR159* was found upregulated at the late stage of cotyledonary embryo development (Li et al. 2013). Zhang et al. (2014) further used RNA-seq to identify sRNAs responsible for synchronous (shared common developmental stages during maturation) or asynchronous SE development (embryos co-existing in different developmental stages and marked by precocious germination). Qualitative and quantitative differences in sRNAs were observed. The majority of non-redundant unknown sRNAs were detected in synchronous embryos, indicating a participation of these sRNAs in the regulation of synchronisation during somatic embryogenesis. On the other hand, the proportion of miRNAs as well as their expression, was higher in asynchronous embryos. Twenty-five miRNAs were found upregulated in synchronous SE and 59 in asynchronous SE. The *miR156* but also *miR167*, *miR397* and *miR398* were identified as some of the best candidates contributing to the regulation of embryo synchronism in larch (see Table 4). The authors assumed that sRNAs are induced by ABA. As a consequence, a precocious expression of differentiation-promoting factors is inhibited and the synchronisation of SE development is regulated.

4.3 Genes related with auxin and ABA signaling

Elbl et al. (2015) focused on a comparative transcriptome analysis of *A. angustifolia* embryogenesis to elucidate differences between distinct embryogenic cell cultures, early ZEs, SEs and unorthodox seed development. In particular, they

compared two embryogenic lines with or without a capacity for cotyledonary SE development. With this approach they identified factors that are assumed to have an influence on cell lines maturation ability and differences between early SE formation and unorthodox seed (ZE) development. Numerous genes were differentially expressed between maturing and blocked embryogenic lines, especially TF genes belonging to the *NAC, WRKY, ERF (ethylene response factor), MYB, HD-ZIP* and *bZIP* families, suggesting their involvement in maturation ability. NAC domain TF family regulated by PIN1 (auxin carrier proteins) such as *CUC* (cup-shaped cotyledon) genes 1 and 2 are known to be crucial for the differentiation of the shoot apical meristem (SAM) as well as formation and separation of cotyledons in conifers (*PaNAC01*, Larsson et al. 2012a; *NAM/NARS2*, Vestman et al. 2011). *WRKY* is known to act downstream of the ABA-insensitive (ABI) protein phosphatases 2C (PP2C)-ABA receptor complex activating Sucrose non-fermenting 1 (Snf1)-related protein kinases 2 (*SnRK2*) and other calcium-dependent kinases (*CDPK*) involved in the ABA signal transduction cascade. *SnRK2* subsequently activates downstream targets, especially the ABA-response elements binding HD leucine zipper (*B-ZIP*) TFs. Several *PP2C* and *SnRK2* transcripts were significantly expressed in *P. pinaster* and the endogenous ABA level increased after 4 weeks maturation suggesting an ontogenetic signal for SE differentiation (Morel et al. 2014a). *PP2C* was proposed as a biomarker for culture adaptive responses to ABA. Similarly Li et al. (2015) found various *CDPKs* genes, likely to be involved in the signaling pathway of cytokinins, upregulated in *P. balfouriana* ET proliferated in optimal conditions, and proposed that these genes may serve as molecular markers of maturation ability of propagated ET. *WRKY* targets ABA-responsive genes (e.g., *ABF2-4*, *ABI4-5*, *MYB2*, *DREB1a-2a*, *RAB18*). Both *WRKY* and ABA-responsive genes involved in response to dehydration (*DREB*), initial leaf morphogenesis (*angustifolia3*/*growth regulating factor1*), and growth (*NAC*) were found regulated during SE development of *P. abies* (Vestman et al. 2011). These data support the idea that priority should be given to the study of TFs involved in ABA-mediated events as a potential source of markers of the maturation ability in conifers.

Elbl et al. (2015) further compared differentially expressed genes between early ZEs and early SEs that did not convert into fully mature cotyledonary embryos. *WUS/WOX* genes were strongly expressed in the SE transcriptome whereas genes related to the auxin-response machinery (*ARFs*), auxin synthesis (IAAs), polar auxin transport and leaf morphology were up-regulated in developing ZEs. It is concluded that regeneration impairment of *A. angustifolia* embryogenic cultures may be the consequence of an auxin signaling failure. Auxin biosynthesis and relocalization by polar auxin transport has a crucial function in activation of the auxin response machinery during plant embryogenesis (apical-basal and radial

embryo patterning) and has a similarly high importance in angiosperms and gymnosperms (Larsson et al. 2012a). Furthermore metabolic profiles of different developmental stages of three embryogenic cell lines (normal, aberrant and blocked SE development) of *P. abies* were established (Businge et al. 2012). Specific metabolites were found for each developmental stage and it was concluded that endogenous auxin and sugar signaling affects the initial stages of SE development. In addition, the results highlight the importance of a timed stress response and the presence of stimulatory metabolites during late embryogenesis. Genes involved in both auxin and ABA signaling and regulation (TFs, miRNAs) may be regarded as putative SE quality-related markers.

4.4 Genes associated with polyamines levels and ethylene production

In addition to the global DNA methylation pattern, Noceda et al. (2009) analyzed the amount of free polyamines (putrescine, spermidine, spermine) in *P. nigra* ET obtained from different genotypes with contrasted maturation ability. It was found that higher contents of long chain polyamines (putrescine, spermidine) have an association with higher methylation levels, which in turn are negatively correlated with the maturation ability. In contrast Klimaszewska et al. (2009) could not correlate maturation ability with hormonal and polyamine profiles in young, aged or secondary lines from the same genotype.

Polyamines are an important class of nitrogen compounds synthesized from S-adenosyl-methionine (SAMet) derivatives while SAMet is also a precursor for ethylene and a methyl donor in transmethylation mechanisms resulting in DNA or histone methylation. Endogenous polyamines (spermine, spermidine) were found to increase during correct SE development in *P. glauca* (Stasolla et al. 2003b). Expression pattern of several genes encoding *ACC oxidase* and *adenosine kinases* were indicative of active ethylene synthesis (Stasolla et al. 2003b) as well as of *ACC synthase* activity in the same species (*PgACS1*, Ralph et al. 2007) and also in *P. sylvestris* (*PsACS2*, Lu et al. 2011). The latter *ACC synthase* gene was proposed as a marker of cotyledonary SE development as its expression was associated with both ethylene production and maturation ability. Expression of these and related genes (*SAH hydrolase*, *methionine synthase*, *SAMet synthase*) during the developmental switch to late embryogenesis in *P. abies* were supporting active transmethylation events, resulting in DNA or histone methylation that may contribute to the global transcriptional repression state observed at specific embryo stages (van Zyl et al. 2003; Stasolla et al. 2004; Vestman et al. 2011). DNA methylation and heterochromatin maintenance through various additional mechanisms were confirmed to be important processes at the onset of *P. pinaster* embryo maturation (de Vega-Bartol et al. 2013). The importance of genes involved in polyamine levels and ethylene production was indicated by proteomic studies

that showed that an increased level of SAMet synthase proteins occurred from the early to late embryo stages of *P. glauca* (Lippert et al. 2005), *A. angustifolia* (Balbuena et al. 2009), and *L. x eurolepis* (Teyssier et al. 2014). In *P. pinaster* the SAMet synthase as well as another protein involved in purine metabolism (adenosine kinase 2) were proposed as markers of the cotyledonary stage of SE (Morel et al. 2014b). The maturation step is still an obstacle in the development of somatic embryogenesis in *A. angustifolia*. Jo et al. (2014) analyzed the protein content during the proliferation phase of one responsive embryogenic line and one blocked line (two different genotypes) using a combination of two-dimensional electrophoresis (2-DE) and MALDI-TOF/TOF mass spectrometry. Eleven proteins were found differentially expressed. Interestingly, SAMet was only found in the responsive cell line whereas a subunit F of NADH dehydrogenase was overexpressed in the non-maturing line, thus suggesting a disturbed cell redox system as NADH dehydrogenase is a component of the plant energy-dissipating mitochondrial system preventing excessive reactive oxygen species (ROS) production. Accordingly, increased ethylene release, lower polyamine content (diamine putrescine) and higher ROS values were revealed in the responsive line. Both biochemical and molecular investigations of polyamines, ethylene, DNA methylation and oxidative stress appeared to be of significant interest for selecting productive embryogenic lines.

4.5 Genes associated with maintenance of redox homeostasis

Various genes involved in the regulation of oxidative stress are differentially expressed during the development of cotyledonary embryos (Stasolla et al. 2003a, 2004; Bonga et al. 2010; Vestman et al. 2011; de Vega-Bartol et al. 2013; Morel et al. 2014a). The maintenance of cellular redox homeostasis is considered to be a generic sensor for controlling embryo development (Stasolla et al. 2004). Antioxidant metabolites such as glutathione are critical in the regulation of oxidative stress and associated production of ROS, free radicals, hydrogen peroxides, and to cope with ATP depletion. ROS were recently shown to be important signaling molecules for activation of PCD and normal SE development in *L. leptolepis* (Zhang et al. 2010b). Glutathione can also interplay with NADP-linked thioredoxin in the frame of auxin transport and signaling. Both maturation yield and SE quality are affected by deregulation of glutathione metabolism in *P. glauca* (Belmonte et al. 2005). A general over-representation of oxidation-reduction processes, with prevalence of glutathione metabolism (glutathione thiolesterase activity, expression of *glutathione transferases*), was observed during early ZE development in *P. pinaster* (de Vega-Bartol et al. 2013). Various genes involved in both detoxification and control of the cellular redox state were downregulated during PEM proliferation in *P. abies* productive lines (*cytosolic*

ascorbate peroxidase, *thioredoxin H*, Stasolla et al. 2004) or regulated from early to late embryo development in *P. glauca* (Stasolla et al. 2003). This involved genes such as *glutathione-S-transferase* (*GST*), *glutathione peroxidase, glutathione reductase, ascorbate peroxidase* and *superoxide dismutase* (*SOD*). In *L. kaempferi* expression of a *Cu/Zn SOD* gene (plastocyanin) putatively regulated by *miR398* increased at the pre-cotyledonary stage (Zhang et al. 2012b). Accordingly, *SOD* was overexpressed early during SE maturation in *P. pinaster* (Morel et al. 2014a) and in sub-optimal SE maturation conditions in *L. x eurolepis* (Teyssier et al. 2011). More recently, culture conditions during ET proliferation were shown to affect the glutathione metabolism pathways in comparative transcriptomic and proteomic approaches in *P. balfouriana* (Li et al. 2015). In this species BAP concentrations in the range 2.5-5.0 µM significantly affected maturation yield and SE germination rate. Proteins of the large family corresponding to GSTs were found upregulated in ET treated with low (2.5 µM) to moderate BAP concentration (3.6 µM) which promoted an increased maturation yield and SE germination rate. GSTs may be involved in the cross-regulation between auxins and cytokinins to control cell proliferation and differentiation.

Various germin-like proteins (GLPs) were also up-regulated in ET with higher maturation ability in *P. balfouriana* (Li et al. 2015). GLPs are extracellular proteins with well-known non-enzymatic activities (auxin-binding protein, serine protease inhibitor) associated to developmental regulation and also to enzymatic activities such as the antioxidant enzyme oxalate oxidase or superoxide dismutase (SOD). GLP enzymatic activities result in production of hydrogen peroxide that may be involved in cell wall remodeling during stress responses and/or development. *GLP* genes were found expressed in ET or early maturing SE in conifers, e.g., in *P. radiata* (Bishop-Hurley et al. 2003), *P. pinaster* (Morel et al. 2014a) and *L. x marschlinsii* (Mathieu et al. 2006). In the latter species expression of *GLP* gene (*LmGER1*) could be associated with SOD activity in apoplastic proteins extracted from early SE (Mathieu et al. 2006). Interestingly, *LmGER1* expression corresponded to the pattern of active PCD during embryo development in conifers. Downregulation of *LmGER1* in proliferating ET resulted in reduced maturation yield, asynchronous SE development and precluded plantlet regeneration. The interest in GLPs, as predictive markers of embryo development as early as after one week on maturation medium, is well supported by combined transcriptomics and proteomics in *P. pinaster* (Morel et al. 2014a).

4.6 Genes associated with other abiotic stresses

A general trend towards regulation of genes involved in response to stress was observed during late SE development with high concordance among transcriptomic, proteomic and metabolomics data sets (Vestman et al. 2011;

Businge et al. 2013; Robinson et al. 2009). The modulation of gene response to stress could, therefore, be of practical interest to improve maturation protocols. Abiotic stress may result mainly from anoxia in suboptimal maturation conditions. Transcriptomic and proteomic profiling revealed that *alcohol dehydrogenase* and *pyruvate decarboxylase* genes were upregulated in *P. pinaster* ET together with *sucrose synthase* (*SuSy3*) during maturation under unfavorable conditions (low gellan gum, Morel et al. 2014a). This is in agreement with activation of both the glycolytic pathway and alcoholic fermentation (anoxia tolerance). *SuSy* genes are responsive to low oxygen level and promote an adequate sugar supply under anaerobic conditions. A submergence induced protein was also overexpressed in *P. glauca* at early SE stage suggesting a possible response to oxygen stress (Lippert et al. 2005). Similarly various *enolase* genes involved in the glycolysis andgluconeogenesis pathways, but also induced by abiotic stress such as low oxygen level in maturation conditions, are regulated in *P. pinaster* (*bifunctional enolase 2*, Morel et al. 2014a), *P. radiata* (Aquea and Arce-Johnson 2008), *P. abies* (Stasolla et al. 2004), *P. glauca* (Lippert et al. 2005), and *L. x eurolepis* (*enolase 1* isoforms, Teyssier et al. 2011). Enolase strongly accumulated in *P. glauca* mature embryos and has been proposed as a putative protein marker of normal embryo development (Lippert et al. 2005). There are additional regulated genes with a likely defense function active during SE maturation in conifers such as *cytochrome P450 monooxygenase* (plant response to PGRs and osmotic stress) in *P. radiata* (Bishop-Hurley et al. 2003) and *P. abies* (Stasolla et al. 2004), various genes from pathways related to secondary metabolisms in *L. kaempferi* (phenylpropanoids, flavonoids, Zhang et al. 2012a), *P. abies (flavanone 3-hydroxylase*, Stasolla et al. 2004) and *P. pinaster* (*flavanone 3-hydroxylase* and genes related to flavonol metabolism, Morel et al. 2014a) and also pathogenesis related protein genes in *P. radiata* (*SN1I*, Aquea and Arce-Johnson 2008) and *P. abies* (Vestman et al. 2011). Defense genes as well as genes involved in cell wall formation and secondary metabolite production were upregulated in an *A. angustifolia* culture capable of maturation (Elbl et al. 2015). Similarly, in *P. balfouriana* ribosomal protein genes were strongly upregulated in productive ET (Li et al. 2015) and could serve as molecular markers of maturation ability. Ribosomal proteins are involved in both regulation of cell growth and apoptosis but also protection against biotic and abiotic stresses.

4.7 Genes related with acquisition of desiccation tolerance and accumulation of storage proteins

Favourable maturation conditions require the balanced combination of carbohydrates and osmotica, in regard to storage reserve accumulation and germination. Businge et al. (2013) analyzed the effects on *P. abies* SE development

of two maturation media containing different carbohydrates (as carbon source) and different osmoticum levels. For this purpose a combination of sugar assays, proteomic and metabolic profiling was used. The addition of PEG (7.5%) and maltose (3%) to the maturation medium resulted in higher maturation ability but lower germination frequencies and higher storage protein content in SE than in SE grown on medium with only sucrose (3%). Embryos resulting from the latter contained, aside from starch, high levels of sucrose, raffinose and late embryogenesis abundant (LEA) proteins, group 2 LEAs (dehydrins), heat shock proteins (HSPs) and small HSPs. The accumulation of these compounds during maturation on medium with sucrose only, may be the reason for improved germination ability by promoting the acquisition of desiccation tolerance. LEA and dehydrins are known to accumulate in plants during late embryogenesis. In *P. pinaster* 5 HSPs, 2 other stress-related proteins and 4 LEAs were proposed as marker of the fresh cotyledonary stage of both SE and ZE (Morel et al. 2014b). In *L. x eurolepis* (Teyssier et al. 2014) proteins belonging to HSPs (cellular protection) or related to protein folding were mostly upregulated at the mature stage (Teyssier et al. 2014) or in sub-optimal conditions (Teyssier et al. 2011). HSPs are induced by ABA and are apparently required throughout embryogenesis up to early seedling growth (Teyssier et al. 2014 and references therein). Therefore, proteomics strengthened both the protective function of HSPs, in response to abiotic stress, and their ubiquitous role in protein folding, assembly translocation and degradation during embryo development.

Routine detection of the main storage proteins can be of practical interest to monitor SE maturity and quality. Proteomic studies confirmed that conifer SE and ZE similarly accumulate globulin (legumin, vicilin) and albumin families as major storage proteins (Klimaszewska et al. 2004, Lippert et al. 2005, Businge et al. 2013, Morel et al. 2014b, Teyssier et al. 2014). Protein accumulation peaks at the cotyledonary stage and are affected by maturation conditions (Klimaszewska et al. 2004; Businge et al. 2013). Candidate biomarkers of the embryo cotyledonary stage were the vicilin-like storage proteins in *P. glauca* (Lippert et al. 2005) and *L. x eurolepis* (Teyssier et al. 2014) or 3 vicilin- and legumin-like and 2 cupin domain-containing storage proteins in *P. pinaster* (Morel et al. 2014b). Similarly, transcripts encoding legumin- and vicilin-like classes of storage proteins increased with similar patterns in *P. taeda* SE and ZE (Lara-Chavez et al. 2012). Expression of these genes can be reduced under suboptimal maturation conditions as was observed with *P. oocarpa* (Lara-Chavez et al. 2012) and *A. angustifolia* (Schlögl et al. 2012). Similarly PEG-containing maturation medium promoted storage protein synthesis in cotyledonary SE of *P. glauca* (Stasolla et al. 2003b). Concomitant upregulation of glutamine synthase (*GS*) and glutamate synthase genes (GS/GOGAT cycle supporting nitrogen assimilation) suggested that storage protein

synthesis benefited from an increased pool of available glutamine. Based on a comparison of cotyledonary SE and ZE in *P. pinaster* and *P. sylvestris* (Pérez-Rodriguez et al. 2006), two GS genes isoforms, expressed in either photosynthetic (*GS1a*) or vascular tissue (*GS1b*), were proposed as markers of cotyledonary SE quality. *GS1b* expression was associated with early differentiation of procambial cells whereas *GS1a* expression could reveal precocious germination of cotyledonary SE that did not reach full maturity.

A proteomic study of mature SE in *L. x eurolepis* further revealed active protein synthesis based on the upregulation of proteins involved in amino-acid metabolism (Teyssier et al. 2014). Differential expression at the mature SE stage of key enzymes for amino-acid synthesis was also reported for *P. abies* (Businge et al. 2013). The importance of controlled proteolysis and protein synthesis during SE development was supported by differential expression of various proteasome subunits in *P. pinaster* (Morel et al. 2014a) and *P. abies* (Lippert et al. 2005) as well as by elongation factor II in *A. angustifolia* (Jo et al. 2014). It was proposed that the proteasome complex could serve as protein markers for monitoring embryo development (Lippert et al. 2005). Products of amino-acid metabolism could possibly provide reliable markers of effective SE development (Teyssier et al. 2014).

5. Some practical outcomes to check or improve embryogenecity and regenerative capacity

In theory, somatic embryogenesis can be established independently from the starting/mother tissue and leads to an unlimited number of genetically identical propagules that develop in a comparable, or even faster way, to their zygotic counterparts. In practice achieving this ideal concept, by “a trial and error strategy”, remains out of reach for most conifer species and this problem precludes a cost-effective integration of somatic embryogenesis into tree improvement programs. There is a growing amount of information about endogenous molecular processes at the different steps of somatic and zygotic embryogenesis (reviewed in Trontin et al. 2015). This molecular knowledge may result in new options to determine or improve the embryogenic potential and maturation ability of propagated ET by applying external stimuli. We briefly review below a few successful, or partially successful approaches to achieve this aim, including comparative “omics” of SEs and ZEs or different culture conditions, modulation of auxin transport (p-chlorophenoxyisobutyric acid, PCIB), alteration of actin cytoskeleton (anti-actin drugs, latrunculin B), chromatin structure (TSA), or redox state (modification of the glutathione pool, GSH, GSSG).

5.1 Improved embryogenecity

Uddenberg et al. (2011) successfully increased the initiation frequency of secondary somatic embryogenesis from somatic seedlings (germinating SE) of *P. abies* by an epigenetic approach using the histone deacetylase inhibitor trichostatin A (TSA) at 10 µM. Initiation frequency was significantly increased in germinating SE treated with TSA for 10 days (85% vs. 35% in untreated control) and also in 10-day-old germinants exposed to TSA for 5 days (22% vs. 5%). Genes with demonstrated impact on maintenance of embryogenic potential in *Arabidopsis* such as *LEC1* and *ABI3* are activated following TSA treatment (Uddenberg et al. 2011). Although expression of related genes in *P. abies* (*PaHAP3A* and *PaVP1*, respectively) was under a reliable detection limit in both control and TSA-treated SE during somatic embryogenesis induction, it was demonstrated that TSA can affect expression of these genes during SE maturation.

Transcriptomics revealed that the maintenance of cellular redox homeostasis is an important process during somatic induction treatment in *P. glauca* (Rutledge et al. 2013). Pullman et al. (2015) studied the effect of an altered redox environment on the induction efficiency of loblolly pine (*P. taeda*) and Douglas-fir (*Pseudotsuga menziesii*) and analyzed the concentrations of reduced and oxidized forms of glutathione (GSH, GSSG), ascorbic acid and dehydroascorbate on a weekly base. The effect of relevant compounds (that were significantly increased in early-stage embryo growth) on the initiation ability was then tested. The response to reducing agents varied in different trials and with different seed sources suggesting medium oxidation in air over time. According to their studies over 4 years, the low-cost reducing agents sodium dithionite and sodium thiosulfate significantly increased ET initiation by 8-99% in *P. taeda* and 5-30% in *P. menziesii* on average.

5.2 New tools to check maturation yield and SE quality

"Substantial equivalence" of SEs and ZEs can be checked using genome-wide technologies such as transcriptomics, proteomics and metabolomics. Even though they are still difficult to perform routinely, SE quality and maturity can be accurately estimated by these technologies, including combined "omic" ones. Cotyledonary SEs obtained by the best maturation condition available were compared with ZEs at different cotyledonary stages, up to the fully desiccated mature embryo, using transcriptomics with *P. taeda* (Pullman et al. 2003) or proteome analysis with *P. pinaster* (Morel et al. 2014b) as well as by a selection of biological and biochemical assays. It was shown that cotyledonary SEs did not conform to fully mature ZE but that they most closely resemble fresh, maturing cotyledonary ZEs. Similarly, comparative transcriptomics of SE and ZE in *A. angustifolia* specifically revealed an auxin signaling failure (Elbl et al. 2015). In all 3 cases the robust data set highlighted a need for putative protocol refinement at the

proliferation, maturation or post-maturation steps. In *P. pinaster* a set of 2 quality markers, derived from *glutamine synthase* gene isoforms (see part 4 in this chapter), similarly revealed precocious germination patterns in cotyledonary SEs suggesting that they did not reach the full maturity stage found in ZE (Pérez-Rodriguez et al. 2006). The expression of 6 developmentally regulated genes (*legumin*- and *vicilin-like*, *group 4 LEA*, *HD-ZIP I*, *26S proteasome regulatory subunit* and *clavata-like*) was monitored in both SE and ZE of P. *taeda* and also in SE of *P. oocarpa* obtained under the maturation conditions developed for *P. taeda* (Lara-Chavez et al. 2012). Differences between SE and ZE as well as between the different pine SE systems suggested that the tested maturation protocol resulted in higher SE quality in *P. taeda* than in *P. oocarpa*. In *P. glauca* the beneficial effect of PEG in the maturation medium (improved SE yield and quality) was demonstrated by using a comparative cDNA array strategy of PEG-treated and control lines (Stasolla et al. 2003b). An integrated transcriptome and proteomic approach was similarly shown to be a robust diagnostic and predictive tool to detect perturbation of pathways critical for normal SE development in *P. pinaster* (Morel et al. 2014a). Differential expression of genes associated to embryo development or culture adaptive response to high vs. low gellan gum levels in the maturation medium could be detected as early as one week after exogenous ABA treatment.

Besides, Robinson et al. (2009) demonstrated that metabolic profiles of proliferating ET from *P. taeda* could be used to accurately predict, in a genotype-independent way, its regenerative capacity. A selected subset of 47 out of the 208 metabolites detected was sufficient to build a descriptive model of maturation ability of embryogenic lines. Their study showed that stress-linked mechanisms may be the reason for interclonal variabilities during maturation. Reduced productivity may either be the consequence of malnutrition or environmental pressure, two reasons that led to an impaired nutrient uptake, subsequent cellular stress and retarded development. Therefore, changes on the transcriptional level are also of specific interest. It is expected that metabolome studies will lead not only to a better understanding of SE development but also to practical tools for monitoring early metabolic events that determine ET physiology at critical stages.

5.3 Improved regenerative capacity (yield and quality)

Find et al. (2002) analyzed the effect of the auxin antagonist PCIB (p-chlorophenoxyisobutyric acid) during SE maturation in *Abies nordmanniana*. PCIB in the range 5.4-21.5 mg l^{-1} reduced ET proliferation and promoted the development of high-quality cotyledonary SEs. This effect was dependent on both the concentration and the application period from low exposure (week 4-8) to full exposure during the entire maturation period. Overexposure to PCIB during maturation caused abnormal embryo development, i.e., a reduced number of

cotyledons. An optimal protocol for PCIB application was strongly influenced by the genotype, thus a general scheme that agreed with all tested cell lines could not be found. These results suggest that endogenously produced auxin may be one reason for low or failing maturation of embryogenic cultures of *A. nordmanniana*, but it implies further that auxin may play a critical role in proper development of cotyledons during the later stages of embryo maturation.

Reorganization of cytoskeletal structures has an important role in PCD. Different waves of PCD are required for the correct development of SE in conifers. A suspensor-specific actin gene expression in *P. abies* was affected by an anti actin drug approach (Schwarzerová et al. 2010). Thereby, the application of low doses (50-100 nM) of latrunculin B during SE maturation predominantly killed suspensor cells, while cells in the meristematic centres remained viable. The treatment resulted in an accelerated maturation of more advanced embryos and the elimination of insufficiently developed embryos (synchronisation of SE development). Latrunculin B led to a decline in the expression of actin gene isoforms, which were predominantly expressed in the suspensor. Vondráková et al. (2015) showed that the strongest effect of latrunculin B occurred when the drug was applied at the beginning of the maturation process. While the total number of cotyledonary embryos was lower than in untreated control cultures, the surviving embryos were of better quality while underdeveloped embryos were eliminated.

Belmonte et al. (2007) used a transgenic approach in *P. abies* to upregulate *HBK3*, a member of the KNOX1 family. Homeobox genes of the KNOTTED1-like class are related to SAM differentiation and formation of organ boundaries. Expression of some *KNOX1* genes in *P. abies* (*HBK2, HBK4*) is specific of ET competent to form cotyledonary embryos (Larsson et al. 2012b). Delayed expression of *HBK2* and *HBK4* in lines treated with the polar auxin transport inhibitor NPA resulted in embryos lacking a SAM. *HBK3* ectopic overexpression resulted in the accelerated differentiation of immature SE from PEMs. Immature SEs showed enlarged embryogenic heads and were able to convert into cotyledonary embryos at a higher frequency. Furthermore, transgenic SEs developed an enlarged SAM with concomitant upregulation of *ARGONAUTE* a gene with specific expression in meristematic cells. The *ARGONAUTE* family takes part in the RNA-induced silencing complex. *AGO* genes are required for proper embryo development and are themselves regulated by miRNAs (e.g. *miR168,* Zhang et al. 2012b). Ectopic expression of *HBK3* and other *HBK* genes (*HBK1, 2, 4*) in *Arabidopsis* revealed similar functions of these genes in spruce and *Arabidopsis* (Belmonte et al. 2007; Larsson et al. 2012b). In addition, it was found that *HBK3* is regulating SE yield through alteration of the glutathione and ascorbate metabolisms (Belmonte and Stasolla 2009).

A modification of the glutathione redox state was also detected in relation

with *HBK1* expression for *P. glauca* (Belmonte et al. 2005). Both SE yield and quality were clearly improved by supplementation of the maturation medium with GSH, followed by a replacement with GSSG during the remaining maturation period. The overall embryo population more than doubled and the percentage of fully developed embryos increased from 22% to almost 70%. These embryos showed an improved post-embryonic growth and conversion frequency. The localisation pattern of the SAM marker gene *HBK1* (apical cells in control embryos) was extended to the subapical cells of treated embryos. Similar improvements of both SE yield and quality was achieved by Belmonte and Stasolla (2007) with application of dl-buthionine-[S,R]-sulfoximine (BSO), which inhibits the biosynthesis of reduced glutathione (GSH), thereby switching the total glutathione pool towards its oxidised form (GSSG).

Similarly to *P. glauca*, the manipulation of the GSH/GSSG ratio in the maturation medium led to an alteration of nitric oxide emission and improved early somatic embryogenesis in *A. angustifolia* (Vieira et al. 2012). Low concentrations of GSH (0.01 and 0.1 mM) increased the yield of early SEs (in suspension culture) in a few days, whereas a longer exposure (> 7 d) led to a loss of early embryo polarisation. Compared with that, high levels of GSH (5 mM in prematuration gelled culture medium) led to the proper development of globular embryos.

Alterations of the redox environment of maturation medium with sodium thiosulfate in *P. taeda* and *P. menziesii* (Pullman et al. 2015) did not result in different GSH or GSSG contents but stimulated early-stage embryo development. In addition, supplementation of the germination medium with GSSG resulted in improved germination ability of SEs. Interestingly, there is conclusive evidence from various conifer species (*P. abies, P. glauca, A. angustifolia, P. taeda, P. menziesii*) that improved regenerative capacity of ET can be obtained by modifications of the redox state in the cultures.

6. Conclusion

Molecular studies are increasingly contributing to a better knowledge of somatic embryogenesis-related genes in conifers, in particular with the recent impact of genome-wide profiling of transcripts (including sRNAs), proteins and metabolites. There are currently only a few studies available for a limited number of species (ca. 25 references targeting two *Picea*, three *Pinus*, three *Larix*, one *Araucaria* and one *Cupressus* species, Trontin et al. 2015) but the corresponding data sets, already highlighted above, indicated the complexity of the genes network involved in both embryogenicity and regenerative capacity of ET. This helps to establish lists of putative marker genes of different somatic embryogenesis steps or SE developmental stages and transitions from ET initiation (Rutledge et al. 2013) to early (Vestman et al. 2011) and late embryo development (Stasolla et al. 2004,

Morel et al. 2014b). Most of the identified genes remain, however, to be validated as robust markers through targeted gene studies in different species across the conifer clade before their operational implementation can be achieved. The simple and sensitive detection of the absence/presence of a marker would be desirable as expression levels could be highly variable depending on many factors including the culture conditions. Besides, it is necessary to develop molecular tools to check for any somaclonal variation of genetic or epigenetic origin as somatic embryogenesis coupled with cryopreservation involved various steps before emblings are produced.

We reviewed here evidence for efficient diagnostic tools to monitor genetic alteration during somatic embryogenesis and after cryopreservation. Estimation of ploidy levels through karyological or flow cytometry studies have been used to detect both genetic and reversible, likely epigenetic, modifications during somatic embryogenesis (von Aderkas et al. 2003). More recently DNA alteration as measured by the frequency of AP sites (apurinic/apyrimidinic) resulting from base excision and repair of oxidized bases could be used to check the effect of different carbohydrates sources in embryogenic cultures (Businge and Egertsdotter 2014). SSRs or even RAPD markers could be used to detect DNA variation in ET and SE but the biological significance of low genetic stability at these loci remains unknown as no large difference in allele frequency could be found compared to in control seedlings families (Burg et al. 2007, Helmersson et al. 2008). In the case of *P. sylvestris* low genetic stability as reflected by SSRs variations were associated with higher maturation ability suggesting that these changes could be more related with genome plasticity to cope with environmental stress such as *in vitro* culture (Burg et al. 2007). Similarly SSRs and RAPDs markers were successfully used to detect DNA mutations in reactivated cultures from cryopreserved stock, particularly in response to mutagenic DMSO treatment, but these changes rarely affected SE normal development and germination. More recent studies further show that episomaclonal variations occurred during somatic embryogenesis resulting in different epitypes, i.e., in different gene expression patterns within the same genotype. Both miRNA and transcriptomic studies were efficient tools to reveal disturbed gene expression profiles resulting, e.g., from ET reversion towards NEC during prolonged subculture (Zhang et al. 2010a) or from different temperatures during SE development (Yakovlev et al. 2014).

At present there are a choice of markers of embryogenicity (Tables 1, 2, 3) that are considered to be reliable, taking into account that they have been found expressed at higher levels in ET vs. NET in independent reports and in more than one species, e.g., *ABI3/VP1*, *WUS*/*WOX2*, *LEC1*/*CHAP3A*, and *SAP2C* (Park et al. 2010; Palovaara et al. 2010; Klimaszewska et al. 2011; Uddenberg et al. 2011; Schlögl et al. 2012). *WOX2* appeared as one of the best targets to check the

embryogenic state as other genes such as *LEC1*-like may have different functions in conifers and could be detected in NET. Other genes of high interest such as *WOX* (e.g. *WOX8/9*, *WOX8A*, *WOX9*, *WOX12*), *germin-like protein* (*GLP)*, β-*expansin*, *cellulase*, *cytochrome P450*, *arabinogalactan protein (AGP),* or some miRNAs (*miR159*, *miR171*) need to be confirmed in different species. Interestingly it became apparent that marker genes of embryogenecity are somewhat different from markers of ET regenerative capacity (Bishop-Hurley et al. 2003).

Specific comparison of productive (plant-forming) and non-productive (non-plant-forming) ET as well as comparative studies of SE and ZE development revealed a number of genes and associated (epi)genetic processes that are candidates biomarkers or tools to check the ET regenerative capacity at the levels of maturation yield, synchronism of SE development or SE quality (Table 4). All 3 major epigenetic processes involved in the regulation of gene expression, i.e., DNA methylation, chromatin modifications and miRNAs, were found associated with ET regenerative capacity.

A low global DNA methylation or specific DNA methylation pattern were associated with an increased maturation ability (Noceda et al. 2009; Klimaszewska et al. 2009). DNA methylation can apparently be modulated by PGRs (Leljak-Levanić et al. 2009) but remains poorly controllable with hypo- or hypermethylating drugs. There is an apparent link between trans-methylation mechanisms, ethylene production and polyamine levels as the S-adenosyl-methionine (SAMet) protein and its derivatives are involved in all these pathways. The expression level of related genes such as *ACC oxidase* was associated with both ethylene production and maturation ability (Lu et al. 2011). Increased ethylene production, lower polyamine content and DNA methylation could be linked with maturation ability in some species (Noceda et al. 2009; Jo et al. 2014). The interest of genes involved in polyamine levels and ethylene production was also strongly supported by proteomic studies (Lippert et al. 2005; Balbuena et al. 2009; Teyssier et al. 2014). Expression of embryogenesis-related genes is apparently driven by chromatin modifications (Uddenberg et al. 2011) and related genes such as ubiquitin-protein ligases were proposed as predictive markers of correct SE development (Morel et al. 2014a). Similarly Zhang et al. (2012b, 2014) showed that several miRNAs contributed to both correct embryo patterning (e.g. *miR159*, *miR166*, *miR168*, *miR171*) and synchronism in development (e.g. *miR156*, *miR167*, *miR397*, *miR398*) targeting important TFs families. Some of these genes should provide excellent biomarkers but it is not yet known if they have similar functions in different species.

Many genes (especially TFs) involved in early organization of embryo patterning driven by polar auxin transport and auxin-mediated, ABA-dependent response machinery during late embryogenesis are also candidate markers of

maturation ability of propagated tissues. We highlighted in this study a few genes from the *PP2C*, *CDPKs*, *SnRK2*, *NAC*, *WRKY*, *ERF*, *MYB*, *HD-ZIP*, *bZIP*, *ARFs* or *IAA* families but many other important genes may be involved in correct embryo development (Trontin et al. 2015), and may be useful for routine monitoring, especially in comparative studies of both SE and ZE.

Cellular redox homeostasis was also shown to be a generic sensor for the regulation of embryo development (Stasolla et al. 2004). Maturation yield and SE quality are affected by deregulation of glutathione metabolism (Belmonte et al. 2005) which in turn can be affected by sub-optimal conditions for ET proliferation (Li et al. 2015) or maturation (Teyssier et al. 2011; Morel et al. 2014a). Various genes involved in both detoxification and control of the cellular redox state can be considered as good candidate predictive markers of embryo development, e.g., *glutathione-S-transferase* (GST), *superoxide dismutase* (*SOD*) and genes encoding other proteins with SOD activities, such as *germin-like protein* (*GLP*). There is also strong concordance of "omics" data sets for the involvement of genes related to other abiotic stresses (especially anoxia) during embryo development with clear implications in the improvement of maturation protocols. Various genes involved in glycolytic, glycolysis and gluconeogenesis pathways, but also in alcoholic fermentation (*alcohol dehydrogenase*, *pyruvate decarboxylase*) or response to oxygen level (*SuSy*, *enolase*), are regulated in sub-optimal maturation conditions (Lippert et al. 2005; Teyssier et al. 2011; Morel et al. 2014a). More surprising is the involvement of defense genes related to secondary metabolism pathways or pathogenesis-related proteins.

Finally it became strongly apparent in this review that proteins involved in both desiccation tolerance and storage reserves are appropriate markers of SE quality. LEA, dehydrins, HSPs and small HSPs accumulation at late stages of embryos development are affected by maturation conditions which in turn impact the SE germination ability (Teyssier et al. 2011, 2014; Businge et al. 2013). Different storage proteins genes of the vicilin-, legumin- and cupin domain-containing families are of high importance in conifers for the development of high-quality SEs. Expression of these genes was found to be reduced in different species in unfavourable maturation conditions (Stasolla et al. 2003b; Lara-Chavez et al. 2012; Schlögl et al. 2012). Similarly, genes related with storage protein synthesis and involved in nitrogen assimilation, controlled proteolysis and amino-acids metabolisms were all revealed as reliable sources of quality-related markers, e.g., glutamine synthase isoforms , glutamate synthase, proteasome subunits (Lippert et al. 2005; Businge et al. 2013; Morel et al. 2014a; Teyssier et al. 2014).

The current molecular knowledge and available technologies have already resulted in a few practical outcomes to estimate SE quality (comparative omics) or to improve embryogenecity or regenerative capacity through modulation of auxin

transport, actin skeleton, chromatin structure or cellular redox homeostasis. Despite the fact that much progress has been achieved in recent years, much improvement is still to come especially with the predicted release of several conifer genomes which may give a boost to developmental genomics as applied to conifer embryo development (Plomion et al. 2015).

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Part 2.

Application of Vegetative Propagation of Forest Trees

Teak

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Abstract

Teak (*Tectona grandis* Linn. f.) is one of the most prized high value timber species. Industrial teak plantations have in recent times rapidly expanded due to shrinking supplies followed by a total ban on harvesting teak from natural stands. The clonal option offers an attractive means of mass producting superior quality planting stock of this species focusing on phenotypic criteria and wood properties. Protocols for large scale propagation by rooted cuttings and *in vitro* microcuttings of mature selected teak trees have been developed to meet the shortage of planting material. The respective pros and cons of these two techniques are reviewed in this chapter, emphasizing the comparative advantages of the efficient tissue culture procedure for meeting increasing requirements of fast growing and premium quality teak planting material in the wet tropical regions worldwide.

Keywords: Axillary budding; Clonal propagation; International dispatch; Meristems; Micropropagation, Rejuvenation; Planting stock improvement.

1. Global status of teak

Tectona grandis Linn. f., commonly known as teak, is a large and long-lived arborescent tree belonging to the *Lamiaceae* family and is native to India, Laos, Myanmar (ex-Burma) and Thailand (Tewari 1992). It remains one of the most prized high value timber due to the outstanding properties of its wood, with special mention for durability and aesthetic features (FAO 2009). This attractiveness has spurred the introduction of the species for timber production in several tropical countries of Asia, starting with Indonesia some 4 to 6 centuries

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ago(Siswamartana 2000; Verhaegen et al. 2010), then Africa and Latin America (Ball et al. 2000). The list of countries that have embarked on industrial teak plantations has rapidly expanded, recently brought about by private investors eager to meet the demand for high grade timber in the wake of declining supplies from natural stands (FAO 2009; Kollert and Cherubini 2012).

Current expectations are to produce teak wood from commercial plantations with much shorter rotations than the longer ones from natural stands, thus giving these establishments a high comparative advantage to become a main source of supply. As demand for plantation-grown teak increases, the private sector has increasingly become involved in commercial plantations. All this has become possible through the judicious use of selected superior, fast growing genetic planting material that produces a high volume of quality wood in the shortest possible time frame (Ugalde 2013).

2. Rationale for propagating teak vegetatively

Sexual propagation through seeds remains for teak, as for most species, the easier and the more natural way to produce new plants, with each seedling being genetically different from another. This creates genetic diversity and is useful for genetic improvement through sound breeding activities. Propagating teak by seeds has been traditionally practiced for centuries, with the possibility of storing the seedlings in the form of "stumps" until the suitable planting season (Kaosa-ard 1986). However, mass production of superior teak planting stock by seeds is impeded by several limitations such as insufficient quantities of fruits produced, low germination rates and a positive correlation between flowering age and forking height (White 1991; Kaosa-ard et al. 1998, Callister 2013). The sooner teak trees produce flowers, the shorter their clear bole length, and hence, the lower their market value. Seed-derived teak trees, even from the same progeny, also show substantial variability for economically important traits that are assumed to be mainly under non-additive control (Kjaer et al. 2000; Callister and Collins 2008; Chaix et al. 2011; Monteuuis et al. 2011).

In 1996, Kjaer and Foster wrote that it will take at least 50 to 70 years before genetically improved teak plantations established from seeds can be harvested while uncertainties associated with the resulting practical genetic gain will remain. Kjaer et al. (2000) further stressed that such breeding strategies will remain heavily penalized by low seed productivity, with average yield of 50 kg per ha from age 15 according to Wellendorf and Kaosa-ard (1988), and overall poor germination rates. The large demand for improved seeds on the one hand and limited productivity of the clonal seed orchards on the other, makes seed procurement a difficult business. At present, a large part of the seed is collected

from the more easily accessible seed sources, e.g. road sides and urban areas, consisting mostly of short branchy trees that are fruiting prematurely and abundantly. This is very likely the main reason for the poor quality of most seed-derived teak planting stock. These aspects have been discussed by White and Gavinlertvatana (1999) who concluded that the "seedling route is outdated and actually represents a deterrent to increased productivity in teak plantations", and as such, to commercial teak plantation investment. According to these authors, the magnitude of the real genetic gain associated with the seedling route has yet to be clearly defined, and the basic question of whether all the efforts invested in it during the past decades are worthwhile, has remained.

In contrast, asexual or vegetative propagation consists in duplicating, theoretically in unlimited numbers, selected genotypes while preserving through mitotic divisions their original genetic make-up, and consequently all their individual characteristics including the economically important traits poorly inherited through seeds. Further, vegetative propagation can be applied to any individual that does not produce fertile seeds, either because it has not yet entered the mature stage or as a result of unfavorable environmental conditions.

Similarly as for other tree species (Zobel and Talbert 1984), applying vegetative propagation to teak can be useful for research as well as for operational activities. Research aspects encompass:

(i) Clonal tests, in order to compare and identify superior clones for operational planting (Goh et al. 2013a, Monteuuis and Goh 2015);

(ii) Genotype X environment interactions for selecting the clones to be deployed according to their adaptibility to planting site conditions (Goh and Monteuuis 2012);

(iii) Genetic parameter estimates, including broad sense heritabilities, genetic correlations between traits and the magnitude of "C effects" (Callister and Collins 2008, Goh et al 2013a); and

(iv) *Ex-situ* conservation of particular genotypes and gene complexes for germplasm enrichment and further use in other environments.

The operational usefulness of vegetative propagation for teak can be for:

(i) Establishing clonal seed orchards, being aware of the limitation of low productivity, but also of the benefits, i.e., the improved and thereby superior genetic quality of the seedlings produced (Chaix et al. 2011, Monteuuis et al. 2011, Goh et al. 2013b); and

(ii) Mass producing rooted cuttings for cutting forestry that can be implemented in the form of monoclonal blocks of various sizes or of clones planted in mixtures (Monteuuis and Ugalde Arias 2013).

In such a situation, mixing the clones at the plantation level reduces the impact of genotypes that may not be well adapted to the site, contrary to large

monoclonal blocks that are more uniform. Nonetheless, in spite of the deployment option, mass propagating the different genotypes separately, regardless of the added constraints of it being more laborious and time-consuming, permits the number of representatives of each clone to be known for a better control of the genetic compostion of the tree populations in the field. This will prevent the risk of having genotypes with the higher multiplication and rooting capacity, but not necessarily the best field performers, supplanting others, thereby leading to the reduction in the genetic base to ultimately only a single clone.

3. Vegetative propagation of teak by *ex vitro* methods

3.1 Grafting and budding

Teak grafting, especially by budding with success rates of nearly 100% in Thailand (Kaosa-ard 1998), has in the past been the most widely practised vegetative propagation technique for establishing clonal seed orchards or *ex-situ* gene banks (Singh and Beniwal 1993). It is a low-cost technology applicable to any teak genotype regardless of its age, providing that the usual basic requisites are met (Hartmann et al. 1997).

However, grafting or budding gives rise to genetically "composite" plants made up of the selected genotype of the grafted scion and of the unselected genotype of the rootstock. This is liable to produce shoots faster than the selected material that was grafted and from which the rootstock shoots cannot be visually distinguished as each looks very much alike to the other, hence frequently resulting in "illegitimates" (Bagchi et al. 1991). The occurrence of such "illegitimates", which may affect a significant proportion of the clonal seed orchards, is likely to depreciate the genetic quality of the seeds produced by illegitimate "mothers" and also by the surrounding "legitimates" fertilized by genetically polluted pollen (Bagchi et al. 1991, Tilakaratna and Dayananda 1994). The consequences can be even more serious when such "illegitimates" are used as stock plants for mass clonal propagation by rooted cuttings for operational planting. Seed producers or stock plants clonally produced on their own roots, for instance, by cuttings or microcuttings prevent such risks: they either grow or die, and illegitimates do not arise. Another aspect associated with the production of clones by grafting or budding is the possible influence of the seed-derived rootstock on the performance of the grafted scion, such as reduced vigor and a branchy architectural development. Lastly, the quality of the connection between the stock and the grafted scion can also become a risk as an additional source of within-clone phenotypic variability.

3.2 Layering

Successful propagation of teak by layering was reported for shoots sprouting from the stump of a 33-year-old felled tree (Lahiri 1985). Mound layering from 5 year-old felled teak trees gave success rates ranging from 45% to 81% depending on the time of the experiment (Monteuuis et al. 1995). However, this technique requires the felling of the donor tree. If it is not cloned, there is a risk of losing the superior genotype, and thus this technique is not practically adaptable to large scale operations.

3.3 Propagation by rooted cuttings and minicuttings

An efficient technique for rooting cuttings from teaks of various ages, including individuals which had entered the flowering stage a long time ago and could therefore be classified as physiologically mature (Hackett 1985; Wareing 1987), was developed during 1992-1994 in Sabah, East Malaysia (Monteuuis 1995; Monteuuis et al. 1995). Subsequently, the successful transfer of this procedure to various tropical countries has confirmed its efficiency for clonally mass propagating by rooted cuttings a wide range of teak Plus trees regardless of their age while preserving their characteristics. Setting sections or "sticks" cut from low branches of the selected tree under shaded and mist system facilties stimulates the production of elongating shoots that can subsequently be used as cuttings for rooting the selected mature genotypes (mobilization phase). Thereafter, the first generation of vegetative copies from the original ortet can be obtained. This method is practically preferred, it being more conservative, to the use of coppice shoots arising from the stump of the selected tree that had been felled (Palanisamy and Subramanian 2001; Singh et al. 2006; Husen and Pal 2007). As in layering, there is indeed always a risk that the felled Plus tree does not produce sprouts from the stump and ultimately dies. Generally represented by a sole individual, which is the case for most seed-derived candidate Plus trees, this technique may result in the loss of the genotype. The "stick" method, further used with success by Surendran and Muralidharan (2007) and thereafter by Akram and Aftab (2009) for cloning 40 to 50 year-old teak Plus trees, has also proven to be more practical and efficient than (serial) grafting or budding onto younger rootstock (Husen and Pal 2003; Shirin et al. 2005).

The few first rooted cuttings obtained from this mobilization phase were then managed intensively as stock plants before embarking on a serial propagation or "cascade process". The capacity for adventitious rooting of the plant material increases gradually with the number of sucessive generations of cascade. Average rooting rates of 70% can be obtained after three cycles of serial propagation (Monteuuis 1995; Monteuuis et al 1995). This apparently is the minimal level of rooting responsiveness required for embarking on large scale production of teak rooted cuttings under cost-efficient conditions.

3.4 Advantages and limitations

Propagation by rooted cuttings of selected teak genotypes of various ages has thus proven to be feasible, with sufficiently high success rates to be compatible with cost-effective large-scale production. For instance, KVTC in Tanzania has routinely used this cloning procedure for producing up to 250,000 rooted cuttings/per annum from mature selected teak genotypes (Hans Lemm, personal communication). Such good results depend, however, on a few basic requirements, such as:

(i) Suitable nursery facilities (Monteuuis et al. 1995), consisting mainly of adjacent shaded areas: one for maintaining the container-grown stock plants under intensive management, especially with regard to watering, feeding, and hedging/pinching operations (Hartmann et al. 1997) and another nearby area equipped with a reliable mist system (Hartmann et al. 1997) for rooting the cuttings, and then facilitating the weaning and hardening processes prior to field planting;

(ii) Efficient mobilisation and rejuvenation techniques as detailed by Monteuuis et al. (1995) for physiologically rejuvenating the mature selected genotypes in order to improve their adventitious rooting ability and suitable shoot-producing capacity for successful rooting; and

(iii) Adapted stock plant management for stimulating the production of shoots with the highest potential for adventitious rooting. Such shoots are characterised by distinctive morphological traits as described previously (Monteuuis 1995, Monteuuis et al. 1995). This must be considered the determining factor for ensuring good rooting rates. It requires special care, skills and techniques, particularly, where attention and observation are concerned and which are often underemined in practice.

More recently, different Latin American countries, in particular, Costa Rica, Brazil, Guatemala, have developed mass clonal production of teak trees using young vegetative minicuttings rooted under aeroponics-fog system conditions (Monteuuis and Ugalde Arias 2013). This system, which has proven to be quite efficient and attractive, requires suitable stock plant management and sophisticated greenhouse facilities equipped with a reliable and high quality fog system. Similar to more traditional methods of propagation by rooted cuttings, stock plants must have a high capacity for adventitious rooting, requiring prior physiological rejuvenation of the genotypes selected from mature teak trees. Nonetheless, the full production cost of these aeroponics-derived minicuttings has to be taken into consideration and might be a limitation under a commercial production set up.

The main limitations compared to tissue culture procedures are:

- an overall lower production efficiency and the effect of climatic changes;

- the bigger space requirement of the facilities;
- the competence of human resources required to collect and maintain the stockplants in an adequate condition for adventitious rooting, particularly if obtained from mature selected genotypes; and
- the limitations, if not impossibility, to export the produced rooted minicuttings to overseas countries owing to stringent phytosanitary requirements of each importing country.

4. *In vitro* micropropagation by axillary budding

Teak can also be vegetatively propagated by tissue culture (Gupta et al. 1980; Mascarenhas and Muralidharan 1993; Sunitibala Devi et al. 1994; Suhaendi 1998). In Sabah, East Malaysia (Monteuuis et al. 1998; Goh and Monteuuis 2001), Thailand (Kaosa-ard et al. 1987; Gavinlertvatana 1998), and in Brazil (Monteuuis and Ugalde Arias 2013), large scale micropropagation activities have been successfully developed for domestic as well as for international markets under the impetus of private companies (http://proteca.com.br; http://www.ysgbiotech.com; http://www.semseo.co.uk/doc/index.cfm?id_doc=575).

Although somatic embryogenesis, particularly of unicellular origin, may be useful for genetic engineering, micropropagation by axillary budding has been preferred to micropropagation by adventitous budding and somatic embryogenesis for large scale *in vitro* production of teak. This is due to higher culture sustainability and genotypic fidelity compared with *de novo* procedures. It is estimated that to date, several millions tissue-cultured teak plantlets have been micropropagated using this axillary budding technology. However, based on the increasing number of plantations that have been established by using clonal planting materials over the past 10 years, particularly in Latin America, the actual scale could very likely be in the double digit million figures. The total amount is difficult to determine accurately as information on sales by the supplier companies generally remain confidential per agreement between supplier and buyer.

The *in vitro* technology described in the subsequent paragraphs was developed by the YSG Biotech Sdn Bhd, Yayasan Sabah Group, where it has been applied with great satisfaction for almost 2 decades from seeds and uppermost, from field-selected Plus trees (Monteuuis et al. 1998; Goh and Monteuuis 2001).

4.1 From seeds

In vitro culture conditions can be very useful for rapidly increasing the number of individuals obtained from seeds of presumably high genetic value but available only in limited number and with low germination capacity (Akram and Aftab 2007). This may be the case of provenances or progenies derived, for

instance, from controlled pollination, or from clonal seed orchards (Yasodha et al. 2005). The beneficial effects of tissue culture is to improve the germination capacity as well as to vegetatively propagate the newly *in vitro* germinated genotypes (Monteuuis et al. 1998). These are mostly propagated as a mixture as they are too young to be reliably selected for individual clonal propagation. This *in vitro* "bulk propagation" can be applied for various lengths of time, depending on needs. However, during the course of the successive propagation cycles, the risk of narrowing the original genetic base owing to the potentially higher multiplication rates of certain genotypes over others could become a problem and should not be underestimated.

Once developed to the right height, the *in vitro* germinated seedlings can be cut into microcuttings to be clonally micropropagated depending on the objectives in mind, for instance, for among- and within-clone variability assessment. In practice, however, this option remains far more cumbersome than "bulk propagation" and is not practically warranted since any teak tree, regardless of its age, can be successfully mass micropropagated.

4.2 From field-selected plus trees

The field-selected phenotypes to be micropropagated *in vitro* can be of any age, including *in situ* individuals as well as nursery stock plants, provided vegetative buds can be collected. About 1cm-long mononodal (single node), and terminal portions from vegetative shoots, preferably actively growing, are routinely used for initiating the *in vitro* cultures with one explant per test tube in order to limit the loss from possible microorganism contaminations. Thereafter, during the subsequent stabilization and production phases, flasks containing 8 to 10 microcuttings each are then used for the mass production. Records from several years of experimentation with different-aged field-grown genotypes established that, subject to the disinfection procedure and depending on the manipulator, 20 to 30% of these primary explants could give rise to contamination-free and responsive *in vitro* cultures (Monteuuis et al. 1998; Goh and Monteuuis 2001). Overall, it takes 6 to 8 months to achieve, through serial subcultures of explants collected from mature selected donor trees, the level of physiological rejuvenation required for large scale production.

Shoot apical meristems or SAMs are big enough (overall size of 0.1 mm) in teak to be used as primary explants, which is not the case for those of most other tree species. The decussate leaf pattern of the species facilitates their excision from the apical buds of the growing donor shoots and skilled people can routinely inoculate 30 to 40 teak SAMs per hour onto proper *in vitro* culture media (Monteuuis et al 1998). In addition to higher success rates and efficiency than

nodal explants for initiating contamination-free cultures (70 vs 20-30%, respectively), especially as far as endogenous contaminants are concerned, SAMs used as primary explants are more efficient for achieving physiological rejuvenation from mature selected genotypes (Monteuuis 1989; Monteuuis and Goh 2015).

The tissue culture protocols used were designed to be as simple as possible in order to be easily applicable, even by non-tissue culture specialists, and to reduce the constraints of large-scale applications. Cost-efficiency and high productivity are in this respect essential. Regardless of the origin of the initial plant material (*in vitro* germinated seedlings or outdoors individuals), or of its age and of the kind of primary explant used (nodal or terminal segments or 0.2mm-long SAMs), the established technology allows for the mass micropropagation under *in vitro* conditions of any genotype, either in bulk or individually, through axillary-produced microshoots with an exponential multiplication rate of 3 to 4 cuttings at every 6 week-long sub-culture. Finally, 50 to 60% of the microcuttings can root spontaneously in the sole multiplication-elongation culture medium during the production phase. Further, the rooting-acclimatisation phase was advantageously achieved in nursery conditions under a mist system with more than 90% success on average in the absence of any application of rooting substance. This confirms that for physiologically rejuvenated material, application of "growth hormones" is not necessary and that the environmental conditions at the acclimatization site are the most important factors at this stage, consistently with previous observations (Bonal and Monteuuis 1997; Monteuuis et al. 1998).

Mortality during the subsequent steps of cultivation in the nursery, before the plants are sufficiently developed to be field-planted, is negligible. To date, millions of microcuttings have been produced by applying this technique, and have developed into vigorous and true-to-type vegetative offspring (Goh and Monteuuis 2012, Goh et al 2013a; Monteuuis and Goh 2015).

4.3 Advantages and limitations

For teak as for any other species that can be tissue cultured (Bonga and von Aderkas 1992), the assets of micropropagation compared to conventional propagation methods, i.e. by rooted cuttings in a nursery, are:

- Year-round production regardless of the local climatic conditions.
- Requirement of only a small space area even for huge numbers of plants produced in flasks.
- Suitably managed stockplants, with nursery facilities and associated competent staff required for their proper maintenance, are not necessary.
- Production and packing of contamination-free plants that meet phytosanitary

requirements for exportation to foreign countries.
- Higher efficiency for achieving the physiological rejuvenation needed for clonally mass propagating mature selected trees true-to-type.

In addition, the comparative advantages of the protocol developed for teak in our case lie in:
- the utilization of a unique elongation-multiplication medium for the production phase, thus reducing the use of resources in relation to time and costs (labor, culture medium, overhead expenses),
- that rooting is easily undertaken at a much cheaper cost under nursery conditions.
- the possibility to use SAMs as primary explants with the above-mentioned benefits, and
- the simplicity of procedure does not require specialized and highly paid staff.

Conversely, and potentially, the few limitations are:
- higher proportion of contaminations and longer delays for physiologically rejuvenating the mature selected plant material when nodal or terminal shoot portions are used as primary explants. SAMs have the advantage of overcoming these problems,
- limited multiplication rates when using the more natural way of multiplication by axillary budding. However, this process offers sustainability, simplicity and efficiency as well as true-to-typeness of the tissue-culture plants produced.

5. Conclusion

From our viewpoint, the basic reasons for the success of micropropagation in teak are:
- the universal reputation of teak as one of the most prized high value timber species,
- the increasing international demand for fast growing planting material that will produce premium quality teak wood in order to achieve higher returns on investment in short time frames,
- the availability of superior clones, greatly preferable to seedlings for meeting investors' goals, which depends on the access to outstanding trees that can be mass propagated true-to-type using a very efficient cloning technique, and
- the optimization of the overall process from initiation to multiplication to exportation, and ultimately, the successful *ex vitro* acclimatization of the microcuttings at the buyers' facility.

These latter conditions were met in Sabah, East Malaysia by the Yayasan Sabah Group Biotech where efficient *in vitro* and nursery protocols for mass cloning true-to-type teak Plus trees selected from highly diverse base and breeding populations (Goh and Monteuuis 2009) were developed. Comparative economic

analyses have clearly shown within this context that for the production of more than 100,000 rooted cuttings per year, the tissue culture procedures developed are more efficient ((Monteuuis et al 1998; Monteuuis 2000; Goh and Monteuuis, 2001). This is mainly due to the fact that although the *in vitro* option must take into account the establishment of a laboratory, it does not require stock plants which need to be intensively managed by competent people as is the case for the nursery option (Monteuuis et al. 1995). The investments required at the nursery level increase in far greater proportions per production target than for micropropagation.

Due to the simplicity of the *in vitro* procedure developed, it can be easily handled by committed low level local workers who are paid less than in countries with a higher standard of living. This makes the production cost of the plantlets cheap and assures a reasonable selling price backed by the renowned quality of the planting material produced when the process is shifted from seedling-derived to clonal planting materials, which is is expected to rise in volume in the future (Ugalde Arias 2013). Market prospects can be further improved with the possibility of sending tissue-cultured plants off to different destinations, at various distances, as a result of phytosanitary immunity, contrary to rooted cuttings (Goh and Monteuuis, 2001). To date, several millions of teak vitroplants have been produced by YSG Biotech and sent to different countries all around the world, including Australia, South America, Africa and within South East Asia. The possibility for such international dispatches lies in having a well-coordinated system in place, from the production of plants per order received to the endorsement by the local quarantine authority in both countries and finally, to the efficient communication among suppliers, buyers and freight agents involved. All this bring about the minimization of untoward risks and the timely arrival of the consignment at the buyer's country within 3 to 5 days, bearing utmostly in mind, the limited shelf life of these live plants.

Unlike many forest tree species, teak plants from cuttings and microcuttings develop true-to-type, in the absence of any phenotypic abnormalities such as undesirable plagiotropic growth patterns that are noted to affect (micro) cuttings of many forest species – the so-called "C effects" (Frampton and Foster 1993). Growth rates are impressive in the first few years, with 4 m of annual increment under evenly distributed high rainfalls in the absence of a long dry season (Goh and Monteuuis 2012, Goh et al. 2013a, Monteuuis and Goh 2015). In addition to this impressive growth, it is noteworthy that the cloned plants developed under such conditions have long clear boles devoid of forks and with very few lateral branches. All these positive features attest to the validity of mass selection based on phenotypic criteria brought about by the efficiency of the developed clonal techniques for teak.

Today, with the ownership of two high quality teak progeny-provenance

plots, comprising of up to 42 families from a broad genetic background, YSG Biotech continues to improve their clonal materials by providing plants that are adapted to different site conditions in tropical and sub-tropical teak-growing regions. Using the developed techniques, the possibility to supply superior quality clonal materials will undoubtedly sustain the establishment of large-scale industrial plantations with more predictable lucrative returns in the near future. Clonal forestry for a high value timber species such as teak and no doubt, other economically-important species, is here for the long haul based on the successful application of vegetative propagation techniques through tissue culture and nursery cuttings.

6. **References**

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In vitro culture of *Eucalyptus*: where do we stand?

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Abstract

Eucalyptus is the second most widely planted multipurpose woody tree species in the world commercially exploited mainly as a source of commercial cellulose fiber and wood and amenity purposes. There are several Eucalyptus tree improvement programs, but Eucalyptus domestication can still be considered as being at an early stage. The recent availability of genomic data for several eucalypt species has generated a strong interest in mastering regeneration and genetic tools since the development of an efficient transformation protocol is necessary to explore eucalypt resources through functional genomics and biotechnology. High rates of plant propagation via axillary shoot proliferation are reported for many *Eucalyptus* species. Somatic embryogenesis is conceptually the most effective propagation method to answer the increasing industrial demand for high-quality uniform planting stock and to rapidly capture the benefits of breeding programs. Most reports use zygotic embryos as the initial explant for somatic embryogenesis induction and plant regeneration but recently a protocol for somatic embryogenesis induction from adult plant material was reported for *Eucalyptus globulus* and one hybrid. Overall, these advances open up new scenarios and possibilities for the deployment of new high-performance clonally replicated planting stock. However, for useful somatic embryogenesis improvement programs of *Eucalyptus*, the

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frequency of somatic embryogenesis initiation, maturation, germination and acclimatisation needs to be improved and controlled. If this technology could be extended to elite germplasm, it would become an economically feasible tool for large-scale clonal production and delivery of improved planting stock, one of the greatest challenges in the era of global climate change. In this review, we revisit the most important aspects of *in vitro* culture of *Eucalyptus* with particular emphasis on somatic embryogenesis.

Keywords: micropropagation; zygotic and mature explants; plant regeneration; somatic embryogenesis; genetic control of somatic embryogenesis

1. Introduction

Eucalyptus spp. are important hardwood trees, native from Australia and the northern offshore islands and they occur under a wide range of environmental conditions (Williams and Woinarski 1997). The remarkable adaptability of eucalypts coupled with their fast growth and superior wood properties has driven their rapid adoption for plantation forestry in more than 100 countries all over the world accounting for more than 20 million ha (Iglesias-Trabad et al. 2009). Small fibers, desirable surface characteristics, smoothness, brightness and low tensile strength make these trees reliable for use in paper industries. Furthermore, eucalypts play an important role in plywood, particle board making and furniture industries due to its tall and straight timber that varies from medium to high density.

This genus, which includes over 800 species, is a member of the Myrtaceae family to which fruit crops such as *Acca sellowiana*, *Psidium guajava* also belong (Bedon et al. 2012; Brooker 2000). The *taxon Eucalyptus* is divided into eight subgenera; the subgenus *Symphyomyrthus* being the largest and containing the majority of the species (Poke et al. 2005). Eldridge et al. (1993) ranked the ten most economically important *Eucalyptus* species cultivated around the world as follows: *E. grandis*, *E. camaldulensis*, *E. tereticornis*, *E. globulus*, *E. urophylla*, *E. viminalis*, *E. saligna*, *E. deglupta*, *E. exserta*, and then either *E. citriodora*, *E. paniculata* or *E. robusta* for pulp production and for its solid wood. From these species, Potts and Dungey (2004) reported that *E. grandis*, *E. urophylla* and their hybrids are the most favoured for pulp and increasingly for solid wood production in tropical and subtropical regions, whereas *E. globulus* is more common in temperate regions (free of severe frosts). In comparison with other *Eucalyptus* species, *E. globulus* has superior fiber morphology and requires fewer chemicals to obtain bleached pulp due to its lower lignin content (Doughty 2000; Eldridge et al. 1993). *E. camaldulensis* is known for its ability to thrive in arid and semi-arid regions, *E. urophylla* is highly productive, and *E. nitens* is recognized for its

adaptability to colder climates (Teulieres and Marque 2007). *Eucalyptus* outstanding diversity, adaptability and growth have made them a global renewable resource of fibre and energy, representing a suitable option to meet the growing wood demands of the world and a great way to prevent natural forests from deforestation. It seems, therefore, inevitable that areas occupied by fast-growing eucalypts will continue to expand worldwide.

Despite their importance as a widely planted crop tree, eucalypt species and hybrids are well known by their recalcitrance to micropropagation and genetic manipulation (Girijashankar 2011). Only a few non-commercial laboratories are proficient at *Eucalyptus* tissue culture and transformation, and have developed robust micropropagation and transformation protocols and routinely produce eucalypts for the public research sector. To take full advantage of the recently sequenced eucalypt genome (Myburg et al. 2014), functional genomics, ecophysiology and biotechnology strategies require an efficient plant regeneration protocol. However, genetic transformation of eucalyptus is still hampered by the lack of an efficient regeneration protocol, which remains restricted to a few species and genotypes (Girijashankar 2011; Teulieres and Marque 2007).

Prior to progress with mass vegetative propagation, all *Eucalyptus* plantations were derived from seeds with varying degrees of establishment and competitive success (e Silva et al. 2004; Watt et al. 2003). Although industrial needs demand an increase in *Eucalyptus* forest productivity, most species remain in an early stage of domestication. For example, most of the genetic parameters reported in *E. globulus* are based on open-pollinated progenies (López et al. 2002). However, due to inbreeding depression from self and/or related mating, observed genetic parameters derived from open pollinated eucalypt populations may be inaccurate. Therefore, most of the more recent breeding programs introduced control-pollinated approaches, which allowed more accurate estimations of the genetic parameters and the separation of additive from non-additive genetic effects (e Silva et al. 2004). Clonal propagation represents the most effective way of capturing both additive and non-additive genetic effects brought about by both traditional tree breeding and biotechnology thus accelerating exploration of genetic gains in plantations (Mullin and Park 1992). Vegetative propagation is a widely used technique in tree breeding programs to efficiently manage populations and to improve planting stock (clonal forestry) faster than is possible with conventional seed orchard procedures (Mullin and Park 1992). *Eucalyptus* vegetative propagation is mainly done with rooted cuttings, a method explored in several clonal propagation programs (de Assis et al. 2004; Watt et al. 2003). However, this strategy is limited by the heterogeneous rooting ability response among clones and decreasing rooting potential because of ageing of parent plants (Eldridge et al. 1993; Mankessi et al. 2010; Watt et al. 2003). Clonal propagation through *in vitro* approaches can provide alternative vegetative multiplication methods, overcoming

some of the difficulties referred to above and providing very high multiplication rates of selected genotypes, with short-term forestry gains.

2. Clonal propagation through *in vitro* culture

Some of the earliest reports of micropropagation methodologies date back to the 1960s and in recent decades some progress has been made in the development of complete plant regeneration protocols. In their review on *Eucalyptus* micropropagation, Le Roux and Van Staden (1991) reported that between 1968 and 1991 only 30 out of 204 publications referring to this genus included protocols for plant regeneration. Since then, several new publications focused on plant regeneration, which shows a substantial increase in the interest of applying such an approach. Protocols for micropropagation of *Eucalyptus* have been developed for several species (Arya et al. 2009; Pinto et al. 2011). Axillary shoot proliferation has been the most used technique of clonal propagation in this genus (Glocke et al. 2006) but organogenesis (Aggarwal et al. 2010) and somatic embryogenesis (Corredoira et al. 2015) are also being applied to propagate selected genotypes. In addition to large-scale micropropagation, axillary shoot proliferation has been applied in programs aiming to: select clones with increased levels of essential oils, namely cineole in *E. polybractea* (Goodger and Woodrow 2008); conserve endangered species, such as *E. phylacis* (Bunn et al. 2005); cryopreserve (Padayachee et al. 2009); propagate selected hybrids (Watt 2014); and even establish clonal micro-gardens (Brondani et al. 2012) by exploring the juvenility of micropropagated plants to produce micro-cuttings. In general, nodal segments or shoot apices are cultured on solid media containing a cytokinin or a low ratio combination of an auxin and a cytokinin (Trindade et al. 1990). However, liquid cultures and particularly temporary immersion systems have proven their effectiveness on *Eucalyptus* micropropagation (McAlister et al. 2005; Watt 2012) and are increasingly used.

Rooting of shoots that result from axillary shoot proliferation is usually achieved following a treatment with IBA or an analogous auxin (Fogaça and Fett-Neto 2005). Histological studies showed that adventitious roots originate from vascular tissues or from newly formed xylem (Baltierra et al. 2004). An origin for the roots near the vascular system is a prerequisite for the success of rooting since in that case the new roots can easily establish connections with the vascular system of the shoots thus promoting further plantlet survival and acclimatization. However, root origin is not the only factor that affects plant development as pointed out by Mokotedi and co-workers (2010) who found that trees propagated *in vitro* present a poorer developed rooting system than trees originated by macropropagation.

Organogenesis is a useful technique not only for clonal propagation but also as a protocol for plant regeneration from genetically transformed cells

(Chauhan et al. 2014; Prakash and Gurumurthi 2009). In *Eucalyptus*, shoot formation has been induced from several types of explants cultured *in vitro* and from lignotubers (Aneja and Atal 1969; Lee and Fossard 1974), which are stem outgrowths that store reserve compounds, but problems such as hyperhydricity and shoot senescence have been reported in *in vitro* cultures (Louro et al. 1999; Whitehouse et al. 2002).

Plant regeneration from protoplasts has also been used in *in vitro* propagation of some *Eucalyptus* species with relative success (Hajari et al. 2006; Le Roux and Van Staden 1991; Pinto et al. 2013; Watt et al. 1999; Watt et al. 2003).

Despite all the techniques available, many of the *in vitro* plant regeneration protocols developed so far still need to be optimised if they are to be considered an economically feasible deployment strategy; besides, obtaining efficient plant regeneration protocols is essential for mass production of improved materials and genetic transformation (Bonga et al. 2010; Poke et al. 2005; Tournier et al. 2003).

2.1 The somatic embryogenesis process in *Eucalyptus:* an old issue with new challenges

Somatic embryogenesis (SE) is another micropropagation technique that has been used to produce plantlets of different *Eucalyptus* species. Somatic embryogenesis is defined as a non-sexual developmental process that produces a bipolar embryo (presenting both shoot and root meristems) from somatic tissues (Merkle et al. 1995; Thorpe 2000). This process was reported as the best example of totipotency in plants (Fehér 2015). Developmental stages similar to zygotic embryogenesis occur and yield an embryo with no vascular connection to the parent tissue (von Arnold et al. 2002). Applying this technique in clonal forestry has multiple advantages, including high multiplication rates, potential for scale-up and delivery via bioreactors, application of synthetic seed technologies, production of suitable target tissue for gene transfer, and maintenance of cultures by low-growth conditions or cryopreservation (essential for field-testing and further selection of the best clones, when seedling material is used). Flexibility to deploy suitable clones depending on breeding aims and/or environmental conditions is of crucial importance to manage genetic diversity and gains, and to establish successful plantations in the current global climatic change context. Moreover, SE allows mass production of selected progenies from relatively small quantities of seeds obtained from controlled crosses, which is particularly useful in the propagation of plants with low flowering and/or seed yield. Somatic embryogenesis also allows exploring the concept of multivarietal forestry by speeding up the deployment of outstanding varieties identified in progeny trials, while integrated in tree breeding programs (Park 2002; Park et al. 1998; Park et al.

2006). Despite the successful use of SE in tree improvement programmes, mass propagation and genetic transformation of eucalypts by SE remains difficult due to the low rates of SE initiation and conversion of somatic embryos into plantlets (Pinto et al. 2013). To date, induction of somatic embryos has been achieved in only few of the more than 800 species in the genus *Eucalyptus*, including *E. grandis*, *E. citriodora*, *E. gunnii*, *E. dunnii*, *E. nitens*, *E. globulus*, *E. tereticornis*, *E. camaldulensis* and some hybrids (Chauhan et al. 2014; Corredoira et al. 2015; Pinto et al. 2013; Pinto et al. 2002). The successful establishment of a SE system is dependent on the correct choice of both plant material and growth conditions. This includes selecting explants with the most appropriate source of competent cells (considering their genotype, age and type), and selecting the best physical and chemical culture conditions (media composition, light, temperature, pH and humidity, among others) that lead to the embryogenic development pathway (Phillips 2004; Thorpe 2000). The interaction between these factors is crucial for the successful induction and expression phases of the SE process and will determine the specific mode of cell differentiation and development (Gaj 2004). The success of any propagation system is set by the quantity and quality of the final product, i.e., the regenerated plants. Any applied research, with commercial or industrial goals, requires large-scale production of emblings (SE-derived plants) and special care during the acclimatisation phase and field implementation, where monitoring of genetic fidelity and performance under *ex vitro* conditions assume particular relevance. This section presents an updated review of all relevant factors impacting the SE processes in *Eucalyptus*. An overview of the different stages of this micropropagation process (from induction to acclimatization) is also presented.

2.2 Primary somatic embryogenesis

As in other woody species, zygotic embryos are the most responsive explants for inducing SE in *Eucalyptus* (Bandyopadhyay et al. 1999; Muralidharan and Mascarenhas 1987; Nugent et al. 2001; Pinto et al. 2002; Pinto et al. 2008; Prakash and Gurumurthi 2005; Termignoni et al. 1996). In the majority of these reports, NAA is the plant growth regulator used for induction of SE (Chauhan et al. 2014; Pinto et al. 2013), but recently picloram has been successfully used with explants obtained from adult trees (Corredoira et al. 2015). Cotyledon explants seem to be more responsive for induction of somatic embryos than other types of explants isolated from zygotic embryos (Pinto et al. 2013). To date, induction of SE in explants derived from mature trees of *Eucalyptus* genus has only been reported in four species and a hybrid (Corredoira et al. 2015).

In *Eucalyptus*, SE was reported for the first time from callus of seedlings of '*E.* x Liechow' (Ouyang et al. 1980, 1981). A few years later, SE was also reported on callus derived from shoots of four-year-old trees of *E. grandis* on MS

medium (Murashige and Skoog 1962) supplemented with 0.1 mg l^{-1} naphthalene acetic acid (NAA) and 5 mg l^{-1} kinetin (KIN) (Lakshmi Sita et al. 1986). Somatic embryos were also obtained by culturing friable callus in liquid medium containing 1 mg l^{-1} BAP, KIN, NAA and 2,4-dichlorophenoxyacetic acid (2,4-D) (Lakshmi Sita et al. 1986). Boulay (1987) achieved SE from hypocotyl and internode *calli* derived from seedlings of *E. gunnii* using two different basal media and a variety of PGR combinations. In *E. citriodora*, somatic embryos were obtained from zygotic embryos grown on B5 medium (Gamborg medium; Gamborg et al. 1968) with 3 mg l^{-1} NAA and 5% (w/v) sucrose (Muralidharan et al. 1989; Muralidharan and Mascarenhas 1987). In addition, Qin and Kirby (1990) were able to induce embryo-like structures in cultures of hypocotyls, cotyledons and leaves of young seedlings of *E. botryoides*, *E. dunnii*, *E. grandis* and *E. rudis*, as well as from young leaves of cultured shoots of adult *E. grandis* clones. These authors used a sequential culture technique with a medium containing MS salts, RV vitamins (reference not given) and amino acids (Qin and Kirby 1990). Slow-growing green protuberances developed from the cut surfaces of the explants after two weeks, and further developed into adventitious shoots and embryo-like structures when transferred to medium supplemented with 1 mg l^{-1} benzylaminopurine (BAP). In 1991, Watt et al. (1991) reported somatic embryo induction on MS medium supplemented with 2,4-D from *E. grandis* leaves of *in vitro* established shoots. With *E. dunnii*, induction was achieved on three-day old seedlings by addition of NAA alone or in combination with 2,4-D (Termignoni et al. 1996). Termignoni et al. (1998) reported the induction of SE in explants of mature trees of *E. saligna* and *E. dunnii*, with a high rate of plant regeneration in the latter (Patent No. PI 9801485-4 INPI). Prakash and Gurumurthi (2005) reported SE and plant regeneration of *E. tereticornis* from embryogenic *calli* obtained from mature zygotic embryos on MS medium supplemented with NAA. When *calli* were transferred to induction medium with BAP, somatic embryos developed after 1–2 weeks. Somatic embryos were successfully germinated and converted on MS PGR-free medium and rooted plants were effectively acclimatised (Prakash and Gurumurthi 2005). Direct organogenesis and a direct SE pathway of plant regeneration were reported as occurring simultaneously in the same nodal explants of *E. camaldulensis* by Girijashankar (2012).

Considering *E. globulus*, a protocol for SE induction was first described by Trindade (1996). Later, Bandyopadhyay et al. (1999) and Nugent et al. (2001) also reported SE induction and embryo formation in this species, but with a very low induction rate. Pinto et al. (2002) reported the regeneration of emblings and studied the effect of the explant origin, type of PGRs and time of exposure on the induction process of SE. These authors were able to induce SE in the presence of NAA from callus derived from cotyledon explants and from mature zygotic embryos (Pinto et al. 2002). Oller et al. (2004) obtained an embryogenic callus phase from leaves of

adult trees cultured in a basal medium with IBA, but further progress of this embryogenic callus was not reported. The most extensively used medium for the induction of SE in woody angiosperms, including several species of *Eucalyptus* (Pinto et al. 2013), is the nitrogen-rich MS medium. Pinto and co-workers (2008) assessed the effectiveness of several media such as MS, ½MS, B5, woody plant medium (WPM (Lloyd and McCown 1980)), DKW/Juglans Medium (Driver and Kuniyuki 1984) and JADS (Correia et al. 1995) during SE induction and expression in *E. globulus*. The results showed that MS and B5 were the best media for SE induction and for embling regeneration (Pinto et al. 2008). Successful induction of somatic embryos in explants derived from adult *E. globulus* trees and a hybrid *E. saligna* x *E. maidenii* has recently been described (Corredoira et al. 2015). Axillary shoot proliferation cultures, previously established from two *E. globulus* trees and one *E. saligna* x *E. maidenii* hybrid tree (all 12-year-old elite trees), were used as sources of initial explants for induction of SE. Shoot apex explants (1-2 mm long) and leaf explants (the two most apical expanding leaves from the apex) were cultured on basal induction medium consisting of MS mineral salts and vitamins, casein hydrolysate (500 mg l^{-1}), 40 mg l^{-1} arabic gum (AG), sucrose (30 g l^{-1}), vitroagar (6 g l^{-1}) and different concentrations of NAA. In a second series of experiments, the basal induction medium was supplemented with different concentrations of picloram (20, 30, and 40 μM; 4-amino-3,5,6-trichloropicolonic acid) and 40 mg l^{-1} AG. All cultures were maintained in darkness at 25ºC for 8 weeks. Somatic embryogenesis was achieved in the shoot apex and in leaf explants of the three genotypes evaluated, although the rate of embryogenesis was significantly influenced by the genotype, auxin and explant type (Corredoira et al. 2015). Picloram was more efficient than NAA for embryo formation. Although picloram has scarcely been tested in *Eucalyptus* species, this compound has been used to stimulate the somatic embryogenic process in material derived from other adult trees (Correia et al. 2011; Steinmacher et al. 2007). The highest rates of induction were obtained in medium containing 40 μM picloram and 40 mg l^{-1} AG, in which 64.0% of the shoot apex explants and 68.8% of the leaf explants yielded somatic embryos. The embryogenic response was higher in the hybrid than in the *E. globulus* genotypes, especially when NAA was added. The embryogenic cultures initiated on picloram-containing medium consisting of nodular embryogenic structures surrounded by a mucilaginous coating layer that emerged from a watery callus tissue developed from the initial explants. This coating was considered to have a protective role against the high levels of phenolic compounds observed in the callus (Corredoira et al. 2015).

Eucalyptus is in fact a rich source of phenolic compounds (Close et al. 2001) and oxidation of polyphenols may represent a limiting factor that prevents proper tissue multiplication and maintenance. Pinto et al. (2008) tested the effect of adding anti-browning compounds (ascorbic acid, charcoal, dithioerythritol,

dithiothreitol, polyvinylpyrrolidone, polyvinylpolypyrrolidone and silver nitrate) to induction and expression media (MS) in controlling tissue oxidation in *E. globulus* during the SE process. The results were discouraging as all tested compounds decreased the SE response on the expression medium. Dithioerythritol, charcoal and silver nitrate were the most effective in reducing browning of the explants when added to the expression medium. When added only during the induction period, anti-browning agents reduced accumulation of phenolic compounds but also severely reduced SE induction and the continuous exposure to antioxidants completely inhibited the SE response (Pinto et al. 2008).

In addition to mineral salts (macro and micronutrients), vitamins and amino acids a source of carbon should also be added to the nutrient medium. In *Eucalyptus*, the recommended concentrations of sucrose for SE induction vary according to different species, but usually range from 2% to 5% (w/v). Although other carbohydrates (e.g. glucose, fructose, maltose, lactose, cellobiose, mannitol, sorbitol and myoinositol) promote SE in other species (Canhoto et al. 1999; Lipavská and Konrádová 2004), the addition of mannitol to the induction medium inhibited the formation of callus on the surface of the explants in *E. globulus* (Pinto et al. 2002).

2.3 Other key factors impacting SE induction

Despite the fact that PGRs, medium composition and explant source are target points in the implementation of any SE strategy, there are other factors that are far less investigated in *Eucalyptus* but should also be highlighted. The genetic influence during the SE process is well known in other species (Merkle et al. 1995), and understanding the genetic control is an important aspect in the improvement of the SE process (Park et al. 1998). The genotype-dependent response to *in vitro* conditions is also currently considered a crucial factor in micropropagation, and particularly on the induction of SE in *Eucalyptus* (Pinto et al. 2008). However, few studies have clearly focused on the magnitude of the genetic control of *in vitro* regeneration in *Eucalyptus* (Bravo et al. 2008) and specifically in SE (Pinto et al. 2008). Such genotypic variability in the embryogenic capacity may reflect differences in the ability to activate key elements of the embryogenic pathway (Merkle et al. 1995). Thus, depending on the type and magnitude of genetic variation, better SE initiation procedures may be possible for recalcitrant species (Bonga et al. 2010; Park 2002). In 2008, Pinto et al. (2008) carried out a study to analyse genetic control in the *E. globulus* SE process. Embryogenic capacity and variability in yearly production (2002, 2003 and 2004) were studied among 13 open-pollinated families, as well as the degree of genetic control during SE. This study implemented a five parent, full-sib diallele mating design and found that SE induction varied among families and across seed production years. It also showed

that SE was under the control of additive genetic effects with 22.0% of the variation in SE initiation due to general combining ability effects and 6.4% due to maternal effects. Neither specific combining ability nor reciprocal effects were significant (Pinto et al. 2008). Such results can be potentially applied to the improvement of the SE response in breeding programs of elite families and, hence, similar studies should be undertaken with other *Eucalyptus* species.

The morphogenic pathway behind the origin of somatic embryos is still poorly understood. Somatic embryos may originate from a single cell or from a small group of cells that differentiate into an organised structure. With some exceptions in myrtaceous species (Canhoto and Cruz 1996; Canhoto et al. 1999) and in *Eucalyptus* in particular, there is a notorious paucity of cytological, histological and ultrastructural information on the different aspects associated with the induction and development of somatic embryos from explant tissues. For *Eucalyptus* a few reports describe that somatic embryos show morphological resemblance to zygotic embryos at various developmental stages (Muralidharan et al. 1989; Watt et al. 1999). According to Watt et al. (1991), embryogenic cells of *E. nitens* present the typical characteristics of those of other embryogenic systems: dense cytoplasm, small volume, prominent nucleus and small vacuoles. These authors presented histological analyses of somatic embryos at different developmental stages, although no further details were given on embryo origin. Similar observations were made for *E. grandis* (Lakshmi Sita et al. 1986) and for *E. globulus* (Trindade 1996).

Bandyopadhyay et al. (1999) examined the ultrastructure of *E. nitens* somatic embryos and compared them with mature zygotic embryos and highlighted the similarities between both structures. Akula et al. (2000) demonstrated the role of calcium in favouring the morphogenic route for SE in *E. urophylla.* In 2010, Pinto and co-workers demonstrated that the available SE protocol for *E. globulus* (Pinto et al. 2002) leads to fluctuations in reserve accumulation in somatic embryos during the SE process up to the stage of embryo maturation. Moreover, these authors showed that reserves within cotyledons of somatic embryos differ from those in zygotic embryos. This reinforces the importance of reserves in the embryogenic process and suggests that manipulating media conditions can improve SE and result in seemingly normal, healthy emblings suitable for industrial production. In that study, starch accumulation increased with time in globular somatic embryos but protein bodies were absent. Cotyledons of zygotic embryos were found to be richer in starch, lipids and proteins than cotyledons of somatic embryos.

2.4 Secondary somatic embryogenesis

After induction of somatic embryos, the next step is to maintain the embryogenic ability. Difficulties in maintaining embryogenic capacity by secondary or repetitive embryogenesis seem inherent to the embryogenic systems reported for different eucalypt species (Pinto et al. 2013). In contrast to primary SE, repetitive, secondary or recurrent SE may occur from somatic embryos in culture, either directly or through callus (for a review see Merkle et al. (1995)). This phenomenon is of key importance both for mass clonal propagation and for gene transfer technology. A much higher efficiency of secondary SE over primary SE is reported in many plant species (Akula et al. 2000; Nair and Gupta 2006; Vasic et al. 2001). In many cases, cultures are able to retain their competence for secondary embryogenesis for many years and thus constitute a very useful material for an array of different studies. Similarly to what was described for the induction stage, the proliferation of embryogenic cells may take a number of forms and is influenced by a variety of factors. In general, repetitive embryogenesis is a very slow process in *Eucalyptus*, with low proliferation rates. To our knowledge, there are only four reports on SE in eucalypts that specifically refer to proliferation and maintenance of embryogenic cultures. Maintenance of embryogenic capacity by subculturing embryogenic *calli* onto MS basal medium and B5 medium supplemented with 5 mg l^{-1} NAA has been described for *E. gunnii* (Boulay 1987) and *E. citriodora* (Muralidharan and Mascarenhas 1995), respectively. Pinto et al. (2004) developed a protocol in which somatic embryos induced in zygotic embryos of *E. globulus* were cultured in MS medium with NAA (3 mg l^{-1}). These authors found that a reduction in NAA levels increased the proliferation of globular somatic embryos and enabled maintenance of SE competence on PGR-free MS (Pinto et al. 2008).

An important topic when considering *in vitro* culture is the genetic stability of the propagation process. Although it is generally accepted that SE is potentially useful in micropopagation, there are concerns over the possibility of producing somaclonal variants, particularly with protocols that rely on indirect morphogenesis (including repetitive SE) and/or high levels of auxins. In the case of *Eucalyptus* SE, this is a poorly researched topic and there are few reports focusing on the assessment of somaclonal variation during this process. Genetic stability and true-to-typeness of *E. globulus* propagation via repetitive SE have been reported for eight-month-old somatic embryos, which were analysed using flow cytometry (FCM) (Pinto et al. 2004). Flow cytometry combined with propidium iodite was used to analyse nuclear DNA content of *E. globulus* somatic and zygotic embryos, and leaves of the parent mother plant in order to determine if SE induced DNA content and ploidy changes in this species (Pinto et al. 2004). Zygotic embryos had a 2C nuclear DNA content of 1.32 pg, somatic embryos had a 2C nuclear DNA content of 1.39 pg, and leaves from the field tree that provided the seeds had a 2C nuclear DNA content of 1.40 pg. These results indicate that no changes (as

detected by FCM) were induced during the embryogenic process. However, other morphological markers, chromosome analysis, breeding behaviour, isoenzymes or DNA markers should be used to detect somaclonal variation to complement the above results. The early assessment of genetic fidelity at various culture stages is highly desirable and may help to identify which culture conditions induce undesired variation (Rani and Raina 2000).

More recently, proliferation of somatic embryos initiated from leaf and shoot explants of adult *E. globulus* and the hybrid *E. saligna* x *E. maidenii* was achieved after subculture of primary somatic embryos on medium containing 3 mg l^{-1} NAA (Corredoira et al. 2015). In their efforts, competence for secondary embryogenesis was maintained for more than 3 years, despite the relatively low rate of embryo proliferation yielded by secondary SE. Differentiation of somatic embryos occurred rapidly, and as a result of the rapid transition to the cotyledonary stage intermediate stages of embryo development were rarely observed. Histological analysis revealed secondary embryos with bipolar organization, with root and shoot apex meristems and differentiation of a cylinder of procambial tissue that bifurcates into small cotyledons.

2.5 From maturation to conversion of somatic embryos

Even when large numbers of somatic embryos are obtained, a common bottleneck for large-scale propagation is the conversion of these somatic embryos into plants. All factors that contribute to the success of this step are also important for high-performing emblings in the subsequent stage of acclimatization to *ex vitro* conditions and field performance. In *Eucalyptus,* mature somatic embryos usually do not develop in the presence of auxin (Pinto et al. 2013) and plant regeneration is either achieved in auxin-free media or, occasionally, in media containing cytokinins and/or gibberellic acid (GA) (Corredoira et al. 2015; Muralidharan and Mascarenhas 1995; Pinto et al. 2004; Watt et al. 1991). Furthermore, additional changes in the composition of the basal medium are often needed, and some species also require the addition of extra compounds such as glutamine, abscisic acid (ABA) and casein hydrolysate (von Arnold et al. 2002). Plant recovery from somatic embryos of the *Eucalyptus* genus is a difficult step and procedures for plant regeneration have scarcely been described and have met with different levels of success. In *E. dunnii*, *E. grandis* and *E. camaldulensis*, germination rates were low (Prakash and Gurumurthi 2009; Watt et al. 1999), whereas a germination rate of 52% was obtained in *E. citrodora* by transferring mature embryos to an auxin-free liquid B5 medium with 20 g l^{-1} sucrose (Muralidharan and Mascarenhas 1995). In the later work, embryos germinated easily and developed healthy shoot and root systems. The same work determined that adding ABA to the medium had a negative effect on the growth of *E. citrodora* embryogenic masses and embryos

died more frequently with increasing concentrations of ABA (Muralidharan and Mascarenhas 1995). Similarly, in *E. grandis*, the addition of ABA and polyethylene glycol (alone or in combination) resulted in a low rate of embling regeneration or no regeneration at all depending on the explant source (Watt et al. 1999).

In *E. globulus*, conversion rates ranged from 2% (Pinto et al. 2008) to 21% (Pinto et al. 2002). The influence of the culture medium (MS and B5), PGRs (auxins and cytokinins) and light on secondary SE was tested (Pinto et al. 2008). These authors reported that MS medium without growth regulators was more efficient for cotyledonary embryo formation and germination than B5 medium. In addition, reducing the levels of auxin (NAA) increased the proliferation of globular somatic embryos and allowed the maintenance of SE competence on a free of PGRs medium. The addition of two cytokinins (BAP and KIN) to the MS medium did not improve proliferation of globular secondary embryos, but was crucial for germination and conversion. Light also played an important role, depending on the SE stage, and influenced the quality of the process (Pinto et al. 2008). In plantlet regeneration from somatic embryos derived from adult material, Corredoira et al. (2015) evaluated the use of liquid germination medium to prevent the rapid drying and browning of isolated embryos that occurred on semisolid germination medium. Cotyledonary-stage embryos (with a well-defined root) were isolated from embryogenic clusters and placed on two filter paper discs (Whatman grade 181) in Petri dishes containing 10 ml of liquid MS germination medium supplemented with 0.1 mg l^{-1} BA and 0.5 mg l^{-1} gibberelic acid. In these conditions, enlargement and greening of the hypocotyl and cotyledons was typically followed by root growth in most of the somatic embryos; however, conversion of embryos with root and shoot development was only minimal (<10%).

2.6 Acclimatization, plant performance and encapsulation of emblings

The ultimate goal of the application of SE to mass-propagated selected individuals is only achieved with the successful acclimatisation of a large number of plants ready to withstand field conditions. Although SE has great potential, there are still technical limitations that need to be removed before the process can be used in tree improvement programs as a deployment strategy. Unfortunately, with respect to *Eucalyptus* emblings, most reports are restricted to germination frequency, conversion into plantlets and survival rates during acclimatisation (Muralidharan et al. 1989; Pinto et al. 2002; Pinto et al. 2008; Prakash and Gurumurthi 2005; Watt et al. 1991), with little focus on the underlying morphological and physiological aspects that occur during the acclimatisation process. Although the behaviour of *Eucalyptus* hybrids propagated by different methods (micropropagation and cuttings) has been compared for traits such as root

stability (Mokotedi et al. 2010) and gas exchange properties (Mokotedi et al. 2010), there is still no information on the behaviour of *Eucalyptus* emblings in the field. Understanding the morpho-physiological behaviour of *in vitro* plants and the changes that occur during the acclimatisation process should facilitate the development of an efficient acclimatisation protocol as well as improve the greenhouse and field performance potential under different environmental conditions (Grossnickle and Folk 2007; Pinto et al. 2011). Embling acclimatisation was reported for *E. grandis* (Watt et al. 1991), *E. citriodora* (Muralidharan et al. 1989; Muralidharan and Mascarenhas 1995) and *E. tereticornis* (Prakash and Gurumurthi 2005). In all of these species, the basic acclimatisation procedure included the transfer to soil substrates (peat, perlite or sand) and gradual reduction in the relative humidity of the environment. However, in these studies, embling survival was the only measure of performance used and thus far no studies have explored important aspects such as the histocytology, physiology or genetic stability of embling acclimatisation after SE in *Eucalyptus*. Later, Pinto and co-workers (2011) reported the complete process from regeneration of secondary emblings (SE-derived plants) to their acclimatisation for *E. globulus* and described the histocytological changes that occur in leaves during *in vitro* to *ex vitro* acclimatisation over a three-month period. After elongation, plants were transferred to pots containing sterilised peat:perlite and acclimatised in a phytotron, with progressive reduction of relative humidity and increased light intensity. Histocytological analyses were performed using light microscopy and electron microscopy (both scanning and transmission), which revealed that significant changes occur during acclimatisation mostly in stomata shape and aperture, starch reserves, chloroplast morphology and mesophyll differentiation. These results demonstrated that during acclimatisation emblings suffered profound changes in leaf morphology in order to successfully adapt to *ex vitro* conditions (Pinto et al. 2011). However, emblings have the advantage of a pre-formed root initials that resemble the taproot of zygotic embryos, which is considered to be one of the most important advantages of SE over organogenesis and cuttings that have adventitious roots (Grossnickle and Major 1994; Kim et al. 2012; Mokotedi et al. 2010). Recovery of somatic embryogenesis derived plantlets with either abnormal morphology or altered physiology during in vitro culture should deserve further attention and it is essential to verify and follow clonal fidelity and field performance of emblings (Kaeppler et al. 2000; Tremblay et al. 1999). As previously mentioned, FCM is the preferred technique to screen for genomic changes in SE-derived plantlets (Pinto et al. 2011). In their study, emblings were obtained from clusters maintained *in vitro* for three years and, therefore, all plantlets were screened for morphological and ploidy abnormalities before starting acclimatisation. This screening revealed no morphological variations and a homogeneous nuclear DNA content, which is in accordance with a previous report

by Pinto et al. (2004). In an earlier study, plants derived from organogenesis were also analysed using FCM for this species and no evidence was found of ploidy changes during *in vitro* culture (Azmi et al. 1997).

The preservation of somatic embryos through encapsulation procedures has only been reported for *E. Citriodora* (Muralidharan and Mascarenhas 1995). The application of this preservation method, together with cryopreservation, is still in its infancy for this genus, but its success strongly depends on the development of reliable SE protocols (Padayachee et al. 2009). Cryopreservation should be considered as a potentially important tool in commercial breeding strategies as it may allow the preservation of selected genotypes while field selection tests are being performed. Padayachee et al. (2009) reviewed the benefits and advantages of cryopreservation and highlighted some of the key challenges that still exist, especially those associated with the cryopreservation of highly hydrated and/or desiccation-sensitive material such as *Eucalyptus* axillary buds.

3. Conclusions

Micropropagation can rapidly capture selection gains developed in traditional tree improvement programs and incorporate the propagules directly into plantations or seed orchards, potentially reducing forest production costs in the long term. Large-scale propagation of superior clones along with accelerated tree improvement programs are necessary for profitable forest breeding programs of *Eucalyptus*. An increasing number of reports have appeared on in vitro propagation of *Eucalyptus* species due to their rising commercial importance on the world market. This chapter reviews the most relevant and recent advances on the in vitro culture of Eucalyptus. Particular emphasis was given to the SE process, from somatic embryo induction to plant acclimatization. In spite of the large amount of research conducted during the last few years, there is still a gap in the knowledge of the mechanisms involved in the regulation of SE. Besides, additional research is needed to identify, and eventually overcome some of the current bottlenecks in the SE process and to devise a successful SE strategy for this economically important forest genus and to establish a SE system capable of applying the technology at an industrial level as well as for gene transfer. Finally, we believe that exchange of experiences among researchers working in this field as well as the release of technical research information possessed by private companies will speed up to the exploitation of eucalypt resources through functional genomics and biotechnology.

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Somatic embryogenesis and plant regeneration of yellow poplar (*Liriodendron tulipifera* L.) at National Institute of Forest Science

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Abstract

A tissue culture study of yellow-poplar (*Liriodendron tulipifera* L.) in Korea was carried out by the National Institute of Forest Science (NIFoS). Somatic embryo clones were induced from embryogenic tissue that had originated from immature seeds that were obtained through artificial breeding between superior trees. Effects of polyamines and activated carbon were investigated for inducing somatic embryos from embryogenic tissue. Normal somatic embryos were acclimated after germination on an artificial soil bed. Mass production of potted emblings was achieved after clarifying various soil bed conditions needed for proper acclimatization. There was no morphological variation between emblings and normal seedlings and they showed very similar growth patterns. About 360,000 emblings have been produced over a three year period since 2010. Meanwhile, the existence of genetic variation in plants that had been regenerated from embryogenic tissue that treated with osmoticum and then cryopreserved was investigated. Transgenic plants were also developed by using *Agrobacterium* bacteria. This paper summarizes the results of the studies with yellow poplar in tissue culture conducted at the NIFoS.

Keywords: Embyogenic cell induction, somatic embryos, conversion, genotypes, cryopreservation, gene transformation

1. Introduction

Yellow-poplar, known as a fossil plant, is a deciduous tree that belongs to the family *Magnoliaceae*. This family can be divided into two species depending on the region. Yellow poplar (*Liriodendron tulipifera* L.) is distributed in eastern

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North America and China-yellow poplar (*L. chinensis* Sarg) is growing in the center of China. These two species are morphologically similar, but the general height of the North American species is more than 30m while the average that of China-yellow poplar is only about 15m. The flower of North American species has a yellow stripe through the middle of the flower while the Chinese species has no stripe (Ryu et al. 2008). There are some written reports that the yellow-poplars were introduced in Korea in the middle of the 1920s, but most of these records were destroyed during the Korean War (1950-1953). Currently, there are yellow poplar trees in Korea that are up to about 70 year-old and it is likely that these trees were introduced from Japan. Large scale afforestation of yellow poplar in Korea was initiated as part of the 4th Forest Master Plan in Korea and the Korea Forest Service has expanded its afforestation efforts with this species on a national scale. Four thousand hectares were afforested during the six years from 2001 to 2007, and more than 20,000 ha have been afforested so far. Most of the yellow poplar seedlings that have been planted in Korea were obtained from the seeds obtained from North America including from Tennessee, North Georgia, Kentucky, Ohio and West Virginia etc. Some of the seedlings were produced from yellow poplar seed harvested from seed orchards in Korea (Ryu et al. 2008).

Most yellow poplar planting stock is produced by seeds. The germination rate of seeds produced in the country of origin is 20-30%, but the seeds obtained from seed orchards in four regions in Korea showed a rate of just 12.9%. Although healthy seeds germinate well, generally seed pretreatment such as stratification is required for 1 to 3 years. It is possible to obtain mass production of planting stock by optimization of in vitro clonal propagation procedures that can be used as an alternative to the traditional propagation methods (Ryu et al. 2003). Somatic embryogenesis technology is considered to be the most efficient one of the various tissue culture techniques and has been used as a means of mass propagation of woody plants (Bonga and von Aderkas 1992; Bonga 2004; Bonga et al. 2010; Merkle et al. 2010).

Somatic embryogenesis of yellow poplar was first attempted by Merkle and Sommer (1986) and plants were obtained from embryogenic tissue protoplasts in 1987 (Merkle and Sommer, 1986). Dai et al. (2001) obtained an efficient embryogenic tissue of hybrid yellow poplar that produced trees . Cryopreservation studies, including of pre-treatment conditions and regeneration of plants from the stored tissues, have been conducted (Montello and Merkle 1995; Vendrame et al. 2001). In Korea, somatic embryogenesis and plant regeneration of yellow poplar, using immature seed as explants has been attempted (Lee 2003; Kim and Moon 2013; Son et al. 2005) while Ahn et al. (2010) tried to regenerate plants from somatic embryos using bioreactors. This overview introduces a summary of studies with yellow poplar which have been conducted at the KFRI.

2. Initiation of embryogenic tissue

The initiation of embryogenic tissue in woody tree explants is strongly dependent on genotypes (Bonga and von Aderkas 1992; Park SY et al. 2011). The rates of induction of embryonic tissue from immature seeds are shown in Figure 1 for four different trees. Based on preliminary tests, the induction medium used in this study consisted of ½MS + 40g/L sucrose, 2.0mg/L 2,4-D, 0.25mg/L BA, 1g/L casein hydrolysate, 0.2% gelrite. Suwon1 showed the highest induction rate (18.2%) of embryogenic tissue and Kangjin3 the lowest (1.2%) after five weeks of cultivation. The average rate of induction was 8.4% and total 491 embryogenic lines were obtained. These genotype-dependent differences occurred annually.

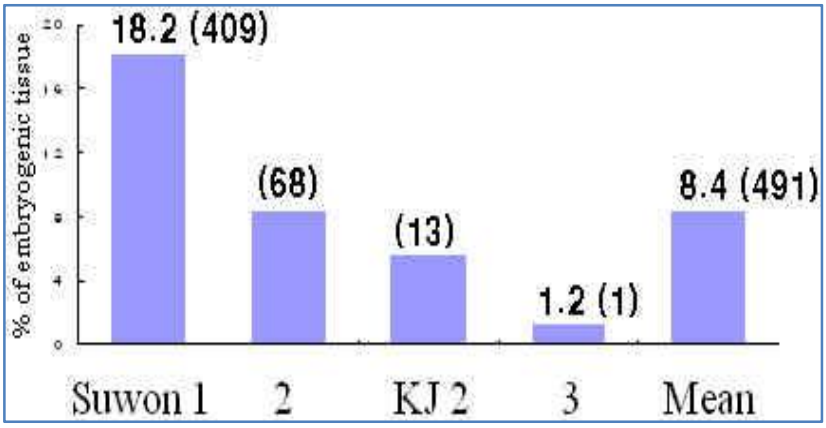

Figure 1. *Comparison of induction rates of embryonic tissue by genotype.*

3. Induction of somatic embryos (SEs)

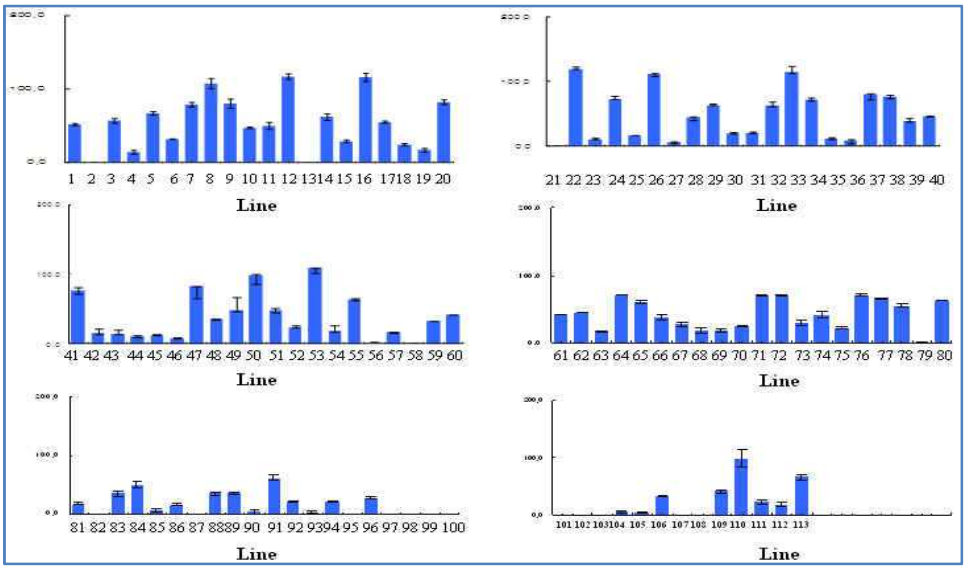

Figure 2. *Production of SEs derived from different embryogenic lines*

Somatic embryos were obtained from various embryogenic lines produced in 2011. The medium that was used contained ½MS + 40g/L sucrose, 500mg/L casamino acid and 0.4% gelrite. A total of 113 embryogenic tissue lines were tested and the frequency of SE development was very different depending on the lines (Figure 2). Interestingly, 17 lines did not produce developing embryos. Therefore, selection of productive lines is required to obtain much amount of SEs in an efficient manner.

4. Germination and plant regeneration

Efficient maturation and germination of somatic embryos and acclimation in soil is one of the most important processes in terms of commercialization. Induction of somatic embryogenesis has been reported for a variety of tree species (Merkle et al. 1998: Moon et al. 2008: Thompson 2014). However, it is often difficult to achieve commercialization because of difficulties in obtaining efficient germination and plant regeneration (Thompson 2014). Significant differences in germination and plant regeneration rates were observed according to genotype (Figure 3). The efficiency of acclamation was also different according to the soil

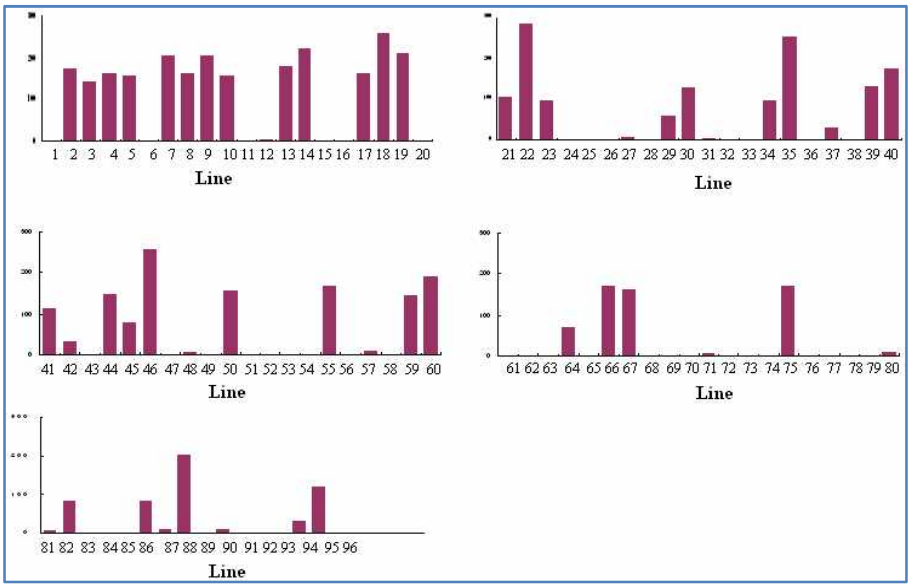

Figure 3. *Comparison of plant production from 96 embryogenic lines*.

bed used since yellow poplar requires a high nutrition level for rapid tree growth. Our results showed that a mix of nutrients for soil beds favors acclimation and growth of yellow poplar (Lee et al. 2003). Based on these results, we established a protocol for yellow poplar somatic embryogenesis and plant regeneration (Figure 4, 5).

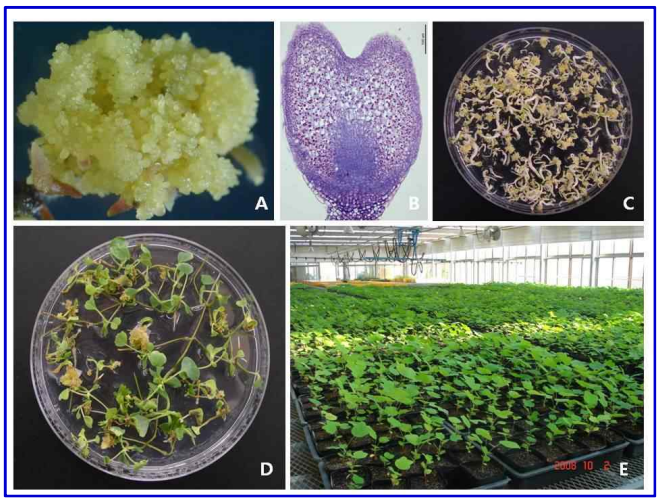

Figure 4. *(Previous page) Somatic embryogenesis and plant regeneration of yellow poplar: A) Embryogenic callus induced from immature zygotic seed; B) Crossed and sectioned somatic embryo; C) Germinating SEs; D) Regenerated plants; E) Acclimatized plants in greenhouse)*

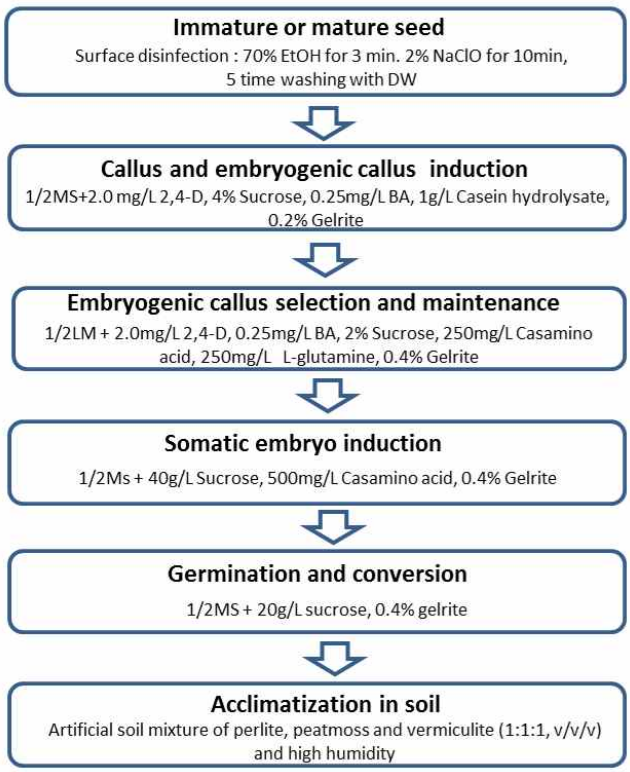

Figure 5. *A protocol of plant propagation via somatic embryogenesis using immature or mature seed of Liriodendron tulipifera*

5. Planting of yellow popular emblings and their subsequent growth

Since 2010, about 360,000 yellow poplar emblings have been distributed to the Northern Forest Service of KFS and seven government institutions in Korea for planting. The yellow poplar tree clones produced at the KFRI were first planted in the mountains in 2004. The growth performance of the clones appears to be similar to that of seedlings and showed a good growth (Figure 6).

6. Cryopreservation of embryogenic tissue

Long-term cryogenic storage and subsequent regeneration of embryonic tissue are an important part of clonal forestry and for increasing productivity (Haggman et al. 2008; Lambardi et al. 2005: Park et al. 1998; Park 2002). Survival and induction of

Figure 6. *11-year-old yellow poplar clones derived via somatic embryogenesis at Eocheon experimental forest in Hwasung, Gyeonggido, Korea.*

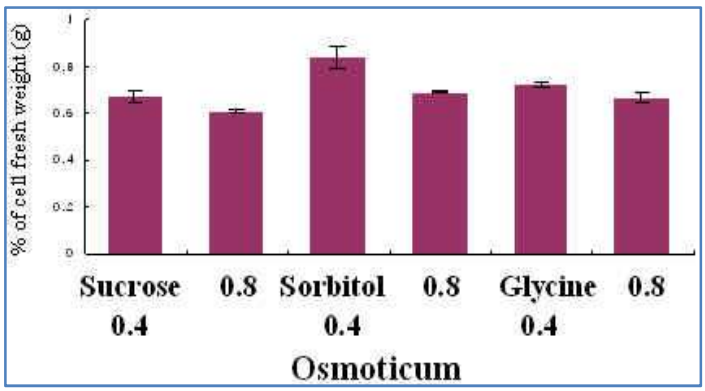

Figure 7. *Comparison of cell proliferation after cryopreservation with different kinds of osmoticums.*

embryonic tissue was tested with different types and concentrations of osmoticum and with DMSO (Dimethyl sulfoxide). The pretreatment with DMSO showed no obvious effect, but with 0.4 M of sorbitol for osmoticum produced the best cell proliferation after cryopreservation (Figure 7). In addition, flow cytometry of the plants recovered after cryo-storage for 6, 12, and 18 months, showed no variation when compared with non-cryopreserved tissue.

7. Transformation of embryogenic tissue

We tried to transform embryogenic callus with the *tzs* gene. Figure 9 shows a schematic diagram of the vector used in the experiment. It was designed to have the gene of *tzs* to be driven by the *pAUX* promoter and the *npt*II gene by *pNOS*. Putative transformed tissues were selected at concentrations of 20 mg/L geneticin. We found that foreign genes were inserted into 9 randomly selected transformed plants of yellow poplar through PCR amplification with two primers (t-gene1, gaggagcatcgtggaaaaag t-gene2, gggaaaaccctggcgttaccca TZ2, 5'-gag ctc acc gaa ttc gcg-3') (Figure 9).

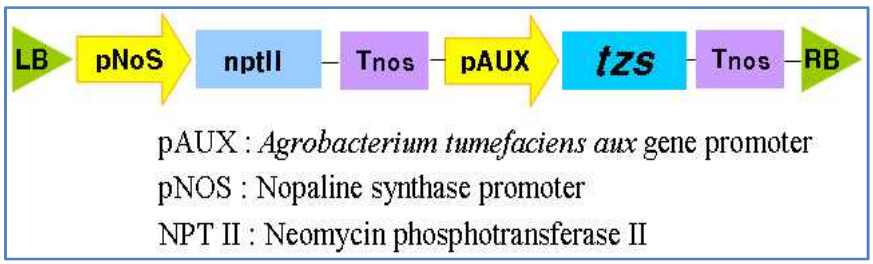

Figure 8. *A schematic diagram of the vector used in present study. tzs: Trans-zeatin secretion. Primer T-gene 1/T-gene 2 Primer T-gene 1/TZS 2*

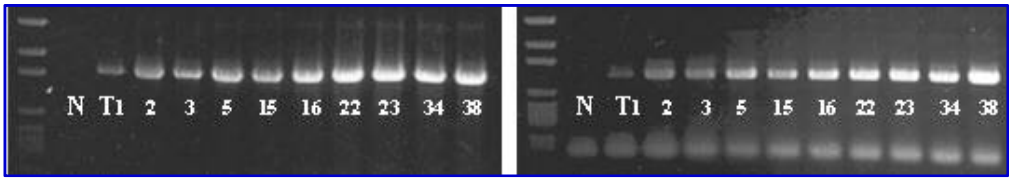

Figure 9. *GM plant conformation by the genomic DNA PCR*

8. Conclusions

It was found that mass production of yellow poplar through somatic embryogenesis, using immature seeds as explants, is possible. However, the frequency of normal somatic embryo formation was low and considerable differences occurred depending on the genotypes. Therefore, cell line selection to

produce efficient somatic clones is necessary. Our protocol can be applied to immature seed that has been obtained by artificial pollination between superior trees. In addition, it has been shown that the emblings provided by our laboratory were not significantly different from seedlings. Therefore, our somatic embryogenesis protocol for various genotypes will enable the efficient propagation of clones of superior trees obtained through our yellow poplar breeding program

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Somatic embryogenesis in *Arbutus unedo* L. and other Ericaceae

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Abstract

The heather or heath family (Ericaceae) is distributed all over the world and includes several well-known species such as the rhododendrons, azaleas, and several members of the genus *Vaccinium* which produce edible berries. In spite of the great number of species included in this family the number of studies related with in vitro culture and micropropagation is unexpectedly low. Protocols for micropropagation have been developed for several species mainly through axillary shoot proliferation and subsequent rooting. For some species in vitro plant regeneration has been obtained through de novo shoot formation. Only in this century (2003) has the first work on somatic embryogenesis induction in an Ericaceae (*Rhododendron catawbiense)* been published. Since then somatic embryogenesis has been achieved in other species such as *Arbutus canariensis* and *A. unedo*, *Conostephium pendulum*, *Elliottia racemosa*, *Erica carnea* and *Leucopogon verticillatus*. In general, leaf segments have been used for somatic embryogenesis induction on a medium containing an auxin and a cytokinin. In this chapter several aspects related with somatic embryogenesis in the Ericaceae are reviewed focusing mainly on the Strawberry tree (*Arbutus unedo*) a species that produces edible berries and that is much appreciated in several Mediterranean countries.

Keywords: Auxins, cytokinins, germination, histology, micropropagation, organogenesis, shoot proliferation, Strawberry tree.

1. The Ericaceae and the genus *Arbutus*

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The heather family (Ericaceae) is a large group of eudicotyledoneous plants comprising 163 recognised genera and about 3350 species according to The Plant List (2015). Distributed all over the world they are more common in the northern hemisphere but are also well represented bellow the equator (Heywood 1993). Several well-known genera belong to this family such as the ornamentals *Rhododendron* and *Azalea* as well as the genus *Vaccinium* which includes several berry producing species. Being such a large family it is not easy to number a set of characteristics common to all the species. However, most members of this family are climbers, shrubs or small trees growing in acidic soils and greatly depending on the association with fungi through the establishment of mycorrhizae to colonize some habitats (Heywood 1993). In some species, like those grouped in the subfamily Monotropoideae, the association with fungi is so important that the plants lack chlorophyll and thrive on decaying organic matter produced by microorganisms. Leaves are usually evergreen and simple, often displaying xerophytic characteristics thus reflecting the harsh conditions where they grow. Flowers are quite diverse in morphology and color especially among the garden genera *Rhododendron* and *Azalea*. However, a common feature is the inverted position of the anthers (Figure 1A) which occurs during flower development. At maturity pollen is shed by pores at the top of the anther which is in fact the base (Figure 1A). Although there are exceptions Ericaceae pollen is usually shed as units of 4 cells (Figure 1B).

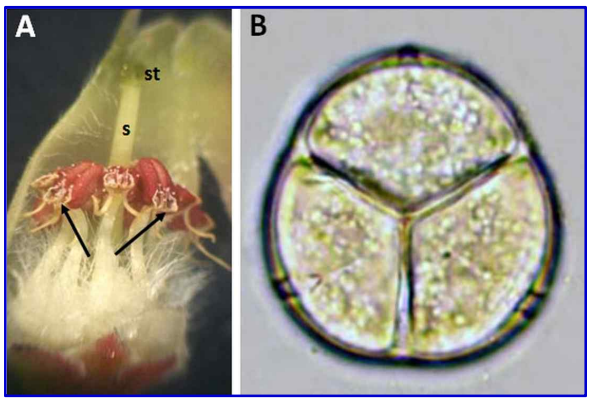

Figure 1. *Aspects of reproductive structures of the Ericaceae.* ***(A)*** *Flower of A. unedo showing the stigma (st) style (s) and the anthers where the open pores allowing shedding can be seen (arrows).* ***(B)*** *Pollen unit of A. unedo formed by 4 pollen grains.*

The fruit is often a capsule but berries are often found as in *Vaccinium*, *Arbutus* or *Corema* (Heywood et al. 2007). Within the Ericaceae there are several members of great economic importance including the above-mentioned garden species of the genus *Rhododendron* and *Azalea* but also the genera *Erica* and *Calluna*. *Vaccinium* is an important genus of fruit species which includes the much

appreciated blueberries, cranberries and bilberries. Some species are explored to produce honey of great quality whereas the wintergreen species (*Gaultheria* spp.) produce essential oils (Bantawa et al. 2011) and others (*Kalmia* spp.) are toxic for farm animals (Heywood, 1993).

Like *Vaccinium*, some members of the genus *Arbutus* also produce edible berries. Although these fruits are not as popular as those produced by several species of *Vaccinium* they are eaten by local populations and are processed to produce different types of products that help the economy of these regions (Gomes 2011). One of the most promising species of *Arbutus* is a plant usually known as strawberry tree due to the somewhat resemblance of its fruits with strawberries.

2. Strawberry tree (*Arbutus unedo* L.)

Strawberry tree (*Arbutus unedo* L.) is a broadleaved bush or small tree (3 – 8 m) native to the maquis of countries bordering the Mediterranean (Piotto et al. 2001) such as Spain, France, Italy, Greece, Turkey, Algeria Morocco, Tunisia and Lybia (Figure 2). It is also found in Portugal, southern Ireland and western France (Figure 2). Beyond this natural area the species also occurs in other regions with a Mediterranean like climate where it has been introduced, mainly in some areas of the USA and Australia (Russell et al. 2007). Strawberry tree is one of the eleven recognized species of the genus *Arbutus* (The Plant List). Examples of other species are *A. andrachne*, *A. arizonica*, *A. canariensis*, *A. menziesii*, *A. xalapensis* and the hybrids *Arbutus* x *andrachnoides* and *Arbutus* x *androsterilis*.

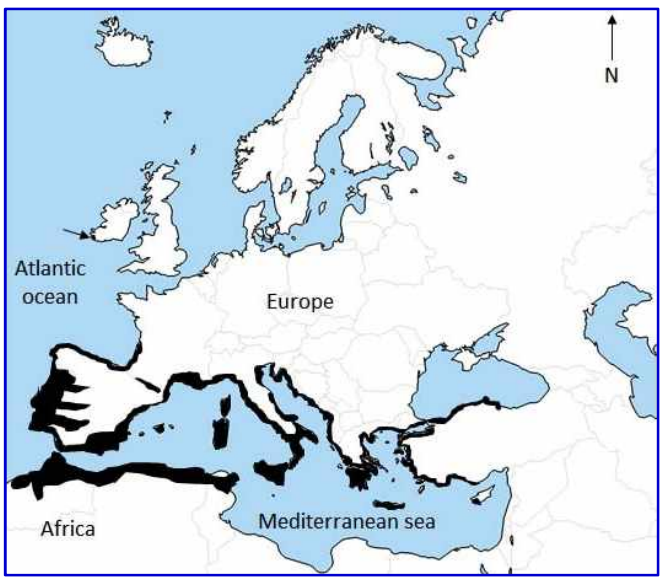

Figure 2. *Distribution map of A. unedo in its native region (adapted from Prada and Arizpe 2008 and Molina et al. 2011).*

A. unedo grows spontaneously in dry areas of rocky and well-drained (Piotto et al. 2001) usually acidic siliceous soils in association with members of the Fagaceae family (Neppi 2001), mainly *Quercus suber* or with maritime or stone pines (Neppi 2001; Godinho-Ferreira et al. 2005; Prada and Arizpe 2008). However, the species shows great plasticity and can be found in soils with other characteristics. The species easily tolerates very dry conditions and it is also quite tolerant to low temperatures (-12 ^{0}C, Piotto et al. 2001). These characteristics make *A. unedo* interesting from an ecological point of view since it can be grown in poor marginal lands where more sensitive species are difficult to cultivate. The strong capability to sprout following fires is also important to the recovery of burned areas in countries such as Portugal, Spain and Greece where summer fires is a scenario repeated year after year (Quevedo et al. 2013).

The reproductive cycle of strawberry tree lasts for over a year. Flowers start to develop during summer, pollination occurs by the end of the year and fruits mature next fall. Thus, *A. unedo* is one of the rare species in which flowers and fruits can be seen at the same time making it an interesting tree for ornamental purposes (Martins 2012). The flowers are white or faintly pink (Figure 3A) with petals fused together to give an urceolate corolla. The fruit (Figure 3B), an almost spherical berry, about 2 cm across, is edible and, as already referred to, matures during the fall. When immature, the fruits are light green, turning yellow and orange as time goes by (Alarcão e Silva et al. 2001) and ending in an appealing bright red color at maturity which attracts not only consumers but also birds that help to spread seeds.

Figure 3. *Flowers (A) and fruits (B) of A. unedo.*

Until recently, strawberry tree has been considered a NUC species (Global Facilitation Unit for Underutilized Species 2015) or what can also be named an emergent fruit crop (Hummer et al. 2012) due to its great economic potential but yet little explored by farmers and fruit producers. However, lately, due to the efforts of researchers, farmers, industrials and other stakeholders as well as to the interest of consumers for red fruits the interest in this species has grown and the area of new plantations has considerably increased. Two main reasons have

contributed to this situation: the support given by funding agencies to research projects with this species and the decline of traditional forest species which has driven the interest of farmers to alternative cultures. In Portugal, the pine nematode disease affecting mainly *Pinus pinaster* and the appearance of pests and diseases on *Eucalyptus globulus* (e.g., *Gonipterus* spp.) are two of the main factors responsible for the renewed interest in the strawberry tree.

The main income for *A. unedo* growers comes from fruit production which, until just a few years ago, was almost exclusively used for the production of a type of brandy called "medronheira" obtained through distillation (Botelho and Galego 2015). This strong alcoholic drink (around 40 % volume of ethanol) is very appreciated in Mediterranean regions and sold at high prices that can reach 20€ a bottle depending on the quality, year and producers. Fruits can be eaten raw (Oliveira and Franco 2015), yet when not completely mature they are a little astringent and might be unpleasant for people not used to eat them. There is an old believe according to which people who have eaten several fruits may get inebriated due to the putative high levels of ethanol present in ripe fruits. However, researches have shown that this is no more than an urban myth (Botelho et al. 2015) and eating strawberry tree berries is as safe as eating any other kind of berries. Thus, the only problem with fresh fruit consumption seems to be postharvest handling due to rapid fruit decay. A solution could be harvesting prior to full maturation. However, *A. unedo* fruits do not mature well when detached from the plant while unripe. Successful attempts to increase shelf-life (up to 15 days) using low temperatures (0 ºC) were carried out by Guerreiro et al. (2013).

A. unedo berries are rich in dietary fibre and vitamin C (Ruiz-Rodríguez et al. 2011), proteins, other vitamins (E and niacin), some minerals like iron and potassium (Özcan and Haciseferoğullai 2007), and polyphenols showing antioxidant properties (Oliveira et al. 2009; Fortalezas et al. 2010; Miguel et al. 2014). However, as pointed out by Ruiz-Rodríguez et al. (2011), the amounts of these components show great variability depending on the origin and season. On average, a single tree can produce 2 – 10 kg fruits a year (Molina et al. 2011) although productions reaching 15 kg have been claimed. To produce 1 L of brandy around 10 kg of fruits are necessary. Honey production (Tuberoso et al. 2010), tanning (Pabuccuoglu et al. 2003), the use of infusions in folk medicine (Pabuccuoglu et al. 2003; Cardoso 2004), biomass for the production of energy, and the use of young branches in the floral industry (Metaxas et al. 2004) are other applications for the plant. A report by Fortalezas et al. (2010) describes the antioxidant properties and neuroprotective capacity of strawberry tree fruits. Finally, and just as a curiosity that deeply reflects the strong relationship between this plant and the history of Mediterranean societies, one should emphasize that a strawberry tree is part of the coat of arms of the city of Madrid.

There is an important lack of information about the susceptibility of *A.*

unedo to pests and diseases. Scales and thrips are small insects that attack leaves and young stems and beyond the direct damage they cause they may also contribute to the propagation of pathogenic fungi. However, detailed studies analyzing the role of these insects on plant development and productivity are absent. Moralejo et al. (2008) have isolated different species of *Phytophthora* from potted strawberry trees. Using the isolates the same authors were able to induce extended lesions in the inner bark of the infected trees. Like in other Ericaceae, rust and anthracnose have been sporadically reported in *A. unedo* trees. However, as in the case of insects, no reliable information exists about the impact of these diseases.

Despite increasing interest in *A. unedo*, most of the fruits are collected from wild trees rather than from orchards. Besides, young trees sold by nurseryman are often from seed origin rather than from clonal selection. This situation has as consequence an irregular fruit production over years and a strong variability in the fruits that can affect production and the income expected by farmers.

Due to the potential economic interest of the plant and the increasing importance that alternative crops are assuming in the global economy (Will 2008; Hummer et al. 2012) as well as in the agriculture and rural development policies and guidelines of the European Union (EAFRD 2013) for 2014-2010, we have been involved in the improvement and breeding of *A. unedo* plants through the application of biotechnological tools. In this chapter we present the results so far obtained concerning in vitro propagation of *A. unedo,* focusing mainly on somatic embryogenesis, and on other members of the Ericaceae family.

3. Conventional propagation

Under natural conditions propagation of strawberry tree occurs through dispersal of seed by frugivorous birds and mammals (Herrera 1982; 1987). Other Ericaceae that produce berries spread using the same strategy. However, in genera in which artificial cross pollination is used to produce new hybrids used in the floral industry, such as *Rhododendron*, asexual methods could be the only process to achieve plant propagation while maintaining quality. *A. unedo* trees damaged by fire or other natural phenomenon can recover from sprouts originating at the basis of stems or from roots.

In nurseries seeds have been used to propagate strawberry trees, in spite the fact that the plants obtained are genetically different. Moreover, seed germination is somewhat difficult (Smiris et al. 2006; Demirsoy et al. 2010) due to strong dormancy either because of the hardness of the seed coat (Smiris et al. 2006) or the increased levels of inhibitory compounds such as abscisic acid (ABA), present in the embryo (Tilki 2004). Nonetheless, stratification can increase the levels of germination to values reaching 100% (Ertekin and Kirdar 2010; Martins 2012). It was also found that the application of gibberellins can substitute for

stratification and promote seed germination (Smiris et al. 2006). Although of limited application until now, sexual reproduction can be used in the breeding of new genotypes displaying new agronomic characteristics (Martins and Canhoto 2014).

The potential of the strawberry tree to form sprouts has been exploited to asexually propagate selected trees. Semi-hard-wood cuttings showed rooting rates over 87% following IBA (indol-3-butyric acid) treatment (Sulusoglu 2012). The results were genotype-dependent, highly variable and strongly affected by the period of the year the cuttings were obtained. It was observed that cuttings gathered in the winter required higher auxin concentrations to reach the same rooting rate than cuttings gathered during summer. Other authors have propagated strawberry trees through cuttings but found that rooting frequencies were reduced, especially when mature cuttings were assayed (Mereti et al. 2002; Metaxas et al. 2004). Treatments with K-IBA were successfully used by Metaxas et al. (2008) to increase rooting ability.

As far as is known, grafting has not yet been consistently applied to *A. unedo* propagation. The dominant exploitation of wild trees rather than selected genotypes can explain this situation. However, the increasing number of farmers that are looking for selected propagated material may justify the development of rootstocks more adapted to particular soil and climatic conditions, thus increasing fruit production and tree productivity as is common practice with other fruit species.

4. *In vitro* culture

Despite the great number of Ericaceae and their economic relevance, the number of studies concerning *in vitro* cloning or the application of other biotechnological tools is surprisingly low (Ratnaparkhe 2007; Cavuşoğlu et al. 2015). Even in species of the genus *Rhododendron* and *Vaccinium* in vitro propagation has been limited to only a few species. In vitro cloning of Ericaceae has been used not only for large scale plant propagation (see Ratnaparkhe 2007) but also for conservation purposes (Almeida et al. 2005; Pereira 2006; Mao et al. 2011; Pereira 2014). Mycorrhization assays have been conducted using in vitro propagated plants (Jansa and Vosátka 2000; Gomes et al. 2013; 2015).

The most used approach for in vitro propagation of Ericaceae has been axillary shoot propagation. Examples of species propagated through this method are *Arbutus unedo* (Mereti et al. 2002; Gomes and Canhoto 2009; Gomes et al. 2010), *Arbutus xalapensis* (Mackay, 1996), *Calluna vulgaris* (Gebhardt and Friedrich 1987), *Conostephium pendulum* (Anthony et al. 2004a), *Gaultheria fragantissima* (Bantawa et al. 2011), *Elliottia racemosa* (Radcliffe et al. 2011), *Kalmia latifolia* (Lloyd and McCown 1980), *Leucopogon verticillatus* (Anthony et al. 2004b), *Oxydendrum arboretum* (Banko and Stefani 1989), *Rhododendron* spp.

(Anderson 1984; Hebert et al. 2009) and a number of *Vaccinium* species (Isutsa et al. 1994; Gajdošová et al. 2007; Ostrolucká et al. 2007; Ratnaparkhe 2007; Cüce et al. 2013; Cüce and Sökmen 2015; Pereira et al. 2015).

For cloning purposes micropropagation is interesting only when adult plants showing particular features can be propagated. In *A. unedo* a protocol has been established to propagate previously selected trees based on fruit production (Gomes and Canhoto 2009; Gomes et al. 2010). Epicormic shoots were used as explant source for the establishment of in vitro cultures. Differences in the propagation rate were observed among the genotypes used (Gomes and Canhoto 2009) as well as in the concentrations and type of plant growth regulators tested (Gomes et al. 2010). Shoots rooted well when treated with IBA and when subcultured on a medium without auxin and with charcoal for root development. Rooting was also achieved following an auxinic shock (15 s) with high IBA concentrations (9.8 x 10^3 µM) followed by culture on a charcoal containing medium (1.5% w/v). Following this protocol about 85% of the rooted plants could be acclimatized.

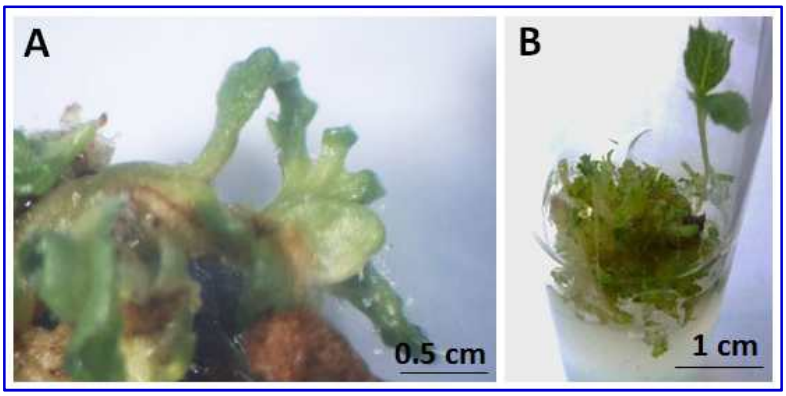

Figure 4. *Adventitious shoot formation in A. unedo.* ***(A)*** *Developing shoots on leaf segments.* ***(B)*** *High organogenic response from a callus formed at the base of a shoot.*

Even though much less used than shoot proliferation, regeneration through organogenesis has also been reported for Ericaceae (McCown and Lloyd 1982; Debnath 2003). For example, with *Elliottia racemosa* it was found that leaf explants cultured on a medium with 10 µM thidiazuron (TDZ) and 5 µM indole-3-acetic acid (IAA) developed adventitious shoots (Woo and Wetzstein, 2008). In shoot proliferating cultures of the strawberry tree de novo shoot meristem formation (Figure 4) was also observed following small callus formation at the base of the shoots (data not published). Highbush blueberry (*Vaccinium corymbosum*) is another example of plant propagation through organogenesis (Billings et al. 1988; Cao and Hammerschlag 2002). In the cultivar Bluecrop pretreatments of the leaf segments first (4 days) on a medium containing TDZ and

NAA (1-naphtaleneacetic acid) and then (3 more days) with zeatin riboside and NAA were crucial for adventitious shoot formation on a final medium containing μM TDZ (Cao and Hammerschlag 2002). Shoot formation has also been reported for the strawberry tree (Martins and Canhoto, 2014) from cultured leaf segments (Figure 4A) or from adventitious buds produced at the base of cultured nodal segments or shoot apices (Figure 4B).

Hence, at least for some Ericaceae species, organogenesis can be an alternative pathway to axillary shoot proliferation. However, the regenerated plants must be carefully evaluated since callus formation is often associated with somaclonal variation of the obtained plantlets (Bairu et al. 2011) although it could also be useful in crop breeding by the creation of new genotypes displaying interesting agronomic characteristics (Acquaah, 2012).

5. Somatic embryogenesis

Like organogenesis or axillary shoot proliferation, somatic embryogenesis is a method to achieve cloning (Park 2002). In comparison with other methods, somatic embryogenesis has several advantages such as the maintenance of embryogenic callus for long periods of time by cryopreservation (Correia et al. 2012) and the regeneration from genetically transformed cells. Furthermore, somatic embryogenesis can be an helpful tool to better understand plant embryogenesis since somatic embryos can be produced in large numbers and the culture conditions can be manipulated to achieve a better understanding of the role of particular factors on embryo development (Yang and Zhang, 2010; Radoeva and Weijers 2014; Smertenko and Bozhkov 2014; Correia et al. 2015), which is difficult to investigate while studying the development of a single zygotic embryo within the complex tissue organization of an ovule. There are a large number of publications concerning the induction of somatic embryogenesis in both angiosperms and gymnosperms. In most of the cases induction has been achieved from whole zygotic embryos or embryo (e.g. cotyledons or hypocotyls) or seedling organs. Even though induction from juvenile tissues offers better possibilities of success, this kind of explants does not allow the propagation of selected genotypes, which impairs cloning of high valuable plants. Somatic embryogenesis induction from explants of adult plants (Klimaszewska et al. 2011) or through indirect approaches in which adult plants are first established in vitro through axillary shoot proliferation after which the leaves from this material are then used for embryo induction are more interesting procedures to achieve effective cloning (Corredoira et al. 2006; Correia et al. 2011).

Somatic embryogenesis in members of the Ericaceae family was first reported in 2003 by Vejsadová and Pretová (2003) for the *Rhododendron*

catawbiense cultivar Grandiflorum (Table 1). Since this pioneer work the number of species of this family in which somatic embryogenesis was achieved has increased but remains relatively low (Table 1) when compared with other families of seed plants. In the following sections the conditions for induction, somatic embryo development and germination in Ericaceae are described.

5.1 Somatic embryogenesis induction

It is well known, since the pioneer works of Steward and coworkers (1958) and Reinert (1958), that an auxin is usually necessary to induce somatic embryogenesis with 2,4-D (2,4-dichlorophenoxyacetic acid) being the most widely tested compound. For a great number of species 2,4-D alone is enough to trigger cell differentiation and further somatic embryo formation. Thus, it is particularly interesting to remark that in none of the Ericaceae in which somatic embryogenesis has been induced 2,4-D has been used (Table 1).

Table 1. Summary of the somatic embryogenesis induction protocols that have been used in Ericaceae.

Species	Type of explant	Basal medium*	Growth regulators	Response	Reference
Arbutus canariensis and *A. unedo*	Leaf segments	Anderson	2 mg/l BA + 5 mg/l NAA	Somatic embryos, germination, plantlets	Canhoto et al. (2007)
Arbutus unedo	Leaf segments	De Fossard major salts and organics + MS micronutrients	8.8 µM BA + 10.7 µM NAA	Somatic embryos, germination, plantlets	Gomes et al. (2009)
Arbutus unedo	Internodal segments	Murashige and Skoog	5 mg/l BA + 5 mg/l NAA	Somatic embryos, plantlets	El-Mahrouk et al. (2010)
Arbutus unedo	Leaf segments	Anderson major salts + MS micronutrients + De Fossard organics	8.8 µM BA and 26.8 µM NAA	Somatic embryos, germination, plantlets, field plants	Martins et al. (2015)
Conostephium pendulum	Leaf explants	B5	10µM zeatin and 5µM indole-3-acetic acid (IAA)	Somatic embryos	Anthony et al. (2004a)
Elliottia racemosa	Leaf segments	B5	10 µM TDZ + 5 µM IAA	Shoot organogenesis, embryo-like structures	Woo and Wetzstein (2008)
Erica carnea	Stem segments	MS, WPM and B5	1 mg/l Zeatin + 0.3 mg/l IBA	Somatic embryos and adventitious buds, plantlets	Li et al. (2012)
Leucopogon verticillatus	Leaf segments	B5	10 µM TDZ + 5 µM IAA	Somatic embryos, rooting of shoots from somatic embryos, plantlets	Anthony et al. (2004b)
Rhododendron catawbiense 'Grandiflorum'	Leaf segments	MS	22.7 µM TDZ + 4.9 µM IBA + 2.3 µM 2,4-D + 59.1 µM 2iP	Shoot organogenesis, embryo-like structures	Vejsadová and Pretová (2003)

* Anderson (Anderson 1984), B5 (Gamborg et al. 1968), De Fossard (De Fossard et al. 1974), MS (Murashige and Skog 1962), WPM (Lloyd and McCown 1980).

In fact, somatic embryogenesis in all the species of Ericaceae has been induced using a combination of an auxin weaker than 2,4-D, such as IAA, IBA or NAA and a cytokinin (2iP, BA, TDZ or zeatin). In *A. unedo* and *A. canariensis* we

have tested 2,4-D alone and combinations of 2,4-D and BA or kinetin and only high proliferative non-embryogenic calli have been obtained (Figure 5A).

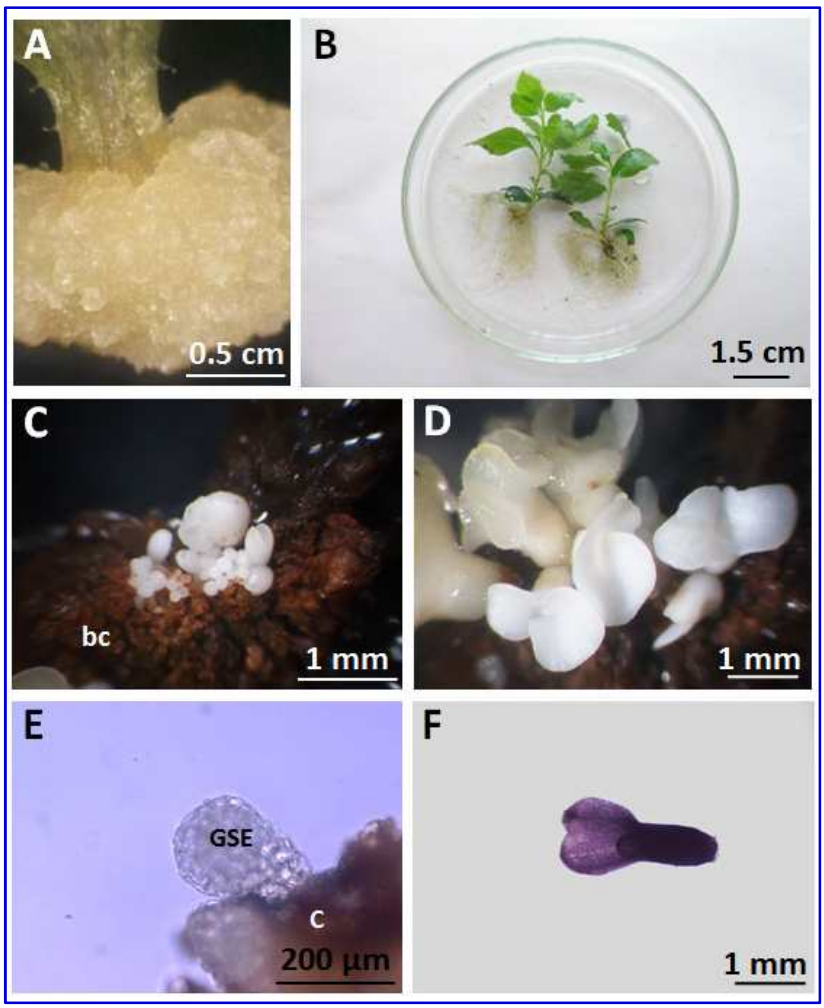

Figure 5. *Somatic embryogenesis induction in A. unedo.* ***(A)*** *Non embryogenic callus formed on a 2,4-D containing medium.* ***(B)*** *Plantlet obtained after shoot rooting. Shoots like this one have been used as a source of leaves for somatic embryogenesis induction.* ***(C)*** *Cluster of somatic embryos arising from a completely browning leaf explant (bc).* ***(D)*** *Cotyledonary somatic embryos at the time they are transferred to the germination medium.* ***(E)*** *Globular somatic embryo (GSE) developing from a callus (c).* ***(E)*** *Isolated cotyledonary somatic embryo.*

With *Leucopogon verticillatus* several sugars (maltose, sucrose, and fructose) were tested on somatic embryogenesis induction (Anthony et al. 2004b) with the best results (about 20 embryos per explant) being obtained on a medium

containing 4% maltose embryos. Differences were also observed with the same species when different pH values of the culture media were tested. In this case, a pH of 7 gave better results (about 15 embryos per explant) than more acidic pH values (4-6).

Another interesting feature concerning Ericaceae somatic embryogenesis is related with the type of explants that have been used. Whereas zygotic embryos are a common type of explant to induce somatic embryogenesis in many species, no reports of somatic embryogenesis induction using embryonary tissues as explant have been published. In all the cases indicated in table 1 leaf or stem segments were the responsive explant. Moreover, they were always obtained from in vitro growing shoots established from seedling or adult plants. In the case of the strawberry tree it was found that somatic embryogenesis can be induced from leaves of in vitro growing shoots (Figure 5B) independently of the initial origin of the shoots (seedlings or adult plants). However, the rates of induction were higher when shoots of seedling origin were the source of the leaves (Martins et al. 2015). Attempts to induce somatic embryogenesis in *A. unedo* through the culture of mature cotyledonary zygotic embryos on a wide range of culture media containing several combinations of 2,4-D or NAA with different cytokinins (BA, kinetin or thidiazuron) were completely unsuccessful and only friable non-embryogenic calli were obtained.

In some embryogenic systems, such as tamarillo (Correia et al. 2012) or carrot, embryogenic calli can be obtained from an initial explant on a medium containing an auxin and by further maintaining the tissue on the same culture medium through periodic subculturing. Somatic embryos and plantlets are usually obtained when the embryogenic calli are transferred to a second medium without auxin a process called two-step embryogenesis. The embryogenic calli can be maintained for several years in culture or can be cryopreserved to be used in further studies. Genetic instability may appear in these long-term cultures (Currais et al. 2013) but a system like this has advantages over embryogenic systems in which somatic embryos are produced from induced cells but a continuous formation of secondary embryos does not occur, which is a process often referred to as one-step embryogenesis. In *A. unedo* (Martins et al. 2015) and other Ericaceae (Table 1) no reports have signaled the formation of embryogenic calli that could be maintained through subcultures. In all the species in which somatic embryogenesis has been reported the embryogenic potential is rapidly lost following the formation of the embryos in the initial culture medium (one-step embryogenesis).

Tissue browning is of common occurrence during somatic embryogenesis induction in leaf segments of *A. unedo* (Figure 5C). A similar situation was reported for *Conostephium pendulum* (Anthony et al. 2004a). To avoid tissue damage due to polyphenol oxidation these authors have added several antioxidant compounds to the culture medium and found that a combination of tripotassium

citrate and citric acid significantly reduced phenolic browning. However, this treatment had no significant effect on the number of somatic embryos formed. In *A. unedo* and *A. canariensis* (data not published) we have observed that tissue browning always precedes somatic embryo formation (Figs. 5C-D). Moreover, browning seems to be a prerequisite condition for true somatic embryo formation since when browning does not occurs, a combination of shoot buds and malformed somatic embryos appear. This seems also to occur in other Ericaceae such as *Elliottia racemosa* (Woo and Wetzstein 2008) and *Rhododendron catawbiense* (Vejsadová and Pretová 2003) in which embryo-like structures resembling shoot bud development have been reported. Browning is the result of polyphenolic oxidation and is usually seen as deleterious to in vitro cultures. However, in the case of somatic embryogenesis induction this may not be the case, since there are several reports relating somatic embryogenesis induction and tissue browning such as for coffee (Neuenschwander and Baumann 1992), pineapple guava (Reis et al. 2008) and tamarillo (Correia et al. 2012) just to name a few. Moreover, in pineapple guava, it was found not only that browning and somatic embryogenesis induction are strongly connected but that the addition of phenolic compounds such as caffeic acid to the induction medium increases the rate of induction (Reis et al. 2009). In a recent work in tamarillo it was observed that the content of ferulic and caffeic acids was higher in embryogenic than in non-embryogenic calli and that the highest levels of these phenolics were found by the 8th week of culture, when proembryogenic masses usually appear (Caeiro, 2015). Taken together, these data seem to indicate that phenolic compounds may play a much more important role in the control of somatic embryogenesis than earlier assumed and that embryogenic systems of the Ericaceae may help to unravel how these phenolics affect somatic embryogenesis induction and embryo development.

Molecular studies related with Ericaceae somatic embryogenesis are scarce. The only known study focusing on the epigenetics of somatic embryo formation was carried out with *Erica carnica* (Yao et al. 2013). Through methylation-sensitive amplified polymorphism (MSAP) analysis it was found that in embryogenic calli the methylation level decreased during somatic embryo induction and recovered in the regenerated seedlings, whose methylation level was close to that of field seedlings. This seems to indicate that DNA methylation might control totipotency acquisition but more studies are needed to confirm these pioneering data.

5.2 Somatic embryo development

Somatic embryogenesis in *A. unedo* is an asynchronous process with embryos at different developmental stages being observed on the same explant (Figures 5C-D).

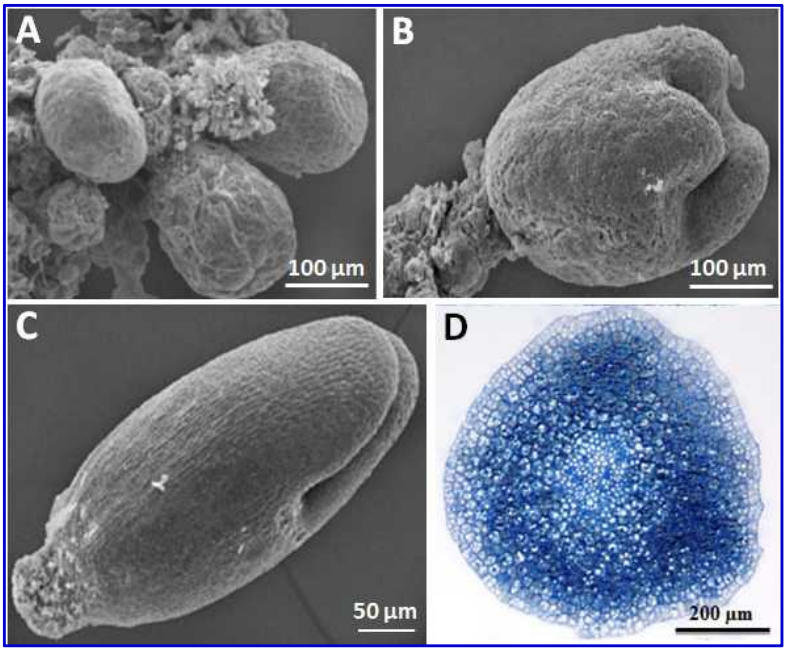

Figure 6. *Histological and scanning electron microscopy analysis of somatic embryogenesis in A. unedo.* ***(A)*** *Early stages of somatic embryo formation.* ***(B)*** *Abnormal somatic embryo with three developing cotyledons.* ***(C)*** *Cotyledonary somatic embryo.* ***(D)*** *Cross section of the hypocotyl of a cotyledonary somatic embryo showing a well-defined protoderm.*

The late stages of embryo development are usually attained after 10 – 12 weeks of culture (Figure 5D). Besides morphologically normal somatic embryos (Figs. 5E-F and 6A) embryos displaying some abnormality were often observed (Figs. 5D and 6B), the most common being the presence of an altered number of cotyledons. Histological sections prepared from morphologically normal somatic embryos (Figure 6C) showed a conspicuous protoderm (Figure 6D) and cells of the hypocotyl filled with storage compounds like in zygotic embryos. A detailed histological study carried out with *Elliottia racemosa* (Woo and Wetzstein, 2008) showed that well-formed shoot apical meristems were present but there was an absence of a defined root apex. This led the authors to conclude that true somatic embryos were not formed. Even though not supported by histological evidence, observations of *Leucopogon verticillatus* in vitro showed that when the embryos were transferred to a germination medium root development did not occur unless specific treatments, such as a 2–5 day pulse treatment of 100 µM IBA, were used (Anthony 2004b). Also, when lower concentrations of NAA are tested (2.2 µM or lower) in cultures of *Arbutus unedo*,, it is difficult to distinguish between shoot formation and somatic embryogenesis at the earlier stages of both morphogenic processes since they can occur in the same explant and look quite similar. These

observations once again highlight the relevance of histological studies during somatic embryo formation to distinguish between organogenesis and somatic embryogenesis. This is particularly important in the case of the Ericaceae since a combination of an auxin and a cytokinin is usually used to induce somatic embryogenesis (Table 1), conditions that are also normally required for *de novo* shoot formation.

5.3 Somatic embryo germination and acclimatisation

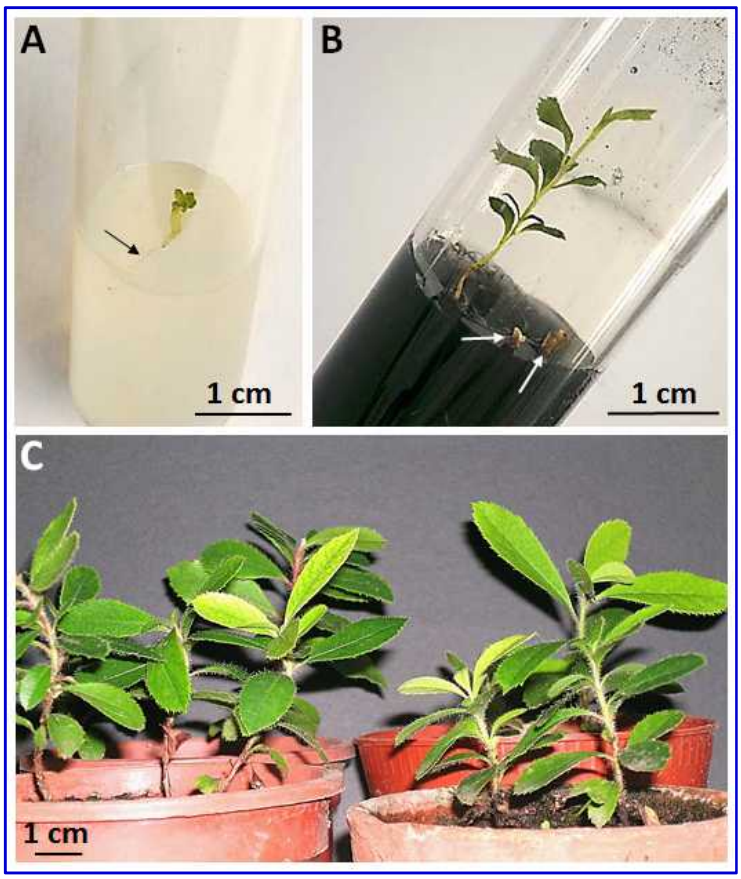

Figure 7. *Somatic embryo germination and plant acclimatization in A. unedo.* ***(A)*** *Developing plantlet following somatic embryo germination. The arrow points to the root.* ***(B)*** *Plantlet at the time of transfer to pots. The arrows indicate somatic embryos that did not germinate.* ***(C)*** *Acclimatised plants obtained through somatic embryogenesis.*

Somatic germination is a critical step to achieve plant regeneration through somatic embryogenesis. *A. unedo* cotyledonary somatic embryos germinate well

when transferred to a medium without growth regulators (Figure 7A) even though some embryos do not germinate (Figure 7B) probably due to morphological abnormalities or physiological or genetics constraints. Further plantlet development can be promoted on a medium containing activated charcoal (Figure 7B).

Acclimatisation of plantlets of somatic embryo origin is not problematic and most of the plants acclimatise well (Figure 7C) which might reflect the tolerance of this species to harsh conditions. Also in *A. unedo*, El-Mahrouk et al. (2010) reported the absolute requirement of light for somatic embryo germination. Under dark conditions germinated embryos became watery, succulent and exhibited abnormal growth. In other species of Ericaceae a rooting step may be necessary to achieve plantlet formation since, as already indicated, root development is often impaired during germination (Anthony et al. 2004b; Woo and Wetzstein, 2008). In these cases a treatment of the developing shoots with IBA is often used to induce root formation (Anthony 2004b).

6. Conclusions and future prospects

Somatic embryogenesis and plant regeneration have been obtained in several members of the Ericaceae family. Common features of the embryogenic systems in Ericaceae are the use of young leaves as explants, the requirement of a combination of an auxin and a cytokinin in the induction medium, the formation of somatic embryos through a one-step method and the close association between somatic embryo formation and tissue browning. In some species somatic embryo germination and plant conversion are problematic. Histological studies have related this drawback to the absence of well-formed meristems in the somatic embryos, mainly of the root pole. Thus, it is necessary to improve the protocols for somatic embryo formation in this family trying to increase the rates of induction and the quality of the somatic embryos formed. Since many Ericaceae are woody long-lived plants it would be interesting to establish protocols for the induction and maintenance of embryogenic calli that could be maintained in slow growth cultures or cryopreserved while plantlets obtained from these are tested in the field. Another aspect that deserves further analysis is the evaluation of genetic diversity of the plants that were propagated through somatic embryogenesis. Molecular markers have been used in other plants and must be tested in Ericaceae to confirm true-to-type somatic embryo formation and the effectiveness of somatic embryogenesis as a cloning technique.

In the case of *A. unedo* a reliable protocol for somatic embryogenesis induction and plant regeneration is available. The clones showed no phenotypic differences between them and the parents. We are now evaluating some physiological parameters of these plants under field conditions and comparing them with plants obtained through other cloning methods.

7. Acknowledgements

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From lab to field – current state of somatic embryogenesis in Scots pine

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Abstract

Scots pine (*Pinus sylvestris* L.) is considered to be a difficult species for vegetative propagation. Somatic embryogenesis of the species has, however, been developed to result in routine plant regeneration. Initiation frequencies remain low when compared with other conifers, but can be compensated by an increased number of explants used. Proliferation of the embryogenic cultures, somatic embryo maturation, germination and conversion into plants, as well as long-term storage of the cultures by cryopreservation already show good performance. There is not much published information available on field performance of Scots pine emblings, and the first results on a Finnish field experiment are presented. Following six growing seasons in the field, the Scots pine emblings grow normally and show genotypic differences. When compared with seedlings of the same genetic background, their height is – depending on the line - either comparable or inferior. At the moment, somatic embryogenesis of Scots pine provides a good tool to produce and use clonal materials for research purposes, as demonstrated, e.g., by several published and ongoing fungal studies. Currently there seems to be, however, no large-scale efforts to apply SE in practical breeding of Scots pine or for producing forest regeneration materials, although the potential of vegetative propagation is recognized in the case of Scots pine.

Keywords: *Pinus sylvestris*, embryogenic cultures, initiation, proliferation, maturation, germination, field performance, cryopreservation, genetic fidelity, fungal studies

1. Introduction

Scots pine (*Pinus sylvestris* L.) is an economically very important and scientifically well investigated forest tree species, having a broad natural range that

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covers large areas with different climate conditions in Eurasia. As reviewed recently by Krakau and co-workers (2013), Scots pine has been the focus of tree improvement, starting around 100 years ago with provenance research. Later on, tree breeding efforts have been initiated in several countries, the most advanced programs being ongoing in Sweden, Finland, Lithuania, and France.

An effective vegetative propagation method can be applied both for enhancing tree breeding and production of a large number of genetically improved plants. Somatic embryogenesis (SE) has become the method of choice for vegetative propagation of conifers (Sutton 2002) due to its high multiplication rate and the maintenance of juvenility *via* cryopreservation. In the case of Scots pine, SE is the only practical choice, propagation through rooted cuttings remaining very difficult and genotype-dependent, despite of extensive efforts to develop a rooting technique for the species (Högberg et al. 2011). Somatic embryogenesis in Scots pine was first reported in 1996 by Keinonen-Mettälä and co-workers. Compared to other pine species, Scots pine appears to be a difficult species also in SE procedures (Klimaszewska et al. 2007).

2. Different steps of somatic embryogenesis show varying performance

2.1 Initiation of embryogenic cultures

In Scots pine, initiation of embryogenic cultures takes place at a rather low frequency, compared with many other coniferous species. Initiation rates published vary from 0.2 to 42%, depending on the material used (Keinonen-Mettälä et al. 1996; Häggman et al. 1999; Lelu-Walter et al. 1999; Niskanen et al. 2004; Burg et al. 2007; Lelu-Walter et al. 2008; Aronen et al. 2009). Only very young explant material can be used, i.e. immature seed embryos. Induction of embryogenic cultures depends on the developmental stage of the zygotic embryo, responding immature embryos being harvested 1-3 weeks after fertilization, depending on the mother tree. In practice, a suitable time for explant collection can be based on degree days (temperature sum with a threshold of +5ºC), 400-650 d.d. showing the best response (Keinonen-Mettälä et al. 1996; Aronen et al. 2009). At this early developmental stage, the most practical method is to take intact megagametophytes containing zygotic embryos (Figure 1a) and place them on the initiation medium for ten weeks without subculturing during the whole initiation period (Lelu-Walter et al. 2008; Aronen et al. 2009).

In Scots pine, SE initiation is significantly affected by explant donor genotype (Lelu-Walter et al. 2008; Aronen et al. 2009). In the controlled crossings, the mother tree effect has been found to be greater than the paternal effect (Niskanen et al. 2004). Cold storage of collected cones before harvesting zygotic

embryo explants from them does not affect SE initiation frequency (Häggman et al. 1999) giving an opportunity to even out the work load over a longer period.

Figure 1. *Somatic embryogenesis in Scots pine: a) Initiation: embryogenic tissue emerging from the explant consisting of zygotic embryo surrounded by megagametophyte. b) Maturing somatic embryos. c) Germination of somatic embryos under in vitro conditions. d) Marking of the Scots pine emblings at greenhouse. e) Emblings at field conditions, Punkaharju experiment in Finland.*

On the other hand, composition of the basal medium may significantly influence the initiation rate (Park et al. 2006; Lu et al. 2012). Modified Litvay's medium (Litvay et al. 1985) has proven to be better than DCR medium (Gupta and Durzan 1985), although they both have successfully been used. Medium with low concentrations of plant growth regulators was more beneficial than medium with standard concentrations (Lelu-Walter et al. 2008).

Initiation of SE has also been attempted using explants from mature Scots pine trees. There is a great interest in clonal propagation of mature trees due to the possibility to propagate selected individuals with known characteristics (i.e. with good growth and wood qualities). The efforts taken for SE induction in mature Scots pines are described in more detail elsewhere in this book (Trontin et al. 2015), but can be shortly summarized: Slices from developing shoot buds were used as explants, and embryogenic-looking tissues induced from these. Microscopical examination revealed proembryo-like structures, most often mixed with undifferentiated callus cells and with a lot of endophytic contamination. Only a few cell lines provided long-term cultures and produced some somatic embryos, either disformed or looking more normal, but without the ability to germinate and convert into plants. Molecular studies of these lines revealed in some cases expression of genes related to embryogenecity, but also some inconsistencies in microsatellite markers in comparison with original donor trees.

2.2 Proliferation of embryogenic cultures

Once obtained, embryogenic cultures are proliferated on maintenance medium that is either the same as the initiation medium (Lelu-Walter et al. 2008) or having a slightly modified plant growth regulator composition (Aronen et al. 2009). The cultures can be maintained as small tissue pieces, but to achieve rapid multiplication of the cultures, proliferation on filter paper can be used, i.e., cells are spread in a thin layer over the surface of a filter paper disk placed on the surface of the culture medium (Lelu-Walter et al. 2008). For example, using 200 mg of culture spread over a disk of 5.5 cm in diameter, the average multiplication rate of 55 SE lines was 24x/6weeks, much more than observed within the same time period for 200 mg tissue pieces, i.e., 9x (Aronen et al. 2009). The choice of proliferation method may also affect the embryo maturation process. According to Aronen and co-workers (2009), proliferation on filter paper may slightly reduce the yield of mature embryos, but improve the quality of the somatic embryos developed.

2.3 Somatic embryo maturation, germination and conversion to plants

Development of cotyledonary somatic embryos depends on different factors, such as plant growth regulators (abscisic acid, ABA) and water availability

(gellam gum concentration) in the culture medium. In Scots pine, embryo maturation requires high, 80-90 µM, ABA and high, 9-10 g/l, gellam gum concentrations (Lelu-Walter et al. 2008; Aronen et al. 2009). The number of somatic embryos produced (Fig. 1b) varies a lot among the lines, the best ones yielding around 1000 embryos per g FW. Using improved protocols for Scots pine, 70-95% of the embryogenic lines in different studies produced mature somatic embryos (Lelu-Walter et al. 2008; Aronen et al. 2009; Krakau et al. 2013).

Harvesting well-developed somatic embryos results in high germination frequencies (Fig. 1c) (>90%, Aronen et al. 2009; 95% for out-cross lines and 85% for self-cross lines, Krakau et al. 2013). For achieving good plant conversion and survival it is necessary to limit duration of embryo maturation to 8-12 weeks, and perform quality control at harvest based on embryo appearance. According to Aronen and co-workers (2009) slim embryos have the best survival and greenhouse growth later on, short and thick embryos being inferior to them. Generally, the performance of Scots pine emblings under *ex vitro* conditions is good (Fig 1d). As reported by Krakau and co-workers (2013), four months after the acclimatization plantlet survival in the shade house was high (almost 80%) whatever the origin of the lines.

3. Field performance of Scots pine emblings

The Finnish Forest Research Institute established a field experiment with Scots pine emblings in 2009 at Punkaharju (61°48'N, 29°17'E, 90m a.s.l.)., and the experiment has been followed yearly to observe performance of the emblings. In this experiment 13 embryogenic lines (10 emblings per line) originating in four donor trees were included, together with seedling controls, as single-tree plots. Both the embryogenic lines and seedlings were of open-pollinated seed origin, produced as described by Aronen and co-workers (2009), and planted as 2-year-olds. In addition, the Punkaharju experiment was planned to study the effect of somatic embryo quality on later field growth of the emblings. Therefore, in five replications, six lines were represented as emblings derived from somatic embryos of different quality; i.e. from embryos classified either as "good", "intermediate", or "inferior" in the beginning of their germination period according to Aronen and co-workers (2009).

Following six years' growth in the field, 95% of the Scots pine emblings and 97% of the control seedlings were alive. The emblings show a normal growth habit when compared with the seedlings (Figure 1e). In a few cases, however, curling of the roots was observed which caused leaning of the individual. This was probably due to a too long nursery period in a small round pot. When examining the height growth, the emblings were compared with the seedlings from the same donor tree (Figure 2).

At the time of planting, the seedlings were bigger than most of the emblings, and this difference remained. After six years, seedlings were significantly bigger than the emblings in three out of four families tested. If the yearly growth is examined, it is seen that for all the families there are both years in which the growth of the emblings and the seedlings differ and years when it does

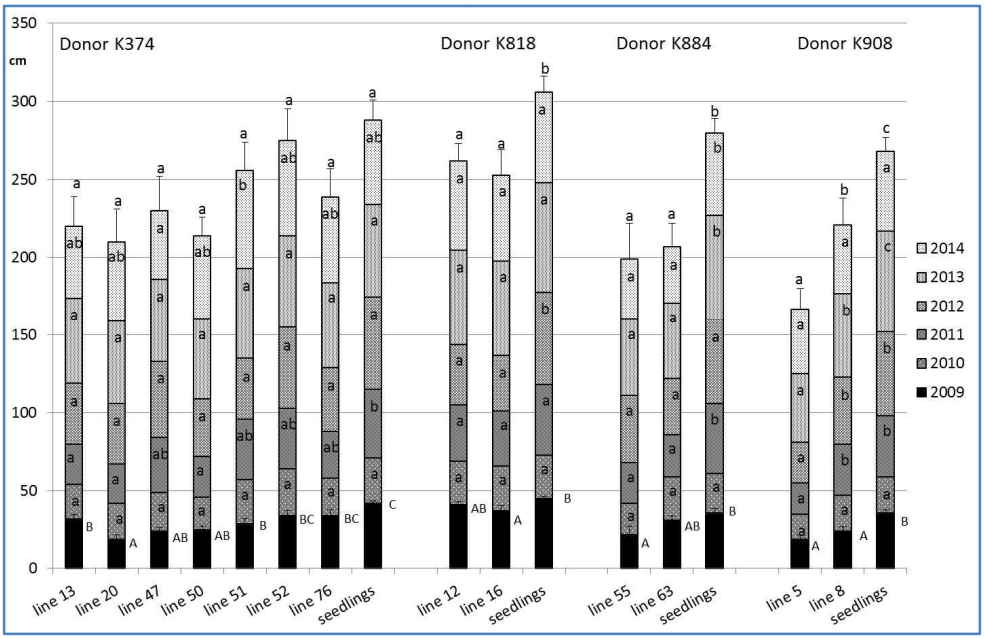

Figure 2. *Growth of Scots pine emblings compared with the seedlings from the same donor tree at the Punkaharju field experiment in Finland. The experiment having ten replications was established with single-tree plots using 2-year-old plants in 2009, and the plant height was measured yearly. Both embryogenic lines and control seedlings are of open-pollinated seed origin, from the donor trees K374, K818, K884, and K908. Within each family, the capital letters indicate significant (S-N-K test, $p<0.05$) differences in plant height in 2009, and the lower case letters in 2014, after six years in the field. The letters within columns indicate significant differences in the yearly growth in each year.*

not. The quality of the original somatic embryos (Figure 3), however, did not affect the height or diameter growth of the emblings (ANOVA $p=0.520$ and $p=0.997$, respectively).

The results of the Punkaharju field experiment show that Scots pine emblings grow normally and show genotypic differences. When compared with the seedlings of the same genetic background, their height following six years at the field is – depending on the line - either comparable or inferior. This might be partly because of the difference in the original size of the planted material. The control seedlings were sown at the same time when germinated somatic embryos were

transferred to the greenhouse. The newly germinated emblings are smaller than newly emerged seedlings, and the emblings also have a rather long lag period in their development following the transfer to greenhouse conditions. This is also reflected in the experimental material. Also the number of lines being tested was small which does not allow making any strong conclusions.

There is not much published information on the field performance of Scots

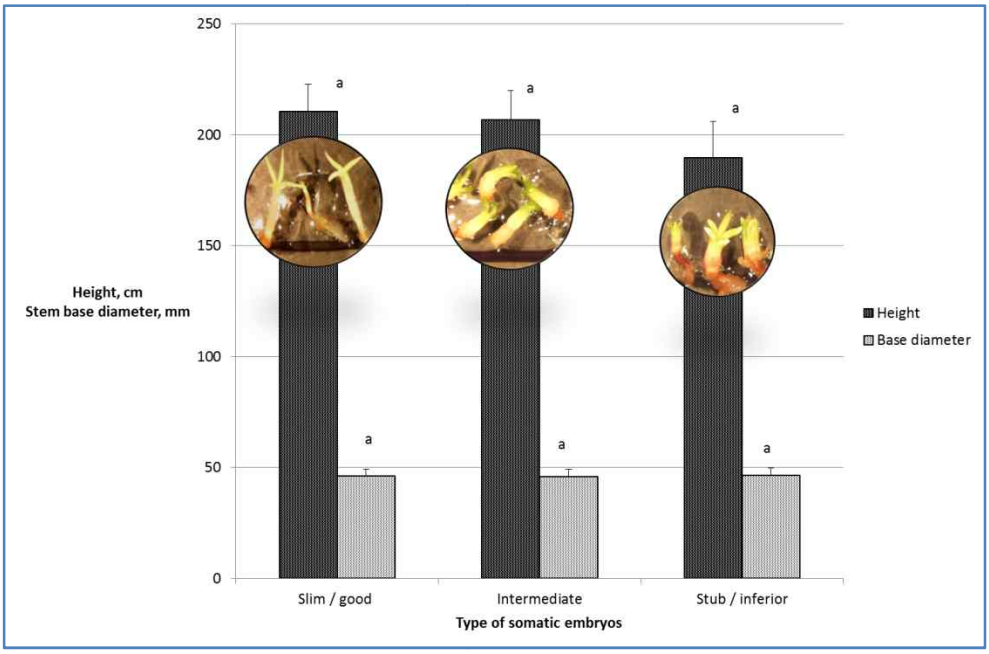

Figure 3. *Field performance of the Scots pine emblings derived from somatic embryos of different quality, following six years' growth in the field. At the beginning of their germination phase, the somatic embryos were classified either as "good", "intermediate", or "inferior" (Aronen et al. 2009). The field experiment having five replications was established at Punkaharju, Finland with single-tree plots using 2-year-old plants from six embryogenic lines in 2009. As the lower case letters indicate, no significant differences in height or stem base diameter were observed in 2014.*

pine emblings. Krakau and co-workers (2013) report that following 10 years' growth in the field in France, emblings show normal behavior, i.e., in growth and shape when compared with seedlings.

4. Storage of embryogenic cultures by cryopreservation

Embryogenic cultures can be stored as cryopreserved – i.e., in liquid nitrogen (-196ºC) - indefinitely without loss of juvenility. Cryopreservation thus offers good perspectives for long-term conservation and reactivation of

embryogenic cultures at any time, e.g., following field testing (Park et al. 1998).

For Scots pine, cryopreservation of embryogenic cultures has been successfully developed. The method is based on usage of a dehydrative pretreatment with increasing sucrose concentration, a cryoprotectant mixture containg 10% polyethylene glycol, 10% glucose, and 10% dimethylsulfoxide (DMSO) applied to samples, and slow cooling in a programmable device for freezing (Häggmann et al. 1998). The original protocol also included cold-hardening of the cultures at +5ºC prior to pretreatment, but this was later proven to be unnecessary. A good recovery rate of over 80 % has been achieved using this cryopreservation protocol after up to 10-years in storage (Latutrie and Aronen 2013). Another, simplified method applying sorbitol as the dehydrative agent, with DMSO (7.5%) as only cryoprotectant, and freezing in Nalgene™ Cryo Containers at -80 ºC in a freezer (Lelu-Walter et al. 2008) has shown good recovery of all the lines tested so far after 9 years of storage (Krakau et al. 2013). The duration of cryostorage had no effect on the growth rate of the recovered cultures, but the yield of somatic embryos per g FW may be reduced after prolonged cryopreservation (Latutrie and Aronen 2013).

5. Genetic fidelity of embryogenic cultures

For the successful integration of vegetative propagation within operational forestry, the SE technology must not only be able to multiply desirable individuals on a large scale, but also preserve superior clones without genetic changes. In this respect, successful cryopreservation of embryogenic cultures is a key requirement.

In the case of Scots pine, genetic stability during somatic embryogenesis has, in a few cases, been studied using DNA markers. Burg and co-workers (2007) observed variation of microsatellite (SSR) markers taking place during both zygotic and somatic embryogenesis, and they found some families having higher mutation rates in tissue culture than in seed embryo development. There is, however, no information if variation in studied marker loci reflects alterations in functional genes. In another study, RAPD markers were used to examine genetic fidelity of embryogenic cultures following cryopreservation by slow-cooling of PGD-cryoprotectant treated samples (Häggman et al. 1998), and no changes either in culture morphology or marker profiles were found suggesting genetic stability of the cultures during cryopreservation and subsequent regeneration.

Among the Scots pine emblings produced during the last few decades at the Finnish Forest Research Institute a couple of lines showed some somaclonal variation among the hundreds grown. In one case, some plants of one clone had a yellow needle color at the beginning of the second growing season (Figure 4a), but appeared normal later on. In another clone, part of the emblings have a bushy growth habit and shorter needles, and this phenotype has remained stable for the

next three years (Figure 4b). These cases are, however, rare and if they are related to mutations or epigenetic regulation, is not known. As summarized by Rani and Raina (2000), the tissue culture environment often is a stress factor that may induce phenotypic changes, e.g., through gene silencing or transposons. Somatic embryogenesis is generally considered more stable than cultures originating from callus or single cells, but careful monitoring of the emblings produced is anyhow needed.

Figure 4. *Somaclonal variation observed in regenerated Scots pine emblings: a) An individual embling showing yellow needle color surrounded by the normal plants of the same clone, at the beginning of the second growing season at the greenhouse. b) Part of the emblings of the line 2169 have a bushy growth habit and shorter needles than the normal plants of the same genotype, photographed at the beginning of the third growing season.*

6. SE applied in fungal research

In the case of Scots pine, interactions between various fungal species and embryogenic cultures or emblings have been studied since the 1990's. The original ideas behind these studies included the examination of plant tissue's reaction to fungal treatments compared to that reaction in whole plants, and the possibility to enchance SE by using, e.g., myccorhizal species.

Niemi and co-workers (1998) studied the effect of several ectomycorrhizal fungi on proliferating embryogenic cultures and obtained both positive and

negative reactions, depending on embryogenic line and fungal species. Slowly-growing embryogenic lines benefitted from the presence of *Laccaria proxima* and *Suillus variegatus* while rapidly proliferating lines cultured with these fungi suffered growth inhibition, browning and necrosis. In later stages of Scots pine somatic embryogenesis, the effect of ectomycorrhiza was more positive: *Pisolithus tinctorius* improved maturation of somatic embryos, if applied together with exogenous spermidine (Niemi et al. 2007), by forming mycorrhiza under *in vitro* -conditions and by enhancing germination of somatic embryos (Niemi and Häggman 2002).

Another ectomyccorhizal fungus, *Tricholoma matsutake,* a species that produces commercially important mushrooms, formed mycorrhiza with Scots pine emblings *in vitro* and enhanced the growth of the emblings (Vaario et al. 2015). The formation of myccorhiza in this study depended on the content of phenolic compounds in the pine genotypes, with only the lines having lower phenolics forming mycorrhiza. This result demonstrates the potential of SE materials in fungal studies and may also give ideas for improving the productivity of mushrooms.

In addition to studying beneficial mycorrhiza and somatic embryogenesis interactions, examination of interactions between pathogenic fungi and Scots pine are also worthwhile. As reported by Lu and co-workers (2011), somatic embryos react differently to elicitors from pathogenic *Heterobasidium annosum* than to elicitors from ectomyccorrhizal *Suillus bovinus* or the weak pathogen *H. parviporum,* not only in their survival and root formation. They also react to these pathogens at the molecular level by altering transcription of genes involved in cell division, cell wall modification and stress responses. This opens the possibility to use *in vitro* emblings and embryogenic cultures in model systems having a well-controlled environment for pathological studies and also potentially for *in vitro s*election of more resistant pine genotypes. In an ongoing project in Finland, the potential of somatic embryogenesis in improving heartwood quality and fungal resistance of Scots pine are currently being studied (Lu et al. 2012).The idea is to produce embryogenic lines from different genetic backgrounds that have variable contents of phenolic substances, and to test *in vitro* selection for fungal resistance with these lines.

7. Conclusions and future views

In conclusion, although Scots pine has been considered as a difficult species for vegetative propagation, significant improvements have been obtained in somatic embryogenesis of the species resulting in routine plant regeneration. Initiation frequencies remain low when compared with other conifers, but can be compensated for by increased numbers of explants used, with the potential

exception of some non-responding families. Later steps of the protocol and also long-term storage by cryopreservation show relatively good performance.

At the moment, somatic embryogenesis of Scots pine provides a good tool to produce and use clonal materials for research purposes, as demonstrated, e.g., by several published and ongoing fungal studies. Currently there is, however, no large-scale effort to apply SE in practical breeding of Scots pine or for producing forest regeneration material. One of the reasons for this is low initiation frequencies combined with strong dependence of initiation success on genetic background that hinders breeding applications. In many countries, seed orchards are well able to provide improved forest regeneration material for Scots pine (Krakau et al. 2013), and, e.g., in Nordic countries the current efforts for developing SE for commercial production of forest regeneration material have thus been focused on Norway spruce because of difficulties in its seed production (Anon. 2014; Högberg and Varis 2015). The potential value of vegetative propagation is recognized for Scots pine, and it has been suggested that SE could be utilized, e.g., to propagate progenies from some exceptionally good plus tree crosses or material showing hybrid vigor in connection with a full-sib breeding strategy (Krakau et al. 2013). Another option may become available with studies designed to find value-added genotypes showing, for example, improved fungal resistance or other valuable traits.

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Towards industrial production of tree varieties through somatic embryogenesis and other vegetative propagation technologies: Nordmanns fir (*Abies nordmanniana* (Steven) Spach) - From research laboratory to production

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Abstract

The main focus of the research on somatic embryogenesis in nordmanns fir has until recently been on improving the basic protocols in each step of the process. However, with recent developments, one single set of methods has shown to be effective for production of plants from more than 500 different untested cell lines. The developed method ensured a very good selection of genotypes at any of the involved steps: initiation, proliferation, cryo preservation, maturation, germination, and nursery culture. Growth and development of clonally propagated plants in the field were similar to those of seed produced plants. For this reason the focus is changing towards improvement of protocols for cost effective large scale production in a commercial set up. The first elite clones have been identified from a small preliminary clonal field trial from 2007, and larger clonal field trials have been established in 2014 and 2015 with 9000 plants from 400 clones.

Keywords: Christmas trees, clonal propagation, automation, field tests, elite trees, somatic embryogenesis

1. Introduction

Nordmanns fir or Caucasian fir (*Abies nordmanniana* (Steven) Spach) originates from the Caucasus Mountains and the Northern Turkey. The species is not widely used for traditional forest products such as timber or pulp and paper. The commercial interest is primarily focussed on the production of Christmas trees

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and greenery. Nordmanns fir is grown as plantation forestry in Denmark and in several other countries in Northern Europe and in the USA. The Christmas tree industry is of steadily growing importance and nordmanns fir has become the economically most important tree species in Danish forestry. The European market is growing from the present state of approximately 50 million trees each year.

The production of Christmas trees is a typical example of agroforestry and it has many similarities with the production of more traditional high value agricultural crops. Christmas trees are grown in mono cultures and the rotation time of 8-10 years is very short compared to traditional forestry. The production is cost and labour intensive, and the single plant represents a sizeable value for the grower.

Propagation of Christmas trees is exclusively from seeds collected in the natural forests in the Caucasus or from Danish seed plantations. The seed supply is unstable and genetic variability is prominent. Nordmanns fir has a generation time of 25-30 years, and traditional breeding programmes are extremely time consuming. The extended generation period is a general problem in breeding programmes for forest trees, but it is particularly a problem in specialized industries, dependent on fast breeding and development of new products.

Even with intensive management and shaping of trees, the growers must expect a loss of 25-35 % of trees due to low quality, when trees are propagated from seeds. Only about 10-15 % of the produced trees are of best quality (Table 1). Clonal propagation of elite material offers the ideal propagation method, with high and uniform quality of trees. The expected gain by use of clonal propagation is estimated to be approximately 3.6 Euro per tree, compared to the present situation with seed propagated material (Table 1). This gain is based on an average better quality of trees. In addition to this, the uniformity in itself provides a predictable production focussed on the specific clone and the possibility of clearcutting the area in one year instead of over several years because of the uniformity of the trees and thus shortening the production time with one or two years.

The application of mono cultures in combination with the 8-10 year rotation time increases the risk of damage caused by insect predation. This is a considerable problem in the Christmas tree production where the form and appearance is of major importance for the final quality and value of the product. At present the problem is mainly met by application of pesticides. However, the public acceptance of this solution is declining, and selection of clones with natural resistance will be the best way of preventing serious damage caused by insects.

Infection by the fungus *neonectria neomacrocarpa* has lately caused severe damage in Danish plantations. The biology behind the infection is not known and the only way of dealing with the problem is to remove infected trees. In 2013, the fungus caused an estimated economic loss of almost 10 million Euros in Denmark, and in future this fungus can cause serious economic losses for the

Table 1. *Nordmanns fir. An example of economic gain by integration of clonal propagation techniques in breeding programs of forest trees. Distribution of trees in categories of quality and estimated sales price when propagated from seedlings or by clonal propagation of elite material (Find et al. 2009). Calculation of average sales price per 100 trees. Estimated numbers were obtained from the Danish Christmas tree Growers Association*

Gain for the grower by use of cloned material					
	%-distribution		Price per tree	Sales price per 100 trees	
Categories, quality	Seeds	Cloning	(Euro)	Seeds	Cloning
Excellent	15	60	12.3	185	738
Standard	40	20	6.9	276	138
Below standard	20	5	3.7	74	185
Rejects on basis of form	20	10	0	0	0
Rejects on basis of damage	5	5	0	0	0
Total	100	100		535	895
Expected gain per tree by clonal propagation: 3.6 Euro					

industry. Resistance towards the fungus seems to have a genetic background, and it is expected that it is possible to select resistant clones (Thomsen et al. 2014). Clonal propagation of resistant trees may be the only protection against serious threats from this and other fungi.

Traditional methods for clonal propagation, such as cuttings, are not possible for nordmanns fir because of a very low rooting rate and plagiotropic growth. Somatic embryogenesis (SE) is at present the only promising method for clonal propagation in nordmanns fir, and the method offers great potential for enhancing gains from intensive Danish tree-breeding programs and for bulk propagation of identified elite trees. Establishment of SE in nordmanns fir was reported for the first time in 1991 (Nørgaard and Krogstrup 1991). Since then the protocols have been further developed (Nørgaard and Krogstrup 1995, Nørgaard 1997 and Find et al. 2002) including development of standard methods for cryopreservation (Nørgaard et al. 1993) and for genetic transformation (Find et al. 2005).

The SE system is developed to a state where it is ready to be tested in a commercial set up. The aim of this chapter is to present the state of the art for this species in our laboratory, and to outline how the technologies are transferred from a research laboratory to a production facility in a commercial set up.

2. Methods in relation to large scale production

2.1 Induction of embryogenic tissue

Embryogenic tissue can be initiated from immature zygotic embryos (Nørgaard and Krogstrup 1991) and from mature zygotic embryos; from fresh or stored and dried seeds (Nørgaard and Krogstrup 1995, Kristensen et al. 2005). Induction of SE is possible all year round. SE is initiated from the hypocotyl after 8-12 weeks of culture (Nørgaard and Krogstrup 1995). The frequency of initiation is very dependent on the quality of seeds. From fresh seeds, initiation rates are from 50-85 % (Kristensen et al. 2005). This rate is large enough to allow setting up clonal tests from selected families or controlled crossings. When the embryogenic tissue has a diameter of approximately 0.5-1.0 cm it is removed from the explant and transferred to fresh medium for proliferation (Figure 1).

Figure 1. *Vegetative propagation of nordmanns fir by somatic embryogenesis.* ***A.*** *Cell culture initiated from post cotyledonary embryos isolated from stored seeds.* ***B.*** *Mature embryos after 12 weeks of maturation.* ***C.*** *Germinated embryos in a petri dish.* ***D.*** *Plants after one growth season in a plug system.* ***E.*** *Plants in the greenhouse. Two years old and ready for transfer to clonal field testing in the fall of 2014. Photo May 2014.* ***F.*** *Three plants from the same clone in the second growth season, May 2014. (Photos E Bihrmann).*

2.2 Maintenance of embryogenic tissue

The embryogenic cultures are maintained on solid proliferation medium by subculture of tissue to fresh medium every 2 weeks. Proliferation of somatic

embryos in nordmanns fir is by continuous cleavage of embryos (Figure 1), and cultures double in size every two weeks. Embryogenic potential will be maintained over many years on solid proliferation medium, but requirements of the maturation medium may change during prolonged periods of proliferation (Find et al. 2002).

2.3 Cryopreservation

All cell lines are stored in liquid nitrogen (Nørgaard and Krogstrup 1995). At present the laboratory holds a gene bank of approximately 900 frozen cell lines of nordmanns fir. The cell lines originated from the Danish breeding program or from selected trees in the natural stands in Georgia.

2.4 Maturation

As opposed to many other conifer SE systems, addition of the growth regulator abscisic acid (ABA) alone does not induce development of high quality cotyledonary embryos in cell lines of nordmanns fir. To increase the number and the quality of mature embryos it is necessary to include an additional step where the auxin antagonist PCIB is added (Find 2001, Find et al. 2002). In a recent test of 400 cell lines from 40 families, high quality mature embryos were produced from 98 % of the cell lines with use of only one standard method including PCIB treatment (unpublished results). Mature embryos are harvested after 12-14 weeks, and a second harvest is possible one week later. Only high quality embryos with no observed abnormalities are harvested. In our experience any type of irregularity in the outer appearance or shape of mature embryos leads to improper development and low performance during germination and plant production (unpublished results). For larger scale production of plants, optimisation of the protocol for each specific cell line will be essential. This will increase the total number of embryos, the average quality of embryos and will importantly improve synchronisation of the development of embryos from the same batch. At present maturation is set up on filter paper on solid medium in 10 cm Petri dishes (Figure 1) (Find et al. 2002). This method is effective for production of a few hundred mature embryos from each of a large number of clones needed for the present establishment of clonal trials. Cheaper and more efficient methods are essential for large scale production from a few selected clones.

2.5 Germination and transfer to soil

Different ways of ‘after ripening’ or pre-treatments have been developed to improve conversion and germination of mature somatic embryos of nordmanns fir. In our experience, the main parameter for successful plant production is the quality

of the mature embryo. Previously, transfer from sterile conditions to soil in the nursery was the main bottleneck for practical application of SE in nordmanns fir. However, lately this has changed and now the growth of clonally propagated plants in the greenhouse is comparable or better than that of similar plants produced from seeds (unpublished results). To reduce production costs, it was tested how early in the process plants can be transferred to the production green house without compromising survival and growth. Interestingly, preliminary tests have shown improved growth of plants in the greenhouse compared to the growth obtained presently in ventilated plastic boxes in the controlled growth room (unpublished results). The reason may be better airflow and increased control of physical factors in a larger scale production facility.

3. Clonal field trials

The establishment of clonal field trials has high priority, as they serve as the basis for the future selection of elite material for establishment of a commercial production. The primary criteria for selection in the Christmas tree production will be the general appearance of trees. Growth rate, form and color are important factors, but the final selection will, to some extent, be based on personal preferences regarding tree form. Additionally, quantitative parameters such as: needle retention, frost tolerance, pest and fungi resistance are very important parameters. Natural resistance towards insects and fungi may increase in importance, because consumer demands for 'natural trees' that are grown without the use of pesticides and fungicides are increasing. Screening for some parameters is possible after a few years of growth, but the final selection may not be possible until a full growth period of 8-10 years has passed.

Figure 2. *Clonal field trial with nordmanns fir established in 2007. After 8 growth seasons. (Photo E. Bihrmann, 2015).*

Our first clonal trial with SE plants of nordmanns fir was established in the fall of 2007 with 379 trees from 9 clones (Find 2014). The aim of this field trial was not primarily to select elite material, but to investigate the growth of SE plants compared to seedlings and to document the phenotypic uniformity of ramets from the same clone. These plants are now eight growth seasons old (Fig 2). The nine clones were phenotypically very different from each other, as one would expect for randomly selected trees with different genetic backgrounds. Opposite to this, phenotypical variation in growth, form, color and time of sprouting was very small between ramets from same clone. Two clones out of the nine clones in total may have commercial potential. One of these clones was fast growing and form and appearance was superior to that of the average for Christmas trees (Fig 3).

Another clone was very slow growing and the appearance was dense and compact (Fig 4). This clone is not suited for normal production of Christmas trees, but has gained interest from producers of potted trees. In 2011, 47 mother trees/families were selected from natural stands in Georgia. Cones were collected

Figure 3 (Left). *Clone selected in 2015 from a field trial established in 2007. The clone is fast growing, and form and appearance is superior to that of the average for Christmas trees. (Photo E. Bihrmann, 2015).*

Figure 4 (Right). *Clone selected in 2015 from a field trial established in 2007. This clone is not suited for normal production of Christmas trees, but has due to its low and compact appearance gained interest from producers of potted trees. Notice the uniform phenotypic appearance of all trees in the row. (Photo E. Bihrmann, 2015).*

from all selected trees and SE was established from 250 cell lines. In 2012, an additional 27 trees were selected from the Danish breeding program. SE was established from 150 cell lines. All clones/cell lines were stored in the cryogenic gene bank.

In the fall of 2014 approximately 4.000 plants at an age of two years, originating from the 250 clones established in 2011, were planted in clonal field trials situated at two different locations in Denmark. All clones were planted on both locations to investigate environmental impact on growth and development. The field trials were randomized and mixed with seedlings of the same age. After one year of growth in the field, survival rate was 90-95 %, and the growth was comparable to the growth of seedlings (unpublished results). Similar field trials with approximately 5.000 plants from an additional 150 clones will be established during the fall of 2015.

4. From research to production

Somatic embryogenesis in nordmanns fir has shown to be effective in the research laboratory and growth of the produced plants is not different from that of seedlings. The next step is to test the methods in commercial scale production. There are many advantages in going from production of a few plants from each of several hundred clones, as in production of plants for clonal field testing, to production of commercial scale amounts of plants from a few selected clones in a production line. In large scale production of a few clones, it is possible to gain experience of the biology of each particular clone/cell line, and each step in the production can be optimized and scheduled to fit to the particular clone/cell line.

There are still unknown biological aspects in SE, which need attention in setting up of a commercial production. The embryogenic cultures of nordmanns fir have shown to be very stable over time in respect to their maturation capacity. This may change during prolonged periods of scale up and large scale production. In order to produce uniform plants and to reduce production costs, developmental steps such as maturation, rooting, and shoot growth must be synchronized in the production. This is not always the case in the present set up, and this must be considered in a larger scale set up. To achieve a cost effective and uniform production, the nursery must have plants delivered during a short period of, e.g., one month each year. To take advantage of the fact that plants can be produced in the laboratory all year, it is necessary to find effective means of arresting the development of mature somatic embryos and ways to store them over longer periods.

At present seedlings at an age of 4 years (2 years in the nursery + 2 years in the field) are sold at approximately 0.5 euro each. Due to better quality of cloned material, the SE produced plants may not need to meet this price, but production

costs must be reduced from the present state in the research laboratory. The existing production is very labor intensive, and automation of specific processes will be an efficient way of reducing costs. For nordmanns fir the two most 'labor expensive' processes are: 1) selection of mature embryos and transfer from maturation medium to germination medium, and 2) transfer of germinated and rooted plants from sterile conditions to soil (Find et al 2009). A previous Danish project aimed at developing automated solutions for these two processes. The conclusion of this work was that it was possible to develop automated handling for the two described processes with a handling time of approximately 4 sec per plant. The most challenging point to establish an automated set up was not the technical aspects or image analysis, but to ensure the required control and synchronization of the biological processes (Find et al 2009).

Integration of the SE production of plants into the existing production of seedlings has been an important objective. The impression was that this was the only way of reducing the additional costs related to the prolonged nursery culture. However, experience in recent years has shown that it is possible to increase growth rate of SE produced plants considerably by intensive management in the nursery. SE produced plants are ready for transfer to the field after 2-3 years of growth, whereas seedlings in the existing production are transferred after 4 years. For this reason it is probably more cost effective to intensify the nursery production of SE plants and to take advantage of the uniformity of clonal propagated plants to optimize growth parameters for each specific clone.

In addition to being a very promising method for enhancing gains from tree-breeding programs and for bulk propagation of elite trees, the SE system offers an excellent basis for development of new methods for future breeding programs. For nordmanns fir the aim is development of protoplast cultures for somatic hybridisation, artificial seeds for improved storage and introduction of new traits by genetic engineering (Find et al 2005).

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Vegetative propagation of Norway spruce: Experiences and present situation in Sweden and Finland

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Abstract

Cutting propagation of Norway spruce is a well-known method requiring juvenile donor plants and a rooting environment with air humidity control. Despite being a relatively simple method, cutting propagation has not reached large-scale commercial plant production due to high production costs. However, cutting propagation is at present routinely used both in Swedish and Finnish breeding programs. A shortened breeding cycle, increased precision and increased selection intensity are the main advantages. Somatic embryogenesis (SE) is at present too expensive to use, both in commercial large-scale propagation and as a tool in breeding. Development of automated somatic seedling production is under way and, if successful, will reduce production costs of superior genetic Norway spruce material. A strong selection takes place during the SE process with a standard protocol, which is needed to produce a material with both reliable genetic gain and sufficient genetic diversity. To obtain ornamental Norway spruce varieties SE plant production can be carried out on a smaller scale, which may provide valuable information that can be used also in large-scale forest plant production.

Keywords: cutting propagation, genetic diversity, genetic gain, ornamentals, somatic embryogenesis

1. Introduction

Norway spruce covers large areas in Sweden and Finland and the species is widely used as raw material in the forest industry. Thus, its economic importance is

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paramount and the species has been subjected to breeding for several decades. Both for making the breeding more efficient and to get a faster exploitation of genetic progress, vegetative propagation can serve as a useful tool (Shaw and Hood 1989; Rosvall et al. 1999). Cutting propagation has been used routinely in the Swedish spruce breeding program since the mid 1990's (Karlsson & Rosvall 1993) and is now used also in Finland as part of the breeding program (Haapanen 2009). The higher price for cuttings compared with seedlings hampers large-scale propagation of superior genetic material. The bold clonal forestry projects that were launched in Sweden in the 1970's and 1980's were stopped before they reached more than a few million cutting-propagated plants per year, primarily because of their high price. No commercial production of rooted cuttings was started in Finland, but experiments have been continued on a small scale (Mikola 2009).

Somatic embryogenesis (SE) of Norway spruce was first obtained in the mid 1980's (Chalupa 1985; Hakman and von Arnold 1985) and was further developed the following decade (von Arnold et al. 1995). This propagation method allows a much faster propagation than by cuttings (Högberg et al. 1998). One obstacle to overcome is, however, the same as for cutting propagation: the high plant production cost. A specific, unfortunate aspect of SE is the substantial loss of genotypes during propagation. The restrictions accompanying the current standard protocol make SE less attractive both as a tool in breeding and for commercial plant production. A shift towards automated processing of SE and thus reduce the cost is necessary to change this picture.

2. Cutting propagation

Rooting of Norway spruce cuttings requires that the donor plants are in a juvenile phase. A freely developed plant can be reliably propagated up to 4-5 years of age. If the donor plants are older, both the average rooting percentage and the number of successfully propagated genotypes drops.

The rooting ability can be kept for a longer time by serial propagation (Kleinschmit et al. 1973) or by hedging (Bentzer 1981). However, genotypes will be lost with both methods and the losses cannot be predicted in advance. The methods to keep the ortets juvenile need large areas for the donor plants which is a drawback for large-scale cutting propagation. Furthermore, the time needed for scaling up selected clones is considerable, putting even more constraints on maintaining juvenility.

Two main propagation periods are used for cutting propagation in Sweden, late summer/early autumn and winter/early spring. The same rooting environment is used in both cases: cool air temperature, high air humidity, soil heating and drainage-allowing soil. The cuttings are typically 8 cm long and inserted in a mixture of peat and perlite in 70/30 proportion. Cuttings are not taken from leaders

of the ortets. Fogging or misting systems, or simpler, fine-dispersing sprinkler jets controlled by a greenhouse computer, normally provide a suitable air humidity to avoid dehydration of the needles. The exact setting varies from greenhouse to greenhouse. Hormone treatment is not necessary to initiate rooting. With a soil temperature of about 20°C, the adventitious roots normally emerge within six to ten weeks. Ten to twelve weeks after insertion the rooting environment can be adjusted to normal growth conditions.

The two propagation periods impose specific handling requirements. In late summer propagation, the time between collection and insertion should be as short as possible. The outdoor air temperature can be 30°C or more in late summer, leading to increased risk for dehydration (greenhouse ventilation is often open during the summer and transpiration and evaporation are high). In these circumstances it is important to frequently check and adjust the greenhouse computer to keep the air moisture high without causing excess of water in the substrate. No shoots develop during late summer propagation.

In winter/early spring propagation, the cuttings are typically collected in November-December when the donor plants are safely hardened. Cuttings are then stored in plastic bags at -3°C until the time for insertion. Shoots flush simultaneously with the adventitious root development.

Propagation with cuttings collected from three to four years old donor plants normally results in 70% rooting or more. This reliable outcome is utilized in breeding programs where candidates for the next breeding generation are routinely cutting-propagated and tested in the field as clones. Time gain and high selection precision are important advantages motivating this strategy. The breeding values estimated after field testing may be biased due to confounding of additive and non-additive effects. However, simulation studies have shown that the genetic gain is substantial and effective even at high levels of non-additive variance (Rosvall et al. 1999).

Besides the routine propagation of candidate genotypes for the breeding program, about one million rooted cuttings per year are produced commercially in Sweden. This production is based on the family forestry concept where parents with high breeding values are crossed and the progeny is propagated without keeping track of specific genotypes. This is a fast, high-value output from the breeding program that has attracted some forest owners. The more robust plant type thus obtained is another feature that contributes to the attraction. The price is still too high for big market production and the rooted cuttings are so far produced only when requested by forest owners, but the volume is slowly increasing. As the breeding programs proceed, genotypes with higher and higher breeding values can be selected and thus motivate a higher price. It is not realistic, however, to assume that cutting propagation will take more than a limited fraction of the forest plant market in the near future.

Attempts to mechanize the insertion during cutting propagation have been made but have failed. The required one-by-one handling appears to be difficult as the needles often attach, thus causing frequent clustering of two or more cuttings that will interrupt the production flow (Högberg et al. 1996).

3. Somatic embryogenesis

The practical experience with SE is limited to propagation rounds using a standard protocol developed during the 1990's and described in Högberg et al. (1998). This protocol describes initiation, proliferation and maturation on solid medium, followed by partial embryo desiccation (Figure 1).

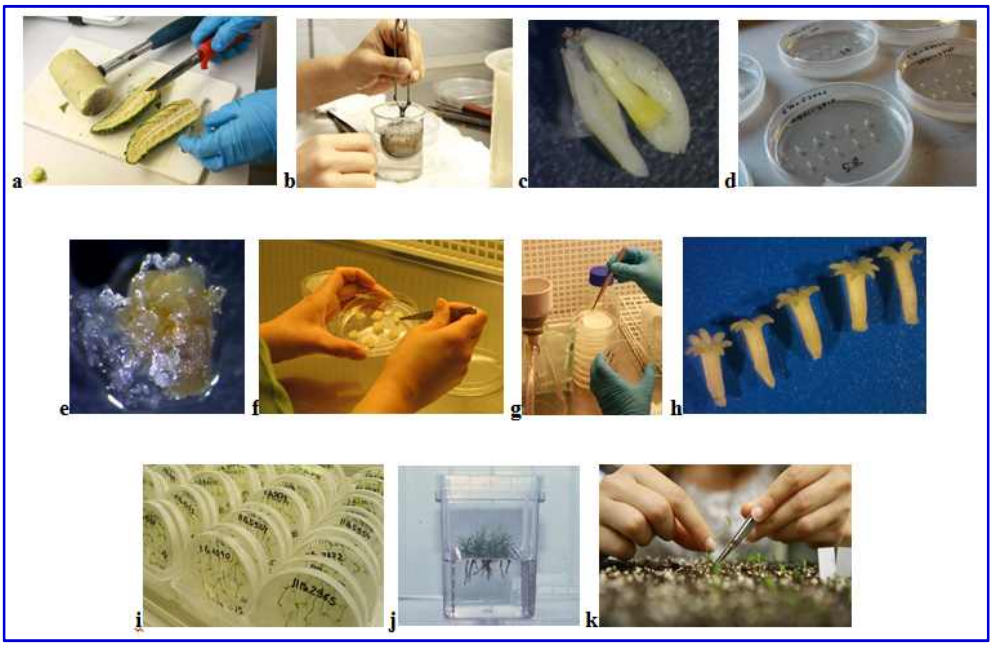

Figure 1. *Production of SE plants of Norway spruce starts with dissecting seeds from immature cones* ***(a)****. Seeds are surface-sterilized* ***(b)*** *and opened* ***(c)****, and embryo is placed on semisolid media* ***(d)****. Growing embryogenic tissue* ***(e)*** *is subcultured every two weeks* ***(f)****. For embryo maturation tissue is suspended in liquid media, suspension is poured onto paper filter, liquid is drained off by suction, and the filter paper is placed on media* ***(g)****. Mature embryos* ***(h)*** *can be moved directly, or after partial desiccation, to semisolid germination medium.* ***(i)****, with a possible subsequent step where germinants are put on a metal mesh allowing root development in liquid* ***(j)****. Small SE plants are cultivated into peat or mixture of peat and perlite* ***(k)****. Photos: Susanne Heiska, Lassi Palmujoki, Saila Varis, Christine Devillard*

After germination on solid medium, small plantlets that develop a proper shoot and root are transferred to a vessel with a metal mesh that allows the root to

be in liquid while the shoot is in air. An alternative method is to use semisolid medium throughout the process until embryos are acclimatized in a greenhouse (Klimaszewska et al. 2001). With this method mature embryos are moved directly to semisolid germination medium from maturation medium without desiccation. It is possible to store mature embryos on maturation media in +4ºC and darkness for up to six months without decrease in germination ability.

Embryos from immature seeds are preferred as explants because the initiation percentages are much lower when using mature seeds. Cryopreservation is used for long term storage of cell masses.

The first attempt of SE with a large number of genotypes performed in Sweden revealed that there is a comparably strong selection for propagation ability (Högberg et al. 1998). Genotypes were lost at all steps and only 13% of the cell lines remained after plant regeneration. Two other propagation rounds involving a large number of genotypes have been made with more or less the same result.

Both family and genotype has a strong impact on the propagation success, both when it comes to how many genotypes can be propagated per family and how many plants can be produced per cell line from a specified amount of tissue. From an economical point-of-view clones that are difficult to produce in large numbers will probably be rejected and selection will be stronger than indicated above.

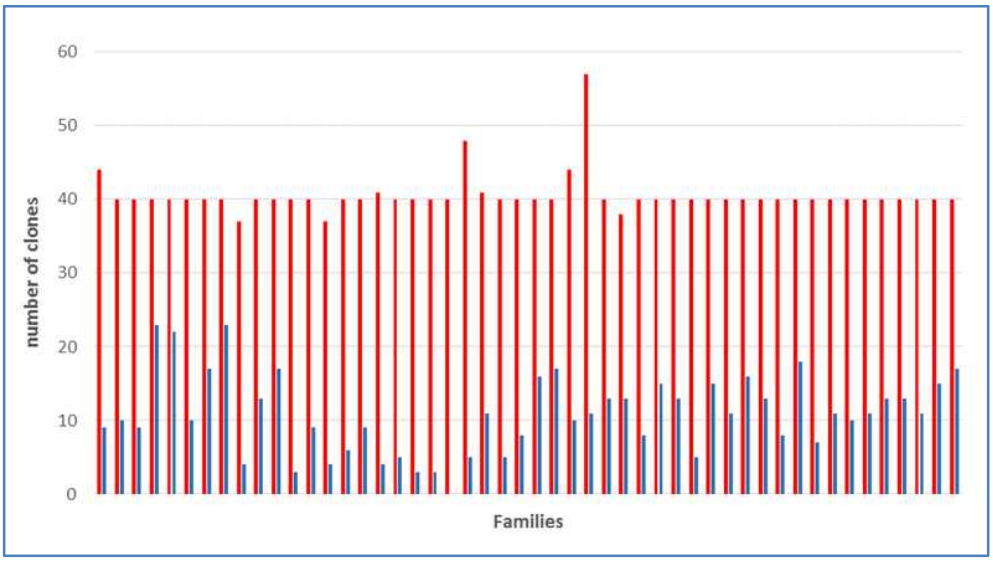

Figure 2. *Reduction of numbers of genotypes during somatic embryogenesis propagation, example from a Swedish SE propagation with half-sib families. Red bars = number of zygotic embryos entering initiation, blue bars = number of clones with plants ready for planting in field trials.*

An important aspect of the genotype loss is the reduction of genotype diversity. Furthermore, the typical uneven number of clones produced per family

(Figure 2) and the uneven plant production capacity among clones (Figure 3) reduce the genetic diversity even more. .

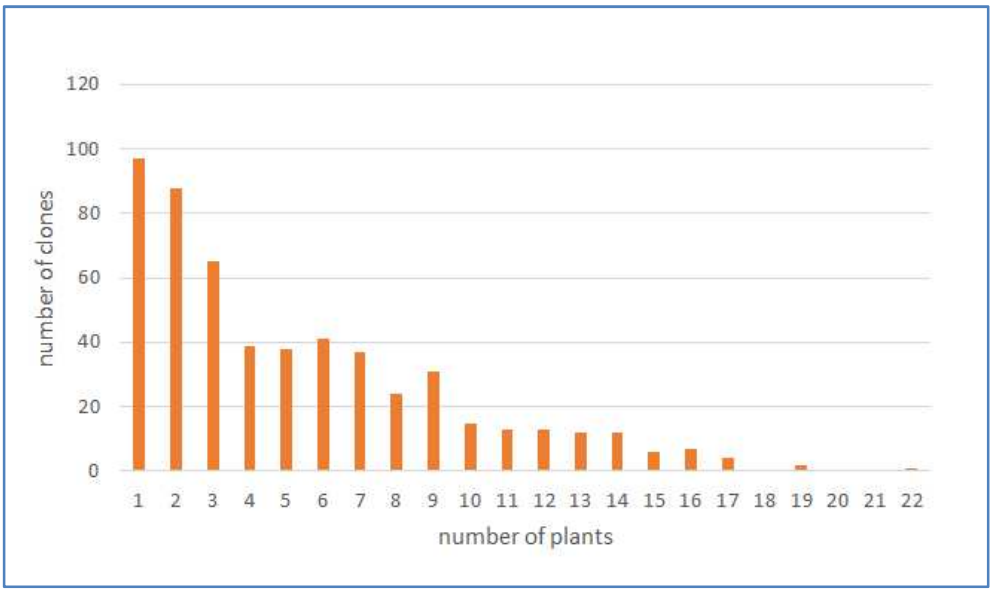

Figure 3. *Example of distribution of plant production capacity on Swedish clones in a somatic embryogenesis propagation program. A majority of the clones produced 4 plants or less. Approximately 3.5 mg embryogenic tissue per cell line entered maturation.*

As in Sweden, SE initiations in Finland vary between families and genotypes; few are good embryo producers (the best line produces almost 700 embryos from a one gram of fresh cell mass) while many do not produce embryos at all (Figure 4). From 5100 initiations made in 2011 and 2012, 258 lines had good embryo productivity and embryo germination rates, which were criteria for selecting lines for cryopreservation. In 2014, more than 2300 lines from over 5000 initiations were first cryopreserved, and embryo productivity will be tested from lines recovered after cryopreservation. This change in order of maturation and cryopreservation was made due to problems in recovery after cryopreservation and the need to cryopreserve cell masses in as fresh a state as possible. In order to improve cryopreservation results, different pretreatment and freezing methods have been studied and pretreatment that involves increasing the sucrose concentration and slow freezing seems to be the best method in Finnish experiments.

One main aim in the ongoing Finnish project is to produce more embryogenic lines for testing in the laboratory for their embryo production capacity for field testing and commercial production. A Finnish tree breeding program will provide the seed to start the SE lines; initiations are made from crossings done with first generation plus trees. So far, the plus trees originated

from southern Finland breeding zones one and two, and SE plants will be marketed for those areas.

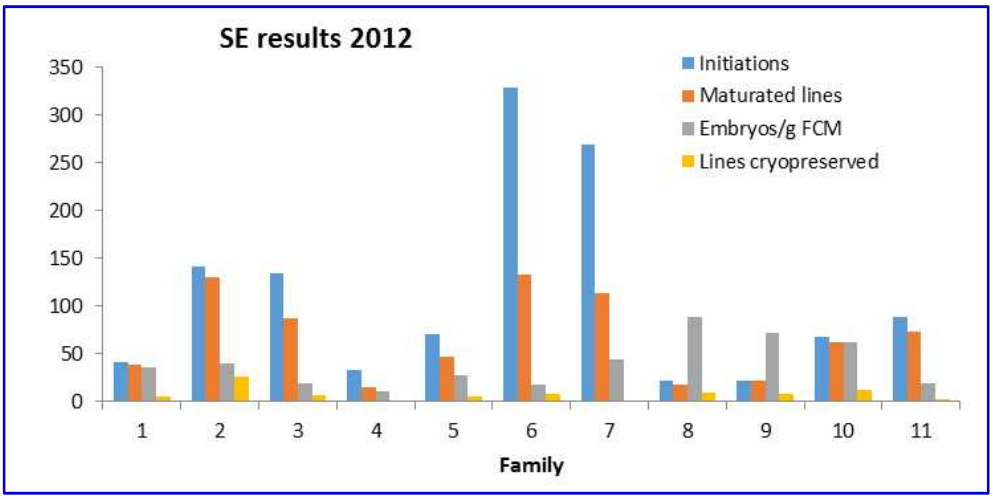

Figure 4. *The number of initiations, maturations, embryos produced and cryopreserved lines from crossings made in 2012 in Finland. FCM= fresh cell mass.*

The cost efficiency at different stages of the SE plant production process has to be improved, and is the focus, e.g., in the ongoing Finnish project. In the research laboratory that is specialized in the production of embryogenic spruce lines this is achieved by the use of bioreactors, applying new lighting based on LED technology, and by developing a system for sample identification and data handling allowing big numbers of embryogenic lines to be processed. At the same time, the field testing process can be shortened by integrating SE with cutting technology, i.e., SE plants are used as donor plants for cutting propagation and both plant types are tested together in the field. This approach could also enhance the tree breeding program, as is being evaluated in the current project.

Commercial mass production of SE spruces in Finland could be realized in a partnership, in which one partner is specialized in plant production in the laboratory and others in greenhouse and nursery cultivation. The potential benefits of this kind of partnership are being evaluated by engaging companies specialized either in mass-production of forest regeneration material or in lab propagation using bioreactors and other forms of automation.

The most critical biological step for practical application appears to be germination and early root development. A good root development during germination leads to plantlets with high probability to successfully acclimatize to normal plant growth conditions.

The most critical step overall for economy is the selection and one-by-one

handling of somatic embryos at the germination start. Attempts to automate SE production have been made for different conifer species and Norway spruce is one of them. So far a cost-effective method has not been presented. A development project for automation of the SE process for Norway spruce is presently run by SweTreeTechnologies to handle proliferation, maturation, one-by-one handling and sorting of embryos in liquid medium. Furthermore it plans to orient the embryos in proper germination positions in a manner currently done by seed sowing machines in forestry nurseries.

A few demonstration trials with SE plants have been growing in the field for the last fifteen years. No deviating performance has been observed when comparing SE plants with seedlings (Figure 5). However, the trials were too small in scale to draw safe conclusions.

Figure 5*. Clonal plot with Norway spruce SE plants.*

In 2009, SE clones were propagated and planted in field tests in a project established by Swedish forest companies and run by Skogforsk. The propagation followed the protocol described above using solid medium. The cell lines were cryopreserved and the traditional clonal test scheme will be followed. The first measurement of the field trials is approaching and aims to coincide with testing of newly developed automated large-scale propagation methods. An alternative to traditional clonal testing is to apply the family forestry concept where seeds from superior families, generated by top parents in the breeding program, are used as

starting material. With this concept cryopreservation can be avoided, but the resulting cell lines cannot be produced repeatedly. This "one-shot" propagation is dependent on correctly calculating the genetic diversity of the starting material in order to provide a sufficient margin for the genotype losses and to end up with a clone mixture that provides sufficient genetic diversity and reliable genetic gain. In this context, it is also important to point out that there will most certainly be a selection of clones with a high plant production capacity (many plants per amount of tissue). This will accentuate the gain and diversity aspects even more (Högberg 2012). The strong selection for SE ability raises questions concerning the selection effects in other traits. Three field trials with 48 half-sib families have been planted, each family represented by both seedlings and SE plants. Hopefully, this will show whether the selection affects important traits for forestry.

4. Legislation for vegetatively propagated forest regeneration material in Sweden and Finland

The Swedish legislation does not prescribe a minimum genetic diversity of vegetative propagated material, leaving the risk consideration to the forest owner. However, an area restriction is imposed saying that only a maximum 5 % of a forest estate can be planted with vegetatively propagated plants. Furthermore, the Swedish Board of Forestry recommends a status number (status number = corresponding census number of genotypes after considering unbalance and relatedness) of 20 (minimum) for plant materials entering the market which may reduce the number of cases where mixtures with a low number of clones are planted. In Finland there are no limitations in the area to be regenerated by clones like in Sweden, but regulations order how to test clones in the field, and how many plants can be produced per clone. To market SE plants as "tested", the superiority of the clonal material must have been demonstrated by comparative testing (at least 8-10 year field testing). In the category "qualified", the value of individual clones shall be established by experience or have been demonstrated by sufficiently prolonged experimentation (3-4 year field testing). The first field trial was established in the spring of 2015 using SE plants and standard seed lots. "Qualified" plants can be produced up to 1 million per clone and 4 million per family, but the production numbers of "tested" clones are not limited. Clonal plants originating from crossings made with already tested trees can be marketed as "mass propagated family". In this case, you have to have clones that represent most of the genetic variation inside the family. The current regulations originated in the 1990's and aimed to manage clonal materials produced by rooting of cuttings. Thus, practical applications of the legislation are currently being discussed with the authorities, and the potential need for revising the regulations and times for testing is being considered.

5. Ornamental forms of Norway spruce

In addition to forest regeneration material, research efforts in Finland have also been focused on the propagation of ornamental forms of Norway spruce. Landscaping is a growing business, both in private and public sectors in modern societies. In Northern Europe, the market now demands consistent and sustainable production of hardy, ornamental conifers. There are decorative forms of native conifers that are hardy and well adapted to harsh Northern conditions. The Natural Resources Institute Finland holds a collection of these naturally born forms found in forests and bred during recent years. To enhance commercial propagation of ornamental forms of spruce, several propagation methods are currently being tested (Figure 6) (Nikkanen et al. 2012). The National Resource Institute Finland has a special "propagation gardens" program for producing shoots of selected decorative genotypes to be propagated either as rooted cuttings or as grafts by commercial plant producers. (Nikkanen 2013).

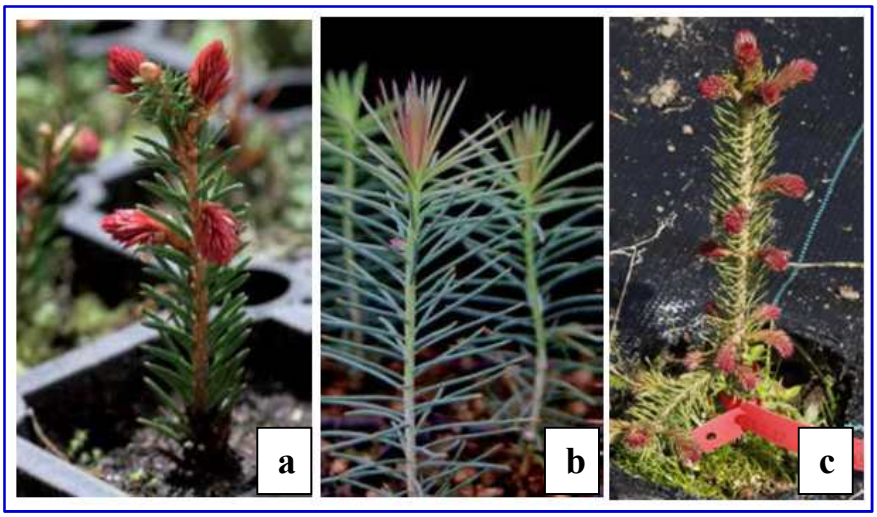

Figure 6. *A red-colored special form of spruce propagated as cuttings (a) or via tissue culture (b, c). Red needle color can be observed already in young tissue-cultured plants (b) but is better expressed in field-grown plants (c). Photo (a) Susanne Heiska (b) Tuija Aronen (c) Teijo Nikkanen*

In addition, SE has been applied for propagation of ornamental forms, using seed embryos originating from controlled crossings among selected forms as explants. So far, crossings have been made between narrow-crowned trees and forms having red needle color in new shoots. The produced SE lines have been planted in field tests for evaluation of their growth habit and other ornamental characteristics, and they will be available for commercial use in the near future. In the case of SE propagation, plant production has also been piloted together with a

company partner. Somatic embryogenesis technology was found to be transferrable to the private laboratory but the acclimation of germinated embryos to greenhouse conditions became a bottleneck in the facilities available for the company partner.

Issues concerning research innovations that enhance propagation and user rights and royalty issues for both natural mutants found and special forms created by breeding have to be settled. As a pilot effort, the application process for getting European plant breeders' rights for one special form of Norway spruce is currently under way.

Marketing and production of Norway spruce special forms is not restricted by legislation in Finland, thus the first step in commercializing SE material could start with them (Figure 7). Also, the price charged for special forms can be much higher than for reforestation plants.

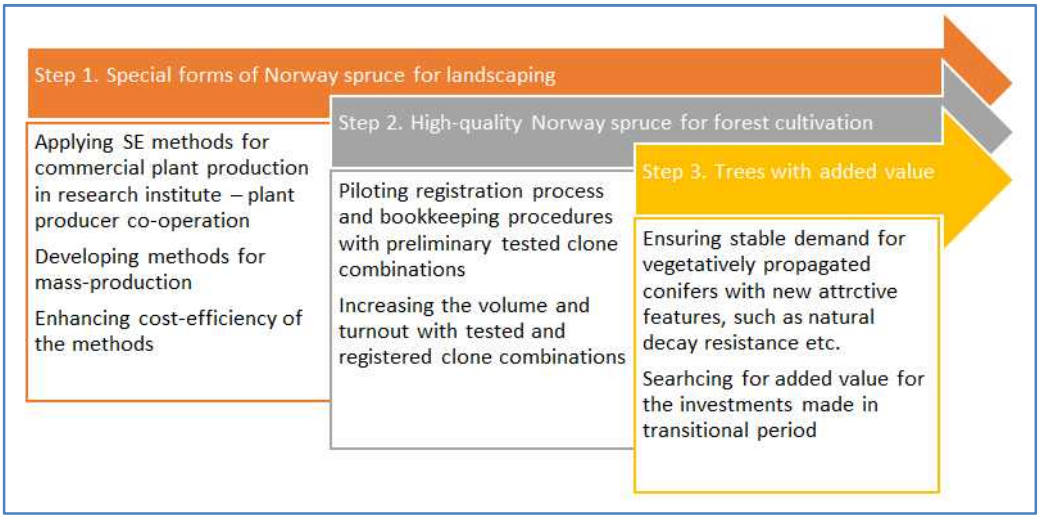

Figure 7. *Three steps of commercializing the Norway spruce SE plant production in Finland (Heiska 2013).*

Clonally propagated materials would greatly benefit, e.g., pathological and entomological studies in Nordic conifers. In the future, demand for regeneration material with valuable traits like fungi resistance is growing. New attractive traits of Norway spruce will be searched for.

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Vegetative propagation of larch species: somatic embryogenesis improvement towards its integration in breeding programs

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Abstract

Vegetative propagation of forest trees offers advantages to both tree breeders and the forest industry. In larch vegetative propagation has chronologically followed three major developments: (i) clonal propagation by cuttings; (ii) 'bulk' vegetative propagation by cuttings and (iii) somatic embryogenesis. The latter has potentially numerous applications such as the production of a large number of genetically improved plants and the amenability of embryogenic cultures to be stored in liquid nitrogen. In *Larix* sp. several improvements of the somatic embryogenesis protocol have been developed. Maturation conditions are now well enough refined to regenerate high quality somatic embryos that are highly similar to zygotic embryos in their anatomy, physiology and protein content. Among conifer species, somatic embryogenesis of *Larix* has become a model for its multiple uses; its integration in a breeding program is now undertaken for clonal propagation of improved material of hybrid larch *Larix* x *eurolepis*. Indeed, somatic embryogenesis assists breeding strategies by offering an alternative tool for at the same time accelerated production of plants for clonal testing and then for mass production; in addition, cryoconservation allows keeping material in a juvenile state and allows mass production of stored material at any time. This review describes the different methods of vegetative propagation of larch sp., in particular the advances in somatic embryogenesis and requirements for its integration into a breeding program.

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Keywords: Breeding; *Larix;* marker; maturation; embryo quality; protein content; zygotic embryo.

1. Larch species

The genus *Larix* is attractive to reforestation programs due to its fast growth, wide ecological plasticity and good wood quality (Gower and Richards 1990). Larch (*Larix* sp.) is one of the major components of coniferous forest in the Northern Hemisphere, where it is represented by around 10 species. In Europe, foresters are interested in the local species European larch (EL, *L. decidua*) -mostly growing in mountainous areas (mainly in the Alps, Tatras, Sudeten Mts)- and by Japanese larch (JP, *L. kaempferi*), a species from high elevation mountains in Honshu-Japan. Other species are also of interest in Northern Scandinavia where Sukaczewi and Siberian larches grow better. Due to their fast juvenile growth, fine architecture and wood properties (mechanical strength and durability), foresters have since long attempted to plant larches well-beyond their native range, in France, Germany and Poland in lowlands, but also in Northern and Western Europe. Success is mitigated. Failure of EL in Western Europe due to larch canker stressed the need for the proper choice of origins and this stimulated provenance research (IUFRO trials). Successful Japanese larch plantations were restricted to oceanic coastal areas where they do not suffer from summer drought. The recent explosion of *Phytophtora ramorum* in the UK and in Ireland in plantations of the very sensitive JL restricts from further plantation expansion (Webber et al. 2010). Problems due to canker (*Lachnellulla willkommii*) on EL (especially from alpine origins) plantations in France have recently raised concerns (Piou et al. 2013). A third larch taxa of prominent interest in Europe but also in North America is the interspecific hybrid between European and Japanese larches (*L.* x *eurolepis*). It is the correspondent in Eastern Asia of the *L. kaempferi* x *L. gmelini* hybrid. Discovered because spontaneous crossings had occurred at Dunkeld, Scotland at the beginning of the 20th century, *L.* x *eurolepis* has quickly attracted foresters but also breeders for its fast growth and benefits gained from complementary traits. Heterosis has often been advocated as an explanation for this superiority over its pure parents but it has only recently been demonstrated that this is in fact correct (Pâques et al. 2013). Danes created their first hybridization orchards in the nineteen forties (some are still producing commercial crops); they were followed by nearly all other European countries that established new seed orchards. The main drawback of open-pollination interspecific hybridization orchards is their unpredictable and changing rate of hybrid seed production from orchard to orchard and even from year to year. Recent results have shown hybrid rates from less than 20% to up to nearly 80% (Pâques et al. 2006). Mismatching of flower phenology

between EL and JL is the main cause. To overcome this instability, separate seed orchards for EL and JL have been established in France and supplemental pollination is used. High hybrid rates are observed (over 90-95%) but because of the extra cost for artificial pollination and of the actual low seed yield in larch compared to other *Pinaceae*, hybrid larch seed is produced at too high a cost. This seriously impedes hybrid larch deployment in plantation.

2. Vegetative propagation

Besides generative reproduction, vegetative mass-production of hybrid plants was attempted to circumvent weaknesses in orchard seed production: vegetative propagation maintains a high level of hybrid purity and attains stabilization of hybrid varieties, higher genetic gains, and better uniformity of crops. Vegetative propagation of larch has followed chronologically three major developments (Pâques et al. 2013): (i) clonal propagation by cuttings; (ii) 'bulk' vegetative propagation by cuttings and (iii) somatic embryogenesis (Figure 1).

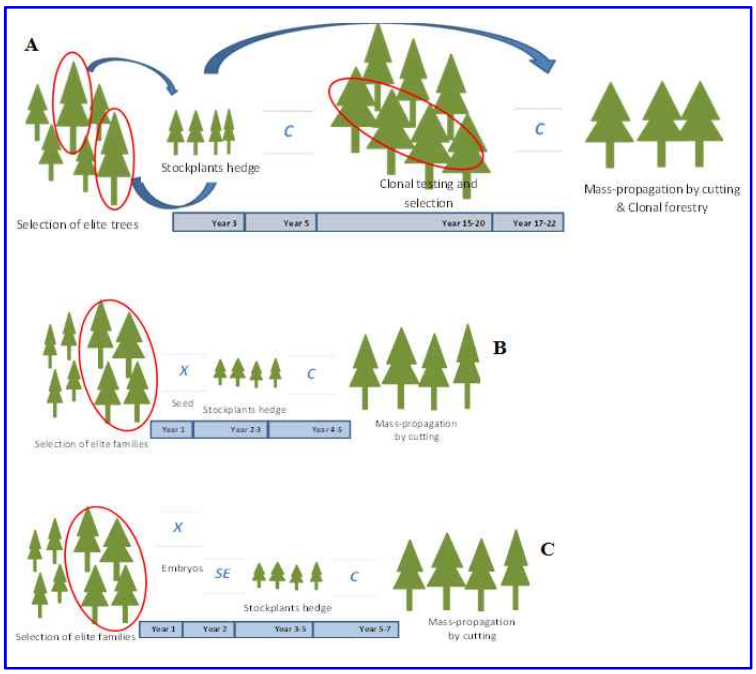

Figure 1. *Hybrid larch vegetative propagation scenarios:* ***A)*** *'clonal' propagation by cuttings,* ***B)*** *'bulk' propagation by cuttings and* ***C)*** *combined somatic embryogenesis and 'bulk' propagation by cuttings. C = cuttings; X = control crossing; SE = somatic embryogenesis*

Clonal propagation by cuttings followed the scenario developed for Norway spruce by Kleinschmit in Germany (Kleinschmit et al. 1973), with its different steps:

selection of young seedlings the in nursery; plantation of ortets in dedicated stockplant banks with appropriate cultivation management of trees; selection of best clones in clonal trials and finally mass-propagation for commercial plantation (clonal forestry). The rapid ageing of stockplants -whatever the technical attempts to maintain juvenility- resulting in low rooting rate and plant plagiotropism led to its commercial failure. Ageing of stockplants and its related problems were circumvented by the so-called 'bulk' propagation by cuttings (Pichon et al. 2001). In this scenario, young seedlings from selected hybrid full-sib families (produced by controlled crossings) are raised as stockplants to feed annual needs of cuttings up to the age where they become physiologically too old for proper rooting (3-4 years). Stockplants are thus regularly renewed over time. Successful results have been obtained in France up to the level of pilot-scale experimental production with nice growing plantations. But because of their higher cost (1.5-2 times that of sexually produced plants), rooted cuttings could hardly compete with seedlings in our context, and the transfer of this technique to commercial private nurseries has not been successful so far. In another forestry and economical context (namely in Québec), several hundreds of thousands of such cuttings are produced yearly and deployed in the forest [https://www.mffp.gouv.qc.ca/forets/semences/semences-production-techniques-resineuses-boutures.jsp]. A third alternative for vegetative propagation is through somatic embryogenesis.

3. Somatic embryogenesis in larch species

Somatic embryogenesis has become a method of choice for clonal propagation of forest trees, due to its high multiplication rate and the amenability of embryogenic cultures to cryogenic storage. This biotechnology constitutes a tool for rapid propagation of material from breeding programs (Lelu-Walter et al. 2013). Advances in conifer somatic embryogenesis in the last 10 years have been reviewed recently (Klimaszewska et al. 2015). In *Larix* somatic embryogenesis has been achieved in several species (Bonga et al. 1995). It was first reported in by Klimaszewska (1989a) for hybrid larches *Larix* x *eurolepis* Henry (*L. decidua* x *L. kaempferi*) and *Larix* x *marschlinsii* Coaz (*L. kaempferi* x *L. decidua*). Since then it has been obtained for European larch (*L. decidua* Mill.) (Cornu and Geoffrion 1990; von Aderkas et al. 1990; Szczygieł 2005), Japanese larch (*L. kaempferi* Gond.) (von Aderkas et al. 1990; Kim et al. 1999), western larch (*L. occidentalis* Nutt.) (Thompson and von Aderkas 1992), eastern larch (*L. laricina*, (Du Roi) K. Koch tamarack) (Klimaszewska et al. 1997) and more recently for Siberian larch (*L. sibirica*) (Tretiakova et al. 2012). Significant improvements in the somatic embryogenesis process have been obtained for Japanese larch (Kim and Moon 2007; Zhang et al. 2010) and hybrid larches (Lelu-Walter and Pâques 2009).

4. Somatic embryogenesis and other biotechnologies

Conifer somatic embryogenesis has potentially numerous applications. Indeed, this efficient method of plant regeneration constitutes a tool for research (study of gene function, reviewed by Trontin et al. (2015)) and for species improvement (production of a large number of genetically improved plants). Embryonal cultures (named embryonal masses in conifers) constitute an interesting material which can be used for other applications than plant regeneration (Figure 2). Efficient protocols have been developed to cryopreserve embryonal masses in liquid nitrogen. Cryopreservation offers real new perspectives for long-term conservation of the embryonal masses without loss of juvenility and their reactivation at any time (Park *et al.* 1998).

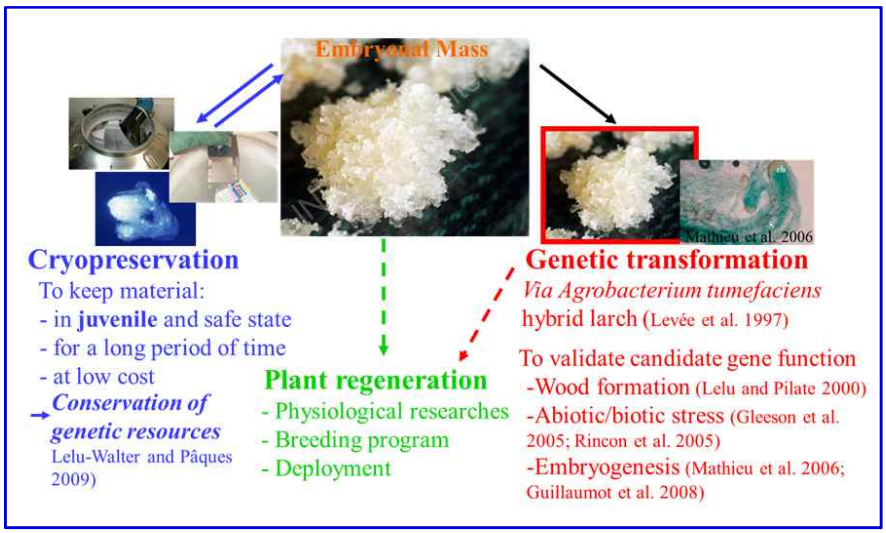

Figure 2. *Somatic embryogenesis of hybrid larches (Larix x eurolepis, L. x marschlinsii): for fundamental and applied research.*

Improvement of tree species using conventional plant breeding techniques is a long process and genetic transformation may help to speed it up. Genetic transformation was first obtained using a microprojectile bombardment protocol but first attempts in *Larix* sp. gave only rise to transient expression (Duchesne et al. 1993); subsequently transgenic tamaracks (*Larix laricina*) were regenerated but the transformation efficiency remained low (Klimaszewska et al. 1997). Transformation via *Agrobacterium tumefaciens* has been successfully achieved for the first time for hybrid larch (*Larix* x *marschlinsii*) giving rise to stable genetic transformation followed by plant regeneration (Levée et al. 1997). The improved procedure gave rise to high transformation rates, an average 108 ± 5.7 (mean ± 95% CI) and 154 ± 19.6 (mean ± 95% CI) resistant embryonal masses per gram fresh

weight (i.e. 15-16 transformation events per Petri dish) for SCOMT (Caffeic acid O-methyltransferase) and SCCR (Cinnamoyl Coenzyme A Reductase) respectively (Lelu and Pilate 2000). In hybrid larch, this efficient genetic transformation has been used to study gene function involved in wood formation (Lelu and Pilate 2000), in abiotic and biotic stress (Gleeson et al. 2005; Rincon et al. 2005) and more recently in embryogenesis (Mathieu et al. 2006; Guillaumot et al. 2008).

Finally we have to mention that embryonal masses have been used as a source of protoplasts (Korlach and Zoglauer 1995) in order to recover somatic embryos and plants as was successfully demonstrated for *Larix* x *eurolepis* (Klimaszeswka 1989b). The ultimate purpose was the fusion of protoplasts to produce hybrids with novel genetic combinations (Pattanavibool et al. 1998). Also of interest is the fact that *Larix decidua* plants have been regenerated from haploid megagametophyte tissue *in vitro* (see von Aderkas et al. in this book).

5. Technical aspects of somatic embryogenesis in larch sp.

5.1 Improvements of the different steps

Recovery of embryonal masses depends on the developmental stage of the zygotic embryo. Research carried out over the last two decades has proven that somatic embryogenesis is initiated most efficiently from immature zygotic embryos (Table 1). Indeed, there has been limited progress towards initiating

Table 1. *Induction of somatic embryogenesis in the Larix sp.*

Species	Explant type	Initiation (% max)	References
Larix decidua	Precotyledonay ZE	21	von Aderkas et al. 1990
	Immature ZE	36	Szczygieł K et al. 2007
	Mature ZE (stored seed)	5	Lelu et al. 1994c
Larix kaempferi	Precotyledonay ZE	17	von Aderkas et al. 1990
		67	Kim *et al.* 1999
Larix laricina	Precotyledonay ZE	44	Klimaszewska et al. 1997
Larix occidentalis	Immature coty. ZE	93	Thompson and von Aderkas 1992
Larix sibirica	Immature coty. ZE	18	Tretiakova et al. 2012
Larix x *eurolepis*	Precotyledonay ZE	15	Klimaszewska 1989a
(L. decidua x *L. kaempferi)*		78	Lelu-Walter and Pâques 2009
Larix x *marschlinsii*	Precotyledonay ZE	25	Klimaszewska 1989a
	Precotyledonay ZE	62	Lelu et al. 1994c
(L. kaempferi x *L. decidua)*	Immature coty. ZE	26	Lelu et al. 1994c
	Cotyledonary SE	98	Saly et al. 2002
	Needle (embling)	3	Lelu et al. 1994c

SE: somatic embryo; ZE: zygotic embryo ; emblings: plant regenerated from a somatic embryo.

somatic embryogenesis from mature seeds. For the hybrid, *Larix* x *marschlinsii*, needles from somatic plantlets (emblings) yielded embryonal masses at a lower frequency (3%) than did mature somatic embryos of the same genotype (83%, Lelu *et al.* 1994c). This decrease in embryogenic ability of the explants could be attributed to differentiation related to maturity of the explant (von Aderkas and Bonga 2000). So far, somatic embryogenesis from mature larch trees has not been obtained and remains a challenge despite attempts giving rise to embryo-like structures (Bonga 1996; 1997).

Once obtained, embryonal masses are transferred onto maintenance medium and sub-cultured every 2 weeks onto fresh medium in order to sustain their proliferation. Embryonal masses can be stored in liquid nitrogen (-196ºC). For *Larix* x *eurolepis* a cryopreservation method was first developed using a programmable freezer (Klimaszewska et al. 1992). Embryonal masses were pre-grown for 24h in medium with 0.4M sorbitol, treated with DMSO 10% before controlled cooling to -40°C. The vials were then submerged and stored in liquid nitrogen. A simplified cryopreservation method (no need for a programmable freezer) was subsequently developed (Lelu-Walter and Pâques 2009). Embryonal masses pre-grown for 24h in medium with 0.4M sucrose, treated with DMSO 10% were placed in a freezer at –80°C for 2 h (in Nalgene™ Cryo 1°C Freezing Containers). The vials were then stored in liquid nitrogen. Cryopreservation techniques have resulted in the recovery of all tested lines. The cryopreservation *per se* and its duration (at least up to 18 years) had no apparent effect on the yield of somatic embryos (Lelu-Walter and Pâques 2009).

For larch sp., as for other conifers, somatic embryo development was improved in the presence of abscisic acid (ABA), its concentration varying according to the species with 40-60µM being the most common concentration range used (Table 2). This resulted in synchronous development of cotyledonary somatic embryos without precocious germination (Lelu and Label 1994). ABA influenced tissue differentiation in larch (Gutmann et al. 1996) and promoted storage products such as lipid and protein accumulation in embryos (Gutmann et al. 1996; von Aderkas et al. 2002). The osmolarity of the culture medium is another important factor. In general the sugar content, either sucrose or maltose is increased (0.2M-0.4M, Table 2); for *L. laricina* and *L. sibirica* polyethylene glycol (PEG) has been added to the maturation medium (Klimaszewska et al. 1997; Tretiakova et al. 2012 respectively). More recently, somatic embryo development has been improved under reduced water availability, i.e. by a high gellan gum concentration, up to 0.8%, for both Japanese (Kim and Moon 2007) and hybrid larches, *Larix* x *eurolepis* and *Larix* x *marschlinsii* (Lelu-Walter and Pâques 2009). For the latter, the number of somatic embryos produced among the embryogenic lines tested (23) is ranging from 8 to over 1500 per g fresh weight of tissue (Lelu-Walter and

Pâques 2009), a phenomenon commonly observed with conifer species. When using the improved protocol for hybrid larch, 94% of the lines produced mature somatic embryos (Figure 3). Recovery of high quality somatic embryos resulted in high germination and plant formation frequencies (96 and 65% respectively, Lelu-Walter and Pâques 2009).

Table 2. *Somatic embryo maturation in the Larix sp: optimal conditions*

Species	ABA (µM)	Sugar (M) / PEG (g L^{-1}) *	Gellan gum (g)	Embryogenic Pot** N° coty SE/ g F W (*N° line tested*)	References
Larix decidua	60	Suc 0.2	4	226 (*6*)	Szczygieł K et al. 2007
Larix kaempferi	60	Mal 0.2	8	392 (*1*)	Kim and Moon 2007
Larix laricina	40	Suc 0.4/50	4	316 (*1*)	Klimaszewska et al. 1997
Larix occidentalis	0.025	nd	4	30 (*7*)	Thompson and von Aderkas 1992
Larix sibirica	121	Suc 0.1/100	4	400 (*7*)	Tretiakova et al. 2012
Larix x eurolepis	60	Suc 0.2	8	1566 (*23*)	Lelu-Walter and Pâques 2009
Larix x marschlinsii	60	Suc 0.2	4	403(*6*)	Lelu et al. 1994a
	60	Suc 0.2	8	2430 (*1*)	Lelu-Walter and Pâques 2009

* Suc : sucrose ; Mal : maltose; PEG: polyethylene glycol; ** Highest response. nd: non disponible.

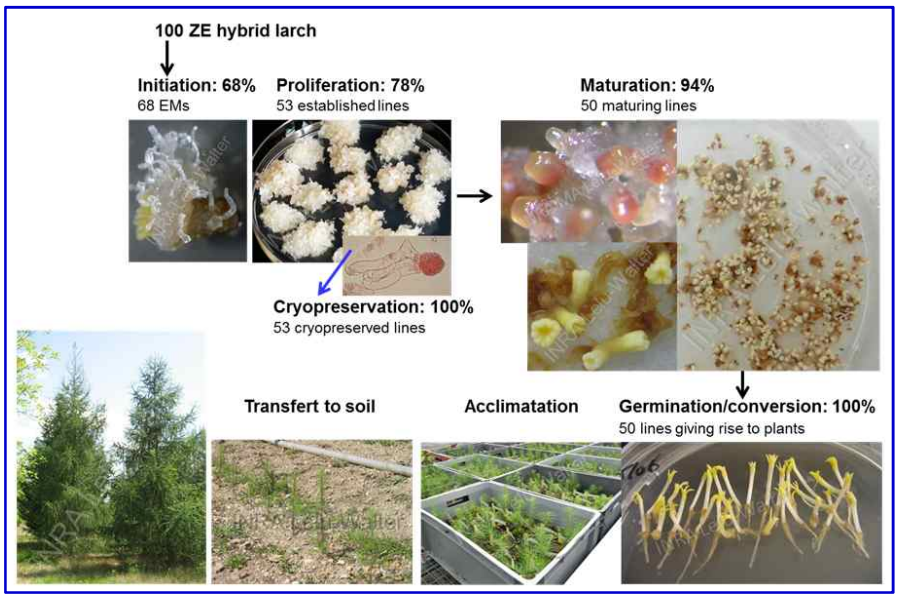

Figure 3. *Hybrid larch (Larix x eurolepis) variety REVE VERT somatic embryogenesis: performance at each step.*

Subsequently plants, during their vigorous growth phase, are directly transferred from the Petri dishes to a potting mix under shade house conditions (Lelu et al. 1994b). This simple procedure allowed high plantlet survival (79% after 8 months in the shade house) before their transfer to the nursery (Lelu-Walter and Pâques 2009).

Somatic embryogenesis has been successfully used to produce the new hybrid larch *Larix* x *eurolepis*, variety REVE-VERT (Figure3) registered in 2005 and produced by a cross of one European larch clone with a polymix of 12 Japanese larch clones (Lelu-Walter and Pâques 2009). Microsatellite markers are useful for searching paternity (checking the heredity), studying genetic diversity (Nardin et al. 2015) and structure of populations (Wagner 2013). For larch two multiplexes with 7 and 6 microsatellite markers have been developed (Wagner et al. 2012). These markers show a gradient of polymorphism from 4 to 15 alleles in our populations. These 13 markers are available to discriminate close individuals, as full-sib lines. For each embryogenic line obtained, the father has been identified without doubt using the 13 markers (Table 3).

5.2 Environmental conditions and physiological status of somatic embryos: are they similar to those of their zygotic counterpart?

Water availability is a key factor for pine somatic embryo maturation (see chapters in this book dealing with pine SE) and appeared to control the development of Japanese and hybrid larch somatic embryos. For hybrid larches, use of a high gellan gum concentration (8 g L^{-1}) in the maturation medium instead of a low one (4 g L^{-1})resulted in an improved physiological status of the somatic embryos considering their dry weight and water content (Teyssier et al. 2011). The accumulation of proteins has also been impacted. After 6 weeks of maturation somatic embryos that developed on 8 g L^{-1} medium had a higher protein content than their counterparts on the 4 g L^{-1}, a difference that was not significant after 8 weeks (Table 4). The identified differentially abundant proteins showed a reduction in abundance of enzymes involved in the glycolysis pathway and HSPs. Interestingly comparing proteomes, the identified proteins suggested that the embryos were more stressed when they were matured on 4 than on 8 g L^{-1} of gellan gum (Teyssier et al. 2011). These results strengthened the choice to mature hybrid larch somatic embryo with 8 g L^{-1} gellan gum.

In conifers the effect of abiotic factors, such as light during somatic embryo maturation, are not commonly studied. Although zygotic embryos develop in the dark, researchers generally specify light conditions for somatic embryogenesis but on what basis? In larch routine maturation protocols are realized either in light (Kim and Moon 2007) or in darkness (Lelu-Walter and Pâques 2009). Recently the effect of environmental conditions (light vs darkness)

has been investigated during somatic embryo maturation of hybrid larch. Morphogenesis of somatic embryos was not different in light or dark: they had a

Table 3.Paternity assignment of embryogenic line N23 of hybrid larch (Larix x *eurolepis) of the REVE VERT variety (Lelu-Walter and Pâques 2009): European larch clone (mother 1) crossed with a polymix of 12 Japanese larch clones (putative father A-L).*

	Identity	bcLK_189	bcLK_211	bcLK_228	bcLK_253	bcLk_263	Ld_101	Ld_30	Ld_31	Ld_42	Ld_45	Ld_50	Ld_56	Ld_58
	LOD score*	0.475	0.641	0.644	0.618	0.724	0.533	0.196	0.509	0.270	0.072	0.170	0.152	0.631
	PIC**	0.793	0.881	0.883	0.872	0.915	0.832	0.554	0.818	0.638	0.348	0.509	0.493	0.878
Mother	1	141 155	null 187	186 196	206 218	214 226	182 187	117 119	136 136	180 182	206 206	null 176	236 238	null 161
Progeny	N23***	154 155	187 205	null 196	214 218	214 216	187 198	117 117	null 136	173 182	206 212	null 169	231 236	141 161
Putative father	A	141 172	187 201	190 192	202 212	192 212	187 204	115 115	114 130	167 175	212 212	157 169	223 223	157 157
Putative father	B	141 172	207 211	null 186	220 222	190 210	187 190	115 117	null 134	167 167	212 212	169 169	231 231	141 143
Putative father	C	141 162	191 199	188 192	218 222	200 200	184 184	115 115	null null	173 175	210 212	157 169	231 231	131 147
Putative father	D	141 172	187 191	190 194	198 202	200 202	184 184	117 117	114 138	175 175	209 212	169 173	231 231	149 149
Putative father	E	154 172	189 201	null 194	218 220	188 200	184 192	115 115	null 132	173 173	212 212	157 157	231 231	141 143
Putative father	F	141 154	191 215	191 200	202 212	202 222	190 204	115 115	132 136	167 173	212 212	169 169	231 231	null null
Putative father	G	141 170	197 211	196 198	204 206	208 234	184 204	115 115	114 138	173 175	212 212	169 169	231 232	null null
Putative father	H	154 174	187 205	null 194	212 214	208 216	198 198	117 117	null 114	173 173	212 212	169 169	231 231	141 143
Putative father	I	141 162	187 191	190 196	206 222	192 212	198 204	null null	114 114	173 173	212 214	157 169	231 231	143 143
Putative father	J	141 141	195 215	193 196	202 210	206 208	187 192	115 115	131 136	173 173	212 212	157 169	231 231	137 145
Putative father	K	152 174	203 203	192 192	202 202	196 212	190 204	115 115	138 138	167 173	212 212	169 169	231 231	141 145
Putative father	L	148 158	205 207	184 184	218 222	196 218	189 200	119 119	134 136	167 175	212 212	157 169	null null	139 145

*LOD: Lod Score; **PIC: Mean polymorphic information content; *** putative father: H.

Table 4*: Quantitative analysis of total proteins in somatic embryos of hybrid larch during maturation according to gellan gum concentration (4 vs 8 g L^{-1}).*

Maturation time	Protein quantity*			
(weeks)	µg mg^{-1} FW		µg $unit^{-1}$	
	4 g L^{-1}	8 g L^{-1}	4 g L^{-1}	8 g L^{-1}
1	16,68 ± 2.7 a	19,45 ± 5.6 a	n.d.	n.d.
3	22,05 ± 6.3 a	39,53 ± 7.8 ab	7,37 ± 2.1 α	7,81 ± 1.5 α
6	66,74 ± 11.6 bc	108,54 ± 9.9 d	68,44 ± 7.9 β	80,45 ± 7.3 βγ
8	83,25 ± 28.9 cd	96,44 ± 23.7 cd	106,26 ± 37 γ	85,58 ± 21.0 βγ

* Values are means ± standard error of six repetitions. In each column, significant differences (P <0.05) in a multiple comparison of means are indicated by different letters. . FW: fresh weight; n.d.: not determined.

full set of organs, i.e., cotyledons, hypocotyl, and embryonal root cap (von Aderkas et al. 2015). However light had a negative effect on protein accumulation but a positive effect on phenol accumulation (quercetrin production, von Aderkas et al. 2015). In hybrid larch, maturation in darkness promoted a development of somatic embryos that was similar to that of zygotic embryos whereas light conditions affected protein and phenolic compound accumulation, especially in the embryonal root cap. Considering the accumulation pattern of storage reserves such as proteins, zygotic embryos and mature somatic embryos showed similarities in (i) their protein profile, (ii) the presence of storage proteins vicilin -like- and legumin -like protein, (iii) their total protein content levels (Teyssier et al. 2014). Thus an improved maturation protocol leads formation of mature somatic embryos that most closely resemble zygotic embryos in their morphology, anatomy, and protein contents. However, the difference between somatic and zygotic embryos in their plant growth regulator content (auxin, cytokinin, ABA) stressed that mature somatic embryos are produced in a fundamentally different physiological context than zygotic embryos (von Aderkas et al. 2001). Indeed somatic embryos lack a storage tissue and require non-physiological levels of ABA to mature properly.

5.3 When to harvest cotyledonary somatic embryos for germination?

Current maturation protocols produce “mature” somatic embryos that morphologically resemble zygotic embryos and are kept in the maturation phase during an arbitrary length of time before subsequent germination. Such an empirical approach does not give any information concerning the quality of somatic embryos needed to achieve maximal plant conversion rates because the potential vigor of the emblings is conditioned by the quality of the embryos (Terskikh et al. 2005; Businge et al. 2013). Therefore, we need to develop markers to assess the quality of somatic embryos. During maturation concomitantly to a dry weight increase, the amount of protein increased reaching a maximum at 8 weeks followed then by a decrease (Teyssier et al. 2014). At this developmental stage, called « late cotyledonary embryo stage», changes in the miRNAs expression appeared very important (Zhang et al. 2012). These proceed to inactivate transcripts involved in various maturation processes such as lignification and thickening of the cell wall, or in energy metabolism during embryogenesis. The activity of certain enzymes can also be used to track changes in metabolism during late embryogenesis and then to identify the molecular status of the embryos (Bailly et al. 2001). In hybrid larch while the activity of enzymes involved in cellular metabolism does not change between 6 and 9 weeks of maturation, the enzymes involved in the anti-oxidative protection of cells have a peak between 6 and 8 weeks of cultures (Table 5); in Japanese and Chinese larches, activities of these enzymes increased as embryos get older (Zhang et al. 2010; Zhao et al. 2015). A

recent proteomic study of the development of somatic embryos gave also novel insights into this process in larch and provided identification of new markers (Zhao et al. 2015).

***Table 5**: Change in enzyme activities in somatic embryos of hybrid larch during maturation on 8 g L^{-1} gellan gum. Results are expressed as the percentage of the activity measured at 6 weeks of maturation. Values are means of 3 measurements ± standard error.*

Enzyme activity	Functional group*	Week of maturation			
		6	7	8	9
enolase	cellular metab.	100 ± 31.0	57.55 ± 31.2	51,90 ± 9.0	79,94 ± 22.1
glyceraldehyde 3 phosphate dehydrogenase	cellular metab.	100 ± 44.3	127,79 ± 24.0	62,57 ± 5.0	66,82 ± 23.8
invertase	cellular metab.	100 ± 40.0	71,60 ± 21.3	204,53 ± 25.7	49,56 ± 19.6
ascorbate peroxydase	antiox. protect.	100 ± 20.3	100,58 ± 6.10	90,95 ± 10.0	101,07 ± 18.40
catalase	antiox. protect.	100 ± 13.2	112,40 ± 14.70	113,25 ± 9.80	83,20 ± 26.4
glutathion peroxydase	antiox. protect.	100 ± 3.4	116,21 ± 15.0	80,50 ± 32.3	113,55 ± 22.10
glutathion reductase	antiox. protect.	100 ± 38.8	190,97 ± 18.4	115,69 ± 28.0	183,05 ± 71.0
pyruvate kinase	antiox. protect.	100 ± 31.1	89,65 ± 27.4	75,36 ± 28.8	133,66 ± 23.8
superoxyde dismutase	antiox. protect.	100 ± 4.2	86,29 ± 11.2	77,14 ± 12.2	83,00 ± 15.6

* Cellular metab: cellular metabolism; antiox. protect.: antioxidative protection.

Maturation duration appeared to influence the subsequent step, i.e., somatic embryo germination. In hybrid larch, extension of the maturation period in the presence of ABA resulted in a significant decrease in both germination and plantlet frequencies (Label and Lelu 1994) that has been correlated with an increase of the *in planta* ABA content (Lelu and Label 1994; Label and Lelu 2000). Consequently a desiccation treatment (1 week at 4°C under a high relative humidity, 98% RH) was applied to cotyledonary somatic embryos matured in presence of ABA. After drying, germination had become synchronised (at a frequency between 89 and 100%) and plantlet recovery had improved (87%, Lelu et al. 1995). Desiccation treatment resulted in a decrease in endogenous ABA content of the somatic embryos (Dronne et al. 1997) while the final water content approximated that of stored seed (Lelu et al. 1995). Desiccation not only enhanced germination capacity but it may also be considered as a method of storage. More recently in Japanese larch gene regulation has been investigated during embryo dormancy and germination showing different expression patterns of miRNAs

(Zhang et al. 2013).

Therefore, it appears that when somatic embryos become cotyledonary, they no longer change morphologically, even though physiological and molecular changes are taking place with accumulation of protein energy reserves and an increase of dry weight and a reduction of the water content (Teyssier et al. 2011). This implies a modification of enzyme activities and of their regulation. For hybrid larch 8 week old somatic embryos seem to be at their maximum quality. Beyond the 8 weeks period phenomena of storage protein hydrolysis and oxidation quickly appear.

6. **Somatic embryogenesis as a tool for breeding programs**

All the progress obtained in hybrid larch SE, have contributed to the development of an improved procedure leading to the routine production of emblings. Among conifer species, somatic embryogenesis of *Larix* is becoming a model for its multiple uses and its integration into breeding programs is now undertaken for the clonal propagation of material improved by breeding of hybrid larch *Larix* x *eurolepis*, variety REVE-VERT (Lelu-Walter and Pâques 2009, Figure 3). We believe that somatic embryogenesis should influence breeding strategies by offering an alternative tool for accelerated mass-production of plants from improved genotypes (full-sib hybrid family). Another use could be to rely on somatic embryogenesis as an alternative to rooted cuttings to increase precision in progeny testing: vegetative propagation of hybrid full-sib families would allow testing them over a significantly greater number of sites for a better genetic evaluation and integration of GxE. Testing the same genotypes in contrasting environments will allow evaluating their phenotypic plasticity. Indeed, due to the low actual reproductive success in larch, the number of sibs per full-sib-family is usually much reduced, especially when one has to use factorial/diallel mating designs.

Another use of somatic embryogenesis in the context of breeding is exemplified by one objective of the Trees4Future Research infrastructure network (http://www.trees4future.eu/), namely to provide genetically stable genotype references to support breeders and genetic research and activities at various levels (e.g. genetic control to estimate genetic gains; provide contrasted genotypes for benchmarking subjective scoring-scales; help to establish pan-European plots to monitor impact of abiotic and biotic factors on tree characteristics). These genotypes can be delivered at the appropriate time through vegetative propagation by somatic embryogenesis thanks to cryopreservation.

Besides its high potential for mass-propagation, the main revolution of somatic embryogenesis in the context of vegetative propagation, is linked to the flexibility offered by the possibility to cryopreserve embryogenic lines and

maintain them juvenile (Figure 2). Concretely, this means that whereas lines (clones) are evaluated in the field for further selection some years or decades later, juvenile stock can be re-activated at any time for mass-propagation.

The strength of somatic embryogenesis is probably best optimized when a few elite-lines are mass-produced at a time. If clonal forestry has found favourable echoes in some countries, within a European forestry context more genetically diverse plantations are favoured, meaning the deployment of several tens of clones at a time. For hybrid larch in France (as for Sitka spruce in UK), full-sib-family forestry is considered to offer an acceptable genetic diversity level. Therefore, to alleviate the difficulty of somatic embryogenesis to handle many lines simultaneously, a combination of somatic embryogenesis and of bulk propagation by cuttings is a possible option. Juvenile stock plants regularly produced from cryopreserved elite-lines by somatic embryogenesis will be mass-propagated by cuttings.

Because genetic gains are often linked to the age at which selection of superior genotypes is possible, the perspective to vegetatively mass-propagate elite adult trees remains for breeders a dream and for biotechnologists a challenge (see chapter by Klimaszewska et al. in this book).

7. Conclusions and perspectives

Somatic embryo maturation is a complex process triggered by many factors. By combining the qualitative and quantitative results obtained with Japanese and hybrid larches, a maturation medium with ABA (60µM) plus 0.2M either sucrose or maltose and 8 g L^{-1} gellan gum is now routinely used to promote somatic embryo maturation. We found that the protein content is a reliable indicator of the physiological maturity of larch somatic embryos. When necessary, desiccation could be applied to somatic embryos in order to synchronize germination and to improve the germination and plantlet formation frequencies. For hybrid larch, the somatic embryogenesis process has been enough refined to be used on a large scale. The rapidity with which new material can be produced and the high potential for amplification make somatic embryogenesis a powerful and flexible tool for release of improved varieties. Full success nevertheless conditioned depends on several requirements posed by breeders. As with any other propagation system, breeders are firstly concerned by the integrity of the propagated, improved variety both in terms of its mean performance and of its genetic diversity. Firstly, as has been demonstrated for rooted cuttings, trees produced by somatic embryogenesis must show no detrimental abnormalities and must behave like or even better than seedlings in terms of growth, architecture, stability and maturation. Confirmation of growth behaviour comparable to that of seedlings is still needed. Tests are now in progress to compare the agronomic

behaviour of emblings with other material (seedlings, cuttings). Secondly, the genetic diversity of the material thus far released (Forest Reproductive Material) has to be enlarged and should not be limited to the few lines available today. A minimum number (10) of successful embryogenic lines that are genetically diverse, should be propagated to compose a multiclonal variety. Finally, practical questions related to logistic aspects (e.g., how many families and individuals per family can be practically managed during the different steps of the technique?) and to the cost of propagation should be properly addressed. As already mentioned above, somatic embryogenesis from adult trees remains a challenge which forces us to try to achieve a better understanding of the molecular biology of embryo development (Vestman et al. 2011, Morel et al. 2014; Yakovlev et al. 2014).

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9. Authors contribution.

MALW conceived in the design of the study and its coordination, and drafted the manuscript. CT carried out the protein analysis and drafted the manuscript. LP conceived the design of the study and drafted the manuscript. VG carried out the paternity assignment analysis and helped to draft the manuscript. All authors read and approved the final manuscript.

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Prospects for new variety deployment through somatic embryogenesis in maritime pine

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Abstract

Maritime pine is a major species in Europe, especially in France, Portugal and Spain. This species has been subjected to advanced forestry and genetic breeding since the early sixties in France. However, there are strong limitations to genetic betterment of the species by traditional improvement methods because of a long generation time, high genetic load as well as a high genetic redundancy within the breeding population. Sudden and drastic socio-economic and environmental changes in recent years would need a significant paradigm shift in current breeding technology to deliver suitably tested tree varieties in plantation forestry, i.e., there is a need for multi-varietal forestry targeting over a wide range of end-products through various sylvicultural regimes. Field comparison of vegetative propagules is a key towards individual clonal selection and efficient capture of the best genetic stocks. Highly efficient clonal propagation technology is also required for scaling up production of improved varieties. Somatic embryogenesis is considered as the key technology to fulfil such requirements in maritime pine. The species is characterized by increased recalcitrance to vegetative propagation through conventional cuttings as trees are reaching their adult vegetative or reproductive phase. Somatic embryogenesis initiation from mature trees is still challenging in conifers. Therefore, the approach developed for maritime pine is postponed propagation of tested trees by combining somatic embryogenesis initiation from immature zygotic embryos and stable cryopreservation of juvenile embryogenic tissue. This review describes recent achievements and challenges towards efficient

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somatic embryogenesis as a key technology for multi-varietal forestry with maritime pine.

Keywords: Acclimatization; embryo quality; emblings; field test; germination; initiation; maturation; *Pinus pinaster*; zygotic embryo

1. Introduction

Maritime pine (*Pinus pinaster* Ait.) is an industrial heavyweight in Europe, especially in France, Portugal and Spain. More than a quarter of European forestry resources are located in France (1.05 Mha, e-IGN 2015) and this species is accounting each year for more than 25% of the national softwood timber (3.7 Mm^3) and pulpwood (2.8 Mm^3) production. Almost all this production (6.5 Mm^3 in 2013) is obtained from intensively managed plantation forests located in the Aquitania region (0.82 Mha). The mean productivity of this species can be high compared to that of other conifers in France (11.8 m^3/ha/year) and about 70% of the marketed harvest is mechanized. More than 38 000 workers are currently employed in this industry (16.5% of the national forest sector) with an annual turnover of around 2.5 billion Euros (36% as exports, INSEE 2006). « *The number of salaried employees, its role in territorial development and cohesiveness, its large contribution to Aquitania's GDP confer to the maritime pine market a leading part in socio-economic development* » (translated quote, Regional Aquitania Council). Two heavy storms recently affected the Aquitania forest (1999 and 2009) and resulted in ca. 300 000 ha being completely cleared, i.e., 30% of the resource plantations were down and 61 Mm^3 of wood (6 years harvesting) were undervalued (IFN 2009). Rapid reforestation, together with increasing forest resilience to major biotic and abiotic risks are thus major objectives for the forthcoming decades (GIP EcoFor, 2010, www.gip-ecofor.org). If harvesting is maintained at up to 95% of the normal annual growth of the maritime pine forest, as observed before the 2009 storm, a severe maritime pine resource shortage is anticipated by 2020.

In this context, the interest for genetically improved varieties has been considerably reinforced in France and there is a high need for improved seeds (ca. 3t/year). As an indicator of the current reforestation program, the production of seedling plants (>90% improved varieties) reached ca. 45 million in 2013 and 2014. French breeding programs launched in the early sixties by FCBA and INRA were federated in 1995 in a joined initiative called “Maritime Pine for the Future” and involved all other major forest actors in France (CPFA, CRPF, ONF). Up to 15% genetic gain was achieved for volume and straightness in the first and second generation varieties (Figure 1). The rather long generation time of this species (15 years) resulted in significant inertia of the conventional breeding program.

Optimizing genetic gain per breeding selection cycle, taking also into consideration

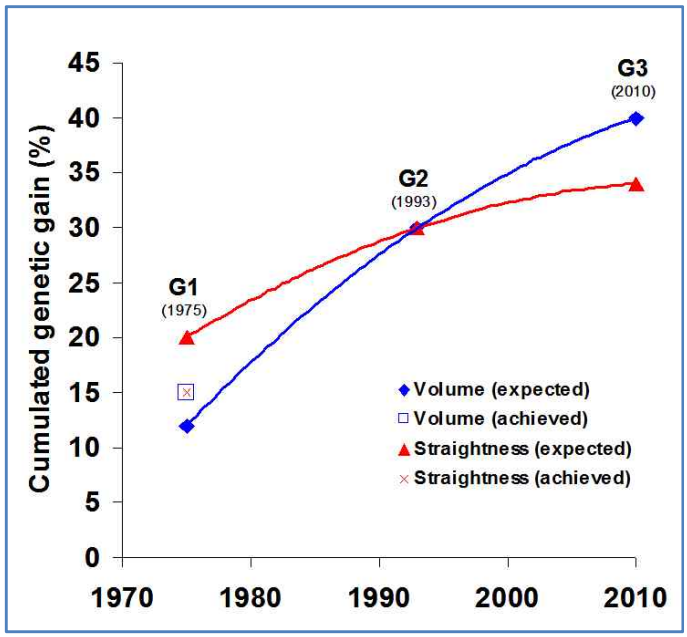

Figure 1. *Cumulated genetic gain (%) expected from first- (G1), second- (G2) or third-generation varieties in maritime pine. Data are expected or achieved genetic gain at age 15 years for growth volume and stem straightness (from Alazard and Raffin 2002). The production of seed orchards started at the date indicated in brackets.*

genetic diversity, is now considered as a requisite for new variety design and deployment. Full productivity of improved-seed orchards for commercial purposes is currently achieved every 17-18 years (Figure 1) and this turnover time is now expected to decrease as a result of improved growth of selected genitors (4^{th} generation expected by 2020). Nevertheless lower genetic gains (10%) are expected by the third round of selection (Figure 1) owing to genetic redundancy within breeding populations. The genetic base of the 4^{th} seed orchard generation has been considerably enlarged to take this phenomenon into consideration (Alazard and Raffin 2002), but sudden and drastic changes in market requirements and environmental constraints in recent years (climate change, abiotic stresses, pests) would need a significant paradigm shift in current breeding technology to deliver suitable tested tree varieties in plantation forestry, i.e., multi-varietal forestry has to be targeted over a wide range of end-products through various sylvicultural regimes. Multi-varietal forestry is simply defined as the deployment of tested tree varieties in plantation forestry (Park 2004; see also Klimaszewska et al. 2007).

Biotechnology offers new opportunities for species improvement, i.e., variety design, conservation and deployment. Advances in genomic- and/or clonally-assisted selection of elite trees would facilitate efficient capture of the best genetic stocks by promoting individual vs. familial selection, i.e., increased selection efficiency of elite genotypes in breeding populations. This can be achieved through accurate and early detection of genes (marker-assisted selection) or genome-wide markers (genomic selection) associated to valuable traits, as well as through field comparison of vegetative propagules (clonal tests). Genomics and particularly genomic selection offers considerable advantages for breeding forest trees over the next decades (Plomion et al. 2015). Efficient clonal propagation would also greatly facilitate deployment of selected varieties. Synergies are, therefore, expected between conventional breeding, early selection and powerful methods for clonal propagation of elite genotypes to implement innovative, multi-varietal forestry in conifers (El-Kassaby and Klápště 2015). Optimized and balanced genetic gain and diversity together with greater flexibility in variety deployment are expected from multi-varietal forestry in pines (Weng et al. 2011; Klimaszewska et al. 2007).

With regard to maritime pine, effective vegetative propagation through conventional cuttings or *in vitro* micropropagation for selection among trees in their adult vegetative or reproductive phase, proved to be difficult to achieve and/or too expensive for being implemented into breeding programs (Trontin et al. 2004; De Diego et al. 2008). In contrast somatic embryogenesis has promising attributes to scale up production of improved varieties in conifers (reviewed in Lelu-Walter et al. 2013; Klimaszewska et al. 2015), particularly in pines (Klimaszewska et al. 2007). Somatic embryogenesis initiation from vegetative explants of adult selected trees has still to be demonstrated for maritime pine as well as for other conifers (see Trontin et al. in this book). Therefore, the ongoing strategy developed for maritime pine throughout Europe involved the postponed propagation of tested trees. This involves large scale regeneration of plants from embryonal masses (EMs) that have been cryopreserved in juvenile form, i.e., these masses were initiated from dissected zygotic embryo (ZE) from immature seed. Somatic embryogenesis initiation was first reported for maritime pine by Jarlet-Hugues (1989) with some insights into both EM initiation and multiplication by Bercetche and Pâques (1995). Full plant regeneration from propagated EM became effective 10 years later (Lelu et al. 1999). Extensive research to improve this process has since been undertaken mainly in France (Ramarosandratana et al. 2001a,b; Jordy and Favre 2003; Breton et al. 2005, 2006; Lelu-Walter et al. 2006; Park et al. 2006; Pérez-Rodríguez et al. 2006; Klimaszewska et al. 2009; Trontin et al. 2011; Morel et al. 2014a,b), but also in Portugal (Miguel et al. 2004; Marum et al. 2009a) and in Spain (Humánez et al. 2012; Álvarez et al. 2013). A synthesis of methodological and scientific aspects of progress up to 2005-2006 can be found in Harvengt (2005)

and Klimaszewska et al. (2007). After more than 20 years of continuous effort, the technology has been sufficiently refined to allow high genotype capture at the initiation step (77%, Park et al. 2006) and to achieve production of somatic embryos (SE) and somatic seedlings (emblings) that have been tested in field tests at FCBA for more than 15 years. Implementation of the technology into multi-varietal forestry would require both high-quality cotyledonary SEs similar to the seedling standard and cost-effective solutions for industrial application of somatic embryogenesis. In this chapter we present recent and major achievements as well as prospects towards procuring high-quality somatic seedlings of maritime pine, particularly at the induction and maturation steps. We also highlight the useful and practical issue of combining somatic embryogenesis with new genomics applications, reverse genetics and cryopreservation. We finally report on some of our first results from field tests indicating that application of somatic embryogenesis in association with both breeding (clonal and varietal tests) and variety deployment should be profitable to the economy of maritime pine forestry.

2. Somatic embryogenesis as a critical enabling biotechnology in maritime pine

Somatic embryogenesis is the core technology in maritime pine to support cryopreservation of genetic resources and genetic, epigenetic, and reverse genetic studies through genetic transformation. It has recently been demonstrated that somatic embryogenesis provides a true *in vitro* model that mimics zygotic embryo development in maritime pine up to the cotyledonary stage (Morel et al. 2014b).

2.1 Somatic embryogenesis vs. reverse genetics

Reverse genetics, defined as ectopic expression or silencing of candidate genes in selected genotypes, has become an indispensable research tool for functional dissection of traits of interest in forest trees. In maritime pine as in other conifers, a long generation time and long life span, high genetic loads as well as high genetic redundancy, are major obstacles to follow standard genetic practices, including association genetics. Validating marker associations with specific properties before transfer into breeding selection models is still challenging. Stable *Agrobacterium*-mediated genetic transformation of maritime pine EMs and transgenic somatic plant regeneration was first reported by Trontin et al. (2002) and further developed in both France (FCBA/INRA collaboration) and at IBET/ITQB in Portugal (reviewed in Trontin et al. 2007). Very similar protocols were also recently developed in Spain (Álvarez and Ordás 2013). The common protocol used by FCBA/INRA has been sufficiently refined to envisage practical application in reverse genetics as an attractive complement to association studies.

The method is based on phosphinothricin selection of reference genotype and has been transferred to 5 teams in Europe (Universities of Málaga, Alcalá, and Valencia in Spain; IBET/ITQB in Portugal; Humboldt University of Berlin in Germany, Trontin et al. 2013) in support of the multinational "Sustainpine" project (http://www.scbi.uma.es/sustainpine/). The maritime pine toolbox for genetic transformation has been further improved by this project. This is one of the greatest efforts worldwide for gene functional analysis in conifers. Transgenic embryogenic lines and somatic plants are currently being investigated for various overexpression and/or RNAi constructs (ihpRNA strategy, intron-spliced hairpin RNA), targeting 39 genes involved in wood formation, carbon and nitrogen metabolisms, ammonium regulation, stress resistance (drought and nutrition) as well as embryogenesis and plant development (de Vega-Bartol et al. 2013a; Hassani et al. 2013; Carneros et al. 2014; Mendoza-Poudereux et al. 2014; Trontin et al. 2014).

2.2 Somatic embryogenesis vs. genomics

In the context of climate change and induced biotic and abiotic stresses, genomics for forest trees is developing rapidly, showing significant achievements and providing new perspectives for breeding (Plomion et al. 2015). In maritime pine much is expected from the full genome sequence that should be available at the end of the European project "ProCoGen" (http://www.procogen.eu/). A reference transcriptome has already been established (Canales et al. 2014) from various tree sources, experimental conditions and tissues, including EMs and cotyledonary SEs. This study provided a large catalogue of more than 26 000 unique transcripts and also a collection of 9641 full-length cDNAs (FLcDNAs). As stated above, the large availability of FLcDNAs paved the way for the collaborative, multinational application of reverse genetics towards functional dissection of traits of economic and ecological interest in maritime pine. Embryogenesis-related genes are among key genes for future application in tree improvement and new variety deployment in conifers (Plomion et al. 2015; detailed review in Trontin et al. 2015). Reference transcriptome (Canales et al. 2014) but also genome-wide transcriptomics (de Vega-Bartol et al. 2013b; Morel et al. 2014a) and proteomic profiling (Morel et al. 2014a,b) of both SEs and ZEs have already provided significant clues for a better knowledge of the molecular aspects of embryo development. In particular, specific genes and master regulators such as transcription factors and genes involved in the epigenetic complex for regulation of gene expression have been found expressed in maritime pine (Gonçalves et al. 2007; de Vega-Bartol et al. 2013b; Morel et al. 2014a). This complex and still fragmented knowledge started to deliver accurate marker genes and new molecular tools to refine somatic embryogenesis in conifers and check for (epi)somaclonal variation throughout the process (reviewed in Miguel et al., in this book). Maritime

pine expression studies of *glutamine synthase* (*GS*) isoform genes, expressed in either photosynthetic (*GS1a*) or vascular tissue (*GS1b*), indicated that these genes could serve as indicators of early differentiation of procambial cells (*GS1b*) or abnormal, early germination of cotyledonary SEs (*GS1a*) that apparently did not reach full maturity (Pérez-Rodríguez et al. 2006). Similarly, in a combined transcriptomic and proteomic study of the molecular responses promoting SE development on maturation medium, Morel et al. (2014a) proposed that *germin-like protein* and *ubiquitin-protein ligase* genes could be used as predictive markers of embryo development (see below part 4) as early as after 1 week on maturation medium (out of 12 weeks to complete maturation). Marker genes could be of great interest to avoid unnecessary expenses associated with sub-optimal culture conditions.

2.3 Somatic embryogenesis vs. cryopreservation

Crypreservation of initiated embryogenic lines is effective in maritime pine using either a programmable freezer with slow cooling (Harvengt 2005) or Nalgene Cryo 1°C Freezing Containers (Marum et al. 2004; Lelu-Walter et al. 2006). Density of the embryogenic suspension was shown to affect recovery of cryopreserved lines and the optimal concentration was estimated to be 250 mg mL^{-1} (Marum et al. 2004). Pre-treatment of the embryogenic suspension is performed in presence of either sucrose 0.5 M (Harvengt 2005), maltose 0.4 M (Marum et al. 2004) or sorbitol 0.4 M (Lelu-Walter et al. 2006). In general a cryoprotectant such as DSMO (dimethyl sulfoxide) is added to the suspension at a final concentration in the range 5-7.5%. Recovery of cryopreserved lines was improved when DMSO was combined with polyethylene glycol (PEG 10%, Marum et al. 2004). A simplified, cost effective method has been developed at INRA (Lelu-Walter et al. 2006). The method involved a single-step pre-treatment of EM with sorbitol and DMSO, slow cooling with no need for a programmable freezer and rapid recovery on filter paper discs. At FCBA a cryo-collection of over 1850 embryogenic lines has been established from 94 families, especially 17 elite families from the FCBA/INRA breeding program developed within the framework of the joined initiative “Maritime Pine for the Future”. The first lines were cryopreserved in the late 1990s and are still used for experiments without any apparent loss in regenerative capacity nor evidence for any (epi)somaclonal variation related to the cryopreservation process. However, a systematic loss of maturation ability within 6 months post reactivation from the cryopreserved stock is observed (Breton et al. 2006). At FCBA virtually all lines are easily recovered from the cryopreserved stock within 2-4 weeks.

3. Somatic embryogenesis in *Pinus pinaster*

3.1 Induction step

Somatic embryogenesis initiation is currently achieved from immature ZEs, either dissected or kept in place within the whole megagametophyte (Table 1). Using dissected immature ZEs allows to choose their developmental stage for culture more carefully. The ZE reaches its highest capacity for initiating somatic embryogenesis in the early stages of late embryogeny, i.e., when it attains dominance and up to the precotyledonary stage. Interestingly, secondary somatic embryogenesis has also been successfully initiated from cotyledonary SEs at high frequency (up to 82%, Klimaszewska et al. 2009). In contrast, the embryogenic potential of ZEs significantly decreases at the early cotyledonary and cotyledonary stages, and fully mature ZEs are no longer responsive in maritime pine as in most other pine species. Similarly somatic embryogenesis from older maritime pine trees in their adult vegetative or reproductive phase remains challenging. Some recent and promising results suggested that EM-like tissue could be obtained from primordial shoot bud explants from mature trees up to 34 years old but these findings need to be further investigated (see Trontin et al. in this book).

Table 1. *Initiation rate of somatic embryogenesis in P. pinaster from different explants and developmental stages.*

Explant type and dev. stage	Seed origin	Basal medium	PGR type	Initiation response (% range)	References
Dissected ZE					
Pre cotyledonary	3 open pollinated trees	mLV	2,4D+BA	26-40	Lelu et al. (1999)
		mLV	No PGR	0-13	
	9 full-sib families	mLV	2,4D+BA	65-100	Lelu-Walter et al. (2006)
	4 open pollinated + 6 controlled crosses	mLV	CPPU	56-75	Park et al. (2006)
Un-dissected ZE					
Dominant ZE	5 open pollinated trees	mLV	2,4D+BA	0-82	Humanez et al. (2012)
Pre cotyledonary	20 open pollinated trees	DCR	2,4D+BA	5-49	Miguel et al. (2004)
Isolated SE					
Cotyledonary	5 embryogenic lines	mLV	2,4D+BA	13-82	Klimaszewska et al. (2009)

BA: benzyladenine; 2,4-D: 2,4-dichlorophenoxyacetic acid; PGR: plant growth regulator; SE: somatic embryo; ZE: zygotic embryo

Two different basal media are commonly used at the induction step depending on the laboratory and the plant material origin or culture conditions. Open-pollinated seed families from Portugal had the highest initiation rate (5-49%, Miguel et al. 2004) on basal DCR medium (Gupta and Durzan 1985). Similarly, in

France, a modified DCR basal medium (mDCR, Breton et al. 2005) containing DCR macroelements and Murashige and Skoog (1962) micronutrients was routinely used to initiate somatic embryogenesis from 31 full-sib and 9 open-pollinated seed families but at a quite low mean initiation rate of 23.3% (Figure 2). In contrast full-sib families from INRA, France (Lelu-Walter et al. 2006) and open-pollinated seed families from Spain (Humánez et al. 2012) had the highest initiation frequencies (82-100%) on a modified Litvay medium (Litvay et al. 1985) that contained half-strength macroelements except iron and EDTA (mLV, Klimaszewska et al. 2000). A comparison of both mDCR and mLV media has been undertaken at FCBA. Considering a large selection of full-sib and open-pollinated families, mLV gave consistently higher initiation rates than mDCR over various initiation efforts from 2000 to 2009 (67.5 vs 23.3%, Figure 2).

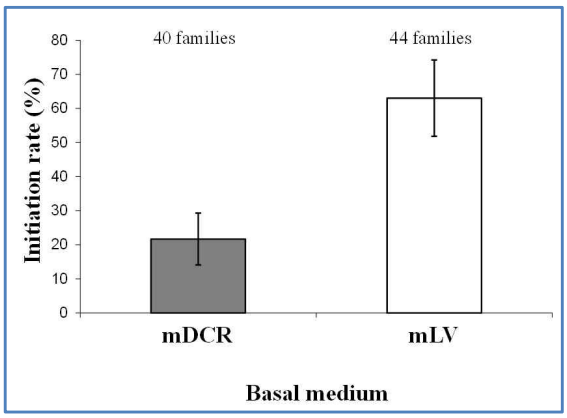

Figure 2. *Initiation rate of somatic embryogenesis from various controlled- and open-seed families in maritime pine as a function of basal medium: mDCR (modified from Gupta and Durzan 1985) and mLV (modified from Litvay et al. 1985). Bars represent 95% confidence limits. Data have been computed from various initiation efforts at FCBA from 2000 to 2009. N = 13758 (mDCR) or 8841 (mLV).*

Most induction media are supplemented with a combination of the auxin 2,4-dichlorophenoxyacetic acid (2,4-D) at 9.0-13.5 µM and the cytokinin benzyladenine (BA) at 2.2-4.4 µM (Miguel et al. 2004; Harvengt 2005; Lelu-Walter et al. 2006; Humánez et al. 2012). Somatic embryogenesis could also be initiated without plant growth regulator (PGR) but the initiation frequency remained comparatively low (0-13%, Lelu et al. 1999). More recently, induction medium was supplemented with the phenylurea CPPU (a potent cytokinin) at 4.0 µM instead of 2,4-D and BA. In combination with Litvay basal salts (mLV), CPPU resulted in significantly improved mean initiation rates compared to the one obtained with 2,4-D and BA (77 *vs.* 34%, Park et al. 2006). This result was

confirmed and strengthened during subsequent initiation efforts at FCBA in 2005, 2006 (Figure 3) and 2007, 2008 (Figure 4). Different mLV-based media and control mDCR were supplemented with various combinations and concentrations of PGR, either 2,4-D and BA or CPPU (Table 2).

***Table 2**. Formulation of mLV- and mDCR-based media used for initiation experiments in P. pinaster at FCBA: fixed and variable components (see Figure 3, 4).*

			Traces (mg l^{-1})		PGR (µM)								
Basal media[a]	Vitamins	Sucrose (g l^{-1})	$NiCl_2$	$CoCl_2$	No PGR	2,4-D 2.2 BA 2.3	2,4-D 9.0 BA 4.4	2,4-D 13.5 BA 2.2	CPPU 0.5	CPPU 1	CPPU 2	CPPU 4	CPPU 6
mLV	LV 1X	30	0	0.125		LV3	LV1,2[b]	LV5				LV4	
mLV	LV 10X	10	0	0.125	LV6,7[b]		LV16		LV8	LV9	LV10	LV11, 12, 14[b]	LV13
mLV	LV 1X	1		0.125								LV17	
mLV	LV 10X	10	0.72	0.125								LV15	
mDCR	DCR 1X	30	0	0.025	DCR1,2[b]			DCR3,4[b]					
mDCR	DCR 1X	30	0.72	0.125								DCR6	

[a]Fixed components; mLV modified from Litvay et al. (1985) = macro LV 0.5X, micro LV 1X, 100 mg l^{-1} meso-inositol, 1 g l^{-1} casein hydrolysate, 0.5 g l^{-1} glutamine, pH 5.8; mDCR modified from Gupta and Durzan (1985) = macro DCR 1X, micro MS 1X, 100 mg l^{-1} meso-inositol, 0.5 g l^{-1} casein hydrolysate, 0.25 g l^{-1} glutamine, pH 5.8; [b]Same medium but different initiation procedures

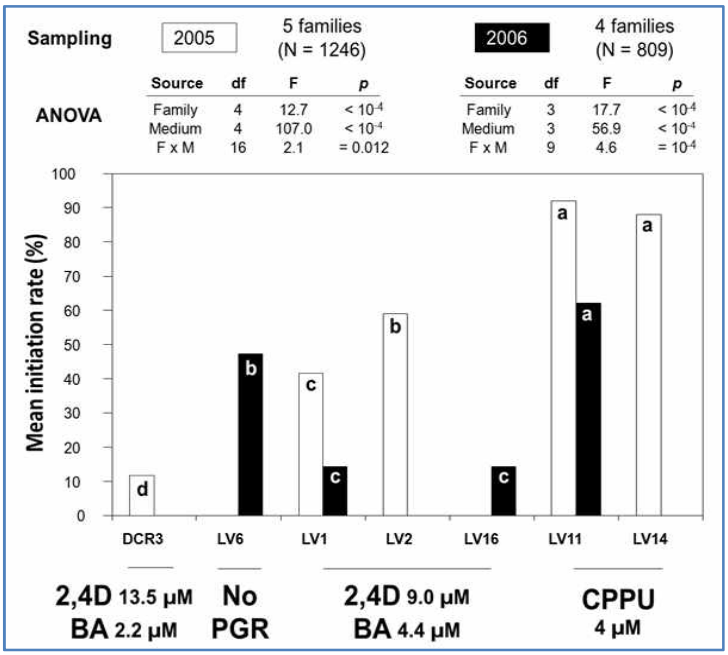

***Figure 3.** Mean initiation rate (%) of somatic embryogenesis in maritime pine with different mLV-based media (LV1,2,6,11,14,16, see Table 2) or control mDCR media (DCR3, Table 2) supplemented with different combinations of PGR, either 2,4-D/BA or CPPU. Sampling and ANOVA are indicated for each initiation effort (2005, 2006) involving 4-5 families. For each year, significant variations between means (SNK tests, α=0.05) are indicated by different letters.*

The "Family" and "Medium" effects were always significant (ANOVA) together with the interaction "Family x Medium" (excepted in 2007). Up to 92% mean initiation rates (2005, Figure 3) were obtained using the mLV/CPPU (4.0 µM) medium with only moderate variation between trial years (62-92%, Figures 3, 4). The optimal CPPU concentration was determined (Figure 4) and it was found that an up to fourfold reduction of the original dose (1.0 µM vs. 4.0 µM used by Park et al. 2006) could be used without any significant decrease in initiation rate.

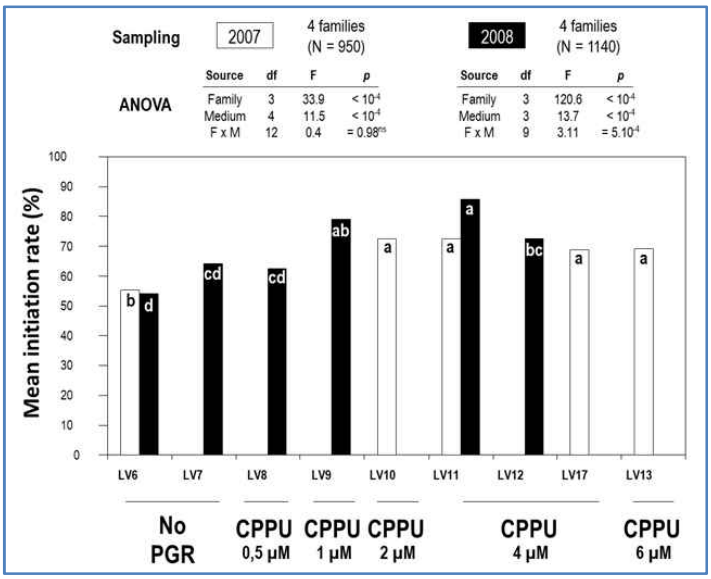

Figure 4. *Mean initiation rate (%) of somatic embryogenesis in maritime pine with different mLV-based media (LV6-13, 17, see Table 2) supplemented with increasing concentration of CPPU. Sampling and ANOVA are indicated for each initiation effort (2007, 2008) involving 4 families. For each year, significant variations between means (SNK tests, α=0.05) are indicated by different letters.*

Interestingly, high initiation rates were also obtained in the case of some families by reducing the exposure time of dissected ZEs on mLV/CPPU (4.0 µM) from 8 weeks (LV11 medium) to only one week (LV12, Figure 4). Initiation rates remained high (47-55%, Figures 3, 4) when initiation media were deprived of CPPU, thus confirming the usefulness of mLV for somatic embryogenesis initiation in maritime pine compared to mDCR. Substituting CPPU (4.0 µM) for the 2,4-D (9.0 µM) and BA (4.4 µM) PGRs in combination with 10 (LV16) or 30 g L^{-1} sucrose (LV1, LV2) significantly reduced the initiation rate (Figure 3). In contrast, CPPU did not have any positive effect in combination with mDCR-based media (data not shown).

In conclusion, CPPU significantly increased the initiation rate to reach a maximum of 65-85% in the range 1-4 µM. We concluded that CPPU at 1 µM was optimal in maritime pine. Family effect was confirmed to be highly significant but

with mean genotype capture within the family of 77%, the variation among genetic backgrounds is now established within acceptable limits and has huge practical implications in breeding programs. Investigation of 3 more families during the FCBA 2009 initiation effort suggested that the mean initiation rate can be further significantly increased by introducing a subculture step of immature explants on induction medium. A 86% mean initiation rate was obtained with a genotype capture rate between 68 and 96% (data not shown).

3.2 EM proliferation and genetic stability

Once obtained, EMs are transferred onto maintenance medium and subcultured weekly or biweekly in clumps onto fresh medium in order to sustain their proliferation. Both maturation yield and SE quality decreased as a function of EM subculture number in maritime pine (Breton et al. 2006; Trontin et al. 2011). Concomitantly, morphological degradation of immature embryos occurred suggesting a progressive loss of regenerative capacity of embryogenic lines. Breton et al. (2005) showed that the aging effect can be lowered by using a high subculture frequency of clumps (7 days instead of 14 days). For rapid EM amplification prior to the maturation treatment, the plating method previously described by

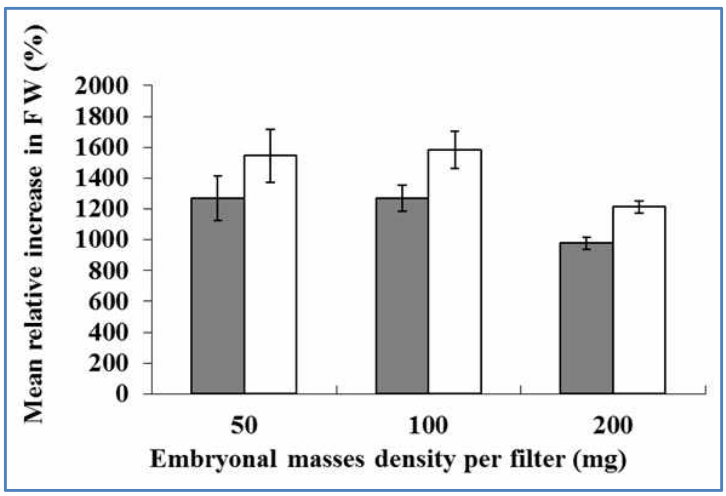

Figure 5. *Mean relative increase in fresh biomass of maritime pine embryonal masses as a function of the density per filter and basal medium during a two-week subculture period.* ▭ *mDCR (modified from Gupta and Durzan 1985);* ▭ *mLV (modified from Litvay et al. 1985). Bars represent 95% confidence limits. FW: fresh weight.*

Klimaszewska and Smith (1997) for *P. strobus* has been adapted for maritime pine (Lelu et al. 1999). EMs dispersed in liquid medium are poured over a filter paper

disc and subcultured every two weeks. A low EMs density of 50-100 mg per filter as inoculum was found optimal (Figure 5). Here again Litvay-based media significantly improved EM growth on filter paper at different cell densities compared to on mDCR (Figure 5). An up to 1500% mean relative increase in biomass can routinely be obtained upon biweekly subculture.

EMs morphology and cytology have been characterized in relation to their proliferation and maturation ability (Ramarosandratana et al. 2001b; Breton et al. 2005). Two different EM morphotypes have been defined, i.e., as smooth or spiky. The spiky morphotype is characterized by the presence of early SEs protruding from the periphery of the EM (Ramarosandratana et al. 2001a) and was associated with increased biomass production (Breton et al. 2005). Proliferation of EMs was also found to be strongly affected by genotype (variation among cell lines) and subculture frequency. During proliferation, microscopic changes of EM occurred, leading to a low occurrence of the early SEs stages, concomitant with a gradual increase in growth rate. EM growth on proliferation medium and early SE development were found to be disconnected. In order to preserve a spiky morphotype, i.e., to retain early embryogenic ability during proliferation, EM should be sub-cultured weekly for a short period (less than 6 months) on a maltose-containing medium without PGRs (Breton et al. 2005). Maltose was found to increase the frequency of well-developed early SE during proliferation.

No major change in ploïdy level was detected in proliferating EM or during early embryo development using flow cytometry (Marum et al. 2009a). In contrast, genetic variation was detected at seven microsatellite loci (SSRs) after prolonged EM subculture for up to 22 months as well as in emblings regenerated from cotyledonary embryos (Marum et al. 2009b). However genetic stability at the analyzed loci could not be associated to abnormal development of emblings.

3.3 EM maturation ability and SE development

Cotyledonary SEs development from embryogenic cultures is under strong genetic control in maritime pine with both maternal and paternal significant effects (Lelu-Walter *et al.* 2006). However, maturation conditions were improved to the point where the variation in cotyledonary SE yield from different genetic backgrounds remained within acceptable limits. The effect of PEG in maturation medium is not clearly established in maritime pine with either no effect (Ramarosandratana et al. 2001a) or a positive effect (Miguel et al 2004). Significant progress was obtained using Litvay-based instead of DCR-based maturation media (Table 3). Mean maturation yield calculated from a large sample of embryogenic lines (238 lines/year on average) matured at FCBA from 2000 to 2009 was 50.5±2.3 SE g^{-1} FW when cultured on mLV, as compared with only

Table 3. *Somatic embryo yield obtained in P. pinaster as a function of different formulations of DCR- and Litvay-based maturation medium.*

Tested lines		Basal	ABA	Sucrose	Gellan	SE yield	Reference
Nb	Maturing[a]	medium[b]	(µM)	(M) / PEG ($g\ l^{-1}$)	gum (g)	g^{-1} FW	
5	5 (100%)	DCR	80	0.17/0	9	8-99	Ramarosandratana et al. (2001a)
896	108 (12%)	DCR	120	0.06/100	10	na	Miguel et al. (2004)
18	18 (100%)	mLV	80	0.2/0	10	2-441	Lelu-Walter et al. (2006)
39	32 (82%)	mLV	80	0.2/0	9	0-192	Trontin et al. (2011)
26	15 (58%)	mLV	80	0.2/0	10	0-274	Hùmanez et al. (2012)

[a]Giving rise to cotyledonary somatic embryos. FW: fresh weight; na: not available

[b]DCR: Gupta and Durzan (1985); mLV: modified from Litvay et al. (1985).

ABA: abscisic acid; PEG: polyethylene glycol; SE: somatic embryo; FW: fresh weight

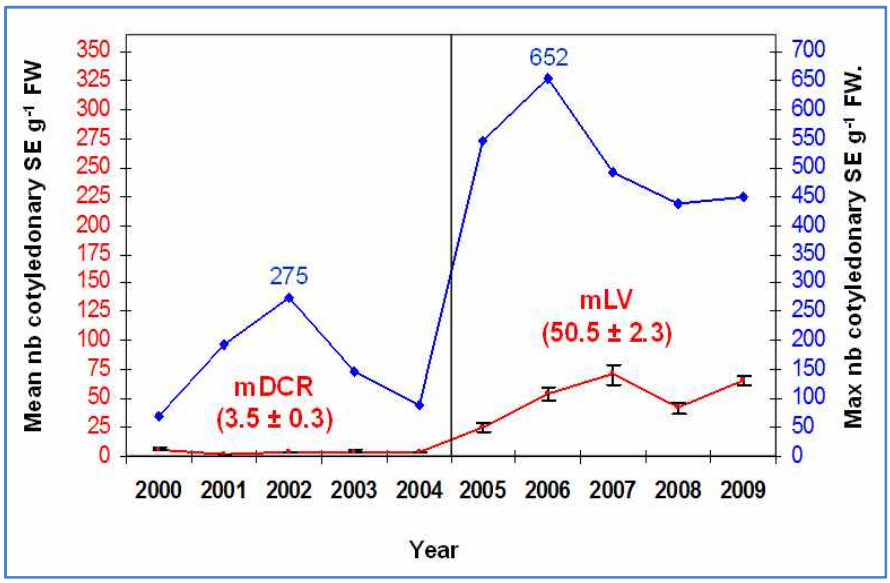

Figure 6. *Maturation ability of maritime pine embryogenic lines as a function of basal medium over 10 years experiments with mDCR (2000-2004) and mLV (2005-2009). Both mean (red curve) and maximum yields (blue curve) in cotyledonary SEs are shown. A mean of 238 lines were investigated per year (18-691 lines). mLV basal medium started to be routinely used in 2005 at FCBA following the results by Park et al. (2006).*
mDCR: modified DCR medium from Gupta and Durzan (1985); mLV: modified LV medium from Litvay et al. (1985). Bars for mean cotyledonary SE yield represent 95% confidence limits. FW: fresh weight.

3.5±.3 SE g^{-1} FW on mDCR (Figure 6). This is a 14-fold increase in mean maturation ability. We also observed a 5-fold increase in mean maximum yield of individual lines from ca. 100 to 500 SE g^{-1} FW on mLV. Changing mDCR for mLV formulations significantly improved genotype capture and performances at

the maturation step. In a recent study of 39 lines from 4 elite families, the genotype capture, calculated as the frequency of lines producing at least 50 cotyledonary SE g^{-1} FW, was estimated to be 43.6% in lines propagated for 18 weeks of subculture (Trontin et al. 2011). However, 10 weeks of subculture later, the genotype capture was strongly reduced to a low 12.8%. An additional consequence of line aging as a function of subculture number was a significant decrease in the size of cotyledonary SEs as measured by embryo or hypocotyl length (Breton et al. 2006). This effect was observed on both mDCR and mLV basal media. As a result, embryo quality significantly decreased, resulting in a lower germination ability (FCBA, unpublished).

As a low genotype capture and reduced SE quality are serious drawbacks for development of somatic embryogenesis at acceptable cost, these results are further evidencing for the need to carefully process both cryopreserved stocks and propagation of embryogenic lines after reactivation from the cryopreserved stock.

Future research in maritime pine should, therefore, focus on achieving a better understanding of the physiological and molecular mechanisms that cause the loss of competence of embryogenic lines to form cotyledonary SEs, resulting in a strong reduction of both maturation yield and genotype capture at this crucial step. Klimaszewska et al. (2009) investigated the young, 3-month-old (productive), and aged, 18-month-old (non-productive) versions of the same embryogenic line (two genotypes tested) with regard to the levels of hormonal and polyamine profiles as well as global and specific (MSAP, methylation-sensitive amplification polymorphism) DNA methylation patterns. Inconsistent hormonal and polyamine profiles were observed in EM cultures of similar phenotypes. Furthermore, the global DNA methylation level measured as the percent of methylated cytosine (% mC) did not significantly change between young and aged cultures (17.8-19.1% mC). Similarly, global DNA methylation was found to be genotype dependent but could not be associated with maturation ability of 3 embryogenic lines with contrasted performances, with the % mC being 12.9% for the line with high maturation ability, 8.5% for the medium and 15.2% for the line with low maturation ability (FCBA, unpublished results).

Treatment of an aged culture with the DNA hypomethylating drug 5-azacytidine (5-azaC) at 10-15 µM greatly reduced growth (cytotoxic effect) but slightly improved EM maturation ability (from 3 to 10-15 cotyledonary SE g^{-1}) suggesting that there is an epigenetic-related origin of the progressive loss of regenerative capacity (Klimaszewska et al. 2009). MSAP profiling of young and aged lines further supported the idea that aging is associated to epigenetic DNA changes (qualitative alterations) involving net demethylation at specific target sequences concomitant with methylation at other sites. A net DNA methylation increase was detected in EM samples of an aged line treated with 5-40 µM 5-azaC for 9 days. In contrast net DNA demethylation was observed in samples subjected

to longer exposure (14 days) at similar 5-azaC concentration, suggesting early cytotoxic and late hypomethylating effects. The consequence of these 5-azaC-induced changes onto the viability and maturation potential of treated tissue remains to be investigated. Overall it suggests that some modulation of DNA methylation could restore the maturation ability. Interestingly this study revealed that secondary EM cultures induced from cotyledonary SEs have better maturation ability than aged EM cultures. It would be interesting to investigate the DNA methylation pattern of these secondary EMs. Secondary somatic embryogenesis appears to be a useful tool to restore maturation ability of embryogenic lines in maritime pine and could also help manage the cryopreserved stock. As stated in the above section, various cultural practices could be used to slow down the aging process, including the use of maltose-based and PGR-free medium and high subculture frequency (Breton et al. 2005). Again it would be interesting to check the DNA methylation pattern of tissues exposed to these cultures conditions.

3.4 SE germination, acclimatization and plant recovery (SE conversion to emblings)

Cotyledonary SEs usually germinated at high frequencies (73% on average Table 4), up to 100% for Spanish provenances depending on the embryogenic line

Table 4. *Somatic embryo germination and plant development in P. pinaster using mLV formulation at INRA*

Cross[a]		Line	Number of SEs			Number of plants	
♀	♂		Isolated	Germinated *(%)*	Ready for acclim. *(%)*	Acclim.[b]	Survival (4 months)
A	D	PM2	183	151 *(82)*	98 *(53)*	50	28
A	E	PM16	123	113 *(92)*	97 *(79)*	56	45
B	D	PM3	337	230 *(68)*	165 *(49)*	60	28
B	D	PM4	114	95 *(83)*	78 *(68)*	30	11
B	E	PM10	65	60 *(92)*	49 *(75)*	27	14
B	E	PM18	90	59 *(65)*	41 *(45)*	13	3
B	F	PM5	375	362 *(96)*	336 *(89)*	252	179
B	F	PM6	120	109 *(91)*	101 *(84)*	70	41
C	D	PM12	332	202 *(61)*	179 *(54)*	84	79
C	D	PM13	197	152 *(77)*	128 *(65)*	61	22
C	E	PM15	83	60 *(72)*	47 *(57)*	22	14
C	F	PM9	112	80 *(71)*	59 *(53)*	26	26
Total		**2302**		**1673** *(73)*	**1378** *(60)*	751	**490** *(65)*

[a]♀: Corsican provenances; ♂: Landes breeding population
[b]Acclimatized plants

(Humánez et al. 2012). SEs germinated in light showed signs of stress (red color of the hypocotyl) whereas those placed the first 10-14 days of germination in darkness developed an elongated hypocotyl. As a result, further handling of germinating SEs is improved (Lelu-Walter et al. 2006). Aerial parts developed after 7 weeks on germination medium.

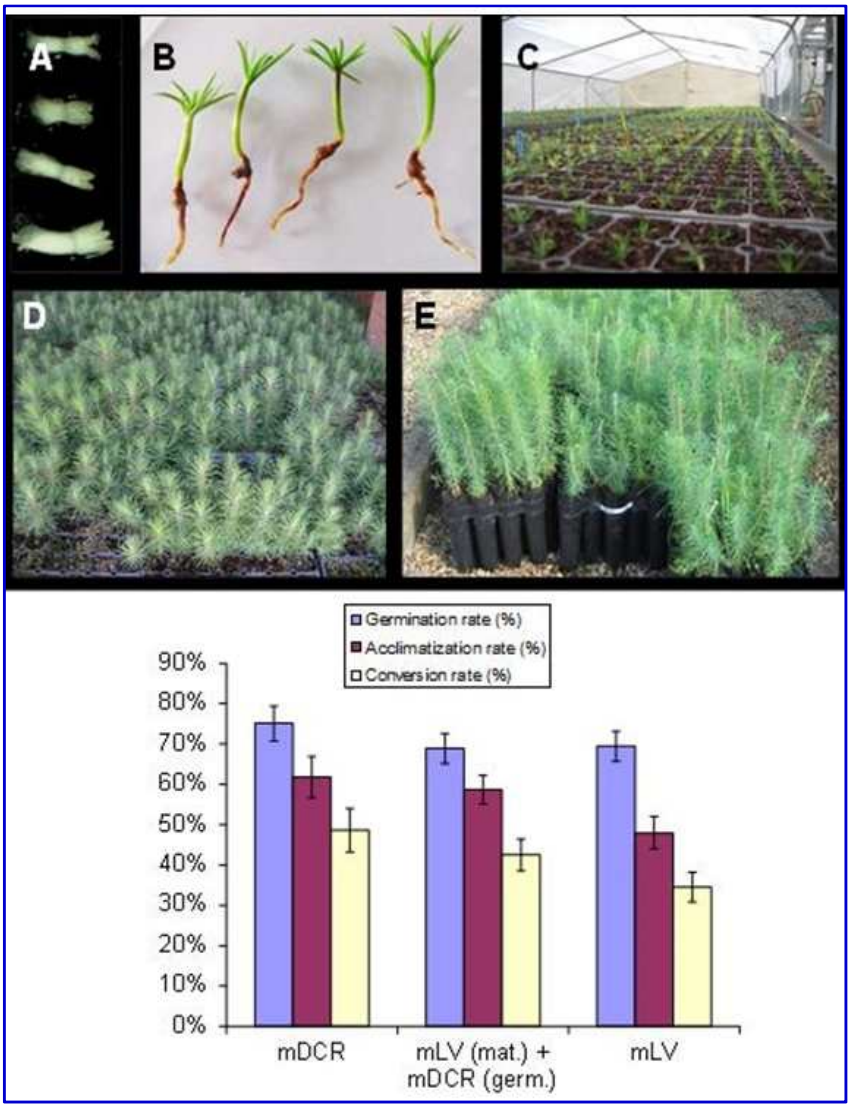

***Figure** 7. Cotyledonary SEs (**A**) germination (**B**), acclimatization (**C**) and conversion into emblings (**D, E**) and corresponding rates (%) in maritime pine as a function of basal medium used at the maturation and germination steps. Data were computed from 6 embryogenic lines originating from 5 unrelated seed families (AAF04005, AAY06006, AB774, NL04045, NL04048, PN519). mDCR: modified DCR medium from Gupta and Durzan (1985); mLV: modified LV medium from Litvay et al. (1985). Bars represent 95% confidence limits.*

Compared to mDCR, the use of Litvay-based medium formulations (mLV), at both the maturation and germination steps, resulted in a lower germination rate (69.4 vs. 75.0%), acclimatization rate (47.9 vs. 61.7%) and conversion rate (34.5 vs. 48.6%) into plantlets capable of proper growth in the greenhouse and then the nursery (Figure 7). Interestingly, when mLV formulations were used during maturation and mDCR during germination, intermediate results were obtained (Figure 7), suggesting that the negative effect of Litvay basal salts on germination rate is partially and early determined during the maturation phase.

4. Physiological and (epi)genetic status of SEs: are they similar to ZEs?

4.1 Reduced water availability induced a shift towards embryogenesis

Significant progress has been made in the development of maritime pine somatic embryogenesis but there are still technical issues that preclude full integration of this powerful vegetative propagation system into the French breeding program. As previously mentioned, maritime pine SEs require a reduction in water availability (high gellan gum concentration in the maturation medium) to reach the cotyledonary stage. This key switch, reported specifically for pine species, is not yet well understood. To facilitate the use of somatic embryogenesis for mass propagation of conifers we need a better understanding of embryo development (Jordy and Favre 2003; Tereso et al. 2007). Recently, a multi-scale, integrated analysis was used to unravel early molecular and physiological events involved in SE development (Morel et al. 2014a). Under conditions unfavourable for SE maturation (4 g L^{-1} of gellan gum in the maturation medium) both transcriptomic and proteomic profiling indicate enhanced glycolysis leading to proliferation of EMs with an increased fresh weight, which may be antagonistic to SE maturation. Under favourable conditions (9 g L^{-1} of gellan gum), we observed adaptive, ABA-mediated molecular and physiological responses to reduced water availability resulting in early transition of EMs from proliferation to the SE developmental pathway as indicated by confocal laser microscope observations, active protein synthesis, overexpression of proteins involved in cell division, embryogenesis and starch synthesis. Specific pathways (e.g. synthesis of protective secondary metabolites, regulation of oxidative stress) are also activated, apparently to overcome constraints due to culture conditions. A protein of germin type and an ubiquitine ligase appear as potential markers of early somatic embryogenesis of maritime pine, while the phosphatase protein 2C stands out as the adaptive answer to the culture environment (Morel et al. 2014a). These results may facilitate monitoring of early EM responses to maturation conditions.

4.2 When to harvest cotyledonary SE for germination?

In maritime pine, improved protocols are now available for the whole somatic embryogenesis process, i.e., from EM initiation to somatic plant regeneration (Figure 7). However, field trials established in France from somatic plant material (emblings) have consistently revealed a lower initial growth rate than the control seedlings (see below part 5). A better understanding of SE maturation is, therefore, required in order to produce high-quality, vigorous somatic plants. SEs are currently matured for 12 weeks to reach the cotyledonary stage before being germinated and converted to plantlets. Although regeneration success is highly dependent on SE quality, the harvesting date is still determined by

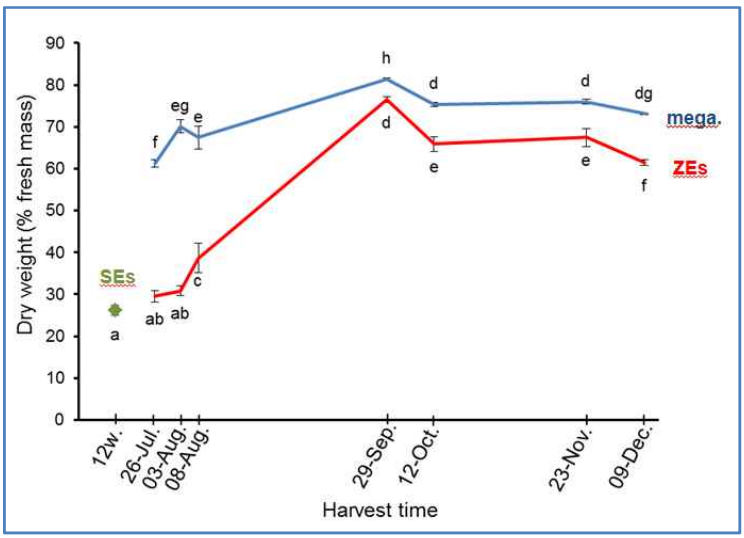

Figure 8. *Dry weight of cotyledonary embryos of somatic (SEs), zygotic origin (ZEs) or megagametophytes (mega.) as a function of harvesting time. Somatic embryos were collected after 12 weeks maturation. Zygotic embryos and megagametophytes were sampled at 7 collection dates from 26 July to 09 December 2011. Bars represent 95% confidence limits. Letters represent statistical groups defined by the Multiple Comparisons of Means method (P<0.05, N = 10).*

their morphological features. This empirical method does not provide any accurate information about embryo quality with respect to their storage compounds (proteins, carbohydrates). SEs matured for 10, 12 and 14 weeks were analyzed by carrying out biological (dry weight, water content) and biochemical measurements (total protein and carbohydrate contents). No significant difference was found between collection dates, suggesting that SE harvesting after 12 weeks of maturation is appropriate (Morel et al. 2014b). Cotyledonary SEs were then compared to various stages of cotyledonary ZEs, from fresh to fully desiccated

(from August to December). The corresponding megagametophytes were also analyzed to evaluate the impact of the maturation on this nutritive tissue. While the megagametophytes presented a slight variation in dry weight (Figure 8), and carbohydrate content (Figure 9) in comparison to that of the ZEs, their respective protein content changed about the same, but in opposite direction, highlighting the transfer of proteins from the nutritive tissue to the embryos (Figure 10). The similarity of the 12-week-old SEs with the fresh cotyledonary ZEs sampled from late July to early August (Figures 8, 9, 10) was confirmed by a hierarchical ascendant cluster analysis with 9 variables. Both types of embryo exhibited similar carbohydrate and protein content and signatures.

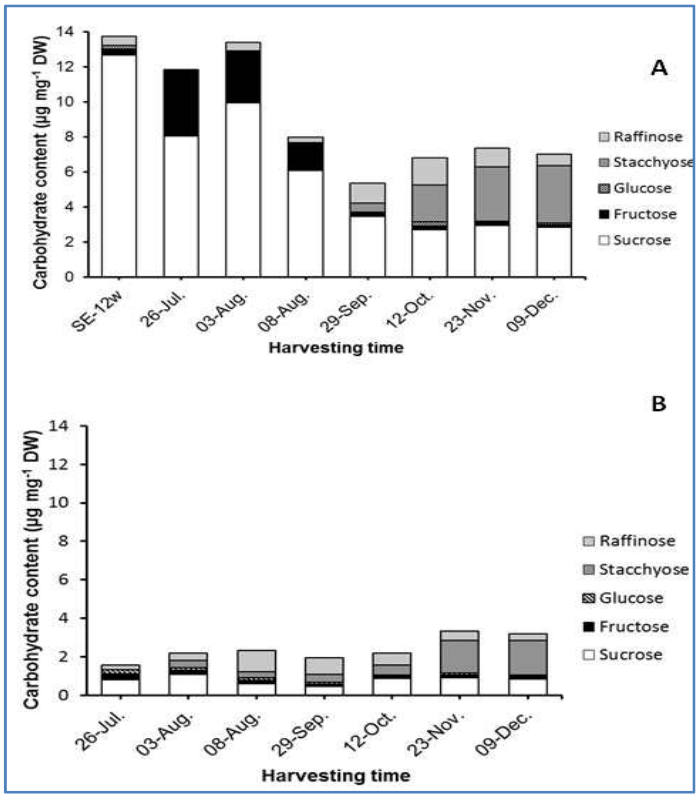

Figure 9. *Changes in carbohydrate content in (**A**) cotyledonary embryos of somatic (SEs) or zygotic origin (ZEs) and in (**B**) megagametophytes as a function of harvesting time. SEs were collected after 12 weeks maturation (SE-12w). ZEs and megagametophytes were sampled at 7 collection dates from 26 July to 09 December, 2011. Bars represent 95% confidence limits.*

This high level of similarity was evaluated at 94.5% according to a proteome profiling test. Highly expressed proteins included storage, stress-related, late embryogenesis abundant (LEA) and energy metabolism proteins. By

comparing overexpressed proteins in developing and cotyledonary SEs or ZEs, some (23 proteins) could be identified as candidate biomarkers for the late, cotyledonary stage. Of these, 18 belonged to five large families of proteins including five HSPs, four LEAs and two other stress-related proteins (aldose reductase, 6-phosphogluconate dehydrogenase), five storage proteins and two proteins involved in purine metabolism (adenosine kinase 2, SAM synthase; Morel et al. 2014b). Results also suggest that improvements of SEs quality may be achieved if the current maturation conditions are refined.

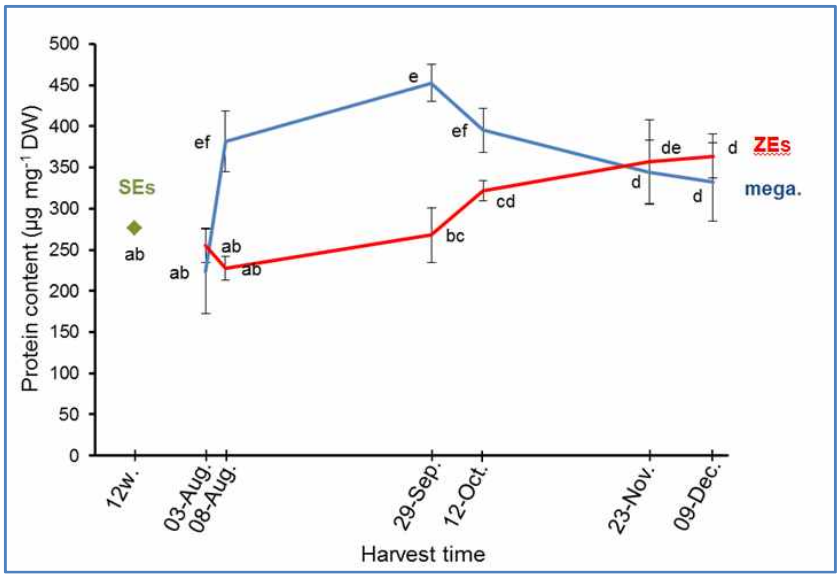

Figure 10. *Quantitative analysis of total proteins in cotyledonary somatic embryo (SEs) matured for 12 weeks, compared to cotyledonary zygotic embryos (ZEs) and their respective megagametophytes (mega.) during maturation. Bars represent 95% confidence limits. Letters represent statistical groups defined by the Multiple Comparisons of Means method (P<0.05, N = 5).*

5. Field testing of somatic seedlings

Data collected from field trials are crucial information to validate the somatic embryogenesis technology, i.e., it is necessary to compare growth and phenology traits of emblings with those of control seedlings. A total of 8 field trials have been established since 1999 by FCBA (7) and INRA (1) with a total of about 3200 somatic plants in test. Each trial is comparing 11 to 78 lines originating from 6 to 12 elite families. First trials were performed in the nursery whereas more recent trials (since 2004) are established in forest conditions. Most plants were initially produced from lines cultivated with DCR-based media, especially at FCBA, but in recent field trials most plants were obtained from lines cultivated

with Litvay-based media. A mean of 91% survival of emblings occurred at age 2 in these field trials. The older trial was planted at a FCBA nursery plot in 1999 (59 plants from 12 clones) and the trees are now entering their adult reproductive phase with regular cone production (Figure 11). Male flowering was classically observed as early as at age 5. Following the last measurement at age 12, somatic trees were 6.5-12.1 m in height with diameters in the range 9-29 cm. These measurements are similar to those of control seedlings at the same plot which were 9.6-11.2 m in height and 19-33 cm in diameter. This trial demonstrates that somatic seedlings could complete the juvenile and adult vegetative growth phases.

Figure 11. *Maritime pine emblings planted by FCBA in 1999 at a nursery plot (Sivaillan, France). The 12-year-old trees are entering their adult reproductive phase (cone production can be observed).*

In another trial planted by FCBA in 2004 (99 plants from 35 lines obtained from 7 families), somatic plants are developing into trees with normal phenotypic behaviour (Figure 12). Mean height at age 7 years (5.71 m) is however significantly lower than that of control seedlings (7.03 m) but no significant difference could be detected for the mean relative increase in height since planting. We conclude that somatic trees developed at a lower initial growth rate than control seedlings. Such a low initial growth was similarly observed in a field trial of 24 somatic clones planted in 2004-2005 (Figure 13). At age 6 years, the height of some clones was found to be similar to that of seedling controls (e.g. clones 25C, 29C, DE737, CM815, ET816). Computing the mean relative increase in height since planting revealed that some clones performed as well as control seedlings and even better in some cases (e.g., clones 29C, PN6128, NM626, NM18c). These results are very encouraging and suggest that initial low growth rate of somatic clones can be overcome after a few years and can be compensated for by selecting within each family the top elite clones. Although full genetic gains cannot be obtained currently with somatic seedlings because of their low initial growth it is expected from field trials that opportunities will exist for selection of clones with improved adaptability and performance to sustain forest productivity in maritime pine plantation forestry (see Wahid et al. 2012).

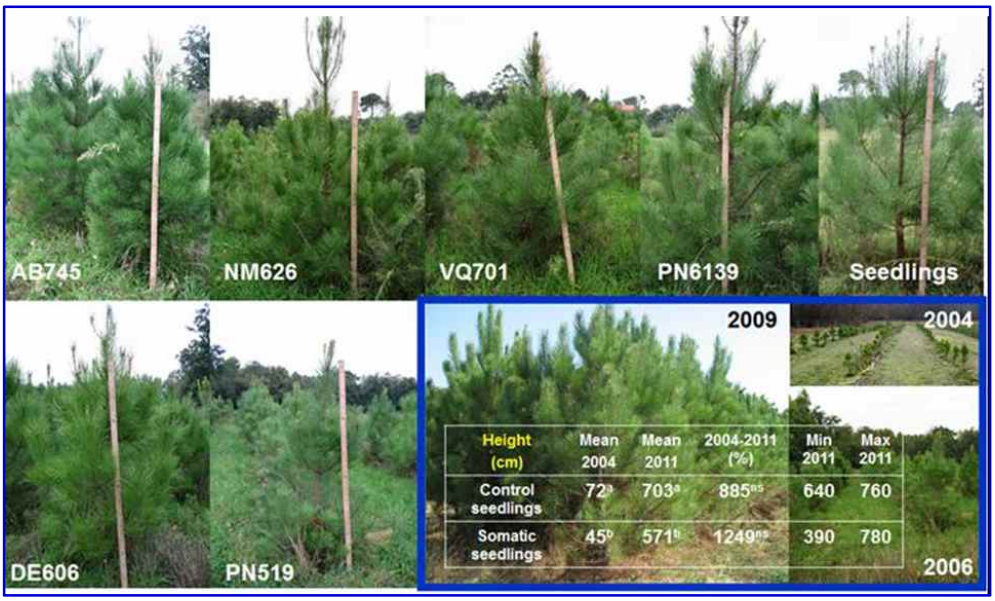

Figure 12. *Somatic clones obtained from 6 embryogenic lines and control seedlings 2.5 years after plantation at a nursery plot (2004, Sivaillan, France). Blue box: a general view of the field trial in 2004, 2006 and 2009. The Table is giving mean height (2004, 2011), mean relative increase between 2004 and 2011 (%), minimal (Min) and maximal (Max) height (2011) for somatic and control seedlings. Significant differences between means are indicated by different letters. ns: non-significant (p< 0.05).*

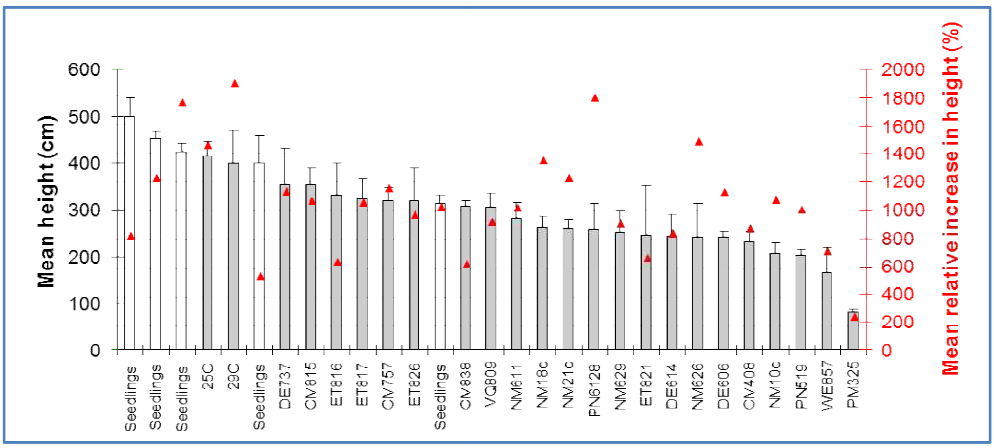

***Figure 13**. Mean height and relative increase in height at age 6 of 24 FCBA or INRA somatic clones (in grey) and 5 control seedlings lots (in white) planted by FCBA in a forest field trial in 2004-2005 (Landriole, France). Control seedlings are either related with clones (genetic controls) or with VF1 varieties (improved controls). Bars represent 95% confidence limits for mean height.*

6. Challenges with somatic embryogenesis in maritime pine

6.1 Aging in embryogenic cultures

Cryopreservation of plant material is not only useful but necessary due to the instability of the physiological and/or DNA methylation state of most embryogenic lines of maritime pine. Regarding short- to mid-term propagation goals, this could easily be compensated for by selecting a few stable lines. In case this instability problem could be solved, or at least significantly reduced, cryopreservation would still be required for securing the long-term availability of lines, particularly for still fragile, freshly initiated ones, and also for line maintenance at low cost when their continuous propagation is not required. In breeding operations, cryopreservation would still be required during the testing of emblings in field plots until the age of final confirmation of their individual clone performance and of their ability to adapt to environmental parameters, i.e., water availability, soil fertility and pest occurrence. Nevertheless, we can anticipate that in the future massive propagation of new clones could directly be launched shortly after initiation from seeds on the basis of marker-assisted or genomic selection. Early phenotype selection among individual immature ZEs may become possible, thus bypassing the need for lengthy initial testing.

The instability of lines has often been described for many plants in terms of "aging" or performance decline (Bhaskaran and Smith 1990). This was described early in the history of conifer somatic embryogenesis in the case of spruce. Degenerating lines, showing an altered microscopic and/or macroscopic

morphology, typically showed a decreased maturation ability. In some cases, this phenomenon was related, or proposed to be related, to the occurrence of mutation in particular genes involved in key steps of embryo morphogenesis (Fourré et al. 1997; Egertsdotter and Von Arnold 1995). Further insights into somatic embryogenesis of model plants and conifers as well (Rose and Nolan 2006; Smertenko and Bozhkov 2014; Zhu et al. 2014; Zhu 2015), pointed out the genetic control of the process. However detailed studies of allelic variability in these genes are still lacking. Regarding maritime pine, Breton et al. (2005, 2006) showed that culture medium as well as various other cultivation parameters like subculture frequency can delay this evolution. It is noteworthy that the same full-sib families could give rise to lines with contrasting behaviour, particularly in terms of sensibility to factors impacting their morphology and associated maturation ability.

6.2 Variability in culture

Tissue culture media vary from lab to lab or even among operators or batches because some components and/or preparation steps are not disclosed or fully described. Different batches of the same medium prepared by different operators could substantially differ in their ability to sustain a proper evolution or reaction of the cultured plant material. Ramarosandratana et al. (2001b) described how establishing cell lines from different EMs can lead to cell populations with different SE maturation ability. Furthermore Breton et al. (2006) reported that variation in maturation yield in maritime pine is independent from spatial factors (the dish) and temporal ones (different sub-lines from the same cryopreserved stock). Spatial heterogeneity probably results in a batch-to-batch or container-to-container variability of maturation yield. Suboptimal conditions can impact the overall cost of the plant production process.

Genetic variation has been shown to represent a major source of variability in somatic embryogenesis of various plant species including conifers (Wareing and Phillips 1970; Henry et al.1994; Nestares et al. 2002; MacKay et al. 2006; Park et al. 1994, 2006; Pinto et al. 2008). This could be seen as a case of the common phenomenon of genetic (relative) recalcitrance to vegetative propagation as described by McCown (2000), Bonga et al. (2010), Elhiti and Stasolla (2012), among many others. Recent results point out the modulation of recalcitrance by both genetic and epigenetic factors (reviewed by Mahdavi-Darvari et al. 2015).

Ramarosandratana et al. (2001a) describe the variable sensitivity among maritime pine lines to three components of the maturation medium (i.e., osmoticum, sugar and gelling agents). Similarly, we showed at FCBA (unpublished) through testing of a few hundred lines the vast initial variability of the response of full-sib lines to alternative maturation protocols.

6.3 Towards industrial application of somatic embryogenesis

The use of bioreactors or other liquid medium-based *in vitro* culture containers has long been proposed as an efficient solution for the production of high quality mature conifer SEs (Attree et al.1994; Tautorus et 1994; Moorhouse et. 1996; Timmis et al. 1998; Gupta and Timmis 2005) but there is no published case of real commercial application. Several companies have claimed some success recently, e.g., Arborgen (Becwar et al 2012), Sweetree (Egertsdotter and Johnson 2014) and Weyerheauser (Swanda 2014).

The biggest challenge toward industrial scale-up of conifer somatic embryogenesis is the genetic as well as physical and temporal heterogeneity of the plant material in response to the treatments aimed at obtaining high quality forest plants. This heterogeneity results in a high variability of results and unpredictability of operational scale production. This problem is less pronounced for some species, like hybrid larch and Norway spruce (Lelu-Walter et al. 2013; Thompson 2014), than for maritime pine.

For maritime pine, the current challenge is the quality of the plant material resulting from the conversion of cotyledonary SEs into somatic seedlings. Indeed, while we produced a huge number of mature embryos over the years at FCBA (nearly half a million) and raised *ex vitro* more than fifty thousand plants, and established several field tests (> 3200 plants, see part 5), the results were unsatisfactory because the initial growth of emblings was slower than that of seedlings. However, as for spruce species (Grossnickle and Major 1994a, b), recovery from slow growth occurred over time.

7. Conclusions: prospects for industrial application

We have described the state and challenges facing the industrial deployment of somatic embryogenesis of forest trees in general and with some details regarding maritime pine (Lelu-Walter et al. 2013). This subject was also dealt with by Thompson (2014), who described the prospects for solving problems such as social acceptance and hesitance of nursery managers and foresters to use SE in their operations.

For maritime pine, most of the technical steps (i.e., initiation, multiplication and cryopreservation) are working sufficiently well with a high rate of capturing genotypes at the initiation step (65-85%). However, subsequent steps towards the regeneration of plants that meet the quality standard required for commercial purposes need further investigation. Low genotype capture at the maturation step (< 50%, see part 3) as well as inadequate embryo quality compared to the quality of fresh cotyledonary ZEs (see part 4) are both currently precluding the regular production, at acceptable cost, of enough plants from a suitable number

of clones. It is likely – but this needs to be validated - that several aspects of these difficulties are interrelated and that improvement at any step will impact positively the yield at other steps. Expected future improvements probably will also help to obtain plant material that is suitable for automatized processing which could result in decreased production cost.

The production of healthy vigorous plants able to sustain harsh weed competition on low fertility sites is a prerequisite for acceptance of SE plant material by breeders, nursery operators and foresters. To achieve such improvements would require close collaboration between academic and private teams. For maritime pine such a collaboration has been in effect since 2004 between INRA and FCBA and has already resulted in significant achievements. Improved knowledge of the somatic embryogenesis process and embryo development, particularly of its molecular aspects (de Vega-Bartol et al. 2013; Morel et al. 2014a,b, Trontin et al. 2015), are thought to provide new opportunities for further refinement of somatic embryogenesis in maritime pine.

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9. Authors contribution

JFT carried out somatic embryogenesis experiments, conceived the design of the study and drafted the manuscript. CT conceived the design of the study, carried out protein analysis and drafted the manuscript. AM carried out protein analysis and helped to draft the manuscript. LH conceived the design of the study and drafted the manuscript. MALW carried out somatic embryogenesis

experiments, conceived the design of the study and its coordination, and drafted the manuscript. All authors red and approved the final manuscript.

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Optimisation of somatic embryogenesis of *Pinus radiata* for production forestry

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Abstract

Conifer somatic embryogenesis (SE) is the primary enabling technology for all conifer biotechnology plant production as well as for the implementation of multi-varietal forestry which is integrated with tree improvement programs. Since the development of this technology in *Pinus radiata* D. Don by Smith (1997) several research projects have been focused on the optimization of the method to increase its efficiency. *Pinus radiata* (Monterrey pine), a coniferous tree from California, USA, is widely planted in New Zealand, Chile, Australia, South Africa and Spain being an important source of wood used in pulp and paper products, construction and furniture. To employ *Pinus radiata* SE commercially, it must work on a wide range of crosses and genotypes. Moreover, it should produce a large number of established plants economically. In this chapter, a description and brief review of the latest methods to optimize all the steps in the SE process from initiation through to germination and subsequent amplification of somatic embryos are shown.

Keywords: embryogenic cell line, embryonal mass, germination, radiata pine, somatic embryos.

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic; BA: benzyladenine; ECLs: Embryogenic Cell Lines; Ems: Embryonal Masses; SE: Somatic embryogenesis; se: somatic embryos

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1. Introduction

Pinus radiata D. Don (Monterey pine, Radiata pine) is a native of California that is widely grown in New Zealand, Chile, Australia, South Africa and Spain. *Pinus radiata* is an important source of wood and pulp that are used in construction, furniture and the paper industry (Cerda et al. 2002).

Forestry productivity can be increased via the planting of high-value trees. Vegetative propagation methods are used to produce large amounts of plant material from control-pollinated seeds where the parent trees have been assessed for growth, wood quality and disease resistance (Menzies et al. 2001). Clonal propagation by somatic embryogenesis (SE) has the ability to enhance this amplification process and capture the benefits of breeding or genetic engineering programs and improve uniformity of planting stock (Pullman et al. 2005). SE is an effective method of propagating superior genotypes when combined with other technologies such as cryopreservation of the embryonal masses (EMs) and thus facilitating the production of field proven genotypes (Park 2002). Furthermore, conifer somatic embryogenesis is enabling the implementation of multi-varietal forestry, defined as the deployment of genetically tested tree varieties integrated with tree improvement programs (Park et al. 2006). Sometimes, SE systems for conifers are not sufficiently optimized to be considered for commercial use (Bonga 2015). The main factors currently limiting commercialization of SE for *Pinus* spp. are:

- The competence window for explant initiation post fertilisation is narrow, lasting around 4 weeks (MacKay et al. 2006);
- Low initiation from high-value crosses (Pullman and Bucalo 2014);
- Low or null proliferation of the initiated Ems;Loss of ability of cultures to be regenerated (Pullman et al. 2005);
- Low success of the process when mature seeds are used to initiate embryogenic cultures (Tang et al. 2001); and
- Abnormalities in embryos produced resulting in low and/or poor quality plantlet production.

Pinus radiata SE was first described by Smith et al. (1994). There have been improvements in different aspects of the SE process in intervening years; the development of optimised protocols remains a difficult process in which several factors have to be carefully studied. Lately, some of these research efforts have been focused on the improvement of initiation (Hargreaves et al. 2009), proliferation (Hargreaves et al 2011; Montalbán et al. 2012), maturation and germination (Montalbán et al. 2010) stages as well as cryopreservation (Hargreaves et al. 2002). Moreover, organogenesis/ SE combined protocols have been

developed in order to increase the number of plants that can be produced from elite clones (Montalbán et al. 2011).

In this chapter, summarised data about the latest improvements aimed to reach the maximum efficiency in *Pinus radiata* SE are given, taking into account the different stages of the process.

2. Initiation of embryonal masses (EMs)

Traditionally, intact cones from open or control pollinated trees are sprayed with 70% (v/v) ethanol, split into quarters and all immature seeds dissected. However, green cones can be stored for several months prior to start initiation procedures (Montalbán et al. 2015). For initiation, immature seeds are surface sterilized in H_2O_2 10% (v/v) plus two drops of Tween 20® for 8 min and then rinsed three times under sterile distilled H_2O in sterile conditions in the laminar flow unit. Seed coats can be aseptically removed and whole megagametophytes provide the initial explant.

Radiata pine SE is most commonly initiated from intact megagametophytes bearing immature zygotic embryos at the pre-cotyledonary stage. The average embryo stage used is similar for all families at any given date and the best physiological stage of the embryos is between stage 2 and 4 (Hargreaves et al. 2009; Montalbán et al. 2012) before the cotyledons start developing. The obtained rates of SE initiation using megagametophytes from 10 control-pollinated families at the optimum collection time showed that 33% of explants gave rise to embryogenic tissue, with a range of 13.6 to 87%, though no detailed data was given (Smith et al. 1994), 17 - 25% for the 19 open-pollinated families tested, irrespective of collection time (Hargreaves et al. 2009) and from 20 % to 67% in 7 open-pollinated families (Montalbán et al. 2012).

EMs initiation (Figure 1A) is in most cases a process of sustained cleavage polyembryony initiated from immature zygotic embryos within the megagametophyte tissues rather than through dedifferentiation from differentiated (true somatic) tissues. One potential problem with this methodology is the risk that the EM arises from multiple fertilisations within one seed. A way to overcome the problem is the use of individual zygotic embryos isolated from megagametophytic tissue as initial explant for SE initiation. This has been done experimentally for radiata pine to determine if multiple fertilisations had taken place. These did occur at low frequency but analyses of established EM derived from zygotic embryo masses within has indicated that the tissue is of one genotype (Hargreaves, unpublished data). Similar work with *Pinus taeda* did indicate multiple paternal genotypes in embryogenic tissue derived from individual immature seed (Becwar et al. 1990). Zygotic embryo mass within an individual megagametophyte can consist of both the dominant and subordinate embryos that resulted from

polyembryony (monozygotic) or simple cleavage polyembryony, where only one fertilization event had occurred. The use of individual dissected cotyledonary embryos of *P. radiata* has been studied and a low level of successful initiation of EMs from this material was achieved (Find et al. 2014) as previously observed in *Picea* spp. (Mo and von Arnold 1991). The Find et al. (2014) study showed that the initiation was possible over a longer period for up to 10 weeks.

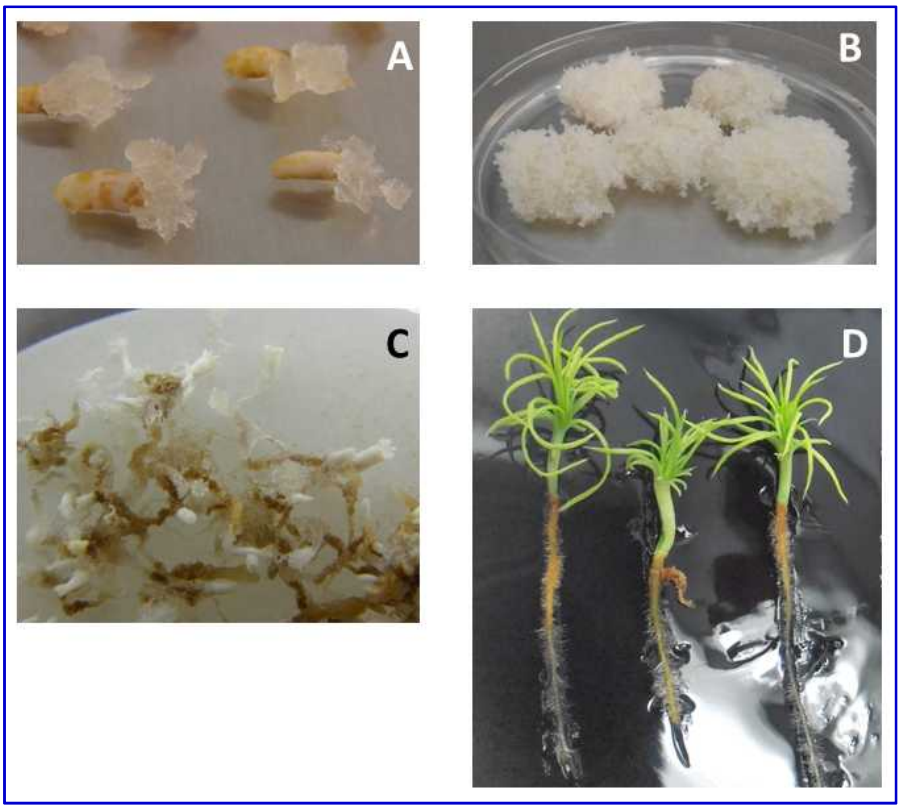

Figure 1. *Pinus radiata somatic embryogenesis process. Embryogenic masses initiation (A) and proliferation (B) in EDM medium (Walter et al. 1998). Somatic embryo maturation (C) cultured on EDM medium supplemented with 60 µM ABA, 6% sucrose and the EDM amino acid mixture for 12 weeks. Somatic embryos germination (D) on half strength modified LP (Aitken-Christie et al. 1988) with activated charcoal.*

Recent significant advances have been made with initiation in radiata pine using pre-cotyledonary zygotic embryos as described earlier in this section with both open and control-pollinated seed (Hargreaves et al. 2009; 2011). Open and control-pollinated seed sampled at optimum developmental stages gave average initiation success rates of 69-70% for all the 39 crosses tested with dissected embryo treatments. However, the procedure of excising the zygotic embryos from

megagametophytes is time consuming and requires sophisticated technical skill not to damage or contaminate the immature zygotic embryo.

Other key issues involved in SE initiation are the culture media. Embryo Development Medium (EDM) (Smith 1996) has traditionally been used for radiata pine initiation and proliferation of EMs although the research group at SCION experimented with Glitz (a modified Litvay medium) obtaining significantly better results than with EDM (Litvay et al. 1985; Hargreaves et al. 2009). The influence of the organic nitrogen source has also been evaluated; in this experiment, EDM and Casein plus Glutamine Medium (CGM), filter-sterilized amino acid solutions adjusted to 5.7 were evaluated (Table 1). The CGM amino acid mixture has been employed in several *Pinus* species (Klimaszewska et al. 2001; Lelu-Walter et al. 2006; Carneros et al. 2009). There were no significant differences in the initiation percentages of EMs initiated on these media although the tissue showed a lack of organization at microscopic level in cultures on CGM (Montalbán et al. 2012). The disorganization of the embryogenic tissue has been associated with EMs ageing and poor maturation yields in *P. pinaster* (Breton et al. 2005; 2006).

Table 1. *Amino acid composition of EDM and CGM media.*

Amino acids	EDM	CGM
L-glutamine	550 mg L^{-1}	500 mg L^{-1}
L-asparatine	525 mg L^{-1}	0
L-arginine	175 mg L^{-1}	0
L-citrulline	19.75 mg L^{-1}	0
L-ornithine	19 mg L^{-1}	0
L-lysine	13.75 mg L^{-1}	0
L-alanine	10 mg L^{-1}	0
L-proline	8.75 mg L^{-1}	0

To obtain the growth of EMs, the culture medium is supplemented with 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 µM benzyladenine (BA) as well as 3% sucrose (w/v) and 3 g L^{-1} gellan gum (Gelrite®). Petri dishes (90 X 20 mm) are used as culture container for the initiation stage.

After 4-8 weeks from the start of the experiment, EMs growing with a size around 3-5 mm in diameter are separated from the megagametophytes and the embryogenic lines can be considered as initiated cell lines, being available for transfer to the proliferation medium. Cultures are incubated in dark or low light (5 µmol m^{-2} s^{-1}) at 23ºC ± 1ºC.

3. Proliferation of EMs

Proliferation of newly initiated EMs is the next important step (Figure 1 B). One of the difficulties encountered in conifer SE for commercial scale is to amass enough material to begin plant production (up to 4 months) (Lelu-Walter et al. 2006). It is necessary to adjust the optimum culture conditions to get the maximum success in a short period of time for this stage. EMs should be sub-cultured to the maintenance (or proliferation) medium every 2 weeks. The maintenance medium has the same composition as chosen for the previous stage but with a higher concentration of Gelrite®, 4.5 g L^{-1}, in order to maintain the spiky morphotype of embryogenic cell lines (ECLs) (Breton et al. 2005). Some metabolites such as amino acids have been correlated with the embryogenic mass proliferation in some conifers (Robinson et al. 2009). In radiata pine, when the effect of amino acid composition of the medium was analysed, significant differences in proliferation rates were observed. Moreover, the micromorphological structure of EMs was also different and embryogenic tissue established on EDM presented a well-organized structure (Montalbán et al. 2012). EMs proliferating on CGM showed a more unorganised structure; to distinguish early embryos was not possible and these lines stopped proliferating after 4 months. Embryogenic lines proliferating on EDM medium showed a more organised morphology and continued proliferating after 6 months. In the same way, the combined effect of excised embryos and Glitz medium showed a positive effect at the early post initiation phase proliferation stage (Hargreaves et al. 2009). In subsequent work Hargreaves and co-workers (Hargreaves et al. 2011) compared a modified Glitz medium (that excluded casein hydrolysate and included an additional 950 mg L^{-1} of L-glutamine and 1000 mg L^{-1} of asparagine (Glitz2) with BLG1 a modified Verhagen and Wann medium (Verhagen and Wann 1989; Find et al. 2002; Walter et al. 2005). After 28 days of growth on the two tested media, Glitz2 and BLG1, the embryogenic mass showed mean increases of 25 and 29 fold respectively which was a major improvement when compared to leaving the tissue on the initiation media of Glitz which was also included as a treatment (in excess of 1700 cell lines from 20 control pollinated families were tested). Different culture morphology was also observed with BLG1 having more organised structures and fewer embryo initials than Glitz2.

4. Maturation of ECLs

Maturation of ECLs is one of the bottlenecks of the SE process (Figure 1 C). Maturation of ECLs into normal cotyledonary somatic embryos is not always successful in radiata pine. Sometimes, it is common to find abnormal morphology (Abrahamsson et al. 2012), asynchronous embryo production as has been described in other species (Yildirim et al. 2006), or poor root development. In this stage, the

composition of the culture medium takes on special importance due to it being the source of adequate amounts of nitrogen and carbon (Lin and Leung 2002). The procedure to maturate EMs starts with its suspension in liquid growth regulator-free EDM medium (Walter et al. 1998) and shaking the culture flask vigorously by hand for a few seconds. Thereafter, a 5 mL aliquot containing between 70 and 150 mg fresh mass of suspended EM is poured onto a filter paper disc in a Büchner funnel. The use of a bigger amount of EMs can stimulate an overgrowth of the tissue that appears to hinder the maturation process. A vacuum pulse is applied for 10 s, and the filter paper with the attached EM is transferred to maturation medium. The maturation medium traditionally used is EDM supplemented with concentrations of abscisic acid between 57 and 90 µM, sucrose at 175 mM, 9 g L^{-1} gellan gum and the previously described amino acid mixture named EDM. The use of activated charcoal has been tested in several *Pinus* species demonstrating a positive effect on maturation (Lelu-Walter et al. 2006). Activated charcoal is not recommended in radiata pine due to embryos showing abnormal morphology and a tendency to germinate precociously (Montalbán et al. 2010).

On the other hand, a common practice to stimulate a shift in the developmental program of the EMs from proliferation to production of embryos is to reduce water availability by increasing the gellam gum and/or sucrose concentration (Ramarosandratana et al. 2001). In fact, the increase of osmolarity seems to be a key factor in improving maturation performance (Montalbán et al. 2010). In previous experiments, we analysed the effect of sucrose concentration and type of amino acid in the maturation medium and the results showed that the media with the highest osmolarity (EDM amino acid mixture and 60 g L^{-1} sucrose) were the best maturation treatments. In the same study, we observed that the amount of sucrose in combination with the organic nitrogen was more critical to somatic embryo development than the concentration of ABA (Montalbán et al. 2010). In this sense, it has been suggested that the composition of the culture medium, particularly the carbohydrate to nitrogen ratio, may represent a key factor responsible for the expression of certain glutamine synthetase-related and photosynthesis-related genes (Pérez-Rodríguez et al. 2006). No subcultures are required during the entire maturation period. Environmental conditions are usually 24 hour dark at 23ºC±1ºC.

5. Germination of somatic embryos

Conversion of somatic embryos (se) into plants is successful when somatic embryos are cultured on half-strength LP medium [(Quorin and Lepoivre 1977), modified by Aitken-Christie et al. (1988)] supplemented with 5.5 g L^{-1} gellan gum and activated charcoal (Hargreaves et al. 2005) (Figure 1D). The use of activated

charcoal has also been recommended for se germination in other species of pines (Salajova and Salaj 2005). The beneficial effect of this component in the germination medium could be attributed to its property of adsorbing residual plant growth regulators (von Aderkas et al. 2002) although it should be combined with a proper mineral combination such as the one in 1/2LP in which germinated embryos become green and present a normal morphology in comparison with the results obtained when using EDM medium (Montalbán et al. 2010). Somatic embryos are cultured in petri dishes with the embryonal root caps pointing downwards, and tilted vertically at an angle of 45º following the protocol described by Klimaszewska et al. (2001). Cultures are maintained at 21 ± 1 ºC under a 16-h photoperiod at 120 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes. Somatic embryos are sub-cultured after 6 weeks onto fresh medium of the same composition. It is also remarkable that the *Pinus radiata* se do not need any pre-treatment due to the high quality of the somatic embryos obtained, resulting in a significant saving of cost and labour (Montalbán et al. 2010).

After 14-16 weeks on germination medium, the plantlets were transferred to sterile peat:perlite (3:1) and acclimatized in a greenhouse with a decrease of the air humidity from 99 to 70% over 5 weeks in controlled environmental conditions.

6. Cryopreservation of embryogenic cell lines

Cryopreservation of EMs is a method to avoid the loss of embryo maturation potential during long-term culture as well as a means to avoid possible somaclonal variation caused by the long-term maintenance of actively-growing embryogenic cultures (Hargreaves et al. 2002). Furthermore, the cryopreservation of EMs permits the maintenance of the juvenile characteristics of the tissue until the clonal field tests have been done (Walter et al. 1998). In *Pinus radiata*, the first protocol to carry out this method was described many years ago (Hargreaves and Smith 1992). However, to achieve this technology on a commercial scale, all the genotypes should be able to survive the process, cultures must retain unaltered genotypes. Find et al. (1998) described some possible reasons for the differences in cryotolerance such us the change of cellular and morphological composition before cryopreservation. Hargreaves and co-workers found that it was important to cryopreserve tissue that was in an active state of cell division and, therefore, EM was cryopreserved after 10 days of sub-culture rather than after the routine 14 days for standard cell line maintenance (Hargreaves et al. 2002).

In summary, for cryopreservation EM (1 g fresh weight) is removed from the maintenance medium and suspended in liquid medium (3 mL), used previously for the proliferation stage, containing 0.4 M sorbitol. Re-suspended tissue is incubated on a shaker at 18-20ºC for 18-24 h under low light conditions. Then DMSO is added to give a final concentration of 10%. After that, aliquots are

transferred to cryovials and placed in a freezing container that had been pre-cooled to 0ºC prior to use. The container should then be stored in a -80ºC freezer for 90 min and after that the vials are transferred to liquid nitrogen storage where they can be stored indefinitely.

For thawing and regrowth, vials are removed from the liquid nitrogen (vapour phase) and introduced in a sterile water bath (45ºC) for 2 min and then the vial is wiped with 70% ethanol and the contents tipped out onto a sterile piece of nylon (Nybolt nylon screen, Scapa Filtration 30 μm diameter mesh) which is placed on top of a stack (4 pieces) of sterile paper towels. After that the nylon with the cells can be transferred to a petri dish containing culture medium with activated charcoal for one hour and are finally cultured in EDM medium. Current (2015) practice at SCION is to use Glitz2 medium for all these steps. Hargreaves et al. (2002) showed survival results from 78 to 100% of different cell lines stored for various years of duration. Moreover, they discovered that by growing the recovered tissue on top of a nurse culture (vigorous tissue maintained for 2 years by routine subculture at 2-weekly intervals; in 2002 the culture medium was EDM6, we now use Glitz2) a complete post-thaw recovery (100% of survival irrespective of storage period) could be obtained. The nurse tissue probably enhances the recovery of cryopreserved cell lines in several ways, i.e., as a physical platform, improved aeration of the cells and possibly by facilitating further removal of remaining cryoprotectants. Growing nurse tissue would also release extracellular proteins and other compounds that may improve the growth of the thawed tissue as has been reported by Chung et al. (1992). Subsequent initiation and post-initiation proliferation studies indicated that EDM6 is a bad treatment for isolated immature zygotic embryos, which further supports the hypothesis that the nurse tissue is modifying the basal medium in a beneficial way (Hargreaves et al. 2009).

7. Combined methods to increase the production of “Elite” embryogenic cell lines

Some of the main bottlenecks in *Pinus radiata* SE are: Low frequencies of initiation making this technique unfeasible for large-scale production (Klimaszewska et al. 2007); Low number of normal quality somatic embryos; and Low rates of germination and establishment in the greenhouse.

In summary, to have some “elite” cell lines with a low embryo quality and/or low germination frequency makes large-scale production of superior genotypes too expensive (Davis and Becwar 2007). To overcome these problems associated with SE, a combined SE and organogenesis protocol can be carried out with high value cell lines. This method was described by Montalbán et al. (2011). Briefly, the method consists on the use of somatic embryos as initial explants for

organogenesis on ½ LP supplemented with 3% sucrose, 8 g L^{-1} Difco Agar® granulated and 4.4 µM BA (pH= 5.8). As container, petri dishes (90 X 15 mm) with 15 mL of medium were used and embryos are cultured in an inverted position with the cotyledons immersed in the induction medium where they form adventitious meristematic tissue (Aitken-Christie et al. 1988). After the meristematic tissue induction, explants are transferred to glass jars with medium without plant growth regulators but with 0.2% activated charcoal (w/v). On this medium the adventitious meristems elongate into well-formed shoots (Figure 2). Cultures are kept at 22±1 ºC under a 16-h photoperiod of 120 mmol m^{-2} s^{-1}.

Figure 2. *Shoot induction in Pinus radiata somatic embryos after 4 weeks growing in modified ½ LP (Aitken-Christie et al. 1988) supplemented with 4.4 µM BA.*

In summary, if the shoots obtained from the somatic embryos have a rooting percentage of around 60%, up to 19 rootable shoots can be obtained from a single somatic embryo. Considering that we can obtain more than 1500 embryos per g of EM (Montalbán et al. 2010), the described method can theoretically produce more than 17000 rooted shoots from that mass of tissue (Montalbán and Moncaleán 2012). Moreover, these shoots can be propagated before rooting and continuously used as a source for plant regeneration. However, as a note of caution, the use of this amplification method could lead to growth differences similar to those observed in plants of adventitious origin or obtained non-adventitiously from axillary meristems of plants that had originated from zygotic embryos. Significant differences in *in vitro* multiplication, adventitious root formation on the shoots after removal from the culture flasks, slower growth and increased physiological signs of maturation were observed on shoots of adventitious origin in comparison to shoots of epicotyl-axillary shoot origin of plants obtained from the same zygotic embryo (Hargreaves et al. 2005). A more useful method is to simply turn the germinated se into organogenic shoot cultures; methods are well developed for organogenesis from epicotyls or from axillary meristems derived from shoot tips of

field grown plants (Hargreaves et al. 2005; Hargreaves and Menzies 2007). No BA is required for this type of organogenesis in radiata pine, elongated shoots are given an auxin treatment to induce adventitious root formation and are transferred to greenhouse conditions prior to root initiation. SCION routinely uses this method to amplify se for research projects and both Forest Genetics and Arborgen in New Zealand use similar methods for commercial production of SE derived clonal material (Figures 3, 4).

Figure 3. *Shoots from Pinus radiata organogenic cultures established from somatic embryos as initial explant showing the root system.*

Figure 4. *Established plants derived from organogenic shoots generated from Pinus radiata in vitro-germinated somatic embryos.*

In summary, for radiata pine, the progress in improving SE protocols as described here has been significant over the past couple of decades and in New Zealand significant numbers of field proven SE origin plants are now being commercially produced on an annual basis (www.forest-genetics.com).

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Somatic embryogenesis and plant propagation in Japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*Pinus densiflora* Zieb. *et* Zucc.)

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Abstract

Somatic embryogenesis in *Pinus thunbergii* and *P. densiflora* was initiated from megagametophytes containing immature zygotic embryos. Embryogenic cultures were maintained and proliferated by 2-to-3-week interval subcultures in medium supplemented with 2,4-dichlorophenoxyacetic acid and 6-benzylaminopurine. Somatic embryo maturation experiments were performed in darkness at 25°C, culturing the embryogenic tissues on maturation media containing 30-50 g l^{-1} maltose, 2 g l^{-1} activated charcoal, 100 µM abscisic acid, and either polyethylene glycol or a high concentration of gellan gum. Low germination frequencies, around 16%, were achieved with somatic embryos after maturation on medium supplemented with polyethylene glycol. In contrast, when somatic embryos were matured on medium containing a high concentration of gellan gum, without polyethylene glycol, the germination frequency recorded for both species was around 80%. Somatic embryos matured with polyethylene glycol were desiccated to improve both germination and plant conversion frequencies. Desiccation of somatic embryos at high relative humidity resulted not only in a marked increment in germination frequency but also subsequently improved the plant conversion rate. In addition, this treatment resulted in a considerable improvement in synchronization of the germinants, compared to the ones of the untreated control. Somatic plants were acclimatized and their growth has been monitored in the field.

Keywords: conifers, desiccation treatment, embryogenic cultures, gellan gum, megagametophytes, plant conversion, polyethylene glycol, post-maturation

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treatments, somatic embryo maturation, somatic plants

1. Introduction

Japanese black pine (*Pinus thunbergii* Parl.) and Japanese red pine (*P. densiflora* Zieb. *et* Zucc.), are two important forest tree species widely used for reforestation and landscaping in Japan. *P. thunbergii* is also important as windbreaks against sand movement and salt spray in coastal areas, and *P. densiflora* as host species of the prized "matsutake" mushroom. Nowadays, the populations of these two species have dramatically decreased as a result of pine wilt disease, caused by the pinewood nematode, *Bursaphelenchus xylophilus* (Steiner et Buhrer) Nickle (Kiyohara and Tokushige 1971), which is transmitted in Japan by two cerambycid beetles, *Monochamus alternates* Hope (Mamiya and Enda 1972) and *M. saltuarius* (Gebler) (Sato et al. 1987). The pine wilt disease is one of the most serious epidemic tree diseases in Japan and has been a critical factor in the mass mortality not only in *P. thunbergii* and *P. densiflora*, but also in other Japanese pine forests such as *P. luchuensis* Mayr (Ryukyu pine) and *P. armandii* Franch. var. *amamiana* (Koidz.) Hatusima (Yakutanegoyou) (Mamiya 1983, Kishi 1995, Kanetani et al. 2001, Maruyama and Hosoi 2012). Therefore, the development of an efficient and stable plant regeneration system is essential for the large-scale propagation of resistant clones derived from plants obtained in long-term breeding programs. Somatic embryogenesis (SE) is the most promising technique for mass propagation of clones, for *ex situ* conservation of genetic resources by cryopreservation, and for plant regeneration after genetic transformation. However, for many species, plant conversion efficiency has been one of the limiting factors in using SE for practical uses. Similarly, for Japanese pines, the plant conversion of mature somatic embryos is limited by the low frequency of root emergence.

Previously, we reported SE and plant regeneration in Japanese black pine (Maruyama et al. 2005a), and Japanese red pine (Maruyama et al. 2005b). In these studies, high maturation frequencies of cotyledonary somatic embryos on maturation media containing polyethylene glycol (PEG) were described; nevertheless, the subsequent germination frequencies achieved remained low. Later, we reported an improved somatic embryo germination protocol for Japanese pines based on the desiccation of somatic embryos after the maturation step (Maruyama and Hosoi 2012). This post-maturation treatment markedly increased germination frequencies and considerably improved synchronization during the germination period. Similarly, in order to improve the somatic embryo germination protocols, post-maturation treatments based on the desiccation of somatic embryos have also been reported to successfully improve germination frequencies in conifers (Hay and Charest 1999, Klimaszewska and Cyr 2002, Stasolla and Yeung 2003). On the

other hand, methods involving reduction in water availability to the cultured cells by increasing the medium gel strength with a high gelling agent concentration have also been reported to efficiently improve somatic embryo germination in several species of pine, spruce, and larch, without any post-maturation treatments (Klimaszewska and Cyr 2002, Klimaszewska et al. 2007, Lelu-Walter et al. 2008, Lelu-Walter and Pâques 2009, Kim and Moon 2014).

This chapter describes the two methods most commonly used for SE in pine species. The germination and plant regeneration results obtained with somatic embryos of *P. thunbergii* and *P. densiflora* after maturation on medium with PEG or a high concentration of gellan gum are compared. The beneficial effect of somatic embryo desiccation after PEG–mediated maturation is also described.

2. Materials and methods

2.1 Source of plant material and embryogenic culture

Embryogenic tissues (ET) were induced from immature seeds collected from open-pollinated sources of Japanese black pine and Japanese red pine, as described by Maruyama et al. (2005a) and Maruyama et al. (2005b), respectively. Excised megagametophytes were cultured on initiation medium (Maruyama et al. 2000), containing 0.5 g l^{-1} casein hydrolysate, 1 g l^{-1} l-glutamine, 10 g l^{-1} sucrose, 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 5 μM 6-benzylaminopurine (BA), and 3 g l^{-1} gellan gum (Gelrite®; Wako Pure Chemical, Osaka, Japan). The pH of the medium was adjusted to 5.8 before sterilization. To promote proliferation, the ET were transferred into the proliferation medium (initiation medium supplemented with 30 g l^{-1} sucrose, 3 μM 2,4-D, 1 μM BA, and 1.5 g l^{-1} glutamine). ET were maintained and proliferated by subculturing at 2- to 3-week intervals, keeping the cultures in the dark at 25°C.

2.2 Maturation of somatic embryos

About 500 mg of freshly weighed ET were suspended in about 3 ml of liquid proliferation medium without plant growth regulators (PGR) and was poured over a 90 × 20 mm plates containing 30–40 ml of semisolid maturation medium. The maturation medium (Maruyama et al. 2000), containing 30-50 g l^{-1} maltose, 2 g l^{-1} activated charcoal (AC), 100 μM abscisic acid (ABA), amino acids (Smith 1996) (in g l^{-1}: glutamine 7.3, asparagine 2.1, arginine 0.7, citrulline 0.079, ornithine 0.076, lysine 0.055, alanine 0.04, and proline 0.035), and 0-150 g l^{-1} PEG (Av. Mol. Wt.: 3000; Wako Pure Chemical, Osaka, Japan) or a high concentration of gellan gum (10 g l^{-1} Gelrite®, without PEG), was used. The plates were sealed with Parafilm and kept in darkness at 25°C for 8–12 weeks.

2.3 Desiccation treatment of somatic embryos after maturation with PEG

For desiccation treatment, cotyledonary somatic embryos from PEG-maturation medium were placed on 30-mm-diameter filter paper disks (Figure 1C) and added into two central wells of a six-well multiplate (Iwaki, AGC Techno Glass Co., Ltd., Chiba, Japan), and the remaining four side wells of the multiplate were filled with 5–6 ml of sterile water, tightly sealed with Parafilm, and kept in the dark at 25°C for 3 weeks (Maruyama and Hosoi 2012). Under these conditions, the generated relative humidity registered with a thermo-hygrometer recorder (RS-10, ESPEC MIC Corporation, Aichi, Japan) was approximately 98%.

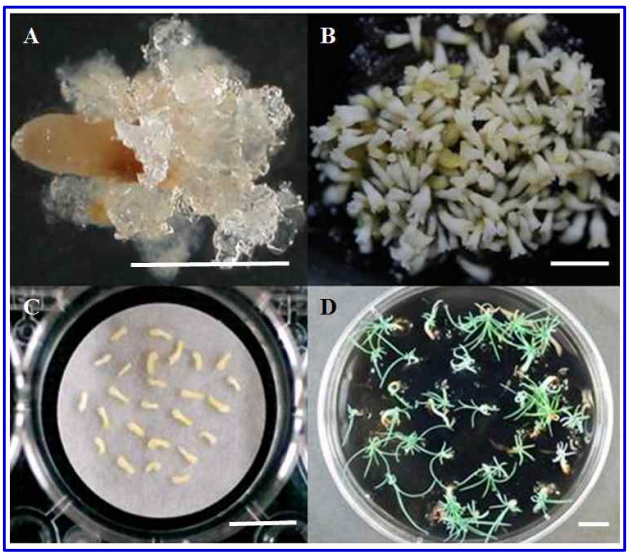

Figure 1. *Somatic embryogenesis in Pinus thunbergii.* ***A*** *Embryogenic tissues.* ***B*** *Cotyledonary somatic embryos.* ***C*** *Desiccation of somatic embryos.* ***D*** *Germination and plant conversion. Bars 1 cm*

2.4 Germination of somatic embryos and plant conversion

Mature somatic embryos were transferred into germination medium containing basal salts at concentrations similar to those used for maintenance and proliferation, but without PGR, and supplemented with 30 g l^{-1} glucose, 2 g l^{-1} AC, 0.4 g l^{-1} glutamine, 0.25 g l^{-1} arginine, and 0.1 g l^{-1} proline, and solidified with 6 g l^{-1} gellan gum. Cultures were kept at 25°C under a photon flux density of about 65 µmol m^{-2} s^{-1} provided by cool, white fluorescent lamps (100 V, 40 W; Toshiba, Tokyo, Japan) for 16 h. The number of somatic embryos that germinated and converted into plantlets was recorded after 6 and 12 weeks, respectively.

2.5 *In vitro* growth and acclimatization of somatic plants

Regenerated plantlets were transferred into 300-ml flasks containing 100 ml of fresh germination medium supplemented with 30 g l^{-1} sucrose, 5 g l^{-1} AC, and 12 g l^{-1} agar (Wako Pure Chemical Industries, Osaka, Japan) or into Magenta® vessels (Sigma, St. Louis, USA) containing Florialite® (Nisshinbo Industries, Inc., Tokyo, Japan) and irrigated with a plant food solution modified from Nagao (1983), which included 143 mg l^{-1} NH_4NO_3, 55.1 mg l^{-1} $NaH_2PO_4 \cdot 2H_2O$, 47.1 mg l^{-1} KCl, 52.5 mg l^{-1} $CaCl_2 \cdot 2H_2O$, 61 mg l^{-1} $MgSO_4 \cdot 7H_2O$, 25 mg l^{-1} Fe(III) EDTA, 0.1 mg l^{-1} Cu EDTA, 0.1 mg l^{-1} Mn EDTA, 0.1 mg l^{-1} Zn EDTA, 1.5 mg l^{-1} H_3BO_3, 0.01 mg l^{-1} KI, 0.005 mg l^{-1} $CoCl_2 \cdot 6H_2O$, and 0.005 mg l^{-1} MoO_3, and kept under the same conditions as described above for 16–20 weeks prior to *ex vitro* acclimatization. The developed plants were transplanted into plastic pots filled with Kanuma soil and acclimatized in a growth cabinet as described by Maruyama et al. (2002).

3. **Results**

3.1 Initiation, maintenance, and proliferation of ET

ET were initiated in 7 out of the 8, and 9 out of the 10 seed families of *P. thunbergii* and *P. densiflora* tested, respectively. The extrusion of ET from the micropylar end of explants occurred mostly after 3–8 weeks of culture. In both species, around 2% of megagametophytes tested had extruded ET. However, SE was initiated mostly after 8–12 weeks of culture, during which time the ET mass increased (Figure 1A). Nineteen explants from 1,104 megagametophytes (1.7%) and 14 from 1,286 (1.1%) with proliferating ET were obtained for *P. thunbergii* and *P. densiflora*, respectively. Although the low SE initiation rates achieved for both species were similar to the results reported elsewhere (Ishii et al. 2001, Taniguchi 2001), and for other pine species such as *P. bankesiana* (Park et al. 1999), *P. patula* (Jones and van Staden 1999), *P. rigida x P. taeda* (Kim and Moon 2007), *P. armandii* var. *amamiana* (Hosoi and Ishii 2001, Maruyama et al. 2007), and *P. luchuensis* (Hosoi and Maruyama 2012), these low initiation frequencies are one of the key problems to resolve before practical application will become possible. In contrast to our results, higher initiation rates were reported for *P. sylvestris* (up to 22%) and *P. pinaster* (up to 40%) (Lelu et al. 1999), *P. strobus* (up to 53%) (Klimaszewska et al. 2001), and *P. taeda* (up to 79%) (Gupta 2014). In several cases, however, the initiation of SE may not result in the capture of stable embryogenic lines because ET growth may cease after the initial extrusion. Therefore, the capture of stable cell lines is the most appropriate criterion by which to compare the ability of SE initiation among species and families (Maruyama et al. 2007).

Initiation medium supplemented with 3 μM 2,4-D and 1 μM BAP

supported the growth of all embryogenic cell lines tested. ET proliferated readily by subculturing at 2- to 3-week intervals, retaining their original translucent and mucilaginous appearance.

3.2 Maturation of somatic embryos

The development and maturation patterns in both species were similar to those described for other pines (Becwar et al. 1990, Smith 1996, Lelu et al. 1999). About 2 weeks after transfer onto maturation media, ET developed gradually and formed an individual and compact mass when approaching the mature stage (Figure 2A-C). Cotyledonary embryos were first observed about 3–4 weeks after transfer of ET (Figure 2D-E), and were completely mature after 8-12 weeks of culture (Figure 2F). As shown in Table 1, the addition of PEG to the medium dramatically stimulated embryo maturation, and the number of mature somatic embryos increased with increasing PEG concentrations up to 100 g l^{-1}, but decreased at 150 g l^{-1}. In contrast, on PEG-free medium, ET proliferation was evident and most of them developed into small embryonal heads with elongated suspensors extending from them and described elsewhere as stage 1 somatic embryos (von Arnold and Hakman 1988). In the absence of PEG, only a few early somatic embryos developed into cotyledonary stage ones. This result is consistent with the results reported for several Japanese conifer species (Maruyama et al. 2000, Ishii et al. 2001, Maruyama et al. 2002, Maruyama et al. 2005a, Maruyama et al. 2005b, Maruyama et al. 2005c, Shoji et al. 2006, Maruyama et al. 2007, Hosoi and Maruyama 2012).

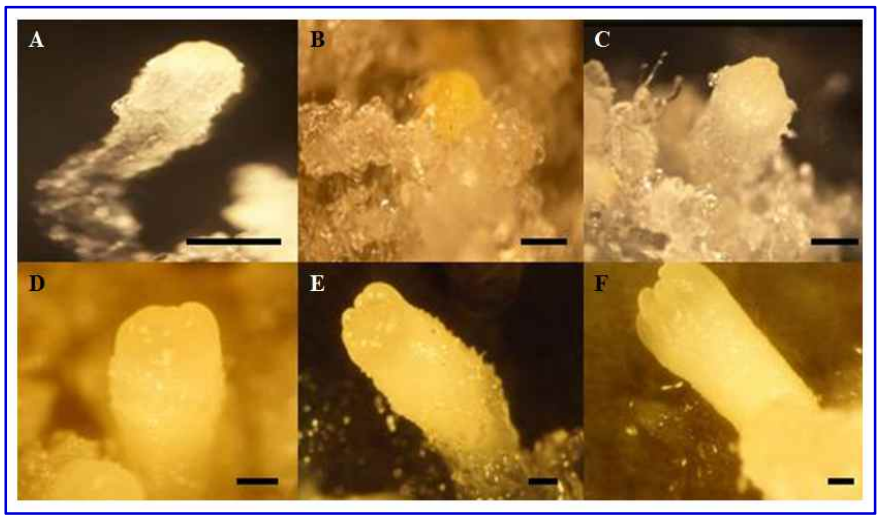

Figure 2. *Somatic embryogenesis in Pinus densiflora.* ***A-F*** *Different developmental maturation stages of somatic embryos. Bars 1 mm*

On the other hand, although the number of somatic embryos produced by the cell line tested was inferior to that of the best results achieved with PEG-media, gellan gum at concentration of 10 g l^{-1} as the gelling agent in the PEG-free maturation media was also effective in the maturation of somatic embryos. Similar to the results obtained on PEG-media, somatic embryo maturation was even more enhanced in the presence of AC (Table 1). Kim and Moon (2014) also reported the beneficial effect of producing somatic embryos of *P. densiflora* on medium supplemented with AC.

Table 1. *Variation in media tested and effect on somatic embryo production in Pinus thunbergii*

Media	PEG (g l^{-1})	AC (g l^{-1})	ABA (µM)	Gellan gum (g l^{-1})	Somatic embryos per plate (SE)
P0	0	0	100	3	8 (5) a
P0AC	0	2	100	3	23 (9) a
P25AC	25	2	100	3	80 (13) ab
P50	50	0	100	3	106 (30) abc
P50AC	50	2	100	3	140 (28) abc
P75	75	0	100	3	157 (40) abc
P75AC	75	2	100	3	455 (85) d
P100	100	0	100	3	135 (20) abc
P100AC	100	2	100	3	468 (32) d
P150AC	150	2	100	3	266 (70) c
P0G10	0	0	100	10	103 (37) abc
P0G10AC	0	2	100	10	191 (64) bc

SE, standard errors of means from five replicates for each treatment

Means followed by same letter are not significantly different at P <0.05

Despite the fact that the average embryo maturation frequency varied according to the species and among cell lines (data not shown), the supplement of 100 g l^{-1} PEG to the medium in combination with 30-50 g l^{-1} maltose, 100 µM ABA, and 2 g l^{-1} AC, was found to be suitable for an efficient somatic embryo production with both species.

3.3 Germination of somatic embryos and plant conversion

As shown in Table 2, when somatic embryos matured on PEG-medium were placed directly on the germination medium, the root emergence of embryos and the subsequent plant conversion occurred at a low frequency (an average of about 16% and 12% for *P. thunbergii* and *P. densiflora*, respectively). In contrast, after somatic embryo maturation had taken place on medium containing a high

concentration of gellan gum without PEG, the germination frequency recorded was around 80% for both species. Subsequently, the frequency of somatic embryos that developed into plantlets was 78% and 70% for *P. thunbergii* and *P. densiflora*, respectively.

3.4 Effect of desiccation treatment on the germination frequency of somatic embryos matured with PEG

Somatic embryos matured on PEG-medium (Figure 1B) were desiccated (Figure 1C) in attempts to improve germination frequencies. Desiccation of

Table 2. *Effect of post-maturation treatment and a high concentration of gellan gum in maturation medium on germination and conversion frequencies in somatic embryos of Pinus thunbergii and P. densiflora*

Species	Cell line	Germination frequency (%)			Conversion frequency (%)		
		Control *1	Post-maturation *2	Gellan gum *3	Control *1	Post-maturation *2	Gellan gum *3
P. thunbergii	T216-2-1	21 (109/530)	71 (556/784)	68 (967/1422)	20 (104/530)	70 (547/784)	67 (946/1422)
	T205-3-3	60 (150/250)	96 (346/361)	70 (171/245)	51 (128/250)	91 (330/361)	65 (159/245)
	T205-3-6	47 (47/100)	70 (70/100)	83 (132/160)	38 (38/100)	61 (61/100)	80 (128/160)
	T216-4-1	2 (4/200)	85 (170/201)	92 (194/210)	1 (2/200)	80 (160/201)	90 (190/210)
	T205-4-1	0 (0/100)	79 (79/100)	94 (94/100)	0 (0/100)	78 (78/100)	93 (93/100)
	T205-4-2	7 (7/100)	70 (105/151)	95 (295/310)	3 (3/100)	67 (100/150)	93 (288/310)
	T205-4-3	9 (9/100)	55 (55/100)	91 (100/110)	5 (5/100)	50 (50/100)	91 (100/110)
	Sm64-6-1	6 (12/200)	93 (296/320)	95 (572/600)	1 (2/200)	90 (288/320)	93 (555/600)
	Tn54-8-5	0 (0/100)	84 (240/287)	66 (119/180)	0 (0/100)	75 (215/287)	64 (115/180)
	Tn54-8-8	1 (1/102)	82 (82/100)	84 (84/100)	0 (0/102)	78 (78/100)	75 (75/100)
	Tn54-8-17	2 (2/100)	67 (80/120)	80 (120/150)	0 (0/100)	65 (78/120)	79 (118/150)
	Tn54-8-20	4 (10/245)	88 (408/466)	77 (154/200)	0 (3/245)	85 (395/466)	70 (140/200)
	Ms90-9-1	5 (5/100)	94 (188/200)	90 (358/400)	1 (1/100)	88 (176/200)	89 (354/400)
	Mn9037-9-1	8 (8/100)	77 (289/374)	81 (323/400)	2 (2/100)	76 (285/374)	80 (318/400)
	Total	15.6 (364/2,327)	80.9 (2,964/3,664)	80.3 (3,683/4,587)	12.4 (288/2,327)	77.6 (2,841/3,663)	78.0 (3,579/4,587)
P. densiflora	D15A	27 (55/202)	85 (169/200)	79 (316/400)	24 (49/202)	80 (159/200)	75 (299/400)
	D19A	13 (13/100)	80 (320/400)	83 (165/200)	10 (10/100)	76 (302/400)	81 (161/200)
	D19-42	20 (20/100)	73 (145/200)	88 (272/310)	10 (10/100)	70 (140/200)	87 (270/310)
	D19-44	4 (7/200)	61 (121/200)	67 (134/200)	1 (1/200)	50 (100/200)	56 (111/200)
	Total	16 (95/602)	76 (755/1000)	80 (887/1110)	12 (70/602)	70 (701/1000)	76 (841/1110)

Values in parentheses represent (germinated or converted somatic embryos / total somatic embryos tested)

*1 Somatic embryos generated on maturation medium supplemented with polyethylene glycol

*2 Somatic embryos generated on maturation medium supplemented with polyethylene glycol were partial desiccated at high relative humidity

*3 Somatic embryos generated on maturation medium supplemented with no polyethylene glycol and a high concentration of gellan gum

somatic embryos at high relative humidity resulted not only in a marked increment in the germination frequencies but also in the subsequent improvement of plant conversion rates in all the cell lines tested. The average germination and conversion frequency improved by around five-fold and six-fold, respectively compared with the frequencies obtained by somatic embryos that were not desiccated (Table 2). Germination started about 1–2 weeks after transfer into the germination medium and the embryos subsequently converted into plantlets after 4–8 weeks of culture (Figure 1D).

3.5 Plant regeneration and establishment in the field

Somatic plants were successfully acclimatized in a growth chamber at 25°C and 80% relative humidity. Subsequently, the acclimatized plants (Figure 3A, C) were transferred to a greenhouse and grown for about 1 year before transplanting to the field. The somatic plants showed 100% survival after being transplanted to the field and their growth is currently being monitored (Figure 3B, D).

Figure 3. *Somatic plants of Pinus thunbergii (**A-B**) and Pinus densiflora (**C-D**). **A**, **C** Acclimatized somatic plants. **B**, **D** Somatic plants growing in the field. Bars 10 cm (**A**, **C**), 1 m (**B**, **D**).*

4. Discussion

SE technology is the most recent vegetative propagation system to be implemented on an operational scale (Grossnicle 2011). In addition, the most important advantage of SE is that the ET can be cryopreserved without changing its genetic make-up and without loss of juvenility (Park et al. 1998). Hence, vegetative propagation by SE on an industrial scale has now been developed for several conifer species (Jain et al. 1995, Klimaszewska and Cyr 2002, Stasolla and Yeung 2003, Jain et al. 2005). However, for many species, such as Japanese pines, the low germination rate hampers efficient large-scale production and is one of the limiting factors for widespread commercial use (Maruyama et al. 2005a, Maruyama et al. 2005b, Maruyama et al. 2007). Efficient maturation and production of high quality somatic embryos that permit a high plant conversion frequency are the most important criteria that have to be met before SE protocols can be used in commercial mass production, breeding programs, and genetic engineering.

In order to improve plant conversion protocols, the desiccation of somatic embryos after PEG-mediated maturation has been recommended for conifer species. For most species, desiccation presumably acts to terminate developmental processes and to initiate those metabolic processes necessary to prepare the seeds for germination and growth (Kermode and Bewley 1985). Despite reports that desiccation of somatic embryos after the maturation process is beneficial in improving the quality of germinants, the germination frequencies vary according to the rate of desiccation and the desiccation tolerance of mature embryos. Desiccation tolerance in conifer somatic embryos generally decreased with increasing rapidity of desiccation (Bomal and Tremblay 1999). Relative humidities of 81% and lower were lethal to the somatic embryos of interior spruce, whereas germination was enhanced following treatments at humidities greater than 95% in comparison to the percentages obtained with untreated controls (Roberts et al. 1990). Similarly, the results of post-maturation treatments in somatic embryos of Japanese black pine indicated that the desiccation of somatic embryos at high relative humidity was most effective in promoting germination (Maruyama and Hosoi 2012). Although specific changes were not explored in this study, the results suggest that desiccation at high relative humidity causes germination-promoting physiological changes in somatic embryos and that the improved performance of somatic embryos after desiccation treatment can be attributed to a change in endogenous hormone levels and accumulation of storage reserves (Ackerson 1984, Dronne et al. 1997, Kong and Yeung 1992, Find 1997, Stasolla et al. 2001, Klimaszewska et al. 2004). The beneficial effect of desiccation treatment after maturation with PEG, improving the germination frequencies and decreasing the time required for germination, was also reported for somatic embryos of interior spruce (Roberts et al. 1990), sitka spruce (Roberts et al. 1991), white spruce (Kong

and Yeung 1992, Kong and Yeung 1995, Attree et al. 1995), hybrid larch (Lelu et al. 1995, Dronne et al. 1997), patula pine (Jones and van Staden 2001), and Ryukyu pine (Hosoi and Maruyama 2012).

On the other hand, in recent years it has been reported that reducing water availability to the cultured cells by increasing the medium gel strength (with a high concentration of gellan gum) in order to produce mature somatic embryos with low water content, has led to improved maturation protocols for several pine species. Restricting water availability has resulted in high germination rates and subsequent high plant conversion frequencies in *P. radiata* (Smith 1996), *P. strobus* (Klimaszewska and Smith 1997, Klimaszewska et al. 2001), *P. sylvestris* (Lelu et al. 1999), *P. monticola* (Percy et al. 2000), *P. pinaster* (Lelu et al. 1999, Lelu et al. 2006), and *P. halepensis* (Montalban et al. 2013). With this method, besides the fact that no pretreatment is required for germination, the ET may remain in the same medium throughout the entire maturation period, which lasts up to 12 weeks (Klimaszewska et al. 2007).

In conclusion, based on our results, the production of somatic embryos and subsequent plant conversion was feasible by both maturation methods for the efficient propagation of Japanese black pine and Japanese red pine. Although this improvement represents a promising perspective for efficient mass propagation of these species, further studies are required to establish an optimal protocol for the commercial production of high quality somatic plants. Evidence that culture conditions during somatic embryo development may affect the quality and the growth performance of somatic plants has been reported for several conifers (Grossnickle et al. 1994, Bozhkov and von Arnold 1998, Hogberg et al. 2001). Therefore, the growth characteristics of somatic embryo–derived plants obtained by both methods will be monitored in the field.

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Initiation of embryogenic suspensor masses and somatic embryogenesis in Japanese red pine (*Pinus densiflora*)

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Abstract

The best embryogenic suspensor mass (ESM) initiation frequencies were obtained from material collected June 28, 2004, Suwon (0.88%), July 1st, 2005, Suwon (1.4%), July 1st, 2005, Anmyeon (2.31%) and July 1st, 2006, Suwon (0.91%), All embryos in the seeds were at the proembryo stage regardless seed collection year (2004, 2005 or 2006) or location (Suwon or Anmyeon). Albeit, it is well known that seed development may vary with climate, from year to year by latitude and elevation. The initiation frequency of ESM and histological results suggest that the optimum yearly seed collection dates are between June 28, and July 5, at least for *Pinus densiflora*, in Korea. Histological analysis of zygotic embryos (proembryos) of seeds, that had been harvested at various times or locations, revealed no significant morphological differences in the stages of development at the various locations. These results show that seed collection time could be critical for obtaining a high rate of ESM initiation.The highest proliferation rate (9.8-fold) of ESM was obtained with ½LM medium supplemented with 3.42 mM L-glutamine. The highest growth ratio with brassinolide (BL) was observed for 1.0 μM (2.3 fold, line 05-21) and 0.05 μM (2.9 fold, line 06-22). However, in the ESM lines 05-21 and 06-22, high ESM growth rates (2.3 fold, line 05-21 and 2.1 fold, line 06-3) were seen without BL when compared with 1.0 μM (05-21) or 0.05 μM (06-22) BL. BL-supplemented medium had a diverse, genotype-specific effect on the degree of ESM proliferation. The highest number (798/g-1 FW) of cotyledonary somatic embryos (line 06-29) was obtained with 0.05% activated charcoal (AC) in the maturation medium. With regard to germination of somatic embryos of the ESM line 05-3 exposed to light-emitting diodes (LED), the frequency was strongly inhibited by both fluorescent and red+blue light (0% germination in both cases). Other lines (05-12, 05-29 and 05-37) showed similar germination patterns when exposed to five different LED sources.

Keywords: germination, glutamine, Japanese red pine, light-emitting diodes, seed

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development, somatic embryos.

1. Introduction

Japanese red pine (*Pinus densiflora*) is an evergreen conifer that grows in East Asia, including Korea, and is ecologically and economically a major forest tree used for reforestation and landscaping. In Korea, it is widely cultivated both for timber and as an ornamental. However, recently, pine wilt disease, caused by the pinewood nematode *Bursaphelenchus xylophilus* has attacked the trees and spread to the entire country, and has become a serious problem in some pines (including this species). Therefore, a long-term pine breeding project was initiated to select resistant clones and propagate them in large numbers. In order to propagate the species, more efficient propagation methods are needed. Among them, somatic embryogenesis (SE) is a promising technique because it offers the capability to produce unlimited numbers of propagules (Sutton 2002).

Initiation of ESM is the most critical step for the application of SE in conifer tree propagation, including for *P. densiflora.* Unfortunately, most *Pinus* species have shown a low initiation frequency of ESM and the limitation in the number of genotypes that can be regenerated through SE may represent a serious constraint to the successful commercial application of this technology. In addition, though various kinds of basal media and PGR combinations have been explored in efforts to increase initiation of ESM formation, the most important factor - the effect of collection time or developmental stage of the zygotic embryos – has not been studied fully. Becwar et al. (1990) reported that several factors play a role in enhancing ESM in immature zygotic embryos of *Pinus* species: genotype (Becwar et al. 1990), collection time or developmental stage of zygotic embryos (Becwar et al. 1990) and levels of Phytagel (Li et al. 1998), brassinolide (Pullman et al. 2003b), abscisic acid (ABA) and silver nitrate (Pullman et al. 2003a) and vitamins B12 and E in the culture medium (Pullman et al. 2006). Although various kinds of basal media and plant growth regulator combinations have been effective in the initiation of ESM, the most important factor

– The relationship between collection time and zygotic embryo development has not been studied fully; nor has the optimal collection date of immature seeds that would result in the highest frequency of ESM initiation been determined. Few reports have been published on ESM initiation in *P. densiflora* (Ishii et al. 2001; Taniguchi 2001; Maruyama et al. 2005; Shoji et al. 2006) and they do not provide sufficiently detailed information on the close relationship between embryo developmental stages (shown by histological sections) and collection dates/sites for successful ESM initiation in this species. Therefore, one of the objective of this study was to determine optimal seed collection dates for the initiation of ESM, by looking at sections of embryos and seed at various developmental stages.

L-Glutamine is a common organic nitrogen source in plant tissue culture media and provides reduced nitrogen in a form that is energetically less costly to assimilate than nitrate or ammonium. In experiments with conifers, cell suspensions of *Pseudotsuga menziesii* grew rapidly in medium with glutamine as the only nitrogen source. The beneficial effect of L-glutamine on somatic

embryogenesis has also been reported elsewhere. Hristoforoglu et al. (1995) reported ESM lines of *Abies alba* proliferated faster and matured better on medium containing L-glutamine and casein hydrolysate than on medium without them. *Picea mariana* somatic embryos matured in medium with glutamine as the sole source of nitrogen (Khlifi and Tremblay, 1995).

Acivated charcoal (AC) is known for its adsorption of residual plant growth regulators and since ESM had been cultured on a medium with 2,4-D and BA prior to transfer onto maturation medium, the beneficial effect of coating the cells with AC particles might be attributed to this particular property.

Finally, the use of LED as a radiation source of plants has attracted considerable interest in recent years because of its vast potential for commercial application. Light conditions play an important role in plant cell and tissue cultures. Light quality may influence callus growth, shoot regeneration and rooting Red light stimulated shoot elongation of geranium and rooting of *Prunus* (Rossi et al. 1993). Blue light promoted rooting and acclimatization of birch (Saebo et al. 1995). However, the effect of various light sources on the growth of embryogenic tissue in Norway spruce (Latkowska et al. 2000) was strongly genotype dependent.

We report here the determination of the optimal seed collection dates by comparing the effect of the embryo developmental stage (determined by micro-sectioning of the seed) on the initiation rates of ESM. In addition we report on the effect of L-glutamine concentration on ESM proliferation, and of activated charcoal treatment on somatic embryo production and finally on the effect of LED on somatic embryo germination of *P. densiflora*.

2. Materials and methods

2.1 Plant material

Immature seeds were collected from four trees grown at the experimental garden of the Korea Forest Research Institute located in Suwon (longitude 126° 57′E, latitude 37° 15′N), Kyeonggi province, Korea from May 31 to July 20 at one week intervals in 2004. In 2005, seed collection was performed at two sites, Suwon and Anmyeon (longitude 126° 23′W, latitude 36° 29′N) at a seed orchard at the Interior Breeding Station of the Korea Forest Research Institute in Chungnam Province, Korea, on June 28, July 1st and July 5. In 2006, immature seeds were harvested only in Suwon, the collection dates were same as in 2005.

2.2 **Microscopic observation of zygotic embryos**

Before the ESM initiation experiments, 15~20 seeds from each collection date were sampled at random, longitudinally dissected with a surgical blade (No 11, Feather), and the stage of zygotic embryo development was monitored for each collection date under a stereomicroscope. The embryo developmental stages were recorded by collection date or location and were used as indicator for the collection of the most responsive explants for initiation of ESM. In addition, immature seeds from different collection dates were fixed in glutaraldehyde (1.5%) and

paraformaldehyde (1.6%) in phosphate buffer (0.05 M, pH 6.8) under refrigeration for 3 months. Dehydration was done at room temperature in a series of different concentrations of ethanol, followed by infiltration with Historesin (Technovit 7100, Kluzer, Germany) at room temperature overnight. Serial sections (3 μm) were prepared with a rotary microtome with a tungsten-carbide knife and the sections were floated in water and dried on a hot plate (40℃). Sections were double stained with Periodic acid-Schiff's (PAS) (0.1%) and Toluidine blue O (0.05%) and observed under a light microscope (Leica D.M.R., Germany).

2.3 ESM initiation from megagametophytes

For sterilization of seeds, the seeds extracted from cones were disinfected with 70% (w/v) ethanol for 2 min and NaClO (2%, w/v) for 10 min, followed by rinsing 5 times with sterile distilled water. The seed coat and nucellus tissue were removed, intact megagametophytes containing zygotic embryos were placed on P6 medium (Teasdale et al. 1986) contained full-strength macro- and micro- salts, vitamins, 1.0 g l-1 L-glutamine, and 30 g l-1 sucrose plus 2.0 mg l-1 2,4-D, 1.0 mg l-1 BA, solidified with 0.2% (w/v) gellan gum (phytagelTM, Sigma). L-glutamine solution was sterilized by filtration, then added to partially cooled medium (45-50℃) after autoclaving. The cultures were kept in darkness at 24±1℃ for 8 weeks without transferring to fresh medium. An ESM may extrude from the corrosion cavity of the megagametophyte at its micropylar end. Frequency of ESM initiation was recorded after 8 weeks of culture.

2.4 Effect of L-glutamine and brassinolide concentration on ESM proliferation

The effect of L-glutamine (0, 1.71, 3.42, 6.84 and 13.68 mM) on ESM proliferation was investigated using ½LM medium supplemented with 9.0 μM 2,4-D, 4.4 μM BA, 58.4 mM sucrose and solidified with 0.4% gellan gum. In addition, the effect of brassinolide (BL) on ESM proliferation was studied. The ESM was suspended onto a filter paper disk (90 mg per disk) which was placed onto the proliferation medium with different concentrations of L-glutamine or BL. The cultures were maintained at 24±1℃ in dark. For each test, there were three replications, each consisting of at least three Petri dishes for each treatment. After 4 weeks of culture, without subculture during the incubation period, the fresh weights of ESM were recorded. The growth rate was calculated on a fresh weight (FW) basis according to: Growth rate = [(FW at the end of treatment minus - FW at the start of treatment) / FW at the start of treatment].

2.5 Effect of AC on somatic embryo maturation

The goal of the somatic embryo maturation experiment was to assess the effects of the presence or absence of activated charcoal (AC, at 0.05%) in the maturation medium. For maturation of somatic embryos, 250 μM (±)-ABA (Sigma), 1.2% gellan gum and 0.05% AC were added to ½LM medium supplemented with 0.2 M maltose and 6.8 mM L-glutamine. The ABA solution

was filter-sterilized (0.22μm, Millipore) and added to the cooling medium after autoclaving. For maturation of somatic embryos, the ESM were weighed and dispersed in liquid ½LM medium without growth regulators. After the ESM suspensions were homogenized, 3 ml of the liquid medium containing 90 mg FW (30 mg/ml) of tissue were poured over a filter paper disk (Whatman #2, 5.5 cm) and placed in a Büchner funnel. After draining the medium with a low pressure pulse vacuum, the filter paper with ESM on it was placed on maturation medium and cultured in darkness for 12 weeks without subculture onto fresh medium. After a total of 12 weeks of culture, the numbers of mature somatic embryos were counted under a stereomicroscope. For each test, there were three replications, each consisting of five Petri dishes for each treatment, at least12 somatic embryos were used for each Petri dish.

2.6 Effect of LED on germination of somatic embryos

To examine the effects of LED light sources on germination, somatic embryo cultures were exposed to LEDs (GF-320, Good Feeling, Sungnam, South Korea). The temperature was 24±2℃ and the photoperiod was adjusted to 16/8 hrs. Somatic embryos were germinated under: fluorescent light (FL) (50 μEm-2s-1, LUMILUX, 40W, OSRAM), which served as the control and four kinds or combinations of LEDs namely 100% red LED (peak wavelength: 660 nm), 100% blue LED (peak wavelength: 450 nm), 50% red+50% blue and 50% red+50% far red (peak wavelength: 730 nm). For each test, there were three replications, each consisting of 30 somatic embryos derived from 4 ESM lines for each treatment.

2.7 Plantlet regeneration and acclimatization

Cotyledonary somatic embryos were selected from embryogenic masses that had been cultured on ABA-containing medium for 12 weeks and were placed horizontally on the surface of ½LM medium containing 60 mM sucrose and 0.4% gellan gum. The cultures were kept for 7 days under dim light (1.5 μEm-2s-1), 16-h photoperiod, 24±1℃, and then transferred to higher light (50 μEm-2s-1). After 8 weeks of germination treatment, plantlets with a well developed epicotyl (at least 20 mm) and roots were transplanted into an artificial soil mixture {perlite: peatmoss: vermiculite (1: 1: 1)} in trays with a transparent lid and were watered once a day. After acclimation for 4-6 weeks, the lid was gradually opened to reduce humidity in the tray. The lid was removed completely when new shoot growth started. Acclimated plants were maintained for a further 4-5 weeks in the tissue culture room (50 μEm-2s-1, 16 h photoperiod, 25±1℃). Thereafter, plants were transferred to the greenhouse.

2.8 Statistical analysis

Data recorded during *in vitro* culture were analysed by ANOVA, and significant differences between means were tested by Duncan's multiple range test at $P = 0.05$.

3. Results and discussion

3.1 ESM initiation frequency based on collection date

The initiation of ESM was critically influenced by the developmental stage of the embryos at the time of collection. As shown in Table 1, in 2004 the ESM

Table 1. *The effect of collection location, dates and developmental stage of the embryo for initiation of ESM in P. densiflora*

Collection Site (year)	Collection Date	Embryo Developmental Stage (%)				
		Proembryo	Globular	Precotyledon	Cotyledon	ESM initiation (%)[a]
Suwon (2004)	May 31	0	0	0	0	0
	June 7	0	0	0	0	0
	June 13	100	0	0	0	0
	June 21	100	0	0	0	0.57de
	June 28	100	0	0	0	0.88cd
	July 5	70	20	10	0	0.33def
	July 13	0	33.3	66.7	0	0
	July 20	0	0	49.1	50.9	0
Suwon (2005)	June 28	100	0	0	0	0.93c
	July 1st	100	0	0	0	1.4b
	July 5	100	0	0	0	0.24f
An Myeon (2005)	June 27	100	0	0	0	0.97c
	July 1st	100	0	0	0	2.31a
	July 5	100	0	0	0	1.65b
Suwon (2006)	June 28	100	0	0	0	0.49de
	July 1st	100	0	0	0	0.91c
	July 5	89.9	10.1	0	0	0.27f

[a] Different letters within columns indicate significant differences at P = 0.05.

initiation frequencies obtained with material collected on June 21, June 28 and July 5 were 0.57%, 0.88% and 0.33%, respectively (Table 1). All explants obtained from material collected June 13, 21 and 28 were at the proembryo stage (100%). After that, the proembryo frequency declined to 70% (July 5) and to 0% in subsequent collections on July 13 and July 20. The latter dates contained globular (33.3%), precotyledonary (66.7%, 49.1%) and cotyledonary (50.9%) embryos. No proembryos were observed in explants collected at July 13 and July 20 and, therefore, no ESM was initiated with material from these two collection dates. As the embryo developed to the precotyledonary stage, the frequency of ESM initiation decreased sharply from 0.88% (June 18) to 0.33% (July 5). Over half (66.7%) of the explants excised from the July 13 collection were at the cotyledonary stage and the rest were globular (33.3 %). The 2.3% initiation rate

was similar to those of *Pinus densiflora* (1.0%) (Ishii et al. 2001) and *P. sylvestris* (Keinonen-Mettala et al. 1996), where proembryo to early-stage embryos were collected in late June and early July for ESM experiments. However, these results disagreed with previous observations for *P. densiflora* (1.4%) (Taniguchi 2001) and *P. pinaster* (Arya et al. 2000; Miguel et al. 2004), where precotyledonary embryos were collected in mid/late July. No embryogenic lines were produced from seeds collected July 13 or 20. Based on the results shown in Table 1, seed collection should be made before the appearance of globular stage embryos of this species. In the experiments of 2005, three collection dates and one more collection location – Anmyeon - were used because of the results obtained the previous year (Table 1). With these experiments the highest frequency recorded was 1.4% (July 1st, Suwon) and 2.31% (July 1st, Anmyeon). In seeds collected from the two different locations in the 2005 test, no differences in the embryo developmental stage were found (Table 1). Finally, in the 2006 test the highest frequency of ESM initiation was 0.91% (Suwon, July 1st) and the developmental stage again was all proembryo (100%) (Table 1). In conclusion, the frequency of ESM initiation was influenced by the developmental stages of the explants. Some attempts have been made to determine the optimal developmental embryo stage for ESM initiation in *Pinus nigra* (Salajova et al. 1999), *P. pinaster* (Miguel et al. 2004), and *P. roxburghii* (Arya et al. 2000). Correlating the developmental stage with strict collection dates is difficult because seed size and embryo developmental stage may differ, even among seeds from a single tree and because of variations between trees due to open pollination (Arya et al. 2000). Even though seed development may vary from year to year by latitude and elevation, histological observation could determine at what date the stage of embryo development is optimal for ESM initiation. As for other conifer species, the developmental stage of the embryo has proven to be a critical factor for ESM initiation in *Pinus densiflora*, and was limited to the proembryo stage. Initiation of ESM from a more advanced stage of embryo development has been previously described as being the precotyledonary stage in *P. pinaster* (Miguel et al. 2004) and *P. roxburghii* (Arya et al. 2000) or even from mature zygotic embryos in *P. koraiensis* (Bozhkov et al. 1997). In a few cases, the fertilization date has been used as a reference point (Keinonen-Mettala et al. 1996). For *P. sylvestris*, the appropriate period was about 2 weeks after fertilization. However, the precise time of fertilization is more difficult to determine than the embryo developmental stage and is further complicated by temporal variations in development among different trees (Lelu et al. 1999).

3.2 Development of zygotic embryos at the sampling dates

For seeds collected May 31, 2004 (Figure 1a), the megagametophytes were translucent with visible archegonia (arrows). No zygotic embryos were observed until the seeds collected June 7 were examined (Figure 1b), where the two archegonia were larger than those of seeds collected May 31 (Figure 1b). In the seeds collected June 13, one early proembryo (resulting from fertilization) was seen in the upper part of one archegonium (Figure 1c), with the embryonic head of

each zygotic embryo being as composed of several cells and with their suspensors linked to one archegonium head (Figure 1c, d) while the other archegonium had started to shrink/degenerate (Figure 1d). In the seeds collected June 21, some zygotic embryos (arrows) were found with their suspensors throughout the corrosion cavity, particularly near the micropylar region (Figure 1e, f). The size of the corrosion cavity was expanded longitudinally towards the chalazal end (Figure 1f). In the seeds collected July 28 (Figure 1g), three late proembryos (arrows) (polyembryogenesis) were found in the corrosion cavity (Figure 1h), one dominant embryo (arrow) had continued to develop and reached the chalazal end while the others had started to degenerate (July 5) (Figure 1i, j). By elongation and development of the apical shoot primordium one dominant embryo and developed to the precotyledonary (July 13) (Figure 1k) or cotyledonary stage (Figure 1l, July 20).

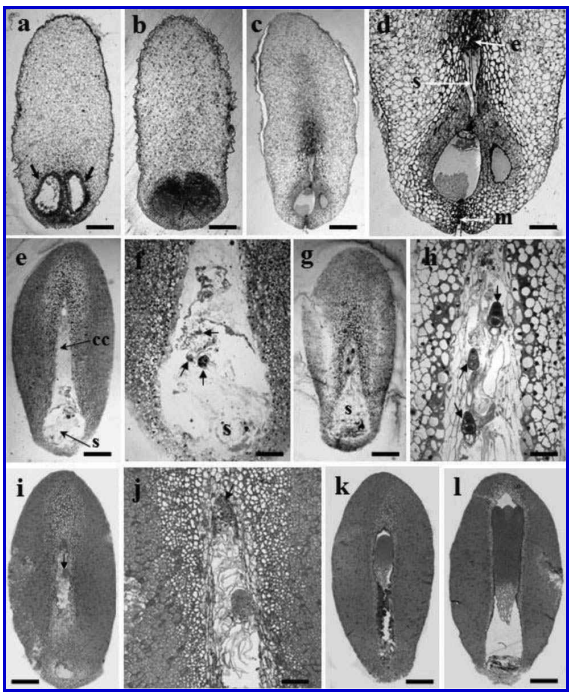

Figure 1. *Developmental embryo stages of P. densiflora. (a) Archegonia (arrows) in a 31 May seed. (b) No embryos found in 7 June seeds. (c) Early-stage embryos in corrosion cavity (13 June). (d) Magnified picture of Figure 1c. One small globular zygotic embryo in corrosion cavity, e: embryonal head, s: suspensor, m: micropyle. (e) Proembryos in the corrosion cavity (cc), s: suspensor (21 June). (f) Numerous suspensors in the corrosion cavity (magnified Figure 1e). (g) Late-stage proembryos in corrosion cavity (28 June). (h) Embryonal head of each embryo (arrow) has more divided cells and longer suspensors than shown in those of Figure g. (i) One surviving dominant embryo (arrow); the other embryos have degenerated (5 July). (j) Magnified picture of Figure c. (k) A zygotic embryo developed more vigorously (13 July). (l) Cotyledon-stage embryo in the corrosion cavity (20 July). Bars: 420mm (a–d), 179mm (e), 159mm (i), 140mm (j), 117mm (k), and 147mm (l).*

3.3 Comparison of microsectioned profiles from seeds collected in 2005 and 2006

In the Suwon seeds collected in 2006 some proembryos were found in the corrosion cavity (Figure 2a). Regardless of the collection date (June 28, July 1st and July 5), the corrosion cavities in seeds collected in Suwon (Figure 2a) or Anmyeon (Figure 2b) had expanded longitudinally towards the chalazal end and were filled with suspensors masses. No large differences were found between seeds collected in 2005 and 2006 in relation with collection date, and year or location in terms of the length and shape of the corrosion cavity or embryo development (Figure 2a, b). In contrast, when compared with seeds collected in 2006, the length of the corrosion cavity was a half of that found in 2005 seeds (Figure 2c, June 28 or July 1st, two in left or right side, respectively) or the corrosion cavity was less developed and some embryos with suspensors were restricted in a round shaped corrosion cavity (Figure 2c, July 1st). However, even though zygotic embryogenesis in the seeds of 2006 lagged behind that of 2005 (Figure 2a, b, c), all seeds collected during those collection dates (i.e., June 28, July 1st or July 5 in 2005 or 2006) had the full potential to initiate ESM and also had a high frequency of ESM initiation (Table 1).

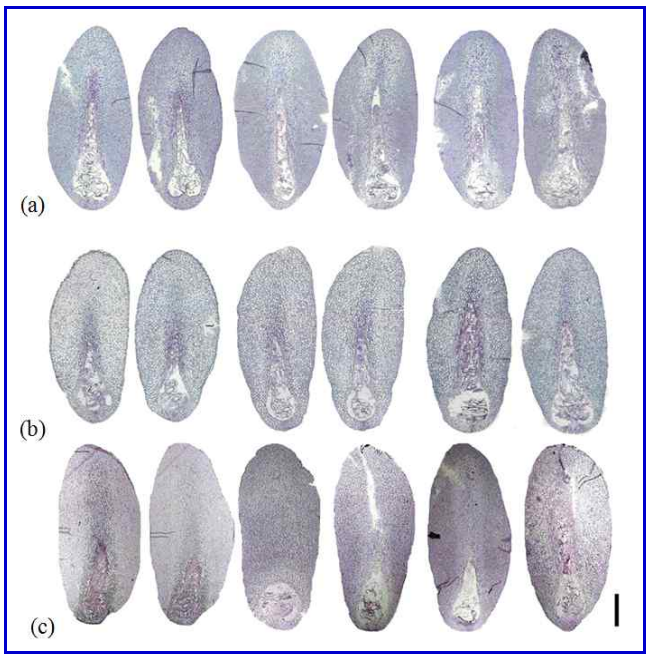

Figure 2. *Comparison of microsection profiles of immature embryo development in relation with seed collection dates and collection locations of P. densiflora. (a) Two on the left (June 28 in 2005, Suwon), two in the middle (July 1st in 2005, Suwon), two on the right (July 5 in 2005, Suwon) (b) Two on the left (June 28 in 2005, Anmyeon), two in the middle (July 1st in 2005, Anmyeon), two on the right (July 5 in 2005, Anmyeon) (c) Two on the left (July 5 in 2006, Suwon), two in the middle (July 5 in 2006, Suwon), two on the right (July 5 in 2006, Suwon) (bar 1.2 mm)*

In addition, although seed development may vary from year to year by latitude and elevation, this histological study suggests that the optimum stage of embryo development for ESM initiation can be determined by micro-sectioning of the seeds collected at different dates. However, the precise time of fertilization is more difficult to establish than the embryo developmental stage which still has to be determined because of the temporal variations in the development among the different trees.

3.4 Effect of L-glutamine on ESM proliferation

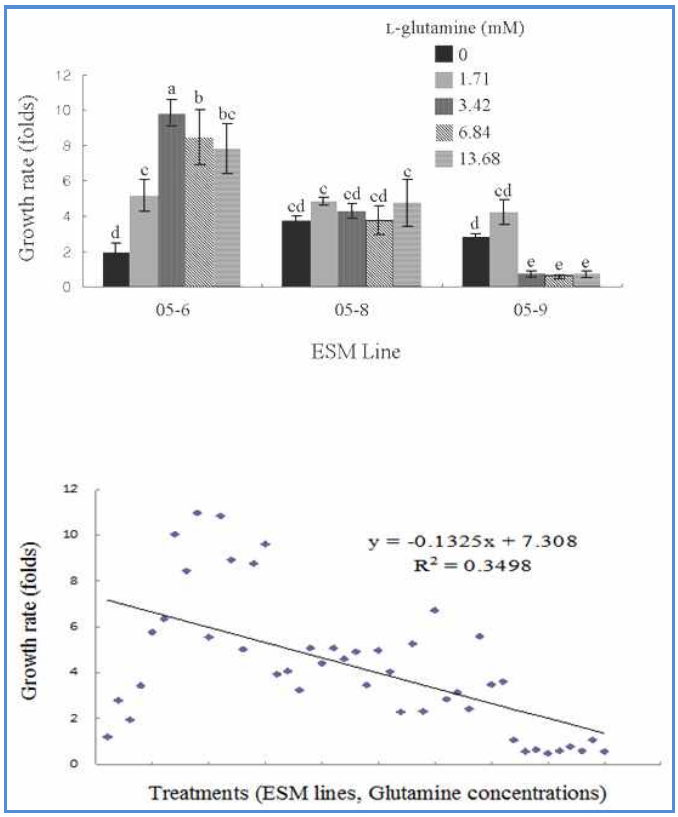

Figure 3. *Effect of various L-glutamine concentrations on weight of the ESM of three genotypes of P. densiflora. Error bars mean standard error of average. Different letters within columns indicate significant differences at P =0.05 (upper graph). Relationship between the treatments (three ESM lines, varying glutamine concentrations) and the growth rate of ESM (lower graph). The treatments were plotted against the growth rate of ESM.*

The effect of L-glutamine concentration on ESM proliferation is shown in Figure 3. The highest proliferation rate of ESM was obtained with the combination of 3.42 mM L-glutamine (9.8 fold, line 05-6) (Figure 3). A lower proliferation rate was obtained on medium with 3.42, 6.84 and 13.48 mM L-glutamine with line 05-9

(0.7, 0.6 fold and 0.7). At the higher level (3.42, 6.84 or 13.68 mM), the ESM weight decreased (05-9) except for line 05-6 and 05-8 (Figure 3). With the line of 05-8, no significant ESM weight increase or decrease was found with the 5 different concentrations of glutamine. Therefore, the fresh weight increment was greatly affected by the nitrogen source and by the genotype of the ESM.

3.5 Effect of BL on ESM proliferation

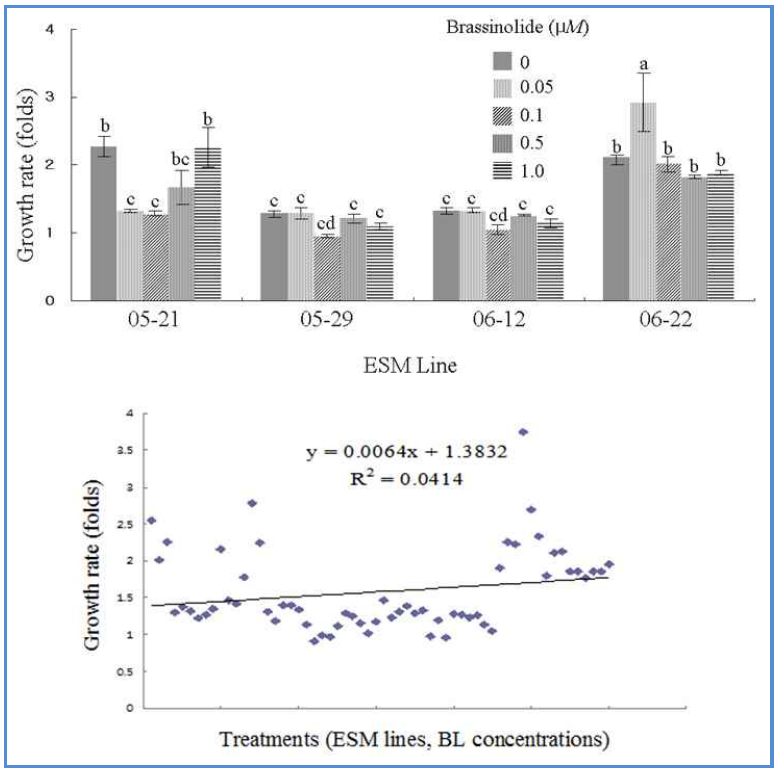

Figure 4. *Effect of BL concentrations on ESM weight of 4 genotypes of P. densiflora (upper graph). Error bars - standard error of the mean. Different letters within columns indicate significant differences at P =0.05. Relationship between the treatments (ESM lines, BL concentrations) and the growth rate of ESM (lower graph). The treatments were plotted against the growth rate of ESM.*

Growth rates for ESM at various concentrations (0, 0.05, 0.1, 0.5 and 1.0 μM) of BL are shown in Figure 4. Based on these results, the highest growth rate was observed for 0.05 μM BL (2.9 fold, line 06-22). However, in the 05-21 line, the treatment without BL also had a high ESM growth rate (2.3 fold). Since the highest BL concentration (1.0 μM) tested showed the greatest ESM growth in the 05-21 line, this suggested that BL concentrations greater than 1.0 μM should be tested. Two ESM lines (05-29 and 06-12) responded poorly compared with the other two lines, regardless of BL concentrations. Therefore, these data showed that

the growth of ESM lines were dependent on the original ESM genotypes, rather than the concentrations of BL. Though little research has been done with BL on conifer species, Pullman et al. (2003b) reported that BL promoted the weight increment of loblolly pine ESM by 66%.

3.6 Effect of AC on somatic embryo maturation

Because the ESM had been cultured for a long time on a medium containing 2,4-D and BA prior to transfer onto maturation medium, the beneficial effect of producing somatic embryos on medium with AC was studied. Addition of AC to the medium is known to adsorb residual plant growth regulators (von Aderkas et al. 2002). Figure 5 showed that somatic embryo yield ranged from 0 (line 05-4, 05-25, 05-31 and 05-58 with/without AC) to 798 (line 05-29 with AC) from plated ESM cells. In general, maturation medium containing AC (line 05-9, 05-21, 05-37 and 05-50) did not produce as many somatic embryos as media without AC leading to the conclusion that somatic embryo production was greatly dependent on the genotype of ESM of *P. densiflora*. Pullman et al. (2005) reported that the addition of AC to the maturation medium resulted in an increase of embryo production for Norway spruce. The same was found for maritime pine (Lelu et al. 2006) and loblolly pine (Pullman and Gupta 1991).

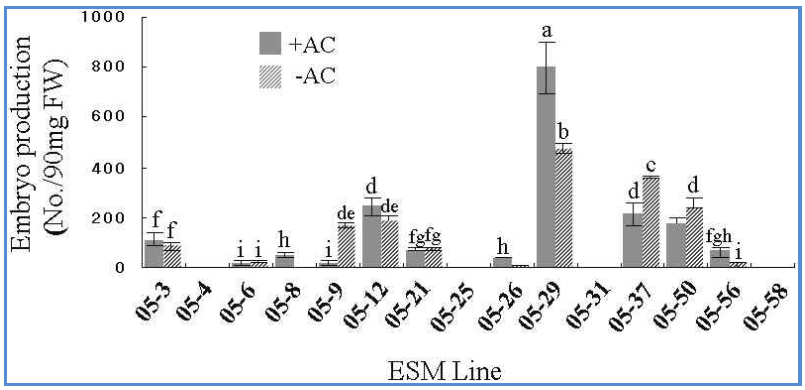

Figure 5. *Effect of AC supplementation on somatic embryo maturation with ESM of 15 genotypes of P. densiflora. Error bars mean standard error of average. Different letters between columns indicate significant differences at P =0.05.*

3.7 Effect of LED on germination of somatic embryos

The various light sources strongly influenced germination frequency of somatic embryos (Figure 6). The highest frequency of germination was obtained with red light (80.9%, line 05-12), other high frequencies were also found with this treatment in other lines (57.1% for lines 05-3 or 05-29 and 67.5 % for line 05-37). Therefore, germination of somatic embryos was positively affected by application of red light. In contrast, lower frequencies were obtained with fluorescent light (0, 12.9, 21.5 and 23.4% for lines 05-3, 05-12, 05-29 and 05-37). In addition, in the

case of red+blue, no germinants were obtained with line 05-3 but a high frequency (72.2%) was obtained with line 05-37. All but line 05-3 responded similarly to the different light sources. Therefore, there were significant interactions between ESM lines and light sources. In conclusion, the germination of somatic embryos of *P. densiflora* was positively affected by application of red light.

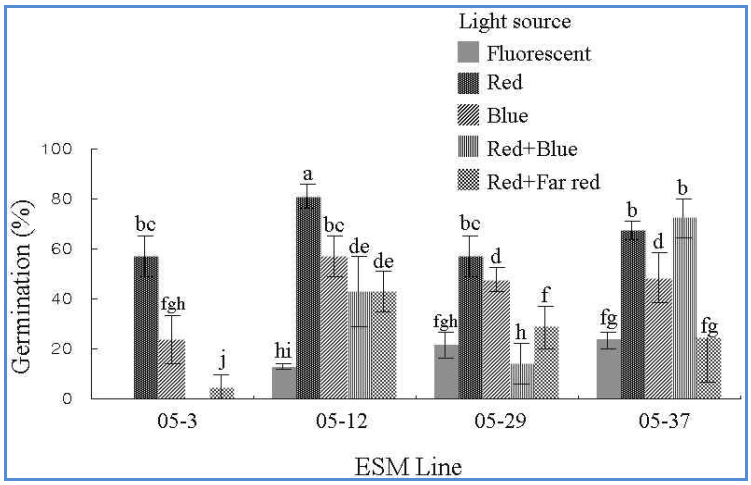

Figure 6. *Effect of light quality on somatic embryo germination with ESM of 4 genotypes of P. densiflora. Error bars mean standard error of average. Different letters between columns indicate significant differences at P =0.05.*

3.8 Somatic embryogenesis and plant regeneration

A mucilaginous cell mass protruded from the micropylar end of the megagametophyte after 6 to 8 weeks in culture (Figure 7a). The translucent and mucilaginous ESM was composed of a few proembryos at an early stage of development (Figure 7b). The ESM lines were proliferated on ½LM medium containing 2,4-D and BA. They proliferated rapidly on the medium and were subcultured weekly onto fresh medium.

After the 6 to 8 weeks of maturation, microscopic observations revealed somatic embryos with cotyledons on the AC-containing medium (Figure 7c). After 2 to 3 additional weeks, a large number of fully developed somatic embryos were produced on the maturation medium with filter-paper (Figure 7d, e).

Two weeks after transfer to germination medium (½LM containing 58.4 mM sucrose, solidified with 0.4% gellan gum) without ABA, mature somatic embryos started to form an epicotyl and shoots (Figure 7f). One week later, the cotyledons turned deep-green and the hypocotyls and roots elongated. Upon transfer to fresh germination medium, plantlets with well-developed cotyledons, elongated hypocotyls and roots developed. When exposed to light, new shoots formed from the terminal bud (Figure 7g). The somatic plants developed, were transplanted into a soil mixture (Figure 7i), The potted plants grew well and could

be transferred to larger pots to foster their growth under greenhouse conditions (Figure 7j).

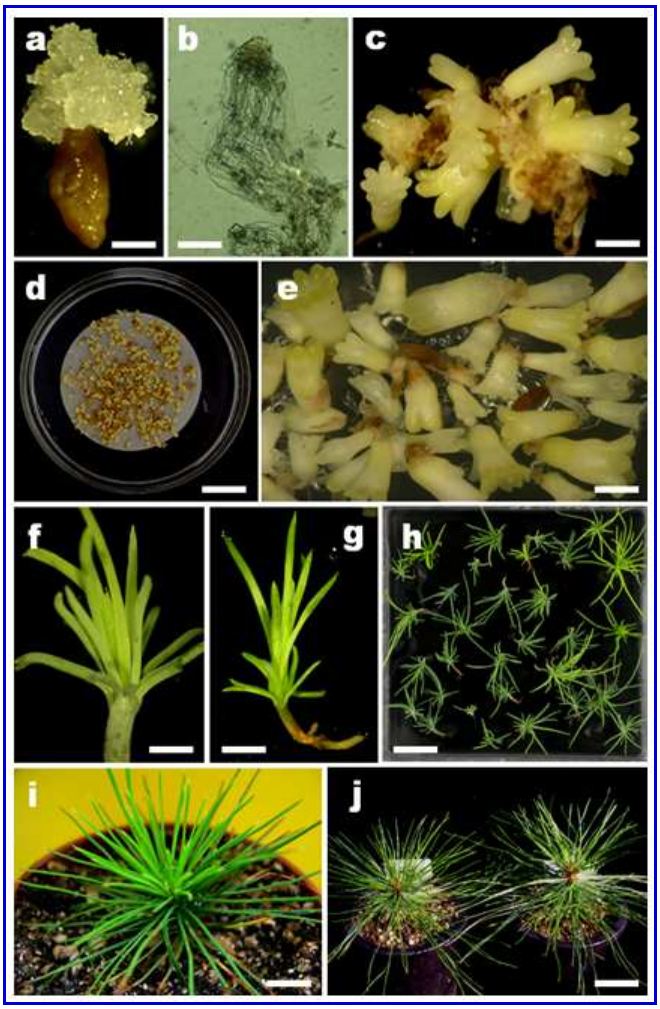

Figure 7. *SE and plant regeneration in P. densiflora. a, White-mucilaginous ESM extruded from micropyle end of megagametophyte after 8 weeks in culture (bar= 2 mm). b, Single proembryo with several long suspensors (bar: 1.3 mm). c, Somatic embryos maturing on 1/2 LM medium with 1.0 % gellan gum, 0.2 M maltose and 250 µM ABA (bar = 0.6 mm). d, Somatic embryos on filter paper placed on maturation medium after 8 weeks in culture (bar = 2.5 cm). e, Collection of cotyledon-stage somatic embryos before germination treatment (bar = 1.2 mm), f, Newly-produced epicotyl shoots from the germinant (bar = 2 mm). g, Germinating somatic embryo after 4 weeks of culture (bar = 2.7 mm). h, More developed germinants after 5 weeks in culture (bar = 1.0 cm). i, Acclimated somatic plant growing in the greenhouse (bar = 1.0 cm). j, Green house-grown 8-month-old somatic plants during a spring flush of new growth (bar = 3.5 cm)*

In conclusion, as shown above, somatic plants were regenerated from somatic embryos of *P. densiflora*. However, there still are problems, i.e., the low initiation rate of ESM, decrease or loss of maturation ability after a long subculture period of ESM, and a low maturation rate. To solve these problems and before the somatic embryogenesis system can be used for genetic transformation, improvement of protocol is needed. Future screening of families to find, ESM lines with a high capacity for embryogenesis is also important.

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Somatic embryogenesis in rigitaeda pine (*Pinus rigida* × *P. taeda*)

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Abstract

The pitch-loblolly pine hybrid (*Pinus rigida*×*P. taeda*) has useful characteristics inherited from both parents but its exploitation is hindered by restrictions posed by conventional breeding and propagation methods. This study was undertaken to establish an effective in vitro system for propagating pitch-loblolly hybrid pine through somatic embryogenesis and to gain insight in the relationship between the efficiency of embryogenic tissue initiation and zygotic embryo development. Zygotic embryos at different developmental stages were tested for their potential in the initiation of ESM (embryogenic suspensor mass) lines using immature seeds of *Pinus rigida*×*P. taeda*. The highest frequency (1.1%) of ESM initiation was obtained with explants from cones collected on July 1. All excised embryos of the July 1 collection were at the early proembryo stage. Two different culture media were compared. Forty eight ESM lines were initiated (0.97%) on *Pinus taeda* Basal Medium (P6) with 13.5 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.4 μ*M* benzyladenine (BA). However, only four ESM lines (0.55%) were obtained on a modified Murashige and Skoog medium (MSG). Most of the ESM arose from seeds that were at the stages ranging from late cleavage polyembryony to the early stage proembryo. Out of 52 lines (0.46%) that were produced from 11,388 explants, only two viable lines (0.018%) (PRT11 and PRT28) survived. As for somatic embryo maturation, the highest number (224/g^{-1} FW) of matured cotyledonary somatic embryos (line PRT 28) was obtained on a medium containing 100 μ*M* abscisic acid (ABA), 0.2 M maltose and 1.2% gellan gum. For germination of the somatic embryos the cotyledonary somatic embryos produced on maturation medium were transferred to half-strength Litvay medium (LM) plus 0.4% gellan gum. The germination rates were high (71.4-96.3%) regardless of the concentrations of either ABA or gellan gum in the maturation medium. Approximately, 500 somatic plants were recovered from the germination medium and transferred to the greenhouse, finally most of them were transplanted successfully to the experimental field.

Keywords: Embryogenic tissue initiation, Rigitaeda pine, Plant regeneration

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1. Introduction

Pitch pine (*Pinus rigida*), native to the northeastern region of United States, was introduced into Korea in 1906 for the purpose of reforestation. This species is hardy and thus has well adapted to barren soils, sandy conditions and harsh winters in Korea. However, this species grows poorly and produces low quality wood. Therefore, experience in Korea over the past several decades has shown that the species should not be used for timber production.

Loblolly pine (*Pinus taeda*), native to the southeastern region of United States, was introduced into Korea in 1925. The trees have good quality wood and grow fast. However, the species tends to be susceptible to cold temperature and thus plantings were largely limited to warmer region of the country.

To overcome these limitations, we initiated a hybrid pine project that was based on artificial hybridization between the two pine species. Attempts have been made to upgrade wood quality, growth rate and cold hardiness by selecting better hybrids from the cross combinations. The pitch-loblolly pine hybrid (*Pinus rigida*×*P. taeda*) has useful characteristics inherited from both parents, such as the cold resistance of pitch pine and the fast growth of loblolly pine. Since the first artificial cross at the Institute of Forest Genetics (Placerville, CA, USA) in 1933 (Righter and Duffield 1951), much research has been conducted in Korea with this hybrid (Hyun 1962). This pine hybrid has been a major reforestation species in Korea, due to its rapid growth, to heights up to 30 m and diameter at breast height up to 1 m, and high timber value. According to the Korea Forest Service (2000), about 10 million trees were successfully planted in reforestation areas covering 32,638 ha by the end of 1987. However, there are constraints on large-scale planting due to pollination problems because of differences in flowering periods of the parents and low fertility rates during F1 hybrid seed production.

The project was a great success in that 1) the hybrids grew as fast as loblolly pine, 2) the quality of wood was almost equal to that of loblolly pine, and 3) the trees were still hardier than loblolly pine (Chong, 1999). However, even with the outstanding success of the hybrids, the F1 seeds produced by artificial hybridization were not suitable reforestation as they are very expensive to produce. Attempts to produce a large quantity of F1 seeds through open pollination by establishing a seed orchard consisting of both pitch pine and loblolly pine were not successful since the two species differed in their flowering time. This was tried in a seed orchard that was established in 1970 with hybrid clones with the idea of mass producing F1 seeds through open pollination. However, as most traits in the thus produced F1 hybrids are more variable than in the parent hybrids, there is still an increasing need for large quantity production of low cost, high value F1 hybrids.

Clonal propagation of high–value forest trees through somatic embryogenesis (SE) has the potential to rapidly capture the benefits obtained by breeding or genetic engineering programs to improve the uniformity and quality of nursery stock, particularly of pine species (Find et al. 1993). Since first reported for spruce (Hakman et al. 1985), SE is favored as a promising tool for mass propagation of coniferous trees. Conifer biotechnology programs can benefit from SE for two major reasons. First, this system offers the capability to produce

unlimited numbers of propagules (Sutton, 2002). Second, the embryogenic culture system could be used for genetic transformation. Some groups have already reported successful regeneration of transgenic trees through SEm e.g., in *P. radiata* (Charity et al. 2005) and *P. strobus* (Levee et al. 1999). Regeneration through somatic embryogenesis in gymnosperms is more difficult than in angiosperms because many species are recalcitrant under in vitro conditions. In many cases, successful regeneration only occurs from immature seeds containing zygotic embryos in early stages of development. These developmental effects have been reported for *Pinus caribaea* (Laine´ and David 1990), *P. pinea* (Carneros et al. 2009), *P. strobus* (Finer et al. 1989; Park et al. 2006), *P. taeda* (Becwar et al. 1990), *Larix decidua* (von Aderkas et al. 1987), *Picea mariana* (Tautorus et al. 1990), and *Pseudotsuga menziesii* (Durzan and Gupta 1987).

It is well known that development of somatic embryos, particularly in pine species, was enhanced by media with a high concentration of gelling agent. Attempts have been made to produce well matured somatic embryos with this technique, i.e., with *P. monticola* (Percy et al. 2000). If this protocol could be made to work with *P. rigida*×*P. taeda*, it would be much easier to produce mature somatic embryos and to convert them into plants. To our best knowledge, there has been only one report on SE in *P. rigida*×*P. taeda* (US patent 5,4313,930, Becwar et al. 1995). However, they did not give detailed information on the relationship between the zygotic embryo developmental stages and seed collection dates with regard to the initiation of ESM. Furthermore, they did not test various concentrations of ABA and gellan gum for somatic embryos maturation or germination with this hybrid species.

The objective of our study was to develop effective SE protocols for *P. rigida*×*P. taeda* for clonal propagation. Particularly, a key objective of our study was to evaluate the effect of the stage of development of the zygotic embryo explants on successful initiation of somatic embryogenesis. For this purpose, seeds with developing zygotic embryos were collected at sequential dates and examined under the microscope. Subsequently, their morphology and developmental stage were correlated with their potential for embryogenic tissue initiation. We tested immature explants (female megagametophyte including the zygotic embryo) at different developmental stages for their potential in the initiation of ESM. In addition, we determined the optimal range of ABA for both maturation and germination of somatic embryos. The development of a protocol that would provide improved SE multiplication rates and high frequencies of converting embryos into somatic plants would be a valuable contribution to *P. rigida*×*P. taeda* propagation and may find applications in genetic transformation of this species.

2. Materials and methods

2.1 Plant material

Open pollinated F1 cones were collected from a seed orchard of *P. rigida*×*P. taeda* at the Interior Breeding Station of the Korea Forest Research Institute located in Chung ju, Korea. They were kept at 4℃ until use. Cones were

disinfected first by a 2 min immersion in 95% ethanol followed by 10 min immersion in 6.0% (w/v) sodium hypochlorite, followed by two 2-min. rinses in sterile distilled water. The cones were then dipped in 95% ethanol and flamed before extracting the seeds. Seed coats of immature seeds were aseptically removed, and whole megagametophytes containing intact zygotic embryos were placed horizontally on an ESM initiation medium.

2.2 Microscopic observation of zygotic embryos of seeds

During the initiation experiments, 15~20 seeds from each collection date were sampled and longitudinally dissected with a surgical blade (No 11, Feather) and the stage of zygotic embryo development was monitored. The developmental stages of the zygotic embryo were classified as described for *P. strobus* (Klimaszewska et al., 2001). The embryo developmental stages were determined for each collection date and were used as indicators for the collection of the most responsive explants for initiation of ESM.

2.3 Micro-section of zygotic embryos

Immature seeds from different collection dates were fixed in glutaraldehyde (1.5%) and paraformaldehyde (1.6%) in phosphate buffer (0.05 M, pH 6.8) under refrigeration for 3 months. Dehydration was done at room temperature in a series of different concentrations of ethanol, followed by infiltration with Historesin (Technovit 7100, Kluzer, Germany) at room temperature overnight. Serial sections (3 μm) were prepared with a rotary microtome with a tungsten-carbide knife, the sections were floated in water and dried on a hot plate (40℃). Sections were double stained with Periodic acid-Schiff's (PAS) (0.1%) and Toluidine blue O (0.05%) and observed under a light microscope (Leica D.M.R., Germany).

2.4 Initiation of ESM and culture medium formulation

Seeds attached to cone scales were carefully extracted and placed in Petri dishes containing distilled water, sterilized in 70% ethanol for 1 min and 15 min in 1% sodium hypochlorite solution with two or three drops of Triton X-100 and then washed four or five times with sterile distilled water. With seed from each collection date the seed coat, megaspore wall and nucellus were removed aseptically and 8–10 excised megagametophytes were placed horizontally on full-strength *Pinus taeda* basal medium (P6, Teasdale et al. 1986) or modified Murashige and Skoog medium (Becwar et al. 1990) solidified with 0.4% (w/v) gellan gum (PhytagelTM, Sigma). Both media contained full-strength macro- and micro-elements, and vitamins, with 10.3 mM L-glutamine (Sigma) and 87.6 mM sucrose and standard concentrations of the plant growth regulators 2,4 dichlorophenoxyacetic acid (2,4-D, 13.5 μM) and 6-benzyladenine (BA, 4.4 μM). The pH of both media was adjusted to 5.7 prior to autoclaving at 121℃ for 20 min. L-Glutamine, which had been filter-sterilized, was added to partially cooled (45–

50℃) medium after autoclaving. After placement on these media the explants were cultured in darkness at 23 ± 2℃ for 12 weeks and examined every 4 weeks for the initiation of embryogenic tissue.

2.5 Proliferation of ESM

For ESM proliferation, tissue on all initiation media were subcultured on ½LM (half-strength salts and full-strength vitamins) medium (Litvay et al. 1985) supplemented with 9.0 μ*M* 2,4-D, 4.4 μM BA, 58.4 mM sucrose, 6.8 mM L-glutamine, and solidified with 0.4% gellan gum. During subculture, each ESM was subdivided into 1.0 cm sized pieces and then cultured in darkness at 24±1℃. The proliferating ESM was subcultured about weekly until the maturation experiments were started. To distinguish embryogenic tissue form non-embryogenic callus each was suspended in LM liquid medium and observed using an inverted microscope (Nikon TE300, Japan).

2.6 Maturation of somatic embryos

Only two out of 52 ESM lines survived and subsequently proliferated on maintenance medium. The two embryogenic lines (PRT 11 and PRT 28) were used to evaluate the effect of ABA and gellan gum concentration on somatic embryo maturation. Various combinations of ABA and gellan gum were tested for optimal maturation of somatic embryos on ½LM medium supplemented with 0.2 M maltose and 6.8 mM L-glutamine. The concentration ranges were 0, 60 or 120 μM for ABA and 0.4, 0.8, 1.0 or 1.2% for gellan gum. ABA was filter-sterilized and added to the cooling medium after autoclaving. The plating technique for maturation of somatic embryos was previously described by Klimaszewska and Smith (1997). The ESM were weighed and dispersed in liquid ½LM medium without growth regulators. After the ESM suspensions were homogenized, 3 ml of the liquid medium containing 225 mg FW (75 mg/ml) of dispersed tissue were poured over a filter paper disk (Whatman #2, 5.5 cm) and placed in a Büchner funnel. After draining the medium with a low pressure pulse vacuum, the filter paper with ESM on it was placed on maturation medium with various concentrations of ABA or gellan gum and cultured in darkness for 12 weeks without subculturing onto fresh medium. After a total of 12 weeks of culture, the numbers of cotyledon-stage somatic embryos were counted under a stereomicroscope. For each test, there were three replications, each consisting of five to seven Petri dishes for each treatment.

2.7 Germination of the somatic embryos

Cotyledonary somatic embryos were selected from embryogenic masses cultured on ABA-containg medium for 12 weeks and placed horizontally on the surface of ½LM medium containing 60 mM sucrose and 0.4% gellan gum without L-glutamine. The cultures were kept for 7 days at 24±1℃ under dim light (1.5

$\mu Em^{-2}s^{-1}$, 16 h photoperiod) and were then transferred to higher light intensity (50 $\mu Em^{-2}s^{-1}$). After 8 weeks of germination treatment, somatic seedlings with a well-developed epicotyl (at least 20 mm) and roots were transplanted into an artificial soil mixture {perlite: peatmoss: vermiculite (1: 1: 1)} in trays with a transparent lid and were watered once a day. After acclimation for 4-6 weeks, the lid was gradually opened to reduce humidity in the tray. The lid was removed completely when new shoot growth started. Acclimated plants were maintained for a further 4-5 weeks in the tissue culture room (50 $\mu Em^{-2}s^{-1}$, 16 h photoperiod, 25±1℃). Thereafter, plants were transferred to the greenhouse.

2.8 Statistical analysis

Effects of the treatments on embryogenic tissue initiation and somatic embryo maturation were explored by one-way analysis of variance, followed by the Duncan test to assess between-treatment differences in means. Differences were considered to be significant if P 0.05 or less. All statistical analyses were conducted using SAS v.8 statistical software (SAS Institute).

3. Results and discussion

3.1 Initiation of ESM

Most megagametophyte explants that contained immature zygotic embryos displayed no detectable response within the first few weeks of culture. However, a few exhibited extrusion of embryogenic tissue from the micropyle, composed of numerous early-stage somatic embryos with embryonal heads and suspensors that could be distinguished from nonembryogenic callus composed of round, iso-diametric cells. Of the 11,388 megagametophytes containing immature zygotic embryos that were cultured, a total of 50 (0.44%) ESM lines were initiated after 8 weeks in culture. The initiation of ESM was critically affected by the developmental stage of the zygotic embryos at the time of culture. As shown in Table 1, the initiation frequency varied with collection dates with the highest frequency (1.1%) obtained with explants from cones collected on July 1. The zygotic embryos in seed collected on July 1 were at the early proembryo stage (85%) (Table 1). This result contradicts previous findings with *P. rigida*×*P. taeda* in US patent 5,4313,930 (Becwar et al. 1995) and *P. taeda* (Becwar et al. 1990) where both precotyledonary and early cotyledonary embryos were more responsive than were proembryo to early-stage embryos. However, our result agrees with that found in *P. sylvestris* where proembryo to early-stage embryos were better in the initiation of embryogenic tissue, suggesting that optimal stages may vary among different species (Keinonen-Mettala et al. 1996). The seeds collected on June 25 contained translucent megagametophytes with embryos at the proembryo stage (8%) (Table 1). Subsequent collections done on July 16, July 30 and July 30 contained globular, precotyledonary and cotyledonary stages, respectively. All our ESM lines were initiated from embryos at the early proembryo to precotyledonary stage (June 25~July 23), not from those at the cotyledonary stage (i.e. July 30

collection). However, as the seeds matured to the precotyledonary stage (July 23), the frequency of ESM initiation decreased sharply from 0.4% (July 16) to 0.08% (July 23). Over half (57.1%) of the excised embryos from July 30 collection were at the cotyledonary stage and the rest were at proembryo (14.3 %), globular (14.3 %) and precotyledonary (10.7 %) stages, respectively. No embryogenic lines were produced from the seeds collected on July 30. These results show that there is an optimal developmental stage of *P. rigida*×*P. taeda* seed for it to be capable of ESM.

The optimal developmental stage of zygotic embryos for ESM in conifer species has often been described. Based on the results shown in Table 1, seed collection should be performed prior to the appearance of distinct pre-cotyledonary stage embryos in *P. rigida*×*P. taeda*. In *P. strobus*, the optimum stage for the initiation of ESM from immature zygotic embryos within intact megagametophytes was also reported as just prior to cotyledon development (Finer et al. 1989).

Table 1. *Relationship between the stage of zygotic embryo development and collection dates of seeds and the initiation of embryogenic tissue in P. rigida*×*P. taeda.*

Collection date	Developmental stage of zygotic embryo (%)				ESM initiation (%)
	Proembryo	Globular	Precotyledonary	Cotyledonary	
June 25	8	0	0	0	0.31 b[a]
July 1	85	0	0	0	1.1 a
July 9	100	0	0	0	0.6 b
July 16	70	30	0	0	0.4 b
July 23	40	40	20	0	0.08 c
July 30	14.3	14.3	10.7	57.1	0 d
August 5	0	0	0	100	0 d

[a] Different letters indicate significant differences at P =0.05.

Thus, the optimum stage of immature zygotic embryo development, being precotyledonary in *Pinus,* differs from that in *Picea* where it tis postcotyledonary (Becwar et al. 1988; Lu and Thorpe, 1987). Attempts have been made to determine the optimal developmental embryo stage for the induction of ESM in other conifers. These include *P. nigra* (Salajova et al. 1999), *P. pinaster* (Miguel et al. 2004), and *P. roxburghii* (Arya et al. 2000). Low initiation rates of ESM formation pose limitations in the application of SE in conifers, particularly in pine species. A low initiation frequency will limit the number of genotypes available for selection and thus prolong the time required to locate elite individuals. Unfortunately, most *Pinus* species have exhibited a low initiation frequency of ESM initiation,

generally in the rage of 0-10%. Combined with other difficulties, such as a low efficiency of embryo maturation and plantlet establishment, *Pinus* species have been regarded as highly recalcitrant to SE. Fortunately, several factors have been reported that enhance initiation of ESM from immature zygotic embryos in *Pinus* species; (1) genotypes (Becwar et al. 1990), (2) collection date or developmental stage of zygotic embryos (Becwar et al. 1990), (3) phytagel level (Li et al. 1998), (4) brassinolide (Pullman et al. 2003b), (5) ABA and silver nitrate (Pullman et al. 2003a), (6) vitamin B_{12} and E (Pullman et al., 2006). Of the two initiation media used, the P6 medium (0.97%) provided a higher rate of EMS formation than the MSG medium (0.55%) (Figure 1). The P6 medium has been reported to be effective for inducing somatic embryos in some conifers (Pullman et al. 2003a). However, in other conifer species including for *P. caribaea* Mor. var. *hondurensis* (Laine and David, 1990) and *Larix×leptoeuropaea* (Lelu et al. 1994), MSG medium was more effective. In our study, P6 medium consistently gave better results than did MSG medium suggesting that our hybrid pine requires different concentrations or combinations of nutrients than do other pine species for optimal expression of their embryogenic potential.

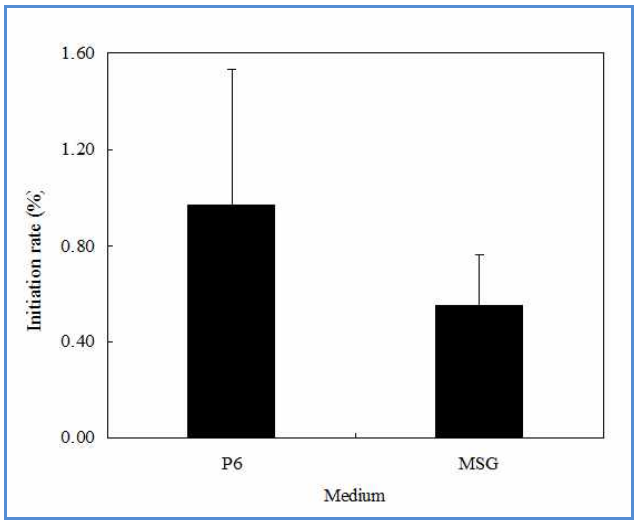

Figure 1. *Effect of culture media on the initiation of ESM from immature megagametophytes of P. rigida×P. taeda. *Error bars = mean standard deviation of average.*

3.2 Microscopic observation of developing zygotic embryos

In pine, zygotic embryogenesis starts from a single fertilization within the ovule, creating a diploid embryo within a haploid megagametophyte. However, a distinctive feature of embryogenesis in pine is two types of polyembryony (Lee 2001) and, accordingly, we found numerous embryos (up to 16 per seed) in early stages of embryogenesis created by simple and cleavage polyembryony. Especially, cleavage polyembryony which occurs in embryos at the 16-cell stage is known to

be related to the success of embryogenic tissue initiation (Cairney and Pullman 2007; Bonga et al. 2010). We microscopically examined 10–20 explants obtained from seeds collected at various dates to determine the developmental stage of zygotic embryos at these dates (Figure 2). In the seeds collected on June 11 and 18, two or three archegonia were found in a single ovule (Figure 2a).

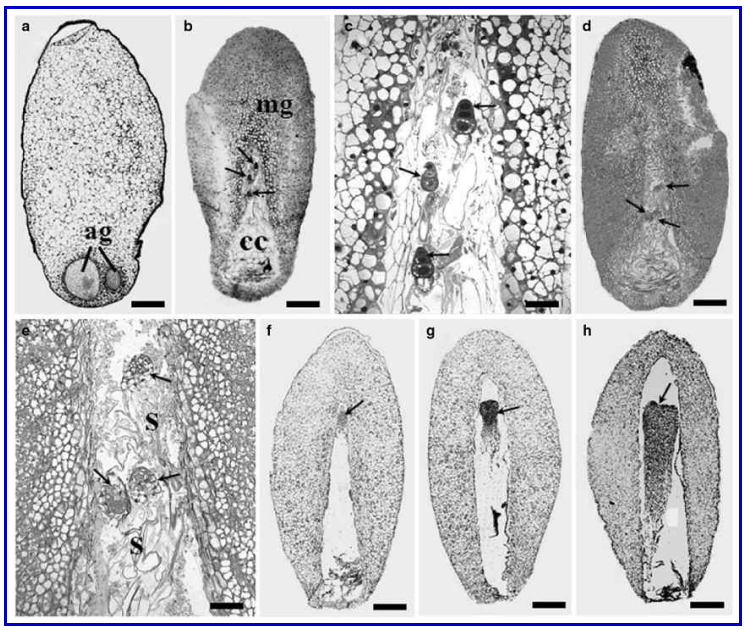

Figure 2. *Developmental stages of zygotic embryos of P. rigida×P. taeda. a. Collected on June 11. A pair of archegonia (ag) are present (bar 117 μm). However, no embryos were found in seeds collected on either June 11 or 18 (not shown here). b. Collected on June 25. Early stage embryos (arrows) within corrosion cavity (cc) were present in the megagametophyte (mg) (bar 117 μm). c. Magnified part of Figure 1b (bar 23.4 μm). The arrows indicate globular zygotic embryos. d. Collected on July 3. More developed zygotic embryos (arrows) were present (bar 117 μm). See suspensors in the corrosion cavity. e. Magnified part of Figure 1d (bar 23.4 μm). Each embryo was composed of a multi-celled embryonal head (arrows) and longer suspensors (s). f. Collected on July 9. One dominant embryo (arrow) had survived and others were denatured (bar 117 μm). g. Collected on July 23. One zygotic embryo (arrow) present, in a precotyledonary stage (bar 117 μm). h. Collected on July 30. Cotyledonary-stage embryo (arrow) present in the corrosion cavity (bar 117 μm)*

At the following collection date (June 25) the ovules had corrosion cavities containing early-stage embryos (Figure 2b, c), indicating that polyembryony had occurred. On July 3, zygotic embryos were further developed and were composed of a multi-celled embryonal head with a longer suspensor (Figure 2d, e). Distinctly dominant embryos were seen in the seed collected on July

16 (Figure 2f) that had developed to the precotyledonary (Figure 2g) and cotyledonary (Figure 2h) stages, while the other embryos had degenerated at the lower end of the corrosion cavity. The relationship between collection date and initiation of embryogenic tissue (Table 1) indicates that only embryos that had undergone polyembryony could induce embryogenic tissue. Hence, polyembryony itself or factors affecting zygotic polyembryony may play important roles in the initiation of embryogenic tissue. The origin of embryogenic tissue is not fully understood. Gupta and Durzan (1986) found that in sugar pine (*Pinus lambertiana* Douglas) somatic embryos could arise directly from suspensor tissue. However, in loblolly pine most somatic embryos are produced by the replication of existing subordinate zygotic embryos (Gupta and Durzan 1987). By contrast, Becwar et al. (1990) found that somatic embryos arose from the suspensor region of loblolly pine embryos, although it was unclear whether they extruded from the suspensor itself or from basal cells of the embryos. Recently, Bonga et al. (2010) hypothesized that embryogenic tissue could be initiated when, in response to 2,4-D in the medium, embryos produced by cleavage polyembryony cleave again when they reach the 16-cell stage.

3.3 Maturation of somatic embryo

This was carried out with two embryogenic lines (PRT 11 and 28) on ½LM medium supplemented with 0.2 M maltose and 6.8 mM L-glutamine, various concentrations of gellan gum and ABA (Table 2). As shown in Table 2, the two tested lines showed different responses to the same maturation conditions. With line PRT 11, the greatest mean number of mature somatic embryos was obtained on the medium containing 120 μ*M* ABA and 1.2% gellan gum ($96/g^{-1}$ FW) but when 120 μ*M* ABA was used with 0.8% gellan gum the response was nil and thus a trend is unclear (Table 2).

On the other hand, line PRT 28 produced more somatic embryos on medium containing 120 μ*M* ABA with 1.0% gellan gum ($224/g^{-1}$ FW). There were no clear optima, either with respect to ABA or gellan gum concentration. However, the number of mature somatic embryos was positively correlated with the relative gellan gum concentrations as is also the case for *P. strobus* (Klimaszewska and Smith, 1997). Likewise, the media with high concentrations of gellan gum (over 0.8%) and high concentrations of ABA (60 or 120 μ*M*) promoted the maturation of a large number of somatic embryos of lines PRT 11 and 28. Only line PRT 28 produced mature somatic embryos on media with 0 to 120 μ*M* ABA and 0.4 to 0.8% gellan gum. Line PRT 11 did not produce mature somatic embryos although a large number of somatic embryos were produced with these treatments. Line PRT 28 produced a large number of mature embryos ($122/ g^{-1}$ FW) even in the absence of ABA in the medium (Table 2). It appears that line PRT 28 is more responsive to the maturation conditions used than is the line PRT 11. A high number of mature somatic embryos was obtained by culturing ESM on maturation medium that provides availability because of its high gellan gum content. Several scientists have established optimal ABA concentrations for the maturation of somatic embryos in *P. strobus* (Klimaszewska and Smith, 1997) and *P. taeda* (Pullman et al., 2003a). According to US patent 5,413,930, yields of harvestable stage 3 somatic embryos

as high as 400 to 500 per gram of ESM have been obtained with *P. rigida*×*P. taeda*

Table. 2. *Effect of various concentrations of ABA and gellan gum on the maturation of somatic embryos in Pinus rigida ×P. taeda.*

Maturation treatments		No. of cotyledonary somatic embryos (g^{-1} FW ESM)	
ABA (μ*M*)	Gellan gum (%)	Line PRT 11	Line PRT 28
0	0.4	0 f[a]	122.1 b
60	0.4	0 f	91.3 d
120	0.4	2.96 e	128.7 b
60	0.8	5.92 e	150.7 b
120	0.8	0 f	115.2 bc
60	1.0	10.4 e	192.5 a
120	1.0	85.8 d	224.4 a
60	1.2	53.3 de	135.3 b
120	1.2	96.1 d	133.9 b

[a] Different letters indicate significant differences at *P* =0.05.

(Becwar et al., 1995). Unfortunately, the patent does not describe what concentration of ABA or gellan gum was used. In addition to the ABA concentration, other factors may have influenced embryo maturation in our experiment. As shown in Table 2, gellan gum concentration was a critical factor in the maturation of the somatic embryos. The highest number of mature somatic embryos per unit fresh weight was obtained when the ESM was cultured on a medium solidified with 1.2% (line PRT 11) or 1.0% (line PRT 28) gellan gum on a filter paper support with the liquid medium completely drained. The present results confirm earlier observations with *P. strobus* (Klimaszewska et al., 2000) and *P. monticola* (Percy et al., 2000) that reduced water availability resulting from high gellan gum concentrations promoted somatic embryo maturation. The germination frequencies ranged from 0 to 93.1% (line PRT 11), and 71.4 to 96.3% (line PRT 28), respectively (Table 3). These results indicate that mature embryos grown on a high gel concentration medium had higher conversion frequencies {except for 60 μ*M ABA* and 1.0% gellan gum which produced a low germination rate (12.5%)} than those grown on low gel concentration medium in the case of line PRT 11. Mature embryos from line PRT 28 which were cultured on medium with the higher gellan gum concentrations (0.8-1.2%) germinated at a high frequency (92.3-96.3%). This germination frequency was much better than that reported for *P. rigida*×*P. taeda* (30%) (Becwar et al. 1995), *P. pinaster* (72%) or *P. sylvestris* (80%) (Lelu et al. 1999) and comparable to that in *P. monticola* (90-95%) (Percy et al. 2000).

3.4 Somatic embryogenesis and plant regeneration

A total of 50 ESM lines were initiated from immature embryos (Figure 3a) during the experiment. However, only two embryogenic lines (PRT 11 and 28) survived and proliferated in subsequent culture. A mucilaginous cell mass protruded from the micropylar end of the megagametophyte within 6~8 weeks in

Table 3. *Effect of various concentrations of ABA and gellan gum on the germination of somatic embryos in P. rigida ×P. taeda.*

Maturation origins		Frequency of germination (%)	
ABA (μ*M*)	Gellan gum (%)	Line PRT 11	Line PRT 28
0	0.4	-[a]	71.4 c[b]
60	0.4	-	87.6 b
120	0.4	0 f	86.4 b
60	0.8	0 f	93.8 a
120	0.8	-	92.3 a
60	1.0	12.5 e	95.7 a
120	1.0	93.1 a	96.3 a
60	1.2	59.6 d	92.4 a
120	1.2	84.7 b	93.6 a

[a] No somatic embryos were produced from this treatment, germination was not accomplished; [b] Different letters indicate significant differences at P =0.05.

culture (Figure 3b). This translucent and mucilaginous ESM was composed of a few somatic embryos at the early stage of development (Figure 3c). The ESM lines were proliferated onto ½LM medium containing 2,4-D and BA (Figure 3d). The transferred ESM proliferated rapidly on this medium and was subcultured weekly on fresh medium. After the 6~8 week maturation treatment from ABA-containing medium, microscopic observations revealed bullet-shaped somatic embryos with prominent embryonal heads, light green in color and with long, translucent suspensors (Figure 3e). After 2~3 more weeks in culture, a large number of fully developed somatic embryos (Figure 3f, g, h) with cotyledons were produced. Mature somatic embryos, produced from the two lines of ESM, started to germinate when transferred to germination medium (½LM containing 58.4 mM sucrose, solidified with 0.4% gellan gum) without ABA (Figure 3i). The subcultured ESM formed numerous somatic embryos with distinct embryonal heads and suspensors. One week after transfer to the germination medium, the cotyledons turned green and the hypocotyls and roots elongated. Upon transfer to fresh germination medium, plantlets with well-developed cotyledons, elongated hypocotyls, and roots developed (Figure 3j). In addition, these plantlets showed a

well-developed apical primordium (Figure 3k). When leaving the plantlets on the germination medium under light, newly developed apical shoots arose from the terminal bud of some them (Figure 3l). No desiccation treatments were needed for the germination of the somatic embryos (Figure 3m, n).More than 500 somatic plants were transplanted into a soil mixture (Figure 3o). The potted plants grew well and could be transferred to larger pots to foster their further growth in the greenhouse (Figure 3p, q). Subsequently they grew well in the nursery (Figure 3r).

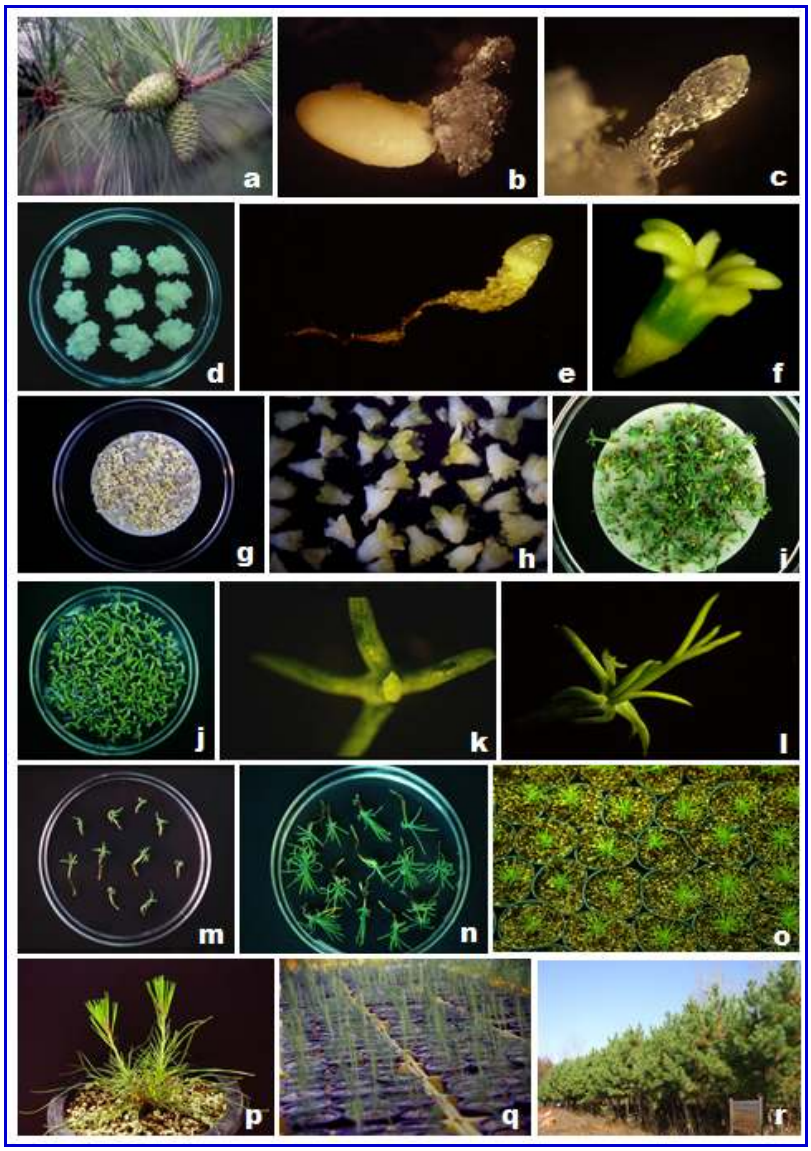

Figure 3. *Somatic embryogenesis in P. rigida×P. taeda. a, Immature cones on a twig of P. rigida×P. taeda. b, White-mucilaginous ESM extruded from the micropyle end of a megagametophyte after 6 week of culture. c, A tiny immature proembryo protruding from a proliferating ESM. d, Well maintained ESM grown*

on medium containing 2,4-D and BA. e, Early stage somatic embryos produced on medium with 120 μM ABA. f, A magnified typical mature cotyledon-stage somatic embryo showing cotyledons and a radicle end. g, Somatic embryos maturing on 1/2 LM medium with 1.0% gellan gum, 0.2 M maltose and 120 μM ABA. h, Cotyledon-stage somatic embryos before germination treatment . i, Somatic plantlets germinated on a filter paper placed on germination medium without plant growth regulators. j, Somatic plantlets obtained from the selected cotyledonary somatic embryos after 3 weeks of culture. k, An apical bud has formed on the top of the embryo. l, A newly produced epicotyl and shoot. m, More developed germinants after 5 weeks of culture. n, Somatic plantlets with a shoot and a primary root after 7 weeks of culture. o, Acclimated somatic plants growing in the greenhouse. p, Green house-grown 8-month-old somatic plants during a spring flush of new growth. q, Greenhouse-grown 2-year-old somatic plants after the first growing season. r, Somatic plants grown at a nursery for 10 years.

4. Conclusion

These results indicate that somatic embryogenesis can be a useful mass propagation technique for this hybrid pine. Another potential benefit of this protocol we developed includes facilitation of genetic transformation for this species. Further research is needed to increase the initiation frequency of ESM. In the near future, the plants obtained by somatic embryogenesis will be compared with seedlings obtained from seed with respect to growth performance, morphology and physiology.

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