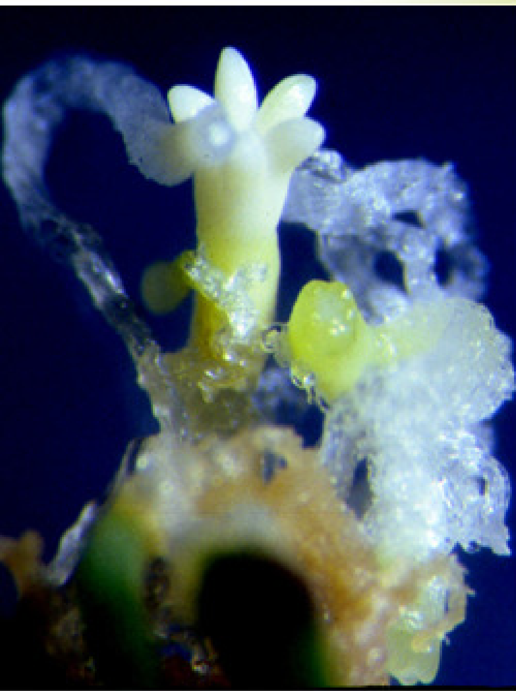
Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management



Proceedings

Second International Conference of the IUFRO Working Party 2.09.02

June 25 – 28, 2012 • Brno, Czech Republic

IUFRO Working Party 2.09.02:
Somatic Embryogenesis and Other Vegetative Propagation Technologies

Conference

PROCEEDINGS

Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management

June 25-28, 2012
Brno, Czech Republic

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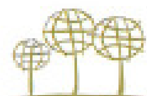

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Preface

Vegetative propagation of woody plants by *in vitro* culture has been attempted since the 1930s; however, it was not until about 20 years later that the first reports appeared of successful plantlet formation in some hardwood species. With conifers this was not achieved until 1975. Since that time, steady progress has been made in propagating woody plants using tissues, organs, cells and protoplasts, which finally led to the discovery of somatic embryogenesis (SE) in conifers in 1985. The successful SE and other clonal propagation methods have, for some tree species, arrived at the point where they can be implemented industrially. This success in vegetative propagation technologies with trees offers new opportunities for tree breeding, genetic conservation and restoration, and sustainable forest management. However, for many important tree species, recalcitrance is still an issue, requiring further research and development.

The IUFRO Working Party 2.09.02 on “Somatic Embryogenesis and Other Vegetative Propagation Technologies” was organized in 2008 with its main objective fostering the development and application of SE and other vegetative propagation technologies in woody plant propagation. The inaugural conference of this Working Party was held in Suwon, Korea in 2010, which brought leading scientists in the field together who enthusiastically embraced the development and the applications of SE and other vegetative propagation technologies. In June 2012, the second conference was hosted by the Mendel University in Brno, Czech Republic. The conference was attended by about 100 scientists and practitioners from the around the world and 38 oral and 54 poster presentations were made. A highlight of this conference was the recognition and awarding of the Mendel Medal (by the Mendel University) to the scientists who contributed significantly to the development of vegetative propagation technology. The honourees were: Drs. Antonio Ballester (Spain), Jan Bonga (Canada), Vladimir Chalupa (Czech Republic), Don Durzan (USA), Inger Hakman (Sweden), Pramod Gupta (USA), Ana Vieitez (Spain), and Sara von Arnold (Sweden). We certainly appreciate their pioneering contributions.

In addition to the scientific program, the participants enjoyed field tours to the Botanical garden and greenhouse in Brno, the Arboretum in Krtiny, caves in the Moravian Karst, a visit to the Austerlitz battle field memorial and the Forestry and Game Management Research Institute’s Kunovice Station. As well, the participants enjoyed the social events at Cerna Hora and Uherske Hradiste!

These proceedings contain the papers and abstracts that were submitted for this proceeding. Abstracts that were not submitted for editing are found in their original format in the “Book of Abstracts” published during the conference (http://www.iufro20902.org/abstracts.pdf). We are grateful to all the contributors to the conference. We would like to acknowledge the contributions of the Executive and Scientific committees that have made this conference such a great experience. In particular, we are grateful to Drs. Petr Horacek (Dean, Faculty of Forestry and Wood Technology) and Jana Krajnakova for making this conference a huge success!

Fredericton, Canada

Yill Sung Park and Jan Bonga

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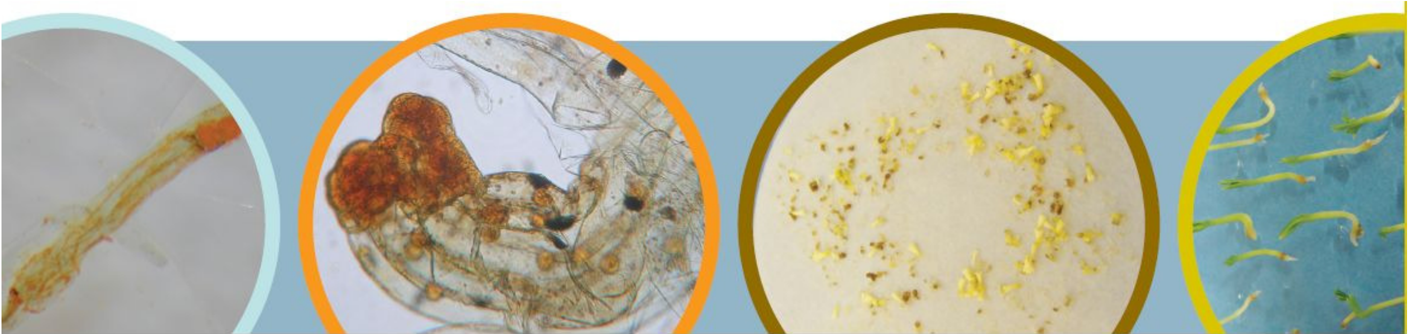

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Interpolated apomictic somatic embryogenesis, androsporogenesis, asexual heterospory, mitosporogenesis and genomic silencing in a gymnosperm artificial sporangium

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Keywords: Pluripotency, artificial sporangium (AS), female parthenogenetic apomixis (fPA), automixis, parthenogenetic androsporogenesis (mAP), mitosporogenesis, somatic embryogenesis, somatic parthenocarpy, experimental evolution, nitric oxide (NO), heterochrony, conifers, gymnosperms

Abstract: The distinctive features of cell cycle development in monoecious and dioecious gymnosperms become altered when the formative factor of gravity is suppressed in the aqueous environment of an artificial sporangium (AS). Embryonal cells of monoecious conifers quickly adapted and reverted to apomixis and automixis. In apomixis, neo-functionalized archegonial tubes dispersed parthenospores, which recapitulated free-nuclear proembryogenesis and somatic embryogenesis. In automixis (meiosis), androsporangial tubes discharged tetrads and other androspores in a display of heteromorphic asexual heterospory. Reproduction in life cycles was interpolated and brought to an earlier stage (progenesis). Eggs of two dioecious gymnosperms developed into oögonial tubes which discharged mitospores. Cells and eggs responded in a fundamental way like their remote ancestors the unicellular Algae. Spore discharges were accompanied by nitric oxide bursts originating from oxygen and arginine N. Silenced cells expulsed nuclear DNA forming micronucleated cells.When embryonal cells returned to unit gravity, cells aggregated on plates into adventitious colonies having different organizations in darkness and light (heterochrony). Protoplasts of embryonal cells were fused to construct heterokaryotic coenocytes which directionally dispersed spores in shallow multiwell plates. Angiosperm flower petiole cells from a *Prunus* rootstock, lacking the ability to form zygotic embryos, reverted to somatic parthenocarpy in an AS. A matrix classified how life and cell cycles relate to fertilization or non-fertilization and to genome reduction or non-reduction. Observations are designed to generate hypotheses and not to prove them. Combinatorial algorithms for the capture of genetic gains employing clonal somatic genotypes in nitrogen-poor boreal soils have yet to be reliably formulated.

Introduction

Karl von Nageli at the University in Munich dismissed Mendel's research published in 1865 for its lack of apparent applicability to anything but peas (Henig 2000). Darwin's supporters strongly believed in blended inheritance. Mendel's observations that genes were conserved rather than changed suggested that they were not open to the variations required for evolution. Not until both views were integrated could genetics and evolution thrive (Henig 2000).

Mendel became convinced that he had not found any universally applicable laws. At Nageli's suggestion he changed to *Hieracium* (hawkweed) and found no similar behavior in common with peas. Unknown to Mendel, hawkweed tended to be apomictic (parthenogenetic). At the turn of that century, the rediscovery of Mendel's laws recognized the importance and applicability of his ideas to their own systems. This launched the genetic revolution.

Franz Unger's book, "Versuch einer Geschichte der Pflanzenwelt", Wien 1852 (Attempt of a History of the Plant World), was one of the many text-books of Mendel (Voipio 1990). Unger had adopted a theory of evolution

characterized by the successive progress from primitive one-celled plant forms to the higher ones.

Unlike life cycles of *Pisum, Heiracium*, and *Arabidopsis*, the complexity, stature, wide adaptive climatic distributions, and long evolutionary history of gymnosperms present great experimental challenges for the capture of genetic gains in forestry. Steward (1958, 1970) demonstrated that single angiosperm cells, suspended in an aqueous culture medium on a clinostat with nutrition and supplemental hormones, are totipotent. Phloem cells from the wild carrot produced somatic embryos. When removed from cultures they formed plants that reproduced sexually. When cells from these plants and their seeds were again passed through a single cell ontogenetic bottleneck, they again produced embryos and plants that repeated their life histories on land.

The same replicated experiments with long-lived gymnosperm cells and eggs might take more than a century to complete. For this reason, pluripotency, or the ability to develop into different cell types, was explored with large and uniform populations of embryonal initials and eggs. Embryonal initials are somatic cells in the developing proembryo that are responsible for the basal developmental plan of seed embryos (Doyle 1963, Dogra 1978). Eggs are female gametes, when fertilized, undergo sexual reproduction forming embryos. Totipotency and pluripotency are not cited as a source of morphological innovation in models for the evolution of land plants (Qui *et al*. 2010).

Pluripotent cell cycles of gymnosperms, portrayed as a long series of interpolated evolutionary changes over hundreds of millions of years, were explored in an artificial sporangium (AS) (Durzan *et al.* 1994, Durzan 2011, 2012). An AS is a bioreactor with an aqueous medium capable of providing basic feed-forward and feedback bioprocess ontogenetic controls under prescribed and controlled laboratory conditions (Dutton *et al.* 1997, Durzan and Durzan 1991, Stephanopoulos 1984).

The effects of gravity, because it is continuous, uniform in intensity, and constant in direction and one of the most important formative factors in tree development, are hydrodynamically removed in the aqueous medium of an AS on a clinostat at 1 rpm. Supporting anatomical structures for the development of a gymnosperm ovule in trees are absent. Structures of a microsporangium, which produce and release pollen (androspores), are also absent. A haploid female gametophyte required for embryo development is no longer needed. The goal is to define suitable measures of pluripotency so that hypotheses of interest to the investigator are tested and yet not so large that the utility of an AS loses focus.

Darkness, a constant narrow temperature range and an aqueous environment are the main environmental factors acting directly on cell and egg populations scaled up in simulated microgravity under controlled and replicated laboratory conditions (genotype x environment). Hormones and nutrition are defined and prescribed in replicated runs lasting no more than 14 to 16 days. Fault-prone cell cycles were either removed or repaired and co-opted for adaptation to an aqueous environment.

Under newly imposed degrees of freedom and constraints of an AS, diploid single-celled embryonal initials and haploid eggs of gymnosperms were capable of neo-functionalization, transdifferentiation and interpolating heteromorphic asexual heterospory and mitosporogenesis. Neo-functionalization, like exaptation and co-option, refers the emergence of new adaptive traits by the exaptation of genes that previously encoded other functions (Blount *et al*. 2012). This includes shifts in the function of a genetic trait. Transdifferentiation refers to the flexibility of cell determination as a consequence of reprogramming already differentiated cells (Okada 1991). Heterospory is considered a key innovation in plant evolution (Bateman and DiMichelle 1994).

Cell lineages were model-referenced to the genotype's cytological and reproductive history, structures, functions, nutrition, physiology, ontogenetic plasticity, and environmental tolerances under field conditions. Repeated runs over one or more annual cycles identified matching, competitive, exclusionary and unexpected ontogenetic outcomes in an AS.

In the terminology of plant evolution, the interpolation and bringing forward of asexual reproductive development into embryonal initials and eggs is referred to as progenesis (Mogie 1992). An interpolation scenario, accounting for the origin of the land plant sporophyte, was postulated by Hemsley (1994). Models for the alternation of generations and evolution of plant life cycles on land are offered by Graham (1993), Haig (1992, 2008), Haig and Wilczek (2006), Qiu *et al*. (2012), and Niklas and Kutscher (2010). Observations relating to the adaptive plasticity of gymnosperm cells in an AS are recorded as follows.

First, pluripotent embryonal initials of monoecious conifers rapidly bypassed embryogenesis, decades of life-cycle maturation, and interpolated female parthenogenetic apomixis (fPA) by transdifferentiating into archegonial tubes which dispersed spores that rapidly initiated free-nuclear conifer proembryogenesis. Embryonal initials are responsible for the development of the basal plan of the seed embryo (Doyle 1963, Dogra 1978).

Embryonal initials also underwent automixis. Automixis is a form of parasexual reproduction involving DNA recombination, restitutional meiosis and parthenogamy (Rieger *et al*. 1976, Suomalainen *et al*. 1978). It bypasses biparental meiosis and fertilization. Embryonal initials undergoing automixis reverted to androsporogenetic parthenogenesis (mAP). They became neo-functionalized and transdifferentiated into androsporangial tubes which dispersed monads, dyad, triad, and tetrads. Products of apomixis and automixis displayed heteromorphic asexual heterospory in an aqueous environment.

Second, multinucleate eggs of dioecious *Ephedra* (Gnetales) and *Taxus* (Taxales) were neo-functionalized into oögonial tubes which discharged and dispersed mitospores. Mitospores are formed asexually by mitosis. Genetic recombination may occur in mitosis but this is rare. Discharged mitopores retained the special morphological characteristics of proembryos in Ephedraceae. Gnetales have practically no fossil record and are far removed morphologically from conifers (Coniferales) and Taxales (Sporne 1965). In *Taxus*, the central cell behaves as the egg cell (Bhatnagar & Moitra 1996, Singh 1978). When fertilized, it develops a 16-nucleate proembryo. In an AS, central cells replicated free nuclei as they became neo-functionalized and transdifferentiated into oögonial tubes with mitospores. Female spores were dispersed in the culture medium.

Third, a lingering fundamental question, relating to the origin of the alternation of generations in the life cycle of plants, was raised by the Linnean Society (1909). "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? This seemed … the essence of the problem which the future had to solve" (*cf.* Bower 1908). Coulter (1914), commenting on the evolution of sex in plants, stated that "…it would be of extreme interest to know the ancient condition, but we know only what it has become".

Cell suspensions of four monoecious and elite conifer genotypes from Europe, North and South America were used to address this fundamental question. Neo-functionalized apomictic embryonal initials (2n) discharged and dispersed female parthenospores. Automixis led to the dispersal of androspores. Neo-functionalized eggs of *Taxus brevifolia* and *Ephedra californica* transdifferentiated into oögonial tubes which dispersed mitospores (Durzan 2011).

Sporogenesis in an AS emulated asexual and sexual reproduction in modern and extinct algae (Bold *et al*. 1980, Maggs and Callow 2002, Stewart and Rothwell 1993). Spore formation in algae occurs by meiosis (meiospores), mitosis (mitospores), asexual reproduction (parthenogenetic gametes) and by motility (nonflagellate spore versus flagellate zoospores) (Maggs and Callow 2002).

Fourth, embryonal cells programmed too far for embryogenesis or incompletely neo-functionalized, displayed Rabl chromosome orientations (bouquets) on nuclear membranes. Bouquets are a visible form of transcriptional silencing leading to the expulsion of independently functioning and non-self governing chromosome parts and methylated DNA sequences (Rieger *et al*. 1976). Their expulsion led to the formation of micronucleated cells.

Fifth, selective forces favoring the evolution of genomic imprinting were implicated in the origin of modern land plants (Haig and Wilczek 2006). Salmine is an arginine-rich protamine in salmonoid fishes which remodels somatic cells to gametes. Conifers have arginine-rich seed proteins. The potential of salmine as a silencing factor in embryogenesis and for imprinting gametogenesis in embryonal initials was explored in an AS (Durzan 2010a). Over a wide range of concentrations, salmine did not remodel embryonal cells into gametes. Embryonal initials no longer differentiated into axial tiers.

Sixth, gravity, water and light are the most important factors in the environment in determining the course of development in woody plants (Zimmermann and Brown 1971). Embryonal initials in an AS, when returned to unit gravity on wet culture plates, assembled into cell colonies displaying different heterochrony in darkness and light. Heterochrony, or change in developmental timing alone, is inadequate to explain the diversity of plant form (Crane and Kendrick 1997). It has utility in experimentally showing how cells in an AS adapt and reorganize when returned to unit gravity.

In darkness, plated cells aggregated into an adventitious cluster. Cells clustered at the center rapidly divided. Colonies were surrounded by spent archegonial tubes. In light, aggregated embryonal initials formed green chloroplasts and assembled into a ring of rapidly diving cells. Mucilaginous factors, released from archegonial and androsporangial tubes and from apoptotic embryonal initials, displayed a wide range of properties associated with the partial enclosure of residual androspores by androsporangial tubes.

Seventh, Klebs (1910) concluded that "sexual and asexual reproduction is independent from one another because they are controlled by different conditions, and it is therefore possible to separate them." "We can replace

the sexual one without difficulty….various stages of development can be produced or prevented as we may desire…the succession (order) of the stages can be modified at will." Although Klebs's conclusions are convincing with plants, they have yet to be applied to products initiated by gymnosperm and angiosperm single cell cycles.

Mendel spent time in the orchard with his father, who worked with fruit trees (Voipio 1990). Fruit tree seeds are enclosed in an ovule which forms the fleshy part (pericarp) of a fruit as in a cherry or apple. Gymnosperms seeds are not enclosed in an ovule. *Prunus cerasus* cv. Vladimir aborts its zygotic embryos under field conditions. It is used as a commercial rootstock for Bing cherry trees. Can flower petiole cell suspensions from this rootstock undergo somatic embryogenesis in an AS? Somatic parthenocarpy in flower petiole cells bypassed flowering and fruit development by directly producing fleshy red pericarps (Durzan 1988a). Unable to undergo somatic embryogenesis, the rootstock was cloned by micropropagation. The utility of an AS is extended to angiosperms having long life cycles.

Eighth, a matrix formulated by Darlington (1937) is updated to classify how reproduction in gymnosperms is related to fertilization or non-fertilization and genome reduction or non-reduction in an AS and to artificial apomixis in crop plants (Marimuthu *et al*. 2011).

Proceedings Abstracts offer alternative methods for cloning somatic embryos in conifers and other woody species. Advances in genomics, molecular biology and bioinformatics have yet to be developed to convincingly characterize clonal reaction norms in field trials especially in N-poor forest soils. Difficult problems relating the origin of cell cycles, pluripotency and totipotency to how gymnosperms might have evolved in the deep past are discussed.

Historical background

The first opportunity to clone conifers from cells emerged at the International Botanical Congress in 1959 at Montreal when F.C. Steward and his colleagues demonstrated plant cell totipotency in carrot cell suspension cultures. In totipotency the "genetic inheritance of the zygote, transmitted at cell division to all daughter cells, conveys to every organelle the inherent capacity to carry out its various functions … each with their own genetic information, may be called into appropriate activity by the great array of simple substances which, singly, or in combination, act as growth regulators: these release, wholly or in part, the inherent totipotency of angiosperm cells … If the new individuals again produces offspring through a complete life cycle, then the originating cells may be considered "totipotent" (Steward 1958, 1970, Steward *et al*. 1958).

Steward's technology was identified in 1960 as having utility to explore how conifer cells might be used to solve problems of unpredictable flowering, seed production and long life cycles, which limited silvicultural practices in N-poor boreal soils (**Fig. 1**).

Earlier, AJP Martin was awarded the Nobel Prize in Chemistry in 1952 for the discovery of paper chromatography (Martin and Synge1941). Steward, knowing Martin, introduced paper chromatography to the USA. His lab used radioactive isotopes and automated column chromatographic methods (*cf* Manning 1993) to identify new amino acids in plants and demonstrated how their metabolism related to protein and DNA synthesis, growth, development and environmental changes (Steward 1963, Steward and Durzan 1975, Durzan and Steward 1967, 1983). Melvin Calvin's group at Berkeley gleaned the utility of paper chromatograph and autoradiography from one of Steward's post docs. This methodology was used to identify the first products of photosynthesis.

Steward's clinostat and cell suspension technology was established in 1963 at the Petawawa Forest Experiment Station in Ontario. Vladimir Chalupa was my first Post-Doc Fellow (1965-1966). White spruce and jack pine cell suspensions were scaled up in one liter nippled flasks on a clinostat rotating at 1 rpm in an environmentally controlled growth chamber (**Fig. 1**). Jan Bonga's tissue culture and cytological research was already in progress at Fredericton. Biochemical analyses evaluated how zygotic embryos and female gametophytes in jack pine seeds across the Canadian Boreal forest were altered by climate at the seed source and in N-poor soils (Durzan and Chalupa 1968, 1972). Canadian Forestry Branch boldly predicted in 1969 (**Fig. 2a**):"From the bud or seed tissue, we grow single living cells in nutrient solutions in flasks. The cells divide and multiply, while we study how cells react to various chemical and nutritional combinations. Radioactive isotopes in the solution enable us to trace and identify the chemical components of living cells in various stages of their development. Nutrition of the tree can thus be examined and controlled under laboratory conditions, so as to grow embryo-like structures that show promise of developing into seedlings with selected characteristics of growth, hardiness, resistance to disease and insects. BIOCHEMICAL RESEARCH INSTITUTE IN FORESTRY – A GIANT STRIDE TOWARDS POSITIVE GROWTH AND QUALITY CONTROL. FORESTRY BRANCH"

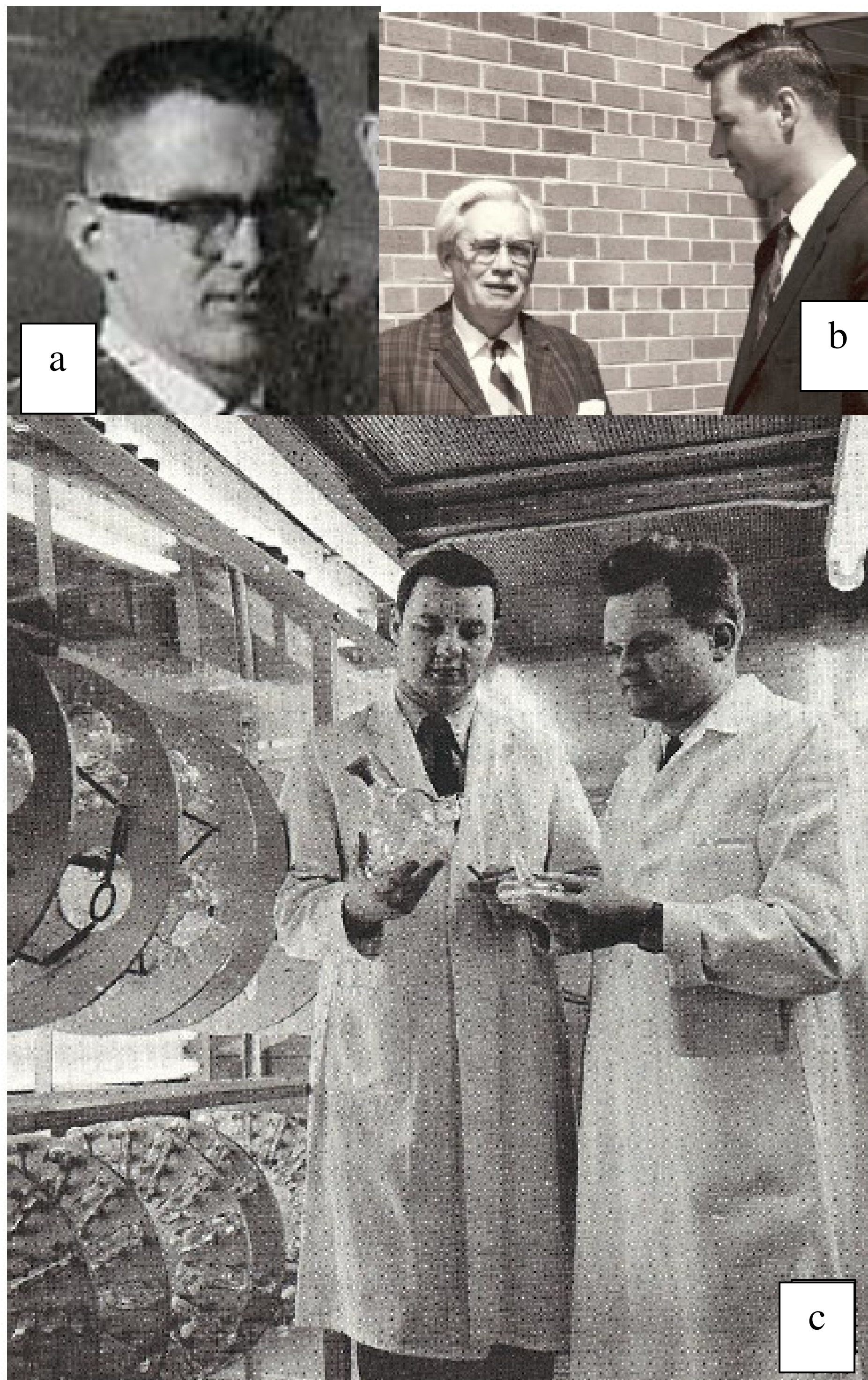

Fig. 1*. *a. *Jan Bonga (inset). Don Durzan and other Canadian forest scientists and Administrators in the Canadian Forest Service visited the Petawawa Forest Experiment Station (PFES) to evaluate future opportunities in forest biology research in support of silviculture in 1964.* ***b***. *FC Steward FRS and Durzan discuss N metabolism, cell and tissue culture strategies for conifers at PFES.* ***c***. *Durzan and Vladimir Chalupa examine cell cultures in a nipple flask from a clinostat in a controlled growth chamber (1965).*

Growth patterns, resembling early proembryo formation, were observed in nippled flasks but not reported until later (Durzan and Steward 1971). At New Brunswick, Bonga and Fowler (1970) and Bonga (1974) examined the utility and developmental properties of gametophyte, micro- and megasporophyll cultures in red pine for tree breeding and improvement. Nuclear changes and diploid nuclei in megasporophyll and microspore cultures were recorded by Bonga (1974, 1981).

Dormant buds from conifers were cultured under different N sources and hormones to initiate apical bud break and shoot formation *in vitro* (Chalupa and Durzan 1973). Metabolic fates of ^{14}C-amino acids in trees under field conditions and the fate of tritiated water during seed germination under aseptic conditions identified which metabolites could best be selected in reformulating culture media supporting DNA replication and overall somatic cell growth rates in nippled flasks (Durzan 1968a, b, c, 1969a, b, 1973a, b, c, d, 1983a, b).

Controlled shading of saplings in canopies under field conditions identified which amino acid families in needles, stem with buds and roots were most responsive to changes in seasonal light intensity, photoperiod and temperature (Durzan 1971). Acid-soluble nucleotides (Durzan *et al*. 1972), metabolic fates ^{14}C-proline and glutamine (Durzan 1973a), uracil (Durzan *et al*. 1973), urea (Durzan 1973b), and tritiated water in the presence of urea (Durzan 1973c) identified metabolic pathways supporting cell, tissue and organ development under controlled laboratory conditions in light and darkness.

Sphaeroblasts, induced by wounding tree trunks, had utility in horticulture to clone fruit trees (Wellensiek 1952). Could cell suspensions from the cambium of mature trees be used to interpolate somatic embryogenesis into cambial cells? Hypocotyl cell suspensions of white spruce responded to synthetic hormones under constant light and temperature by forming sphaeroblasts in nippled flasks (Durzan *et al*. 1973). By contrast, diurnal changes in temperature and photoperiod of a late spring day, recorded at the genotype's forest site, increased sugars in cells, produced tannins in vacuoles and disrupted sphaeroblast organization in nippled flasks (Chafe and Durzan 1973). Cambial initials, dedifferentiated and scaled up in darkness at a constant temperature in an AS, might be used with hormones to initiate somatic embryogenesis, apomixis and automixis in mature genotypes.

Prospects for the mass propagations of improved stock of forest trees by cell and tissue culture and other methods were evaluated (Durzan and Campbell 1974). Methods, having utility at that time, were sequential treatments with growth regulators, nutrient formulations model-referenced to ontogeny, and progressive simplification of media. Mutations, transfer of genes from one cell to another, acquisition of bacterial genes, organelle uptake, nitrogen fixation, transduction, transformation, somatic cell hybridization, chimeras, production of homozygous specimens, predictions of phenotype expressions, disease-free genotypes, host-parasite relations, cultivation with mycorrhizae and cryopreservation were identified as having utility in forest biological research.

The recovery of forty liters of cell biomass from hypocotyls and radicles in nippled flasks was characterized by Richards's comprehensive growth function and by conditioning factors released from cells in the culture medium (Chalupa *et al*. 1976, Durzan *et al*. 1976). Autocatalytic and logarithmic growth functions in light and darkness (Durzan and Chalupa 1976a, 1976d) were related to compositional changes in free amino acid N in cells, conditioning of the culture medium (Durzan and Chalupa 1976b), to sequential anabolic and catabolic changes and to a model describing the release and dispersion of free cells (Chalupa *et al*. 1976, Durzan and Chalupa 1976c).

Spruce budworm outbreaks threatened Canadian forests and contributed to serious fire outbreaks near cities. Research was diverted to show that forest fertilizer use and amino acids in the conifer food supply were correlated with spruce budworm development (Shaw *et al* 1978). *N*-phosphoryl arginine was isolated and identified in the spruce budworm (Durzan and Pitel 1977). This high energy phosphagen served as ATP throughout all stages of instar development.

Tracking radioactively labeled metabolites required the development of new automated and analytical methods integrated with liquid scintillation counters (Durzan 1969b, Ventimiglia and Durzan 1986). Products of ^{14}C-arginine, urea, proline, glutamine and guanidino acetic in conifers characterized how N metabolism related to adaptation and survival under N-poor boreal field conditions (Durzan 1968a, b, c, d, 1973a, b, c, 1983a, 2009a, 2010b) and to metabolic engineering in a space environment (Durzan 2000). Advances in carbon and nitrogen metabolism were summarized in a Symposium honoring FC Steward (Bidwell and Durzan1975).

Canada enters the clonal age

White spruce was micropropagated in vitro (Campbell and Durzan 1975, 1976 a, b). The American elm was regenerated from cell cultures (Durzan and Lopushanski 1975). Canadian Broadcast Corporation (CBC) Radio Archives produced in January 27, 1975 an audio file announcing that Canada has entered the clonal age in forestry. The file is accessible at http://www.cbc.ca/archives/Categories/Science-technology/biotechnology/Canada.

Laser red and far-red light were used to regulate germination in jack pine seeds (Campbell and Durzan 1979). Brief exposures to red light at night inhibited flowering in a white spruce seed orchard (Durzan *et al*. 1979). Embryonic cell development in darkness was selected to avoid precocious developmental changes induced by light intensity and quality.

"Tissue culture in forestry" (Bonga and Durzan 1981) and "Cell and tissue culture in forestry" in three volumes (Bonga and Durzan 1987) were published. Bonga (1981) reviewed the cytology of haploid conifer cells. Petawawa Experiment Station was closed (Place 2002). Somatic embryos and regeneration of plants was obtained from primordial shoots of 10-year-old white spruce (Klimaszewska *et al*. 2011a). Expression profiles of 11 genes tracked the developmental process. A chronology of subsequent cell and tissue culture research in Canada was summarized by Klimaszewska *et al*. (2011b). Problems relating to the experimental recall of residual and past developmental history of cells were outlined by Durzan (1984).

Somatic embryogenesis

Vladimir Chalupa (1985) first reported somatic embryogenesis in a conifer (Norway spruce) followed by Hakman *et al*. (1985). At Davis, embryo rescue methods (Ramming 1983) were being modified to isolate conifer embryonal suspensor masses (ESMs, Fig. 2c) and clone elite genotypes by somatic embryogenesis and cleavage polyembryogenesis (Gupta and Durzan 1986a, Hong *et al*. 1991). Patterns of ESM development and mucilage formation by Norway spruce ESMs vary by genotype (Jokinen and Durzan 1994). Mucilage from embryogenic cultures, when spread on young needles from shoots rejuvenated *in vitro,* increase mitotic activity and produced embryoid-like structures (Durzan unpublished).

Acetocarmine and Evans blue tracked the cytological development of axial tiers from embryonal initials and visualized various stages of cleavage polyembryogenesis in somatic embryos (Dogra 1967, 1978, Durzan 2008). Acetocarmine reacts with cytoplasmic and nuclear proteins and with peroxisomes in embryonal initials (Durzan 1996b). Cell regulatory protein turnover along the axial tier of early somatic embryos was demonstrated and coordinated by protein ubiquitination and apoptosis (Durzan 1996b, 2010a). Ubiquitination and turnover are linked with plant hormonal signals in cells (Santner and Estelle 2009).

Rescued residual ESMs in seeds of sugar pine, stored for 5-years in a USDA Forest Service drawer, were sufficiently viable to initiate somatic embryogenesis (Gupta and Durzan 1986b). Protoplasts from loblolly pine embryonal initials regenerated somatic embryos (Gupta and Durzan 1987, 1991).

Somatic embryogenesis in Douglas-fir was first demonstrated with embryonal cell suspensions (Boulay *et al*. 1988, Durzan and Gupta 1987). Abscisic acid was added to overcome the problem of cleaving early embryos adhering to one another. Cryopreservation and thawing removed damaged cells and selected for cells capable of regenerating somatic embryos (Gupta *et al*. 1987). Douglas-fir and Norway spruce clones were planted in soil and cryopreserved (Durzan and Gupta 1987, 1998). Progress for cloning viable somatic embryos in support of forest research and capturing genetic gains was periodically summarized (Durzan 1981, 1985, 1988e, 1991, 2008).

Foreign gene expression was demonstrated in micropropagated Douglas-fir (Dandekar *et al*.1987). A firefly luciferase gene was expressed in Douglas-fir and loblolly pine protoplasts (Gupta *et al*.1988). A double haploid genotype of larch was produced by protoplast fusion (Pattanavibool *et al*. 1998, von Aderkas and Bonga 1993).

Somatic polyembryogenesis was patented by the University of California (Durzan and Gupta 1998) and quickly licensed by Weyerhaeuser. The first cloned specimen of loblolly pine was donated to Weyerhaeuser. Patent terms and conditions stipulated that Weyerhaeuser's progress could not be revealed in my publications. Pramod Gupta was hired by Weyerhaeuser to develop this technology for their use (Gupta, these Proceedings). Simplified hybrid seed production by latent diploid parthenogenesis (*cf*. Durzan *et al*. 1994) and parthenote cleavage was patented (Durzan 1988d) but not licensed.

Cell cycles, multicellularity and neo-functionalization in an artificial sporangium

Plant life cycles begin and end by passing through a single-cell bottleneck (Dawkins 1982). Without knowledge of cell cycles, it is not possible to understand how genes on chromosomes and environments direct development. Bottleneck cycles switch genes on and off. Genomic replicators reset and establish a new ontogenetic calendar with a particular time.

Dividing cells at the G1phase prepare for the S phase (DNA replication). G1 provides time for the cell to monitor the internal and external environment to ensure that conditions are suitable and preparations are complete

before the cell commits to DNA synthesis and cell replication. Synthetic plant hormones stimulate DNA synthesis and increase the probability that plant eggs or egg-equivalents will develop parthenogenetically (Bell 1992, Durzan *et al*. 1994).

Cell cycling in and out of G1 becomes the main determinant for the rates of DNA synthesis, repair, and cell proliferation in reproduction and development (Murray and Hunt 1993). Ubiquitin-mediated cyclin turnover serves as an oscillator that determines cell cycle progression (Hershko 1991). Epigenetic mechanisms regulate gene expressions for specific cell fates. Diploidy exploits mutations that are overdominant. It permits the efficient repair of double-strand breaks (Crow 1994).

Cell cycles are terminated by programmed cell death (apoptosis) (Bell 1994, 1996, Durzan 2010a, Havel and Durzan 1996a, b, 1999a, b). Apoptosis is detected cytologically by the TUNEL reaction using a terminal deoxynucleotidyl transferase (Havel and Durzan 1995). Early embryos released mucilage that digested external food sources and products of apoptosis. This renewed a nitrogen food supply and other factors supporting embryo survival and development (Durzan and Steward 1983).

The first comprehensive treatise on gymnosperm embryology was provided by Strasburger (1872). Doyle wrote Steward in 1965 suggesting that conifer embryonal initials could be considered totipotent based on their ability to generate the basal plan for embryogenesis in seeds (Doyle1963). Uniform cell populations of embryonal initials were subsequently selected as "ontogenetic set points" to explore mitosis, amitosis (apomixis), meiosis (automixis), free-nuclear replications (coenocytes), genomic duplications (polyploidy) and apoptosis in nippled flasks on a clinostat. Stebbins (1965, 1988) cited nucleic acid synthesis, mitotic rhythms, and morphogenesis as factors having utility in making evolutionary comparisons. He encouraged studies with an artificial sporangium at Davis.

Clinostats

Clinostats were first used to explore artificial rhythms in plant development (Darwin and Pertz 1881). When rotations were removed, artificially imposed rhythms continued as an after-effect in plants. Development became linked to memory (Russell1930). The internal transmission of growth-stimulating signals from site of perception of an environmental stimulus to sites of subsequent response was postulated. The analogy of development with habitual action with past memory in plants also required an explanation. Darwin and Pertz's experiments led to the classic auxin studies by Boysen-Jensen and F. W. Went using oat coleoptiles. Auxin responses became a favorite model system of Charles and Francis Darwin (Ayres 2008).

Steward's inclined clinostat rotating at 1 rpm removes the formative effects of gravity on cells (**Fig. 2b**). When plant growth in an outer space environment is compared to a clinostat only small differences are detected (Space Studies Board 1998). The best way to simulate weightlessness is obtained with a 3-D clinostat rather than a horizontal slightly inclined one. The speed of rotation must be increased above 1 rpm. Weightlessness can be achieved in rotating wall vessels (**Fig. 2d**) but not in air-lift and stirred-tank bioreactors.

Cells under simulated microgravity and in an aqueous medium of nippled flasks are exposed to mild centrifugal forces and oscillations having mass-dependent amplitudes of speed and phase shifts relative to the clinorotation (Albrecht-Buehler 1992, van Loon 2007). The direction of gravity in single cells is perceived by the sedimentation of statoliths, tethered by the cytoskeleton. If cells are forcefully perturbed, amyloplasts in embryonal initials respond by releasing bursts of nitric oxide (see below).

Mild turbulent flow facilitated buoyancy and gas exchanges among cells. Convective currents generated mass-dependent movements as cells and their products entered and exited each of the nipples of the AS (**Fig. 2b**). Hydrostatic pressures, which are independent of the direction of gravity, were imposed in the presence of synthetic growth hormones. Anabolic feed-forward and catabolic adaptive feedback reactions (Durzan and Durzan 1991, Stephanopoulos 1984) neo-functionalized cell cycles for adaptation to forces and factors in an AS.

Controlled temperatures in an AS varied no more than 25 ± 3 °C. When Araucariaceae were dominant in the Triassic, ocean temperatures ranged between 21°C to 36 °C (Sun *et al*. 2012). A suspended micro-bathymetric imaging and environmental monitoring system might be designed to observe how cell cycles and ontogeny respond to temperatures, pH, osmolality and imposed hydrodynamic forces in an aqueous medium (Durzan 1989).

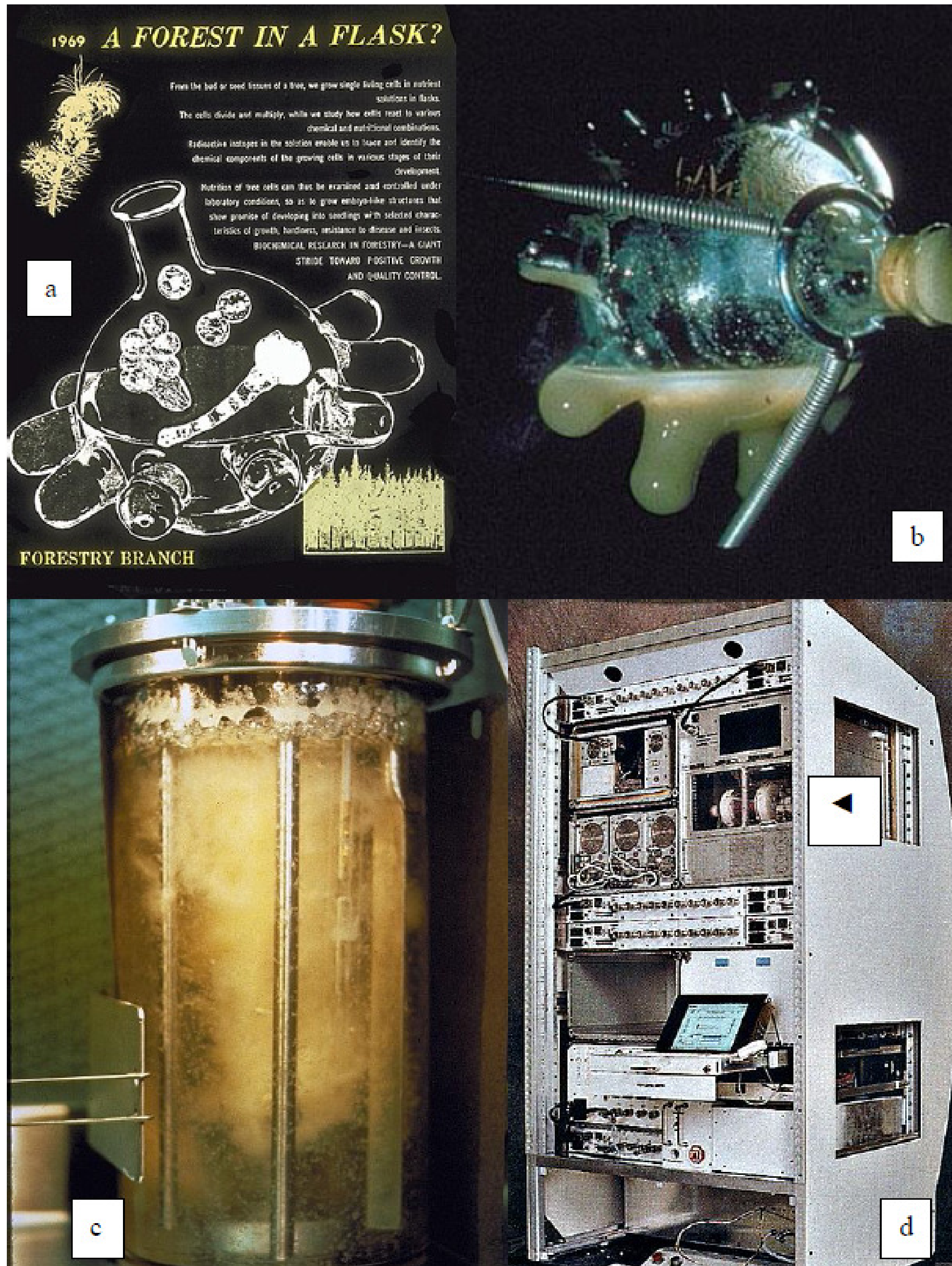

Fig. 2*. *a*.** *After cloning white spruce and regenerating the American elm from cell cultures at the Forest Research Institute in Ottawa, the Forestry Branch advertised in 1969 that cell and tissue culture research might someday contribute to silviculture.* ***b. *One-liter nippled flask with a uniform cell suspension of Norway spruce embryonal initials.* ***c***. *Parthenogenetic apomixis and somatic embryo formation was reported and scaled-up in 1.4 L stirred tank Multigen bioreactor (Durzan et al. 1994). The bioreactor shows embryonal suspensor masses (ESMs) of Norway spruce around the main spindle. ESMs were later converted into clones for field planting. Somatic polyembryogenesis was patented by University of California and licensed by Weyerhaeuser (1997-2007).* ***d***. *Rotating wall bioreactors (◄) in the minipayload integration modules in a NASA space shuttle. (cf. Durzan. Abstract). Rotating wall bioreactors and nippled flasks were used to culture egg suspensions of Ephedra californica and Taxus brevifolia.*

Apomixis in conifers occurs under field conditions

Phases in the sexual life cycle of a monoecious conifer are illustrated in **Fig. 3**. Apomixis occurs in conifers (Allen 1942, Dogra 1966, Orr-Ewing 1957a, b, Saxton 1909). Apomixis produces unreduced gametes that retain the genotype of the parent. It is followed by parthenogenetic development of the diploid nucleus in a cell into an embryo. Experiments that prevented pollination in Douglas-fir seed orchards provided proof for apomixis in seeds.

Four requirements are identified for asexual reproduction in plants (Mogie 1992). These are: the capacity for parthenogenesis must be present. Fertilization of eggs or egg-equivalents must be prevented. Meiotic reduction is avoided. All conditions must be met simultaneously. Under laboratory conditions, these requirements were first met in Norway spruce cell suspensions using nippled flasks and a stirred tank bioreactor (Durzan *et al.*1994).

The level of DNA in an egg nucleus influences the probability of parthenogenetic development. An egg cell or nucleus can be replaced with another cell of the archegonium to fulfill egg function (Mogie 1992, Dogra 1966). The capacity for mitosis (endomitosis, amitosis) by a substituted cell becomes an efficient, effective and parsimonious way of establishing parthenogenesis. Short-term selection now acts on the total genetic variance and not just the additive component in asexual species (Crow 1994).

Batygina (2009) reviewed apomixis in angiosperms. She identified sudden changes in environment, photo-thermal genes, duplicate gene asynchrony, polyploidy and polygenic traits as factors associated with apomixis. "What is not clear is why, when or how the transition to asexuality occurs" (Mogie 1992).

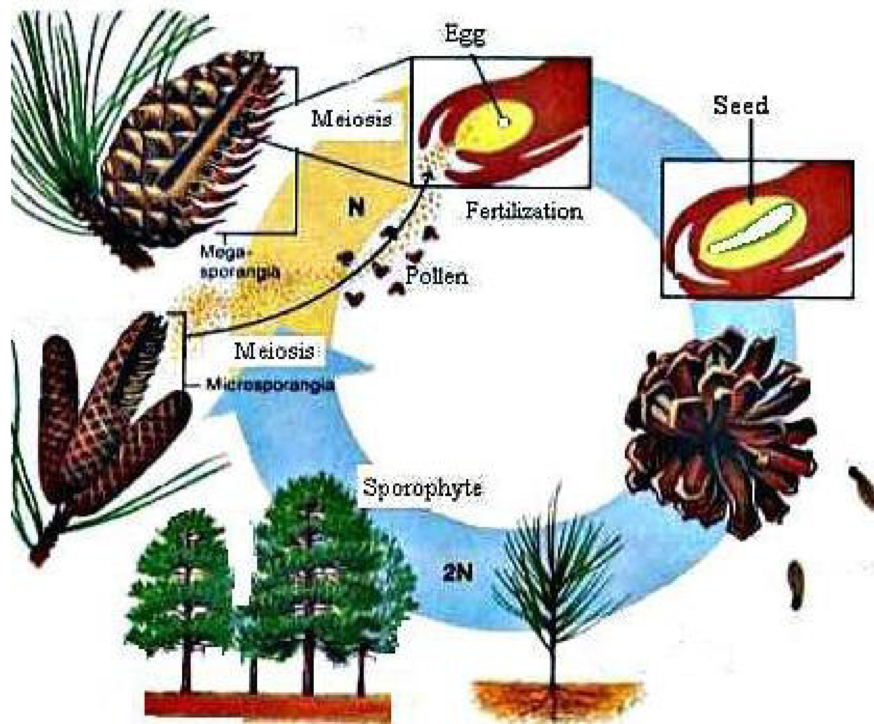

Fig. 3. *Apomixis and automixis were interpolated into cell cycles of embryonal initials (2n) and nuclear cycles of eggs (n) and scaled up in an AS. In progenesis, asexual reproduction is brought to an earlier developmental stage of the life cycle. Decades of sporophyte development, reproductive maturation and gene x environment are bypassed (blue arrow) in an AS under controlled laboratory conditions. In asexual heterospory, embryonal initials from monoecious conifers are neo-functionalized and transdifferentiated into archegonial or androsporangial tubes which discharge female parthenospores or androspores into the aqueous environment of an AS. Apomictic sporophytic development is completed outside the AS and evaluated under field conditions (g x e). Egg suspension cultures from dioecious gymnosperms replicate their free nuclei by mitosis in transdifferentiated oögonial tubes that discharge mitospores. All observations are modeled to asexual and sexual reproduction in modern algae (Maggs and Callow 2002) and to paleobotanical records (Stewart and Rothwell 1993).*

Reproductive neo-functionalization and interpolation of asexual heterospory

Transitions to apomixis and parasexual reproduction (automixis) were demonstrated with cell suspensions of embryonal initials from elite genotypes of Norway spruce (Durzan *et al*. 1994), Douglas-fir (Durzan 2010), *Araucaria angustifolia* (Durzan 2012) and with egg suspensions cultures from female *Taxus brevifolia* and *Ephedra californica* (Durzan 2012).

Stock cultures were maintained for several years in dark controlled growth chambers purged of ethylene at 25 ± 3 ^{o}C. Experimental protocols required brief exposures of cells to weak cool and incandescent white light. Exposures never exceeded 0.2 mmoles/m^2/sec. A fresh supply of hormones in a predefined medium was required to initiate apomixis and automixis in embryonal initials and eggs.

Female parthenogenetic apomixis (fPA) and androsporogenetic parthenogenesis (mAP) were cytologically model-referenced to the reproductive development of each monoecious conifer and its development under field conditions. Asexual reproduction in eggs was referenced to the life cycle of each female dioecious genotype. Spores dispersed in an aqueous medium are classified and model-referenced to reproduction in algae (Bold *et al*. 1980, Maggs and Callow 2002, Steward and Rothwell 1993). Asexual heterospory was not observed in the absence of synthetic plant growth hormones.

Reproductive neo-functionalization of embryonal initials from monoecious conifers required that each embryonal initial undergoes progenesis and interpolates fPA and mAP into subsequent nuclear and cell divisions. Neo-functionalized embryonal initials were transdifferentiated into archegonial and androsporangial tubes that developed, discharged and dispersed heteromorphic female and male spores into the AS. Female parthenospores rapidly underwent free-nuclear somatic proembryogenesis (Durzan *et al*. 1994, Durzan 2011, 2012). Somatic embryogenesis was completed outside the AS and in the laboratory.

Embryonal initials undergoing automixis were neo-functionalized and transdifferentiated into androsporangial tubes that discharged and dispersed monads, dyad, triads, tetrads and polyads. This expression of asexual heterospory bypassed the juvenile and maturation phases of the life cycle (**Fig 3**).

Rescued eggs of *Ephedra* and *Taxus* replicated free nuclei. Eggs were neo-functionalized and transdifferentiated into multinucleated oögonial tubes. Free nuclei developed into mitospores which were discharged and dispersed in an aqueous AS (see below).

Female parthenogenetic apomixis, somatic embryogenesis and cleavage polyembryony

When model-referenced to the development of archegonia in conifers under field conditions (Håkansson1956), apomictic nuclei in embryonal initials of Norway spruce, Douglas-fir and *Araucaria angustifolia* responded like central cells in archegonia. In the absence of fertilization, their nuclei replicated DNA by endomitosis (**Fig. 4**). Chromosomes were duplicated without cell or nuclear division. If an existing gene were to mutate at this time and take on new functions, it might contribute to evolutionary changes (Pennisi 2012).

Endomitosis (4n) was followed by an amitotic dumb-bell shaped nuclear cleavage (*cf.* Chamberlain 1935, p.122). Embryonal initials became neo-functionalized and transdifferentiated into archegonial tubes with a diploid egg-equivalent nucleus and a ventral canal nucleus (vcn). Vcn formation may contain residual transposable elements and genomic silencing factors found in embryonal initials. Blackman and Chamberlain considered the vcn as an arrested gamete because it was observed to fuse with a microgamete (egg) in *Pinus* (Johansen 1950, p. 12).

The vcn moves to a distal pole of the archegonial tube and undergoes apoptosis (Havel and Durzan *et al*. 1994, Durzan 1996a, b, 1999a and b). Apoptotic products from the degenerating vcn may provide signals, necrohormones and supplemental nutrition for the development of the diploid egg-equivalent nucleus and its neocytoplasm into a proembryonal parthenospore (Bell 1994, 1996).

Traction fibers in the cytoplasm of archegonial tubes became compressed around the prospective female parthenospore. Fibers were retained in the tube when the spore was discharged (Durzan 2012). The dispersed female parthenospore rapidly underwent a series of free-nuclear replication characteristic of coenocytic stages in seed proembryogenesis (*cf.* Johansen 1950). Nuclear replications and cell regulatory protein turnover (Durzan 1988b, 1996b, 2010a) become linked to the signaling actions of plant hormones (Santner and Estelle 2009).

In Pinaceae seeds, the free-nuclear stage in zygotic proembryogenesis results in two free nuclei. Both divide once more to form four small free nuclei which increase in size. Free nuclei are pulled to the base of the archegonium by traction exerted by fibrils. Nuclei are walled off to initiate proembryo development (Berlyn and Passof 1962).

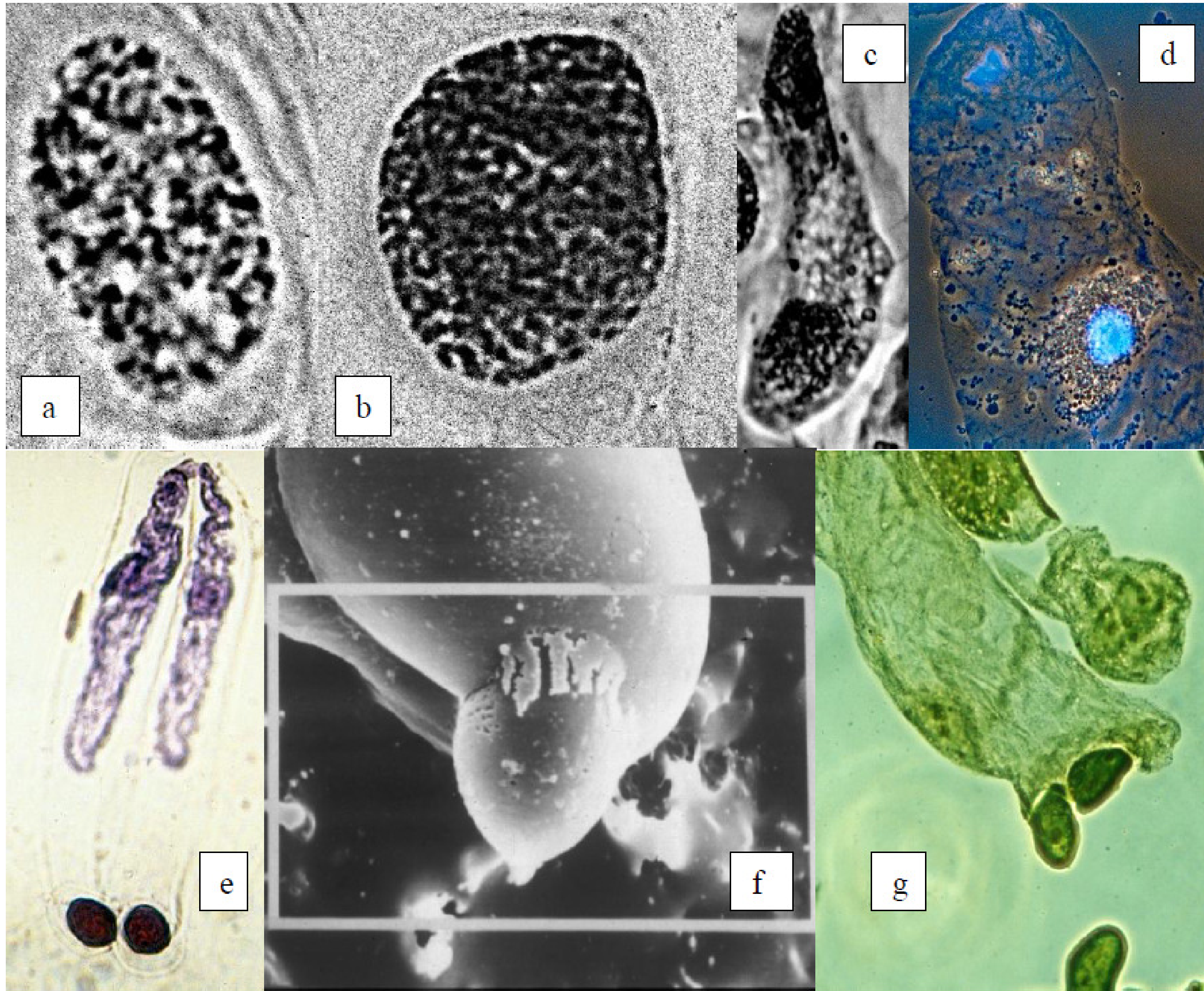

***Fig. 4**. Embryonal initials of monoecious conifers emulated archegonia formation in trees under field conditions. (Araucaria **a** to **c**). An embryonal initial nucleus (2n) (**a**) replicates (4n) (**b**) and undergoes amitosis (**c**). Initials are neo-functionalized into archegonial tubes with an egg-equivalent nucleus and a vcn. (Feulgen-Giemsa). (Norway spruce **d**). An egg-equivalent nucleus in a transdifferentiated archegonial tube is surrounded by a neocytoplasm. The small apoptotic vcn (top) deteriorates (DAPI). (Douglas-fir **e**, **f**).Twin archegonial tubes (**e**). The prospective diploid female parthenospore with its neocytoplasm is separated from the archegonial cytoplasm and moves basally before discharge. The upper vcn deteriorates (acetocarmine, Evans blue). The parthenospore (**f**) emerges through an archegonial tube before dispersal into the aqueous culture medium (SEM). Dispersed female parthenospores rapidly undergo free-nuclear proembryogenesis. (Norway spruce automixis and androsporogenesis **g**). Monads are discharged from transdifferentiated androsporangial tubes. Monads develop into dyads, triads, tetrads and polyads (silver nitrate, Giemsa).*

Cross-pollinated Douglas-fir produced high numbers of free nuclei which yield vigorous proembryos in seeds. Self-pollinated seeds produced proembryos with few free-nuclei and embryos aborted (Orr-Ewing 1957a, b). Dispersed parthenospores of Douglas-fir developed into cleaving early embryos. Individual somatic embryo development was completed outside an AS (**Fig. 5**). Protoplasts of embryonal initials were also able to generate somatic embryos (Gupta and Durzan 1987).

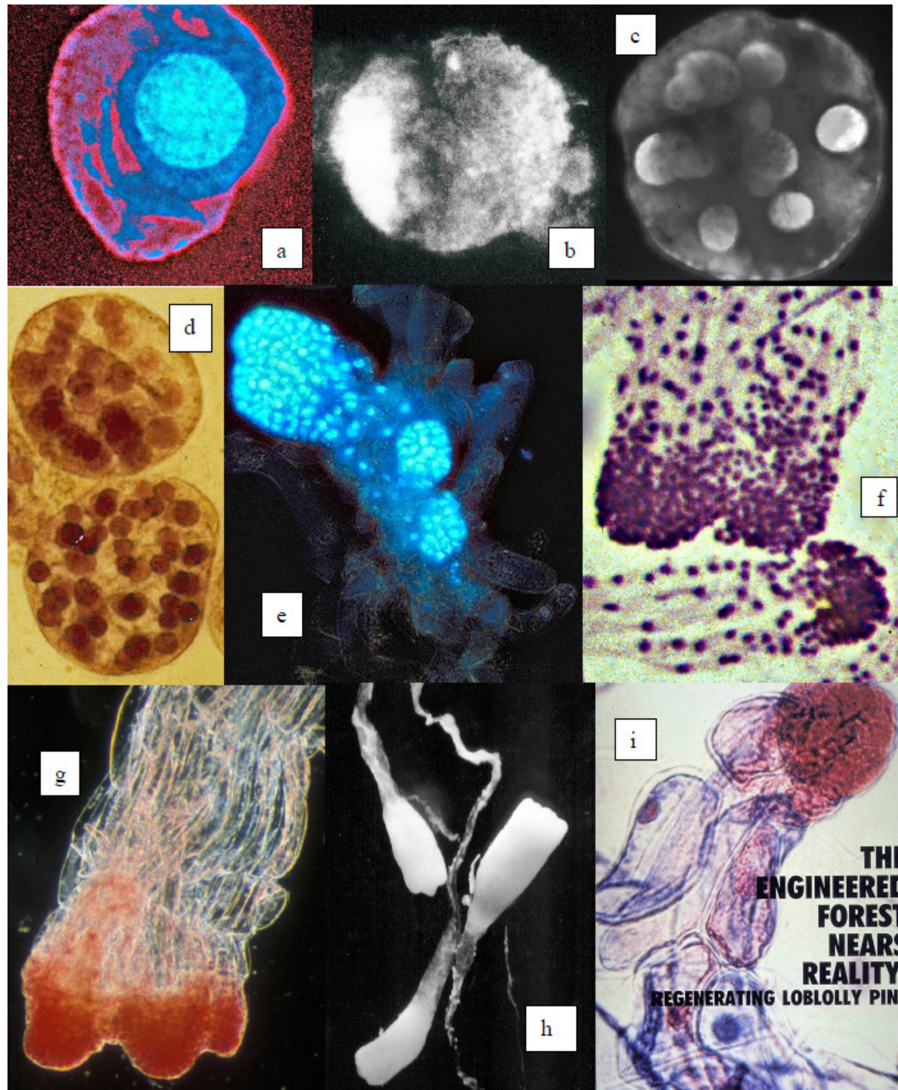

Fig.5*. Discharged female parthenospores of conifers rapidly undergo free-nuclear replication emulating proembryogenesis in fertilized seeds. In seeds the release of the proembryo is restrained by enclosure by the female gametophyte. (Araucaria* ***a****,* ***b****,* ***c****).* ***a****. A parthenospore of Araucaria produces a burst of nitric oxide when discharged (red NO fluorescence in spore and in culture medium) (DAPI, DAF-2DA).* ***b****. First free-nuclear division in the parthenospore occurs rapidly. Daughter nuclei are stained for the proliferating cell nuclear antigen (PCNA) which is a cofactor in DNA replication. Note the unequal distribution of PCNA activity which would account for the variable sizes of free nuclei reported in Araucariaceae (anti-PCNA FITC fluorescence in nuclei).* ***c****. Free-nuclear volumes commonly vary in Araucaria proembryogenesis (DAPI). (Norway spruce* ***d*** *to* ***g****).* ***d****. Free-nuclear replication in two female parthenospores (orcein).* ***e****. Developing parthenogenetic proembryos display cleavage polyembryogenesis in an AS (DAPI fluorescence). Seed proembryos in Norway spruce are classified as not showing monozygotic cleavage polyembryogenesis (Dogra 1967, 1978).* ***f****. Colchicine added to the culture medium increases free-nuclear replication in proembryos (Durzan et al. 1974).* ***g****. Cleaving embryonal initials (acetocarmine) adhere and their separation can be controlled by abscisic acid in the culture medium. (Douglas-fir* ***h****). Early embryos are developed in culture plates having a aqueous culture medium. They were removed to light and cultured in soil (Durzan and Gupta 1987a). (Cover of Nature Biotechnology 1987* ***i*** *). The first report of regenerating loblolly pine by somatic polyembryogenesis was published. Parthenogenetic development was not mentioned nor included in the patent for somatic polyembryogenesis licensed by Weyerhaeuser*

Cleavage polyembryony is one of several conditions responsible for non-recurrent parthenogenesis in seeds (Darlington 1958). The University of California patented "Simplified hybrid seed production by latent diploid parthenogenesis and parthenote cleavage" (Durzan 1988d) but it was never licensed.

Apomictic somatic embryogenesis differs significantly from culture systems employing proembryogenic masses (PEMS) as a source of clonal somatic embryos (Filonova *et al.* 2000) and from other methods reporting somatic embryogenesis in these Proceedings.

Arginine, oxygen and nitric oxide (NO) bursts

The discharge and dispersion of female parthenospores was accompanied by NO bursts in an aqueous medium (**Fig. 5a**). NO production in plants was first visualized using DAF2DA fluorescence in cytological preparations (Magalhaes *et al.* 1999). NO signals mediate and integrate adaptive functions in organelles and responses to environmental stresses (Durzan 2002, Durzan and Pedroso 2002, Youle and van der Bliek 2012). NO was subsequently recognized as a plant hormone (Santner and Estelle 2009). NO represses the transition to flowering in *Arabidopsis* (He *et al.* 2004). Its role in initiating flowering in conifers is unknown.

NO biosynthesis requires the participation of mitochondria of male origin (Birky 1995, Whittle and Johnston 2002) and enzymes in the cytosol (**Fig. 6**). NO is produced by a poorly characterized nitric oxide synthase (NOS). This enzyme uses the amidino N of L-arginine and dissolved oxygen as substrates to rapidly synthesize NO and L-citrulline. NO can be released into air and dispersed in the aqueous medium.

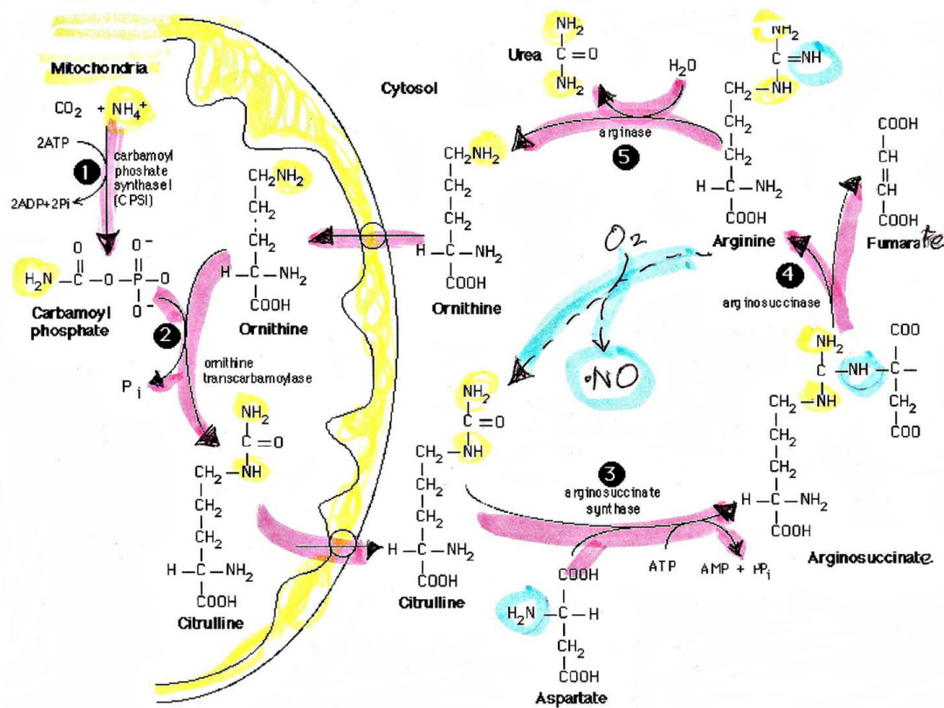

***Fig.6**. Biosynthesis of NO (blue arrow). NO is derived from arginine and oxygen. Arginine is synthesized via the partial reaction of a urea cycle (purple arrows). This requires enzymes in mitochondria of male parental origin. Ammonium and carbon dioxide react to form carbamoyl phosphate (Durzan 1983a) which combines with ornithine from the cytosol to produce citrulline (steps 1 and 2). Dark respiration increases the levels of carbon dioxide in the AS. Citrulline exits mitochondria and in the cytosol uses N from aspartic acid to form arginine (steps 3 and 4).*

In a dark aqueous AS, respiratory carbon dioxide is quickly distributed in equal amounts per unit volume, between air and water. Increased respiration enriches carbon dioxide levels and bicarbonate ions in water. Mitochondria link the conversion of bicarbonate and ammonium into carbamoyl phosphate (Durzan 1983a). Carbamoyl phosphate and ornithine are required for the synthesis of free arginine via the partial reactions of a urea cycle (Durzan 1968a, 1969a and 1973b). Carbon metabolism in the citric acid cycle is linked with N metabolism through an aspartate-argininosuccinate shunt. The shuttle supports protein and DNA synthesis in cells.

Arginine, not used in NO synthesis, is cleaved by urease to ornithine and urea. Ornithine is re-circulated to mitochondria to sustain arginine production for metabolism and protein synthesis. Carbamoyl phosphate and ornithine yield citrulline which moves to the cytosol and becomes available for the synthesis of free arginine.

NO has beneficial and harmful effects depending on its concentrations and milieu (Durzan and Pedroso 2002). At low concentrations, it is a chemical messenger that directly integrates and differentiates time-dependent responses for irritability, defense, adaptation, and survival. Prime direct targets of NO are hemes that shuttle NO, carbon dioxide, and oxygen. These reactions mediate cellular stresses, intermediary metabolism, respiration, development, aging, and may alter photosynthesis in light. High NO levels in the presence of reactive oxygen species generate reactive nitrogen species that initiate oxidative and nitrosative stresses. This contributes to DNA damage and apoptosis (Durzan 2000, 2002, 2006, Durzan and Pedroso 2002, Pedroso and Durzan 1999, Pedroso *et al*. 2000a, b).

In the deep Canadian winter of 1536, NO, originating from arginine in decoctions from coniferous trees, could have accounted for the recovery of sailors near death and suffering from scurvy in Jacque Cartier's crew (Durzan 2009b). Arginine, NO and derived guanidino compounds in conifers have significance in ethnobotanical and medicinal practices by indigenous peoples in eastern Canada.

Androsporogenesis (mAP) in an AS

Androsporogenesis is sometimes used to replace microsporogenesis because the importance of size (mega, micro) in spore evolution in relation to food supply has been debatable (Thomson 1948). Sterling (1963) even suggested that the microspore be called an embryonal cell. This view was disputed by Fernando *et al*. (2010) and others because it implied an untested relationship with the embryo.

Altered hormonal signals, reduced food supplies and catabolic changes promoted mAP. Embryonal cells stopped developing early embryos and were neo-functionalized and transdifferentiated into narrow androsporangial tubes with replicating nuclei undergoing automixis. Automixis produced monads and aberrant meiotic restitutional products. Asyndesis, due to sticky chromosomes and incomplete pairing and meiotic errors, is common under field conditions (Andersson 1947, Andersson *et al*. 1969) and in cell suspensions. Androspores were discharged and dispersed as monads, dyads, triad, tetrads and polyads (Durzan 2010, 2012) (**Fig.** 7). Tetrad formation in Norway spruce cultures was illustrated but not discussed (Durzan *et al*. 1994).

Genes that initiate DNA recombinations contribute to evolution and exploit DNA repair mechanisms (Wilkins and Holliday 2009). Automictic DNA recombinations occur in plant cell and tissue cultures (Nuti Ronchi 1995). Accurate alignments during homolog synapsis increase recombinations between fully matched long sequences.

Recombination activating genes (RAG-1 and RAG-2) encode enzymes that rearrange and recombine genes. Recombinant DNA nodules in automictic nuclei were detected by anti-RAG-1 fluorescence (**Fig. 7**) (Durzan 2012). Recombinant nodules occur on the central element of the synaptonemal complex during zygotene and pachytene. They reside at sites of reciprocal recombination and play a role in homologous chromosome synapsis, crossing over, and cross-over interference. RAG-1 activity alone is generally recognized as insufficient to distinguish between mitotic and meiotic crossing-over. An antibody to synaptonemal complexes (unpublished) provided supporting evidence for automixis in Norway spruce.

Dichogamy and asexual heterospory

The separation of gender expression over an annual cycle (dichogamy) occurs in conifers under field conditions (Owens 2006). Heterospory is the formation of two different types of spores. Homospory describes the formation of spores having approximately the same size. Heterospory is considered the most important key innovation in the evolutionary history of the plant kingdom and is controlled by environmental factors (Bateman and DiMichelle 1994). Gender expression may have originated epigenetically in response to environment factors.

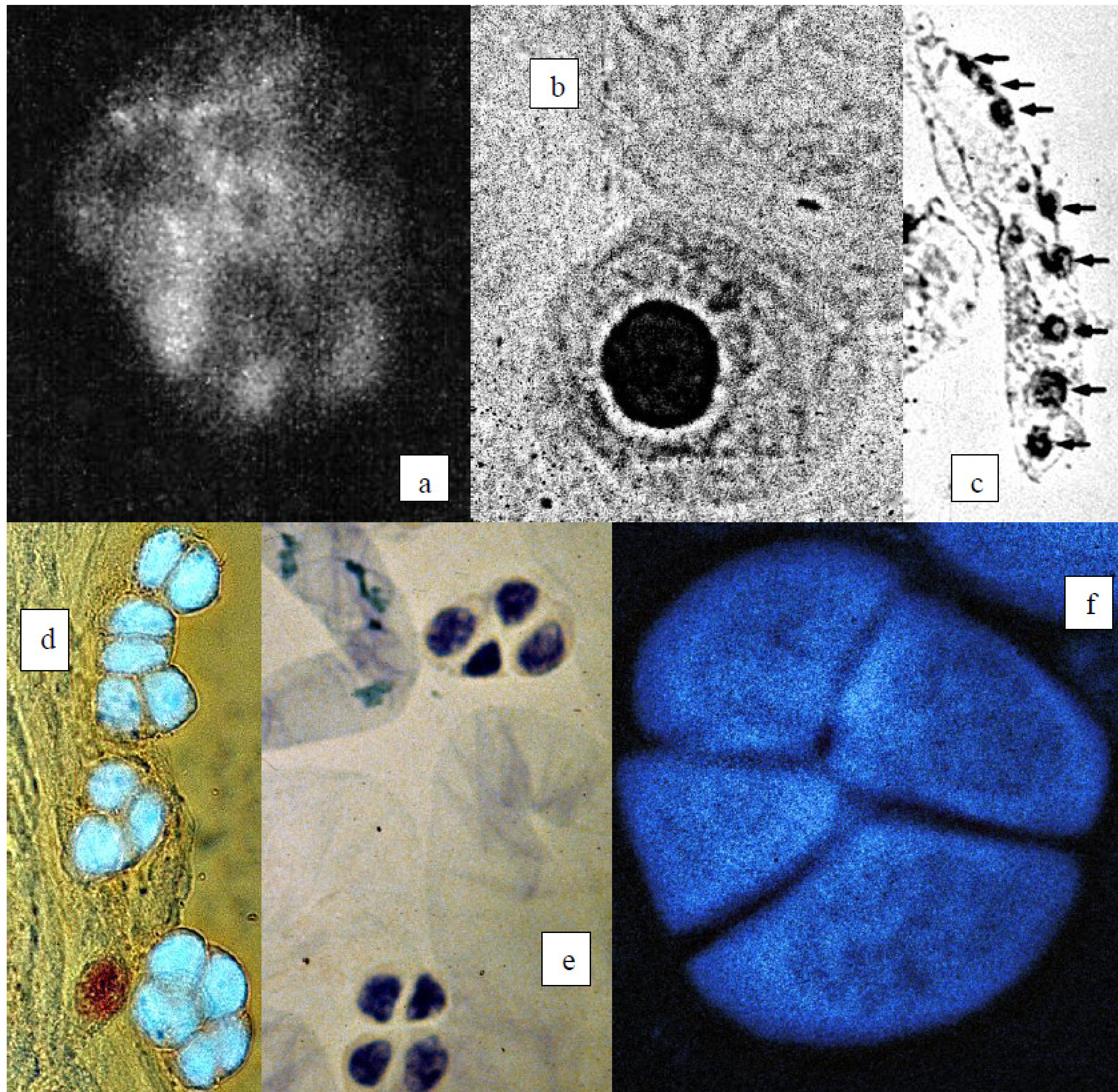

***Fig. 7**. Automixis and parthenogenetic androsporogenesis. (Araucaria **a**, **b**). **a**. Nuclei of embryonal initials undergo parasexual reproduction (meiosis in one form or another) and display recombinant DNA nodules in nuclei (Anti-RAG1 fluorescence). Recombination is known to occur in mitosis but this rare. **b**. Discharged monad with a densely staining nucleus (Feulgen-Giemsa). (Norway spruce **c** to **f**). **c**. Transdifferentiated androsporangial tube with multiple nuclei (arrows) **d**. An apoptotic monad (red, TUNEL) and viable dyad, triads and polyads (DAPI fluorescence) from spent androsporangial tubes. **e**. Tetrads (Feulgen-Giemsa). **f**. Polyamine distribution in a tetrad (o-phalaldehyde, blue fluorescence).*

Dichogamy in Douglas-fir embryonal initials (Durzan 2010) was observed in an AS within a narrow but constant temperature range. The formation and release of dyads, triads and tetrads occurred mainly between January and April. Female PA was prevalent July to November. It is not known if dichogamy would have been expressed the following year.

Thermostat functions in plant cells are located on nucleosomes with the H2A.Z protein (Deal and Henikoff 2010). According to the authors, this protein is conserved throughout evolution. With rising temperature the H2A.Z-carrying nucleosomes alters gene-specific transcriptional responses. A relatively constant temperature of an AS would maintain a stable putative chromatin thermostat. Imprinted endogenous rhythms, controlled epigenetically, are postulated as responsible for expressions of dichogamy.

Lags (hysteresis) between apomictic and automictic cell cycles and among expressions of fPA, mAP can be modeled by Volterra's mathematical descriptions of growth in competing cell populations. Runs at different times of the year might reveal how darkness, light and endogenous rhythms characterize expressions of dichogamy under laboratory conditions. A curve relating to asexual heterospory and dichogamy in an AS would display a closed path in an x and y plane.

Genome silencing

Vitoria Nuti Ronchi (1965) demonstrated how mitosis and meiosis contributed to the formation of variant cell types and gamete-like cells in angiosperm cultures. While visiting Davis, she pointed out that parasexual reproduction, similar to that reported for carrot cell suspension cultures, was evident in cell suspensions of conifer embryonal initials.

Her colleagues, Giorgetti *et al*. (2007) linked RNA interference and heterochromatinization to the extrusion of DNA sequences during meiotic prophase in pollen mother cells of barley and oil palm. An analysis of extruded DNA sequences implicated gene amplifications which removed accumulated multicopy somatic DNA sequences. Removal of these multicopy sequences re-established totipotency (pluripotency) in cells before entering gametogenesis.

Embryonal initials of Norway spruce, Douglas-fir and especially *Araucaria*, stuck in incompletely reprogrammed states displayed Rabl chromosome orientations on nuclear membranes. Rabl orientations were originally thought to comprise highly repetitive DNA in the heterochromatin of different chromosomes (Sybenga 1992).These orientations are responsible for the formation and extrusion of bouquets (**Fig. 8a, b**).

Bouquets are comprised of independently functioning and non-self governing chromosome parts (Rieger *et al*. 1976). They are a visible form of transcriptional silencing by removal of methylated DNA sequences. Expulsion is an expression of genome silencing. It contributed to the formation of micronucleated cells (**Fig. 8c**).

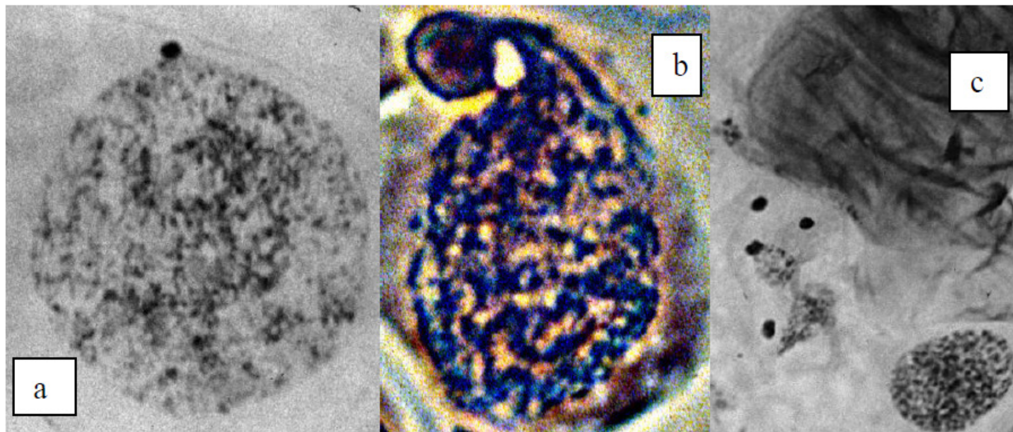

Fig. 8. *Genomic silencing of embryonal initials involves chromatin Rabl formation, excision (cytomixis) and the formation of pycnotic micronucleated cells (Araucaria angustifolia, Feulgen-Giemsa).* ***a***. *Rabl bouquet formation occurs at telomere sites on the nuclear membrane.* ***b***. *Excision of silenced DNA from nucleus.* ***c***. *Field of four micronucleated cells below a spent androsporangial tube.*

Micronuclei arise from acentric chromosome fragments or from chromatids and whole chromosomes that are not incorporated by mitosis or meiosis into daughter nuclei at telophase (Rieger *et al*. 1976). Variations in micro-nuclear DNA are characterized by low copy numbers, unique and repetitive sequences, distributions of families of transposable elements, and re-arranged highly repetitive DNA sequences.

Salmine blocks the remodeling embryonal initials into gametes

Selective forces, favoring the evolution of genomic imprinting and silencing, may have been present during the origin of modern land plants (Haig and Wilczek 2006). Salmine is an arginine-rich protamine responsible for the remodeling of cells into gametes in salmonoid fishes. Seed storage proteins in conifers are also rich in arginine (Schulze 1896, Mothes 1929). Salmine's potential as a chromatin silencing factor in homeotic somatic embryogenesis and in imprinting gametogenesis in Norway spruce was explored in an AS (Durzan 2010a).

Synthetic hormones, co-option, apomictic and automictic DNA changes neo-functionalized and transdifferentiated embryonal initials into archegonial or androsporangial tubes. Transcriptome changes (acetocarmine reactive), feed-forward and feedback (anabolic and catabolic) phases, and altered food supplies characterized fPA and mAP. In the presence of salmine, embryonal initials no longer differentiated into axial tiers. Proembryos with developing tiers no longer formed embryonal suspensors. Nuclear and cytoplasmic organization in embryonal initials was disrupted. Nucleoli became highly vacuolated. PCNA (cyclin) activity, required for DNA synthesis, was blocked. PCNA is needed for DNA replication, repair and epigenetic inheritance. Cleavage polyembryony was no longer evident in early embryos. Biomass loss and gluconeogenesis of amino acids led to the accumulation of free arginine N. Salmine did not remodel cells into gametes as in salmonoid fishes. In the absence of salmine, proembryos developed into normal axial tiers showing cleavage polyembryony.

Eggs discharge mitospores from transdifferentiated oögonial tubes

Ephedra californica and *Taxus brevifolia* are dioecious gymnosperms. They require a partner of the opposite sex for completion of their sexual cycle. Lethal genes in haploid cells, when expressed, are quickly removed from populations.

Ephedra californica (Gnetales): Gnetales have practically no fossil record and are far removed morphologically from conifers and Taxales (Sporne 1965). *Ephedra* is considered unique among gymnosperms having a distinct type of seed proembryogeny. After fertilization, zygotes produce eight proembryonal nuclei by three successive divisions (Bhatnagar and Moitra 1996). Four of the eight free nuclei in the lower half of the archegonium are produced from the zygote. The remaining four nuclei in the upper half are derived from the fusion product of the vcn and a second sperm nucleus (second fertilization).

In the absence of fertilization and in an AS, eggs were neo-functionalized and transdifferentiated into elongated oögonial tubes which discharged and dispersed five mitospores. Mitopores retained the special morphological characteristics of proembryos in Ephedraceae (**Fig. 9**).

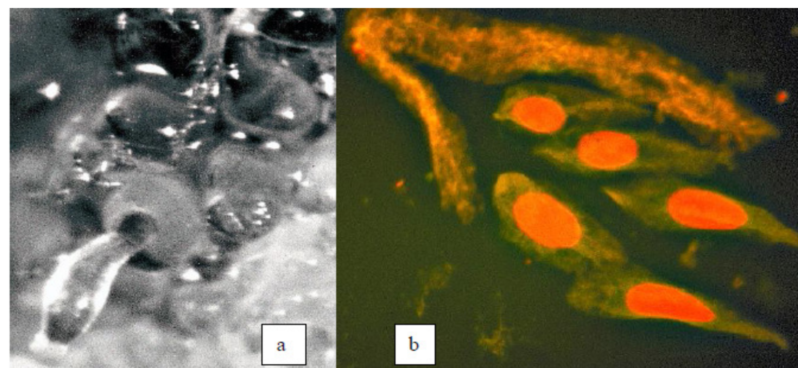

Fig.9. *Rescued eggs from a female genotype of Ephedra californica S. Wats, are transdifferentiated into oögonial tubes (**a**) that discharge parthenospores into an aqueous medium (**b**) (Havel and Durzan, unpublished). A zygote usually produces eight proembryonal nuclei by successive divisions. Four nuclei (lower half of the archegonium) are derived from the zygote. Four nuclei (upper half) are derived from the fusion product of the vcn and a second sperm nucleus (second fertilization). In the absence of fertilization only five "parthenospores" were discharged into the aqueous medium. Spent tubes floated to the air-water interface in the AS. Ephedra is considered unique among gymnosperms having a distinct type of proembryogenesis (Acridine orange fluorescence).*

Taxus brevifolia (Taxales, Taxaceae): In gymnosperms lacking a ventral canal cell, the central cell behaves as the egg cell. The ventral canal cell is not formed in *Taxus*, *Torreya* and *Widdringtonia* (Bhatnagar & Moitra 1996, Singh 1978). The central cell nucleus, when fertilized, replicates and develops a 16-nucleate proembryo. The separation of Taxales from Coniferales is based on the discovery of fertile specimens of *Paleotaxus* in the Triassic and of *Taxus jurassica* in Jurassic rocks. Both specimens have terminal and solitary ovules (Sporne 1965).

Central cells, like those described by Sterling (1948), underwent free-nuclear mitoses and were neo-functionalized and transdifferentiated into oögonial tubes with mitospores (**Fig. 10a, b, c**). Tubes discharged and dispersed female mitospores in an AS. Gametic genome doubling (complete meiotic automixis) was not ruled out.

Paclitaxel and taxane biosynthesis by eggs suspended in an AS was controlled by regulating NO production (**Figs 10d**, **e**) (*cf*. Durzan 2006).The natural occurrence and significance of paclitaxel and taxane formation in *Taxus* sp. is reviewed by Appendino (1994).

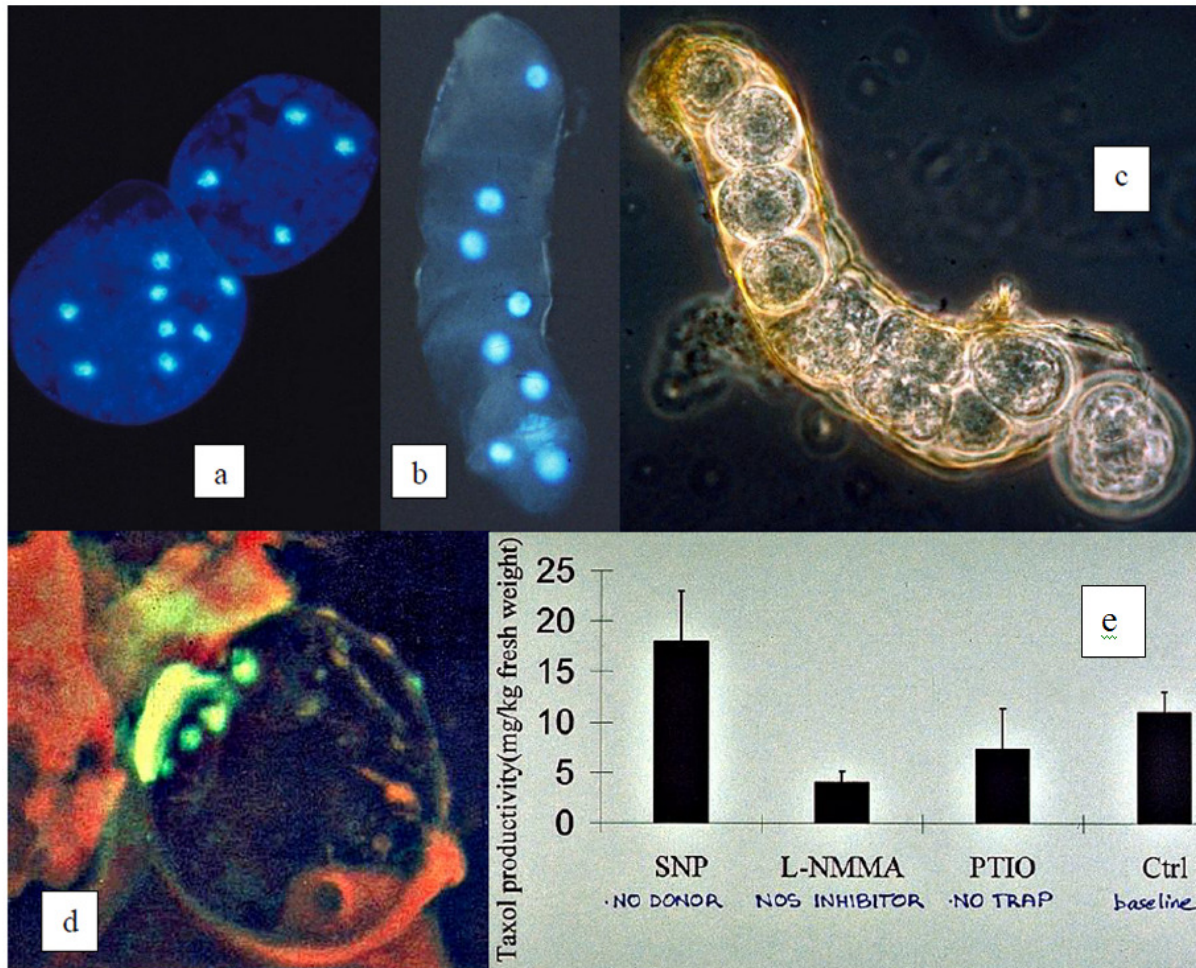

Fig. 10. *Rescued eggs (**a**) of Taxus brevifolia (female dioecious genotype) in an AS are neo-functionalized and initiate mitotic free-nuclear replications (**b**) during the transdifferentiation of oögonial tubes (**c**)(DAPI). Free nuclei develop into mitospores (**c**) before being discharged into an aqueous medium. Eggs synthesize paclitaxel (Taxol®), an anticancer compound used in the treatment of ovarian cancer. Fluorescent labeled antibody precursors of paclitaxel comprise of a side-chain (red) and a taxane ring (green). The biosynthetic relation of newly synthesized paclitaxel (yellow) to its known precursors was displayed on the surface of an egg (Fig. **d**). Taxol production and recovery were a function of nitric oxide (NO) levels in cultures. An inhibitor of nitric oxide synthase (NOS) and a NO trap (PTIO) reduced Taxol production (Fig. **e**). Paclitaxel recovery was increased when a NO donor (sodium nitroprusside SNP) was introduced into cultures (Durzan 2006). NO is formed from arginine N and oxygen.*

Heterospory in an aqueous environment emulated algal reproduction

The feasibility of experimentally emulating evolutionary expressions with gymnosperm cells in an aqueous environment was previously contemplated (Durzan 1991, 1996a). Water is a metabolic substrate for the enzymatic synthesis and replication of DNA (Kornberg and Baker 1992). Based on the laws of mass action, water as a substrate is not in short supply in an AS.

Under aseptic conditions the OH group in the 2' position of ribose in ribonucleotides can be replaced by tritium from tritiated water for the biosynthesis of tritiated deoxyribonucleotides. The latter were incorporated into nuclear DNA of imbibing jack pine embryos (Durzan *et al*. 1971a, Durzan 1983b). All exchangeable tritium in embryos and substrates was removed from and replaced by hydrogen ions *before* biochemical and autoradiographic analysis. Covalently bound tritium was recovered from newly synthesized DNA. Chemical degradation of DNA revealed tritium only in the deoxyribose moiety of DNA.

Autoradiography of nuclear sections located bound tritium in replicating DNA at Okazaki sites. These are short fragments of DNA produced on the lagging strand during DNA replication. Strands are rapidly joined by DNA ligase to form a continuous DNA strand. Covalent tritium could be used to label, locate and analyze genes in nuclear and organelle DNA responsible for the interpolation of heteromorphic asexual sporogenesis. Identification of tritiated DNA-labeled genes expressing heteromorphic asexual sporogenesis would enable comparisons with genes responsible for spore formation in algae.

Spore formation in algae occurs by meiosis (meiospores), mitosis (mitospores), asexual reproduction (parthenogenetic gametes) and by motility (nonflagellate spore versus flagellate zoospores) (Maggs and Callow 2002). Coenocyst formation and spore discharge occurs in motile unicellular and colonial algae (Bold *et al*. 1980).

Apomictic and parasexual spore dispersal by gymnosperms emulated (≈) asexual and sexual reproduction in modern and extinct algae (Maggs and Callow 2002, Stewart and Rothwell 1993) as follows:

APOMIXIS → fPA (♀ parthenospores) ≈ APOMIXIS → Algal parthenogenetic spores

MITOSIS* → Mitospores/oöspores) ≈ MITOSIS → Algal mitospores/oöspores
Ephedra & Taxus*

MEIOSIS **→ mAP (monads, dyads, triads tetrads) ≈ MEIOSIS → Algal meiospores
****Genetic recombination occurs in automixis, meiotic restitution and rarely in mitosis.**

COENOCYTE DEVELOPMENT * ≈ → COENOCYTIC Algae (subtropical & tropical)**
*****Free-nuclear replications in proembryonal parthenospores**

In *Scenedesmus*, the rupture and liberation of the daughter coenobium from the mother cell lasts less than 3 seconds (Březina *et al*. 1972). This is within the time frame for the release of parthenospores in an AS. A coenobium in algae is a colony where the number of cells is fixed at the time of reproduction.

Niklas and Kutscher (2010) postulated that the multicellular reproductive structures of extant charophytes and embryophytes are developmentally homologous. Reproductive structures of the embryophytes evolved by virtue of the co-option and re-deployment of ancient algal homeodomain gene networks. In an AS, there is no multicellular reproductive structure but only the glass of a nippled flask or bioreactor.

Evidence for feedback-loops in ecological change and rapid evolution is substantial (Schoener 2011). Favorable alleles are established in asexual populations only if they have an advantage that outweighs the loss of fitness due to the deletion of associated alleles (Barton 1998). In an AS, deletion may have been avoided by progenesis and interpolation of apomixis and automixis. Proembryos, derived from dispersed spores, continued somatic embryogenesis outside the AS. Factors, thought responsible for global warming, could be use to explore the adaptive plasticity of cell cycles of monoecious and dioecious genotypes in an AS.

Heterochrony, polarity, morphogenesis and the return to unit gravity

"The task of physiology is to study the changes which go on in plants at present living, to measure their extent, and to investigate their causes. From that study one may draw conclusions as to the phenomena which took

place in earlier periods of the earth's history, and so it is possible for physiology and morphology to work hand in hand in this field of inquiry" (Jost 1907).

Heterochrony, or the change in timing or rate of development, is implicated in the evolution of gymnosperms (Miller 1988, Sporne 1965). While much has been postulated about the transition of plants to land (Crane and Kenrick 1997, Graham 1993, Haig 1992, 2008, Haig and Wilczek 2006, Niklas and Kutscher 2010), no experimental information was available to indicate how gymnosperm cells undergoing neo-functionalization in an aqueous environment would respond developmentally when returned to unit gravity in darkness or light. Light, water and gravity are the most important factors in the environment in determining the course of development in woody plants (Zimmermann and Brown 1971).

Aborted embryonal initials and apoptotic cells comprised up to ca. 5.0 % of the biomass in an AS. Degraded chromatin and chromosomes were dispersed in the culture medium and reacted strongly with an antibody for ubiquitin (Durzan 1996b, Durzan *et al*. 1994). Ubiquitin controls the metabolic turnover of specific cell regulatory proteins in embryonal initials. Ubiquitination labels enzymes and nucleolar proteins for destruction along the axial tiers of early embryos (Durzan 1987, 1996b, Durzan 2008). Mucilaginous proteins, PCNA and other biomimetic factors are dispersed in the culture medium.

When cell populations from an AS were returned to unit gravity and spread in culture plates (darkness or light) cells were reorganized into new colonies (Fig. 11). Plates contained a fresh culture medium for fPA and mAP. Colonies in darkness and light displayed new and distinguishing morphological growth patterns (**Fig. 11a, b**). Embryonal initials of Norway spruce assembled into "adventitious" meristem-like colonies.

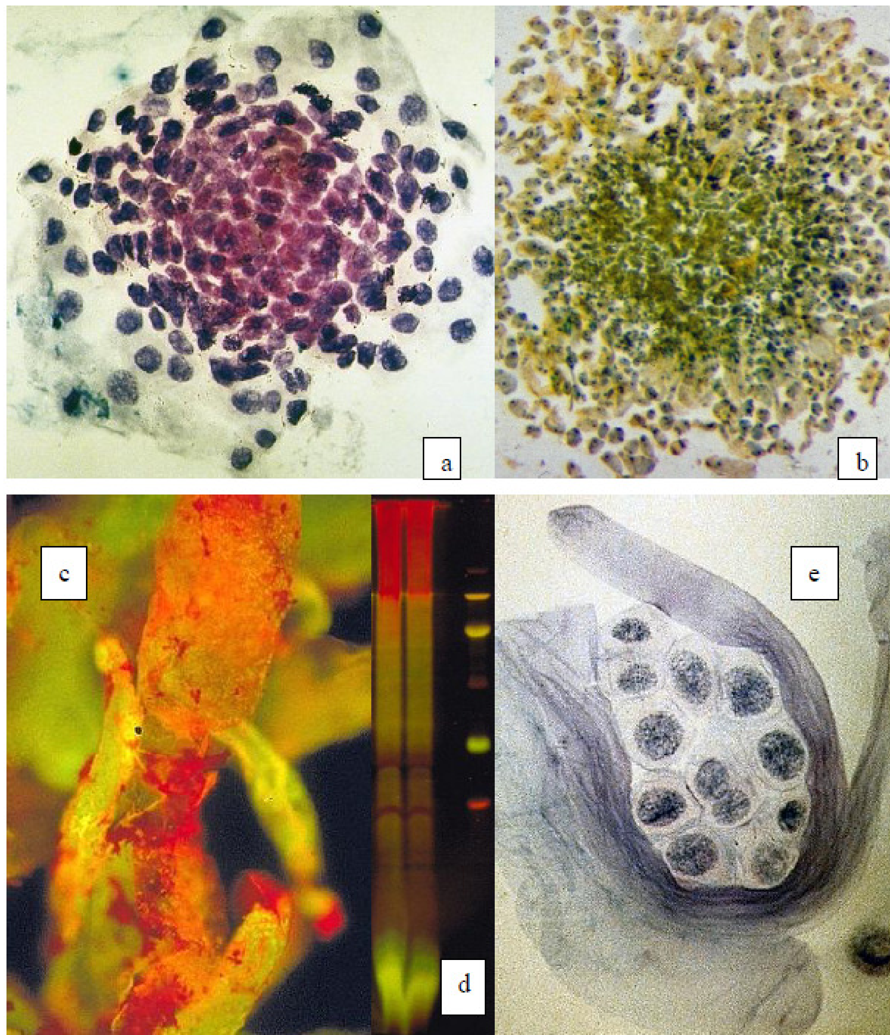

Fig. 11. Heterochrony. Transdifferentiating Norway spruce and Douglas-fir embryonal cell suspensions when returned to unit gravity and cultivated in a shallow culture plate released mucilaginous and proteinaceous factors. Cells now aggregated into planar colonies in a moist medium. ***a****. After 30 d in darkness, Norway spruce archegonial tubes and cells self-organized into weak fibonacci-like patterns. (Nuclei 10 to 12 μm dia.). Fibonacci morphogenetic lineages occur in conifer shoot apices and seed cones. Centrally located replicating nuclei reacted red rather than deep blue to Feulgen-Giemsa.* ***b****. After 2 months in white light, cells aggregated and also formed a colony. Amyloplasts in cells at the center now produce chlorophyll in chloroplasts. Lingering peripheral archegonial tubes and cell were not yet adapted to light.* ***c****. Archegonial and narrow androsporangial tubes release mucilaginous factors which are stained by Stains-all (Green and Pastewka 1974) and viewed under UV light.* ***d****. Mucilage was recovered and separated on polyacrylamide gels, stained and photographed under UV light for comparison with Fig. c. The bright red and low mw green fractions have acetocarmine reactivity.* ***e****. Dividing androspores of Douglas-fir were surrounded by a spent and compressed androsporangial tube. A residual archegonial tube lingers at the lower right. Feulgen-Giemsa. Nuclei 8 to 10 μm dia.*

In darkness (**Fig. 11a**) neo-functionalized tubes of Norway spruce no longer discharged spores. Embryonal cells divided into lineages directed toward the centre of the colony. Rapid cell cycling in centrally localized cells altered the Feulgen-Giemsa staining of nuclei. Colonies were surrounded by weakly staining and spent tubes, some retaining nuclei.

In white light (**Fig. 11b**), Douglas-fir archegonial and androsporangial tubes and embryonal initials aggregated forming an adventitious, quasi-morphogenetic cell colony. Amyloplasts, in a ring of centrally located cells, differentiated into chloroplasts with chlorophyll. Greening occurs within two weeks. Cells replicated faster than in darkness.

Tubes and cells released mucilaginous products, PCNA and other factors into the culture medium (**Fig. 11c, d, and e**) that appear responsible for promoting rapid cell divisions and expressions of heterochrony in light and darkness. It is not known if surrounding spent tubes enclosing spores have significance for interpolating reproductive structures in the evolution of the land sporophyte, *cf.* Hemsley (1994). The total possible colony configurations, their fates and potential for organized development, phyllotaxis, cambial formation and longevity remain unknown.

Somatic embryogenesis and interpolated "somatic parthenocarpy"

Gymnosperm seeds are not enclosed in an ovule. Angiosperm seeds are enclosed in an ovule which forms the fleshy part (pericarp) of a fruit. Tree fruits are composed of living cells deprived of nutrition from the parent tree. Cells are genetically programmed to age, disintegrate and die. Ripening is the first phase in a fruit's progression to death characterized by changes in texture, flavor and color. While fruit cells in liquid culture are not expected to completely resemble their counterparts in trees, they offer ease of manipulation and year-round availability to explore fruit senescence at biochemical level for the development of improved postharvest technologies (Romani and Hess 1986).

Prunus cerasus cv. Vladimir is a commercial cultivar used as a rootstock to support the vigor of Bing cherry trees. Its flower petiole cells (**Fig. 12a**) were scaled up in nippled flasks to demonstrate somatic parthenocarpy (Durzan 1988a, Durzan *et al*. 1991).

A MS medium (MS, Murashige-Skoog 1962) with 2 mg/l NAA formed callus in darkness. Callus was transferred to MS medium with 1 mg/l 2,4-D and 0.5 mg/l BAP to establish cell suspensions in nippled flasks. Cells were screened through a 1250 μm mesh and placed in multiwell plates (MP) with a MS or a M2 medium. The M2 contained urea, which replaced the ammonium nitrate and potassium nitrate in the MS medium. Calcium and magnesium were supplied as glycerophosphates. After 12 d in light, cells in the M2 medium produced anthocyanins and produced dense cell clusters. The MS medium and exposure to light blocked anthocyanin formation and cells remained green (**Fig. 12b**).

When cells were cultured in nippled flasks with M2, compact red nodules of pericarp were developed (**Fig. 12c, d**). In cross section nodules resembled the flesh of cherry fruit (**Fig. 12e, f**). Through progenesis, petiole cells were differentiated into parthenocarpic tissue. The interpolation of parthenocarpic development bypassed flowering and fruit development by directly producing a pericarp without seeds.

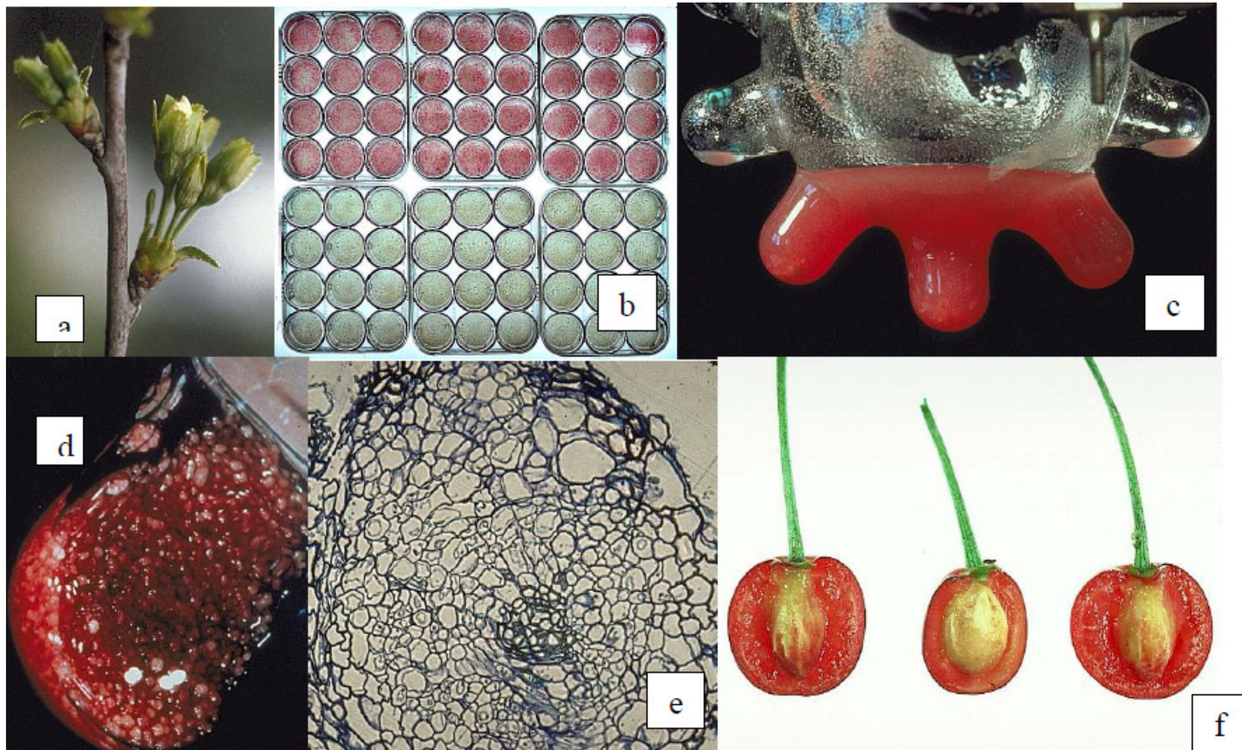

Fig. 12. *Flower petioles excised from Prunus cerasus cv. Vladimir (**a**) were used to establish cell suspensions in Delong and nippled flasks with a Murashig-Skoog (1962) medium. Flask contents were screened to collect cells which were assayed in multiwell plates with growth hormones that promoted development in light or darkness (**b**). Culture in darkness without nitrate but with reduced N (M2 medium) produced red cells which were scaled up in nippled flasks at 25 ^{o}C (**c**). Culture in light and with reduced N source showed evidence of early embryo formation but further development was aborted. After three weeks of growth, red parthenocarpic nodules developed (**d**). When sectioned they showed signs of a central core of cell where seeds would normally develop (e). Cherries from a cultivar growing on this rootstock in the field were cut for comparison with parthenocarpic nodules in nippled flasks (**f**). Data from C Peng, MSc Thesis (1988), Kieth Hansen (unpublished) Department of Pomology and Durzan (1988).*

This genotype aborts zygotic embryogenesis after fertilization. It is vegetatively propagated. Somatic early embryos generated in a MS medium reached a heart-shaped stage then aborted and turned brown. When the rootstock was micropropagated on a MS medium (Peng 1988, MSc Thesis) and planted in the field, clones of the rootstock after 8 years displayed precocious flowering (Durzan unpublished).

Cytological classification of reproduction and apomixis

Darlington (1937) used a matrix, based on cytological and chromosomal studies, to classify how reproduction in life cycles of versatile species relate to fertilization or non-fertilization (rows) and genome reduction or non-reduction (columns). This matrix can be modified to include gymnosperm apomixis, automixis, heteromorphic asexual heterospory and mitosporogenesis, nuclear transfers, neo-functionalization of protoplasts and cells, and artificial apomixis.

Neo-functionalization of protoplasts was demonstrated when the chimeric luciferase gene (*luc*) from the firefly was electroporated into protoplasts of embryonal initials of Douglas-fir and loblolly pine (Gupta *et al*. 1988) (**Fig. 13A**). The *luc* gene was expressed when luciferin was added as a substrate. Light flashes were measured in a luminometer. Protoplast fusions and artificially created heterokaryotic coenocytic free-nuclear proembryos and embryos, if established under field conditions, could be designated as “coeno-species” (**Fig. 13b, c**). It is not yet known whether dominant genes can be activated by nuclear transplantation or by the creation of heterokaryons.

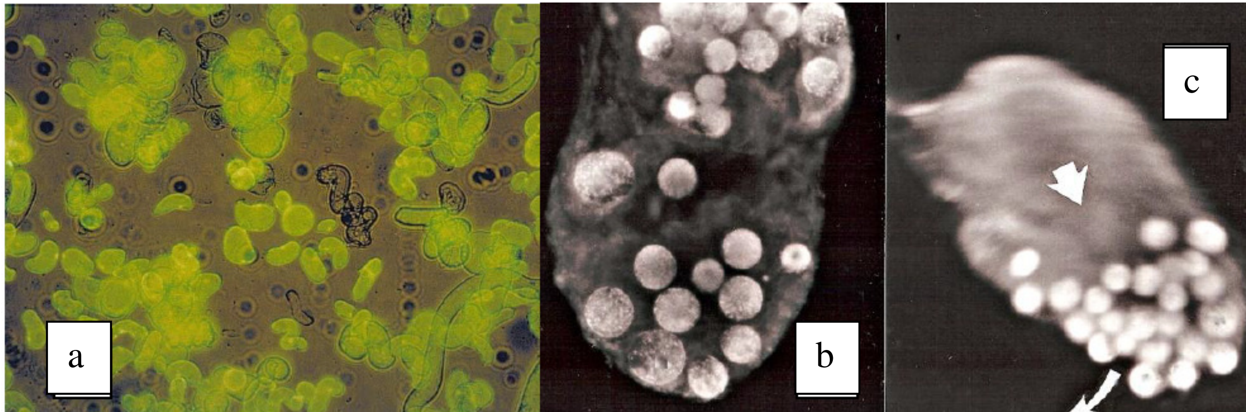

Fig. 13. Genetic neo-functionalization. a. Firefly luc genes, when electroporated into protoplasts of Douglas-fir and Norway spruce and given luciferin, produce yellow light flashes in darkness (Gupta et al.1988). b and c. Fused coenocytes (Araucaria) were neo-functionalized into a heterokaryotic archegonial protoplast which rapidly assembled and discharged female parthenospores. Parthenospores, if regenerated into somatic embryos and planted in the field would introduce genetically variable population of "coeno-species" (Durzan 2012).

Models for the evolution of simple asexual organisms, such as bacteria or viruses, are based on self-replicating RNA and DNA (Eigen and Schuster 1979, Popa 2004). Different types of sequences, arising by exaptation, apomixis and automixis, would replicate at different rates and favor sequences that replicate faster. DNA sequences that replicate slowly might not sustain their abundance in the genome. These sequences could benefit by cell-cycles which replicate and incorporate lineages quickly.

Gamete formation without meiosis (apomeiosis) occurs in *Arabidopsis* (Maruthachalam *et al*. 2008). Mutation of *DYAD/SWITCH1*, a regulator of meiotic chromosome organization, brings about sporogenesis without reduction of chromosome and initiates apomixis.

"Artificial apomixis" was reported in *Arabidopsis* (Marimuthu *et al*. 2011). *Arabidopsis* MiMe and dyad mutants, which produce diploid clonal gametes, were crossed with a strain whose chromosomes are eliminated after fertilization. Up to 34% of the progeny were clones of their parent. The first-generation cloned plants were cloned again. Diploid seeds from one clonal parent were produced. The goal was to develop crop plants that can fertilize themselves and produce clonal seeds displaying hybrid vigor. Gamete formation has yet to be explored with gymnosperms. Eggs may someday be developed in the laboratory from embryonal initials.

Somatic embryogenesis: clonal evaluation of conifers in field trials

Asexually multiplied clones may adapt to a number of environments. But rapidly changing environments may require a genotype not found in any of the clones. The capture of genetic gains in different locations requires mathematical modeling. Mixed clonal and seedling quality assessments offer comparable measures for evaluating field performance and capturing genetic gains over a production cycle (Ritchie 1984, Timmis 1998). Combinatorial algorithms for genotype exclusions and matching over several decades have yet to be reliably formulated.

Problematic assessments would benefit from causal analyses employing molecular phenogenetics (Durzan 1990). This includes: 1. Description of the problem, symptoms and phenotype; 2. Proof of its heritable nature and mode of transmission; 3. Identity and characterization of responsible genes; 4. Epigenetic profiling of genotypes in different locations; and 5. Characterization of non-cooperative g x e reaction norms.

Survival in boreal soils and in northern latitudes (g x e) requires that meristems prepare for the onset of winter, survive very low temperatures, and prepare for bud break in spring when the snow melts. In nitrogen poor soils, this sequence is mediated by N-rich arginine in storage proteins and arginine in physiological fluids. N-rich free guanidino compounds, some being respiratory inhibitors are formed by transamidination reactions of arginine with amines in buds, cambium and roots which prepare meristems for winter dormancy (Durzan 2010b). In spring and when conditions allow, guanidino N is returned to arginine N so that N can be redirected via the partial reaction of the urea cycle (**Fig. 6c**) to amides and amino acids for protein and nucleic acid synthesis (Durzan 2009a). These

metabolic pathways might help explain why problematic clones in field trials do not survive severe winters.

Nucleic acid, chromosomal protein, low mw RNAs and ribosomal RNA analyses have an early history in conifers (Pitel and Durzan 1978a, b, c, d). We now know that conifers have unique small RNA silencing signatures (Dolgosheina *et al*. 2008). Genomic DNA is transcribed into various types of RNA but not all RNAs are transcribed into proteins. RNA is involved in homologous chromosome pairing during meiosis (Dernberg 2012). Non-coding RNAs are associated with abnormal, inconsequential transcriptions in cell differentiation and development. It is not clear how they relate to abnormal, inconsequential transcriptions and to patterns of cell differentiation and development under field conditions. Genetic systems often behave unpredictably due to structural interactions among DNA, RNA, protein components and functional interactions with various metabolites.

Our ability to quantify and program multigenic events and metabolic pathways remains limited. Transcription, translation and degradation of RNA transcripts are factors in gene expression. All three are controlled by a combination of promoters, ribosome binding sites and encoded regulatory signals. These unpredictably interact with each other through the formation of RNA structures and the recruitment of factors that affect transcript accessibility and stability. Improved methods are needed to distinguish among genetic elements at the transcript level and to track and identify predictable genetic outcomes.

Several DNA profiling methods and reporter genes are available to track gene expression under continuous selection (Bergthorsson *et al*. 2007). Profiling methods include restriction and amplified fragment length polymorphisms, random amplified polymorphic DNA, micro- and mini-satellites, simple sequence repeats, cleaved amplified polymorphic sequences, single nucleotide polymorphisms, expressed sequence tags, genetic linkage maps, and quantitative trait loci. RNA-Seq with picograms of mRNA would aid the profiling of gene expressions (Goetz and Trimarchi 2012).

It should now be possible to use profiling methods and reporter genes to track how nuclear and cell cycles rapidly initiate asexual reproduction in cells, eggs and explants under controlled, defined, replicated and prescribed nutritional and environmental conditions. Lasers were used to prime phytochrome and synchronize seeds for germination and to experimentally photo-induce or block reproductive development in a seed orchard (Campbell and Durzan 1979, Durzan 1980, p. 54, Durzan *et al*. 1979). Hyperspectral imaging of temperatures and light reflectance could be used to track phenotypic changes and adaptations in the field (Gong *et al*. 1997). Radio-frequency identification detectors (RFIDs) would automatically transfer, store and retrieve data related to clonal performance and health status as seasons change.

Difficult problems in evolutionary history

Modifications of the phenotype alone, brought about by alternations of the environment, do not constitute evolution, unless they are accompanied by some genotype change (Dobzhansky 1951). Any unit "that reproduces and maintains its integrity long enough to permit the action of selection is an evolutionary entity" (Williams 1966, 1976). Asexual populations incorporate a "rachet mechanism" (Muller 1964). They can never get to contain, in any of its lines, a load of mutations smaller than that already existing in its present least-loaded lines.

A most difficult problem is to decide how far it is justifiable to extrapolate backwards and relate laboratory observations to how gymnosperm cells, coenocytic cycles and sexual cycles might have evolved under conditions of a deep past evolutionary cradle. Clade traits do not distinguish among functional, incidental or neutral traits and have no readily identifiable means of inheritance (Vermeij 1996).

Did gymnosperm cells originate, not primarily by cell-division, but by differentiation from a dividing multinucleate continuum (coenocytes in **Fig. 5**) followed by cell wall formation as proposed by Rohde (1923)? Assimilation, growth, and differentiation continue in the absence of cells walls. When cell walls and tissues develop after free-nuclear divisions, a multinucleate protoplasmic continuum becomes maintained through plasmodesmata.

Could the first diploids have arisen via endomitotic errors (**Fig. 4**) or by cell fusion and predated regular sexual life cycles (Hurst and Nurse 1991)? Endomitosis and amitosis were responsible for rapidly interpolating asexual fPA and somatic embryogenesis (**Fig. 3**).

Could meiosis have originated from mitosis? Parasexual meiotic reproduction (automixis, **Fig. 7**) was expressed by mitotic embryonal initials with a prescribed food supply, synthetic hormones and narrow temperature range in darkness. Conditions from the deep past could be simulated to manipulate cell cycles and define threshold conditions showing how asexual and sexual reproduction might have originated.

Would fine tuning of a molecular clock, circadian rhythms and simulations of primitive environments recall more primitive phasic expression in early development? Clock proteins, when translocated to the nucleus, could be

used to inhibit transcriptional activity and initiate or recall primitive and simple pleomorphic cell cycles, i.e., the occurrence of two or more cycles in one life cycle. This would impose a set point for ontogeny having a generative factor proportional to the cell population, an inhibiting factor proportional to factors in the population, and a hereditary component composed of the sum of individual factors responsible for the process.

Interpolated neo-functionalization of embryonal initials in the aqueous environment of an AS was genetic (automixis), epigenetic (apomixis), and a function of g x e (reaction norm). Epigenetic changes accrue over an organism's lifetime and leave a permanent heritable mark on the genome. Could these changes be explained by noncoding RNAs as transcripts from DNA which do not code for proteins? Did interpolated apomictic and automictic epigenetic genomic changes leave a permanent heritable mark in heteromorphic asexual spores in an AS?

Inbreeding depression in conifers remains among the highest in any group of organisms. Plating of eggs and their protoplast fusion products would remove the heavy load of recessive lethal genes. Variations in copy numbers, unique and repetitive sequences, transposable elements, silenced genes, and re-arrangements of highly repetitive sequence would identify some factors which contribute to coenocytic proembryogenesis, survival, diversity and reaction norms in gymnosperm reproduction.

Recent literature dealing with evolution of the life cycle in plants contains numerous conclusions (Qiu *et al*. 2012) but not many experiments that tie them together. Observations with an AS suggest that other phase-specific renewal signals might be incorporated into pluripotent (totipotent) cells. This could have wide applications for exploring ontogenetic, regenerative and evolutionary plasticity. A major impediment to the exploitation of experimental evolutionary science is the absence of reference genome sequences or an appropriate enabling alternative. Providing either remains a primary challenge.

Every great advance in science has issued from a new audacity of imagination (Dewey 1958).When dealing with pluripotency, totipotency and evolutionary changes under laboratory conditions there always remains a danger to describe complex observations beyond which terminology, definitions and observations all lose precision and meaning.

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References

Albrecht-Buehler G (1992) The simulation of microgravity conditions on the ground. *Am Soc Grav Space Biol Bulletin* 5 (2): 3-10

Allen GS (1942) Parthenocarpy, parthenogenesis, and self-sterility of Douglas-fir. J Forestry 40: 642-644

Andersson E (1947) A case of asyndesis in *Picea abies*. Hereditas 33, 301-347

Andersson E, Ekberg I, Eriksson C (1969) A summary of meiotic investigations in conifers. Studia Forestalia Suecica 70: 1-20

Appendino G (1994) The phytochemistry of the yew tree. *Nat Prod Repts* 12: 349-360

Ayres P (2008) The aliveness of plants: the Darwins at the dawn of plant science. Pickering and Chatto, London, 227 pp

Barton N (1998) The geometry of adaptation. Nature 395:751-752

Bateman RM, DiMichelle WA (1994) Heterospory: the most important key innovation in the evolutionary history of the plant kingdom. Biol Revs 69:345-417

Batygina TB (2009) Amphimixis and automixis. In: Embryology of flowering plants. Terminology and concepts. Vol 3: Reproductive systems. Batygina TB (ed) pp 101-181

Bell PR (1992) Apospory and apogamy: implications for understanding the plant life cycle. Intl J Plant Sci 153:S123-S136.

Bell PR (1994) Apomictic features revealed in a conifer. Intl J Plant Sci 155: 621-622

Bell PR (1996) Have necrohormones a role in embryogenesis? Acta Societatis Botanicorum Poloniae. 65: 1-2

Bergthorsson U, Andersson D, Roth JR (2007) Ohno's dilemma: evolution of new genes under continuous selection. Proc Natl Acad Sci USA 104:17004-17009

Berlyn GP, Passof PC (1965) Cytoplasmic fibrils in proembryo formation in *Pinus*. Can J Bot 43:175-177

Bhatnagar SP, Moitra A (1996) Gymnosperms. New Age International Ltd, New Delhi

Bidwell RGS, Durzan DJ (1975) Some recent aspects of nitrogen metabolism. In: Historical and Recent Aspects of Plant Physiology: A symposium honoring FC Steward, Cornell Univ Press, Coll Agric Life Sci, pp. 162-227

Birky W (1995) Uniparental inheritance of mitochondria and chloroplast genes: mechanism and evolution. Proc Natl Acad Sci USA 92:11331-11338

Bold HC, Alexopoulos CJ, Delevoryas T (1980) Morphology of plants and fungi. Fourth ed. Harper & Row, New York

Bower FO (1908) The origin of a land flora: a theory based upon the facts of alternation. Macmillan, London

Blount ZD, Barrick JE, Davidson CJ, Lenski RE (2012) Genomic analysis of a key innovation in an experimental *Escherichia coli* population. Nature 489:513-518

Blume Y, Durzan DJ, Smertenko P (2006) Cell biology and instrumentation: UV radiation, nitric oxide and cell death in plants. NATO Science Series, IOS press, Amsterdam

Bonga JM (1974) In vitro culture of microsporophylls and megagametophyte tissue of *Pinus*. In Vitro 9:270-277

Bonga JM (1981) Haploid tissue culture and cytology of conifers. Coll Intl sur la Culture “In Vitro” des Essences Forestières. Fontainebleau, France. AFOCEL, Nangis, France 283-292

Bonga JM, Durzan DJ, eds (1982) Tissue culture in forestry. Martinus Nijhoff, Dordrecht.

Bonga JM, Durzan DJ, eds (1987) Cell and tissue culture in forestry. Vol 1. General principles in biotechnology, Vol 2. Specific principles and methods: growth and development. Vol 3. Case histories: Gymnosperms, angiosperms and palms. Kluwer Academic Publ, Hingham, MA

Bonga JM, Fowler DP (1970) Growth and differentiation of gametophytes of *Pinus resinosa* cultured in vitro. Can J Bot 48:2205-2207

Boulay MP, Gupta PK, Krogstrup P, Durzan DJ (1988) Development of somatic embryos from cell suspension culture of Norway spruce (*Picea abies* Karst.) Plant Cell Repts 7:134-137

Březina V, Hřib J, Komárek J (1972) Morphological changes in the cells of *Scenedesmus quadricauda* during ontogeny. Arch Hydrobiol Suppl 41:57-71

Campbell RA, Durzan DJ (1975) Induction of multiple buds and needles in tissue cultures of *Picea glauca*. Can J Bot 53:1652-1657

Campbell RA, Durzan DJ (1976a) Vegetative propagation of *Picea glauca* by tissue culture. Can J For Res 6:240-243

Campbell RA, Durzan DJ (1976b) The potential for cloning white spruce via tissue culture. Proceedings of the 12th Lake States Forest Tree Improvement Conference, August 18-22, USDA Forest Service Tech Gen Rept NC-26, p. 158-166

Campbell RA, Durzan DJ (1979) Laser activation of phytochrome-controlled germination in *Pinus banksiana*. Can J For Res 9:522-524

Chafe SC, Durzan DJ (1973) Tannin inclusions in cell suspension cultures of white spruce. Planta (Berl.) 113:251-262

Chalupa V (1985) Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. Commun Inst Forest Czech Republic 14, pp 57-63

Chalupa V, Durzan DJ (1972) Differences in the seed size and in the biochemical composition of seed of various provenances of jack pine (*Pinus banksiana* Lamb.). Lesnictvi (Czech. Forestry) 18:1089-1106

Chalupa V, Durzan DJ (1973) Growth and development of resting buds of conifers *in vitro*. Can J For Res 3: 196-208

Chalupa V, Durzan DJ, Vithayasai C (1976) Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 2. The quantitative analysis of the growth of callus from hypocotyls and radicles. Can J Bot 54:446-455

Chamberlain CJ (1935) Gymnosperms. Structure and evolution. Dover Publ Inc, NY

Coulter JM (1914) The evolution of sex in plants. Univ Chicago Press, Chicago

Crane PR, Kenrick P (1997) Diverted development of reproductive organs: a source of morphological innovation in land plants. Pl Syst Evol 206:161-174

Crow JF (1994) Advantages of sexual reproduction. Dev Gen 15:205-213
Dandekar AM, Gupta PK, Durzan DJ, Knauf V (1987) Transformation and foreign gene expression in micropropagated Douglas-fir (*Pseudotsuga menziesii*). Bio/Technology 5:587 -590
Darlington CD (1937) Recent advances in cytology. P Blakiston & Son Co, Philadelphia PA
Darlington CD (1958) Evolution of genetic systems. Oliver & Boyd, Edinburgh. pp 157-168
Darwin F, Pertz DFM (1903) On the artificial production of rhythm in plants. With a note on the position of maximum stimulation. Ann Bot XVII:93-106
Dawkins R (1982) The extended phenotype. The gene as a unit of selection. WH Freeman, Oxford
Deal RB, Henikoff S (2010) A chromatin thermostat. Nature 463: 887-888
Dernberg AF (2012) RNA plays meiotic matchmaker. Science 336:681-682
Dewey J (1958) The quest for certainty: A study of the relation of knowledge and action. Kessinger Publ, Whitefish MT
Dobzhansky T (1951) Genetics and the origin of species. Columbia Univ Press, New York
Dolgosheina EV, Morin RD, Gozde Aksay, *et al*. (2008) Conifers have a unique small RNA silencing signature. RNA 14:1508–1515
Dogra PD (1966) Observations on *Abies pindrow* with a discussion on the question of occurrence of apomixis in gymnosperms. Silvae Genetica 15:11-20
Dogra PD (1967) Seed sterility and disturbances in embryogeny in conifers with particular reference to seed testing and tree breeding in Pinaceae. Stud Forst Suecica 45:1-97
Dogra PD (1978) Morphology, development and nomenclature of conifer embryo. Phytomorphology 28: 307-322
Doyle J (1963) Proembryogeny in *Pinus* in relation to that in other conifers – a survey. Proc. Roy Irish Acad 62B:181-216
Durzan DJ (1968a) Nitrogen metabolism of *Picea glauca*. I. Seasonal changes of free amino acids in buds, shoot apices and leaves, and the metabolism of uniformly labeled ^{14}C-L-arginine by buds during the onset of dormancy. Can J Bot 46:909-919
Durzan DJ (1968b) Nitrogen metabolism of *Picea glauca*. II. Diurnal changes of free amino acids, amides, and guanidino compounds in roots, buds, and leaves during the onset of dormancy of white spruce saplings. Can J Bot 46:921-928
Durzan DJ (1968c) Nitrogen metabolism of *Picea glauca*. III. Diurnal changes of amino acids, amides, protein, and chlorophyll in leaves of expanding buds. Can J Bot 46:929-937
Durzan DJ (1969a) Nitrogen metabolism of *Picea glauca*. IV. Metabolism of uniformly labeled ^{14}C-L-arginine, [carbamyl-^{14}C]-L-citrulline, and [1,2,3,4-^{14}C]-γ-guanidinobutyric acid during diurnal changes in the soluble and protein nitrogen associated with the onset of expansion of spruce buds. Can J Biochem 47:771-783
Durzan DJ (1969b) Automated chromatographic analysis of free monosubstituted guanidines in physiological fluids. Can J Biochem 47:657-664
Durzan DJ (1971) Free amino acids as affected by light intensity and the relation of responses to the shade-tolerance of white spruce and shade intolerance of jack pine. Can J For Res 1:131-140
Durzan DJ (1973a) Nitrogen metabolism of *Picea glauca*. V. Metabolism of uniformly labeled ^{14}C-L-proline and ^{14}C-L-glutamine by dormant buds in late fall. Can J Bot 51:359-369
Durzan DJ (1973b) The metabolism of ^{14}C-urea by white spruce seedlings in light and darkness. Can J Bot 51:1197-1211
Durzan DJ (1973c) The incorporation of tritiated water into amino acids in the presence of urea by white spruce seedlings in light and darkness. Can J Bot 51:351-358
Durzan DJ (1980d) Progress and promise in forest genetics. In: Paper Science and Technology: The Cutting Edge, 50th Anniversary, May 8-10, 1979, Institute of Paper Chemistry, Appleton, WI, pp 31-60
Durzan DJ (1983a) The fate of carbon during the assimilation of carbamoyl phosphate in white spruce seedlings as revealed by [^{14}C]-carbamoyl phosphate, [^{14}C]-cyanate, and [^{14}C]-bicarbonate labeling patterns. Physiol Plant 59: -241
Durzan DJ (1983b) Metabolism of tritiated water during imbibition and germination of jack pine seeds. Can J For Res13:1204-1218
Durzan DJ (1984) Special problems: Adult vs juvenile explants. In: Chapter 17, Handbook of plant cell culture, Crop species, Eds, Sharp WR, Evans DA, Ammirato PV, Yamada Y, Macmillan, NY, 2:471-503
Durzan DJ (1985) Biotechnology and the cell cultures of woody perennials. Biotechnology in Forest Science Workshop, sponsored by the Science Council of Canada, February 21-22, 1985. University of British

Columbia, Vancouver, Forestry Chronicle 61(5):439-447
Durzan DJ (1987) Plant growth regulations in cell and tissue culture of woody perennials. In: Hormonal control of tree growth. Kossuth SV, Ross SD, eds. Martinus Nijhoff, Dordrect, pp 95-111
Durzan DJ (1988a) Applications of cell and tissue culture in tree improvement. In: Applications of Plant Cell and Tissue Culture. J Wiley Chichester (Ciba Foundation Symp 137, pp. 36-58
Durzan DJ (1988b) Plant growth regulators in cell and tissue culture of woody perennials. J Plant Growth Regulation 6:95-111
Durzan DJ (1988c) Metabolic phenotypes in somatic embryogenesis and polyembryogenesis. In: Genetic Manipulation of Woody Plants. Hanover J *et al*. (eds), Plenum Press, NY, pp 293-312
Durzan DJ (1998d) **S**implified hybrid seed production by latent diploid parthenogenesis and parthenote cleavage. University of California, US Patent 5840567Durzan DJ (1988e) Somatic polyembryogenesis for the multiplication of tree crops. Biotech Genetic Eng Revs 6:339-376
Durzan DJ (1989) Physiological aspects of somatic polyembryogenesis in suspension cultures of conifers. In: International Symposium on Forest Tree Physiology. Ann Sci Forestières 46 (suppl): 101-107
Durzan DJ (1990) Molecular phenogenetics as an aid to fruit breeding. Proc Intl Symp *In Vitro* Culture and Horticultural Breeding. May 30 - June 3, 1989, Cesena, Italy. Acta Hort 280:547-556
Durzan DJ (1991) Improving tree growth and quality through biotechnology. Kemia-Kemi (Finnish Chemistry) 18:192-199
Durzan DJ (1996a) Asexual reproductive adaptation to simulated Cretaceous climatic variables by Norway spruce cells *in vitro.* Chemosphere 33:1655-1673
Durzan DJ (1996b) Protein ubiquitination in diploid parthenotes of Norway spruce. Intl J Plant Sci 157:17-26
Durzan DJ (2000) Metabolic engineering plant cells in a space environment. Biotechnology and Genetic Engineering Revs 17:349-383
Durzan DJ (2002) Stress-induced nitric oxide and adaptive plasticity in conifers. J Forest Sci 48:281-291
Durzan DJ (2006) Nitric oxide, cell death and increased taxol recovery. In: Cell Biology and Instrumentation: UV Radiation, Nitric Oxide and Cell Death in Plants. Blume YA, Durzan DJ, Smertenko P (eds) IOS Press, NATO Advanced Research Workshop, Yalta, Ukraine, September 8 to 11, pp 234-25
Durzan DJ (2008) Monozygotic cleavage polyembryogenesis. Cytology and Genetics 42:159–173 (English and Russian versions). DOI 10.3103/S0095452710020015
Durzan DJ (2009a) Transamidination-based recycling of the respiratory inhibitor [1-^{14}C]- guanidinoacetic acid to ^{14}C-glycine in white spruce shoot primordia entering winter dormancy (*Picea glauca* Moench Voss). Trees-Structure and Function 24: 335-341. DOI: 10.1007/s00468-009-0403-3
Durzan (2009b) Arginine, scurvy, and Jacques Cartier's "tree of life". J. Ethnobiology & Ethnomedicine 5: 5 doi:10.1186/1746-4269-5-5
Durzan DJ (2010a) Salmine and the homeotic integrity of early embryos of Norway Spruce. Cytology and Genetics 44: 67-78 (Russian and English)
Durzan DJ (2010b) Arginine and guanidino compounds characterize the shade tolerance of white spruce saplings entering winter dormancy. J Forest Science 56:77-83
Durzan DJ (2011) Parthenogenetic apomixis and androsporogenesis in a Douglas-fir artificial sporangium. Sexual Plant Reproduction 24: 283-296, DOI 10.1007/s00497-011-0171-2
Durzan DJ (2012) Female parthenogenetic apomixis and androsporogenetic parthenogenesis in embryonal cells of *Araucaria angustifolia*: interpolation of progenesis and asexual heterospory in an artificial sporangium. Sexual Plant Reproduction 25:227-246. Online DOI 10.1007/s 004797-012-0189-0
Durzan DJ, Bennett DR (1968) Observations on single freely-suspended single cells of jack pine growing in cell culture. Proc 11th Mtg Committee on Forest Tree Breeding in Canada, pp 19-21
Durzan DJ, Bourgon G (1976) Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 7. Observations on cytoplasmic streaming and effects of L-glutamine and its analogues on subcellular activities. Can J Bot 54:507-517
Durzan DJ, Campbell RA (1974) Prospects for the mass production of improved stock of forest trees by cell and tissue culture. Can J For Res 4:151-174
Durzan DJ, Campbell, RA, Wilson A (1979) Inhibition of female cone production in white spruce by red light treatment during night under field conditions. Env Exptl Bot 19:133-144
Durzan DJ, Chafe SC, Lopushanski SM (1973) Effects of environmental changes on sugars, tannins, and organized growth in cell suspension cultures of white spruce. Planta (Berl.) 113:241-249

Durzan DJ, Chalupa V (1968) Free sugars, amino acids, and soluble proteins in the embryo and female gametophyte of jack pine as related to climate at the seed source. Can J Bot 46:417-428

Durzan DJ, Chalupa V (1976a) Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 3. Growth of cells in liquid suspension cultures in light and darkness. Can J Bot 54:456-467

Durzan DJ, Chalupa V (1976b) Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 4. Changes in amino acids of callus and in seedlings of similar genetic origin. Can J Bot 54:468-482

Durzan DJ, Chalupa V (1976c) Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 5. Changes in free arginine and Sakaguchi-reactive compounds during callus growth and in germinating seedlings of similar genetic origin. Can J Bot 54:483-495

Durzan DJ, Chalupa V (1976d) Growth and metabolism of cells and tissue of jack pine (*Pinus banksiana*). 6. Free nitrogenous compounds in cell suspension cultures of jack pine as affected by light and darkness. Can J Bot 54:496-506

Durzan DJ, Chalupa V, Mia AJ (1976) Growth and metabolism of cells and tissues of jack pine (*Pinus banksiana*). 1. The establishment and some characteristics of a proliferated callus from jack pine seedlings. Can J Bot 54:437-445

Durzan DJ, Durzan PE (1991) Future technologies: Model-reference control systems for the scale-up of embryogenesis and polyembryogenesis in cell suspension cultures. In: Micropropagation. P. Debergh and R.H. Zimmerman (eds), Kluwer, Amsterdam, pp 398-423

Durzan DJ, Gupta PK (1987) Somatic embryogenesis and polyembryogenesis in Douglas-fir cell suspension cultures. Plant Sci 52:229-235

Durzan DJ, Gupta PK (1998) Method for clonal propagation of gymnosperms by somatic polyembryogenesis. US Patent No. **5,821,126.** Oct. 13, 1998

Durzan DJ, Hansen K, Peng C (1991) Anthocyanin production in cell suspension cultures of *Prunus cerasus* cv. Vladimir. Adv Hort Sci 5:3-10

Durzan DJ, Jokinen K, Guerra M, Santerre A, Chalupa V, Havel L (1994) Latent diploid parthenogenesis and parthenote cleavage in egg-equivalents in Norway spruce. Intl J Plant Science 155: 677-688

Durzan DJ, Mia AJ, Wang BSP (1971a) Effects of tritiated water on the metabolism and germination of jack pine seeds. Can J Bot 49:2139-2149

Durzan DJ, Mia AJ, Ramaiah PK (1971b) The metabolism and subcellular organization of jack pine embryo (*Pinus banksiana*) during germination. Can J Bot 49:927-938

Durzan DJ, Lopushanski SM (1975) Propagation of American elm via cell suspension cultures. Can J For Res 5:273-277

Durzan DJ, Pedroso MC (2002) Nitric oxide and reactive nitrogen oxide species in plants. Biotechnology and Genetic Engineering Reviews 19: 293-337

Durzan DJ, Pitel JA (1977) Occurrence of *N*-phosphoryl-arginine in the spruce budworm (*Choristoneura fumiferana*). J Insect Biochem 7:11-13

Durzan DJ, Pitel JA, Mia A, Ramaiah PK (1973) Metabolism of uracil by germinating jack pine seedlings. Can J For Res 3:209-221

Durzan DJ, Pitel JA, Ramaiah PK (1972) Acid soluble nucleotides and ribonucleic acids from germinating jack pine seeds. Can J For Res 2:206-216

Durzan DJ, Steward FC (1967) The nitrogen metabolism of *Picea glauca* (Moench) Voss and *Pinus banksiana* Lamb. as influenced by mineral nutrition. Can J Bot 45:695-710

Durzan DJ, Steward FC (1971) Morphogenesis in cell cultures of gymnosperms: some growth patterns. Invited paper, International Union of Forest Research Organizations. Section 22. Workshop held May 28-June 5, Helsinki, Finland, 20 pp plus 8 plates. Abstr Comm Inst For Fenn 74(6):16

Durzan DJ, Steward FC (1983) Nitrogen metabolism, Chap. 2. In: Plant Physiology, An Advanced Treatise, F.C. Steward (ed.), Academic Press, Inc, New York 8:55-265

Durzan DJ, Ventimiglia F (1994) Free taxanes and the release of bound compounds having taxane antibody reactivity by xylanase in female, haploid-derived cell suspension cultures of *Taxus brevifolia.* In Vitro Plant Cellular and Developmental Biology 30P: 219-227

Durzan DJ, Ventimiglia F, Havel L (1998) Taxane recovery from cells of *Taxus* in micro- and hypergravity. Acta Astronautica 42: 455-463

Dutton K, Thompson S, Barraclough B (1997) The art of control engineering. Addison-Wesley, New York
Eigen M, Schuster P (1979) The hypercycle: a principle of natural self-organization. Springer, Berlin
Fernando DD, Quinn CR, Brenner ED, Owens JN (2010) Male gametophyte development and evolution in extant gymnosperms. Intl J Plant Dev Biol (special issue) 4:47-63
Filonova LH, Bozhkov PV, von Arnold S (2000). Developmental pathways of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. J Exptl Bot 51:249-264
Goetz JJ, Trimarchi JM (2012) Transcriptome sequencing of single cell with Smart-Seq. Nature Biotechnology 30:763-765
Gong P, Pu R, Yu P (1997) Conifer species recognition: An exploratory analysis of in situ hyperspectral data. Remote Sensing of the Environment 62:189-200
Graham LE (1993) Origin of land plants. John Wiley, New York
Green MR, Pastewka JV (1974) Simultaneous differential staining by a cationic carbocyanine dye of nucleic acids, proteins and conjugated proteins. II. Carbohydrate and sulfate carbohydrate containing proteins. J Histochem Cytochem 22:774-781
Gupta PK, Dandekar AM, Durzan DJ (1988) Somatic polyembryogenesis and luciferase gene expression in Douglas fir and loblolly pine protoplasts. Plant Science 58:85-92
Gupta PK, Durzan DJ (1986a) Plantlet regeneration via somatic embryogenesis from subcultured callus of mature embryos of *Picea abies* (Norway spruce). *In Vitro* Cellular and Development Biology 22:685-688
Gupta PK, Durzan DJ (1986b). Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Technology, 4:643-645
Gupta PK, Durzan DJ (1987) Somatic embryos from protoplasts of loblolly pine proembryonal cells. Bio/Technology 5:710-712
Gupta PK, Durzan DJ (1991) Loblolly pine (*Pinus taeda* L.). In: Biotechnology in Agriculture and Forestry. III. Trees. YSP Bajaj (ed) Springer-Verlag, NY pp. 383-407
Gupta PK, Durzan DJ, Finkle BJ (1987) Somatic polyembryogenesis in embryogenic cell masses of *Picea abies* (Norway spruce) and *Pinus taeda* (loblolly pine) after thawing from liquid nitrogen. Can J For Res 17:1130-1134
Haig D (1992) Brood reduction in gymnosperms. In: Cannibalism: ecology and evolution among diverse taxa. Eds, Elgar M, Crespi B, Oxford University Press, Oxford, pp 63-84
Haig D (2008) Homologous vs antithetic alternation of generations and the origin of sporophytes. Bot Rev 74: 395-418
Haig D, Wilczek A (2006) Sexual conflict and the alternation of haploid and diploid generations. Phil Trans R Soc B 361:335-343
Haines RJ (1983) Embryo development and anatomy in *Araucaria* Juss. Aust J Bot 31: 125-140
Håkansson, A. 1956. Seed development of *Picea abies* and *Pinus silvestris*. Med. från Statens Skogsforsk. 46:1-23
Hakman I, Fowke LC, von Arnold S, Eriksson T (1985). The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). Plant Science 38:53-59
Havel L, Durzan DJ (1996a) Apoptosis in plants. Bot Acta 109:268-277
Havel L, Durzan DJ (1996b) Apoptosis during diploid parthenogenesis and early somatic embryogenesis of Norway spruce. Intl J Plant Sci 157:8-16
Havel L, Durzan DJ (1999a) Apoptosis during early somatic embryogenesis in *Picea* sp. In: Somatic embryogenesis in woody plants. Jain SM, Gupta PK, Newton RJ (eds) Kluwer Academic, Dordrecht 4:125-147
Havel L, Durzan DK (1999b) Programmed cell death in plant development. In: Advances in Regulation of Plant Growth and Development. Strnad M, Peč P, and Beck E (eds) Peres Co. Prague.203-212
He Y, Tang R-H, Hao Y, Stevens RD, Cook CW, Ahn SM, Jing L, Yang Z, Chen L, Guo F, Fiorani F, Jackson RB, Crawford NM, Pei Z-M (2004) Nitric oxide represses the *Arabidopsis* floral transition. Science 305: 1968-1971
Hemsley AR (1994) The origin of the land plant sporophyte: an interpolation scenario. Biol Rev 69: 263-273
Henig RM (2000) The monk in the garden: the lost and found genius of Gregor Mendel, the father of genetics. 292 pp, Houghton Mifflin Co, New York

Hershko A (1991) The ubiquitin pathway of protein degradation and proteolysis of ubiquitin-protein conjugates. Trans Biochem Soc 19:726-729

Hong, L, Boulay M, Gupta PK, Durzan DJ (1991) Variations in somatic polyembryogenesis: induction of adventitious embryonal-suspensor masses on developing Douglas fir embryos. In: Woody Plant Biotechnology. Ahuja MR (ed), Plenum Publ NY, pp 105-121

Hurst L, Nurse, P (1991) A note on the evolution of meiosis. J Theoretical Biology 150:561–563

Johansen DA (1950) Plant Embryology. Chronica Botanica, Waltham Mass

Jokinen K, Durzan DJ (1994) Properties of rescued embryonal suspensor masses of Norway spruce (*Picea abies* L. Karst.) determined by the genotype and the environment *in vitro*. Silva Fennica 28: 95-106

Jost L (1907) Plant physiology. English translation by Harvey Gibson. Oxford, Clarendon Press

Klebs G (1910) Croonian lecture: Alternations in the development and forms of plants as a result of environment. Proc R Soc Lond B 82:547-558

Klimaszewska K, Overton C, Stewart D and Rutledge RG (2011a) Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old somatic white spruce and expression profiles of 11 genes followed during the tissue culture process. Planta 233: 635-647

Klimaszewska K, Park YS, Bonga JM (2011b) Tissue culture research at the CFS. Natural Resources Canada, Canadian Forestry Service, Laurentian Forestry Center, Quebec

Kornberg A, Baker TA (1992) DNA replication. WH Freeman, New York

Lang WH (1909) A Theory of Alternation of Generations in Archegoniate Plants Based Upon the Ontogeny. New Phytol 8: 1-12

Linnean Society (1909) Alternation of Generations. Meeting of the Linnean Society on February 18th 1909 following a paper by Dr. WH Lang. New Phytol 8: 104-116

Magalhaes JR, Pedroso MC, Durzan DJ (1999) Nitric oxide, apoptosis and plant stresses. Physiology and Plant Molecular Biology 5:115-125

Maggs CA, Callow ME (2002) Algal spores. Encyclopedia of life sciences. Nature Publishing Group

Manning JM (1993) The contributions of Stein and Moore to protein science. Protein Science 2:1188-1191

Maruthachalam R, Mohan PA, Marimuthu MPA, Siddiqi I (2008) Gamete formation without meiosis in *Arabidopsis*. Nature 451:1121-1124

Marimuthu MPA, Jolivet S, Ravi M, Pereira L, Davda JN, Cromer L, Wang L, Nogue F, Chan SWL, Siddiqi I, Mercier R (2011) Synthetic Clonal Reproduction Through Seeds**.** Science 331:876 DOI: 10.1126/science.1199682

Martin AJP, Synge RLM (1941) A new form of chromatogram employing two liquid phases. 1. A theory of chromatography. II Application to the determination of the higher monoamino acids in proteins. Biochem J 35:1358-1368

Miller CN (1988) The origin of modern conifer families. In: Origin and evolution of gymnosperms, ed, Beck CB, Columbia University Press, NY, pp 448-486

Muller HJ (1964) The relation of recombination to mutational advance. Mutation Res1:2-9

Mogie M (1992) The evolution of asexual reproduction in plants. Chapman & Hall, London

Mothes K (1929) Physiologische Untersuchungen űber das Asparagin und das Arginin in Coniferen. *Planta* 7: 585-649

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio-assays with tobacco tissue cultures. Physiol Plant 15:473-497

Murray A, Hunt T (1993) The cell cycle. WH Freeman & Co. New York

Niklas KJ, Kutscher U (2010) The evolution of the land plant sporophyte. New Phytol 185:27-41

Nuti Ronchi V (1995) Mitosis and meiosis in cultured plant cells and their relationship to variant cell types arising in culture. Intl Rev Cytol 158:65-140

Okada TS (1991) Transdifferentiation. Flexibility in cell differentiation. Oxford Univ Press, New York

Orr-Ewing AL (1957a) A cytological study of the effects of self-pollination on *Pseudotsuga menziesii* (Mirb.) Franco. Silvae Genetica 6:179-185

Orr-Ewing AL (1957b) Possible occurrence of viable unfertilized seeds in Douglas-fir. Forest Science 3: 243-248

Owens J (2006) The reproductive biology of loblolly pine. Forest Genetics Research Council of British Columbia. FCG Extension note 07. ISBN 0-7726-5342-9

Pattanavibool R, Klimaszewska K, von Aderkas P (1998) Interspecies protoplast fusion in *Larix*: comparison of electric and chemical methods. In Vitro Cell Dev Biol Plant 34:212-217
Pedroso MC, Durzan DJ (1999) Detection of apoptosis in chloroplasts and nuclei in different gravitational environments. J Gravitational Physiol 6:19-20
Pedroso MC, Magalhaes JR, Durzan DJ (2000a) A nitric oxide burst precedes apoptosis in an angiosperm and a gymnosperm. J Exptl Bot 51:1027-1036
Pedroso MC, Magalhaes JR, Durzan DJ (2000b) Nitric oxide induces cell death in *Taxus* cells. Plant Sci 157:173-181
Pennisi E (2012) Gene duplication's role in evolution gets richer, more complex. Science 338:316-317
Pitel JA, Durzan DJ (1978 a) Changes in the composition of the soluble and chromosomal Proteins of jack pine (*Pinus banksiana* Lamb.) seedlings induced by fenitrothion. Envir Expt Bot 18:153-162
Pitel JA, Durzan DJ (1978 b) Low molecular weight RNA in jack pine (*Pinus banksiana* Lamb.) seedlings. Bi-monthly Research Notes. Fisheries and Environment Canada, Canadian Forestry Service, 34:15-16
Pitel JA, Durzan DJ (1978 c) Chromosomal proteins of conifers. 1. Comparison of histones and nonhistone chromosomal proteins from dry seeds of conifers. Can J Bot 56:1915-1927
Pitel JA, Durzan DJ (1978 d) Chromosomal proteins of conifers. 2. Tissue-specificity of the chromosomal proteins of jack pine (*Pinus banksiana*). Can J Bot 56:1928-1931
Place ICM (2002) 75 years of research in the woods. A history of Petawawa Forest Experiment Station and Petawawa National Forestry Institute. General Store Publishing House, Burnstown, Ontario, Canada
Popa R (2004) Between necessity and probability: Searching for the definition and origin of life. Springer Verlag, New York
Qui Y-L, Taylor AB, McManus HA (2012) Evolution of the life cycle in land plants. J Systematics and Evoln 50:171-194
Ramming D (1983) Embryo culture. In: Methods in fruit breeding. Moore JN, Janick J, Eds, pp 136-144, Purdue Univ Press, Indiana
Rieger R, Michaelis A, Green MM (1976) Glossary of genetics. Fourth ed. Springer-Verlag, Berlin
Ritchie GA (1984) Assessing seedling quality. In: Forest nursery manual. Production of bareroot seedlings. In: Duryea ML, Landis TD (eds) Martinus Nijhoff, The Hague and Boston, 243-259
Rohde E (1923) Der plasmodiale Aufbau des Tier- und Pflanzenkörpers. Zeitschrift für wissenshaftliche Zoologie 120:325-535
Romani R, Hess B (1986) Suspension cultured pear fruit cells are being used as a model system to study ripening and cellular senescence. California Agriculture, July-August, p. 15
Russell ES (1930) The interpretation of development and heredity. A study in biological method. Oxford Univ Press, Oxford
Santner A, Estelle M (2009) Recent Advances and Emerging Trends in Plant Hormone Signaling. Nature (London) 459: 1071-1078
Saxton WT (1909) Parthenogenesis in *Pinus pinaster*. Bot Gaz 47:406-409
Schoener TW (2011) The newest synthesis: understanding the interplay of evolutionary and ecological dynamics. Science 331:426-429
Shaw GG, Little CHA, Durzan DJ (1978) Effect of fertilization of balsam fir trees on spruce budworm nutrition and development. Can J For Res 8:364-374
Schulze E (1896) Über die beim Umsatz der Proteinstoffe in den Keimpflanzen einiger Coniferen Arten entstehanden Stickstoffverbindungen. Zeit physiol Chem 122:435-448
Singh H (1978) Embryology of gymnosperms. Handbuch der Pflanzenatomie. Gebrüder, Borntraeger, Berlin, Vol 10, Part 2
Space Studies Board (1998) A strategy for research in space biology and medicine in the new century. National Academy Press, Washington, DC, pp 49-62
Sporne KR (1965) The morphology of gymnosperms. The structure and evolution of primitive seed-plants. Hutchinson University Library, London
Strasburger E (1872) Die Coniferen und Gnetaceen. Jena
Stebbins GL (1965) Some relations between mitotic rhythms, nucleic acid synthesis and morphogenesis in higher plants. Brookhaven Symp Biol 18:204-221

Stebbins GL (1988) Essays in comparative evolution. The need for evolutionary comparisons. In: Plant Evolutionary Biology, Eds, Gottlieb L, Jain SK, Chapman and Hall, New York, 3-20

Stephanopoulos G (1984) Chemical process control. An introduction to theory and practice. Prentice-Hall, Englewood Cliffs, NJ

Sterling C (1948) Gametophyte development in *Taxus cuspidata*. Bull Torry Bot Club 75:147-165

Steward FC (1958) Growth and organization in cultured cells. III. Interpretations of the growth from free cells to plants. Am J Botany 45:709-713

Steward FC (1963) Effects of environment on metabolic patterns. In: Evans LT, Ed, Environmental control of plant growth. Academic Press, NY, 195-214

Steward FC (1968) Growth and organization in plants. Structure, development, metabolism, physiology. Addison-Wesley, London

Steward FC (1970) Croonian lecture. From cultured cells to whole plants: the induction and control of their growth and morphogenesis. Proc Roy Soc London B 175:1-30

Steward FC, Durzan DJ (1965) Metabolism of nitrogenous compounds. In: Plant Physiology. An Advanced Treatise," Steward FC (ed) Academic Press, NY, Vol. 4, Part A, pp 379-686

Steward FC, Mapes MO, Smith J (1958) Growth and organized development of cultured cells. I. Growth and division of freely suspended cells. Am J Bot 45:693-713

Stewart WN, Rothwell GW (1993) Paleobotany and the evolution of plants. Cambridge Univ Press, Cambridge Strasburger E (1872) Die Coniferen und die Gnetaceen. H. Dabis, Jena

Sun Y, Joachimski MM, Wignall PB, Yan C, Chen Y, Jiang H, Wang L, Lai X (2012) Lethally hot temperatures during the early Triassic greenhouse. Science 338:366-370

Suomalainen E, Saura A, Lokki J (1987) Cytology and evolution in parthenogenesis. CRC Press, Boca Raton, Florida

Thomson RB (1945) Polyembryony. Sexual and asexual embryo initiation and food supply. Trans Royal Soc Canada 34:143-169

Sybenga J (1992) Cytology in plant breeding. Springer-Verlag, Berlin

Timmis R (1998) Bioprocessing for tree production in the forest industry. Conifer somatic embryogenesis. Biotech Progress 14:156-166

Ventimiglia FM, Durzan DJ (1986) The determination of monosubstituted guanidines using a dedicated amino acid analyzer. Liquid chromatography/Gas chromatography 4:1121-1124

Vermeij GJ (1996). Adaptation of clades: Resistance and response. In: Adaptation, Rose MR, Lauder GV Eds, Academic Press, New York, pp 363-379

Wellensiek SJ (1952) Rejuventation of woody plants by formation of sphaeroblasts. Proc Konenklyke Ned Acad v Wetenschappen Ser C 55:567-573

Williams GC (1966) Adaptation and natural selection. Princeton Univ Press

Williams GC (1976) Sex and evolution. Princeton Univ Press

van Loon JWA (2007) Biology in space and life on earth. Effects of spaceflight on biological systems. Brinckmann E (ed), Wiley-VCH Verlage GmbH & Co, KGaA, Weinheim, pp 17-32

Voipio P (1990) When and how did Mendel become convinced of the idea of general, successive evolution? Hereditas 113: 179-181

von Aderkas P, Bonga JM (1993) Plants from haploid tissue culture of *Larix deciduas*. Theor Appl Genet 87:225-228

Whittle C-A, Johnston MO (2002). Male-driven evolution of mitochondrial and chloroplast DNA sequences in plants. Molecular Biology and Evolution 19: 938-942

Youle RJ, van der Bliek AM (2012). Mitochondrial fission, fusion, and stress. Science 337:1062-1065

Zimmermann MH, Brown CL (1971). Trees, structure and function, Springer-Verlag, Berlin

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Recalcitrance in the *in vitro* propagation of trees

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Abstract: Recalcitrance is common when we attempt to achieve *in vitro* propagation of trees. It is a problem that is multi faceted and has plagued researchers for a long time. Several aspects of recalcitrance have been dealt with in earlier reviews. The purpose of the current discourse is to deal with aspects not or insufficiently dealt with in these earlier reviews. For several tree species, in particular conifers, recalcitrance is still a problem even if the explant material is highly juvenile. Once the adult stage has been reached *in vitro* propagation is possible for only a limited number of species. When attempting propagation of adult trees a number of potential solutions are suggested, including miniaturization of the explant, selecting explants near the site of meiosis, using shoot explants at specific moments during their annual cycle and making use of asymmetric divisions. The role played by epigenesis in recalcitrance is also discussed.

Introduction

Recalcitrance is a long-time problem that has frustrated efforts to propagate many species by *in vitro* means, in particular when trying to propagate adult tree specimens. Recently we published a review (Bonga et al. 2010) that dealt with various aspects of the problem. In the current manuscript I will discuss new information that has come to light since then and expand on some of the issues discussed earlier.

Recalcitrance, which is defined as the inability to propagate a plant by commonly used *in vitro* techniques, is a common problem with tree species, especially with conifers (Bonga et al. 2010). Especially somatic embryogenesis (SE), which is the most desired method of clonal propagation, is often difficult or impossible to achieve and often, when possible, only at initiation rates too low to be practical. Recalcitrance in explants increases with the age of the plants from which we initiate our cultures.

As a consequence clonal propagation is often restricted to the use of explants excised from juvenile sources. In the following I will discuss some of the causes of recalcitrance and suggest means of tackling the problem.

What is recalcitrance?

Recalcitrance to vegetative propagation has evolved over time and, therefore, has survival value. This is perhaps well illustrated by such long-lived individuals as 800-year-old Douglas-fir trees. If one considers the cambium near the base of such trees one realizes that this cambium over that long time span has produced year after year nothing but phloem and xylem. The genetic information in its cells for any other developmental activity, be it, among others, root, shoot, or embryo formation, never needed to be expressed. It is no wonder that mechanisms have evolved to repress specific genetic information in positions within the plant where expression of such information is not beneficial. Recalcitrance generally increases as the plant develops and even though sometimes partially

reversible by experimental means (Arnaud et al. 1993, Monteuuis et al. 2011) its complete reversal generally occurs only during the sexual process.

In the growing plant, in particular in conifers, recalcitrance is expressed in such traits as a reduced rooting capacity of cuttings, plagiotropic behavior of cuttings and grafts, changes in foliage morphology and eventually in sexual maturity as the plant ages. As has been shown by nuclear transplant experiments in animals and microorganisms (review Bonga et al. 2010) many of the age related changes are due to epigenetic factors that accumulate in the cytoplasm (e.g. yeast Shcheprova et al. 2008, Barral 2010). During the sexual process, phase changed is reversed from the mature to the embryonic phase. Much of this reprogramming occurs during meiosis. It has been observed that during the meiotic prophase methylated heterochromatic bodies are extruded from pollen mother cell nuclei (Giorgetti et al. 2007). These authors speculate that that this extrusion of accumulated multicopy DNA sequences is required to regain totipotency before entering gametogenesis.

In maturing trees growth behavior is to a more or lesser degree determined by (shoot) apical meristems. An extreme example of this is provided by rooted cuttings of *Araucaria excelsa*. This species displays a rigid growth architecture with an orthotropic trunk and no branching beyond a first and second order of branching. Rooted cuttings of the orthotropic leader will form normal orthotropic plants; rooted cuttings of a first order branch terminal will form a plagiotropic plant with first and second order branches while rooted cuttings of a second order branch will be plagiotropic and form nothing but a string of second order branch internodes (Meins and Binns 1979). Most tree species show more flexibility than that but rooted cuttings and grafts, especially in the conifers, often still show abnormalities if the cuttings are taken from trees more than a few years old.

Recalcitrance of tree species *in vitro*

True-to-type organogenesis, and certainly SE in conifers, is similarly confined to young individuals. In fact SE is often limited to immature zygotic embryos. In a cooperative international study of SE of various *Picea* and *Pinus* species (Park and Bonga 2011) it was found that most *Picea* species had high initiation rates if immature seeds were used as explants. For the *Pinus* species the results were more varied with *Pinus strobus* and *P. pinaster* showing high initiation rates and the other species much lower ones with immature seed as explants. The induction rates for the *Picea* species was much reduced by the use of mature instead of immature seed as explant source; for all the *Pinus* species by Park and Bonga (2011) the initiation rates were nil or near nil when mature seeds provided the explants. However, this is not universal for all pine species. Gupta and Durzan (1986) were able to initiate somatic embryos from sugar pine seeds that had been stored for 5 years.

Over the years extensive studies have been carried out with jack pine (*Pinus banksiana)* a rather recalcitrant species with regard to SE induction (Park et al. 1999, 2006). Over the years we have obtained induction rates no higher than 3-4%, using immature seed from trees that we had identified as being responsive. Initiation proved to be possible only with seed in which the embryo is at the poly-cleavage stage. There are many types of cleavage in conifers (Dogra 1978, Singh 1978). In *Pinus banksiana* at this stage of development the 16-cell zygotic embryo cleaves into four 4-cell embryos of which one will develop while the other three eventually abort. This is labeled as indeterminate cleavage polyembryony by Dogra (1978) and Singh (1978). We assume that as long as the explant is on a 2,4-D containing medium the 4-cell embryos will cleave again when they reach the 16-cell stage and that this process repeats itself for as long as the explant is exposed to 2,4-D. In this manner a large number of 4-cell embryos are formed each of which will develop into a multi-celled embryo once the culture is transferred to a plant growth regulator free medium. These embryos mature and germinate easily thus forming a large number of clonal plants. This process is not unique to jack pine; a few other species (*Juniperus communis*) similarly depend on a continuation of the poly-cleavage process to obtain large numbers of somatic embryos (Helmersson and von Arnold 2009).

With *Picea* spp. reasonably high initiation rates are generally obtained if immature seed is used as explant source with much lower rates occurring if the seed or excised zygotic embryo explant is mature. With *Pinus* spp. the initiation rates are more varied. Of a number of tested species the rates were high, using immature seed as explant, for *P. strobus* and *P. pinaster* but much lower for others while the rates were mostly nil when mature seed or their excised embryos were used (Park and Bonga 2011). Clearly, for many conifer species the initiation and proper maturation and conversion rates are still too low for practical use of SE in combination with breeding/ selection systems that have been used for species with reasonably high SE initiation rates (Park and Bonga 2011). With some species, for example Douglas fir, callus formation will supplant the formation of embryonal suspensor masses *in* vitro when seed, from which the embryo explant is excised, matures (Hong et al. 1991).

For mature conifers the results in attempts to obtain SE have been largely dismal. There has been only one publication that described SE in a sexually mature conifer (*Picea glauca*, Klimaszewska et al. 2011) and in that case only from shoots excised from buds of trees obtained by SE rather than by sexual means. One problem with either organogenesis or SE in explants from adult conifers is that with the exception of a very few species coniferales do not produce juvenile root or stump sprouts, the exceptions being *Sequoia sempervirens* (Bon et al. 1994), *Pinus serotina* (Bramlett 1990), *Taxodium distichum* (Wilhite and Toliver 1990) and *Cunninghamia lanceolata* (Minghe and Faxin 2001). This does not mean that adult conifers do not have cells that are pluri- or toti- potent. One would expect that the stem cells present in the shoot apical meristems are pluri- or toti-potent. However, these stem cells are difficult to access. It has been found that shoot apices can only express their morphogenetic capabilities if most of their cells are removed leaving only the very apex with a minimal number of leaf or needle primordia attached.

Interesting experiments have been carried out in that respect by Irish and McMurray (2006) with corn. When miniaturized shoot apices with only one or two leaf primordia left attached were excised from mature plants these apices produced juvenile plants when cultured *in vitro*. When apices with more leaf primordia were cultured plants arose that formed mature type internodes from the start. This demonstrates that there are cells in the shoot apex capable of regenerating juvenile plants but most of the surrounding tissue has to be removed before these cells can express their juvenility. Such miniaturization of the shoot apex has been effective with the conifer *Sequiadendron giganteum*. Monteuuis (1991) excised tiny apices with only one needle primordium left attached from a 100 year old tree and cultured these *in vitro*. Juvenile plantlets were obtained from these cultures. Furthermore, this line has retained its juvenility *in vitro* since its initiation (Monteuuis et al. 2011). However, a similar procedure with adult *Sequioa sempervirens* resulted in only a partial recovery of juvenile characters (Arnaud et al. 1993). I have attempted to culture tiny shoot apices of *Larix decidua* but these died in culture presumably because of excessive wounding during excision.

Perhaps a better way of proceeding would be to produce protoplasts from shoots excised from buds, followed by selective separation of those protoplasts that originated from the cells that have morphogenic potential. For conifers shoot tips are generally not the preferred source for protoplasts; generally tissues with a less compact cell structure such as embryogenic cultures or cell suspensions thereof (Maruyama et al. 2000) are preferred. Nevertheless, *Pinus pinaster* seedling shoot tips have been used successfully for protoplast generation (Gomez-Maldonado et al. 2001). Another potential way of making use of miniaturized apices is by micrografting them onto seedlings, a graft providing a more natural environment than artificial nutrient media (Arnaud 1993, Ewald 1998, Monteuuis et al. 2011). Furthermore, grafting often appears to have a partial rejuvenating or re-invigorating effect which may in part be due to placing the scion close to the root system (Arnaud et al. 1993, Monteuuis et al. 2011). In addition, similar effects have been observed after forcing of proventitious buds or spraying with BA (Monteuuis et al. 2011). Unfortunately, all these procedures are laborious and often only have a low success rate.

In our work we have not been able to keep miniaturized shoot apices of *Larix decidua* alive. We had to employ the total shoot (apical cone with needle primordia after removal of the bud scales and referred to here as primordial shoots). These will show some response of interest, primarily if excised from buds collected in early April (just before bud break) or during the last week in August and the first week in September (about one month before the buds become dormant). Initially the excised primordial shoots were exposed to auxin and cytokinin for longer or shorter duration (Bonga 1994). In particular if pulsed with picloram a fast growing mucilaginous tissue emerged that contained micro-calli with bundles of elongated cells attached that resembled very early stage somatic embryos (Figures 1-3). In addition, coenocytic cells with either two or four free nuclei were also noted, suggesting that the early phase of an embryogenic pathway more akin to the zygotic one had also been initiated (Figure 4). However, none of these cellular arrangements nor the coenocytes developed into proper embryos. (For comparison and a more detailed account see Durzan in these proceedings).

Of more interest was a very soft, olive green tissue that developed without exposure to plant growth regulators while exposed to low light intensity. It contained a large number of intensely green, small dots that under the microscope appeared to be small spheres surrounded by a distinct epidermal layer (Bonga 1996, 2004). These spheres formed putative embryos that in appearance were often near normal with a hypocotyl, a whorl of cotyledons, an epicotyl and a distinct shoot apex. However, sectioning of these revealed that the root apex was not fully developed. Upon germination they formed shoots that elongated and formed stems up to several centimeters long but rooting, like in earlier experiments (Bonga and von Aderkas 1988) that produced adventitious shoots, was rare. Clearly, my use of *Larix decidua* primordial shoots has not resulted in true-to-type propagules even when collected in early April or late August. Are there any explant sources other than dormant and developing buds that could be used? In the past we discovered that slices of the female cones of *Larix decidua* are capable of adventitious shoot

formation if these cones are collected at the time when meiosis is taking place in the cones (Bonga and von Aderkas 1988). These shoots appeared in various positions on the cone slices, i.e. on exposed ovular, ovuliferous scale and bract tissues. Similarly, Wang et al. (1991) obtained adventitious shoot formation from slices of female cones of *Picea abies* collected at the time of meiosis. Therefore, it appears possible that the process of meiosis, by an as yet unknown mechanism, has a temporary rejuvenating effect on neighboring tissues. Somatic tissues of anthers

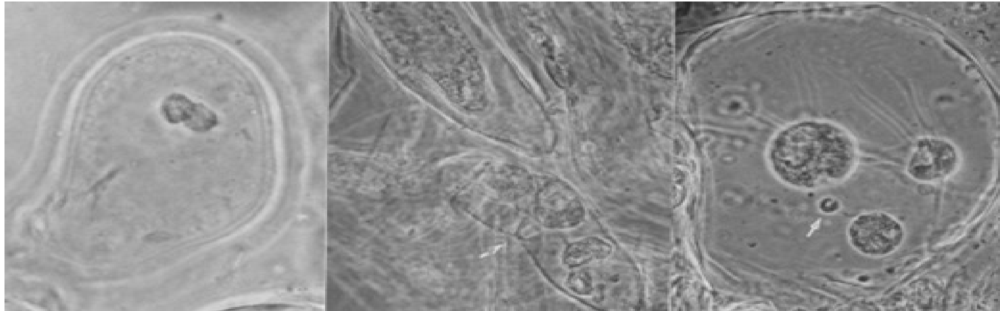

Fig. 1. *A cell with two nuclei: A linear tetrad?: A cell with four nuclei (arrow = icronucleus); a cell like this obviously cannot produce an embryo even though a small part of the programming required for SE may have occurred.*

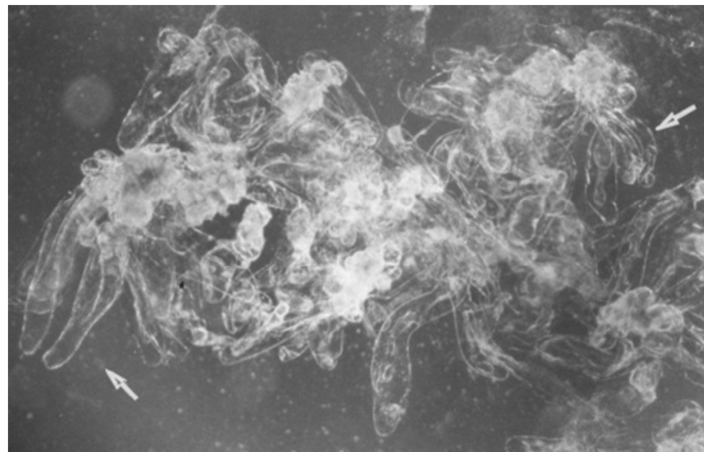

Fig. 2. *Microcalli with long cells attached. Notice bundling of long cells in some of the arrangements (arrows).*

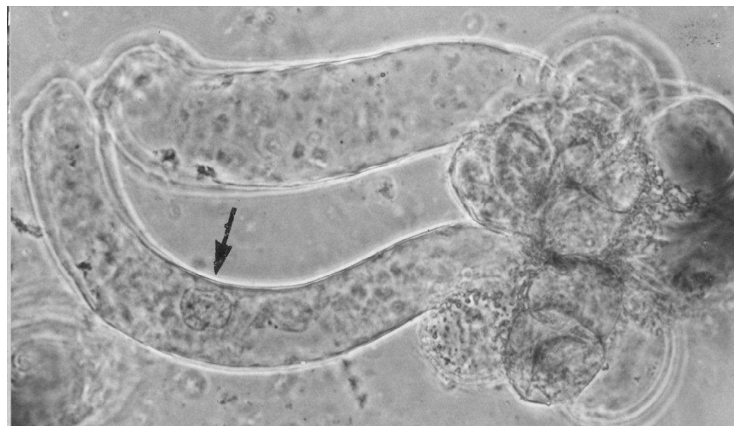

Fig. 3. *Microcallus with a bundle of long cells. Notice that long cells contain cytoplasm and a nucleus (arrow).*

and inflorescences of several hardwood species and palms also have initiated SE (reviewed by Merkle et al. 1998). SE induction generally was highest in immature explants, i.e., in explants that were close to the time of meiosis when they were excised.

Another aspect that deserves attention is asymmetric division. Such divisions are of frequent occurrence during the sexual process. During this process in conifers asymmetric divisions occur in pollen, the female tetrad, zygote and pro-embryo formation (Allen and Owens 1972, also see Durzan in these Proceedings). Similarly they occur in Angiosperm zygotic embryo formation (Feher et al. 2003, Zhang and Laux 2011). Furthermore, asymmetric divisions are often noted in the initiation of SE of conifers. In cultures of haploid as well as of diploid explants of *Larix* spp., SE was initiated by first producing an elongated cell that by an asymmetric division formed a small cell (von Aderkas et al. 1991). Similarly, asymmetric divisions have been observed during initiation of SE in *Picea abies* (Filonova et al. 2000) and *Picea omorika* (Budimir 2003) (also see Durzan in these Proceedings). Furthermore, such division is also an early event in SE arising from protoplasts, provided that agarose is present in the culture medium (Flores Berrios et al. 2000). In general, SE in cultures of many species arises from an initial asymmetric division (Feher et al. 2003).

Asymmetric divisions also initiate the formation of stomata on leaves (Petricka et al. 2009). In that respect it is noteworthy that SE has been induced from very young leaf explants of 6-7 year old *Quercus alba* trees (Corredoira et al. 2012). In these leaves the stomata were in the early stages of being formed, i.e., asymmetric divisions were occurring. All the observations above suggest that there may be an important connection between the induction of SE and the occurrence of asymmetric divisions.

Discussion

Despite the many recent advances in conifer SE technology, using zygotic embryos or such embryos left encased in the megagametophyte, there are still many problems. Even though large-scale industrial application of the technology is presently practiced for some species, for many others there are still limitations. Success has primarily been limited to several *Picea, Larix* and a few *Pinus* species. The problems remaining for many conifers are low initiation rates, SE being restricted to only a few genotypes within the species and problems proper maturation and germination of the SEs. Obviously, a lot of research is still required to improve SE protocols to the point where they will become industrially applicable to all commercial conifer species. Even more problematic is micropropagation of adult conifer trees. Although such propagation has been accomplished for a few species (see above), it has been achieved in a few instances and on only a very small scale.

With regard to improving SE from immature or mature zygotic embryos not many potential solutions present themselves. New plant growth regulators appear on the market from time to time and some of these may prove to be more effective than the currently used ones. For example, a promising new cytokinin is meta-topolin which acts much like benzyladenine but is metabolized more rapidly thus avoiding the persistent residual effects of the latter (Aremu et al. 2012). Culture media and environmental conditions have not yet been optimized for many species. Further efforts in that direction may eventually result in some improvement. Perhaps more attention to some of the gaseous stimulants is warranted. For example, nitric oxide (NO) is of some interest. Together with auxin it has been found to play a role during embryogenesis, including in conifer species where it is involved in apoptosis, monozygotic cleavage polyembryogenesis and gravisensing (Durzan and Pedroso 2002, Durzan these Proceedings) and in the establishment of polarity (Siveira et al. 2006).

With regard to clonal propagation of adult trees, what approach could possibly lead to fruitful results? As was pointed out above, miniaturization of the shoot tip promises to be effective. There is a considerable literature dealing with stem cells in shoot apices (review Barton 2010, Sablowski 2011) and it is presumably these stem cells that are pluri- or toti- potent and that have to be freed from restraining influences of neighboring cells before they can express their morphogenetic capacity. We can assume that indeed it are the stem cells that regenerated the juvenile adventitious corn and *Sequoiadendron* shoots mentioned above. The main problem is our current inability to remove all needle primordia and other tissues that inhibit the stem cells from expressing their pluri- or toti-potency from the shoot apex while keeping the tiny explants alive.

Our efforts to have this pluri- or toti-potentent capacity expressed in primordial bud explants of mature *Larix decidua* without removal of the restraining cells may have resulted in the initiation of the very early stages of SE. As mentioned earlier, when the explants were cultured on auxin containing medium, a fast growing mucilaginous callus appeared with cell clumps that looked like early SEs. However, one has to be careful in claiming

such a status because the observed structures, instead of being repressed somatic embryos could be random arrangements of cells. If one looks at a large number of randomly arranged cells in a tissue squash a few patterns resembling somatic embryos are bound to show up even when these are structures composed of nothing but non-embryogenic callus cells. Clearly, a claim of true embryo initiation cannot be based solely on formation of a few embryo-like cell clumps appearing in tissue squashes.

A more convincing observation of SE occurred in our cultures of *Larix decidua* primordial shoots that were grown on plant growth regulator free medium with embryos arising from well structured globular nodules. These embryos were capable of forming shoots but alas lacked a proper root meristem. This experiment suggests that explants from adult *Larix decidua* are self sufficient in producing the hormones required for SE. Not all conifers require auxin or cytokinin for SE induction (e.g. *Juniperus communis* (Helmerson and von Arnold 2009).

One peculiar aspect of these embryos was that several of them showed secondary embryogenesis. Not only did the shoot apex frequently form a new embryo rather than a shoot, embryos also arose from meristematic centers in the hypocotyl. Secondary embryos also arose from the stems and needles of some of the elongating shoots formed by the embryos (Bonga 1996). The fact that propagules obtained by SE *in vitro* sometimes retain a capacity for embryo formation whereas this capacity is lost in their equally aged zygotic counterpart, has also been observed for a few other Gymnosperm species (Harvengt et al. 2001, Helmersson and von Arnold 2009, Klimaszewska et al. 2011). Some plants derived from somatic embryos of *Picea glauca* retain a capacity for somatic embryogenesis even up to the time of flowering of these individuals (Klimaszewska et al. 2011). Therefore, it appears that the capacity for embryogenesis is not shut down in these. This is unlike to what happens in the zygotic embryo in which the capacity for embryogenesis is suppressed while it matures within the seed and after germination. Therefore, because of their continued SE ability, propagules obtained by SE are not entirely "true-to-type" clones of the zygotic embryos they are obtained from. Perhaps this is a long-lasting epigenetic effect created by the initial *in vitro* environment. Epigenetic reprogramming occurring *in vitro* can lead to major abnormalities and are mostly ascribed to DNA methylation and chromatin remodeling (Miguel and Marum 2011). Recent research has shown that a major role in epigenetic modification of the cell is also played by microRNA modification. Such modification provides cells with the means to adapt to changes in the microenvironment that surrounds the cells (Jiang et al. 2012).

Some long-lasting epigenetic effects can deliberately be introduced to create desired developmental attributes. For example, Kvaalen and Johnsen (2008) reported that height growth and the date of bud set in two-year-old *Picea abies* plants produced by SE is affected by the temperature under which the seed that provided the explant for SE induction was raised. Somatic plants raised from SEs initiated from zygotic embryos from seed that had matured at high temperature were taller and had a later bud set than those from seed matured at low temperature. Somatic plants obtained from the latter were more suited for planting at more northerly latitudes than the somatic plants obtained from seed matured at high temperature while the latter were more suited for planting further south. This demonstrates that epigenetic variation purposely induced can have beneficial practical effects. Of course these epigenetic effects should not be confused with variation or poor growth performance that has resulted from sub-optimal *in vitro* culture practices. For example, Hogberg et al. (2001) observed that unnecessarily long periods of exposure to abscisic acid and constant illumination during SE maturation negatively affected subsequent height growth over two growing seasons. Such problems can be avoided.

Whatever the cause of the various epigenetic effects mentioned above, it warrants a new assessment of what we call a clone. A proper clone has always been assumed to be a true-to-type, i.e. a genetically identical copy of the parent. This may still largely be correct but perhaps we have to consider clones to be normal when they show variation that is not dramatically affecting overall growth rates or other important characteristics. However, another recent observation also poses a problem to our traditional concept of a clone. A study of *Populus trichocarpa* clones has shown that leaves are genetically different from roots and that the crowns of clones arising from root sprouts are, therefore, genetically different from those of the parent tree (Yong 2012). Apparently, variation in somatic mutations has resulted in genetic variation within the tree that is as large as the variation occurring across unrelated trees. In addition, it has been postulated that within the crowns of highly branched trees genetic variation occurs among the shoot meristems (Cherfas 1985, Gill et al. 1995).This again may not have a large affect on those growth characteristics that we are mostly interested in, but, nevertheless, our concept of what constitutes a clone may need correction.

As noted earlier in this paper, organogenesis or SE has occurred in conifer female flower parts and in anther tissues of several hardwoods. In each case the flower parts were immature and, therefore, in meiosis or close to the time of meiosis. In both female and male flower development there are strong interactions between the cells that follow the sexual and those that follow the sporophytic pathway. Strong epigenetic signaling between the two

occurs in particular during the pre-meiotic and meiotic phase (Gutierrez-Marcos and Dickinson 2012, Tucker et al. 2012). Perhaps, this strong back and forth signaling predisposes the somatic tissues of the ovule and anthers to a state that is more able to express its capacity for organogenesis or SE than at times when there is less cell-to-cell signaling. Of particular interest is the observation that SERK genes are strongly expressed in the somatic tissues of developing anthers at meiosis (Colcombet et al. 2005) and in developing ovules as well (Kwaaitaal et al. 2005). SERK genes, if over-expressed *in vitro,* promote SE (Hecht et al. 2001). This could perhaps explain why somatic tissues of immature anthers and ovules are more capable of organogenesis or SE than other tissues.

As noted before, SE is often initiated by asymmetric division. Research with yeast has shown that asymmetric divisions counteract the effect of aging in their cell lines. Yeast cell lines go through about 50 divisions and then die. Somewhere along that line of divisions, cells will form a small bud that separates (asymmetric division) from the original much larger cell. Shcheprova et al. (2008) and Barral (2010) describe the function of a septin ring around the neck of the attachment between the large and the small cell. This septin ring prevents extra chromosomal ribosomal DNA circles that are associated with aging from passing from the large to the small cell. Therefore, when the small cell separates from the large one it starts a new cell line that is free of these age associated DNA circles (and presumably other age related particles as well) and thus forms a line capable of another about 50 divisions. Making the neck between the large and small cell leaky by experimental means resulted in some of the circular DNA entering the small cell which reduced the life span of the new cell line while the old cell line lived longer than expected because it had lost circular DNA to the small cell. This provides an interesting model for a quantitative assessment of aging and rejuvenation of these cell lines. Similarly, a fluorescent probe of age-associated aggregates in yeast showed that these were retained in the large cells during budding (Zhou et al. 2011). Presumably somewhat similar mechanisms operate during some of the asymmetric divisions occurring in plants. Furthermore, transcription and cell cycle factors that are involved in the asymmetric divisions leading to the formation of stomata have been identified (De Veylder et al. 2007).

As noted earlier young leaves of 6-7 year old *Quercus alba* in which stomata are just being formed are capable of SE. In that respect the guard cells of the stomata, formed by asymmetric division, are of particular interest. Guard cells are highly differentiated, yet they can also be totipotent. For example, guard cell protoplasts of sugar beets have initiated SE whereas protoplasts of the cells of all the other tissues of the leaves failed to do so (Hall et al. 1996, 1997). Regeneration of plants from protoplasts through SE has also been noted for *Gossypium hirsutum* (Nobre et al. 2001).

Another interesting aspect of guard cells is that they lack plasmodesmata (Hall et al. 1997), i.e., they cannot communicate with neighboring cells. The concept that isolation fosters de-differentiation is an old one. Steward (1968) was the first to state that cell isolation may be required before they could become totipotent. Cell isolation, by callose depositing on the cell wall or by other means of disruption of plasmodesmata, occurs at several stages of the sexual process and *in vitro* de-differentiation (review Bonga 1987, Feher 2003). It is also of interest to note that guard cells express the same SERK genes (Colcombet et al. 2005, Leonhardt et al. 2004) that play a major role in somatic embryogenesis (Hecht et al. 2001). Furthermore, it has been stated that guard cells possess a "transient stem-cell-like property" (Pillitteri et al. 2007).

Conclusions

One of the trends in the observations described so far that I would like to emphasize is the following. It is noticeable that in those cases where plant regeneration by SE or organogenesis has been successful, a change in developmental programming may have occurred at or near the explant site just prior to explant excision.

1)For conifers SE from zygotic embryos was most easily achieved when these embryos were immature, which is at the time the developmental program changes from formation of globular embryos to torpedo and cotyledonary shaped ones with meristem formation also occurring during that time. Subsequent to formation of the cotyledons the embryo enters a maturation and finally a dormancy mode, a period where developmental changes are far less dramatic.

2) For adult conifers there are two collection periods when cultures of primordial shoots (buds minus bud scales) are capable of forming adventitious shoots or putative somatic embryos. These short windows of opportunity occur just before bud break, when there is a shift from the development of needle primordia to the initiation of lateral buds, and in late August when there is a shift from bud scale formation to the development of an apical dome with needle primordia.

3) The somatic tissues of conifer female cones will form adventitious shoots if cone slices are excised for culture at the time that meiosis occurs, i.e., the period when a shift from mitosis to meiosis occurs with many attendant developmental changes.

4) The switch from symmetric to asymmetric division also appears to be a developmental change associated with an opportunity to initiate regeneration of plants.

Besides timing of explant excision, there are many other ways of dealing with recalcitrance. Of these only some have been touched upon briefly in this presentation. Recalcitrance is a complex issue and much further research is required before we will gain a better understanding of the problem.

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References

Allen GS and Owens JN (1972) The life history of Douglas fir. Environment Canada, Forest Service, Ottawa

Aremu AO, Bairu MW, Dolezal K, Finney JF and Van Staden J (2012) Toplolins: A panacea to plant tissue culture challenges? Plant Cell Tissue Organ Cult 108: 1-16

Arnaud Y, Franclet A, Tranvan H and Jacques M (1993) Micropropagation and rejuvenation of *Sequoia sempervirens* (Lamb) Endl: a review. Ann Sci For 50:273-295

Barral Y (2010) The gates of immortality. The Scientist 24: 38 (www.the-scientist.com/2010/10/138/1)

Barton MK (2010) Twenty years on: The inner workings of the shoot apical meristem, a developmental dynamo. Dev Biol 341: 95-113

Bon MC, Riccardi F and Monteuuis O (1994) Influence of phase change within a 90-year-old *Sequoia sempervirens* on its *in vitro* organogenic capacity and protein patterns. Trees 8:283-287

Bonga JM (1987) Clonal propagation of mature trees: Problems and possible solutions. In: Bonga JM and Durzan DJ (Eds) Cell and tissue culture in forestry, Vol 1, General principles and biotechnology. Martinus Nijhoff Publishers, Dordrecht, pp 249-271

Bonga JM (1994) Formation of organized structures in mucilaginous tissue derived from explants of 35-year-old *Larix eurolepis* and *L. decidua*. In: Pardos JA, Ahuja MR and Rossello RE (eds) Biotechnology of trees: Proc IUFRO Working Party S2.04-07, Valsain Spain Oct 18-22 1993, pp 79-89

Bonga JM (1996) Frozen storage stimulates the formation of embryo-like structures and elongating shoots in explants from mature *Larix decidua* and *L. x eurolepis* trees. Plant Cell Tissue Organ Cult 46:91-101

Bonga JM (2004) The effect of various culture media on the formation of embryo-like structures in cultures derived from explants taken from mature *Larix decidua*. Plant Cell Tissue Organ Cult 77:43-48

Bonga JM and von Aderkas P (1988) Attempts to micropropagate mature *Larix decidua* Mill. In: Ahuja MR (ed) Somatic cell genetics of woody plants. Kluwer Academic Publishers pp 155-168

Bonga JM, Klimaszewska KK and von Aderkas P (2010) Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tissue Organ Cult 100:241-254

Bramlett DL (1990) *Pinus serotina* (Michx.) Pond Pine. In: Burns RM and Honkala BH (eds) Silvics of North America. Vol 1. Conifers. USDA For Serv Agric Handbook 654, pp 470-475

Budimir S (2003) Developmental histology of organogenic and embryogenic tissue in *Picea omorika* culture. Biol Plant 47:467-470

Cherfas J (1985) When is a tree more than a tree? New Scientist, June 20, 106: 42-45

Colcombet J, Boisson-Dernier A, Ros-Palau R, Vera CE and Schroeder JI (2005) *Arabidopsis* somatic embryogenesis receptor kinases1 and 2 are essential for tapetum development and microspore maturation. Plant Cell 17: 3350-3361 ((Anther SE (1))

Corredoira E, San-Jose MC and Vieitez AM (2012) Induction of somatic embryogenesis from different explants of shoot cultures derived from young *Quercus alba* trees.Trees 26: 881-891 DOI 10.1007/s00468-011-0662-7

De Veylder L, Beeckman T and Inze D (2007) The ins and outs of the plant cell cycle. Nature Rev/Mol Cell Biol 8: 655-665

Dogra PD (1978) Morphology, development and nomenclature of conifer embryo. Phytomorphology 28: 307-322

Durzan DJ and Pedroso MC (2002) Nitric oxide and reactive nitrogen oxide species in plants. Biotech Genet Engin Rev 19: 293-337

Ewald D (1998) Advances in tissue culture of adult larch. In Vitro Cell Dev Biol - Plant 34:325-330

Feher A, Pasternak TP and Dudits D (2003) Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult 74: 201-228

Filonova LH, Bozhkov PV and von Arnold S (2000) Developmental pathway of somatic embryogenesis in *Picea abies* as revealed by time-lapse tracking. J Exp Bot 51: 249-264

Flores Berrios E, Getzbittel L, Mokrani L, Alibert G and Sarrafi A (2000) Genetic control of early events in protoplast division and regeneration pathways in sunflower. Theor Appl Genet 101: 606-612

Gill DE, Chao L, Perkins SL and Wolf JB (1995) Genetic mosaicism inplants and clonal animals. Ann Rev Ecol Syst 26: 423-444 ((Clones (1))

Giorgetti L, Castiglione MR, Martini G, Geri C and Nuti Ronchi V (2007) Methylated DNA sequence extrusion during plant early meiotic prophase. Caryologia 60: 279-289

Gomez-Maldonado J, Crespillo R, Avilla C and Canovas M (2001) Efficient preparation of Maritime pine (*Pinus pinaster*) protoplasts suitable for transgene expression analysis. Plant Mol Biol Rep 19: 361-366

Gupta PK and DJ Durzan (1986). Somatic polyembryogenesis from callus of mature sugar pine embryos. Bio/Technol 4:643-645

Gutierrez-Marcos JF and Dickinson HG (2012) Epigenetic reprogramming in plant reproductive lineages. Plant Cell Physiol 53:817-823

Hall RD, Riksen-Bruinsma T, Weyens G, Lefebvre M, Dunwell JM and Krens FA (1996) Stomatal guard cells are totipotent. Plant Physiol 112: 889-892

Hall RD, Riksen-Bruinsma T, Weyens G, Lefebvre M, Dunwell JM, van Tunen A and Krens FA (1997) Sugar beet guard cell protoplats demonstrate a remarkable capacity for cell division enabling applications in stomatal physiology and molecular breeding. J Exp Bot 48: 255-263

Harvengt L, Trontin JF, Reymond I, Canlet F and Paques M (2001) Molecular evidence of true-to-type propagation of a 3-year-old Norway spruce through somatic embryogenesis. Planta 213: 828-832

Hecht V, Vielle-Calzada J-P, Hartog MV, Schmidt EDL and Boutilier K (2001) The *Arabidopsis* Somatic Embryogenesis Receptor Kinase 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. Plant Physiol 127: 803-816

Helmersson A and von Arnold S (2009) Embryogenic cell lines of *Juniperus communis*; easy establishment and embryo maturation, limited germination. Plant Cell Tissue Organ Cult 96: 211-217

Hogberg KA, Bozhkov PV, Gronroos R and von Arnold S (2001) Critical factors affecting *ex vitro* performance of somatic embryo plants of *Picea abies*. Scand J For Res 16: 295-304

Hong L, Boulay M, Gupta PK and Durzan DJ (1991) Variations in somatic polyembryogenesis: Induction of adventitious embryonal-suspensor masses on developing Douglas fir embryos. In: Ahuja MR (ed) Woody plant biotechnology. Plenum Publ Corp NY pp 105-121

Irish EE and McMurray D (2006) Rejuvenation by shoot apex culture recapitulates the developmental increase of methylation at the maize gene *Pl-Blotched*. Plant Mol Biol 60: 747-758

Jiang XH, Zhang JT and Chan HC (2012) Ion channels/transporters as epigenetic regulators? A microRNA perspective. Sci China Life Sci 55: 753-760 doi: 10.1007/s11427-012-4369-9

Klimaszewska K, Overton C, Stewart D and Rutledge RG (2011) Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old somatic white spruce and expression profiles of 11 genes followed during the tissue culture process. Planta 233: 635-647

Kvaalen H and Johnsen O (2008) Timing of bud set in *Picea abies* is regulated by memory of temperature during zygotic and somatic embryogenesis. New Phytol 177: 49-59

Kwaaitaal MACJ, de Vries SC and Russinova E (2005) *Arabidopsis thaliana* Somatic Embryogenesis Receptor Kinase 1 protein is present in sporophytic and gametophytic cells and undergoes endocytosis. Protoplasma 226:55-65

Miguel C and Marum L (2011) An epigenetic view of plant cells cultured *in vitro*: somaclonal variation and beyond. J Exp Bot 62: 3713-3725

Minghe L and Faxin H (2001) Performance of Chinese-fir (*Cunninghamia lanceolata* (Lamb.) Hook.) plantlets from upper-crown and basal origins as modified by grafting and development as buried ramets before explant harvest. Silvae Genet 50:37-44

Monteuuis O (1991) Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through *in vitro* meristem culture. I Organogenic and morphological arguments. Physiol Plant 81: 111-115

Monteuuis O, Lardet L, Montoro P, Berthouly M and Verdeil J-L (2011) Somatic embryogenesis and phase change in trees. In: Park YS, Bonga JM, Park SY and Moon HK (eds) Proc IUFRO Working Party 2.09.02 "Somatic embryogenesis of trees" conference on "Advances in somatic embryogenesis of trees and its application for the future forests and plantations" August 19-21, 2010, Suwon, Republic of Korea, pp 21-29

Nobre J, Keith DJ and Dunwell JM (2001) Morphogenesis and regeneration from stomatal guard cell complexes of cotton (*Gossypium hirsutum* L.). Plant Cell Rep 20: 8-15

Park YS, Bonga JM, Cameron SI, Barrett JD, Forbes K, DeVerno LL and Klimaszewska K (1999) Somatic embryogenesis in jack pine (*Pinus banksiana* Lamb). In: Mohan Jain S, Gupta PK and Newton RJ (eds) Somatic embryogenesis in woody plants, Vol 4, Kluwer Academic Publishers Dordrecht, pp 491-504

Park YS, Lelu-Walter MA, Harvengt L, Trontin JF, MacEacheron I, Klimaszewska K and Bonga JM (2006) Initiation of somatic embryogenesis in *Pinus banksiana, P. Strobus, P. pinaster,* and *P. s ylvestris* at three laboratories in Canada and France. Plant Cell Tissue Organ Cult 86: 87-101

Park YS and Bonga JM (2011) Application of somatic embryogenesis in forest management and research. In: Park YS, Bonga JM, Park SY and Moon HK (eds) Proc IUFRO Working Party 2.09.02 "Somatic embryogenesis of trees" conference on "Advances in somatic embryogenesis of trees and its application for the future forests and plantations" August 19-21, 2010, Suwon, Republic of Korea, pp 3-8

Petricka JJ, van Norman JM and Benfey PN (2009) Symmetry breaking in plants: molecular mechanisms regulating asymmetric cell divisions in *Arabidopsis*. Cold Spring Harb Perspect Biol 2009;1:a000497

Pillitteri LJ, Sloan DB, Bogenschutz NL and Torii KU (2007) Termination of asymmetric cell division and differentiation of stomata. Nature 445: 501-505 doi:10.1038/nature05467

Sablowski R (2011) Plant stem cell niches: From signalling to execution. Curr Opinion Plant Biol 14: 4-9

Siveira V, Santa-Catarina C, Tun NN, Scherer GFE, Handro W, Guerra MP and Floh EIS (2006) Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert.) O. Ktze. Plant Sci 171: 91-98

Shcheprova Z, Baldi S, Buvelot Frei S, Gonnet G and Barral Y (2008) A mechanism for asymmetric segregation of age during yeast budding. Nature 454: 728-734 doi:10.1038/nature07212

Singh H (1978) Embryology of gymnosperms. Encyclopedia of plant anatomy. Borntraeger, Berlin, Stutgart

Steward FC (1968) Growth and organization in plants. Addison-Wesley Publ Cy, Reading, Massachusetts, USA

Tucker MR, Okada T, Hu Y, Scholefield A, Taylor JM and Koltunov AMG (2012) Somatic small RNA pathways promote the mitotic events of megagametogenesis during female reproductive development in *Arabidopsis*. Development 139:1399-1404

von Aderkas P, Bonga JM, Klimaszewska K and Owens J (1991) Comaparison of larch embryogeny *in vivo* and *in vitro*. In: Ahuja MR (ed) Woody plant biotechnology, Plenum Press, New York, pp139-155

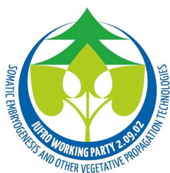
Wang KX, Karnosky DF and Timmis R (1991) Adventitious bud production from mature *Picea abies*: rejuvenation associated with female strobili formation. In: Ahuja MR (ed) Woody plant biotechnology, Plenum Press, New York, pp 83-90

Wilhite LP and Toliver JR (1990) *Taxodium distichum* (L.) Rich. Baldcypress. In:Burns RM and Honkala BH (eds) Silvics of North America. Vol 1. Conifers. USDA For Serv Agric Handbook 654, pp 470-475

Yong E (2012) Tree's leaves genetically different from its roots. www.nature.com/news/tree-s-leaves genetically-different-from-its-roots-1.11156

Zhang Z and Laux T (2011) The asymmetric division of the *Arabidopsis* zygote: from cell polarity to an embryo axis. Sex Plant Reprod 24:161-169 DOI 10.1007/s00497-010-0160-x

Zhou C, Slaughter BD, Unruh JR, Eldakak A, Rubinstein B and Li R (2011) Motility and segregation of Hsp 104-associated protein aggregates in budding yeast. Cell 147: 1186-1196



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Partial rejuvenation of mature hardwood trees through somatic embryogenesis: The example of pedunculate oak

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Abstract: Mature trees, exhibiting desirable traits, are generally recalcitrant to clonal propagation. In hardwoods, the use of plant material retaining juvenile physiological characteristics, such as epicormic shoots, root suckers or stump sprouts, may facilitate the in vitro propagation of elite genotypes. Even using this approach, micropropagation of mature trees may be hindered by relatively low rates of multiplication and poor rooting abilities, decline of proliferation capacity over time, propagation being limited to specific genotypes within species, etc. Theoretically, somatic embryogenesis (SE) is the most efficient procedure for mass propagation of forest trees and, at the same time, may be the best method for inducing rejuvenation of mature material. However, major difficulties must still be overcome, as the main problem reported for many hardwood species (including *Fagaceae*) is the relatively low conversion rate of the SE into plantlets. In many cases, only shoot development is available as consequence of the germination process. The shoots produced may then be multiplied through axillary shoot culture, allowing testing of the rejuvenation hypothesis. The aim of this work was to test this hypothesis by developing an appropriate experimental model which was applied to plant material collected from three centenarian genotypes of pedunculate oak (*Quercus robur* L.). Axillary shoot cultures were established in vitro, SE was induced from these cultures and later axillary shoot cultures were again established in vitro from embryo-derived plantlets. The morphogenetic response (multiplication, rooting) of the plant material from the different origins was evaluated and compared. The results obtained showed that morphogenetic values were higher (mainly in the rooting experiments) in SE-derived shoots compared with their corresponding mature shoot cultures. Clearly, some level of rejuvenation of the mature material was achieved through the embryogenic process although the response was related to both the type of the plant material, initially established in vitro, and the genotype. The experimental model proposed would be an interesting tool for biochemical and molecular studies of phase change in hardwood trees.

Introduction

Mature trees, exhibiting desirable traits, are generally recalcitrant to clonal propagation, hindering the application of multivarietal clonal forestry. Multivarietal clonal forestry is defined as the clonal deployment of tested tree varieties in plantation forestry, which may dramatically increase forest productivity (Park and Bonga 2010).

Problems associated to recalcitrance in vegetative propagation include the observation that propagation is often limited to only a few genotypes within the species, that propagation is in many species restricted to the use of very juvenile propagules and that often only low propagation rates are achieved by using in vitro technologies. In hardwoods, these limitations would be partially alleviated if mature material retaining physiological juvenile conditions are used to establish in vitro cultures. Our experience working with *Fagaceae* indicates that *Castanea, Quercus* and *Fagus* species may be propagated in vitro using both organogenesis and somatic embryogenesis (SE). Theoretically, SE is the most efficient procedure for mass propagation of forest trees. However, major difficulties must still be overcome in order to made SE commercially viable in these species, particularly when cultures originate from mature trees. Once induced, the main problem reported for most of the *Fagaceae* species is the very low rate of conversion of somatic embryos into plants, which remains difficult in many genotypes, resulting in a

limited production of viable plants. Consequently, much further effort is needed to improve the maturation, germination, and plantlet conversion steps of the embryogenic process.

Searching for alternatives to circumvent these difficulties in mass propagation of elite trees is an interesting approach. Many attempts were carried out to rejuvenate mature material through in vitro culture but we will mention only a few examples. One of the first species in which rejuvenation was studied was *Sequoiadendron giganteum* where the possibility to rejuvenate old trees by small shoot apical meristem culture has been shown to be possible (Monteuuis and Bon 1989, Bon and Monteuuis 1991, Monteuuis 1991). The response at the molecular level was also studied in this experiment (Bon 1988). Rejuvenation through micrografting of shoot meristems of 140-year-old European larch trees onto larch seedling rootstocks has been demonstrated in Germany (Ewald and Kretzschmar, 1996). Variations of dome shape in *Eucalyptus urophylla x Eucalyptus grandis* shoot apical meristems have been related to the phase change (Mankessi et al. 2010, 2011) while the embryogenic response depended of the origin of the initial explants. The effects of chronologic, ontogenetic and physiologic aging on explant capacity for SE and on the overall efficiency of the process in *Hevea brasiliensis* were discussed (Lardet et al. 2009).

According to Pierik (1990) the induction of SE in vitro is, in principle, the most efficient method for inducing rejuvenation and may be the only method of regenerating truly juvenile propagules (Bonga *et al*. 2010). Furthermore, Monteuuis et al. (2010) stated that SE is the only way of achieving complete ontogenetic rejuvenation as its resets the ontogenetic process to zero through the formation of embryos. If this hypothesis is true, plants derived from somatic embryos should exhibit juvenile characteristics, including a high capacity for micropropagation through organogenesis. However, data demonstrating rejuvenation in plants derived from germinated somatic embryos of mature tree origin are scarce. SE has been induced in mature specimens of *Hevea brasiliensis* and rejuvenation was reflected in the re-acquisition of the micropropagation capacity in material from somatic embryo-derived plants (Carron et al. 1995). The ability of SE-derived clones to undergo organogenesis in vitro has become of great interest in *Theobroma cacao* (Traore et al., 2003), although the work is not directly related to rejuvenation. The induction of SE in mature trees has been reported for only a limited number of conifers (Klimaszewska et al. 2011) and broad-leaved species. Preconditioning plant material through in vitro procedures should be considered when attempting to succeed in the induction of SE from mature trees. With these techniques, reviewed by Monteuuis et al. (2010), meaningful results have been obtained for different tree species in terms of rejuvenation.

The potential level of rejuvenation achieved after certain treatments should not be only based on the in vitro micropropagation (morphogenetic responses) criteria but also on genetic/epigenetic and molecular studies. Changes in DNA methylation were associated with maturational changes during ontogeny of *S. giganteum* (Monteuuis et al. 2008). Measurements of global DNA methylation or polyamine content in *Pinus nigra* was correlated with embryogenic potential (Noceda et al. 2009). Qualitative differences of genomic DNA methylation in juvenile and mature *Acacia mangium* has also been reported (Baurens et al. 2004). Similar results have been mentioned for the biological characterization of young and aged embryogenic cultures of *P. pinaster* (Klimaszewska et al. 2009). Recent studies have begun to reveal the molecular mechanism of phase change in the annual species *Arabidopsis* and maize, but the molecular mechanism of phase change in perennial woody species is still largely unknown. However, the juvenile phase of *Populus x canadensis* was drastically prolonged by overexpression of the micro RNAs miR156 and miR172 (Wang et al., 2011), a result which clearly opens a new way to understand the complex problem of phase change in trees.

During the last 20 years our research group has been involved in the micropropagation of hardwood trees (mainly *Castanea* and *Quercus*) from material with juvenile and mature characteristics, not only by organogenesis but also by SE (Corredoira et al. 2006a; Ballester et al. 2009; Vieitez et al. 2012). We also studied the feasibility to rejuvenate mature material through the somatic embryogenic process (Martínez et al. 2012) in order to test whether or not SE is the most efficient method for inducing rejuvenation. The objective of the present review is to summarize the results achieved.

The experimental model

To micropropagate mature hardwood trees it should be taken into consideration that zones exist within trees that are more morphogenetically competent than other parts of the tree (Bonga 1985), suggesting the use of root suckers, epicormic shoots, sphaeroblasts, stump sprouts and/or new shoots derived from severe pruning as the most reactive explants to initiate in vitro cultures from mature trees instead of material collected from the crown (Bonga et al. 2010). Accordingly, we established shoot cultures from plant material simultaneously collected from basal

shoots (BS) and braches from the crown (C) of the same mature trees in both European chestnut (*Castanea sativa*) and pedunculate oak (*Quercus robur*) (Fig. 1a). The age of the selected chestnut mother trees ranged from 30 to 80-year-old while those of oak ranged from 100 to 300-year-old. Segments (about 30 cm long) of basal shoots and branches were forced to flush in a growth chamber and both shoot tips and nodal explants of the new flushed shoots (Fig. 1 b) were used as explants to establish in vitro cultures (Vieitez et al. 1994). Using this procedure, 6 genotypes of chestnut (in addition, two genotypes of seedling origin, used as juvenile controls, were also established in vitro, Fernández-Lorenzo et al. 1999) and 2 genotypes of pedunculate oak were established in vitro (Fig. 1 c) (Vidal et al. 2003). The differential morphogenetic response between materials derived from basal shoots and crown branches was very clear, not only in the shoot multiplication capacity but also in the rooting rates recorded: the BS shoots exhibited morphologically juvenile characteristics in both stems and leaves, and showed significant differences in most of the parameters analyzed. Furthermore, BS material rooted more easily than C shoots regardless of the species and the genotype used. Genotype differences in rooting capacity were evident but rooting rates were significantly higher in BS shoots than in their C counterparts in all of the genotypes studied. The great stability of these cultures should be emphasized: the differences in rooting ability have been maintained for more than 15 years of in vitro establishment, as demonstrated by yearly rooting experiments. Even in the oak genotype Sainza, which was established in vitro at three different dates within a period of 15 years, similar differences in rooting ability of BS and C shoots were obtained regardless of the date of establishment. It seems that, after a long period of in vitro culture, the chestnut and oak materials used still "remember" their original position within the tree. Even after this long passage of time, the correlative effects are retained. This stability allowed us to use this experimental system to study the anatomical, biochemical and molecular responses of the shoots according to their topophysical origin.

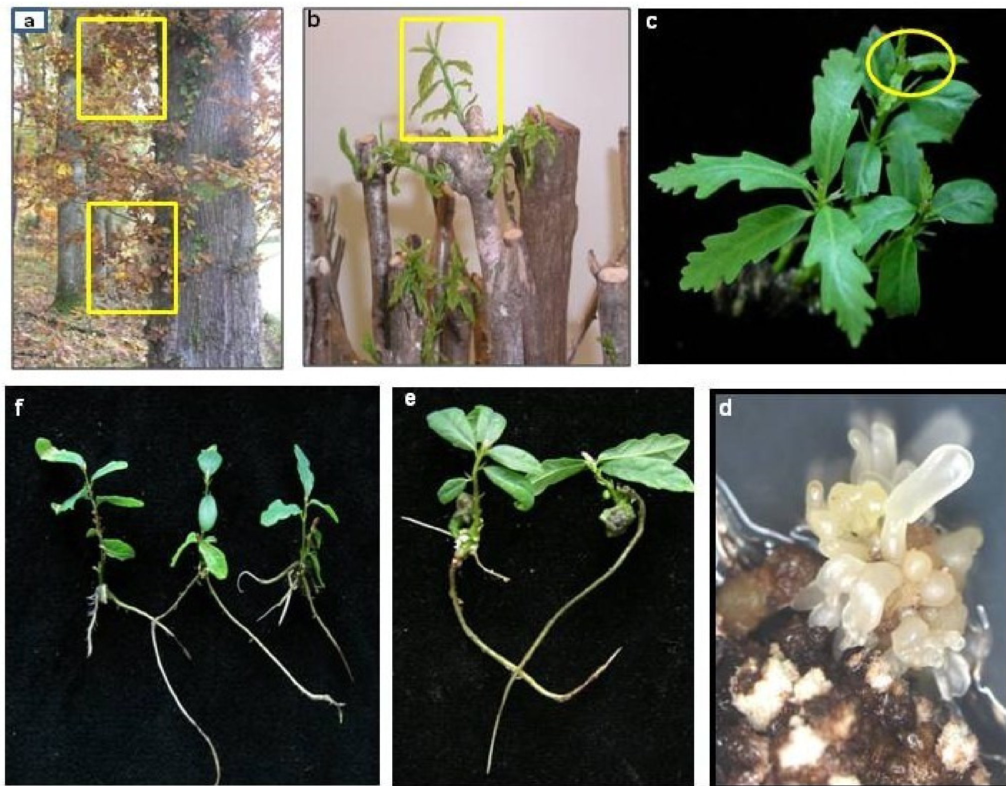

Figure 1. *Micropropagation of mature Quercus robur trees through organogenesis and somatic embryogenesis. (a) Selection of plant material from mother trees; (b) forced flushing in a growth cabinet to be used as source of explants to establish axillary shoot cultures; (c) leaves and shoot tips explants which are used to induce somatic embryogenesis (d); (e) germination of oak somatic embryos; (f) rooted shoots derived from axillary shoot cultures initiated from germinated somatic embryos.*

An anatomical study of the rooting process comparing BS and C shoots was carried out after treatment of the microcuttings with indole-3-butyric acid (IBA) (Ballester et al. 1999, 2009; Vidal et al. 2003). In both species (chestnut and oak) the response was similar: initially, mitotic periclinal divisions were observed in the phloem region of the basal part of both BS and C microcuttings 24 hours following the IBA treatment. After 4 days, the occurrence of histological events differed between BS shoots and C shoots. Certain cells of the cambial and phloem region of BS shoots became meristematic, giving rise to meristemoids and then root primordia, whereas no differentiation of root meristemoids occurred in cells of C shoots. The biochemical analyses carried out during the rooting process demonstrated that the endogenous auxin level at the time of excision from shoot cultures was very low and similar in both types of microcuttings; after 1-2 days of IBA treatment, the concentration of endogenous indole-3-acetic acid (IAA) increased rapidly, and reached a transient peak. The amount of IAA was significantly higher in C shoots than in BS shoots. After 3-4 days, the concentration of auxin decreased to lower levels but was always higher in C than in BS shoots, with values similar to those measured at excision in the case of chestnut. We concluded that, during the time course of the rooting process, more endogenous IAA was detected in mature (C) than in juvenile (BS) shoots, suggesting that the level of IAA is not the limiting factor accounting for the lack of rooting capacity in C shoots. It is well-accepted that a higher concentration of endogenous auxin in the base of the cutting results in higher rooting capacity but this correlation does not occur in chestnut and oak under our experimental conditions. Factors involved in this differential response may be related to the organization of root primordia which may consume more IAA than non-meristematic growth. This applies not only to IAA provided by basipetal transport but also to rooting inhibitors, auxin receptors, etc.

By using the same experimental model, two genes, *CsSCL1* and *QrCPE*, were isolated from chestnut and oak, respectively (Gil et al. 2003; Vielba et al. 2011). The different *CsSCL1* expression patterns in rooting-competent (BS) and –incompetent (C) microshoots, together with the specific location of transcripts (by in situ hybridization) in cell types involved in root meristemoids initiation and in root primordia development, indicate an important role for this gene in determining whether or not certain cells will enter the root differentiation pathway and its involvement in meristem formation. These genes could be considered as potential markers for maturation-related characteristics (Vielba et al. 2011).

Taking these results into consideration, we have also used this experimental system for induction of SE. Previously, we developed consistent procedures to initiate SE from leaf explants (Toribio et al. 2004; Valladares et al. 2006) which may be excised from forced shoots of basal shoots and branch segments collected directly from mature trees. However, given that BS and C material were already established in vitro, we also used shoot proliferating cultures as a source of leaf explants and shoot apex explants to induce SE (San-José et al. 2010). Obviously, the use of explants from in vitro cultures is advantageous, as sterilization is not required and the experiments can be repeated all year around. Following this approach, SE was induced in different *Quercus* species (Fig. 1d) and different embryogenic lines were established (Vieitez et al. 2012, Corredoira et al. 2012). Induction of SE followed an indirect pathway from a callus tissue formed in the initial explants. After 4-6 weeks of culture initiation, meristematic cells originated in superficial layers of callus protuberances, but these cells evolved into differentiated vacuolated cells. A subsequent dedifferentiation into embryogenic cells occurred after 9-12 weeks of culture and successive divisions of these cells led to the formation of proembryos and embryogenic cell aggregates. Somatic embryos were asynchronously differentiated through the typical developmental stages: globular, hearth-, torpedo- and cotyledon-shaped embryos. Nodular structures, also referred to as proembryogenic masses (PEMs), that generated somatic embryos of both uni- and multicellular origin were also evident (Corredoira et al. 2006b). PEMs and somatic embryos were mainly produced on the callus generated on the abaxial side of leaf explants, especially in the thickening midvein and the margins of the *Quercus alba* leaf blade (Corredoira et al. 2012). Histological studies confirmed an indirect SE process from shoot apex explants, in which leaf primordia and the corresponding axial zones were involved in generating callus, whereas the apical meristem itself did not proliferate (San-José et al. 2010). The somatic embryos originated in leaf and shoot apex explants were isolated to establish embryogenic lines that are maintained by repetitive embryogenesis. Once induced, the main problem reported for oak embryogenic systems is the low rate of conversion of somatic embryos into plants (Fig. 1e), which remains difficult in most genotypes, resulting in a limited production of plants (Vieitez et al. 2012).

Alternatively, SE could be used to test the rejuvenation hypothesis. The possible rejuvenation achieved by somatic embryos could be applied to vegetative propagation of mature trees by using somatic-embryo-derived plants and/or partially germinated embryos with only shoot development. These could then be multiplied by axillary

branching with subsequent rooting of shoots (Fig. 1f). This procedure opens the possibility to compare the morphogenetic capacity of oak genotypes established in vitro from selected mature trees and from the plants derived from somatic embryos induced from material of the same trees. The experimental model proposed is shown in Fig. 2 and was applied to 3 centenarian pedunculate oaks of different genotype. Axillary shoot proliferating cultures were

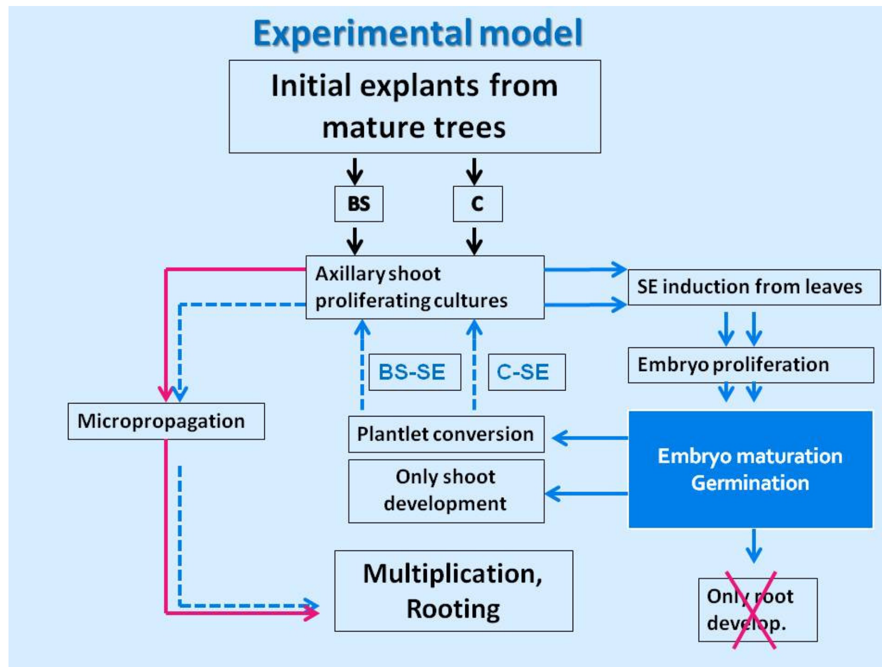

Figure 2. *Experimental model to compare the morphogenetic response of axillary shoot cultures initiated from mature oak trees (BS and C lines) and their corresponding axillary shoot lines derived from the shoots collected from germinated somatic embryos (BS-SE and C-SE lines) induced from the same plant material (see text for a complete explanation).*

established from basal shoots (BS) and crown branches (C) from mature oak trees and their multiplication rates and rooting capacity were determined. On the other hand, SE was induced from these BS and C proliferating cultures using leaves and shoot tips as initial explants. After embryo maturation and germination, shoot proliferating cultures were established from somatic embryo-derived plants and denominated as BS-SE and C-SE lines. After stabilization of these cultures (depending on the genotype, Martínez et al. 2012), the multiplication and rooting rates were recorded and compared with those obtained for their BS and C counterparts (Fig. 2). The characteristics of the plant material used in this study are shown in the Table 1. Basal shoots (BS) were only available for Sainza and this isogenic shoot culture may be considered as "control juvenile". In the case of Sainza-C-SE material, two shoot culture lines were established from two different germinated embryos (Sainza-C-SE-1 and Sainza-C-SE-2); in the case of B-17 genotype, only the shoot culture line from somatic embryo-derived plants was available, as we could not establish the shoot culture line from B-17 mature tree because a problem related to its identification in the field.

Results and Discussion

Data obtained on shoot proliferation and rooting ability of the 3 tested genotypes are shown in Table 2. Compared to Sainza-BS, Sainza-BS-SE line produced a similar number of shoots per explant and a similar percentage of rootable shoots (shoots longer than 14 mm). Similar values for all the parameters were obtained in both Sainza-C-SE lines. The frequency of rootable shoots was not significantly different from that of the Sainza-BS line although the mean number of rootable shoots per explant was significantly higher in Sainza-C-SE1 line compared with the Sainza-BS line (Martínez et al. 2012). This indicates that the variables related to shoot elongation were enhanced in cultures derived from somatic-embryo-derived plants of mature origin, and are similar to those of the physiologically juvenile line, Sainza-BS, suggesting that some rejuvenation had occurred. More interesting are

the differences in rooting capacity observed in the different Sainza lines. The rooting ability of the "juvenile control" Sainza-BS (71.5%) was significantly higher than that of the Sainza-C line (3.3%) confirming previous results when these materials were compared (Vidal et al. 2003). The rooting rate of the line derived from the somatic embryo (Sainza-BS-SE) increased up to 92.7%, which seems to indicate that even the "juvenile control" Sainza-BS achieved

Table 1. Plant material used in the study (*Quercus robur*)

Genotype	Age (years)	Origin	Line
Sainza	≈ 300	Basal shoots (BS) from mother tree	BS "juvenile control"
		Somatic embryo-derived plants (SE) from BS cultures	BS-SE
		Crown branches (C) from mother tree	C
		Somatic embryo-derived plants (SE) from C cultures	C-SE1
		Somatic embryo-derived plant (SE) from C cultures	C-SE2
CR-O	≈ 100	Crown branches (C) from mother tree	C
		Somatic embryo-derived plants (SE) from C cultures	C-SE
B-17	≈ 100	Somatic embryo-derived plants (SE) from C flushed shoots	C-SE

rejuvenation through the embryogenic process. The two lines Sainza-C-SE-1 and –C-SE-2 rooted similarly and better than the mature line Sainza-C, which may suggest the acquisition of at least a certain level of juvenility for the shoots derived from the somatic plants. However, the rooting data recorded for these two embryo-derived lines are far from those recorded for the "juvenile control" lines Sainza-BS and Sainza-BS-SE. Rooted plantlets of Sainza-BS-SE and Sainza-C-SE-1 were acclimatized and grown in the greenhouse. After 12 months, significant differences in plant growth between the two origins were recorded with a mean length of 40.4 cm for BS-SE plants and 23.0 cm for C-SE-1 plants (Martínez et al. 2012). This result, in addition to the longer internodes and the pale green colour of the leaves seem to indicate differences in juvenility between the two lines, showing that BS-SE plants have the most juvenile characteristics.

Table 2. Morphogenetic response of micropropagated cultures of pedunculate oak through somatic embryogenesis (Modified from Martínez et al., 2011)

Genotype	Line	Number of shoots/explant	% shoots >14 mm	Rooting %	Root number/ rooted shoot
Sainza	BS	5.5 ± 0.5 c	42.0 ± 2.5 a	71.5 ± 1.8 b	3.0 ± 0.3 ab
	BS-SE	5.9 ± 0.3 bc	42.5 ± 2.4 a	92.7 ± 5.0 a	4.3 ± 0.5 a
	C	7.4 ± 0.5 ab	26.0 ± 1.2 b	3.3 ± 1.9 d	1.0 ± 0.0 c
	C-SE-1	8.7 ± 0.5 a	37.6 ± 2.9a	23.2 ± 1.9 c	1.4 ± 0.4 bc
	C-SE-2	8.3 ± 0.3 a	36.8 ± 3.3 a	24.3 ± 2.9 c	1.3 ± 0.1 bc
CR-0	C	7.5 ± 0.5 a	31.6 ± 2.3 b	45.6 ± 9.5 b	1.7± 0.1 a
	C-SE	7.5 ± 0.2 a	40.5 ± 1.6 a	89.8 ± 3.5 a	2.5 ± 0.3 a
B-17	C-SE	5.2 ± 0.3	49.4 ± 2.1	90.0 ± 2.4	2.1± 0.2

In the CR-0 genotype, a similar number of shoots was obtained in the CR-0-C-SE line and the mature line CR-0-C, although the percentage of rootable shoots is higher in the former. Also the rooting rate of CR-0-SE line (89.8%) was significantly higher than that of CR-0-C line (45.6%). This reinforces the hypothesis of rejuvenation as the rooting achieved in the embryogenic-derived line is close to the values obtained in the Sainza-BS-SE line and higher than the “juvenile control” of the same genotype. Furthermore, the high rooting percentage obtained in B-17-C-SE (90.0%) suggests an improvement in rooting capacity due to SE involvement in the establishment of this line, as the value recorded is close to those recorded for the lines Sainza-BS-SE and CR-0-SE. However, we could not demonstrate that this parameter was associated with the acquisition of juvenility, as the shoot culture line from B-17 mature tree was not established in vitro.

The results reported indicate that the proposed experimental model used in this research is valid to test the rejuvenation hypothesis which may occur through the somatic embryogenic process. The results are very consistent as they are the result of several years of work including numerous repetitions of the experiments. The significant improvement in rooting rates obtained in stock shoot cultures of somatic plantlet origin in comparison with those derived from mature trees is solid and could be used in other hardwood species. However, according to the results obtained in our work, a complete rejuvenation has not been demonstrated, as the rooting rates of Sainza-C-SE-1 and Sainza-C-SE-2 lines are far from those recorded for the “control juvenile” BS and BS-SE lines. The results obtained within Sainza lines seem to indicate that there may be a gradient of physiological juvenility, with Sainza-BS-SE displaying the maximum degree of juvenility and Sainza-C the lowest. This result would also be valid for other genotypes.

Regardless the level of juvenility achieved through the embryogenic process, the proposed experimental model would be useful to improve the micropropagation capacity of recalcitrant genotypes. Our results seems to be on line with those of Carron et al. (1995) working with *Hevea brasiliensis*, as partial rejuvenation was also obtained through SE in their experiments. These authors reported rooting frequencies of 43-55% in shoots from two genotypes regenerated from somatic-embryo derived plants, whereas it was not possible to initiate rooting from mature material.

As mentioned above, a valuation of the level of rejuvenation achieved after any treatment should not only be based on in vitro micropropagation, but also on biochemical and genetic studies. In this respect, a full-length cDNA clone (*QrCPE*) was differentially expressed in the same Sainza-BS and Sainza-C shoot lines used in the present work (Gil et al. 2003). In addition, changes in DNA methylation were associated with mature/juvenile changes in different species (Monteuuis et al. 2008, Baurens et al. 2004, Klimaszewska et al. 2009). The possibility to modify the juvenile phase in trees by overexpression of specific genes (Wang et al. 2011) open new possibilities for a better understanding of the phase change in trees. The experimental model developed in this work would be an interesting tool to be applied in this type of studies.

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References

Ballester A, San-José MC, Vidal N, Fernández-Lorenzo JL, Vieitez AM (1999) Anatomical and biochemical events during in vitro rooting of microcuttings from juvenile and mature phases of chestnut. Ann Bot 83:619-629

Ballester A, Vidal N, Vieitez AM (2009) Developmental stages during in vitro rooting of hardwood trees from material with juvenile and mature characteristics. In: Niemi K, Scagel C (eds) Adventitious root formation of forest trees and horticultural plants—from genes to applications. Research Signpost, Kerala, 277–296

Baurens F-C, Nicolleau J, Legavre T, Verdeil J-L, Monteuuis O (2004) Genomic DNA methylation of juvenile and mature *Acacia mangium* micropropagated in vitro with reference to leaf morphology as a phase change marker. Tree Physiol 24:401–407

Bon MC (1988) J16: an apex protein associated with juvenility of *Sequoiadendron giganteum*. Tree Physiol 4:381-387

Bon MC, Monteuuis O (1991) Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through in vitro meristem culture. II. Biochemical arguments. Physiol Plant 81:116-120

Bonga JM (1985) Vegetative propagation in relation to juvenility, maturity and rejuvenation. Martinus Nihoff, The Hague, 387-412

Bonga JM, Klimaszewska KK, von Aderkas P (2010) Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tissue Organ Cult 100:241–254

Carron MP, Etienne H, Lardet L, Campagna S, Perrin Y, Leconte A, Chaine C (1995) Somatic embryogenesis in rubber (*Hevea brasiliensis* Müll. Arg.). In: Jain S, Gupta P, Newton R (eds) Somatic Embryogenesis in Woody Plants, vol 2. Kluwer, Dordrecht, 117–136

Corredoira E, Ballester A, Vieitez J, Vieitez AM (2006a) Somatic embryogenesis in chestnut. In: Mujib A, Samaj J (eds) Plant Cell Monographs, Vol. 2, Somatic Embryogenesis., Springer, Berlin, Heidelberg, 177-199

Corredoira E, Valladares S, Vieitez AM (2006b) Morphohistological analysis of the origin and development of somatic embryos from leaves of mature *Quercus robur*. In Vitro Cell Dev Biol Plant 42:525-533

Corredoira E, San-José MC, Vieitez AM (2012) Induction of somatic embryogenesis from different explants of shoot cultures derived from young *Quercus alba* trees. Trees 26:881-891

Ewald D, Kretzschmar U (1996) The influence of micrografting in vitro on tissue culture behaviour and vegetative propagation of old European larch trees. Plant Cell Tissue Organ Cult 44:249-252

Fernández-Lorenzo JL, Rigueiro A, Ballester A (1999) Polyphenols as potential markers to differentiate juvenile and mature chestnut shoot cultures. Tree Physiol 19:461-466

Gil B, Pastoriza E, Ballester A, Sánchez C (2003) Isolation and characterization of a cDNA from *Quercus robur* differentially expressed in juvenile-like and mature shoots. Tree Physiol 23:633–640

Klimaszewska K, Noceda C, Pelletier G, Label P, Rodríguez R, Lelu-Walter MA (2009) Biological characterization of young and aged embryogenic cultures of *Pinus pinaster*. In Vitro Cell Dev Biol Plant 45:20–33

Klimaszewska K, Overton C, Stewart D, Rutledge RG (2011) Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old somatic white spruce and expression profiles of 11 genes followed during the tissue culture process. Planta 233:635–647

Lardet L, Dessailly F, Carron MP, Montoro P, Monteuuis O (2009) Influences of aging and cloning methods on the capacity for somatic embryogenesis of a mature *Hevea brasiliensis* genotype. Tree Physiol 29:291-298

Mankessi F, Saya AR, Boudon F, Guedon Y, Montes F, Lartaud M, Verdeil JL, Monteuuis O (2010) Phase change-related variations of dome shape in *Eucalyptus urophylla x Eucalyptus grandis* shoot apical meristems. Trees 24:743-752

Mankessi F, Saya AR, Montes F, Lartaud M, Verdeil JL, Monteuuis O (2011) Hystocitological characteristics of *Eucalyptus urophylla x Eucalyptus grandis* shoot apical meristems of different physiological ages. Trees 25:415-424

Martínez T, Vidal N, Ballester A, Vieitez AM (2012) Improved organogenic capacity of shoot cultures from mature pedunculate oak trees through somatic embryogenesis as rejuvenation technique. Trees 26:321-330

Monteuuis O (1991) Rejuvenation of a 100-year-old *Sequoiadendron giganteum* through in vitro meristem culture. I. Organogenic and morphological arguments. Physiol Plant 81:111-115

Monteuuis O, Bon MC (1989) Rejuvenation of a 100 yr old giant sequoia (*Sequoiadendron giganteum* Buchholz) through in vitro meristem culture. Ann Sci For 46(suppl):183s–186s

Monteuuis O, Doulbeau S, Verdeil JL (2008) DNA methylation in different origin clonal offspring from mature *Sequoiadendron giganteum* genotype. Trees 22:779–784

Monteuuis O, Lardet L, Montoro P, Berthouly M, Verdeil J-L (2010) Somatic embryogenesis and phase change in trees. In: Park YS, Bonga JM, Park SY, Moon HK (eds). Proceedings of the IUFRO Working Party 2.09.02: “Somatic Embryogenesis of Trees” conference on “Advances in Somatic Embryogenesis of Trees and Its Application for the Future Forests and Plantations”, August 19-21, 2010, Suwon, Republic of Korea, 21-29

Noceda C, Salaj T, Perez M, Viejo M, Canal MJ, Salaj J, Rodriguez R (2009) DNA demethylation and decrease on free polyamines is associated with the embryogenic capacity of *Pinus nigra* Arn. cell cultures. Trees 23:1285–1293

Park YS, Bonga JM (2010) Application of somatic embryogenesis in forest management and research. In: Park YS, Bonga JM, Park SY, Moon HK (eds). Proceedings of the IUFRO Working Party 2.09.02: “Somatic Embryogenesis of Trees” conference on “Advances in Somatic Embryogenesis of Trees and Its Application for the Future Forests and Plantations”, August 19-21, 2010, Suwon, Republic of Korea, 3-8

Pierik RLM (1990) Rejuvenation and micropropagation. In: Nikkamp HJJ, Van der Plas LHW, Van Aartrijk J (eds.). Progress in plant cellular and molecular biology. Procc. of the VIIth International Congress on Plant Tissue and Cell Culture. Kluwer Academic, Amsterdam, The Netherlands, 91-101

San-José MC, Corredoira E, Martínez MT, Vidal N, Valladares S, Mallón R, Vieitez AM (2010) Shoot apex explants for induction of somatic embryogenesis in mature *Quercus robur* L. trees. Plant Cell Rep 29:661-671

Toribio M, Fernández C, Celestino C, Martínez MT, San-José MC, Vieitez AM (2004) Somatic embryogenesis in mature *Quercus robur* trees. Plant Cell Tissue Organ Cult 76:283-287

Traore A, Maximova SN, Guiltinan MJ (2003) Micropropagation of *Theobroma cacao* L. using somatic embryo-derived plants. In Vitro Cell Dev Biol Plant 39:332–337

Valladares S, Sánchez C, Martínez MT, Ballester A, Vieitez AM (2006) Plant regeneration through somatic embryogenesis from tissues of mature oak trees: true-to-type conformity of plantlets by RAPD analysis. Plant Cell Rep 25:879-886

Vidal N, Arellano G, San-José MC, Vieitez AM, Ballester A (2003) Developmental stages during the rooting of in vitro cultured *Quercus robur* shoots from material of juvenile and mature origin. Tree Physiol 23:1247–1254

Vieitez AM, Sánchez MC, Amo-Marco JB, Ballester A (1994) Forced flushing of branch segments as a method for obtaining reactive explants of mature *Quercus robur* trees for micropropagation. Plant Cell Tissue Organ Cult 37:287–295

Vieitez AM, Corredoira E, Martínez T, San-José MC, Sánchez C, Valladares S, Vidal N, Ballester A (2012) Application of biotechnological tools to *Quercus* improvement. Eur J Forest Res 131:519-539

Vielba JM, Díaz-Sala C, Ferro E, Rico S, Lamprecht M, Abarca D, Ballester A, Sánchez C (2011) *CsSCL1* is differentially regulated upon maturation in chestnut microshoots and is specifically expressed in rooting-competent cells. Tree Physiol 31:1152-1160

Wang JW, Park MY, Wang LJ, Koo YJ, Chen XY, Weigel D, Poethig RS (2011) MiRNA control of vegetative phase changes in trees. PLOS Gen 7:e1002012

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Initiation of embryogenic suspensor masses (ESM) and somatic embryogenesis in Japanese red pine (*Pinus densiflora*)

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Key words: germination, glutamine, light-emitting diodes, seed development, somatic embryos,

Abstract: The best 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%), and 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 collection time for seeds can be based on the collection dates (June 28, July 1st and July 5), at least for *Pinus densiflora*, in Korea. The highest proliferation rate (9.8-fold) of ESM was obtained from ½LM medium supplemented with 3.42 mM L-glutamine. 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 from ESM line 05-3 with 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 with five LED sources.

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. In order to propagate the species, more efficient propagation methods are needed. Among them, somatic embryogenesis (SE) is a most promising technique, because this system offers the capability to produce unlimited numbers of propagules.

Initiation of ESM is the most critical step for the application of SE in conifer tree forestry including this species. Unfortunately, most *Pinus* species have shown a very low initiation frequency of ESM, 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 initiate ESM formation, the most important factor - the effect of collection time or developmental stage of the zygotic embryos – has not been studied fully.

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 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).

Activated 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 light of quality 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. Effect of L-glutamine concentration on ESM proliferation, and activated charcoal treatment on somatic embryo production and finally effect of LED on somatic embryos germination in this species.

Materials and Methods

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 (Table 1).

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 analyzed 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, 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).

ESM initiation: For sterilization of seeds, the seeds extracted from cones were disinfected by 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 the micropylar end of the seed. Frequency of ESM initiation was recorded after 8 weeks of culture.

Effect of L-glutamine 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 filter paper (90 mg per each) and 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 total 4 weeks of culture without subculture during incubation, the fresh weights of ESM derived from each treatment were recorded. Growth rate was calculated on a fresh weight (FW) basis according to: Growth rate (folds) = [(FW at the end of treatment - FW at the start of treatment) / FW at the start of treatment]

Effect of AC on somatic embryo maturation: The goal of this somatic embryo maturation experiment was to asses the effects of the presence or absence of activated charcoal (AC, at 0.05%) in the maturation medium. For maturation of somatic embryo, 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. 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 dispersed tissue were poured over a filter paper (Whatman #2, 5.5 cm) and placed in a Büchner funnel. After draining the medium with 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 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.

Effect of LED on germination from somatic embryo: To examine the effects of LED light sources on the germination, somatic embryos were cultured in a LED system (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) and four kinds of LED treatments which consisted of four types- 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). Fluorescent lamps were used as the control. For each test, there were three replications, each consisting of 30 somatic embryos derived from 4 ESM lines for each treatment.

Plantlet regeneration and acclimatization: Cotyledonary somatic embryos were selected from embryogenic masses cultured on ABA-containing medium for 12 weeks and placed horizontally on the surface of ½LM medium containing 60 mM sucrose and 0.4% gellan gum. The cultures were kept for 7 d 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, the 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 further for 4-5 weeks in the tissue culture room (50 µEm-2s-1, 16 h photoperiod, 25±1□). Thereafter, plants were transferred to the greenhouse.

Results and Discussion

Relationship between the developmental stage of embryo and collection dates for ESM initiation: Developmental stages and collection dates of seeds were checked to determine the optimal stage in ESM initiation. The initiation of ESM was critically influenced by the developmental stage of the embryos at the time of culture. As shown in Table 1, in the 2004 study, the ESM initiation frequency obtained with collection dates June 21, June 28 and July 5 were 0.57%, 0.88% and 0.33%, respectively (Table 1). In observance of embryo developmental stages, all explants collected on June 13, 21 and 28 were at the proembryo stage (100%) (Table 1). After that, the proembryo frequency declined to 70% (July 5) and to 0% on subsequent collections on July 13 and July 20. The latter dates corresponded to globular (33.3%), precotyledonary (66.7%, 49.1%) and cotyledonary (50.9%) stages. No proembryos were observed in explants collected at July 13 and July 20 and, therefore, no ESM was initiated from these two collection dates (Table 1). As the seeds 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 excised explants from July 13 collection were at the cotyledonary stage and the rest were globular (33.3 %) (Table 1). No embryogenic lines could be produced from the seeds collected on July 13 or 20. Based on the results shown in Table 1, seed collection should be made before appearance of globular stage embryos in this species. In the experiments of 2005, three collection dates and one more collection location – Anmyeon - were because of the results of previous year experiment (Table 1). From these experiments the highest frequency was recorded with 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 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 explants apparently.

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 $P = 0.05$.*

Comparison of microsectioned profiles from collected seeds in 2005 and 2006: In the Suwon 2006 seeds some proembryos were found in the corrosion cavity (Fig. 2a). Regardless of collection time (June 28, July 1st and July 5), the corrosion cavity in the seeds was expanded longitudinally to the chalaza end and showed an opulent amount of suspensors throughout the corrosion cavity of seeds collected from Suwon (Fig. 2a) or Anmyeon (Fig. 2b). No large differences were found in the seeds between 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 (Fig. 2a, b). In contrast, when compared with those of seeds in 2006, the length of the corrosion cavity was a half of that in 2005 (Fig. 2c, June 28 or July 1st, two in left or right side, respectively) or much less developed, and some embryos with suspensors were restricted in the round shaped corrosion cavity (Fig. 2c, July 1st). However, even though zygotic embryogenesis in the seeds of 2006 lagged behind that of 2005 (Fig. 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). 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 microsectioning of the seeds from different collection 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 mother trees.

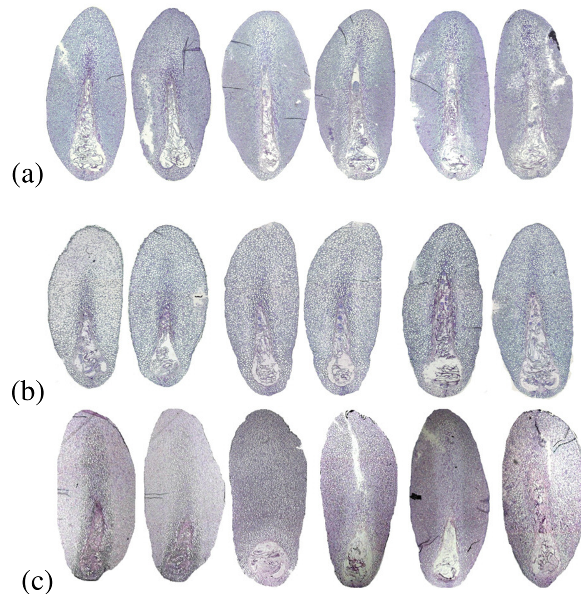

Fig. 2. *Comparison of microsection profiles of immature embryo in relation with seed collection dates and collection locations in P. densiflora. (a) Two on the left (June 28 in 2005, Suwon), two in the middle (July 1st in 2005, Suwon), two on the right (July 5 in 2005, Suwon) (b) Two on the left (June 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)*

Effect of L-glutamine on ESM proliferation: The effect of L-glutamine concentrations on ESM proliferation is shown in Fig. 3. The highest proliferation rate of ESM was obtained with the combination of 3.42 mM L-glutamine (9.8 fold, line 05-6) (Fig. 3). However, a lower proliferation rate was obtained on the medium with 3.42, 6.84 and 13.48 mM L-glutamine in the line of 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 the line 05-6 and 05-8 (Fig. 3). In the line of 05-8, no significant ESM weight increase or decrease was found with the 5 different concentrations. Therefore, the fresh weight increment was greatly affected by the nitrogen source and by the genotype of ESM (Fig. 3).

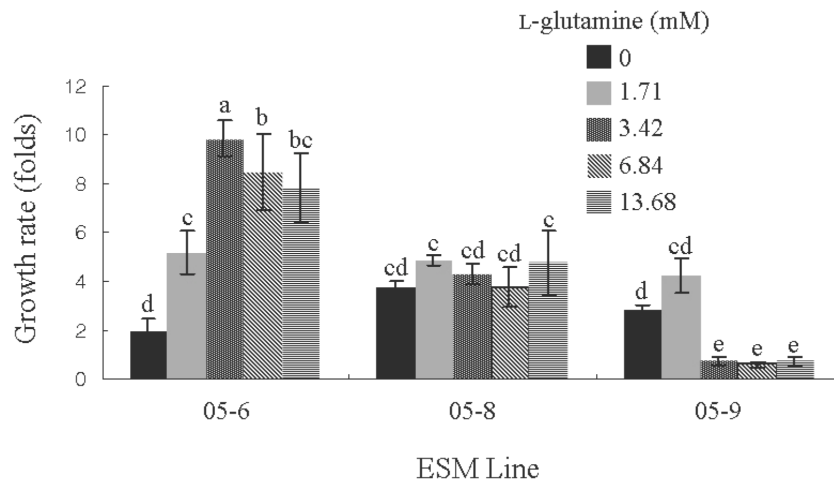

Fig. 3. *Effect of various L-glutamine concentrations on weight of the ESM from 3 genotypes in P. densiflora. Error bars mean standard error of average. Different letters within columns indicate significant differences at P =0.05.*

Effect of AC on somatic embryo maturation: Since 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 AC in the medium on producing somatic embryos was studied because the addition of AC in the medium is well known for its adsorption of residual plant growth regulators. In our experiment, somatic embryo yields ranged from 0 (05-4, 05-25, 05-31 and 05-58 with/without AC) to 798 (05-29 with AC) from the plated cells of ESM (Fig.4). In general, maturation medium with AC (05-9, 05-21, 05-37 and 05-50) did not produce as many somatic embryos media as the media without AC (Fig. 4). Furthermore, we concluded that the somatic embryo production was greatly dependent on the genotype of ESM in *P. densiflora* (Fig. 4).

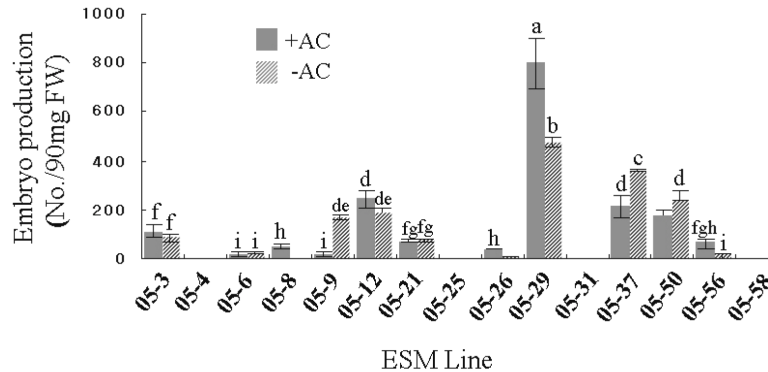

Fig.4. *Effect of AC supplementation on somatic embryo maturation with ESM of 15 genotypes in P. densiflora. Error bars mean standard error of average.Different letters within columns indicate significant differences at P =0.05.*

Effect of LED on germination from somatic embryo: The various light sources strongly influenced germination frequency of somatic embryos (Fig. 5). The highest frequency of germination was obtained with red light (80.9%, line 05-12), other high frequencies were also found with this treatment (57.1% for 05-3 or 05-29 and 67.5 % for 05-37). Therefore, germination of somatic embryos was positively affected by application of red light. In contrast, some lower frequencies were marked from the fluorescent light (0, 12.9, 21.5 and 23.4% for 05-3, 05-12, 05-29 and 05-37) regardless of the ESM lines tested. In addition, in the case of red+blue, no germinants were obtained from the line of 05-3 but a high frequency (72.2%) was obtained from line 05-37 (Fig. 5). All but line 05-3 responded similarly in the different light sources (Fig. 5). Therefore, there were some 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 (Fig. 5).

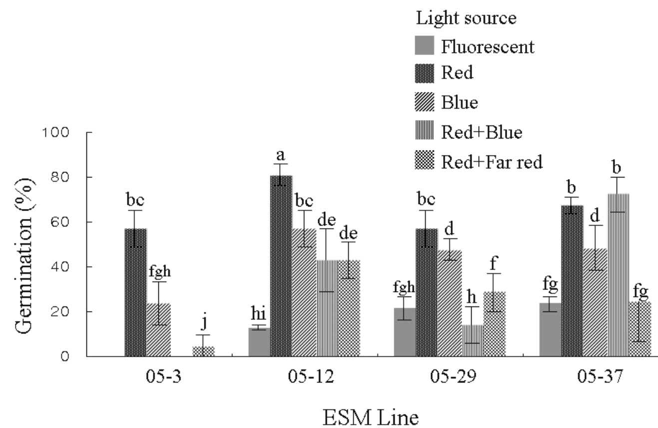

***Fig. 5**. Effect of light quality on somatic embryo germination with ESM of 4 genotypes in P. densiflora. Error bars mean standard error of average. Different letters within columns indicate significant differences at P =0.05.*

Germination of somatic embryos and plant regeneration: Two weeks after transfer to the germination medium (½LM containing 58.4 mM sucrose, solidified with 0.4% gellan gum) without ABA, mature somatic embryos, produced from the ESM, started to form an epicotyl and shoots. 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. Under light conditions, some newly induced apical shoots formed from the terminal bud. The somatic plants developed, were transplanted into a soil mixture. The potted plants grew well and could be transferred to larger pots to foster their growth under greenhouse conditions.

References

Hristoforoglu, K.; Schmidt, J.; Bolhar-Nordenkampf, H. (1995) Development and germination of *Abies alba* somatic embryos. Plant Cell Tiss. Org. Cult. 40:277-284

Khlifi, S.; Tremblay, F.M. (1995) Maturation of black spruce somatic embryos. I. Effect of L-glutamine on the number and germinability of somatic embryos. Plant Cell Tiss. Org. Cult. 41:23-32

Latkowska, M.J.; Kvaalen, H.; Appelgren, M. (2000) Genotype dependent blue and red light inhibition of the proliferation of the embryogenic tissue of Norway spruce. In Vitro Cell. Dev. Biol. -Plant 36:57-60

Rossi, F.; Baraldt, R.; Facini, O. Photomorphogenic effects on in vitro rooting of *Prunus* rootstock GF 655- 2. Plant Cell Tiss. Org. Cult. 32:145-151; 1993.

Saeba, A.; Skjeseth, G.; Appelgren, M. (1995) Light quality of the in vitro stage affects the subsequent rooting and field performance of *Betula pendula* (Roth). Scand. J. For. Res. 10:155-160

Teasdale, R.D.; Dawson, P.A.; Woolhouse, H.W. (1986) Mineral nutrient requirements of a loblolly pine (*Pinus taeda*) cell suspension culture. Plant Physiol. 82:942-945

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A combined method to increase somatic embryogenesis efficiency in valuable cell lines of *Pinus* spp.

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Keywords: Auxins, Cytokinins, somatic embryos, organogenesis.

Abbreviations: EFS - embryos forming shoots; ET - embryogenic tissue; G – germinated; NG - -non germinating; SE - somatic embryogenesis; SEC - shoot elongation capacity

Abstract: This work describes a combined plant regeneration system that includes somatic embryogenesis and organogenesis from immature seeds of radiata pine as well as a cold storage step to conserve the embryos. We have developed a preservation method for somatic embryos at non-freezing temperatures (5ºC), which maintained the viability of the explants for more than a year, and a multiplication system of these somatic embryos through organogenesis. By means of cold preservation and *in vitro* organogenesis somatic plantlet production is not restricted to a certain moment of the year and the material obtained can be multiplied and rooted when demanded with no detrimental effect on the plants produced.

Introduction

The level of domestication in forest trees is significantly lower than that of agricultural plants. However, there is a great potential for improvement through genetic breeding. Although conventional seed orchards provide genetically improved seeds, traditional breeding strategies combined with *in vitro* vegetative propagation have shown advantages such as additional genetic gain achieved by capturing non-additive genetic variation. Furthermore, the speed with which clones may be introduced to meet market goals and the ability to program diversity into a clonal plantation are advantages (Park et al. 1998). The Neiker-Tecnalia research group has successfully achieved adult clonal propagation in pine species (Cortizo et al. 2009; De Diego et al. 2008, 2010) but changes in the attributes of the resulting plants have sometimes been observed and the reinvigoration of the material has been transitory in *in vitro* conditions. On the other hand, vegetative propagation from physiologically juvenile tissue has been successful in a number of conifer species (Bergmann and Stomp 1992; Moncaleán et al. 2005)

The two main *in vitro* techniques used routinely for plant micropropagation are somatic embryogenesis (SE) and organogenesis (Giri et al. 2004). To assist *in vitro* research, somatic embryogenesis and organogenesis have been used as model systems to study the structural, physiological, and molecular bases of development. Studies of organogenesis and SE have shown the fundamental role of plant growth regulators in *in vitro* culture (Jiménez 2005); this basic understanding has greatly contributed to the extension of tissue culture for commercial applications (Stasolla and Thorpe 2010). In this sense, propagation via SE is an effective method in propagating elite plants when it is combined with other technologies, such as cryopreserving the embryogenic tissue (ET) and selecting elite clones in field tests (Park 2002). Due to the importance of these technologies, SE in pines has been widely reported in the past few years (Klimaszewska et al. 2007). In the majority of these reports, the induction of ET is carried out from immature seeds. The problem is that the competence window for this type of explant is narrow, lasting around 4 weeks (MacKay et al. 2006; Yildirim et al. 2006). ET initiation has also been obtained from mature embryos

(Tang et al. 2001). Even though mature seed explants overcome the problem of the short competence window of immature seeds, the low success rate makes this approach unfeasible for large-scale production (Klimaszewska et al. 2007). Furthermore, some genotypes produce only a low number of viable embryos among many of poor quality. This results in a low germination frequency and makes large-scale production of the genotypes too expensive, which necessitates the elimination of these genotypes from production. Moreover, there are some additional problems related with these techniques such as the recalcitrance of some embryogenic lines to be cryopreserved and sometimes the low regeneration capacity of the cryopreserved tissue after clonal tests.

Having in mind all the problems associated to somatic embryogenesis and cryopreservation as well as the high cost and sometimes low efficiency of the latter technique, we present a combined SE and organogenesis system including a cold storage step when required (Fig. 1). This approach could overcome the aforementioned seasonality of ET initiation, the low embryo production and the limited success in the maturation and germination steps in *Pinus radiata* D. Don.

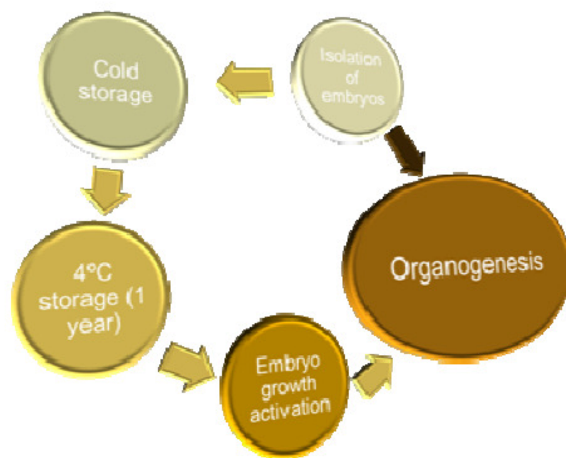

Figure 1*. Different options to follow after the isolation of Pinus radiata somatic embryos in order to obtain an efficient system of in vitro propagate plants by organogenesis, including or not a cold storage step.*

Material and Methods

Plant material:

Pinus radiata ET was initiated following the methods described by Montalbán et al. (2012). Subsequently, somatic embryos were obtained following the protocol described by Montalbán et al. (2010). After 12 weeks on maturation medium, mature somatic embryos were selected and isolated from ET. At that point, two pathways can be followed:

Reactivate the embryogenic process or initiate organogenesis from growing embryos that were isolated from the ET (Fig. 2 A). For the embryogenic process, the somatic embryos collected were white to yellowish, non-germinating, with a distinct hypocotyl region, and at least three cotyledons. Two types of explants were used for induction experiments: freshly collected (NG) somatic embryos (Fig. 2 A), and somatic embryos germinated for 1 week (G) (Fig. 2 B). Germination medium was half-strength macronutrients (except for the iron) and complete micronutrients and vitamins from LP medium (Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988) (1/2 LP), supplemented with 3% sucrose, 0.2% activated charcoal (w/v) and 10 g L^{-1} Difco Agar granulated (LPE). Cultures were kept under dim light (40 µmol m^{-2} s^{-1}) at 21 ± 1ºC.

Cold storage:

As Figure 1 shows, once somatic embryos were obtained the cold storage procedure can be initiated. The embryos (Fig. 2 A) were poured onto a filter paper disc (Whatman no.2) in a Petri dish containing 1/2 LP medium without plant growth regulators. The culture medium was supplemented with 3% sucrose. Then, the Petri dishes were stored at 5ºC in the dark. After one year, isolated embryos can be used in two different ways: growth reactivation and germination or shoot induction to follow the organogenic process.

Figure 2. *Pinus radiata Non Germinated (NG) embryos (A) (bar 3 mm) and germinated embryo (G) of Pinus radiata after 1 week in LP medium without plant growth regulators and with activated charcoal (bar 4 mm).*

Growth reactivation:

Embryos were cultured on LPE. Cultures were kept under dim light (40 µmol m^{-2} s^{-1}) at 21 ± 1ºC. After 2 months, the percentage of germinated embryos was evaluated and then the explants were transferred to a wet sterile peat:perlite mixture (3:1) and acclimatized in the greenhouse under controlled conditions, at 21 ± 2ºC and progressively decreasing humidity from 95 to 80%. After 60 days under *ex vitro* conditions, the survival percentage was estimated.

Organogenic process:

Shoot induction was carried out following the method of Montalbán et al. (2011). The basal medium was 1/2 LP supplemented with 3% sucrose, different BA concentrations (1, 4.4 or 22 µM) and 8 g L^{-1} Difco Agar. When NG embryos were used as initial explants, different induction periods and BA concentrations were assayed: 1 µM BA for 2 weeks (2BA1), 1 or 4.4 µM BA for 3 weeks (3BA1 and 3BA4.4) and 1, 4.4 or 22 µM BA for 4 weeks (4BA1, 4BA4.4 and 4BA22). When G embryos were used as initial explants, the induction treatments assayed were 1 or 4.4 µM BA for 3 weeks. Embryos were placed in 90x15 mm Petri dishes containing 15 mL of induction medium. Six to eight embryos per Petri dish were cultured in an inverted position with the cotyledons immersed in the induction medium (Aitken-Christie et al. 1988). After induction treatments, the explants were transferred to glass jars with the previously described LP medium but lacking BA for 4 weeks. Then, they were subcultured monthly on LPE. Cultures were kept at 21 ± 1ºC under a 16-h photoperiod of 120 µmol m^{-2} s^{-1}.
After 3 months on LPE, the percentage of necrosed, hyperhydric embryos and embryos forming shoots (EFS) was recorded. After 6 months on LPE, the number of shoots > 3 cm per embryo was registered and the shoot elongation capacity (SEC) index was calculated (Lambardi et al. 1993).

Root induction:

Elongated shoots (15 mm) were transferred to glass jars with rooting medium. Based on previous experiments, the basal medium chosen for rooting was modified LP medium with quarter-strength macronutrients except for the iron at half-strength, micronutrients and vitamins, supplemented with 3% sucrose and 8 g L^{-1} Difco Agar granulated (1/4LP). This basal medium was supplemented with three different auxin treatments: 1.5 mg L^{-1} 1-naphthalene acetic acid (NAA), 1.5 mg L^{-1} indole-3-butyric acid (IBA) and 1 mg L^{-1} IBA with 0.5 mg L^{-1} NAA (MIX). The effect of the photon flux [120 µmol m^{-2} s^{-1} (L) and darkness (D)] during the first week of the explants in the rooting media was also tested; the root induction experiments comprised three auxin treatments and two light regimes, a total of six combinations. Thus, NAA-D is 1/4LP supplemented with 1.5 mg L^{-1} NAA and kept in darkness the first week. Four shoots were cultured in each culture vessel and five glass jars per treatment were used. After 3 weeks in root induction medium, the shoots were transferred to LPE medium. Embryos were subcultured in LPE medium and kept under the same conditions described above. After a month on LPE medium, the rooting percentage was scored and explants with and without visible roots were transferred to a wet sterile peat:perlite mixture (3:1) and acclimatized in the greenhouse under controlled conditions at 21±2ºC and progressively decreasing humidity. After two months under ex vitro conditions, the acclimatization percentage was recorded.

Results

After the cold storage period and in order to analyse the viability of the embryos, we analysed the survival percentage. Figure 3 shows the percentage of plantlets growing after two months in germination medium. Ninety five percent of the embryos survived and reactivated their growth (Fig. 3). Then, plantlets with a well-formed root were transferred to the greenhouse. After 1 month under controlled conditions, eighty five percent of the plants were healthy (Fig. 3).

Increasing the BA concentration or the induction period decreased the number of necrosed embryos. The lowest percentage for this parameter was obtained in G embryos cultured in induction media with 1 or 4.4 μM BA for 3 weeks (11.1 and 27.8%, respectively) (Data not shown) (Fig. 4 A). The highest percentage of embryos that did not produce shoots and died shortly after the induction period was obtained in NG embryos cultured in induction media with 1 μM BA for 2 weeks (70.8%) (Data not shown). All surviving explants showed an organogenic response and produced shoots.

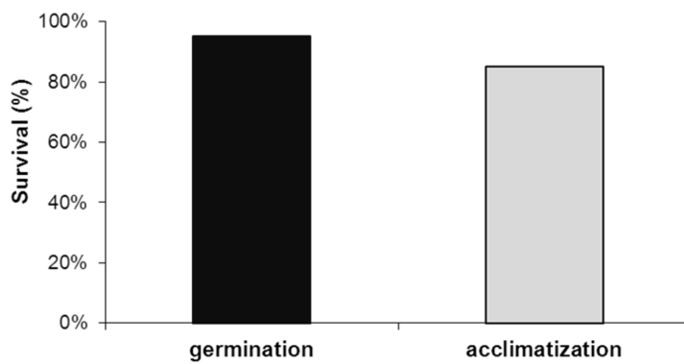

Figure 3*. Germination (%) and acclimatization (%) of Pinus radiata somatic embryos after cold storage (4º C) and the subsequent growth reactivation in LP medium with activated charcoal.*

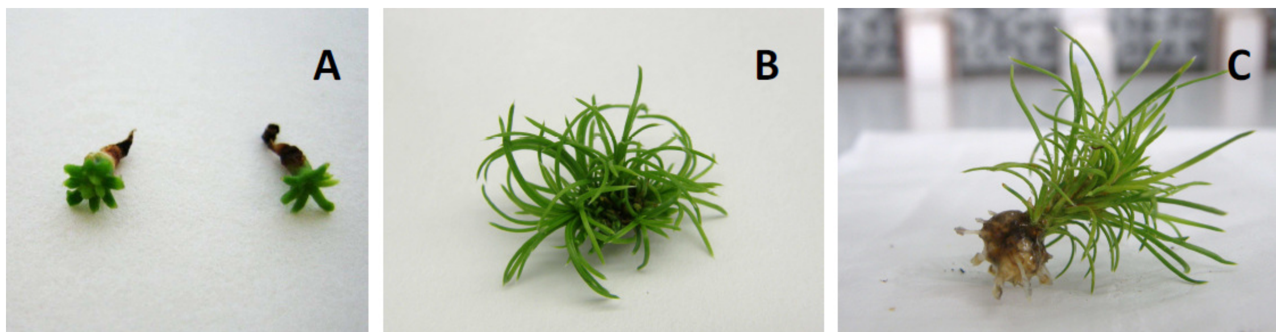

Figure 4*. (A) Pinus radiata somatic embryos after 3 weeks of culture in 4.4 µM BA (bar 8 mm). (B) Pinus radiata explant from somatic embryo cultured without cytokinins during 8 weeks after its culture in in BA 4.4 µM BA during the induction period (3 weeks) (bar 11 mm). (C) Pinus radiata explant cultured for 3 weeks on modified ¼ LP medium supplemented with 1 mg L-1 IBA and 0.5 mg L-1 NAA (MIX) (bar 14 mm).*

Neither the induction time nor the concentration of BA showed a clear caulogenic response (Fig. 5). The lowest number of shoots in NG embryos was obtained when 1 μM BA was applied for 2 and 3 weeks to the induction medium (Fig. 5). Longer induction periods (3 or 4 weeks) or higher BA concentrations (4.4 or 22 μM) produced a significantly higher number of shoots per embryo (Fig. 4 B; Fig. 5). When NG and G embryos were compared, the number of shoots per embryo in NG explants after treatment 3BA4.4 (19.3 shoots per embryo) was significantly better than the number of shoots obtained in NG embryos after treatment 3BA1 and in G embryos after treatments 3BA1 and 3BA4.4 (Fig. 5). NG embryos cultured with 3BA4.4, 4BA4.4 and 4BA22 achieved the best SEC indexes (9.1, 10.2 and 7.4, respectively) (Data not shown).

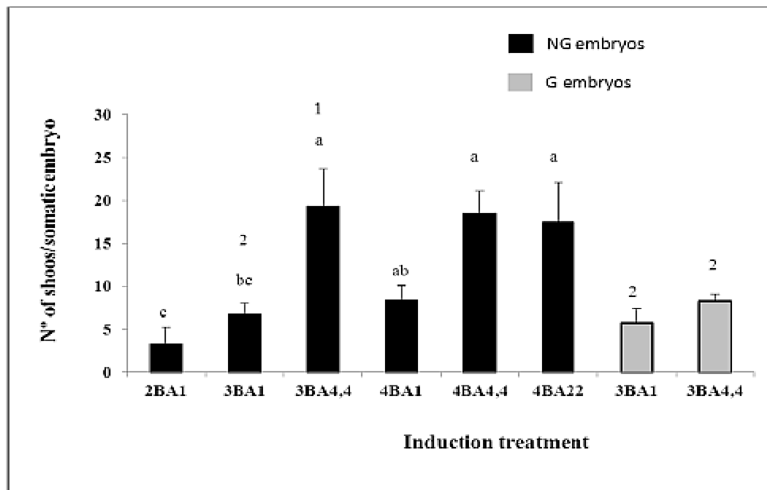

Figure 5. Number of shoots per embryo in non-germinated (black) and germinated (grey) Pinus radiata somatic embryos. Different letters (a, b) show significant differences between different treatments in NG embryos; an different numbers (1, 2) show significant differences between NG and G embryos for treatments with 1 or 4.4 µM BA for 3 weeks (3BA1, 3BA4.4) by Duncan's post hoc test (p≤0.05).

There were significant differences for the percentage of shoots with roots among root induction treatments. The percentage of explants rooted with NAA or the mixture of IBA and NAA (MIX) under a 16-h photoperiod of 120 µmmolm^{-2}s^{-1} was significantly higher than the percentage of rooted explants obtained when shoots were cultured with IBA under the same light conditions (Table 1; Fig. 4 C). Darkness for the first week of the root induction stage was not beneficial for the percentage of rooted explants, and these percentages were not significantly different for the different root induction treatments tested (IBA, MIX and NAA) (Table 1).

Table 1*. Rooting (%) of shoots from somatic embryos of Pinus radiata after different root induction treatments on modified ¼ LP supplemented with 1.5 mgL-1 IBA, 1.5 mgL-1 NAA or 1 mg L-1 NAA with 0.5 mgL-1 IBA (MIX) and under different light regimes for the first week of the root stage. Light (L), Darkness (D).*

Rooting treatment	Rooting (%)
IBA-L	5.0±3.3 b
IBA-D	5.0±3.3 b
MIX-L	50.0±10.0 a
MIX-D	20.0±5.0 b
NAA-L	60.0±12.7 a
NAA-D	25.0±7.9 b

Discussion

In this study, we have tried to overcome the low frequency of plantlet regeneration often encountered in pines by means of shoot organogenesis. Our results indicate that explant type, cytokinin induction period and BA concentration significantly affect the percentage of responding somatic embryos and the number of shoots per embryo obtained. The success in the induction of organogenesis from somatic embryos resembled the results obtained when the same methodology was used in zygotic embryos of the same species (Montalbán et al. 2011). It has been proposed that in *Pinus radiata* zygotic embryos the high bud-forming capacity of cotyledons is related to the undifferentiated state of cotyledonary cells at the time of culture (Aitken-Christie et al. 1985). But in several pine species, it is a current practice to germinate whole embryos or elongate cotyledons for a certain time (Valdés et al. 2001), ranging from hours to days, before applying any induction treatment (Hargreaves et al. 2005; Moncaleán et al. 2005). In our experiments, G embryos showed the highest percentage of EFS (88.9%) whereas NG ones did not present percentages higher than 47% for the same treatment. On the contrary, In *Pinus ayacahuite*, Saborio et al. (1997) suggested that most cotyledonary cells of embryos kept in culture for 6 or more days might have lost the ability to dedifferentiate and therefore were unable to respond to the bud induction conditions.

In our experiments, the presence of activated charcoal in the germination medium could have had a beneficial effect due to its property of adsorbing residual plant growth regulators (von Aderkas et al. 2002). The germination step before shoot induction could have made the G somatic embryos prone to an organogenic response, eliminating a growth regulator excess and preparing the explants for shoot induction.

In NG embryos, the percentage of organogenic embryos was higher when the BA concentration was increased from 1 to 4.4 µM. Higher BA concentrations did not give better percentages. Similarly, Moncaleán et al. (2005) found in *P. pinea* that increasing the exposure time from 2 to 5 weeks did not affect the percentage of responding embryos. In our work, the source of material (G vs. NG embryos) seemed to be the most critical factor. The highest number of shoots per embryo was obtained in NG embryos induced with treatments 3BA4.4, 4BA4.4 and 4BA22 (19.2, 18.5 and 17.5, respectively). In previous experiments with *P. radiata* zygotic embryos cultured under the same conditions, we found treatment 3BA4.4 the most productive (Montalbán et al. 2011). The number of shoots per embryo from G embryos was not statistically different from the values obtained in NG embryos cultured with 1 µM BA. Webb et al. (1988) also found that a low BA concentration and germination for longer than 6 days negatively affected the production of shoots in zygotic cotyledons of eastern white pine. In this context, Valdés et al. (2001) reported a drop in the number of buds per cotyledon in *P. pinea* when the explants were germinated before the induction treatment.

Rooting percentage of shoots was significantly affected by the rooting treatment used. In accordance with our results in organogenesis from zygotic embryos, explants treated with NAA-L and MIX-L showed higher rooting percentages (60 and 50%, respectively) than explants treated with IBA (5%). In radiate pine *in vitro* organogenesis it is a common practice to use a mixture of IBA and NAA for rooting (Hargreaves et al. 2005). NAA alone has also been reported to be effective in *in vitro* rooting of several pine species such as *P. ayacahuite* (Saborio et al. 1997) and *P. pinaster* (Alvarez et al. 2009).

The light regime also influenced the process: in conifers a dark period at the beginning of the rooting stage is often required for root formation and generally a reduction of light intensity favours adventitious root development (Alonso et al. 2006). Our results are in agreement with other reports dealing with the *Pinus* genus where rooting of explants is higher under a 16-h photoperiod (Tang and Guo 2001).

A few protocols combining SE and organogenesis have been described for angiosperms (Khan et al. 2006; Zdravković-Korać et al. 2008; Sriskandarajah and Lundquist 2009) to maximise plant production from genetically transformed ET (Kim et al. 2009) or to regenerate endangered species (Siva et al. 2009). For conifers, there are neither reports focused on that subject nor protocols that include a cold storage step. However, such a methodology could possibly overcome some problems associated with SE such as the aforementioned seasonality of ET initiation and a low embryo production obtained from the ET. The latter problem is especially important in the case of genetically transformed ETs, where the plantlet regeneration is often low (Giri et al. 2004).

We have established a plant regeneration system via a combined pathway of SE and organogenesis and Figure 6 shows a hypothetical scheme about the efficiency of the system. In summary, once the somatic embryos are obtained and with a rooting percentage of these at around 60%, up to 19 rootable shoots can be obtained from a single embryo. Considering that we can obtain more than 150 embryos per 100 mg of ET (Montalbán et al. 2010), the described method can theoretically produce more than 1700 rooted shoots from that mass of tissue. Moreover, these shoots can be propagated before rooting and continuously used as a source for plant regeneration.

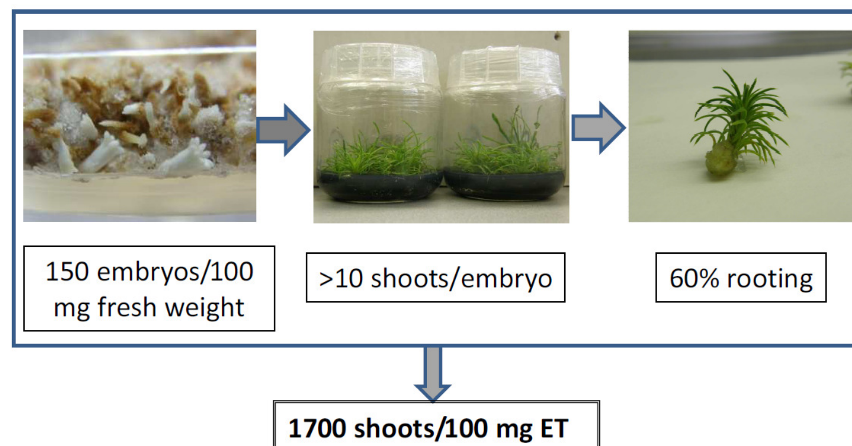

Figure 6*. Theoretical efficiency of plant regeneration system consists in a combined method of Pinus radiata somatic embryogenesis and organogenesis.*

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References

Aitken-Christie J, Singh AP, Horgan KJ, Thorpe TA (1985) Explant developmental state and shoot formation in *Pinus radiate* cotyledons. Bot Gaz 146:196–203.

Aitken-Christie J, Singh AP, Davies H (1988) Multiplication of meristematic tissue: a new tissue culture system for radiata pine. In: Hanover JW, Keathley DE (eds) Genetic manipulation of woody plants. Plenum, New York, pp 413–432.

Alonso P, Moncaleán P, Fernández B, Rodríguez A, Centeno ML, Ordás RJ (2006) An improved micropropagation protocol for stone pine (*Pinus pinea* L.). Ann For Sci 63:1–7.

Álvarez JM, Majada J, Ordás RJ (2009) An improved micropropagation protocol for maritime pine (*Pinus pinaster* Ait.) isolated cotyledons. For 82:175–184.

Bergmann BA, Stomp AM (1992) Influence of taxonomic relatedness and medium composition on meristematic nodule and adventitious shoot formation in nine species. Can J For Res 22:750–755.

Cortizo M, de Diego N, Moncaleán P, Ordás RJ (2009) Micropropagation of adult Stone pine (*Pinus pinea* L.). Trees 23:835–842

Davis JM, Becwar MR (2007) Developments in tree cloning. PIRA International Ltd, Surrey, UK, 69 pp.

De Diego N, Montalbán IA, Fernández de Larrinoa E, Moncaleán P (2008) *In vitro* regeneration of *Pinus pinaster* adult trees. Can J For Res 38:2607–2615

De Diego N, Montalbán IA, Moncaleán P (2010) *In vitro* regeneration of adult *Pinus sylvestris* trees. S Afr J For Res 76:158–162

Giri CC, Shyamkumar B, Anjaneyulu C (2004) Progress in tissue culture, genetic transformation and applications of biotechnology to trees: an overview. Trees 18:115–135.

Hargreaves CL, Grace LJ, van der Maas SA, Menzies MI, Kumar S, Holden DG, Foggo MN, Low CB, Dibley MJ (2005) Comparative *in vitro* and early nursery performance of adventitious shoots from cryopreserved cotyledons and axillary shoots from epicotyls of the same zygotic embryo of control-pollinated *Pinus radiata*. Can J For Res 35:2629–2641

Jiménez VM (2005) Involvement of plant hormones and plant growth regulators on *in vitro* somatic embryogenesis. Plant Growth Regul 47:91–110.

Khan T, Singh A, Pant RC (2006) Regeneration via somatic embryogenesis and organogenesis in different cultivars of cotton (*Gossypium* spp.). In Vitro Cell Dev Biol Plant 42:498–501.

Kim SW, Oh MJ, Liu JR (2009) Plant regeneration from the root derived embryonic tissues of *Rosa hybrida* L. cv. charming via a combined pathway of somatic embryogenesis and organogenesis. Plant Biotechnol Rep 3:341–345

Klimaszewska K, Trontin JF, Becwar MR, Devillard C, Park YS, Lelu-Walter MA (2007) Recent progress in somatic embryogenesis of four *Pinus* spp. Tree For Sci Biotechnol 1:11–25.

Lambardi M, Sharma KK, Thorpe TA (1993) Optimization of *in vitro* bud induction and plantlet formation from mature embryos of aleppo pine (*Pinus halepensis* Mill.). In Vitro Cell Dev Biol 29:189–199.

MacKay JJ, Beckwar MR, Park YS, Corderro JP, Pullman GS (2006) Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. Tree Genet Genomes 2:1–9.

Moncaleán P, Alonso P, Centeno ML, Cortizo M, Rodríguez A, Fernández B, Ordás RJ (2005) Organogenic responses of *Pinus pinea* cotyledons to hormonal treatments: BA metabolism and cytokinin content. Tree Physiol 25:1–9.

Montalbán IA, De Diego N, Moncaleán P (2010) Bottlenecks in *Pinus radiata* somatic embryogenesis: improving maturation and germination. Trees 24:1061–1071.

Montalbán IA, De Diego N, Moncaleán P (2011) Testing novel cytokinins for improved *in vitro* adventitious shoots formation and subsequent *ex vitro* performance in *Pinus radiata*. For 84 (4):363-373.

Montalbán IA, De Diego N, Moncaleán P (2012) Enhancing initiation and proliferation in radiata pine (*Pinus*

radiata D. Don) somatic embryogenesis through seed family screening, zygotic embryo staging and media adjustments. Acta Physiol Plant 34 (2):451-460.

Park YS (2002) Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Ann For Sci 59:651–656.

Park YS, Barrett JD, Bonga JM (1998) Application of somatic embryogenesis in high-value clonal forestry: deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell Dev Biol Plant 34:231–239.

Park YS (2002) Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. Ann For Sci 59:651-656.

Quoirin M, Lepoivre P (1977) Études des milieux adaptés aux cultures *in vitro* de *Prunus*. Acta Hortic 78:437–442.

Saborio F, Dvorak WS, Donahue JK, Thorpe T (1997) *In vitro* regeneration of plantlets from mature embryos of *Pinus ayacahuite*. Tree Physiol 17:787–796.

Siva R, Rajasekaran C, Mudgal G (2009) Induction of somatic embryogenesis and organogenesis in *Oldenlandia umbellata* L., a dye-yielding medicinal plant. Plant Cell Tissue Organ Cult 98:205-211.

Stasolla C, Thorpe TA (2010) Tissue culture: historical perspectives and applications. In: Kumar A, Sopory SK (eds) Applications of plant biotechnology: *in vitro* propagation, plant transformations and secondary metabolite production. IK International, India, pp 1–39

Sriskandarajah S, Lundquist PO (2009) High frequency shoot organogenesis and somatic embryogenesis in juvenile and adult tissues of seabuckthorn (*Hippophae rhamnoides* L.). Plant Cell Tissue Organ Cult 99:259–268.

Tang W, Guo Z (2001) *In vitro* propagation of loblolly pine via direct somatic organogenesis from mature cotyledons and hypocotyls. Plant Growth Regul 33:25–31.

Tang W, Guo Z, Ouyang F (2001) Plant regeneration from embryogenic cultures initiated from mature loblolly pine zygotic embryos. In Vitro Cell Dev Biol Plant 37:558–563.

Valdés AE, Centeno ML, Ordás RJ, Fernández B (2001) Relationships between hormonal contents and organogenic response in *Pinus pinea* L. cotyledons taken from plantlets at different germination periods. Plant Physiol Biochem 39:377–384.

von Aderkas P, Label P, Lelu MA (2002) Charcoal affects early development and hormonal concentrations of somatic embryos of hybrid larch. Tree Physiol 22:431–434.

Webb DT, Flinn BS, Georgis W (1988) Micropropagation of eastern white pine (*Pinus strobus* L.). Can J For Res 18:1570–1580.

Yildirim T, Kaya Z, Isik K (2006) Induction of embryogenic tissue and maturation of somatic embryos in *Pinus brutia* TEN. Plant Cell Tissue OrganCult 87:67–76.

Zdravković-Korać S, Ćalić-Dragosavac D, Uzelac B, Janošević, Budimir S, Vinterhalter B, Vinterhalter D (2008) Secondary somatic embryogenesis versus caulogenesis from somatic embryos of *Aesculus carnea* Hayne.: developmental stage impact. Plant Cell Tissue Organ Cult 94:225–231.

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The Embryogenic Lines and Somatic Embryogenesis of Coniferous Species in Siberia

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Keywords: embryogenic lines, somatic embryogenesis, *Larix sibirica, Larix gmelinii, Pinus sibirica, Pinus pumila.*

Abbreviations: ABA - Abscisic acid; PEG – polyethylene glycol

Abstract: Experiments that involved culturing of immature isolated embryos of Siberian coniferous species were carried out on different modified media: *Pinus sibirica, Pinus pumila* on ½LV medium, *Larix sibirica, Larix gmelinii* on AI medium (patent №2456344). For induction of embryogenic callus every species needs an optimized medium supplemented with L-glutamine, casein hydrolysate, ascorbic acid and hormones at different concentrations and in different proportions. The active proliferation of embryonal masses is observed on the same medium with a reduced concentration of cytokinins. The maturation of somatic embryos took place on basal medium with abscisic acid (ABA) (120-200 µM) and polyethylene glycol (PEG) (8-10%). We found that cell elongation of the hypocotyls and asymmetric division, as well as development of globules of somatic embryos and their maturation are the key stages of somatic embryogenesis. The embryogenic cell lines were obtained in *L. sibirica* and *P. pumila*. The embryogenic lines were characterized by different proliferative activity. The number of somatic embryos varied from 210 to 410 per 500 mg of callus fresh weight. The cell lines are capable of long-term (over three years) self-maintenance and mass production of somatic embryos and plants, which will become a highly promising material for establishing conifer forest plantations in Siberia.

Introduction

The problem of gene conservation of the main forest species may be solved by using a combination of classical selection methods as well as modern innovation biotechnological methods such as somatic embryogenesis which is widely used abroad in plantation forestry. Somatic embryogenesis has some benefits as compared to other methods of cloning. This efficient method of plant regeneration enables us to obtain and retain genetic resources during a long period of time due to the high productivity of proliferating embryonic masses (EM) (Lelu et al., 1994; Park, 2002, 2006; Lelu-Walter and Pâques, 2009; Carneros et al., 2009; Klimaszewska et al., 2001, 2009).

In Russia the studies of somatic embryogenesis of coniferous woody species were started in the V. N. Sukachev Institute of Forest SB RAS (Krasnoyarsk) at the beginning of the 21th century (Belorussova and Tretyakova, 2008; Tretyakova and Barsukova, 2012). The technology to obtain somatic embryos and plantlets is being developed for incorporation with the different selection programs: i.e., hybridization by controlled pollination of coniferous species using improved parent trees to obtain heterosis and retain the hybrids for posterity. The purpose is selection and testing of valuable hybrid tree genotypes *in vitro* and *in vivo*; the mass replication of obtained hybrids through somatic embryogenesis *in vitro* followed by transplanting the plants to the forest nursery.

This study was focused on obtaining enbryogenic cell lines (Cl), investigating somatic embryo morphogenesis, and analyzing possibilities to control this process in conifers growing in Siberia

Materials and Methods

The objects of study were 34 trees of *Larix sibirica* Ledeb., 10 trees of *Larix gmelinii* Rupr, 13 trees and 7 clones *of Pinus sibirica* Du Tour and 40 trees of *Pinus pumila* Pall., growing in a natural stand in the western Sayan Mountains, near the Baikal lake and Khakassia. With trees of *P. sibirica* control pollination was conducted by using pollen of trees-accelerates with annual cone development.

Isolated zygotic embryos at the stage of cotyledon initiation were used for somatic embryogenesis induction. The female cones were collected in July from 2007 to 2011. The seeds were scaled, surface-sterilized with a 5% alcoholic solution of iodine for three minutes. After three replicated flushes with sterile distilled water, the megagametophytes were treated with hydrogen peroxide for 5-10 min. The embryos were extracted from the megagametophytes under sterile conditions, placed on moistened filter paper in Petri dishes, and then transferred onto a culture medium.

Callus induction

The immature isolated embryos of Siberian coniferous species were cultured on different modified media: *Pinus sibirica, Pinus pumila* on ½ LV medium (Litvay et al., 1985)*, Larix sibirica, Larix gmelenii* on AI medium (patent N2456344). For induction of embryogenic callus every species needs an optimized medium supplemented with 0,1-1,0 gl^{-1} mesoinositol, 0,4 gl^{-1} ascorbic acid, 1,0 gl^{-1} casein, 0,5 gl^{-1} L-glutamine, 30 gl^{-1} sucrose, and 7gl^{-1} agar. The growth regulators used were 2,0 mgl^{-1} 2,4-D and 1,0 mgl^{-1} BA. pH was adjusted to 5,8 prior to autoclaving 121 ºC, 110 kPa, for 20 min. Five embryos were cultured on 20 ml of inductive media in each Petri dish in darkness at 25±1 ºC.

Embryonal mass (EM) proliferation

To obtain EM proliferation, we used the above basal media with 2,0 mgl^{-1} 2,4-D, 0,5 mgl^{-1} BA, and 20 gl^{-1} sucrose. The cultures were incubated in the dark at 24±1ºC. The calli were transferred to a fresh culture medium every 14 days.

Somatic embryo pre-maturation

Seven days after subculture on the proliferation medium, pieces of intensively growing EM each weighing 100-300 mg were transferred onto hormone-free liquid basal medium with 10mgl^{-1} activated carbon and increased (34 gl^{-1}) sucrose content to stop EM proliferation and to induce somatic embryo maturation. The explants were cultured on a shaker (60 revolutions per minute) under low-intensity (10 µmol m^{-2} s^{-1}) light and 16-h photoperiod during a week.

Somatic embryo maturation

Larch somatic embryo maturation was done using basal medium containing different combinations of 40-60 gl^{-1} sucrose, 120-200 µM ABA, 1µM IBA, and 8-10% PEG, with 3-4 gl^{-1} Gelrite as a gelling agent. Culture was performed under low-intensity (20 µmol m^{-2} s^{-1}) light, 16-h photoperiod at 25±1ºC. Growth regulators (ABA and IBA) and L-glutamine were sterilized by filtering and added to the cooled culture medium after autoclaving.

Somatic embryo germination

To obtain larch somatic embryo germination, they were grown in a climate camera (AWTech, GC-300) on growth regulator-free basal medium with 10 gl^{-1} activated carbon and 34 gl^{-1} sucrose. A somatic embryo was considered as germinated as soon as radicle emergence was observed. The plantlets were then transferred to a moistened soil substrate (sand/vermiculite/peat; 1/1/1; v/v/v).

Cytological analysis

Cytological analysis was done using squash preparations. To obtain these preparations, explants were placed onto a slide and kept in a dye (safranin with methylene blue) for 1-2 minutes. Glycerin was then added and the preparation was covered by a cover glass.

The micro-samples were analyzed with a LOMO MICMED-6 microscope. Statistical data processing was carried out by standard methodologies using Microsoft Excel-2003. Single-variate variance analysis was used to evaluate reliability of the results obtained.

Results

Somatic embryogenesis of *Larix sibirica and Larix gmelinii*

Callus development in *L. sibirica* and *L. gmelenii* depended on explant development stage. The best callus growth (in 93% of the *Larix sibirica* and in 81% of the *L. gmelinii* explants) was observed at the cotyledon initiation stage. Morphological observation of the larch species showed that EC induction took place on the 8th -14th days of culture. EC developed either uniformly across an explant surface, or in the radicle and hypocotyl region. The callus obtained was of white color and had knobby structure (Fig.1A).

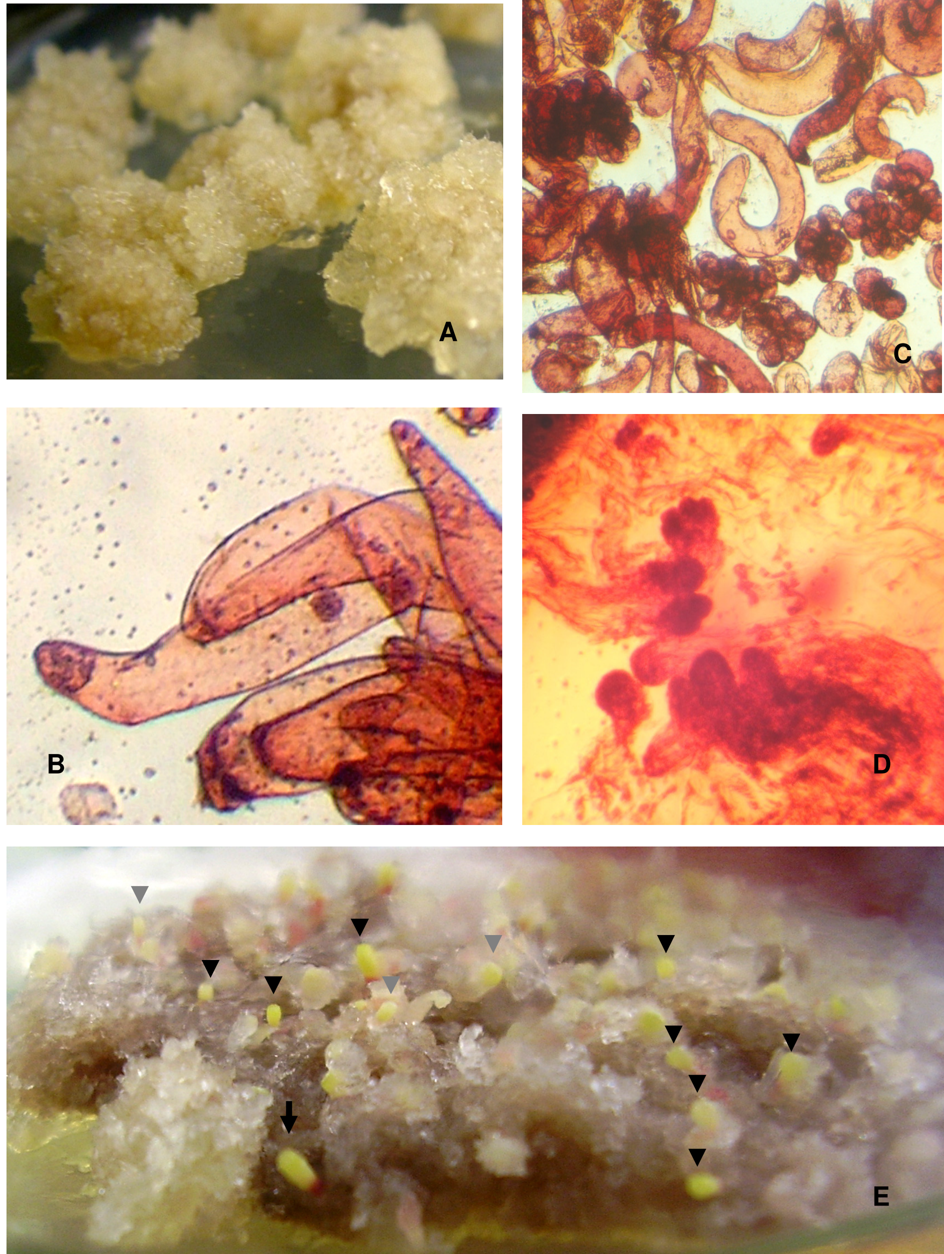

Fig.1. *Somatic embryogenesis in vitro of Siberian larch: A – embryogenic callus; B - formation of initial cells and embryonal tube; C – proliferating embryonal mass; D – globular somatic embryos; E – mature somatic embryos.*

The development of EM in callus occurred after 25-35 days of cultivation in *Larix sibirica* and *L. gmelinii* on the media AI with a reduced concentration of BA (0,5 mgl^{-1}). However, after 3-5 months of cultivation development of callus stopped and formation of EM occurred in 18% of the explants of *L. sibirica*. Active proliferation of EM was observed in genotype № 4C, resistant to larch bud midge. Seven cell lines (Cl) were obtained in this genotype. Cl 1 *(08-03-00-01),* 2 *(09-03-00-02),* 3 *(09-03-00-03)*, 4 (*09-03-00-04),* 6 *(11-03-00-06),* 7 *(11-03-00-07) w*ere obtained in larch as a result of open pollination. Age of cultures is 14-38 months. Cell line 5 *(09-03-01-05)* was obtained as a result of cross pollination of *L. sibirica* x *L. sukaszewii*. Age of culture is 26 months.These Cl varied in EM proliferation activity. After four weeks of proliferation Cl 1 and Cl3 explant-derived EM reached 12,2 g and 16,5 g, respectively. EC rates ranged from 0,165±0,005 to 0,467±0,005 g in *L. gmelinii.* Weight of callus of *L. sibirica* after 10 weeks was 110-580 g in different lines. Somatic embryo amounts ranged 210 (CL 3) to 390 (CL 1) per 500 g of embryogenic mass (Fig.2). After 1 year the weight of callus was 550-3000 g. The number of somatic embryos (torpedo stage) per 500 mg of callus was 220-410.

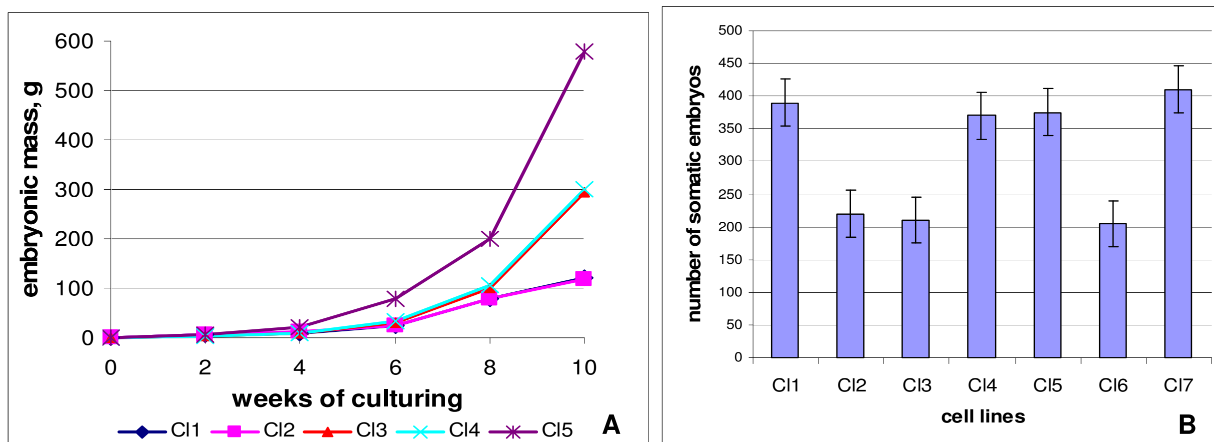

Fig. 2. *Proliferation of embryogenic mass of Siberian larch cell lines (Cl): A – Fresh weight; B - number of somatic embryos in 500 mg of EM after 10 weeks of culturing*

Cytoembryological Control of Somatic Embryogenesis

The formation of EM in all the larch species under study follows the same scheme and begins with elongation of explant cells and their asymmetric division, such as described for Siberian larch (Belorussova and Tret'yakova, 2008). Asymmetric division of cells is crucial for the whole process of somatic embryogenesis in general. As a result, embryonic tubes appeared with an embryonic initial at the other end. As in zygotic embryogenesis, the embryonic initial underwent serial divisions within both planes. Somatic embryos consist of embryonic (round meristematic cells) and suspensor (highly elongated) cells. After 30–40 days, somatic embryos at the early stages of embryonal development (globular embryos) were observed in the callus EM of Dahurian and Siberian larches (Figs. 1 B, C). Torpedo-like somatic embryos (the next stage of development) were obtained only in Siberian larch on AI medium.

No formation of mature embryos was observed when the calli of Dahurian larch were inoculated into the nutrient media for development of mature embryos. The medium with a low concentration of ABA (5,3 - 24 mgl^{-1}) decreased embryogenic activity of Siberian larch, and the calli became green. Therefore, no formation of mature somatic embryos capable of further development into plants in Siberian and Dahurian larches was observed on the media recommended for maturation of somatic embryos of European larch and its hybrids (Lelu-Walter et al., 2006, 2008). AI medium supplemented with various concentrations of ABA, PEG, Gelrite, and sucrose was applied to initiate maturation of somatic embryos of Siberian larch.

The medium containing 32 mgl^{-1} ABA, 10% PEG, 40 gl^{-1} sucrose, and 4 gl^{-1} Gelrite was optimal for the development of Siberian larch somatic embryos. This medium favored the formation of cotyledonary somatic embryos as early as after three or four weeks of culturing. A cotyledonary ring was initiated and started to develop. After 50 days of culture on the maturation medium, the somatic embryos reached from 1,1 to 1,5 mm in length and had a clear bipolar structure of body and fully developed cotyledons.

To induce development of mature somatic embryos in the Cl of Siberian larch, EM were kept in liquid basal medium during a week. After such a treatment, we managed to obtain the mass formation of mature somatic embryos. Maturation of somatic embryos in Cl of Siberian larch lasted for 40–60 days.

Germination of Somatic Embryos

The somatic embryos with well-developed cotyledons were transferred to the germination medium (basal AI medium without plant growth regulators and supplemented with activated carbon (10 mgl^{-1})).
Decreasing the concentration of macro- and microelements and iron (two times), as well as exclusion of organic nitrogen and vitamins had a positive influence on the germination of somatic embryos, i.e., 70% of somatic embryos developed into normal plantlets. On day 5–7, hypocotyl elongation was observed, and a root appeared.
The epicotyl appeared two or three weeks after the cultivating on the germination medium. The somatic embryos with a well-developed root and epicotyl were considered as good plants and transferred to the ecosoil (Fig. 3).
Therefore, six embryogenic cell lines of Siberian larch and one cell line of the hybrid of Siberian larch with Sukachev larch that mass-produced somatic embryos and plants, were for the first time obtained.

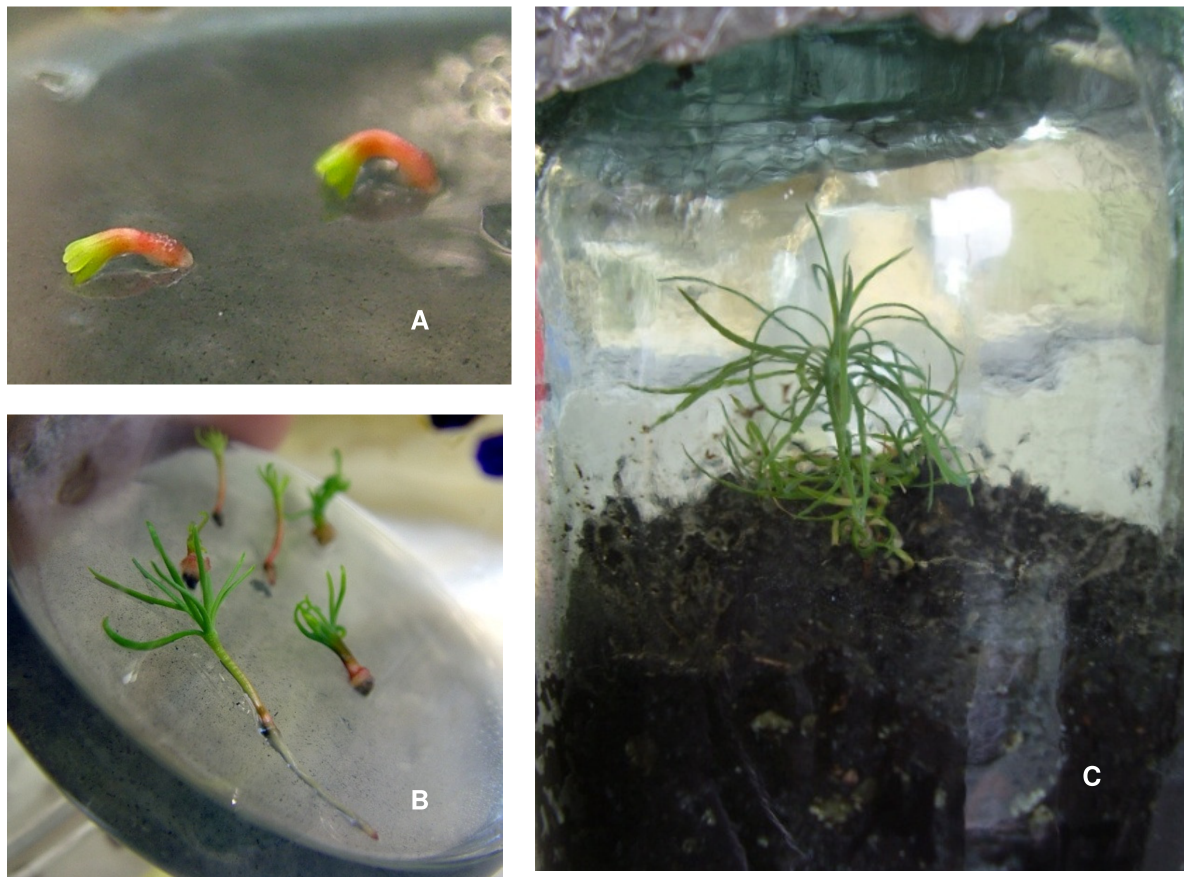

Fig.3*. Development of Siberian larch plantlets: A – germination of somatic embryos; B – plantlets on the medium in vitro; C – plantlets in ecosoil.*

Somatic embryogenesis in *Pinus pumila*

Culturing *P. pumila* (Mountain pine) embryos at the pre-cotyledon stage of the plant embryo development on ½ LV medium, to which 2,4-D and BA hormones (2:1) were added, resulted in callus development in 90-94% of explants. Of all the calluses obtained, 82% began to proliferate, 48% formed EM. Two to five months of culture produced seven intensively proliferating cell lines of two genotypes (№24 and №64), with one gram of callus containing 620 ± 47 somatic embryos. When keeping EM immersed in LV basic medium, in the presence of 32 mgl^{-}

ABA, it took 60 days for somatic embryos to mature. The number of mature somatic embryos varied from 12 to 38 per 1 g of callus among the cell lines. On hormone-free ¼LV medium, 0,7-2,0% of all somatic embryos germinated and formed plantlets. A cytoembryological analysis revealed that somatic embryo initiation and development in *P. pumila* occurred the same way as in larch (Fig. 4). Elongation of cells near the radicles and hypocotyls, the earliest indicators of somatic embryogenesis induction, were followed by asymmetric cell division, development of globules of embryos and suspensors. On the medium containing ABA, histodifferentiation and development of embryo organs were observed.

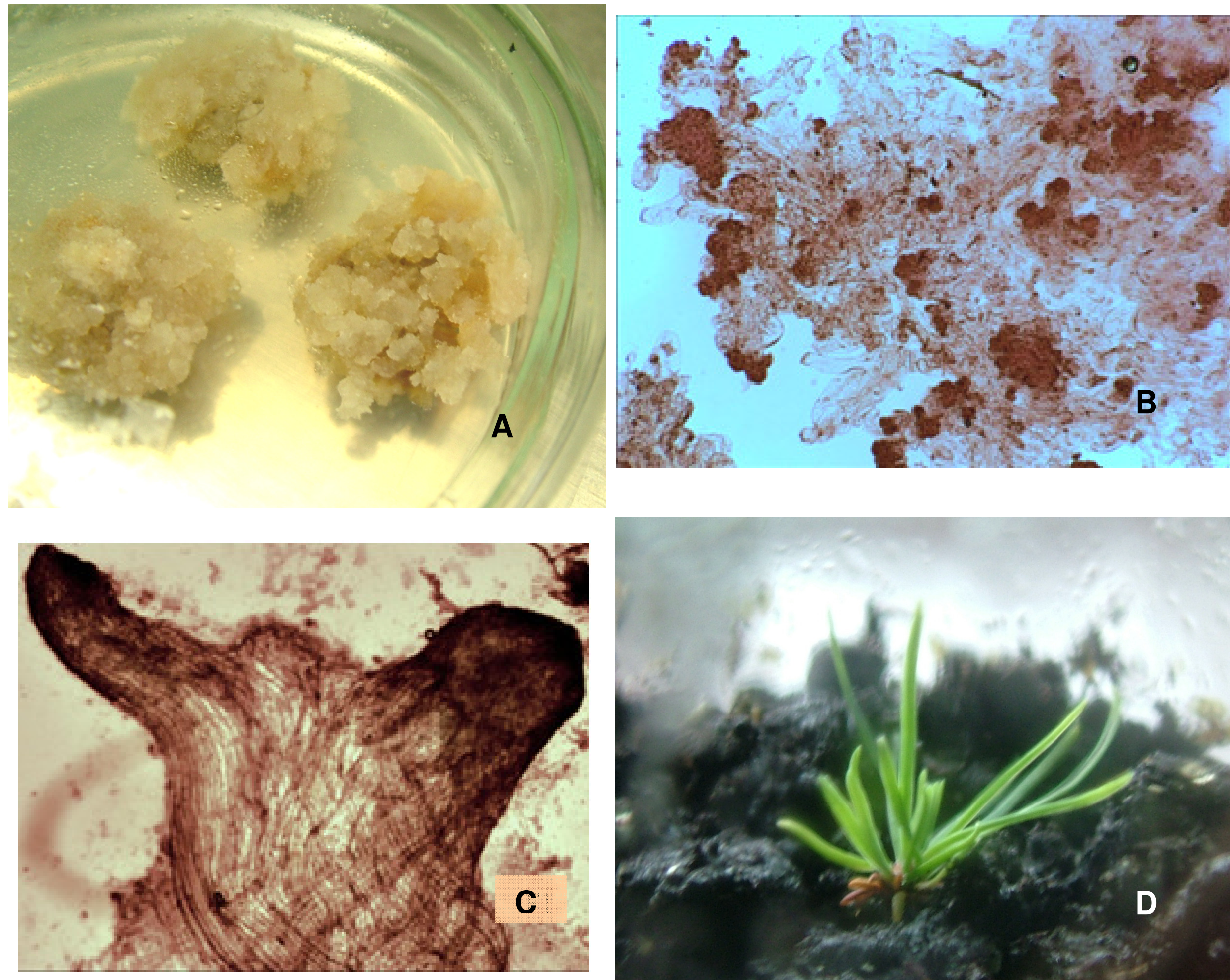

Fig.4 *Somatic embryogenesis in in vitro culture in Mountain pine: A – embryogenic callus; B, C - globular somatic embryos; D – plantlets in ecosoil.*

Somatic embryogenesis in *Pinus sibirica*

When culturing isolated immature *Pinus sibirica* (Siberian pine) embryos on ½ LV medium, callus development appeared to be most intensive (in 75-80% of explants), like in the larch species and in *P. pumila*, when cultured at the pre-cotyledon and later development stages until zygotic embryo lengths exceeded 2 mm.

Callus growth on 1/2LV medium was monitored to show that callus initiation and proliferation in the embryos of the seeds collected in a natural *P. sibirica* stand differed from those in a graft clone plantation of the species. After 1-month of culture, callus weight varied from 0,10 to 0,76 mg among the explants of the naturally growing trees and clones (Fig.5). During further culturing on proliferation medium, callus growth slowed down and necrosis occurred in the callus. A cytological analysis applied throughout the entire culture period revealed no

embryonal structures. The explants from both the natural stand and the open-pollinated clones developed non-embryogenic calli.

Culture of the embryos of the hybrid seeds obtained through controlled pollination of seven *P. sibirica* clones by the pollen of an accelerated-growth individual with an annual female cone development cycle resulted in intensively growing embryogenic callus, which was, in certain years, 5-6 times the weight of the calli in the explants from the natural stand and from the open-pollinated clones (Fig. 5 B, C).

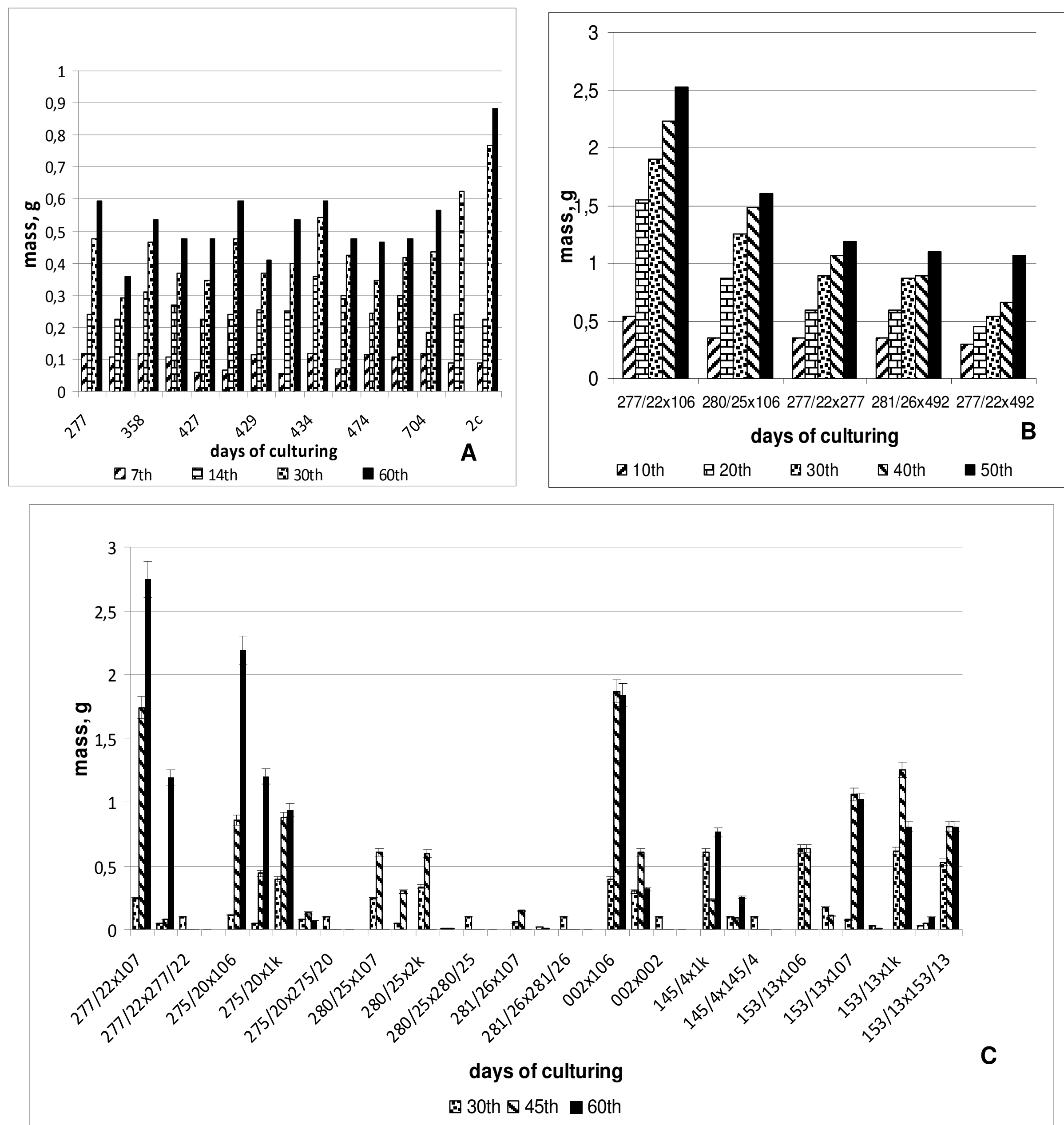

Fig. 5 *Fresh weight of Siberian pine callus (explants – zygotic embryos): A – from natural stands, B-C - from clones pollinated by pollen of tree-accelerate (№ 106, 107) and by pollen from plus-trees (№277, 492, 1k, 2k). B – in 2007, C – in 2011.*

During further culturing, the highest increases in fresh callus weight were exhibited by two clones (№153/13 and №002) pollinated by pollen from tree-accellerate. The calli of these two clones had a loose structure characteristic of embryogenic callus. A cytological analysis revealed rapid EM formation in these calli, with somatic cells beginning to elongate on the 5th – 8th day of culture and reaching a length of 700-800 mkm on the 20th day. Elongated cells grew wider, became vacuolated, and two to three nuclei became visible in them. The calli of the hybrid seeds were made up by elongated cells and remained unchanged till the end of their initiation, which lasted for 30 days. After the calli were transferred onto the proliferation medium, the two clones pollinated by the pollen of the tree with an annual female cone development cycle and by the pollen of №2k plus tree exhibited asymmetric cell division resulting in the development of embryonal initials and tubes. In the proliferation medium, somatic embryo globules and suspensors composed of several embryonal tubes occurred in the embryogenic calli on the 12th – 15th day. After 1-month culture on this medium, somatic embryos began to appear. Our experiments on somatic embryo maturation on 1/2LV medium in presence of ABA are still underway.

It should be noted that, elongated cells formed in the calli by the end of their initiation remained unchanged in the majority of the explants of individuals subjected to different controlled pollination variants. Such calli remained non-embryogenic and died in time.

Our experiments on *in vitro* culturing of *L. sibirica, L. gmelenii*, *Pinus pumila* and *P. sibirica* showed that 3% of the genotypes of interest were capable of EM formation. Embryogenic Cl can thus be obtained from explants of a limited number of trees. It was found (Lelu-Walter, 2008; Carneros et al. 2009) that only the explants of certain donor-trees are able to form somatic embryos and plants. Somatic embryogenesis in conifers is strongly controlled genetically (MacKay et al. 2006). Our study showed that individuals with a high reproduction potential can be used as such donors.

Although somatic embryos of *Larix* and *Pinus* species are of vegetative origin, they develop according to the zygotic embryogenesis scenario (Lelu et al. 1994; Stasolla and Yeung 2003; Lelu-Walter and Pâgues 2009). We found that hypocotyl cell elongation and asymmetric division, as well as the development of globules of somatic embryos and their maturation are the key stages of somatic embryogenesis. All these processes are controlled by phytohormones. The cell lines we obtained are capable of long-term (over three years) self-maintenance and mass production of somatic embryos and plants, which will become highly promising material for establishing conifer forest plantations in Siberia.

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References

Belorussova AS and Tretyakova IN (2008) Characteristics of *Larix Sibirica* somatic embryo development: embryologic issues. Russian J Dev Biol 39 : 1-10.

Carneros E, Celestino C, Klimaszewska K, Park Y-S, Toribio M and Bonga JM (2009) Plant regeneration in Stone pine (*Pinus pinea* L.) by somatic embryogenesis. Plant Cell Tiss. Organ Cult. 98 : 165-178.

Klimaszewska K, Park, Y-S, Overton C, MacEacheron I and Bonga JM (2001) Optimized Somatic Embryogenesis in *Pinus strobes* L. In vitro Cell Dev Biol._Plant 37 : 392–399.

Klimaszewska K, Noceda C, Pellitier G, Label P, Rodrigues R and Lelu-Walter M-A (2009) Biological Characterization of young and aged embryogenic cultures of *Pinus pinaster.* Ait. In Vitro Cell Dev Biol Plant 45 : 20-33.

Lelu M-A, Klimaszewska K and Charest PJ (1994) Somatic embryogenesis from immature and mature zygotic embryos and from cotyledons and needles of somatic plantlets of *Larix*. Can J For Res 24 : 100-106.

Lelu-Walter M-A, Bernier-Cardou M and Klimaszewska K (2008) Clonal Plant Production from Self and Cross-Pollinated Seed Families of *Pinus sylvestris* (L.) through Somatic Embryogenesis. Plant Cell Tiss Organ Cult 92 : 31–45.

Lelu-Walter M-A and Pâques LE (2009) Simplified and improved somatic embryogenesis of hybrid larches (*Larix x eurolepis* and *Larix* × *marschlinsii*). Perspectives for breeding. Ann. For. Sci. 66 : 104.

Litvay JD, Verma DC, Johnson MA (1985) Influence of Loblolly Pine (*Pinus taeda* L.) Culture Medium and its Components on Growth Somatic Embryogenesis of the Wild Carrot (*Daucus carota* L.). Plant Cell Rep 4 : 325-328.

Park Y-S (2002) Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and

deployment considerations. Ann For Sci 59 : 651-656.
Park Y-S., Lelu-Walter MA, Harvengt L, Trontin JF, MacEacheron I, Klimaszewska K, and Bonga JM (2006) Initiation of Somatic Embryogenesis in *Pinus banksiana, P. strobus, P. pinaster*, and *P. sylvestris* at Three Laboratories in Canada and France, Plant Cell Tiss Organ Cult 86 : 87–101.
Stasolla C and Yeung EC (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tiss Organ Cult 74 : 15-35.
Tretyakova IN and Barsukova AB (2012) Somatic embryogenesis in culture in vitro 3-th Larix species. Russian J Dev Biol 43 : 1-11.

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Towards scaling-up the micropropagation of *Juglans major* (Torrey) Heller var. 209 x *J. regia* L., a hybrid walnut of commercial interest

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Key words Acclimation, Phloroglucinol, iron source, recalcitrant, rooting, temporary immersion system, sucrose

Abstract: As a result of the program for quality-wood production conducted by Bosques Naturales SA, several walnut *èlite* genotypes have been selected. Whereas it is important to establish an efficient clonal propagation system for such hybrids, doing it has been hampered by the well-known recalcitrance of walnut to *in vitro* manipulation. As a few general micropropagation protocols are available, they must be adapted to the specific genotypes and conditions of each laboratory. Here, we present some recent findings that have allowed us to improve the current micropropagation strategies for *Juglans major* (Torrey) Heller var. 209 x *J. regia* L., a hybrid of commercial interest. We specifically investigated the influence of phloroglucinol (Phl) during multiplication; the benefits of using FeEDDHA instead of FeEDTA; the influence of subculture length; the optimal doses of sucrose for root pre-induction and the use of a Temporary Immersion System (TIS) to promote elongation. Phl was a key factor to promote basal-*calli* formation and to increase microshoot length. There was no statistically significant interaction between genotype and iron source used, a relevant finding taking into consideration that substitution of FeEDTA by FeEDDHA was a determinant for rooting and for survival. Despite genotype-specific effects on *in vitro* behaviour, there was a high influence of sucrose concentration during root pre-induction. Microshoots growing in TISs were taller than those multiplied in gelled culture media. More than 90 % of the vitroplants that survived the first 4 weeks under *ex vitro* conditions could reach nursery and field plantation. These results allowed us to clone successfully 8 *èlite* genotypes since 2010.

Introduction

Establishment of multi-clonal plantations of highly valuable genotypes is an important requisite in forestry to reduce the risks caused by the genetic variability of seed orchards. Micropropagation is the best choice for massive multiplication of *èlite* trees, however, due their high recalcitrance only a few woody species are commercially propagated.

Juglans are considered amongst the most recalcitrant genera for *in vitro* culture. Few walnut species and/or varieties are mass micropropagated, most of them used as rootstocks or fruit trees. Low survival percentage during *in vitro* establishment; high influence of genotype, mainly characterized by long stationary phases, as well as a reduced ability for rooting; chlorosis of microshoots and low survival rates during acclimation, are amongst the most critical factors that determine the success of walnut micropropagation.

In a walnut selection program, associated to the accelerated annual propagation of several highly valuable trees for wood production, a standardized and functional micropropagation protocol is needed. However, the high genotypic determinism and the well-known recalcitrance of walnuts hinder the obtaining of a common system for all them.

A functional micropropagation protocol should be based on the obtaining of healthy and highly reactive microshoots as a manifestation of their "right" physiological state: an adequate mineral composition of culture medium is important to reach this goal. Microshoots that had been multiplied in the corrected DKW formulation (McGranahan *et al*. 1987) had a healthy appearance except for the pale green colour of the leaves. A similar disorder was observed by Najafian Ashrafi *et al.* (2010) in Persian walnuts. As chlorosis might have been caused by iron deficiency we decided to replace FeEDTA by FeEDDHA, considered a more stable and more suitable chelate for field growing fruit trees (Pestana *et al*. 2003) and for micropropagation. Moreover, FeEDTA is photosensitive, provoking chlorosis by iron deficiency (Van der Salm *et al*. 1994).

Once survival to *ex vitro* conditions was greatly enhanced using rooted microshoots, our work addressed the improvement of the rooting ability for all clones. The root formation is closely related with the utilization of quality microshoots. Microshoots good for rooting were defined as those that came only from apical explants that reached more than 20 mm in height, with at least 8 nodes and green and healthy leaves. A key factor to obtaining a quality walnut microshoot is the formation of a big basal callus in the first 2 weeks of every subculture. In this process Phl has an important role. Phl is used *in vitro* as growth regulator, promoting the multiplication of apple (Rustaei *et al*. 2009) and *Ficus religiosa* L. (Siwach and Rani Gill 2011) and improving the rooting of almond (Ainsley *et al.* 2001).

Here a compilation of the main results on walnut hybrid micropropagation since 2005 is presented. The introduction of Phloroglucinol (Phl) on multiplication and the substitution of FeEDTA by the more stable chelate FeEDDHA are the most important changes introduced in the culture medium proposed by Driver and Kuniyuki (1984) and corrected by McGranahan *et al.* (1987). Other factors, such as the influence of sucrose dose during root pre-induction phase and the length of subculture prior to rooting were also assessed. The use of TISs to promote the elongation of microshoots from highly recalcitrant genotypes was also investigated.

Materials and Methods

Plant material and in vitro *introduction*

Our micropropagation protocol was developed for the walnut hybrid (*Juglans major* var. 209 x *J. regia* L.) for wood production. Epicormic branches from *èlite* field growing trees, selected from Bosques Naturales SA (Spain) orchards, were used for *in vitro* culture initiation. Two different methods were followed for introduction. For the first one, epicormic branches were collected during late spring and beginning of summer in 2008 and 2009; segments bearing at least 1 dormant bud were washed for 10 minutes with sterilized water and detergent. Afterwards they were dipped for 60 seconds in alcohol (96 %), followed by a 20 min treatment with NaOCl (1%, pH were adjusted approximately to 7.5) and some drops of Tween 20 and finally the explants were rinsed 3 times before inoculation in test tubes containing 10 ml of culture medium. For the second method, the basal part of epicormic segments, 20-25 cm long, collected in late winter and early spring of 2011 and 2012, were introduced in sterilized water and stored under thermo and photoperiod conditions in the laboratory to promote bud breaking. Then the softwoods formed were used as starting material, superficially disinfected only with NaOCl (pH was not adjusted), while the rest of the procedure was kept the same. Before being incorporated in the general micropropagation schedule, microshoots successfully introduced were tested to be free from endogenic actively-growing *bacteria* by inoculating a small part of callus formed on a culture medium composed of meat and yeast extract (5 g/L), glucose (5 g/L), KH_2PO_4 (2 g/L) and agar (7 g/L). Eight genotypes that behaved differently through all the phases of micropropagation were used. Non recalcitrants were considered to be those that were easily introduced, whose average size of microshoots was above 35 mm and had a high ability (>80 %) for rooting (DA, D53 and D15). Recalcitrants were those that had some problems for *in vitro* establishment, microshoots were between 25 and 34 mm high and/or had an average of 50 up to 80 percent of root induction (DM, DN, D49 and D51). In this group were some genotypes that could be included in the first one if some particular characteristics were considered (like DM, with little microshoots (<20 mm) but a high ability for rooting). High recalcitrants were those that needed several attempts during introduction and had low rooting percentages, usually less than 50 % (G3 and D48).

Culture medium and general conditions

DKW (McGranahan *et al.* 1987) formulation was used in all phases of micropropagation. For

multiplication 8 microshoots were inoculated in 380 ml vessels with 80 ml of culture medium supplemented with 1.0 and 1.5 mg/L of BAP for introduction and multiplication, respectively. Two doses of agar (Industrial Agar 707469, Pronadisa, Spain) were used, 5.5 or 6 g/L, depending if the iron source was FeEDDHA or FeEDTA. The introduction culture medium was additionally supplemented with 75 mg/L of Phl. Microshoots grew under a 16/8 photoperiod (PPFD 45-50 µm. m^{-2}. s^{-1}) in a growth chamber with a temperature of 24± 0.2ºC.

Substitution of FeEDTA as iron source

An equivalent dose (119 mg/L) of ethylenediamine di-2-hydroxy-phenyl acetate ferric (FeEDDHA, Duchefa Biochemie) was used instead of the less stable iron source FeEDTA. The influence of FeEDDHA on multiplication, rooting and survival were assessed.

Use of Phl during multiplication

The effects of several doses of Phl (0, 25, 50 and 75 mg/L) during multiplication were assessed. *Calli* weight and length and nodes per microshoots were measured.

Rooting conditions

Rooting was divided into two separated steps: pre-induction and expression as were recommended by McGranahan *et al*. (1987). For pre-induction the macroelements were reduced to 50 % and IBA (5 or 10 mg/L) was used as hormone. Microshoots, more than 20 mm long, were inoculated in this culture medium and incubated for 5 days in the dark. The expression phase was conducted for 2 weeks under photoperiod conditions (16 h light), with IBA being removed from the culture medium and replacing agar by vermiculite.

Length of subculture prior to root pre-induction

The influence of different ages of microshoots (4, 5 and 6 weeks) during the phase pervious to root pre-induction was assessed. Rooting percentage, number of roots, length of the longest root and height of microshoots were measured.

Dose of sucrose during pre-induction phase

Three concentrations of sucrose (20, 40 and 60 g/L) were used as carbon source in pre-induction culture medium. The effects of each were assessed at the end of the expression phase. Rooting percentage, number of roots, length of the longest root and height of microshoots were measured.

Temporary immersion systems (TIS)

A TIS, similar to that proposed by Lorenzo *et al.* (1998) and Escalona *et al*. (1999), was built. To determine what explants were most suitable for initiation, an experiment was conducted: B1, apical segments with 4-6 nodes and approximately 10 mm in length; B2, apical segments with 8-10 nodes and approximately 20 mm in length; A1, apical segments with 4-6 nodes and approximately 10 mm in length cultured in gelled medium for 1 week and A2, which is equal to A1 but is cultured for 2 weeks in gelled medium prior to inoculation in TIS. Length of microshoots and nodes/microshoots were assessed. Microshoots growing in gelled medium were used as control. After some previous assays, it was established that 45 seconds of immersion per day was enough and the dose of BAP was reduced 50%, the other conditions were the same. In order to avoid overestimation of the results, the same relation of culture medium per explant (10:1) for conventional micropropagation was used. For TIS, fifteen explants were inoculated in vessels with 750 ml volume.

Acclimation

Rooted microshoots were potted in a mix of peat (90 %) and vermiculite (10 %). Temperature ranged from 32 (day) to 16ºC (night). The illumination was at a maximum of 300 µm. m^{-2}. s^{-1} during the first 3 weeks and was kept below 500 µm. m^{-2}. s^{-1} the next 4-5 weeks. Relative humidity went from 60 up to 90 % .

Experimental conditions

Each vessel was selected as experimental unit, and consequently all data are averages obtained from all the microshoots in the vessel. The vessels were randomly distributed in a growth chamber. Successful experiments were repeated at least 2 times. A Bifactorial design was used to discard, or retain, a hypothetic genotypic answer. Statistical significance was determined by means of ANOVAs and post-hoc tests were performed to know the differences between the different levels of each factor. Percentage data were transformed with $\arcsin\sqrt{x}$.

Results and Discussion

In vitro introduction

The fact that our starting material for *in vitro* introduction are branches collected from single-selected trees, highly hampered the performance of standardized experiments. Therefore we have, since 2005, based the protocol on previous experiences. When direct introduction from field grown trees was used, low success was reached.

Moreover, it is a time consuming process, as the culture medium should continuously be renewed due to the profuse releasing of phenolics. This necessitated, in some cases, the subculture every 24 h and, in general, at least every 2 days during the first 5 to 6 weeks. Only a few trees (DA, D15 and D53) could be routinely introduced using this procedure. However, most of genotypes were highly recalcitrant and several attempts were necessary, as well as a high quantity of starting material. Additionally other trees, like G27, were not able to be *in vitro* cultured after 3 years using this method. During the summer of 2008 we tried to introduce 30 *èlite* trees. For that purpose we managed 1264 explants in 61 attempts. Only 83 segments, belonging to 7 genotypes, were finally established *in vitro*, the other 1181 (93.4 %) were discarded for fungi and *bacteria* contamination, for death as a consequence of the high release of phenolics to culture medium and for their inability to respond to *in vitro* conditions. Due to the limited success of this method, we found it necessary to use a more efficient alternative that would include both rejuvenation and growing under controlled conditions of starting material prior to initiation as was recommended by Bonga and Von Aderkas (1992). Hence, segments of epicormic branches approximately 20 cm long, bearing dormant buds, were placed in tap water and stored under controlled conditions (light and temperature) simulating the beginning of spring. The bases of the sticks were removed and the water was replaced weekly. Despite the genotypic influence, after 3 weeks bud sprouting was observed and healthy shoots formed. When these reached at least 20 mm in length, they were collected for initiation (fig 1). Smaller segments were not suitable because they normally form a big callus covering the explants completely, which prevented bud break and shoot growth.

Fig 1 *Forcing softwood formation of walnut hybrid from dormant bud under controlled conditions*

During 2011 and 2012 we had 16 walnut hybrid genotypes introduced successfully; despite the microbiological contamination being still present, the losses associated to this factor were less than those obtained with the other method. However, the most important achievement was that phenolization was completely removed probably due to the use of rejuvenated explants. Moreover, it allowed the reduction of the number of subcultures and the manipulation of explants, facilitating *in vitro* establishment. As a result in 2012 only 211 explants were managed from which 113 (53.5 %) were lost by different causes such as bacterial (30.8 %) and fungal (20.4 %) contamination and only 2.4 % of them died later. Finally, we were able to introduce 8 out of 11 genotypes: 5 walnut hybrids, 1 Persian walnut, 1 black walnut (*J. nigra* L.) as well as the Paradox clone Vlach. The 3 genotypes that did not respond to *in vitro* culture had problems associated to poor bud break and formation of small shoots. This emphasizes the importance of using only healthy shoots with a length of at least 15 mm as starting material. The advantages of this procedure are based probably on the fact that epicormic sprouts arise from inactive meristems produced during the juvenile phase. Therefore it is expected that these explants would be established more easily *in vitro* than more adult meristems (Van Sambeek *et al.* 2002).

Substitution of FeEDTA as iron source

As a first result the microshoots growing in a culture medium supplemented with FeEDDHA had greener leaves and were more vigorous than the ones cultured with FeEDTA (fig 3B). Although growth was stimulated we did not find any statistical influence on the length of microshoots during multiplication as has also been observed in micropropagation of *Rosa hybrida* (Van der Salm e*t al*. 1994). Noteworthy results were reached on rooting and

acclimation. Microshoots growing in the presence of FeEDDHA had a higher ability to root with a higher number and length of the roots than did the ones growing in the presence of FeEDTA (fig 2). The influence of different iron sources on pear micropropagation was also studied and although FeEDDHA increased the number of shoots per

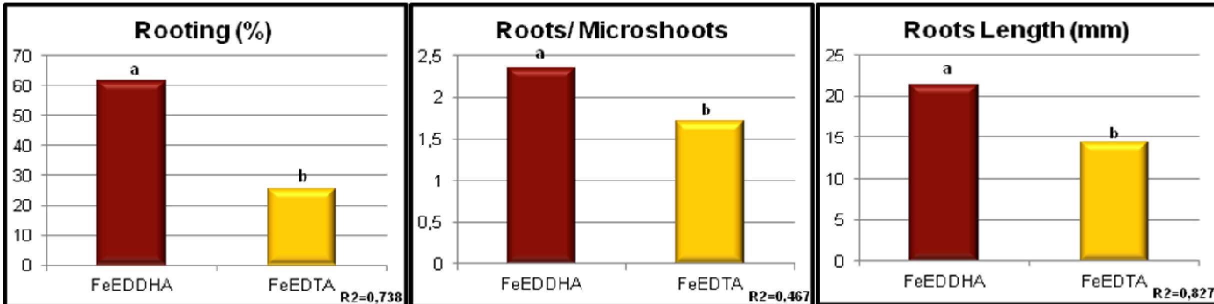

Fig 2 *Effects of substitution of FeEDTA by FeEDDHA on rooting of walnut hybrid microshoots ($p \leq 0.05$)*

explant and fresh mass production, FeEDTA was more suitable than FeEDDHA for rooting (Sotiropoulos *et al.* 2006). If the behaviour of a plant is a manifestation of its metabolic state, with an important influence of nutrition, we assumed that these results are a probable consequence not only of a higher and better availability of iron but of the other mineral components (Sotiropoulos *et al.* 2006) as well. Furthermore, the influence on chemical (pH) and physical (strength) properties of the culture medium has to be considered. Van der Salm *et al*. (1994) did not find important differences in pH in culture medium using both chelates, but under our conditions great variations were observed. The pH of culture medium supplemented with FeEDDHA showed a slight reduction after sterilization, kept stable from the beginning to the second week, on the third week the pH fell down to 4.5, recovering its higher value a week later; at the end it rose up to 6.6 approximately. On the other hand, when FeEDTA is used, the pH drops down to 5.1 after autoclaving, increasing steadily to above 7.0 at the end of the subculture cycle. This high value of pH may be the cause of the decay of microshoots at the end of the subculture, precisely when the differences in pH effect between both chelates are more evident.

The definitive influence on improving the quality of microshoots by replacing FeEDTA by FeEDDHA was observed during acclimation. After acclimation only 1 % of the vitroplants that reached field plantation come from cultures grown on FeEDTA. These were affected not only by chlorosis but also by complete defoliation and apical death (fig 3). McGranahan and Leslie (1988) also found that the retention of leaves was important in promoting the explant survival and growth. An important conclusion was that there was no statistical interaction between genotype and the iron source used. It seems to show that FeEDDHA is more suitable for micropropagation of walnut hybrid than FeEDTA.

Fig 3 *Abnormalities of walnut hybrid microshoots growing in culture medium supplemented with FeEDTA: chlorosis (A, B, and C), defoliation (A and C) and apical death (D)*

The use of Phl during multiplication

In this process Phl has an important role, without it in the culture medium, the microshoots failed to grow, probably as a consequence of the formation only of little *calli* (fig 4). The incorporation of 25 mg/L was enough to promote *calli* formation and growth (table 1). Despite the Leslie *et al.* (2004) finding that when 1 mM of Phl is added to multiplication medium the rooting is promoted, we did not observe any important advantage of increasing the Phl dose to 75 mg/L (table 1). On the other hand, some experiments conducted with higher doses (800 µM) showed a more unstable behaviour of cultures although the auxiliary bud formation was clearly stimulated, especially in species that possesses as high an apical dominance as the walnut hybrid (data not shown). Like with the iron source, there was no interaction between genotype and Phl dose, another important result if we take into account that our main goal is to set up a commercial walnut micropropagation protocol. As Sharifian *et al.* (2009) showed that the effect of Phl on rooting is different for each genotype of *J. regia* L., further experiments should be done under our conditions to determine if the Phl dose has some influence on rooting and/or on the results of acclimation.

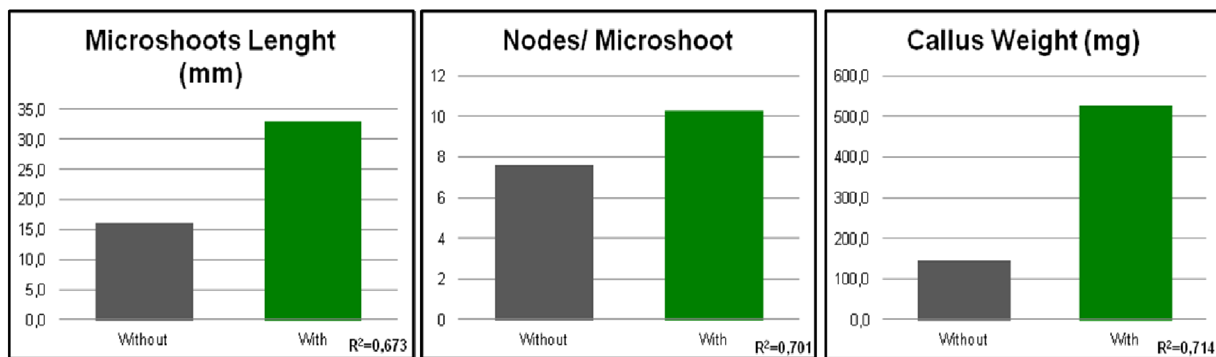

Fig 4. *Influence of Phloroglucinol on calli formation and walnut hybrid microshoots growth (p≤0.05): gray=Without Phloroglucinol; green= with Phloroglucinol.*

Table 1 *Effects of different doses of Phloroglucinol on calli formation and growth of in vitro walnut hybrid microshoots (p≤0.05)*

Phl (mg/L)	*Callus* Weight/ Microshoot (mg)	Nodes/ Microshoot	Length of Microshoots (mm)
0	145,4[b]	8[b]	16,2[b]
25	371,1[a]	10[a]	27,3[a]
50	526,2[a]	10[a]	32,9[a]
75	593,5[a]	11[a]	31,6[a]

Influence of the length of subculture prior to root pre-induction

It is has been stated that rooting has a close relationship with lignin accumulation. Fu *et at.* (2011) found changes in phenylalanine ammonia lyase (PAL) activity during rooting of peony trees as well as differences in the content of total phenolics between easy and difficult-to-root genotypes. Santos Macedo *et al.* (2012) also demonstrated that a significant increase in the accumulation of total phenolics, flavonoids and lignin is observed preceding root initiation in IBA-treated olive explants. At the same time lignin accumulation is associated with aging. In nature, the amounts of acid-insoluble Klason lignin increase with the age of micropropagated regenerants of *Morus nigra* L. (Ďurkovič *et al.* 2012). *In vitro* gardenia microshoots also show an increase in lignin content during adventitious root formation from day 0 to day 20 of induction (Hatzilazarou *et al.* 2006). In walnut lignin accumulation was lower in the shoot population that did not show visible signs of rooting (Bisbis *et al*. 2003). Vahdati *et al*. (2004) found in Persian walnut that the stimulation of growth has a negative influence on rooting

ability, a process that is probably associated with an actively vegetative state, once 3 weeks-old microshoots were used. Other important elements are that auxins only accumulate in the basal part of walnut shoots where new roots will be formed (Falasca *et al*. 2000). The timing for root formation in elm was significantly delayed in the apical part in comparison with the basal part where the first roots appeared (Malá *et al.* 2005). For these reasons and based on the fact that BA (6-benzylaminopurine) concentration strongly affected the deposition of lignin in the vascular cells (Quiala *et al.* 2012), we hypothesized that rooting ability is highly influenced by the age of microshoots. In fact, we found that rooting percentage and number of roots formed improved with the age of the microshoots (fig 5) independently of genotype. Microshoots that are 6 weeks old are more suitable for rooting than those that are 4 weeks old. For some genotypes, for instance D53, the difference goes from 0 % of rooting, in microshoots after the 4th week of subculture to more than 90 % 2 weeks later. These results are be probably influenced by the reduction of the active growth, aging and lignin accumulation in the microshoots.

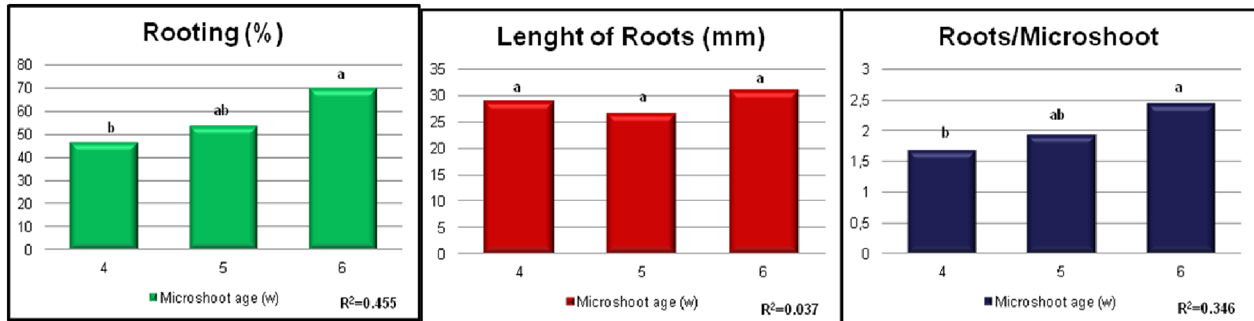

Fig 5 *Influence of the length of subculture on rooting of micropropagated walnut hybrids (p≤0.05)*

Dose of sucrose during pre-induction phase

Osmolytes, like sucrose, have a great influence on water potential of the culture medium. They also act as carbon source and help in determining the morphogenic response (George 1993). We did not find any reference that dealt specifically with the influence of different carbon sources and/or concentrations during pre-induction. Of the two rooting phases experiments with carbon sources have only been done during the expression phase (Dolcet-

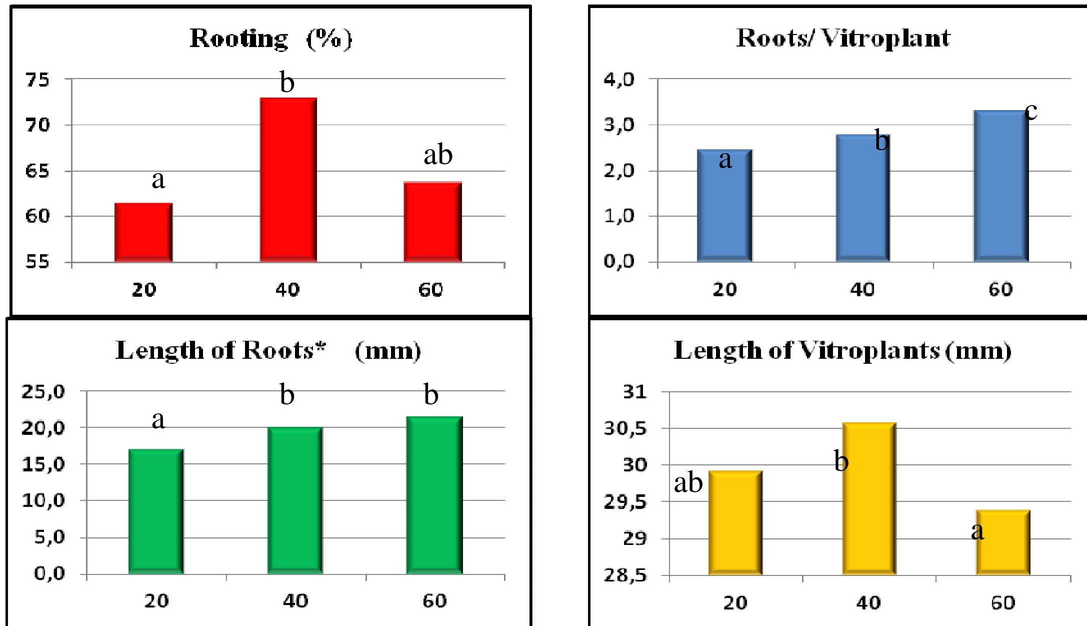

Fig 6 *Effects of different doses of sucrose (g/L) during pre-induction phase on rooting and growth of walnut hybrid microshoots (p≤0.05), * longest root*

Sanjuan *et al.* 2004). Based on our previous experience, and the recommendation of McGranahan *et al.* (1987) to use 53 mg/L of sucrose, we decided to assess the influence of 3 doses in this phase. Like for the other factors there was a genotypic influence and the dose of sucrose determined the rooting rate and the length of microshoots. As our goal is to seek generalities towards the establishment of a commercial walnut micropropagation protocol we will not analyse the genetic contribution. The dependent variables were affected in different ways but the most positive influence was obtained with 40 and/or 60 g/L of sucrose (Fig 6). Although rooting percentage was statistically similar for both doses, the number of roots was higher when 60 g/L was used. Microshoots were smaller with this dose than with 40 g/L, due, probably, to the fact that shoot and root growth are opposite and energy-consuming processes. Hence, under some conditions, when a particular treatment is favoured, for instance rooting, root growth is comparatively higher than shoot development. More details can be seen in Table 2, where, into each genotype group, a clear contraposition between these two variables is observed. Vahdati *et a*l. (2004) also obtained similar results for "Vina" walnut, although they did not find statistically significant differences among doses. These results may suggest that the higher the size of the microshoots the lower the ability for rooting. However, a more precise explanation would be that as they seem to be sharing growth resources, conditions that boost the growth of one of these two systems prevents the other one from maximun development. Supporting this hypothesis is the finding of Nobel *et al*. (1989) in *Agave lechuguilla* where conditions that favour shoot growth (high nitrogen and phosphorus supply) the dry weight of roots was approximately constant while shoot dry weight was increased. Smith *et al*. (1991) also found for several woody species that as long as lateral roots did not develop, *in vitro* plants maintained vigorous shoot growth, whereas shoot growth was temporarily suspended while plants initiated roots *ex vitro*.

Table 2 *Results on rooting and growth of different walnut hybrid vitroplants and sucrose doses ($p \leq 0.05$)*

Gentoype	Sucrose (g/L)	Rooting (%)	Roots/ Vitroplant	Length of Roots* (mm)	Length of Vitroplants (mm)
DM	20	$69{,}0^{cd}$	$1{,}8^{a}$	$15{,}7^{bc}$	$18{,}7^{a}$
DM	40	$90{,}9^{ef}$	$2{,}8^{bcd}$	$19{,}0^{cd}$	$19{,}2^{a}$
DM	60	$98{,}7^{f}$	$4{,}7^{e}$	$26{,}9^{ef}$	$18{,}65^{a}$
DA	20	$73{,}1^{cd}$	$2{,}6^{bc}$	$18{,}3^{c}$	$32{,}44^{b}$
DA	40	$81{,}3^{de}$	$3{,}0^{cd}$	$19{,}1^{cd}$	$30{,}84^{b}$
DA	60	$45{,}3^{a}$	$2{,}1^{ab}$	$15{,}1^{b}$	$31{,}58^{b}$
D51	20	$54{,}7^{ab}$	$2{,}1^{a}$	$11{,}7^{a}$	$35{,}79^{de}$
D51	40	$57{,}4^{bc}$	$2{,}2^{ab}$	$12{,}7^{ab}$	$37{,}08^{e}$
D51	60	$59{,}5^{bc}$	$2{,}9^{cd}$	$15{,}3^{b}$	$35{,}08^{cd}$
D53	20	$75{,}7^{d}$	$3{,}2^{cd}$	$22{,}3^{de}$	$32{,}75^{bc}$
D53	40	$73{,}0^{cd}$	$3{,}4^{d}$	$29{,}2^{f}$	$35{,}2^{cde}$
D53	60	$74{,}3^{cd}$	$3{,}6^{d}$	$28{,}5^{f}$	$32{,}27^{b}$

*Longest root

Although a statistical interaction between genotype and sucrose dose was observed, the worst results were obtained with 20 g/L, except for clone DA (table 2). Both doses, 40 and 60 g/L, seem to be more suitable for rooting and growth, in general, though some extra experiments should be done when abnormal behaviour is observed for a new particular genotype.

Temporary immersion system (TIS)

The advantages of TISs have been demonstrated on micropropagation for banana (Alvard *et al.* 1993), sugarcane (Lorenzo *et al*. 1998), pineapple (Escalona *et al.* 1999), *Eucalyptus* (McAlister *et al.* 2005) and teak (Quiala *et al.* 2012). However, there is only one reference on micropropagation of walnut using a liquid culture

(Roschkel and Pijut 2007). This shows the feasibility to clone black walnut using this system, although we did not succeed with shake cultures. The main goal was to know if our walnuts could be successfully cultured in TISs and if there was a positive influence on elongation of highly recalcitrant genotypes. A former assay gave us the first input regarding the necessity of reduction of the dose of BAP since 1.5 mg/L promoted the formation of hyperhydric microshoots. After correcting the immersion time, the volume of culture medium and the vessel size we decided to determine what kind of explant was suitable for TIS. In a first approach, abnormal microshoots were obtained when the same explant kind that was used for traditional micropropagation was inoculated in TISs. The growth was highly influenced by the explant source, affecting both length and number of nodes, as well as the proportion of suitable microshoots for rooting (table 3). The best option was to use explants cultured for at least 2 weeks in gelled medium (A2), which had a pre-formed basal-callus as well as opened leaves, that could contribute to the absorption of the components of the culture medium. As a consequence, microshoots formed from A2 had a normal appearance similar to that obtained from GM (fig 7C).

Table 3 *Influence of the explant kind on elongation of walnut hybrid microshoots in TISs ($p \leq 0.05$)*

Explant Kind	Microshoot Length (mm)	Microshoot >20 mm (%)	Microshoot >25 mm (%)	Nodes/ Microshoot	Normal Shoots (%)
GM	$20,9^{c}$	$61,3^{c}$	$28,8^{d}$	-	$100,0^{a}$
B1	$20,0^{c}$	$37,8^{e}$	$24,9^{e}$	7^{c}	$94,8^{a}$
B2	$31,1^{a}$	$82,5^{a}$	$59,8^{a}$	8^{b}	$96,9^{a}$
A1	$24,3^{b}$	$55,2^{d}$	$44,8^{c}$	9^{a}	$98,5^{a}$
A2	$31,1^{a}$	$67,7^{b}$	$56,3^{b}$	11^{a}	$98,5^{a}$

The results obtained with B2 are, in general, an overestimation because the size of initial explants was almost double that of the one under the other treatments. The microshoots obtained with this treatment were curled in the base (fig 7B) which is an important limitation to root induction and transplantation to *ex vitro* conditions. The quality of microshoots from A1 are similar to A2 regarding their morphology although they certainly are less

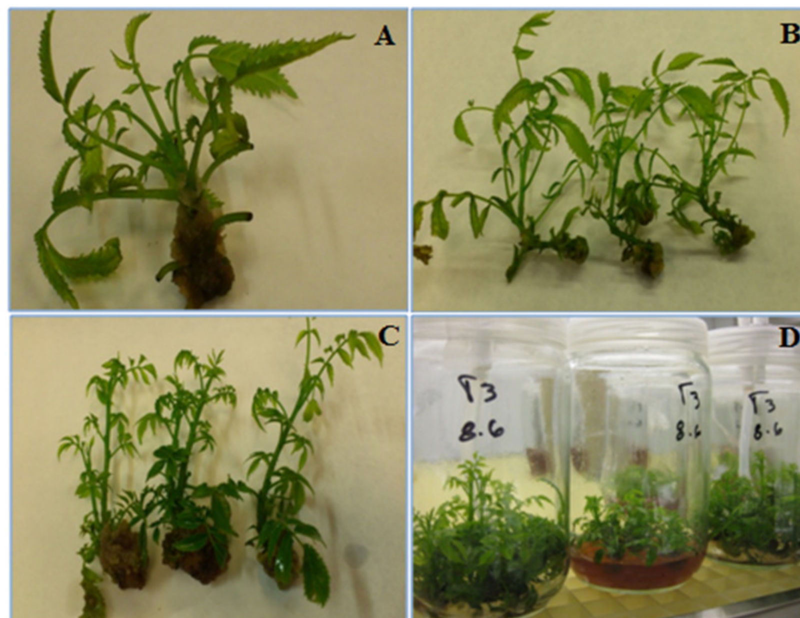

Fig 7 *Behaviour of different kinds of explants in a temporary immersion system (A) B1, (B) B2 and (C) A2, compared to (D) traditionally micropropagated walnut hybrids in gelled medium*

developed. This point to the need of previous adaptation and/or a complete formation of callus prior to the inoculation in TISs. Despite the genotype used, B1was the worst explant, with the lowest growth as well as a profuse formation of *calli*, in some cases covering almost all the stem (fig 7A). Hyperhydricity is a physiological disorder commonly associated to the use of liquid culture, including in TISs (Etienne and Berthouly 2002). Although there were no statistical differences for the percentage of normal shoots, some degree of hyperhydricity appeared in TISs. Therefore further experiments (volume of culture medium, use of growth retardants, extra aeration, time and number of immersion per day) should be done to set more suitable conditions for walnut in TISs.

Resumption of walnut micropropagation and main results obtained

Some key factors were analyzed, that helped us to set the bases for a commercial protocol for walnut hybrid micropropagation at Bosques Naturales S. A. The introduction of Phl and the replacement of FeEDTA by FeEDDHA were the most important changes that allowed us to produce suitable microshoots for rooting and acclimation. Other factors, such as age of microshoots and sucrose dose during root pre-induction, also helped us to enhance rooting ability for all genotypes used.

Acclimated vitroplants showed a high vigour developing a profuse radical system in a few weeks which contributed to obtaining high survival percentages in the green house and field plantations. Similar results were obtained by Dolcet-Sanjuan et al. (2004) with selected genotypes of *J. regia* and several walnut interspecies hybrids. The applications of these results allowed us to clone up to 8 genotypes from 2010 to 2012 (fig 8). This protocol has also been used to micropropagate some other walnut species as *J. nigra*, *J. regia* and Vlach, a hybrid Paradox.

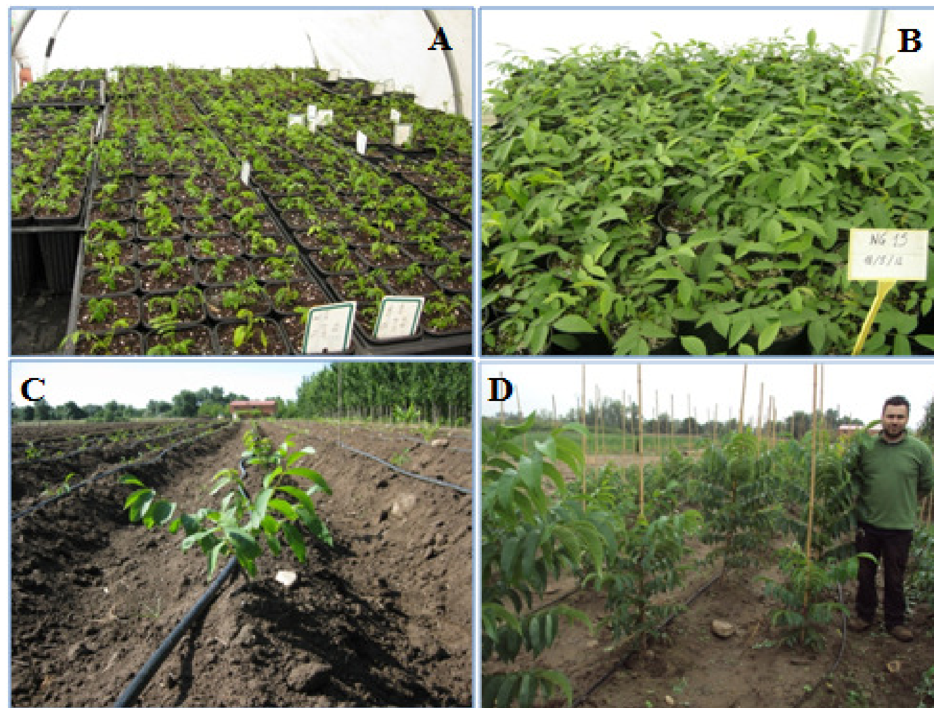

Fig 8 *Walnut hybrid vitroplants after transplantation to ex vitro conditions (A), 3 months old vitroplants in greenhouse (B); field planted in May, 2012 (C) and the same vitroplants 4 months later (D), photo taken September, 26th, 2012*

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References

Ainsley P J, Collins G G, Sedgley M. 2001. *In vitro* Rooting of Almond (*Prunus dulcis* Mill.) *In vitro*. Cell. Dev. Biol.-Plant. 37:778-785

Alvard D, Cote F, Teisson C. 1993. Comparison of methods of liquid medium culture for banana micropropagation. Effects of temporary immersion of explants. Plant Cell, Tissue and Organ Culture. 32: 55-60

Bisbis B, Kevers C, Crevecoeur M, Dommes J, Gaspar T. 2003. Restart of lignification in micropropagated walnut shoots coincides with rooting induction. Biologia Plantarum. 47 (1): 1-5

Bonga J M, Von Aderkas P. 1992. *In vitro* culture of trees. Kluwer Academic Publishers, Dordrecht, The Netherlands:1-232

Docet-Sanjuan R, Claveria E, Gruselle A, Meier-Dinkel A, Jay-Allemand C, Gaspar T, 2004. Practical Factors Controlling *In vitro* Adventitious Root Formation from Walnut Shoots Microcuttings. Journal of the American Society for Horticultural Science. 129 (2): 198-203

Driver J A, Kuniyuki A. 1984. *In vitro* propagation of Paradox walnut rootstock. Hortscience. 19: 507-509

Ďurkovič J, Kaňuchová A, Kačik F, Solár R, Langyelová A. 2012. Genotype- and age-dependent patterns of lignin and cellulose in regenerants derived from 80-year-old tress of black mulberry (*Morus nigra* L.). Plant Cell, Tissue and Organ Culture. 108: 359-370. DOI 10.1007/s11240-011-0047-z

Escalona M, Lorenzo J C, Gonzalez B, Daquinta M, Gonzalez J L, Desjardins Y, Borroto C G. 1999. Pineapple (*Ananas comosus* L.) micropropagation in temporary immersion systems. Plant Cell Reports. 18: 743-748

Etienne H, Berthouly M. 2002. Temporary immersion systems in plant micropropagation. Plant Cell, Tissue and Organ Culture. 69: 215-231

Falasca G, Reverberi M, Lauri P, Caboni E, de Stradis A, Altamura M M, 2000. How *Agrobacterium rhizogenes* triggers *de novo* root formation in a recalcitrant woody plant: an integrated histological, ultraestructural and molecular analysis. New Phytol. 145: 77-93

Fu Z, Xu P, He S, Teixeira da Silva J A, Tanaka M. 2011. Dynamic changes in enzyme activities and phenolic content during *in vitro* rooting of tree peony (*Paeonia suffruticosa* Andr.) plantlets. Maejo Int. J. Sci. Technol. 5 (02): 252-265

George E F. 1993. Plant Propagation by Tissue Culture. Part1. The Technology. 2nd Edition. Exegetics Limited: 1-574

Hatzilazarou S P, Syros T D, Yupsanis T A, Bosabalidis A M, Economou A S. 2006. Peroxidases, lignin and anatomy during in vitro and ex vitro rooting of gardenia (*Gardenia jasminoides* Ellis) microshoots. Journal of Plant Physiology. 163: 827-836. DOI 10.1016/j.jplph.2005.06.018

Leslie C A, Hackett W P, Bujazha D, Hirbod S, McGranahan G H. 2005. Adventitious Rooting and Clonal Plant Production of Hybrid Walnut (*Juglans*) Rootstock Selections. Acta Hort. (ISHS) 705: 325-328

Malá J, Gaudinová A, Dobrev P, Eder J, Cvriková M. 2005. Role of phytohormones in organogenic ability of elm multiplicated shoots. Biologia Plantarum. 50 (1): 8-14

McAlister B, Finnie, Watt M P, Blakeway F. 2005. Use of the temporary immersion bioreactor system (RITA®) for production of commercial *Eucalyptus* clones in Mondi Forest (SA). Plant Cell, Tissue and Organ Culture. 81: 347-358

McGranahan G H, Driver J A, Tulecke W. 1988. Tissue Culture of *Juglans.* In: Cell and Tissue Culture in Forestry. Vol. 3. Case Histories: Gymnosperms, Angiosperms and Palms. Eds. J. M. Bonga and D. J. Durzan. Martinus Nijhoff Publishers: 261-271

McGranahan G, Leslie C A. 1988. *In vitro* Propagation of Mature Persian Walnut Cultivars. HortScience. 23 (1):220

Nobel P S, Quero E, Linares H. 1989. Root versus shoot biomass: responses to water, nitrogen and phosphorus application for *Agave lechuguilla*. Bot. Gaz. 150 (4): 411-416

Pestana M, de Verennes A, Araujo Faria E. 2003. Diagnosis and correction of iron chlorosis in fruit trees: a review. Food, Agriculture and Environment. 1 (1): 46-51

Quiala E, Cañal M J, Meijón M, Rodríguez R, Chávez M, Valledor L, de Feria M, Barbón R. 2012. Morphological and physiological responses of proliferating shoots of teak to temporary immersion and BA treatments. Plant Cell, Tissue and Organ Culture. 109: 223-234

Roschkel C, Pijut P M. 2007. Micropropagation of *Juglans nigra* L. in liquid culture. Society for *In vitro* Biology Annual Meeting, Indianapolis, IN. *In vitro* Cellular and Developmental Biology-Plant. 43: S46

Rustaei M, Nazeri S, Ghadimzadeh M, Hemmaty S. 2009. Effect of Phloroglucinol, Medium Type and Some Component on *In vitro* Proliferation of Dwarf Rootstock of Apple (*Mallus domestica*). International Journal of

Agriculture and Biology. 11 (2): 193-196

Santos Macedo E, Sircar D, Cardoso H G, Peixe A, Arnholdt-Schmitt B. 2012. Involvement of alternative oxidase (AOX) in adventitious rooting of *Olea europea* L. microshoots is linked to adaptive phenylpropanoid and lignin metabolism. Plant Cell Report. 31: 1581-590. DOI 10.1007/s00299-012-1272-6

Sharifian S, Vahdati K, Mirmasoumi M, Ghaem Maghami S A. 2009. Assessment of Phloroglucino Effect on Rooting of Tissue Cultured Persian Walnut. Acta Hort. (ISHS) 812:189-196

Siwach P, Rani Gill A. 2011. Enhanced Shoot Multiplication in *Ficus religiosa* L. in the Presence of Adenine Sulphate, Glutamine and Phloroglucinol. Physiol. Mol. Biol. Plants. 17 (3): 271-280. DOI 10.1007/s12298-011-0074-6

Smith M A L, McClelland M T, Timmermann R. 1991. Anomalous Root Structure on Woody Plants *In Vitro*. J. Environ. Hort. 9 (2): 61-64

Sotiropoulos T E, Almaliotis D, Papadakis I, Dimassi K N, Therios I N. 2006. Effects of Different Iron Sources and Concentrations on *In vitro* Multiplication, Rooting and Nutritional Status of the Pear Rootstock “OHF 333”. European Journal of Horticultural Sciences. 71 (5): 222-226

Vahdati K, _Leslie C, Zamani Z, McGranahan G. 2004. Rooting and acclimatization of *in vitro* grown shoots from mature trees of three Persian walnut cultivars. HortScience. 39 (2): 324-327

Van de Salm T P M, Van der Toorn C J G, Hänish Ten Cate C H, Dubois L A M, De Vries D P, Dons H J M. 1994. Importance of the iron chelate formula for micropropagation of *Rosa hybrid* L. “Moneyway”. Plant Cell, Tissue and Organ Culture. 37: 73-77

Van Sambeek J W, Preece J E, Coggeshall M V. 2002. Forcing Epicormic Sprouts on Branch Segments of Adult Hardwoods for Softwood Cuttings. In: Combined Proceedings International Plant Propagators´ Society. 52: 417-424

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"Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management"
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Building the "pipeline": Applying somatic embryogenesis, bioreactors and transgenic technology to restore the American chestnut

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Keywords: *Castanea dentata*, chestnut blight, gene transfer, somatic seedlings

Abstract: For the past three years, our lab has been part of the Forest Health Initiative (FHI), a multi-institution research project that has as its mission to demonstrate the application of biotechnological tools to address forest health threats in the U.S. The first FHI target species is American chestnut (*Castanea dentata*) and we have been employing embryogenic chestnut cultures to address several FHI objectives focused on restoration of the species. Somatic embryogenesis (SE) is being used to propagate chestnut blight-resistant hybrid backcross-derived material from The American Chestnut Foundation's (TACF) breeding program for clonal testing. SE is also providing target material to test candidate genes (CGs) from Chinese chestnut and heterologous sources that may provide resistance to the blight fungus (*Cryphonectria parasitica*) and/or Phytophthora. New embryogenic cultures have been initiated from several American chestnut full-sib and half-sib families and, for the first time, from hybrid backcross material, including TACF B3F3 families. Airlift bioreactors, tested as an alternative to growing embryogenic suspension cultures in shaken flasks, have greatly accelerated the production of embryogenic material for both somatic embryo production and *Agrobacterium*-mediated genetic transformation, producing sufficient target material for transformation experiments every two weeks. Using new modular vectors constructed specifically for the FHI project, 28 CGs are already in the transformation "pipeline." Transformation frequencies for some target lines have been very high, producing almost 700 putative transformation events per 50 mg of inoculated tissue. The first somatic seedlings carrying the FHI CGs from Chinese chestnut and from heterologous sources are currently in production.

Introduction

At the beginning of the 20th century, American chestnut (*Castanea dentata*) was one of the most important forest trees in the eastern United States, accounting for one-quarter of the trees in the southern Appalachian forest. It was especially dominant on the ridges and benches in the mountains, in some cases forming almost pure stands. The tree grew to tremendous size, with some trees exceeding 2 m in diameter and 34 m in height. American chestnut was an excellent example of a multiple-use forest species. Its timber was both relatively light and strong and was famous for its durability, which was due to its high tannin content. This property and its propensity to split cleanly (i.e. without the need for a saw to make boards) made it the wood of choice for outdoor purposes in rural settings. Huge volumes of chestnut were harvested for utility poles, fences, shingles and siding, as well as for pulp and tannins. The nuts not only provided food for wildlife, but also for the local human population, who consumed them and sold them for income. The accidental introduction of the chestnut blight fungus, *Cryphonectria parasitica*, on Asian chestnut stock around the turn of the century resulted in the death of virtually every mature American chestnut tree within 40 years (Anagnostakis, 1987). The necrotrophic fungus invades through any wound in the bark and kills the living tissue (phloem, vascular cambium, xylem) beneath, forming a sunken canker and eventually girdling and killing the tree. The fungus was first discovered on chestnut trees in the New York Zoological Park in the Bronx, New York City, and eventually spread throughout the natural range of the tree. The resulting epidemic is

widely cited as the worst forest tree health disaster in recorded history. Fortunately, the fungus fails to infect the roots of American chestnut and stem death is followed by re-sprouting from the root collar. Eventually, the new stems are infected and killed back, resulting in repeated cycles of infection, stem dieback and re-sprouting. Most of the stump sprouts are shrub-like and remain in the forest understory, but in some cases, trees have survived for decades and grown sufficiently large to flower and produce nuts. There is evidence that some of these large, surviving Americans ("LSAs"), such as the Amherst Tree in Virginia, have low levels of blight resistance that, combined with other factors, such as site fertility and colonization of cankers with hypovirulent strains of the blight fungus, have allowed them to survive despite being infected with the fungus for several years (Griffin et al., 2006). Unfortunately for the tree, another deadly disease had already eliminated American chestnuts from lower elevation eastern U.S. forests before the blight appeared. *Phytophthora cinnamomi*, probably introduced into the U.S. from Asia on ornamental woody plants in the early 19th Century, causes a number of diseases in forest trees, and specifically "ink disease" in chestnut. Unlike chestnut blight, this Oomycete infects the roots of the tree, killing it quickly and preventing any regeneration via re-sprouting.

Following the initial outbreak of blight in New York City, a variety of approaches were tried to stop the spread of the blight and restore American chestnut. Initial attempts to stop the spread of the fungus included pruning infected branches, spraying with fungicide and establishment of quarantine's or "immune zones." None of these approaches were effective. While initial searches for natural blight resistance within the species were unsuccessful, over the past few decades, the American Chestnut Cooperators Foundation (http://www.accf-online.org/) has identified several LSAs, such as the Amherst Tree mentioned above, some of which appear to have low levels of natural resistance, and have been breeding among these LSAs to try to enhance levels for resistance in the progeny (Griffin et al., 2006). Another attempt at producing blight-resistant trees involved induction of mutations by gamma-irradiating nuts (Dietz 1978). Extensive work has been done by several labs on the application of hypovirulent strains of the blight fungus, which are themselves infected with a hypovirus that attenuates the fungus and makes it possible for infected trees to survive infection with virulent fungus strains (Anagnostakis, 1978). The most effective progress towards generating blight-resistant trees has been made by hybridizing American chestnut with Chinese chestnut (*Castanea mollissima*), which is blight-resistant, but not a large, canopy tree like American chestnut. While initial attempts by the USDA to use this approach failed and were abandoned in the 1960s, a renewed effort by The American Chestnut Foundation (TACF) beginning in the 1980s employed hybridization followed by multiple generations of backcrossing to American chestnut parents, to retain American chestnut traits along with the Chinese chestnut resistance genes (Hebard 2006). Finally, intercrosses were made among the backcrossed trees to bring the Chinese chestnut resistance genes into homozygosity. Trees of this final "B3F3" generation are now being tested in the field for blight resistance and growth performance. However, variability in B3F3 progeny with regard to blight resistance and growth performance will likely remain, since they result from open-pollination in the seed orchard.

As an alternative to conventional breeding, the Merkle Lab at the University of Georgia (UGA) and the Powell and Maynard Labs at the State University of New York – Environmental Science and Forestry (SUNY-ESF) began trying to develop in vitro propagation and gene transfer systems for the American chestnut in the late 1980s, with the idea that candidate genes for blight resistance would probably be identified within a decade and having a gene transfer system for the chestnut already in place would facilitate engineering these genes into the tree.

Development of in vitro propagation and gene transfer for American chestnut

We began working on American chestnut in 1989, with the goal of developing the following tools: (1) A robust *in vitro* propagation American chestnut via somatic embryogenesis, (2) A cryopreservation protocol for American chestnut germplasm and (3) A reliable transformation system for American chestnut. We first reported the induction of somatic embryogenesis (SE) from zygotic embryo explants of American chestnut in 1991 (Merkle et al., 1991), although no somatic seedlings were produced in that study. The embryogenesis induction protocol we use today is very similar to that reported in Merkle et al. (1991). Briefly, green burs are collected in early August and dissected to remove the immature nuts, which are surface-disinfested and dissected to remove the immature seeds. The seeds are cultured on a semisolid induction-maintenance medium (IMM), which is a modified woody plant medium (Lloyd and McCown, 1980) with 4 mg/l 2,4-D and 0.5 g/l L-glutamine, with transfer to IMM with 2 mg/l after one month. The explants that produce embryogenic material, which may be repetitive embryos, proembryogenic masses or, in rare cases, embryogenic callus (Fig. 1), are maintained by monthly transfer to fresh medium. On average, only about 2 percent of explanted seeds produce embryogenic cultures and the period

between explanting and establishment or "capture" of embryogenic cultures is about 5 months.

Conversion of American chestnut somatic embryos proved to be problematic and American chestnut somatic seedling production remained very low until a suspension culture/size fractionation/plating system was developed by Andrade et al. (2005). We currently employ this as our standard protocol in the lab. Suspension cultures are initiated by inoculating approximately 0.5 g of embryogenic cell clusters into 125 ml Erlenmeyer flasks containing 30 ml of liquid IMM (with 2 mg/l 2,4-D). Suspensions are maintained by shaking on a gyratory shaker at 100 rpm in the dark at 25° C. Cultures are fed every two weeks by aspirating out the old medium and adding 30 ml of fresh IMM. For embryo production from the suspension cultures, cell clusters are collected by size-fractionating approximately 45 day-old suspensions on stainless steel sieves. Suspensions are poured through nested sieves with pore sizes of 860 µm and 38 µm, such that cell clumps with diameters between the two pore sizes are collected on

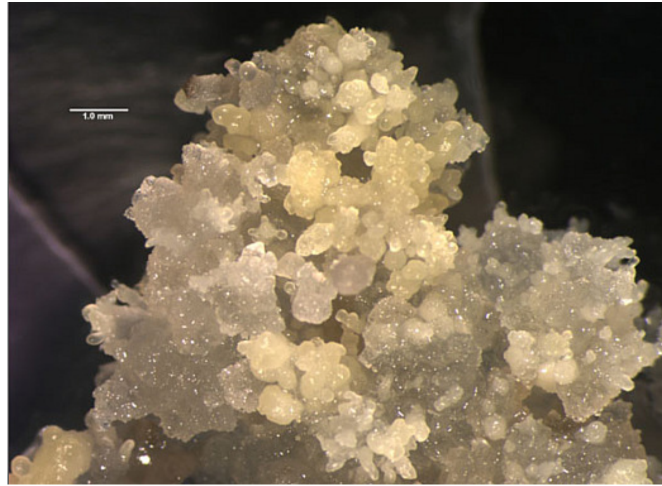

Fig. 1. Embryogenic American chestnut callus.

the 38 µm sieve. Cell clusters collected on the 38 µm sieve are backwashed from the sieve with liquid embryo development medium (EDM; IMM lacking 2,4-D) using a pipette, and collected on nylon mesh using a Büchner funnel under mild vacuum. Cell clusters are cultured along with the nylon mesh on semi-solid EDM to allow somatic embryo development from the cell clusters, which usually takes 2-4 weeks. Then, individual somatic embryos are picked from the nylon mesh and transferred to 100 mm plastic Petri plates (about 25 per plate) with semi-solid EDM and incubated in the dark at 25 C for a few weeks to allow them to enlarge to 0.5 – 1 cm in diameter. Plates of enlarged embryos are wrapped in aluminum foil and stored at 8° C for at least 12 weeks. Following cold treatment, the embryos are transferred to Magenta GA7 vessels containing 100 ml of germination medium (GM; EDM lacking L-glutamine but supplemented with 0.5 g/L activated charcoal) and incubated under cool white fluorescent light (100 µmol•m-2•s-1) with a 16 h photoperiod at 23° C. Following germination, somatic seedlings are transferred to peat-based potting mix and hardened off in a humidifying chamber before transfer to the greenhouse.

Embryogenic American chestnut cultures are very amenable to cryostorage and we routinely store multiple copies of all new embryogenic cultures as well as copies of any transgenic events we produce. As described in Holliday and Merkle (2000), embryogenic chestnut cultures are pretreated in IMM supplemented with 0.4 M sorbitol and are stored in IMM supplemented with 5% DMSO as cryoprotectant. Cryovials with embryogenic material are loaded into Nalgene "Mr. Frosty" containers containing isopropanol, which, when stored in an ultra-low freezer, allow gradual lowering of the temperature of the cryovials to -80°, after which the cryovials are removed and stored in the cryofreezer. Re-growth frequencies following cryostorage are generally higher than 95 percent.Andrade et al. (2009) reported a high-frequency *Agrobacterium*-mediated transformation system for American chestnut using selection in liquid medium, which we still employ in the lab today, with some modification. Briefly, embryogenic suspension cultures are size-fractionated on a stainless steel screens as described above for somatic embryo production and the size fraction of embryogenic cell clumps less than 860 µm is collected and co-cultivated with Agrobacterium. Following co-cultivation, cell clumps are cultured in flasks of

liquid selection medium, which is IMM supplemented with Geneticin, the concentration of which varies with the target line, depending on its sensitivity to the antibiotic. Within a few weeks, Geneticin-resistant colonies appear in the flasks of selection medium against a background of stagnated, non-transformed cell clumps and these resistant colonies are removed and cultured individually as putative independent transgenic events. Each transgenic event is grown up in its own flask of selection medium for somatic embryo and somatic seedling production following the protocol described above for non-transgenic material. Andrade et al. (2009) reported that this protocol had an average efficiency of four independent transformation events per 50 mg of target tissue, with minimal escapes. The time between co-cultivation and transgenic somatic seedlings ready for the greenhouse is approximately 10 months.

Applying chestnut somatic embryogenesis for the Forest Health Initiative

Soon after we reported the chestnut transformation system described above, significant new support for chestnut biotechnology research was provided by the Forest Health Initiative (FHI; www.foresthealthinitiative.org/). FHI chose American chestnut as its first target for research in its mission to demonstrate the application of biotechnological tools to address forest health threats in the U.S. The application of in vitro clonal propagation and transgenics is part of a "braided" approach to bring the tools of biotechnology to bear on the chestnut blight problem, which also includes efforts in the areas of germplasm, breeding, genomics and gene discovery. In addition to biological sciences research, the FHI also includes teams focusing on social and environmental issues and on regulatory and legal affairs associated with biotechnology and forest health. As part of this effort, we are collaborating with scientists from multiple universities (SUNY-ESF, Penn State, Clemson), The American Chestnut Foundation (TACF) and the USDA Forest Service to employ SE for several project objectives. The FHI goals of the UGA group were to: (1) collect chestnut germplasm to initiate new embryogenic cultures, (2) test somatic embryo/somatic seedling production abilities of new cultures to identify best lines for transformation and clonal testing. (3) cryostore copies of all embryogenic cultures, (4) construct vectors for cloning and expression of candidate genes (CGs) for *Cryphonectria* and *Phytophthora* resistance, (5) generate at least 20 transgenic events with at least 10 trees per event for each of 30 CGs for resistance screening, and (6) establish field sites with transgenic lines for disease resistance screening. The last 3 steps are known as the "transgenic pipeline" for the project. Over the first three years of the FHI project (2009-2011), we have used the culture initiation protocol described above to culture thousands of chestnut seed explant provided by multiple cooperators, including The American Chestnut Foundation, the American Chestnut Cooperators Foundation, and the Virginia Department of Forestry. We have established over 450 new embryogenic cultures from American chestnut, Chinese chestnut and TACF B3F3 hybrid backcross chestnuts. Initiation frequencies ranged as high as 6 percent for some families. It is notable that this is first time that TACF B3F3 material has been cultured and that initiation frequencies for B3F3 material using our protocol were not significantly different from those for pure American chestnuts. We have screened the majority of the new cultures for their ability to produce somatic seedlings and have identified several promising pure American chestnut and B3F3 lines with potential to be used for clonal testing and/or for transformation targets. The fact that our SE protocol works for propagating B3F3 material (Fig. 2) is an indication that the combination of breeding programs with SE technology and cryostorage, as already employed for some top commercial conifers by companies such as ArborGen and CellFor, could also be applied to TACF's breeding program. Thus, compared to the somewhat variable output from TACF's open-pollinated seed orchards, SE technology makes possible mass propagation of elite varietal chestnuts for timber or nut production.

The "transgenic pipeline" for testing candidate genes (CGs) for their potential to confer resistance to *Cryphonectria* or *Phytophthora* has been running since the beginning of the project. CGs have come from both Chinese chestnut and heterologous sources. Chinese chestnut CGs were identified by the FHI Genomics and Gene Discovery group working with comparative transcriptomics of canker tissue from blight resistant Chinese chestnut and blight-susceptible American chestnut. Some of these CGs were also known to be in the genomic intervals to which blight resistance has been mapped in Chinese chestnut. CGs were cloned from chestnut libraries by Dr. Bill Powell at SUNY-ESF and cloned into the *pFHI-03* vector in Dr. Joe Nairn's lab at UGA. This vector has modular construction for insertion of different genes and promoters at rare restriction sites. Both the Powell/Maynard labs at SUNY-ESF and the Merkle Lab at UGA have transformed the CG vectors into American chestnut cultures and begun to regenerate transgenic plants that will eventually be screened for disease resistance. To date, 27 Asian chestnut, 4 heterologous and 3 reporter genes have been transformed into multiple American chestnut backgrounds using this system. During this work, transformation frequencies have risen from those previously reported by Andrade et al. (2009) to almost 700 putative transformation events per 50 mg of inoculated tissue for some target

Fig. 2. Somatic seedling derived from B3F3 chestnut hybrid backcross culture.

Fig. 3. Airlift bioreactors.

Fig. 4. Transgenic American chestnut trees planted in the nursery

lines. Thousands of transgenic somatic embryos have been produced. Currently, there are transgenic trees in the greenhouse for eight of the candidate genes, with several more genes at the stage of *in vitro* somatic seedlings. In order to reach this level of productivity, we found that we had to grow chestnut target material faster than the shaken-flask method we had previously employed. Airlift bioreactors, which employ a simple construction (1000 mL Kimax bottles with liquid IMM, aerated by a vacuum pump; Fig. 3), greatly accelerated the production of embryogenic culture material, generating sufficient target material to conduct transformations every two weeks (Kong et al., 2011).

While the first of the trees transformed with FHI CGs have yet to be planted in field tests, some transgenic chestnuts from previous work that pre-dates the FHI have been planted out in Georgia. Over 100 trees representing different transgenic events with the ESF39A synthetic anti-microbial peptide gene (Newhouse et al., 2007) were planted in our Whitehall Nursery near Athens, GA in May 2011 and some of these are expected to reach a size suitable for blight screening in 2013 (Fig. 4).

Clearly, our work for The Forest Health Initiative has really just begun. Over the coming years, we hope to grow thousands of chestnut somatic seedlings, both from conventionally bred material and transgenic material to the size where they can be screened for disease resistance and other performance characteristics. Thus it is likely that somatic embryogenesis will have a significant role in restoring the American chestnut to the forests of the eastern United States.

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References

Anagnostakis SL (1978) American experience with hypovirulence in *Endothia parasitica*. In: MacDonald WL, Cech FC, Luchock J & Smith C (eds.), Proceedings of the American Chestnut Symposium, West Virginia University, Morgantown, West Virginia, January 4-5, 1978, (pp. 37-39).

Anagnostakis SL (1987). Chestnut blight: the classical problem of an introduced pathogen. Mycologia 79:23-37

Andrade GM & Merkle SA (2005) Enhancement of American chestnut somatic seedling production. Plant Cell Rep. 24: 326-334

Andrade GM, Nairn CJ, Le HT & Merkle SA (2009) Sexually mature transgenic American chestnut trees via embryogenic suspension-based transformation. Plant Cell Rep. 28:1385-1397

Dietz A (1978) The use of ionizing radiation to develop a blight resistant American chestnut, *Castanea dentata*, through induced mutations. In: MacDonald WL, Cech FC, Luchock J & Smith C (eds.) P roceedings of the American Chestnut Symposium, West Virginia University, Morgantown, West Virginia, January 4-5, 1978, (pp.17-19).

Griffin G, Elkins J, McCurdy D & Griffin L (2006) Integrated use of resistance, hypovirulence, and forest management to control blight on American chestnut. In: Steiner KC & Carlson JE (eds.) Restoration of American Chestnut To Forest Lands - Proceedings of a Conference and Workshop, May 4-6, 2004, The North Carolina Arboretum, Natural Resources Report NPS/NCR/CUE/NRR - 2006/001 (pp. 97-107). USDI National Park Service, Washington, DC

Hebard FV (2006) The backcross breeding program of the American Chestnut Foundation. In: Steiner KC & Carlson JE (eds.) Restoration of American Chestnut To Forest Lands - Proceedings of a Conference and Workshop, May 4-6, 2004, The North Carolina Arboretum, Natural Resources Report NPS/NCR/CUE/NRR - 2006/001 (pp. 61-77). USDI National Park Service, Washington, DC

Holliday CP & Merkle SA (2000) Preservation of American chestnut germplasm by cryostorage of embryogenic cultures. J. American Chestnut Foundation 14(1):46-52.

Kong L, Tull R, Holtz CT, Montello PM, Sakowski R, Nairn CJ & Merkle SA (2011) Application of airlift bioreactors for highly efficient genetic transformation of American chestnut. In: Nelson CD, Rousseau, RJ & Yuceer C (eds.) Proceedings of the 31st Southern Forest Tree Improvement Conference, June 13-16, 2011, Biloxi, MS (p. 102)

Lloyd G & McCown B (1980) Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot-tip culture. Proc. Inter. Plant Prop. Soc. 30:421-427

Merkle SA, Wiecko AT & Watson-Pauley BA (1991) Somatic embryogenesis in American chestnut. Can. J. For. Res. 21:1698-1701

Newhouse AE, Schrodt F, Liang HY, Maynard CA & Powell WA (2007) Transgenic American elm shows reduced Dutch elm disease symptoms and normal mycorrhizal colonization. Plant Cell Rep. 26:977-987

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New ornamental conifers for harsh northern conditions through cutting propagation of special forms of Norway spruce

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Key words: ageing, genotypic variation, ornamental tree, propagation time, rooting substrate

Abstract: The propagation ability of 17 taxa of Norway spruce was examined with the aim of evaluating their potential as new cultivars for landscaping under the harsh conditions of Northern Europe. Rooting of shoot cuttings was chosen as propagation method in order to provide easily accessible and low-cost application for plant producers. The effects of donor age (varying from 5 to 55 years), timing of propagation (winter versus summer cuttings), type of cutting (plain or with the heel), length of cutting, and rooting substrates (peat-vermiculite or Spruce-Rhododendron soil™-bark-vermiculite) on the rooting success were studied. The rooting success varied highly among the taxa. Generally, the forms having normal height growth but colored needles rooted more easily than the forms without apical dominance or the ones showing reduced or pendulous growth. The overall rooting success of the winter cuttings highly exceeded that of the summer cuttings. Moreover, the late-summer cuttings needed a period of dormancy to be able to form roots. Amongst the winter cuttings, the use of peat-vermiculite as substrate clearly increased the average rooting (22.6 %) compared with that of Spruce-Rhododendron soil™-bark-vermiculite (10.6 %). The relatively high rooting success of the cuttings originating in the 45-50-year-old donor trees, 51-53 %, was achieved with the best combination of the treatments (i.e. winter cuttings propagated in peat-vermiculite) and the clones (*P.a.* f. *aurea* clone K219, *P.a.* f. *cruenta* clone U2080, and the regular and dense-crowned clone K359). The satisfactory rooting success of 52 and 42 % was also achieved in the 20-year-old hybrids of *P.a*. f. *globosa* × *P.a.* f. *cruenta* and *P.a*. f. *cruenta* × *P.a*. f. *pendula*, respectively.

Introduction

Landscaping is a growing business, both in private and public sectors in modern societies. In Finland, for example, the import of woody ornamentals has more than doubled in the 2000´s, comprising the economical value of circa five million Euros currently (Uimonen 2011). In Northern Europe, the market now demands consistent and sustainable production of hardy, ornamental conifers. These could replace the less hardy imports and would be suitable for use in landscaping under the harsh conditions, in which deciduous tree species appear without leaves for half a year (Nikkanen 2009). For urban tree selection, an ideal conifer tree species should not only have a good overall adaptation to local climate, but also a reasonable tolerance to the urban environment and stresses (Dirr 1998, Sæbo et al. 2005). Trees with these qualities, combined with additional ornamental traits and a growth habit that requires a minimum of maintenance work should be preferred (Raisio 2009).

There are ornamental forms of native conifers that are hardy and well adapted to harsh Northern conditions. These naturally born forms found in forests have been collected and registered e.g. in Finland (Oskarsson and Nikkanen 2001, Nikkanen 2009) and Latvia (Zilins et al. 2009). Recently, also a small number of crossings between certain forms have been produced in order to find new hybrids for ornamental use. New forms raised this way are crossings between the red coloured spruce (*Picea abies* f. *cruenta*), the weeping spruce (*P. abies* f. *pendula*) and the compact globe spruce (*P. abies* f. *globosa*) (Lehtonen and Nikkanen 2008).

For the cloning of ornamental conifers, potential vegetative propagation methods include grafting techniques, rooted cuttings and tissue culture (Hartmann et al. 2011). Grafting as a propagation method generally works well in coniferous species, but is labor intensive and has a moderate multiplication rate (Hartmann et al. 2011,

Dirr and Heuser 2006). Compared with grafting, higher multiplication rates can be achieved by rooting shoot cuttings. However, the applicability of the method varies remarkably among the coniferous species (Dirr and Heuser 2006) and is dependent on the age of the donor trees (Bonga 1982). Compared with grafting and rooting shoot cuttings, tissue culture has the highest multiplication rate (Hartmann et al. 2011). The method is, however, not yet well developed for routine multiplication of adult conifers with well-known characteristics (Bonga and von Aderkas 1992), despite recent improvement of the technique (Klimaszewska et al. 2011).

Of the Nordic conifers, Norway spruce (*Picea abies*) is the species having the biggest number of both commercial cultivars and special forms (Krüsmann 1985, Oskarsson and Nikkanen 2001, Nikkanen and Velling 2011); thus also providing the best opportunities for selecting new ornamental trees for harsh conditions. Propagation of the cuttings in Norway spruce has already been studied for several decades (see e.g. Farrar 1939), in an effort to apply it for tree breeding and reforestation purposes (Kleinschmit et al. 1973, Kleinschmit and Schmidt 1977, Sonesson and Hannerzt 2002, Mikola 2009). There is, however, only some information on propagation of ornamental cultivars published (Kelly 1972, Oliver and Nelson 1957, Iseli and Van Meter 1980). Generally, the conditions recommended for rooting shoot cuttings of spruce and the results of rooting following different treatments vary extensively and thus it can be concluded that local conditions and genotypes should always be considered in order to achieve commercially satisfactory results (Dirr and Heuser 2006).

The aim of the present study was to examine the propagation ability of Norway spruce genotypes having potential to be used in landscaping in a harsh environment but that have not been used commercially earlier. Rooting of shoot cuttings was chosen as propagation method in order to provide easily accessible and low-cost application for plant producers. In addition to different taxa, donor age, timing of propagation, type and length of cutting, and rooting substrates were examined in order to find the best options for cloning of new, hardy ornamental conifers for Northern conditions.

Material and methods

Plant material

In its genetic register, the Finnish Forest Research Institute (Metla) has records of 1850 individual trees that are genetic deviants of the tree species native to Finland. The number of the deviant Norway spruce trees in the register is 950. Many of these deviant trees have also been conserved in clone archives and arboretums, some of which served as a source of material for this study.

Altogether 17 different taxa of Norway spruce, described in detail in the Table 1, were used for the present study. Fifteen of them were clones of the registered natural genetic deviants, and two were hybrids originating in the controlled crosses between special forms of Norway spruce (Table 1).

Table 1. *The Norway spruce material used in the rooting experiments.*

Ornamental form	Taxon	Origin of taxon	Propagation material		Growth habit	Needle color	Included in experiment
			Origin	Age			
Picea abies f. *tabulaeformis*	Clone E2165	Suomusjärvi N60°24', E23°47'	Imatra, Pelkola N61°08', E28°49'	45	no apical dominance	green	March August
Pendulous form with no top	Clone K1487	Tohmajärvi N62°20', E30°08'	Imatra, Pelkola	45	no apical dominance	green	March August
Dwarfish growth	Clone E228	Orivesi N61°43', E24°25'	Imatra, Pelkola	45	reduced growth, dense branching	green	March August
Dwarfish growth	Clone E440	Askola N60°31', E25°35'	Imatra, Pelkola	45	reduced growth, dense branching	green	August
P.a. f. *globosa*	Clone E1730	Somero N60°35', E23°40'	Punkaharju, propagation garden N61°48', E29°20'	12	reduced growth, dense branching	green	August
P.a. f. *globosa*	Clone E2491	Loppi N60°37', E24°26'	Punkaharju, propagation garden	12	reduced growth, dense branching	green	August
Progeny of *P.a.* f. *globosa*	Clone E11387	Lohja	Punkaharju, field experiment N61°48', E29°19'	55	reduced growth, dense branching	green	March August
Progeny of *P.a.* f. *globosa*	Clone E11387	Lohja	Punkaharju, propagation garden	5	reduced growth, dense branching	green	August
Progeny of *P.a.* f. *globosa*	Clone E11389	Lohja	Punkaharju, field experiment	55	reduced growth, dense branching	green	March

Progeny of *P.a. f. globosa*	Clone E11387	Lohja	Punkaharju, propagation garden	5	reduced growth, dense branching	green	August
Progeny of *P.a. f. globosa*	Clone E11389	Lohja	Punkaharju, field experiment	55	reduced growth, dense branching	green	March
Regular and dense crown	Clone K359	Rautalampi N62°44', E26°41'	Imatra, Pelkola	45	normal	green	March August
P. a. f. pendula	Clone E475	Mäntsälä	Imatra, Pelkola	45	pendulous	green	August
P. a. f. pendula	Clone E479	Mäntsälä N60°42', 25°07'	Imatra, Pelkola	45	pendulous	green	March August
P. a. f. pendula	Clone E11601	Mäntsälä	Tuusula, Ruotsinkylä	20	pendulous	green	August
P. a. f. pendula	Clone E11602	Mäntsälä	Tuusula, Ruotsinkylä N60°21', E25°00'	20	pendulous	green	March August
P. a. f. aurea	Clone K219	Viitasaari N63°00', E26°00'	Punkaharju, old clone archive N61°48', E29°20'	50	normal	yellow	March August
P. a. f. cruenta	Clone U2080	Jämtland, S N63°48', E16°25'	Punkaharju, old clone archive	50	normal	red	March
Globular growth with red color	Full-sib family	*P.a. f. globosa* (Hyvinkää) × *P.a. f. cruenta* (Jämtland, S)	Mäntsälä, Ohkola N60°32', E25°11'	20	reduced growth, dense branching	red	March August
Pendulous growth with red color	Full-sib family	*P.a. f. cruenta* (Jämtland, S) × *P.a. f. pendula* (Mäntsälä)	Mäntsälä, Ohkola	20	pendulous	red	March August

The main criteria for the selection of these taxa were their ornamental qualities i.e. low growing individuals with no apical dominance, erect individuals with pendulous crown, individuals having globular crown with reduced growth, and individuals with special color of needles were chosen (Fig. 1). Selection was performed among the genotypes that were available in numbers allowing collection of hundreds or thousands of shoots for cutting propagation yearly. Shoot material used as cuttings was collected from old or rejuvenated (by grafting on seedling stock) donor trees (Table 1).

Fig 1 *Ornamental forms of Norway spruce tested for rooting winter cuttings in the March experiment.*

Preparation of cuttings

Branches for two experiments studying cutting propagation were collected from six different locations (Table 1). From upright growing forms of more than five meters in height, branches were cut from the second quarter of the crown from the top, and from shorter and dwarf trees all the crown was used.

For the rooting experiment initiated in March, the branches were collected in advance in order to avoid deep snow and severe frost. The collection took place on 15th and 17th of December and on 11th and 18th of January. The branches were stored in boxes with snow at the temperature of -5°C to prevent their dehydration. The experiment was established within four days, from 9th to 12th of March, 2011.

For the August experiment, the branches were collected between 11th and 16th of August, and stored under moist conditions in cold storage, at the temperature of +2-+4°C. The cutting preparation and insertion into the substrate took place from 17th to 19th of August, 2011.

Only lateral shoot tips representing the youngest growth were used for cuttings. In each taxon, half of the cuttings were prepared by excising the shoot near to base (plain cuttings), while the other half was torn off so that a heel of the previous year's growth was left on the cutting (cuttings with the heel). Finally, the heel was partially removed by careful trimming with a knife.

Rooting experiments

Two types of rooting substrates were used for both experiments: the mixture of fertilized peat (White 420 W F6, Kekkilä, Finland) and vermiculite in proportion of 50:50 (v/v) and the mixture of Spruce-Rhododendron Soil™ (Kekkilä), bark and vermiculite in proportion of 15:15:70 (v/v).

The experiments were conducted using a split-split-plot design with the rooting substrate as a main plot factor, the taxon as a sub-plot factor and the cutting type as split sub-plot factor. To control the variation in rooting conditions within the greenhouse, the experiment was split into two blocks.

In the March experiment, both blocks were divided into two main plots, one containing seven growing boxes of peat-vermiculite substrate and the other containing seven boxes of Spruce- Rhododendron soil-bark-vermiculite substrate, i.e. altogether of 14 boxes within the block and 28 in the whole experiment. In the August experiment, the main plots consisted of eight boxes of each substrate, i.e. altogether the total of 32 boxes in the whole experiment. In each box, there was room for 200 cuttings. The box was, in most cases, divided into two taxa (sub-plot factor). In every taxon, half of the cuttings were plain and the other half with heel (split sub-plot factor). The number of the cuttings per taxon-cutting type-substrate –combination was 50, i.e. 400 cuttings were rooted per each form, except in a few cases, in which there was not enough material for that. In these cases the number of the cuttings in some split sub-plots was 25 at the minimum. Location of the cutting types was randomized within the taxa, and the location of forms was randomized within the boxes containing the same substrate. Location of the substrates was randomized within the blocks.

Rooting experiment was established in a greenhouse equipped with bottom heat and controllable rooting conditions. The air temperature was set to 15°C, and the temperature of rooting media to 22°C. The air humidity was regulated by an automatic mist system, and targeted to 80-90%. Additional light was applied during rooting when necessary, and set to 12h/12h day/night photoperiod so that the minimum of 214 µMol s-1m-2 of light during the day period was achieved.

Fungicide treatment of the cuttings was carried out by spraying them in two-week intervals with 0.07 % Topsin M® (Nippon Soda Ltd, Japan). If fungal growth was observed on the substrate, additional spraying with 0.3 % Tirama50™® (Kemira Agro, Finland) was carried out.

Observations and measurements

Observations and measurements started 12 weeks after the establishment of the experiments. The measured traits were vitality and length of cutting, rooting of cutting and number of roots. Vitality was classified in three categories: alive and new shoot growth, alive but no new shoot growth, and dead. Length of cutting was measured, and the number of roots was counted. All the rooted cuttings were transplanted into fertilized peat (White 420 W F6, Kekkilä), and their cultivation was continued in normal green house conditions.

In the August experiments, observations made after 12 weeks of rooting revealed hardly any rooted cuttings. Therefore the material was transferred to a cold storage (+4ºC, dim light for 6 h/day) for overwintering, and taken back to the rooting conditions defined above in the March 2012. New observations were then done after 12 more weeks of rooting.

Statistical analyses

Statistical analyses were computed only for the data collected from the March experiment, due to low rooting success in the August experiment. Rooting percent for each split sub-plot was calculated as the proportion of the rooted cuttings of all cuttings inserted.

To evaluate the effect of the rooting substrate and cutting type on the rooting amongst the studied taxa, a mixed model analysis of variance (ANOVA) was computed for the rooting percent using a model adjusted for a split split-plot design randomized on complete blocks. The statistical model used, consisted of block (two levels), substrate (two levels), taxon (12 levels), cutting type (two levels) and their interactions as fixed factors (Table 2). The model was built by testing block by substrate and block by taxon within the substrate as random factors. The block by substrate factor was omitted from the model as redundant, and thus, only block by taxon within substrate was used as a random factor in the final model.

Also, a model for probability of rooting was developed using logistic regression analysis. Rooting substrate, taxon, needle color, growth habit, age of donor tree, cutting type and block were tested as covariants for binary response variant (rooted or not). The best fit of the model was evaluated by stepwise addition of the covariants into the model, and by comparing the coefficient of determination (Nagelkerke's R2) and goodness of fit test variables (Hosmer and Lemeshow $\chi 2$) computed for the different models. The best fitting model included substrate, needle color, growth habit, and age of donor tree as factors (see Table 3). Kendall's coefficient of concordance (W) was computed to evaluate the dependency between the rooting success and the number of roots. Mean values of the taxa were used for the analysis (n=12). The relationship between the length of cuttings and rooting percent was tested with Pearson Correlation analysis. For the analysis, the mean cutting length per each split sub-blot was used. Prior to the parametric analyses, the distribution of the data was evaluated from residual plots. All the statistical analyses were performed using PASW (version 17.0).

Results and discussion

The cuttings represented 17 different taxa that were classified according to their growth habit and needle color. The rooting success varied remarkably among and within these classes. Timing of propagation had the greatest influence on the rooting percentage of the cuttings. Also the age of the donor tree and rooting substrate used were found to affect the rooting success.

Timing of propagation

In the present study, there was a striking difference in the rooting success between March and August experiments. In March, the overall rooting percentage was 16.7 %, but in August, only 44 of the 6250 cuttings i.e. 0.7 % had rooted after 12 weeks. Following the cold storage, 2174 cuttings i.e. 35 % of the August material was still alive, and 4.7 % of them rooted. Because of this low rooting success, the effects of the other factors were examined using only the data from the March experiment.

The propagation times tested in the present study were chosen based on the recommendations of earlier studies. Farrar (1939), Oliver and Nelson (1957), and Girouard (1975) recommended the use of winter cuttings collected from dormant donors, while Kelly (1972), and Iseli and Van Meter (1980) preferred summer cuttings collected at the end of the active growing period but before cold acclimatization of the donors takes place.

In Farrar's (1939) study in Connecticut, US, the timing of winter cutting collection was critical for rooting success: from October to January, the best rooting was achieved in the cuttings taken in December. The same collection time was the best also for the ornamental cultivars of *P. abies* f. *ohlendorffii, P. a.* f. *pygmaea*, and *P.a.* f. *remontii* studied in Ottawa, Canada (Oliver and Nelson 1957). Girouard (1975) made an extensive study on timing of cutting propagation in Norway spruce in Quebec, Canada. He collected shoots from 7-year-old donors with the interval of 2-3 weeks throughout two consecutive years and found the highest rooting percentages in cuttings taken in April and May just before or during bud burst. The second most favorable collection period was during October and November when the donor plants had been subjected to cool temperatures.

Kelly (1972) studied dwarf cultivars of Norway spruce in Ireland and found that summer cuttings rooted better than the winter cuttings taken in March. He collected shoots at two week intervals from late July to the beginning of September, and observed the best rooting at mid-August, although the optimum time for each genotype varied. Iseli and Van Meter (1980) studied dwarf cultivars of Norway spruce in Oregon, US. They found summer cuttings collected from the beginning of August to mid-September rooted better than the ones taken in winter, from January to mid-March.

In the present study, only the winter cuttings rooted reasonably. This might be related to the Northern origin (N60º24' – N63º48') of the present taxa and their adaptation to a short growing season. Growth cessation in Norway spruce that is induced by the critical night length and temperature sum needed, is dependent on the latitude of origin (Partanen 2004, Gyllenstrand et al. 2007). Thus, mid-August as collection time was not optimal for the present genotypes, although recommended by earlier studies performed in more Southern origins. Probably the physiological status of the Northern trees at mid-August is already turned towards dormancy, which inhibits rooting. This is also reflected by the fact that after overwintering, part of the August cuttings in the present study could be rooted. Considering the overall shortness of the growing season at high latitudes, the time window for collecting summer cuttings of Norway spruce is presumably rather narrow, and earlier timing for collection of the cuttings should be tested.

Rooting success of the different taxa

There was remarkable variation in the rooting percentage of the cuttings among the tested taxa (Fig. 2-3, Table 2). As studied with the material in the March experiment, significant differences in rooting success were also found among the groups of different taxa, classified according to their growth habit and needle color (Table 3).

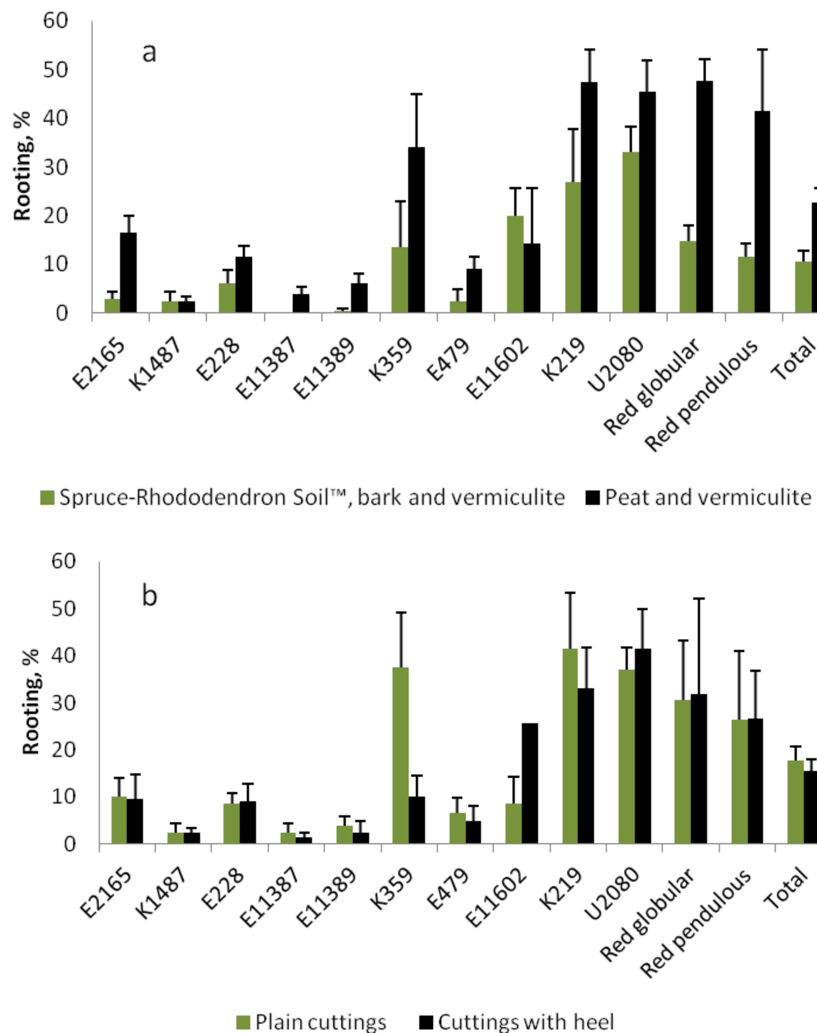

Fig 2 The average rooting percent (±SE) of the shoot cuttings in the different Norway spruce taxa in the March experiment. (a) Rooting on different substrates. (b) Rooting of different cutting types.

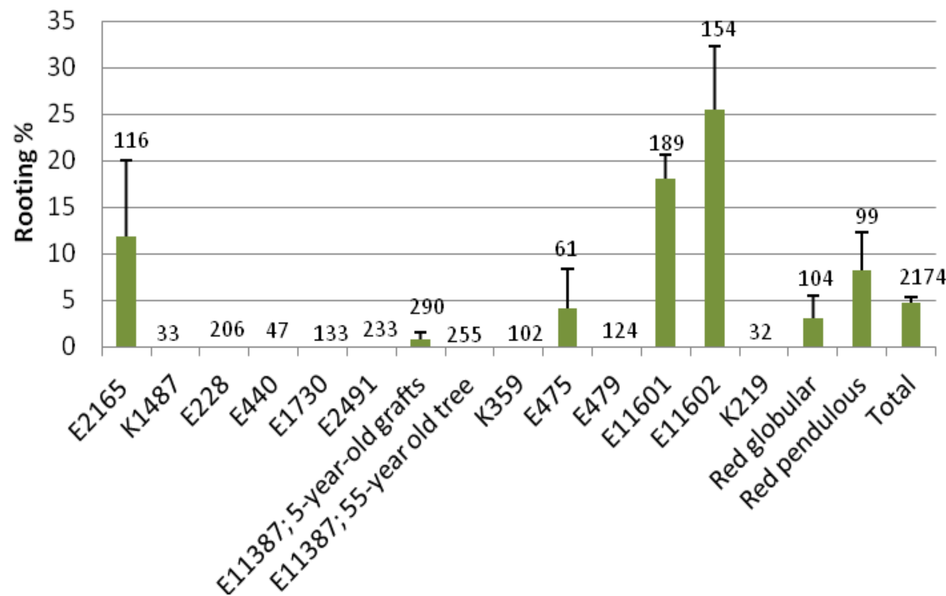

Fig 3 *The average rooting percent (±SE) of the shoot cuttings in the different Norway spruce taxa in the August experiment after overwintering at cold storage. The number of cuttings in each taxon is shown on the bars.*

Table 2. *Results of the ANOVA for the differences in the rooting percent among the studied taxa, affected by the different substrates and cutting type. Asterisks, *, ** and *** indicate, that the difference between the means is significant in the levels of 0.05, 0.01 and 0.001, respectively*

Source of variation	**Degrees of freedom**		**F-value**	***p***	**Sig.**
	Hypothesis	**Error**			
Intercept	1	19	171.197	0.000	***
Block	1	19	13.150	0.002	**
Substrate (S)	1	19	21.188	0.000	***
Taxon (T)	11	19	9.987	0.000	***
S by T	11	19	1.484	0.217	ns
Cutting type (C)	1	20	0.608	0.445	ns
S by C	1	20	0.930	0.346	ns
T bt C	11	20	2.604	0.030	*
S by T by C	11	20	0.442	0.917	ns

In the March experiment, the taxa with the highest rooting success were *P.a.* f. *aurea* clone K219, *P.a*. f. *cruenta* clone U2080, and the regular and dense-crowned clone K359 (Fig.2), in which 51-53 % of the cuttings rooted with the best combination of treatments (the peat-vermiculite substrate and the cutting type preferred by the taxon). Also in the hybrids of *P.a.* f. *globosa* × *P.a.* f. *cruenta* and *P.a*. f. *cruenta* × *P.a*. f. *pendula*, satisfactory rooting success, 52 and 42 %, respectively, was achieved. Following overwintering of the cuttings of the August experiment, the taxa showing the highest rooting response were *P.a*. f. *pendula* clone E11602 with the rooting success of 26 %, and E11601 with that of 18 %.

The number of the roots in the rooted cuttings varied from 1 to 8, and there was a significant dependence (Kendall's W 1.0 and p=0.001) between the root number and the rooting percent of the taxon. After the first growing season, 98.3 % of the rooted cuttings were alive. New shoots were observed in 33 % of them, i.e. the majority of the cuttings had roots developed, but no shoot elongation had taken place in the first growing season.

Genotypic variation in the rooting success of the Norway spruce cuttings is well known, and the rooting success may also vary depending on the growth habit (Hartmann et al. 2011). Kelly (1972) studied twelve dwarf cultivars and found remarkable differences in their rooting percentage. In *P. a.* 'Nidiformis', *P.a.* 'Microsperma', and *P.a.* 'Prostata' rooting around 80% could be achieved, while in other genotypes the best results varied from 10 to 68 %. Also Roulund and Pellett (1974), Kleinschmit et al. (1973), and Hannerz et al. (1999) reported wide differences in rooting success among Norway spruce clones.

In the present study, the rooting success varied among the taxa representing different growth habits. In the material of the March experiment, the taxa with normal height growth were among the best-rooting ones, independently of their age or needle color (Fig. 2, Table 2). The lower rooting success of the other taxa, i.e. the ones without apical dominance, the ones with reduced growth, and the pendulous ones, may be related to their internal phytohormone balance. Formation of adventitious roots is enhanced by auxins, and cutting propagation utilizes a natural phenomenon of polar transport of endogenous auxin. Auxin is synthesized especially in apical shoot meristems and young leaves, and is then transported to roots (Taiz and Zeiger 1991), as shown also in conifers (Sundberg and Uggla 1998). The polar transport accumulates auxin just above to any wound site, such as the cutting base, where it then promotes the initiation of root primordia (Taiz and Zeiger 1991). Upward branches of conifers are characterised by a higher auxin transport capacity than horizontal ones (Veierskov et al. 2007). Thus, the absence of upward shoots or small amount of young needles with active auxin biosynthesis could contribute to lower rooting success in the taxa growing slowly and /or without the apical dominance.

Cutting size

In the present study, there was a connection between the length of the cuttings and the rooting success: the taxa having the longest cuttings were also the ones with the highest rooting percentages (Fig. 4). The bigger cuttings have more photosynthetic capacity and storage carbohydrates available for the formation of adventitious roots. Farrar (1939) found longer (> 10 cm) cuttings to root better than shorter ones. Roulund and Pellett (1974) preferred cuttings 9-12 cm in length. Hannerz et al. (1999) studied cuttings of 3.5-7.5 cm in length and observed a positive correlation between rooting percentage and shoot length. In dwarf cultivars annual shoots are often very short. Iseli and Van Meter (1980) reported that bigger cuttings of dwarfs having a leader and two tiers of side branches root as well as smaller cuttings consisting of a single shoot. This observation can be utilized in commercial propagation in order to get saleable plants faster. According to Kelly (1972), production of a saleable dwarf spruce (12-24 cm in diameter, 8-12 branches) from the rooted cutting normally takes three growing seasons.

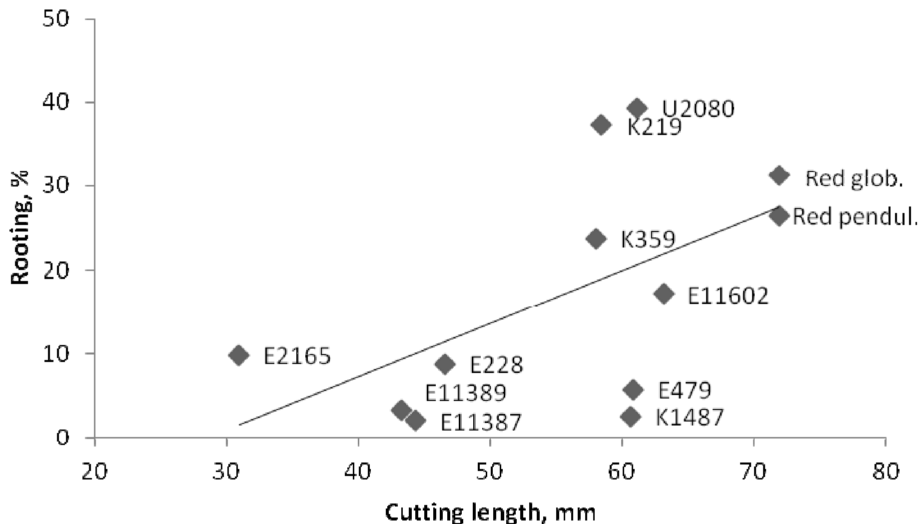

Fig 4 *Relationship between the length of the cuttings and the rooting percent of the taxa of Norway spruce tested in the March experiment. In the plot, each dot represents the mean value of the taxon. Pearson correlation test indicated that the relationship is significant (r=0.30, p=0.005). The correlation test was computed for the uncombined data (n=88).*

Cutting type

The cutting type – plain cuttings versus the ones with the heel – had no significant effect on the rooting percentage in the present material, although there were single genotypes that preferred one cutting type over the other. (Fig. 2b, Table 2). In all, plain cuttings were faster to produce and can thus be recommended from a practical point of view. Roulund and Pellett (1974) recommended plain cuttings, too. They also stated that no removal of basal needles is advised because of its adverse effect on rooting and increased costs of propagation. Farrar (1939) suggested that the more abundant resin flow from the plain cuttings is favorable for their rooting, the resins protecting the cuttings from bacteria and fungi during the rooting process. In Girouard's (1973) study, cutting type had no effect on the rooting, but the presence of the heel seemed to hinder shoot formation and elongation.

Position of cutting in the donor tree

Only lateral shoot tips were used as cuttings in the present study. Oliver and Nelson (1957) and Ferguson (1968) reported them rooting more readily than terminal shoots, but noted that a plagiotropic growth habit is more probable in lateral than in apical cuttings. On the other hand Girouard (1975) considered both lateral and terminal shoots suitable as propagation material.

Cuttings collected for the present study, originated either at the upper part of the tree crown, or were collected all over the crown in the case of dwarfish taxa. Use of upper crown shoots might have decreased rooting percentage, because Hannerz et al. (1999) showed that rooting of the cuttings taken from lower crown positions of the 10-year-old Norway spruce trees was 4-5 times higher (45 %) than of cuttings taken from the upper crown (9.8 %). The same phenomenon was also observed by Roulund (1975) who reported an average increase of rooting of 2.5 % per whorl from the top to the lower parts in the 6-21 –year-old donors. When considering growth habit of the rooted cuttings, the use of upper crown shoots can be justified. Due to topophysis, the cuttings often maintain the growth habit that they had as shoots on the donor tree (Olesen 1978), especially in the case of older trees (Kleinschmit et al. 1973, Roulund 1975, Pulkkinen 1992).Therefore the risk of plagiotropic growth is smaller when cuttings are taken from the apical, upward growing parts of the crown.

Donor tree age

The age of the donor tree had a significant effect on rooting success of the cuttings in the March experiment (Table 3). The average rooting percentage of the cuttings originating in the 20-year-old trees was 26.4, while of that of the cuttings from the 45-55 –year-old trees was only 13.8. However, some of the older donors (K219, U2080, K359) had a high rooting success (Fig. 2). In the August experiment, in the taxa having the highest rooting success after overwintering, the cuttings were taken from 20-year-old donors. In the clone E11387, some cuttings collected from 5-year-old grafts rooted, while none of the cuttings taken from the 55-year-old original donor tree did (Fig 3).

Table 3. *Significance of the factors affecting rooting success of Norway spruce shoot cuttings collected in March together with the odd ratios obtained for the different factor classes. The logistic regression model generated was* $\log(p/1-p) = 0.451 - 1.139m + 0.569c - 1.729h_1 - 1.294h_2 - 1.562h_3 - 1.019a$, *in which the design variables are:* m *= rooting substrate,* c *= needle color,* h_1, h_2, *and* h_3 *= growth habit classes, and* a *= age class.*

Factor	***p***	**Odds ratio (95 % confidence interval)**
Rooting substrate	0.000	
Peat-vermiculite		1
Spruce-Rhododendron soil-bark-vermiculite		0.320 (0268-0.382)
Needle color	0.023	
Green		1
Yellow or red		1.767 (1.083-2.883)
Growth habit class	0.000	
Normal		1
No apical dominance		0.177 (0.098-0.321)
Reduced growth and dense branching		0.274 (0.161-0.466)
Pendulous		0.210 (0.113-0.389)
Age class	0.000	
20-year-old		1
45-55 –year-old		0.361 (0.215-0.605)

Roulund and Pellett (1974) reported that the rooting success of Norway spruce cuttings decreases with the increasing age of the donor tree: approximately 4% per year at age of 6-9 years, 6% per year for donors of 9-13 years of age, and 1% per year for 13-21 year –old donors. Good rooting percentages have, however, been reported even for older trees: In Farrar's (1939) study with 40-year-old trees with a normal growth habit, the optimum treatment of winter cuttings resulted in 89% of them producing roots. Also Oliver and Nelson (1957) found cuttings taken from the 20-30 year-old donors of *P. abies* f. *ohlendorffii, P. a.* f. *remontii* and *P.a.* f. *nidiformis,* rooting up to 90% in optimum conditions.

Harmful effects of donor tree ageing on commercial propagation can be reduced by rejuvenating techniques. In serial propagation new cuttings are taken from the rooted cuttings at a few years intervals, and thus more juvenile donor plants will continuously be available (St. Clair et al. 1985). Also repeated re-grafting onto juvenile rootstock can be used. The results from the clone E11387 in the August experiment suggest that this approach could work also for ornamental forms of Norway spruce. In order to facilitate the propagation of the selected Norway spruce taxa, the Finnish Forest Research Institute has started to establish propagation gardens consisting of rejuvenated materials.

Rooting substrate

Of the two substrates tested in the March experiments the peat-vermiculite mixture (50:50) was shown to be significantly better , with the average rooting of 22.6%, than the 15:15:70 mixture of Spruce-Rhododendron Soil™ -bark-vermiculite, on which the average rooting was only 10.6% (Fig 2a, Tables 2-3). In the August experiment, the overall rooting percentage on the peat-vermiculite was 6.2% and on the Spruce-Rhododendron Soil™ -bark-vermiculite 3.6 %. Water-retention ability of the vermiculite is, however, high, and it can be speculated that with more airy mixtures (e.g. with perlite with no water-retention ability used instead of vermiculite) the rooting percentages could be improved.

Many different types of substrates have successfully been used for rooting of Norway spruce cuttings. Often used are peat mixed with another material to provide more air space in the medium. Avoidance of over-watering is important to prevent decay of cutting bases, and the substrates have been developed for this. Ferguson (1968) described the mixture of sand and peat (1:1) being the best choice. Girouard (1973) used the same mixture. Hannerz et al. (1999) mixed peat and Leca grains 1:1. Kelly (1972) used a 2:1 mixture of peat moss and sand, while Roulund and Pellett (1974) preferred a 1:1 mixture of fresh spaghnum moss and sand. They also reported that rockwool, sand and gravel gave acceptable rooting results. Also pure perlite (Girouard 1975, Iseli and Van Meter 1980), vermiculite (Oliver and Nelson 1957), and coarse sand (Farrar 1939) have all successfully been used.

Greenhouse conditions

The greenhouse conditions of the present study were adjusted as recommended by the earlier studies. An automatic mist system (Iseli and Van Meter 1980) to control air humidity of 80-90 % (Farrar 1939), additional illumination (Roulund and Pellett 1974, Girouard 1975), and bottom heat (Iseli and Van Meter 1980, Hannerzt et al. 1999) were applied with the air temperature set to 15ºC and the substrate temperature set to 22ºC. In both experiments, the targeted conditions were achieved, except that on some sunny days, the temperature of air raised temporarily up to 20ºC. No fertilization was applied during the rooting period in the present study, as recommended by Roulund and Pellett (1974) to reduce development of fungi and algae. Several fungicide treatments were, however, needed to prevent fungal growth, potentially because of the pre-fertilized rooting media used.

Other factors contributing to rooting success

Besides the factors studied in the present work, nutrient status of the donor plant and hormonal treatments are also considered important for rooting of Norway spruce shoot cuttings (Ferguson 1968, Dirr and Heuser 2006). Nutrient status of the present donors was not examined because most of them were big trees growing in distant clone archives. To optimize the rooted cutting production, however, the donor plants should be grown in containers and maintained at optimum nutrition level, with good control of weeds, pests and diseases (Iseli and van Meter 1980).

The published reports on the effect of phytohormone treatments on the rooting of the Norway spruce cuttings are contradictory. As reviewed by Ferguson (1968) and Dirr and Heuser (2006), auxin treatments have resulted in various outcomes, from rooting inhibition with increased mortality, and no effect at all, to increased rooting. In the preliminary experiments, performed partly with the same genotypes of Norway spruce as in the present study, IBA treatment of the winter cuttings was found to have either an inhibitory or no effect at all on rooting success (Teivonen 2010). Based on this result, no auxin treatment was applied in the present study.

This is in agreement with the observations of Farrar (1939) who achieved better rooting in winter cuttings without auxin treatment rather than by using IBA. A similar result with summer cuttings was reported by Girouard (1973), who also noticed that the auxin treatment had a negative effect on the growth of the new shoots in rooted cuttings.

Besides the auxins, there is only a little information about the effect of the other phytohormones on rooting. Rönsch et al. (1993) tested (22S, 23S)-homobrassinolide, a synthetic phytohormone belonging to the group of brassinosteroids, for treating winter cuttings of Norway spruce, and found that the treatment improved the rooting percentage from 50 to 92%.

As a conclusion, the present results show that ornamental forms of Norway spruce of Northern origin can be propagated as rooted shoot cuttings, but for commercial production, careful selection of genotypes is needed. Rooting success varied tremendously among the tested 17 taxa. The taxa having normal height growth but colored needles rooted more easily than the taxa without apical dominance or showing reduced or pendulous growth. The timing of propagation proved to be highly important for the Northern origins. The shoots collected from dormant donors during the winter rooted much better than the ones collected at late summer. Moreover, the late-summer cuttings needed a period of dormancy to be able to form roots. Of the tested rooting substrates, the peat-vermiculite mixture was the best choice. Commercial nurseries specialized in ornamental tree production would like to have rooting of more than 50 % (pers. comm. Jari Mäntynen, Taimityllilä Co). Thus, a few of the taxa tested in the present study can be recommended for propagation. These include the clones of *P.a.* f. *aurea* and *P.a.* f. *cruenta*, but also hybrids having globular or pendulous growth habit and red needle color.

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References

Bonga JM (1982). Vegetative propagation in relation to juvenility, maturity, and rejuvenation. In: Bonga JM, Durzan DJ (Eds) Tissue Culture in Forestry, Martinus Nijhoff, Dr. W. Junk Publishers, The Hague, pp 387-412

Bonga JM, von Aderkas P (1992) In vitro culture of trees. Kluwer Academic Publishers, Dordrect/Boston/London

Dirr MA (1998) Manual of woody landscape plants: Their identification, ornamental characteristics, culture, propagation and uses. Stipes Publishing LLC, Champaign, Illinois

Dirr M, Heuser C (2006) The Reference Manual of Woody Plant Propagation - From Seed to Tissue Culture. Varsity Press Inc. Cary North Carolina, USA

Farrar JL (1939) Rooting of Norway spruce cuttings. For Chronicle: 152-163.

Ferguson DC (1968) Propagation of Picea abies by cuttings. Plant Propagator 14(2): 5-9.

Girouard RM (1973) Rooting, survivel, shoot formation and elongation of Norway spruce stem cuttings as affected by cutting types and auxin treatments. Plant Propagator 19(6): 16-17.

Girouard RM (1975) Seasonal rooting response of Norway spruce stem cuttings. Plant Propagator 21(3): 9.

Gyllenstrand N, Clapham D, Källman T, Lagercrantz U (2007) A Norway spruce FLOWERING LOCUS T homolog is implicated in control of growth rhythm in conifers. Plant Physiol 144: 248-257.

Hannerz M, Almqvist C, Ekberg I (1999) Rooting success of cuttings from young Picea abies in transition to flowering competent phase. Scand J For Res 14: 498-504.

Hartmann HT, Kester DE, Davies FT, Geneve RL (2011) Hartnann and Kester’s plant propagation. Principles and practices. Prentice Hall, Upper Saddle River, New Jersey

Iseli J, Van Meter M (1980) Propagation of Picea abies cultivars by cuttings. Plant Propagator 26(3): 9-11.

Kelly JC (1972) Propagation of dwarf Piceas at Kinsealy. Proc Intern Plant Propagator’s Soc 22: 238-240.

Kleinschmidt J, Műller W, Schmidt J, Racz, J (1973) Entwicklung der Stecklingsvermehrung von Fichte (*Picea abies* Kart.) zur praxisreife. Silvae Genet 22: 4-15.

Kleinschmidt JW, Schmidt J (1977) Experiences with Picea abies cuttings propagation in Germany and problems connected with large scale application. Silvae Genet 26: 197-203.

Klimaszewska K, Overton C, Stewart D, Rutledge RG (2011) Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old somatic white spruce and expression profiles of 11 genes followed during the tissue culture process. Planta 233: 635–647

Krüsmann G (1985) Manual of cultivated conifers. Warda HD (Ed), Epp M (Translator). Timber press, Portland, Oregon

Lehtonen J, Nikkanen T (2008) Metsäpuiden erikoismuotojen jalostus Suomessa. (Breeding of special forms of forest trees in Finland, in Finnish with English summary). Sorbifolia 39(2): 3-10.

Mikola J (2009) Successes and failures in forest tree cutting production in Finland. Working Papers of the Finnish Forest Research Institute 114: 27-30

Nikkanen T (2009) Hardy options for landscaping – domesticated exotics and special forms of Nordic conifers. Working Papers of the Finnish Forest Research Institute 114: 27-30

Nikkanen T, Velling P (2011) Metsäpuiden erikoismuodot – koristepuita viherrakentamiseen. Metsäkustannus Oy, Helsinki

Oliver RW, Nelson SH (1957) Propagation of spruce from cuttings. Proc Intern Plant Propagators Soc 7: 41-48.

Olesen PO (1978) On cyclophysis and topophysis. Silvae Genet 27: 173-178.

Oskarsson O, Nikkanen T (2001) Metsäpuiden erikoismuotoja kultakuusesta luutakoivuun. Metsäntutkimuslaitoksen tiedonantoja 670

Partanen J (2004) Dependence of photoperiodic response of growth cessation on the stage of development in *Picea abies* and *Betula pendula* seedlings. For Ecol Management 188: 137-148.

Pulkkinen P (1992) The effect of crown form and plagiotrophy on the growth of Picea abies f. pendula cuttings. Scand J For Res 7: 71-81.

Raisio J (2009) Conifers in landscaping – Potentials and problems. Working Papers of the Finnish Forest Research Institute 114: 23-26.

Roulund H (1975) The effect of the cyclophysis and the topophysis on the tooring and behavior of Norway spruce cuttings. Acta Horticult 54: 39-50.

Roulund H, Pellett NE (1974) Propagation of Norway spruce (Pice abies L. Karst) by stem cuttings. Plant Propagator 20(1): 20-26.

Rönsch H, Adam G, Matschke J, Schachler G (1993) Influence of (22S, 23S)-homobrassinolide on rooting capacity and survival of adult Norway spruce cuttings. Tree Physiol 12: 71-80.

Sæbo A, Borzan Z, Ducatillion C, Hatzistathis A, Lagerström T, Supuka J, Garcia-Valdecanthos JL, Rego F,

Slycken Van J (2005) The selection of plant materials for street trees, park trees and urban woodland. In: Konijnendijk CC, Nilsson C, Randrup T, Schipperijn J (Eds) Urban Forests and Trees. Springer, pp 257-280

Sonesson J, Hannerz M (2002) Mellansvenska klonskogsbruksprojektet -slutrapport. Skogforsk Arbetsrapport 505

St. Clair B, Kleinschmit J, Svolba J (1985) Juvenility and serial propagation of Norway spruce clones (*Picea abies* Karst.). Silvae Genet 34: 42-48.

Sundberg B, Uggla C (1998) Origin and dynamics of indoleacetic acid under polar transport in *Pinus sylvestris*. Physiol Plant 104: 22-29.

Taiz L, Zeiger E (1991) Plant Physiology. The Benjamin / Cummings Publ. Company, Inc., Redwood City, California, US

Teivonen S (2010) Havupuiden erikoismuotojen lisäysmenetelmät. Propagation of conifer selections (in Finnish with English summary), M.Sc. Thesis, Faculty of Agriculture and Forestry, University of Helsinki, 46 pp.

Uimonen J (2011) Koristepuiden tuotanto, tuonti ja kauppa. In: Nikkanen T, Velling P (Eds) Metsäpuiden erikoismuodot – koristepuita viherrakentamiseen. Metsäkustannus Oy, Helsinki, pp 118-131

Veierskov B, Rasmussen H, Eriksen B, Hansen-Møller J (2007) Plagiotropism and auxin in Abies nordmanniana. Tree Physiol 27: 149-153.

Zilins J, Lapse J, Svilans A (2009) Investigation of conifers mutation forms in Latvia and the possibilities in selecting new sorts. Working Papers of the Finnish Forest Research Institute 114: 31-33.

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Effect of auxins on the induction of somatic embryos from immature zygotic embryonic axes of *Ocotea porosa* (Nees ex Mart.) Liberato Barroso

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Keywords: NAA, 2,4-D, hydrolyzed casein, immature zygotic embryonic axes, immature seeds, picloram.

Abstract: *Ocotea porosa* (Ness ex Martius) Liberato Barroso, known as "imbuia", belongs to the Lauraceae family and is a tree native to the Mixed Ombrophilous Forest (Araucaria Forest) where it was heavily exploited due to the high quality and worldwide value of its hardwood that is used in luxury furnishing manufacturing. The aim of this study was to establish a protocol of induction of somatic embryogenesis of *Ocotea porosa* from tissue of immature material. Seed disinfestation was performed through immersion in ethanol 70% (v/v) for 5 min, followed by 20 min in a sodium hypochlorite solution (NaOCl) 4% (v/v) supplemented with 0,1% of Tween® 20. After that, the seeds were rinsed five times with sterile water. Immature zygotic embryonic axes and whole immature seeds (cotyledon and zygotic embryonic axes) were as explants used. The culture medium used was the WPM. In the first experiment three auxins were compared: 2,4-D, NAA and picloram (200 or 400 µM) during 120 days. In the second and third experiments immature embryonic axes and immature seeds were cultured on a medium supplemented with 2,4-D (200 or 400 µM) combined with hydrolyzed casein (0.5 and 1.0 g/l). In these experiments 2,4-D induced the highest percentage of somatic embryos (10.6) in immature zygotic embryonic axes. In immature seeds, the combination of 200 µM 2,4-D and 1 g/l hydrolyzed casein induced the highest percentage of somatic embryos (24.4). The percentage was much lower (up to 3.6) when isolated immature embryonic axes were used as explants.

Introduction

Ocotea porosa (Ness ex Martius) Liberato Barroso, known as "imbuia", belongs to the *Lauraceae* family and is native to the Mixed Ombrophilous Forest (Araucaria Forest). In this habitat it was heavily exploited due to the high quality and worldwide value of its hardwood that is exported in large quantities for luxury furnishing manufacturing (Carvalho 2003). As a result, this species is an endangered one and is quoted in the official list of endangered Brazilian flora species, in the vulnerable category (IBAMA, 1992) and in the Red List of Parana, in the rare category (Sema 1995).

The sexual propagation of *Ocotea porosa* at its natural occurrence area is difficult, due to the strong tegumentary dormancy and its irregular germination (Carvalho 2003). Moreover, the seed viability is short by virtue of it being a recalcitrant species, showing a high level of humidity (Carvalho 2003). Another limiting factor for its vegetative propagation is the low response of cuttings to the induction of adventitious roots (4%) (Inoue and Putton 2007).

Somatic embryogenesis is a process by which the somatic cells, under inductive conditions, generate embryogenic cells, which undergo a series of morphological and biochemical alterations, resulting in formation of somatic embryos (Santos et al. 2002; Komamine et al. 2005). Somatic embryogenesis is a model for the study of morphological, physiological, molecular characteristics and biochemical events that occur during the embryogenesis in higher plants. Beyond that, it has potential biotechnological applications, such as artificial seed production and propagation of elite plants or genetically modified plants (Santos et al. 2002; Queiroz-Figueroa 2006).

The formation of somatic embryos varies with the type, concentration and time exposure to the growth regulators present in the culture medium. Auxins are considered important for cell division and differentiation, as well as for the induction of somatic embryogenesis (Pasternak et al. 2002). Among the synthetic auxin used in somatic embryogenesis, 2,4-D (2,4-dichlorophenoxyacetic acid) is reported as the most efficient and therefore is the most used in many embryogenic cultures (Fehér et al. 2001, 2002). The influence of exogenous auxins, especially 2,4-D, has been extensively studied in the induction of somatic embryogenesis, acting indirectly through the increase in endogenous levels of IAA (indole acetic acid) or altering the metabolism of auxins. Besides 2,4-D, other auxins are used in somatic embryogenesis induction, such as naphthalene acetic acid (NAA) (27%), indoleacetic acid (IAA) (6%), indole butyric acid (IBA) (6%), picloram (5%) and dicamba (5%) (Fehér et al. 2003).

Under *in vitro* conditions, induction of somatic embryogenesis occurs in explants whose cells are determined or pre-embryogenic. The first phase begins with the dedifferentiation of cells which become competent and determined for embryogenesis. These processes involve complex mechanisms of reactivation cell division and reprogramming of metabolism and development (Feher et al. 2003). Two routes are possible to obtain somatic embryos: the direct and the indirect ones. Somatic embryogenesis can occur directly from pre-determined cells. In contrast, indirect embryogenesis occurs in non-determined and non-differentiated cells forming an embryogenic callus or not (Von Arnold et al. 2002).

For many forest species, including several members of Lauraceae family, such as *Laurus nobilis* (Canhoto et al. 1999), *Persea americana* Mill (Sanchez-Romero 2006), *Ocotea catharinensis* (Viana and Mantell 1999) and *Ocotea odorifera* (Santa-Catarina et al. 2005), somatic embryogenesis induction protocols have been established. However, for *Ocotea catharinensis*, Santa-Catarina et al. (2005) found difficulties in optimizing the conversion stage of somatic embryos into plants.

The aim of this study was to test the effect of three auxins on the formation of somatic embryos from immature zygotic embryonic axes and immature seeds of *Ocotea porosa*.

Material and Methods

Plant material

To conduct these experiments fruits were used (green colored) collected in Campina Grande do Sul and Colombo (Embrapa Forestry), Parana, Brazil, in December 2010 and January 2011. The seed disinfestation was performed in a laminar flow chamber, through immersion in ethanol 70 % (v/v) for 5 min, followed by 20 min in a sodium hypochlorite solution (NaOCl) 4 % (v/v) supplemented with 0,1 % of Tween® 20. After that, the seeds were rinsed five times with sterile water. Immature zygotic embryonic axes and whole immature seeds (cotyledon and zygotic embryonic axes) were used as explants.

Effect of three auxins on somatic embryos formation in immature embryonic axes

The immature embryonic axes (0.2 to 0.4 cm) were cultured in an induction medium consisting of WPM (“Woody Plant Medium”, Lloyd and McCown, 1980) vitamins and salts, supplemented with sucrose (20 g/l), activated charcoal (1.5 g/l) and Vetec® agar (3.5 g/l). Three auxins, 2,4-D, NAA or picloram (200 or 400 µM) were supplemented to this medium and the cultures were maintained for 120 days. The experimental design was completely randomized with six treatments and five repetitions with 10 embryonic axes per plot.

Effect of 2,4-D on the formation of somatic embryos from zygotic embryonic axes

The immature embryonic axes (0.2 to 0.4 cm) were inoculated in an induction medium consisting of WPM vitamins and salts, supplemented with sucrose (20 g/l), activated charcoal (1.5 g/l), agar Vetec® (3.5 g/l) and 2,4-D (200 or 400 µM) combined with hydrolyzed casein (0.5 and 1.0 g/l). The explants were kept in the dark for 120 days. The evaluation was performed every 30 days. The experimental design was completely randomized with four treatments and five repetitions with 10 embryonic axes per plot.

Induction of somatic embryos from immature seeds

The immature seeds (0.8 to 1.0 cm) were inoculated in an induction medium consisting of WPM vitamins and salts, supplemented with sucrose (20 g/l), activated charcoal (1.5 g/l), agar Vetec® (3.5 g/l) and 2,4-D (200 or 400 µM) combined with hydrolyzed casein (0.5 and 1.0 g/l). The explants were kept in the dark for 120 days. The experimental design was completely randomized with four treatments and six repetitions with 10 immature seeds per plot.

Media and culture conditions

The pH of all culture media was adjusted to 5.8 prior to autoclaving. Test tubes (25 x 150 mm) containing 10 ml of culture media were used. The cultures were placed in the dark at 27±2 ºC (day) and 18±2 ºC (night).

Experimental design and statistical analysis

The data were subjected to Bartlett test at 5% probability, using the Assistat program. The mean number of somatic embryos per explant, embryogenic mass containing somatic embryos, yellowish white callus, dark callus (black or brown), and necrosis of zygotic embryonic axes were evaluated every 30 days.

Results and Discussion

Effect of three auxins on somatic embryos formation in immature embryonic axes

Patterns of indirect induction of somatic embryos were observed. The maximum percentage of somatic embryos formation (10.6) and highest number of globular embryos (4.8) were obtained when immature zygotic embryonic axes were cultured in WPM medium containing 200 µM 2,4-D (Table 1). In medium containing 400 µM 2,4-D, somatic embryos were not formed. The percentages of somatic embryos formed on medium containing 200 or 400 µM NAA were 3.5 and 2.5, respectively. In the presence of 200 or 400 µM picloram it was 3.1 and 2.8, respectively, similar to those observed on culture medium containing NAA (Table 1). Similar results were found with *Ocotea odorifera* that presented a mean of 5.1 % of explants with somatic embryos formed on MS medium containing 144 µM 2,4-D (Santa-Catarina et al. 2001).

Somatic embryogenesis was induced by the three auxins tested. However, new experiments should be carried out in order to improve the percentage of induction, and other concentrations of these regulators should be tested, in order to replace the 2.4-D. In general, in the protocols for somatic embryogenesis induction for *Ocotea* species, 2,4-D is the auxin source mostly used in the culture medium. Furtado (2010) tested the effect of 2,4-D and NAA in cultures of *Ocotea catharinensis* and globular somatic embryos were observed in mature zygotic embryonic axes cultured in WPM medium supplemented with NAA (36 µM) or 2,4 D (72 µM). These results are similar to those found with *O. porosa* in which both 2,4-D and NAA induced somatic embryos.

The purpose of replacing the 2,4-D by picloram or NAA is mainly to avoid the inhibitory effect of 2,4-D on the development of somatic embryos caused by its prolonged use. According to Machakova et al. (2008) the most frequently used auxin for induction of embryogenic cultures is 2,4-D. However, since cultures maintained on 2,4-D can become genetically variable, some researchers have preferred to use NAA or IAA or a transfer of the callus to a medium containing one of these compounds once it has been initiated in a medium supplemented with 2,4-D.

The presence of calluses with black or yellowish white color occurred in all treatments. The highest percentage of yellowish white calluses was observed in culture medium containing 400 µM picloram while black calluses were mainly observed in the presence of 400 µM 2,4-D. The percentage of immature zygotic embryonic axes that did not form callus or somatic embryos was low, ranging from 2.5 to 13.1 (Table 1).

***Table 1**. Effect of three auxins on the formation of somatic embryos in immature embryonic axes of Ocotea porosa, after 120 days of culture in WPM medium.*

Auxin	Treatment (µM)	NSE	EMSE	YWC	DC	NEZA
2,4-D	200	4.8 a	10.6 a	24.9 ab	56.9 abc	7.5 b
	400	0.0 c	0.0 b	20.0 b	80.0 a	0.0 c
ANA	200	1.0 bc	3.5 b	21.1 b	62.2 abc	13.1 a
	400	1.2 bc	2.5 b	16.3 b	78.9 ab	2.5 bc
PIC	200	3.8 ab	3.1 b	54.4 ab	424 bc	0.0 c
	400	1.2 bc	2.8 b	63.2 a	31.2 c	2.8 bc

NSE: mean number of somatic embryos per explant; EMSE: embryogenic mass containing somatic embryos (%); YWC: yellowish white callus (%); DC: dark callus (black or brown) (%); NEZA: necrosis of zygotic embryonic axes (%). Means followed by the same letter do not differ significantly by Tukey test at 5% probability.

Effect of 2,4-D on the formation of somatic embryos from zygotic embryonic axes

The expression pattern of somatic embryos occurred indirectly from white embryogenic masses. The average time for starting the events of induction and expression of somatic embryos from immature embryonic axes was 8 to 12 weeks after inoculation in culture medium. The percentage of explants forming embryogenic masses ranged between 2.5 and 3.6 (Table 2). Induction of somatic embryos was observed in WPM culture medium supplemented with 200 µM 2,4-D and 1 g/l hydrolyzed casein, or with 400 µM 2,4-D and 0,5 or 1 g/l hydrolyzed casein. The latter also showed a higher average number of globular somatic embryos (20) and 5 cordiform embryos were formed (data not shown) (Table 2).

Table 2. *Effect of combinations of 2,4-D and hydrolyzed casein (CH) on the induction of somatic embryos of Ocotea porosa from immature zygotic embryonic axes, after 120 days in WPM medium.*

Treatment (µM)	CH (g/l)	NSE	EMSE	YWC	DC	NEZA
200	0.5	0.0 b	0.0 b	5.2 b	66.7 ns	28.1ns
200	1.0	2.0 b	2.5 a	15.0 ab	65.0 ns	17.5 ns
400	0.5	2.0 b	3.2 a	35.0 a	39.2 ns	22.6 ns
400	1.0	20 a	3.6 a	28.6 a	49.1 ns	18.8 ns

NSE: mean number of somatic embryos per explant; EMSE: embryogenic mass containing somatic embryos (%); YWC: yellowish white callus (%); DC: dark callus (black or brown) (%); NEZA: necrosis of zygotic embryonic axes (%). Means followed by the same letter do not differ significantly by Tukey test at 5% probability.

Induction of somatic embryos from immature seeds

Induction of somatic embryos occurred in all treatments (Table 3). The average time for starting the events of induction and expression of somatic embryos from whole immature seeds was 8 to 12 weeks after inoculation in culture medium. This period was similar to that observed when immature embryogenic axes were used as explants. This induction occurred directly in all explants, without callus formation. In the present study, the formation of somatic embryos was only observed on zygotic embryonic axes, i.e., there was no induction of somatic embryos in the cotyledonary tissues. Ribas et al. (2000) also observed that the expression of somatic embryogenesis in *Aspidosperma polyneuron* immature zygotic embryos occurred directly. The same results were reported for *Euterpe edulis* (Guerra 1989).

The highest formation of embryogenic mass containing somatic embryos (24.4%) was observed when immature seeds were cultured in WPM culture medium containing 400 µM 2,4-D and 0.5 g/l hydrolyzed casein. In this treatment the average number of somatic embryos formed per explant was high (4.0) in comparison with other treatments (Table 3).

Table 3. *Effect of combinations of 2,4-D and hydrolyzed casein (CH) on the induction of somatic embryos of Ocotea porosa from immature seeds, after 120 days on WPM medium.*

Treatment (µM)	CH (g/l)	NSE	EMSE	YWC	NEZA
200	0.5	2.8 ab	15.3 ab	29.1 ns	55.6 ns
200	1.0	4.0 a	22.1 ab	26.3 ns	51.6 ns
400	0.5	4.0 a	24.4 a	29.9 ns	45.7 ns
400	1.0	1.0 b	10.8 b	25.9 ns	63.6 ns

NSE: mean number of somatic embryos per explant; EMSE: embryogenic mass containing somatic embryos (%); YWC: yellowish white callus (%); NEZA: necrosis of zygotic embryonic axes (%). Means followed by the same letter do not differ significantly by Tukey test at 5% probability.

The formation of yellowish white callus was detected only after formation of somatic embryos and the percentage was similar in all treatments (Table 3).

Increase in the percentage of somatic embryos formation (up to 24%) obtained in this experiment, may have been positively influenced by the presence of the cotyledons, i.e., the cotyledons provided an additional source of nutrients and hormones. In addition, the use of immature tissues to initiate the embryogenesis process is frequently cited in the literature as they are more responsive to plant growth regulators (Schmidt et al. 1997). These authors reported that the tissues from zygotic embryos are embryogenic in nature, showing meristematic conditions that favor the induction of somatic embryos. In this case, the tissues have competent cells (determined for embryogenesis), i.e. are capable of reacting to specific signals of development.

Conclusion

The results obtained indicate that the formation of somatic embryos of *Ocotea porosa* can be carried out in WPM culture medium supplemented with 2,4-D (200 µM) using immature seeds as explants.

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References

Canhoto JM, Lopes ML, Cruz GS (1999) Somatic embryogenesis in Bay Laurel (*Laurus nobilis*). In: Jain SM, Gupta PK, Newton R J (eds) Somatic embryogenesis in woody plants. Kluwer Academic Publishers, Dordrecht pp 341-367.

Carvalho PER (2003) Espécies arbóreas brasileiras. Volume 1. Colombo: Embrapa Florestas, 1040pp.

Fehér A, Pasternak T, Miskolczi P, Ayaydin F, Dudits D (2001) Induction of the embryogenic pathway in somatic plant cells. Acta Hortic 15: 485-496.

Fehér A, Pasternak T, Ötvös K, Miskolczi P, Dudits D (2002) Induction of embryogenic competence in somatic plant cells: a review. Biologia 57:5-12.

Fehér A, Pasternak TP, Dudits D (2003)Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult 74: 201-228.

Ibama – Instituto Brasileiro do Meio Ambiente. Portaria Nº 37-N, de 3 de abril de 1992. Lista oficial de espécies da flora brasileira ameaçadas de extinção.

Inoue MT, Putton V (2007) Macropropagação de 12 espécies arbóreas da Floresta Ombrófila Mista. Floresta 37:55-61.

Furtado MB (2010) Indução e controle da embriogênese somática em *Ocotea catharinensis* e *Ocotea odorifera* (Lauraceae). Dissertação, MSc in Biotechnology, São Paulo University, Brazil.

Komamine A, Murata N, Nomura K (2005) Mechanisms of somatic embryogenesis in carrot suspension cultures – morphology, physiology, biochemistry, and molecular biology. *In vitro* Cell Develop Biol – Plant 41:6-10.

Lloyd G, McCown B (1980) Commercially feasible micropropagation of mountain laurel, *Kalmia latifolia*, by use of shoot tip culture**.** Proceed. Int. Plant Propagation Soc 30:421-427.

Machakova I, Zazimalova E, George EF (2008) Plant Growth Regulators. I: Introduction; Auxins, their Analogues and Inhibitors. In: George EF, Hall MA, De Klerk J (eds). Plant Propagation by Tissue Culture, 3rd edition, 504pp.

Queiroz-Figueroa FR, Rojas-Herrera F, Galaz-Avalos RM, Loyola-Vargas VM (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. Plant Cell Tissue Organ Cult 86:285-301.

Ribas LLF, Guerra MP, Zanette F, Kulchetscki L (2000) Somatic embryogenesis in *Aspidosperma polyneuron* Mull. Arg. In: Jain SM, Gupta PK, Newton RJ (eds). Somatic embryogenesis in woody plant pp 509-537.

Sanchez-Romero C, Marques-Martín B, Pliego-Alfaro F (2006) Somatic and zygotic embryogenesis in Avocado. In: Meyib A, Samay J (eds) Somatic embryogenesis. Plant Cell Monographs 2:271-284.

Santa-Catarina C, Maciel SC, Pedrotti EL (2001) Germinação *in vitro* e embriogênese somática a partir de embriões imaturos de canela sassafrás (*Ocotea odorifera* Mez). Rev. Bras. Bot. 24:501-510

Santa-Catarina C, Moser JR, Bouson ZL, Floh EIS, Maraschin M, Viana AM. (2005) Protocol of somatic

embryogenesis: *Ocotea catharinensis* Mez (Lauraceae). In: Jain SM, Gupta PK (eds) Protocol for somatic embryogenesis in woody plants. Springer, Dordrecht pp. 427-443.

Santos ALW, Silveira V, Steiner N, Vidor V, Guerra MP (2002) Somatic embryogenesis in parana pine (*Araucaria angustifolia* (Bert.) O. Kuntze). Braz. Arch. Biol. Technol.45:97-106.

Schmidt ED, Guzzo F, Toonen MA, De Vries SC (1997) A leucine-rich repeat containing receptor-like kinase marks somatic plant cells competent to form embryos. Development 124:2049-2062.

SEMA - Secretaria de Estado do Meio Ambiente. Lista vermelha das plantas ameaçadas de extinção no Estado do Paraná. Curitiba: SEMA: GTZ, 1995.

Viana AM, Mantell SH (1999) Somatic embryogenesis of *Ocotea catharinensis*, an endangered tree of the mata atlântica (South Brazil). In: Jain SM, Gupta P K, Newton RJ. (eds) Somatic embryogenesis in woody plants. Kluwer Academic Publishers, Dordrecht, pp 197-214.

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Effect of picloram and silver nitrate on embryogenic callus induction in *Acrocomia aculeata* (Jacq.) Lodd. ex Mart. by thin cell layer culture

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Keywords: somatic embryogenesis, macaw palm, oil production, ethylene inhibitor

Abstract: *Acrocomia aculeata* is a native palm of South America that produces fruits with great potential for oil production. The quality of this oil is similar to that of olive oil. Commercial planting of this species has to deal with the difficulties of propagation by seeds that have a low germination rate. Somatic embryogenesis could be a valuable tool for propagation of *A.aculeata*. The purpose of this study was the callogenesis and somatic embryo production using the TCL (Thin Cell Layer) technique. Leaves of plants produced *in vitro* were transversally cut into five TCLs from the base and placed in culture medium for callus induction. This medium was composed of Y3 salts and Morel´s vitamins and supplemented with 150 or 300 µM of picloram, with or without 1 µM of silver nitrate and with active charcoal. The production of primary callus was evaluated after 12 weeks and the callus were then transferred to a multiplication medium. The results obtained with 150 µM picloram were better than with 300 µM, with 68 % of the explants forming callus. The primary callus showed growth of embryogenic callus after 4 weeks in the second medium and, after 8 weeks, the first clusters of somatic embryos appeared. Somatic embryos, in the globular stage, appeared in some calluses (6 %). However, the conversion of somatic embryos into plants failed. In conclusion, the lower concentration of picloram, without silver nitrate, was the best treatment for callus induction in leaf TCLs of *A. aculeata*.

Introduction

Acrocomia aculeata (Jacq.) Lodd. ex Mart., known as macaw palm, belongs to Arecaceae family and is a perennial palm native to South and Central America. In Brazil, it occurs in the states of Minas Gerais, Goiás, Mato Grosso, Mato Grosso do Sul, São Paulo and Parana (Scariot and Lleras 1995; Lorenzi, 2006). It has a high fruit production rate and its fruits have a great potential for oil production. The quality of this oil is similar to that of olive oil; it may be used as edible oil and has potential for the pharmaceutical industry (Bélen-Camacho et al. 2005). The oil extraction, moreover, generates by-products, like mesocarp cake, rich in soluble fibers of high value and used in animal feed and fabrication of cakes and biscuits (Ramos et al 2008).

Actually, the *A. aculeata* production is extrativist and the seedlings are obtained through seed that has a low germination rate. For high-scale commercial utilization, it is necessary to establish a more productive cultivation system. Somatic embryogenesis is a plant regeneration process whereby somatic embryos develop from somatic cells (Bhojwani and Razdan 1996). This process is important for mass propagation, especially in species whose seeds have a long dormancy and low germination rate, as occurs in several palm species. Somatic embryos of *A. aculeata* were obtained by Moura et al (2009), using zygotic embryos, however, the conversion rate into plants was low.

One of the auxins most utilized for the induction of somatic embryogenesis is picloram. It showed good results for callus induction in cassava (Groll et al. 2001); oil palm (Teixeira et al. 1995; Scherwinski-Pereira et al.

2009) and macaw palm (Moura et al. 2009). The auxins and stress signaling pathway may be responsible for the cellular reprogramming concerning embryogenesis (Quiroz-Figueroa et al 2006).

Silver nitrate is a powerful inhibitor of ethylene action, but might be toxic depending on the concentration, species or explant type. Ethylene affects callus growth and somatic embryogenesis (Kumar et al., 2009), acting either as a promoter or as an inhibitor (Kong et al. 2011). The role of ethylene in plant tissue culture is not well understood but it affects many aspects of *in vitro* morphogenesis and specially somatic embryo formation (Ptak et al 2010). It is known that ethylene reduces the competence in somatic embryogenesis of many plant species (Kong et al. 2011), but its effect is genotype dependent (Al-Khayri, 2004).

The objective of this study was to evaluate the effects of picloram and silver nitrate on primary and embryogenic callus induction in leaf thin cell layers of *A. aculeata* and analyze the morphoanatomical aspects involved in this process.

Materials and Methods

Plant material, medium and culture conditions

Mature fruits were collected at Bodoquena, Mato Grosso do Sul, Brazil. The endocarp was removed and the kernels were surface-sterilized under aseptic conditions, by a one min immersion in 70% ethanol, followed by a 15 min immersion in a 6% sodium hypoclorite solution plus one drop of Tween 20® in each 100 ml. Thereafter, the kernels were rinsed 3 times in sterile distilled water. Zygotic embryos were aseptically removed from the kernels and then cultured in test tubes with 10 ml of Y3 culture medium (Eeuwens 1976) plus Morel’s vitamins (Morel and Wetmore 1951), 30 $g.L^{-1}$ sucrose, 1 $g.L^{-1}$ activated charcoal and gelled with 5,8 $g.L^{-1}$ agar (Vetec®). The cultures were kept at 25±2 °C in the dark for 4 weeks and then in a 16 h photoperiod for 8 weeks.

Seedlings of 2-4 cm had their roots, cotyledon petioles and most external leaves removed. The remaining leaf portion was transversally cut from the base into five slices about 1 mm thick (TCLs) and the explants were inoculated in Petri dishes containing 40 ml of culture medium each. The induction medium contained Y3 salts, Morel´s vitamins, 30 $g.L^{-1}$ sucrose, 1,5 $g.L^{-1}$ activated charcoal, 2 $g.L^{-1}$ Gelzan (Sigma), 500 $mg.L^{-1}$ glutamine, 150 or 300 µM picloram, with or without 1 µM silver nitrate. The explants were cultivated in the dark in a growth room for 12 weeks. No subculture was applied. The callus formation was evaluated after 12 weeks.

The primary calli obtained were transferred to multiplication medium. In this step, the activated charcoal was reduced to 0,3 $g.L^{-1}$ and 500 $mg.L^{-1}$ hydrolysed casein was added. Two treatments were made: (1) 75 µM picloram and 25 µM 2-iP were added but without silver nitrate and (2) with 25 µM 2-iP and 1 µM silver nitrate but without picloram. Subcultures on the same fresh culture medium were done at 4 week intervals for 8 weeks.

Embryogenic calli were maintained in the maturation medium that was described previously, but with 40 µM 2,4-D, 10 µM 2-iP, 1,0 $g.L^{-1}$ activated charcoal and 500 $mg.L^{-1}$ hydrolysed casein. Subcultures on the same fresh medium were done at 4 week intervals.

Clusters of somatic embryos were cultivated in a conversion medium containing Y3 salts, Morel´s vitamins, 30 $g.L^{-1}$ sucrose, 2 $g.L^{-1}$ Gelzan (Sigma), 500 $mg.L^{-1}$ glutamine, 12,5 µM 2-iP and 0,5 µM NAA. The explants were maintained in the light for 4 weeks and then transferred to the first medium described without plant regulators.

Histological analyses

To evaluate the histological alterations associated with somatic embryogenesis, calli were fixed in 1% glutaraldehyde solution and 4% formaldehyde solution (Karnovsky, 1965). Thereafter, the samples were dehydrated in a graded alcohol series (5–95%), one hour in each solution and infiltrated with Leica Historesin® and alcohol (1:1, v/v) for 12 h. The material was then infiltrated with pure resin for 24 h and polymerized following the manufacturer’s instructions. 5–7 µm sections were obtained with a manual microtome, placed on a slide and stained with 0.5% toluidine blue in 0.1 M phosphate buffer (O´Brien et al, 1964) or 0,5% toluidine blue in bórax and 0,05% fuchsin.

Statistical analysis

The effect of two picloram concentrations and the presence or not of silver nitrate were evaluated on primary callus production and the presence or not of picloram and silver nitrate on the embryogenic callus formation. The experiment was a factorial one, with a completely randomized design with ten replicates. Each replicate was composed of one Petri dish with five explants each. Data were transformed by (arsin $x^{1/2}$ /100) and

submitted to analysis of variance (ANOVA) and the means compared with Tukey's multiple range test at 95% significance.

Results

The calli appeared on the explants after 3 weeks of culture. They were whitish and had a friable appearance (Figure 1 a). After 12 weeks of induction, statistical differences between callus formation rates caused by different combinations of picloram and silver nitrate were observed (Table 1). The result of primary callus induction obtained for explants culture on medium containing 150 µM picloram alone was significantly higher than that obtained with other treatments (Table 1). Visually, a high oxidation of the explants was observed in the treatments with silver nitrate after 4 weeks, leading to necrosis of the explants. The same was observed in the treatment with 300 µM picloram, after 8 weeks.

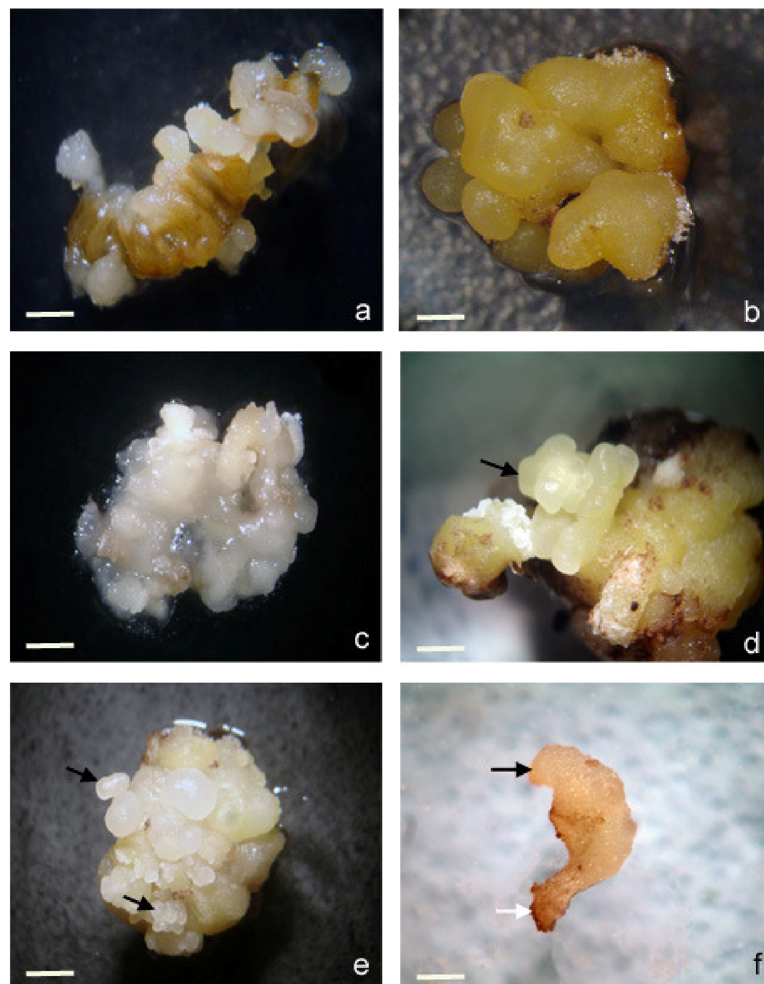

Fig 1 *(a) Primary callus formed after 12 weeks of in vitro culture, bar=15 mm (b) Yellow compact callus, bar=17 mm (c) Friable callus with gelatinous aspect, bar=29 mm (d) Compact callus with embryonic cluster (arrow), bar=14 mm (e) Globular embryos (arrows) formed on nodular callus in maturation medium, bar=28 mm (f) Failed somatic embryo in conversion medium. Note the primary root (white arrow) and malformed plumule (black arrow), bar=63 mm*

***Table 2** Effect of picloram and silver nitrate on callus induction on A. aculeata TCLs after 12 weeks of in vitro culture*

$AgNO_3$ / Picloram	150 µM	300 µM	Mean
0 µM	68,0 Aa	25,0 Ab	46,5 A
1 µM	26,0 Ba	34,0 Aa	30,0 B
Mean	47,0 a	29,5 b	

Lower case letters represent statistical differences among the picloram concentrations and upper case letters represent differences among silver nitrate concentrations, according to Tukey's test ($p > 0.05$).

With the transfer of primary callus to embryogenic callus formation medium, two types of calli were observed. The compact callus is characterized by the hard appearance (Figure 1 b) and the friable callus is soft and whitish (Figure 1 c). There was no significant difference in the results of friable callus and compact callus formation between the treatments with 75 µM picloram and 25 µM 2-iP and without picloram (Table 2). The friable callus, after 8 weeks of culture, showed gelatinous appearance. The calli cultured in the medium without picloram did not form embryogenic clusters. Thereafter, embryogenic callus appeared on some compact calli (Figure 1 d) in the medium with picloram.

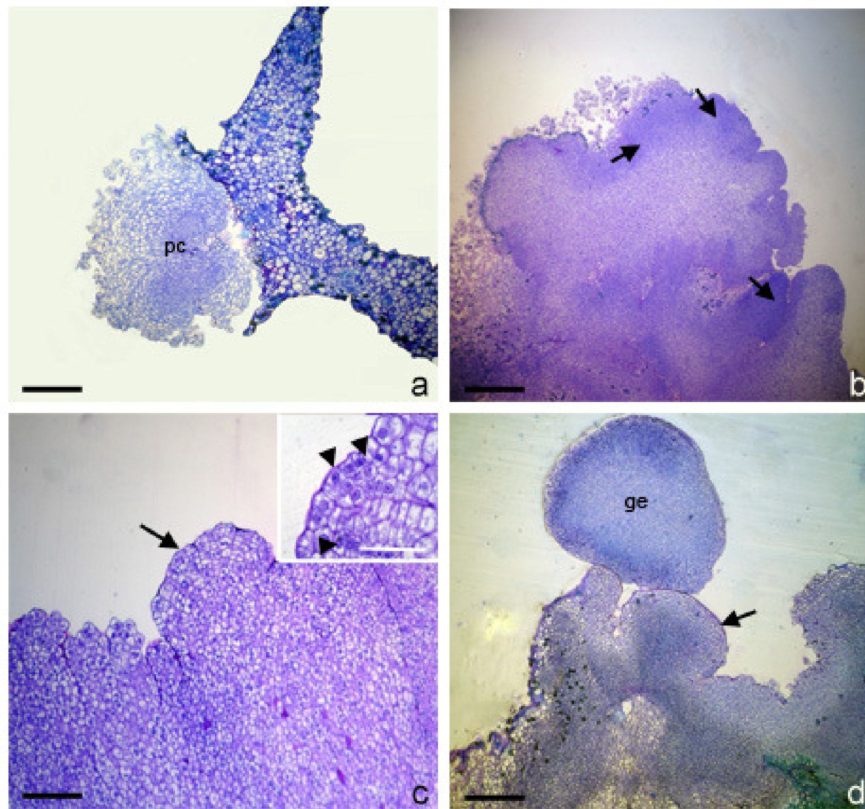

***Fig 2** (a) Histological section of primary callus (pc) formed after 12 weeks of in vitro culture, bar=100 µm (b) Section of compact callus with meristematic zones (arrows), where embryonic clusters formed, bar=500 µm (c) Embryonic cluster showing a proembryo (arrow). In detail, embryogenic cells, showing small cells with evident nuclei and anticlinal divisions (head arrows), black bar= 100 µm, white bar= 50 µm (d) Globular embryo (ge) without a defined protoderm and a proembryo (arrow), bar=500 µm*

Table 2 *Effect of picloram and 2-iP on consistency of calli formed on A. aculeata TCLs after 16 weeks of in vitro culture*

Picloram	2-iP	Friable	Compact
75	25	54,43 A	45,57 A
0	25	50,88 A	49,12 A

Means with the same letters are not significantly different according to the Tukey´s test (p > 0.05).

The embryogenic calli were transferred to maturation medium and showed formation of clusters of somatic embryos (Figure 1e). Those at the globular stage appeared in some callus (6%), named nodular callus. Afterwards, the somatic embryos (mean of 8.5 embryos per nodular callus) cultured on conversion medium failed to convert and oxidized when placed in the light. They formed a primary root and plumule, but were chlorotic and malformed (Figure 1f).

Histological sections showed the primary callus formation (Figure 2a) after 12 weeks of induction. The compact callus developed meristematic zones, with cells characterized by dense cytoplasm and evident nucleoli, where the embryogenic clusters appeared (Figure 2b). Proembryos developed on the surface of embryogenic callus with many anticlinal divisions (Figure 2c). The globular somatic embryos that formed after 6 months were placed in the light, but they did not form a defined protoderm, as shown in Figure 2d.

Discussion

Silver nitrate, an ethylene action inhibitor, affects somatic embryogenesis by increasing or decreasing the *in vitro* response of explants, depending on the species (Kong et al. 2011; Al-Khayri and Al-Bahrany 2004). Therefore, the effect of silver nitrate on somatic embryogenesis must be evaluated for each species. The silver nitrate used in a culture medium might increase somatic embryogenesis and this response may be related to its antagonistic effect to ethylene (Gaspar et al 1996).

However, in *A. aculeata* explants, silver nitrate decreased callus formation during the induction phase and inhibited the embryogenic callus formation in the second medium. The same was observed by Hatanaka et al. (1995) in leaf explants of *Coffea canephora,* where the addition of silver nitrate to the medium inhibited somatic embryogenesis. Likewise, in *Rubus* ssp., silver nitrate addition decreased callus induction in leaf explants (Tsao and Reed 2002).

Picloram has been reported as being efficient for callus induction in palm species (Goh et al. 2001; Steinmacher et al. 2007; Scherwinski-Pereira et al. 2010; 2012; Silva et al 2012). In *A. aculeata*, Moura et al. (2009) obtained somatic embryos from zygotic embryos in MS medium with 9 µM picloram and without activated charcoal. In this work, picloram was efficient in Y3 medium supplemented with activated charcoal. Steinmacher et al. (2007), using the TCL technique in peach palm, obtained a higher number of calli and somatic embryos when MS medium contained 300 or 600 µM picloram. In *Elaeis guineensis*, picloram at 450 µM proved to be superior to 2,4-D for callus induction (Scherwinski-Pereira et al 2010).

The embryogenic callus formation occurred when picloram was reduced and maintained in the culture medium. After transfer to maturation medium, the formation of globular somatic embryos was observed. Nevertheless, after transfer to the conversion medium the embryos oxidized. A low conversion rate is a recurring problem in somatic embryogenesis protocols. Low rates of somatic embryo conversion in plants might be associated with morphological abnormalities or somatic embryo immaturity (Ammirato et al. 1987; Suhasini et al. 1996). A defined protoderm was not observed in *A. aculeata* when the somatic embryos were cultured on conversion medium and this may explain the lack of conversion.

In conclusion, the lowest concentration of picloram was the most efficient for callus induction in leaf TCLs of *A. aculeata.* Silver nitrate in the medium decreased callus induction and inhibited embryogenic callus formation. The lack of conversion was due to immaturity of embryos.

References

Al-Khayri JM, Al-Bahrany AM (2004) Genotype-dependent in vitro response of date palm (*Phoenix dactylifera* L.) cultivars to silver nitrate. Sci Hort 99:153–162

Ammirato PV (1987) Organization events during somatic embryogenesis. In: Green CE, Somers DA, Hackett WP, Biesboer DD (Eds) Plant Tissue and Cell Culture, Plant Biol, Vol 3 (pp 57-81), Alan R. Liss. Inc. New York

Belén-Camacho DR, López I, García D, Gonzaléz M, Moreno-Alvarez MJ, Medina C (2005) Evaluación físico-química de la semilla y del aceite de corozo (*A. aculeata* Jacq.). Grasas y Aceites 56:311-316

Bhojwani SS, Razdan MK (1996) Plant Tissue Culture: Theory and Practice Elselvier Science

Eeuwens CJ (1976) Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera* L.) and culture *in vitro.* Physiol Plant, 36:23-28

Gaspar T, Kevers C, Reid DM, Greppin H, Thorpe TA (1996) Plant hormones and plant growth regulators in plant tissue culture. In Vitro Cell Dev Biol Plant, 32:272–289

Goh DKS, Bon MC, Alioti F, Escoute J, Ferrière N, Monteuuis O (2001) In vitro somatic embryogenesis in two major rattan species: *Calamus merrellii* and *Calamus subinermis*. In Vitro Cell Dev Biol Plant, 37:375-381

Groll J, Mycock DJ, Gray VM, Laminski S (2001) Secondary somatic embryogenesis of cassava on picloram supplemented media. Plant Cell Tissue Organ Cult 65:201-210

Hatanaka T, Sawabe E, Azuma T, Uchida N, Yasuda T (1995) The role of ethylene in somatic embryogenesis from leaf discs of *Coffea canephora*. Plant Sci 107:199–204

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137-138

Kong, D., Shen, H. & Li, N. (2011) Influence of AgNO3 on somatic embryo induction and development in Manchurian ash (*Fraxinus mandshurica* Rupr.). Afric J Biotechnol 11**:**120–125

Kumar V, Parvatam G, Ravishankar GA (2009) AgNO3 - a potential regulator of ethylene activity and plant growth modulator. Electronic J Biotechnol doi: 10.2225/vol12-issue2-fulltext-1

Lorenzi GMAC (2006) *Acrococomia aculeata* (Jacq.) Lodd. ex Mart. - Arecaceae: bases para oextrativismo sustentável. Thesis, Federal University of Parana.

Morel G, Wetmore RH (1951) Tissue culture of monocotyledons. Am J Bot 38: 138-140

Moura EF, Motoike SY, Ventrella MC, Junior AQS, Carvalho M (2009) Somatic embryogenesis in macaw palm (*Acrocomia aculeata*) from zygotic embryos. Sci Hort, 119:447–454 Morel G, Wetmore RH. 1951. Tissue culture of monocotyledons. Am J Bot 38: 138–140

O'Brien TP, Feder N, McCully ME (1964) Polychromatic staining of plant cell walls by toluidine blue. Protoplasma, 59:368-373

Ptak A, Tahchy AE, Wyzgolik G, Henry M, Laurain-Mattar D (2010) Effects of ethylene on somatic embryogenesis and galanthamine content in *Leucojum aestivum* L. cultures. Plant Cell Tissue Organ Cult, 102: 61-67.

Quiroz-Figueroa FR, Rojas-Herrera R, Galaz-Avalos RM, Loyola-Vargas VM (2006) Embryo production through somatic embryogenesis can be used to study cell differentiation in plants. Plant Cell Tissue Organ Cult doi:10.1007/s11240-006-9139-6

Ramos MIL, Ramos-Filho MM, Hiane PA, Braga-Neto JA, Siqueira EMA (2008) Qualidade nutricional da polpa de bocaiúva *Acrocomia aculeata* (Jacq.) Lodd. Ciência e Tecnologia de Alimentos, 28:90-94

Scariot AO, Lleras E, Hay JD (1991) Reproductive biology of the palm *Acrocomia aculeata* in Central Brazil. Biotropica 23:12-22.

Scherwinski-Pereira JE, Guedes RS, Fermino PC, Dilva, TL Costa FH (2010) Somatic embryogenesis and plant regeneration in oil palm using the thin cell layer technique. In Vitro Cell Dev Biol Plant, 46:378-385

Scherwinski-Pereira JE, Silva RA, Fermino PCP, Luis ZG, Freitas EO (2012) Somatic embryogenesis and plant regeneration in açaí palm (*Euterpe oleracea*) Plant Cell Tissue Organ Cult doi 10.1007/s11240-012-0115-z

Silva RC, Luis ZG, Scherwinski-Pereira, JE (2012) Differential responses to somatic embryogenesis of different genotypes of Brazilian oil palm (*Elaeis guineensis* Jacq.) Plant Cell Tissue Organ Cult doi:10.1007/s11240-012-0170-5.

Steinmacher DA, Krohn NG, Dantas ACM, Stefenon VM, Clement, CR, Guerra, MP (2007) Somatic Embryogenesis in Peach Palm Using the Thin Cell Layer Technique: Induction, Morpho-histological Aspects and AFLP Analysis of Somaclonal Variation. Ann Bot. doi:10.1093/annbot/mcm153

Suhasini K, Sagare AP, Krishnamurthy KV (1996) Study of aberrant morphologies and lack of conversion of somatic embryos of chickpea (*Cicer arietinum* L). In vitro Cell Dev Biol Plant 32:6–10

Teixeira JB, Sondahl MR, Nakamura T, Kirby EG (1995) Establishment of oil palm cell suspensions and plant regeneration. Plant Cell Tissue Organ Cult 40:105–111

Tsao CWV, Reed BM, (2002) Gelling agent, silver nitrate, and sequestrene iron influence adventitious shoot and callus formation from *Rubus* leaves. In Vitro Cell Dev Biol - Plant 38:29–32

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Somatic Embryogenesis in Brazilian pine: establishment of biochemical markers for selection of cell lines with high embryogenic potential

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Abstract: Embryogenesis is a complex, highly organized biological process that plays a central role in the life cycle of an organism. More specifically for conifers, zygotic and somatic embryogenesis (SE) models have been proven to be promising tools in elementary studies on cell biology, physiology and biochemistry as well as in large-scale vegetative propagation and the establishment of *in vitro* germplasm banks. Although clonal propagation and *ex situ* conservation of elite genotypes based on SE is now a consolidated method for a number of conifers species, the adoption of suboptimal conditions during *in vitro* growth of somatic embryos has restricted its use in Brazilian pine. The present work is dedicated to analyze the current state of SE technology in Brazilian pine. Some issues concerning the use of SE, including cell signaling molecules (polyamines -PAs, nitric oxide -NO, and reactive oxygen species -ROS) and establishment of biochemical markers for embryogenic potential are discussed.

Introduction

Brazilian pine (*Araucaria angustifolia* Bert O. Ktze) is the only native conifer of economic importance in Brazil, representing the most exploited timber source up to the 1970s (Mattos, 1994; Shimizu and Oliveira, 1981). As Brazilian pine exploitation progressed, the natural reserves decreased almost to the point of elimination, due mainly to clear-cutting for timber export (Ondro et al. 1995). Today, only 2 % of the 20 million hectares originally covered with *Araucaria* forest are present in South Brazil (Guerra et al. 2000). Even after inclusion of Brazilian pine in the official list of endangered Brazilian plant species, the remaining populations are still under pressure of exploitation by the timber industry (Stefenon et al. 2009).

This species has a high nutrient demand during plant development, being much more exigent in soil fertility than most other conifers (Handro, 1986). This factor along with the long life cycle and the long period of time between pollination and seed formation (typically three years) (Haines et al. 1984), hamper the establishment of breeding and large scale reforestation programs with this species. The deployment of natural reserves of Brazilian pine obliged the Brazilian government to encourage reforestation programs with exotic coniferous species, mainly *Pinus elliottii* and *Pinus taeda* (Guerra et al. 2002).

The development of technologies for conservation and genetic improvement of *A. angustifolia* is required in order to establish reforestation programs with this species. For *A. angustifolia*, the priority now is the conservation of natural remnant populations and the establishment of a breeding program for competitive reforestation programs (dos Santos et al. 2008). Biotechnological approaches have the potential to provide efficient methods of micropropagation, genetic improvement, and for germplasm conservation of tropical and subtropical trees (Reed et al. 2011; Lara-Chavez et al. 2011). Among the techniques available, somatic embryogenesis (SE) is a

micropropagation technique that has become widely utilized in forest biotechnology (Durzan 2008; Klimaszewska et al. 2011). However, protocols for plant regeneration through SE in gymnosperms have so far been established mainly for members of Pinaceae family, and none yet for Brazilian pine.

Attempts at using somatic embryogenesis for mass clonal propagation and *ex-situ* conservation of elite genotypes in Brazilian pine are underway since 1992 (Guerra & Kemper, 1992). However, still today there are numerous biological unknowns regarding the complex developmental pathway in this species (Durzan 2012). Therefore, there is a need to research stimuli and conditions necessary for the correct somatic embryo formation, and to develop markers, which can be used for identification of highly responsive Brazilian pine embyogenic cell lines.

Somatic embryogenesis in Brazilian pine: induction, proliferation and maturation of somatic embryos

In spite of the numerous reports published about induction and development of somatic embryos, no standard protocol to induce embryogenic cultures of all plant species has ever been conceived. The induction of SE in conifers is usually conducted in a culture medium supplemented with different concentrations and combinations of growth regulators auxin and cytokinin, a carbon source (usually saccharose) and a gelling agent in the dark and under constant temperature (Stasolla & Yeung, 2003). For some species, like Brazilian pine, growth regulators may be excluded, both in the induction and proliferation phases using liquid and semi-solid medium (dos Santos et al., 2002; dos Santos et al., 2008).

For induction of embryogenic cultures (EC) in Brazilian pine, immature zygotic embryos are expressly required as an explant source (dos Santos et al., 2008). The first signs of cell organization in EC are usually seen in the first weeks of growth. EC may present competent cells for embryogenesis next to cells that do not exhibit this trait. The coexistence of cells with these opposing characteristics has been reported for Brazilian Pine by *in situ* hybridization of the *SERK* gene (Steiner et al., 2012).

Similarly to other conifer species, embryogenic cultures of Brazilian pine may be identified during the proliferation phase based on their typical translucent and mucilaginous appearance and by the presence of stage I somatic embryos (Fig. 1a), though cytochemical characteristics are also used for this purpose (Fig. 1b) (Guerra et al., 2000; dos Santos et al., 2008). In conifers, cells that are strongly stained with carmin and acetic acid but weakly stained with Evans blue are considered embryogenic, whereas a weak staining with the former and intense color with the latter indicate suspensor cells (Durzan et al., 2008). Additionally, during the proliferation phase biochemical analysis of embryogenic and non-embryogenic cultures of Brazilian pine revealed the development of different protein profiles (dos Santos et al., 2008), PA profiles of putrescine (Put), spermidine (Spd), and spermine (Spm), NO, and ROS (Silveira et al., 2006; Steiner et al., 2007).

Similarly to what happens in zygotic embryos, maturation is one of the most important stages in SE. At this stage, the somatic embryo undergoes morphological and biochemical changes mediated by an accurate genetic control, resulting in the deposition of reserve substances, repression of germination and development of tolerance to desiccation (Bewley & Black, 1994; Cairney& Pullman, 2007). In conifers, although some papers have suggested that polyetilene glycol (PEG), by itself, promotes maturation (Svobodová et al., 1999), other studies have demonstrated that the combined use of PEG and other maturation promoters, especially abscisic acid (ABA), or even low molecular weight osmotic agents like maltose, are essential when the aim is to increase quality and quantity of somatic embryos obtained with maturation (Klimaszewska & Smith, 1997; Stasolla & Yeung, 2003).

In general, ABA concentrations between 10 and 150 µM are used, while PEG and carbohydrates vary between 2 and 10% (w/v). It should be underlined that the combination of different concentrations of ABA, PEG and maltose has been successfully used in the maturation of somatic embryos in several plant systems, including Brazilian pine (Santos et al., 2002; Steiner et al., 2008) (Fig. 1c and 1d). However, up to now there is no report about plant regeneration from mature somatic embryos of Brazilian pine.

Biochemical and physiological studies on Brazilian pine somatic embryogenesis

Apart from allowing the mass cloning of plants, SE has also been used as an experimental system in biochemical, physiological and molecular research during the different embryo developmental stages, which are difficult to assess *in situ* (Durzan et al., 2008). In this context, great attention has been devoted to the redox system (Stasolla et al., 2010; Vieira et al., 2012) and PA metabolism (Silveira et al., 2006; Gemperlová et al., 2009). Similarly to what happens in animals, the initial stages of embryogenesis in plants are characterized by intense metabolic activity, high cell division rates, increased NO and ROS production (Silveira et al., 2006; Santa- Catarina

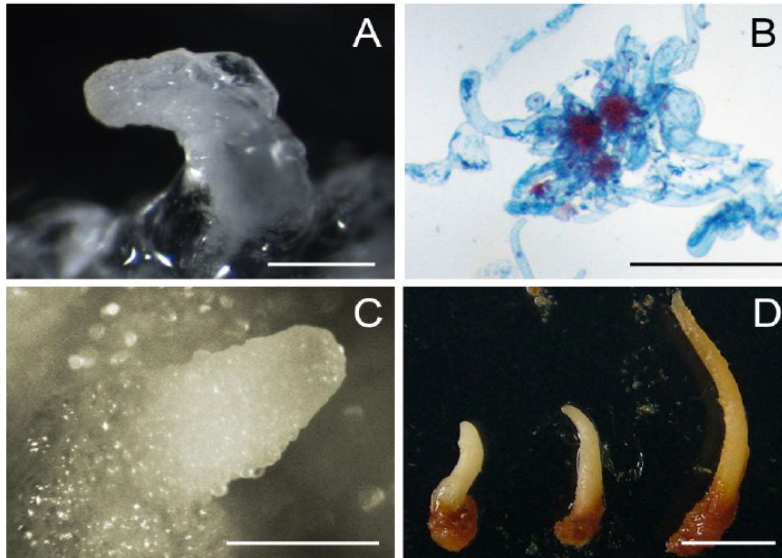

Fig. 1*- Somatic embryo development in Brazilian pine. (A) stage I somatic embryo developed in proliferation medium B5; (B) early somatic embryo stained with acetocarmin and Evans blue; (C) stage II somatic embryo developed on maturation medium (120 µM ABA, 7% PEG 4000, and 9% maltose) after 60 days of subculture; (D) stage III somatic embryo developed on maturation medium after 120 days of subculture [bar 0.3 mm (a, b, and c), 1.5 mm (d)]*

et al., 2007; Zhang et al., 2010; Osti et al., 2010). Previous studies have shown that ROS may be associated to the osmotic properties of the culture medium and plant regeneration, through SE. Inappropriate conditions during *in vitro* cultivation of embryogenic cultures may increase ROS and NO production, leading to the development of defective embryo morphology (Zhang et al., 2010). Therefore, the maintenance of an equilibrium between the pro-oxidant and antioxidant systems, which produce and scavenge NO and ROS (Fig. 2a-d) respectively, has been considered an important aspect in cell differentiation processes, like SE (Stasolla & Young, 2001; Silveira et al., 2006; Zhang et al., 2010).

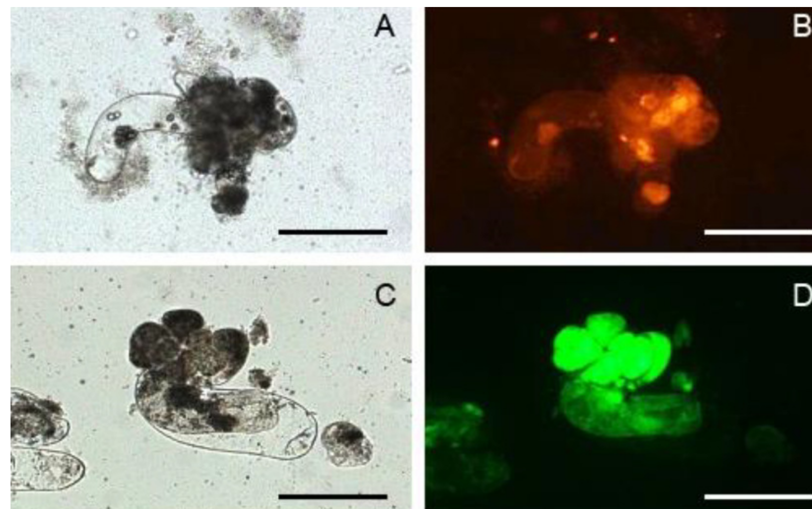

Figure 2*- NO and ROS production in embryogenic cultures of Brazilian pine (A-C) Embryogenic culture proliferated in MSG liquid medium (B) NO detection using DAR-4M AM (D) ROS detection using H2DCFDA [bar. 200 µm (a-d)].*

Recently, the first reports of a relationship between PAs and NO were published in the literature (Tun et al., 2006; Silveira et al., 2006; Santa-Catarina et al., 2007). A correlation between NO metabolism and PAs in *A. angustifolia* embryogenic cultures (Silveira et al., 2006), showing that a drop in endogenous levels of NO induced by incubation in a culture medium containing Spd and Spm improve embryogenic culture growth. Oppositely, incubation with Put (Silveira et al., 2006) and NO donor (Osti et al., 2010) increase levels of endogenous NO which, in turn, stimulates cell division at the same time that morphogenetic evolution is inhibited.

Polyamines (PAs) are widely regarded to function as regulators of embryogenesis in angiosperms and gymnosperms (Thorpe & Stasolla, 2011), as well as SE (Silveira et al., 2006; Steiner et al., 2007; Santa-Catarina et al., 2007). In Brazilian pine, studies have shown that exogenous PAs, especially Spd and Spm, influence embryogenic competence, affording morphogenetic evolution of somatic embryos (Silveira et al., 2006). In addition, levels of PAs can be used in Brazilian pine as biochemical markers for differentiation of EC with high embryogenic potential. EC responsive to maturation conditions (with development of mature somatic embryos) showed significantly lower Put/Spd ratios, when compared to non-responsive EC (Jo et al. 2011) (Fig. 3a-b).

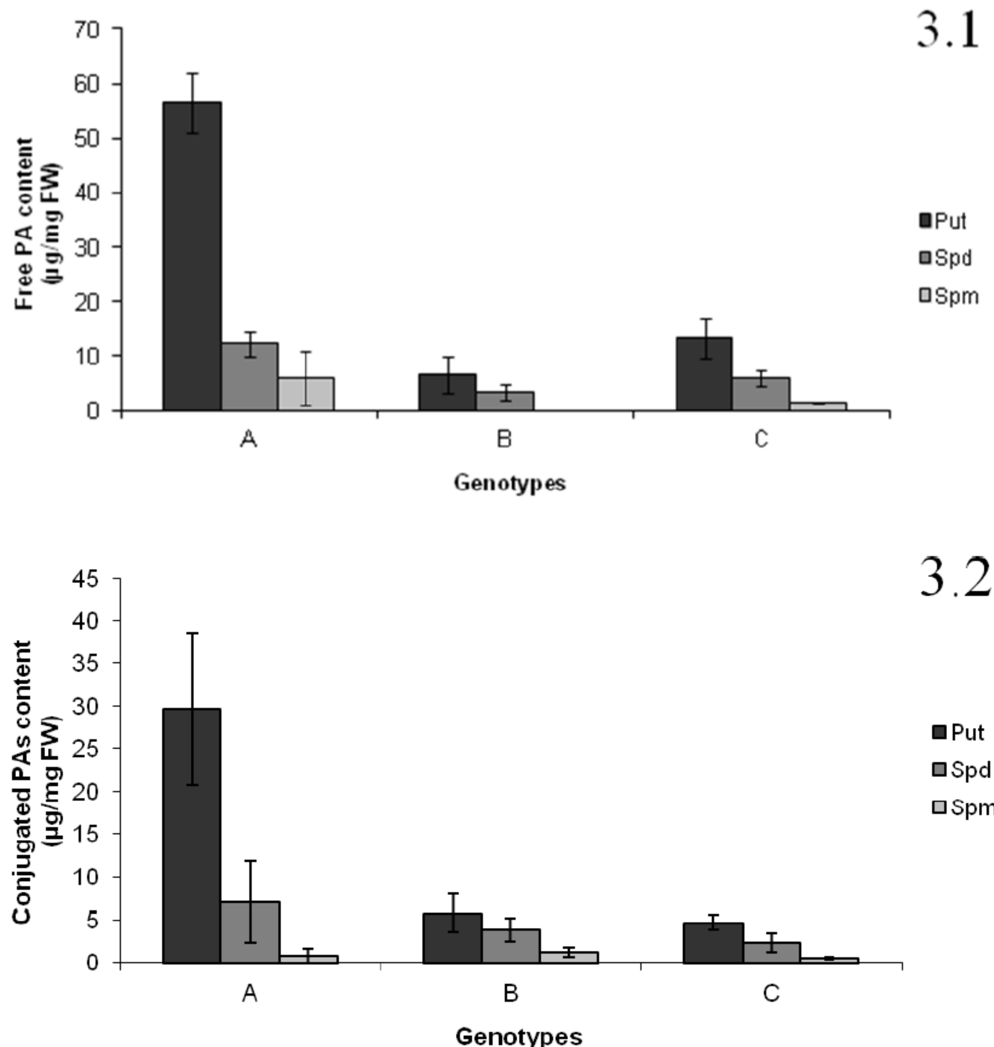

Fig. 3 *Free (3.1) and conjugated (3.2) PAs levels in Brazilian pine embryogenic cultures with different embryogenic potential. A – genotype non-responsive to ABA and osmotic agents, B and C – genotypes responsive to ABA and osmotic agents.*

Conclusion

In recent years, the increasing use of wood and products obtained from forest species demands higher productivity from the forest industry. However, this greater demand cannot be met based only on the exploitation of areas containing native populations or on the use of endangered species like Brazilian pine. Biotechnology tools like SE may allow the forest industry to increase productivity figures so as to meet market demands, apart from affording the sustainable use and the *ex situ* conservation of remaining Brazilian pine populations. For this scenario to become a reality, efficient protocols of regeneration of Brazilian pine using SE still need to be perfected.

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References

Baron K, Stasolla C (2008) The role of polyamines during *in vivo* and *in vitro* development. In Vitro Cell. Dev. Biol.-Plant 44:384-395

Bewley JD, Black M (1994) Seeds: Physiology of development and germination. Plenum Press, New York, pp 341

Cairney J, Pullman GS (2007) The cellular and molecular biology of conifer embryogenesis. New Phytol 176:511–536

dos Santos ALW, Silveira V, Steiner N, Vidor M, Guerra MP (2002) Somatic embryogenesis in Parana Pine (*Araucaria angustifolia* (Bert.) O. Kuntze). Brazi Archi Bio/Tech 45(1):97-105

dos Santos ALW, Steiner N, Guerra MP, Zoglauer K, Moerschbacher BM (2008) Somatic embryogenesis in *Araucaria angustifolia*. Biol Plant 52(1):195-199

Durzan DJ (2008) Monozygotic cleavage polyembryogenesis and conifer tree improvement. Tsitol Genet 2(3): 159-173

Durzan DJ (2012) Female parthenogenetic apomixis and androsporogenetic parthenogenesis in embryonal cells of *Araucaria angustifolia*: interpolation of progenesis and asexual heterospory in an artificial sporangium. Sex Plant Reprod 25(3): 227-246

Guerra MP, Kemper E (1992) Tecnologias Futuras: Aplicação da poliembriogênese somática para a propagação massal de plantas elite de *Araucaria angustifolia* (Bert) O Ktze. Anais do Congresso Nacional Sobre Essências, São Paulo, pp 1233-1236

Guerra MP, Silveira S, dos Santos ALW, Astarita LV, Nodari RO (2000) Somatic embryogenesis in *Araucaria angustifolia* (Bert) O. Ktze. In: Jain SM, Gupta P, Newton R (eds) Somatic Embryogenesis in Woody Plants, Vol. 6, Kluwer Academic Publishers, Dordrecht, pp 457-478

Guerra MP, Silveira V, Reis MS, Schneider L (2002) Exploração, Manejo e Conservação da Araucária (*Araucaria angustifolia*). In: Simões LL; Lino CF (eds) Manejo Sustentável da Mata Atlântica: A Exploração de seus Recursos Florestais. SENAC, São Paulo, pp 85-101

Haines RJ, Prakasha N, Nikles DG (1984) Pollination in *Araucaria juss*. Aust J Bot 32:583-594

Handro W, Ferreira A (1986) Araucaria (*Araucaria spp.*). In Bajaj YPS (ed). Biotechnology in Agriculture and Forestry, Tree I. Springer-Verlag, Heildelberg, pp 310-315

Jo L, dos Santos ALW, Schlögl PS, Guerra MP, Rossi MM, Floh EIS (2011) Establishment of molecular markers for early selection of embryogenic cultures with high embryogenic potential in brazilian pine (*Araucaria angustifolia* (BERT) O. KTZE). BMC Proc 5(7):138

Klimaszewska K, Smith DR (1997) Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. Physiol Plant 100:949–957

Klimaszewska K, Overton C, Stewart D, Rutledge RG (2011) Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old somatic white spruce and expression profiles of 11 genes followed during the tissue culture process. Planta 233:635-647

Lara-Chavez A, Flinn B, Egertsdotter U (2011) Initiation of somatic embryogenesis from immature zygotic embryos of Oocarpa pine (*Pinus oocarpa* Schiede ex Schlectendal). Tree Physiol 31(5):539-554

Mattos JR (1994) O pinheiro brasileiro. Artes gráficas princesa Ltda., Lages, pp 225

Ondro WJ, Couto L, Betters DR (1995) The status and practice of forestry in Brazil in the early 1990s. Forest Chron 71: 106-119

Osti RZ, Balbuena TS, Guerra MP, Franco DW, Floh EIS (2010) Nitrosyl ethylenediaminetetraacetate ruthenium (II) complex promotes cellular growth and could be used as nitric oxide donor in plants. Plant Sci 178:448-453

Reed BM, Kane M, Bunn E, Pense VC (2011) Biodiversity conservation and conservation tools. In Vitro Cell Dev Biol – Plant 47:1-4

Santa-Catarina C, Silveira V, Scherer GFE, Floh EIS (2007) Polyamine and nitric oxide levels correlate with morphogenetic evolution in somatic embryogenesis of *Ocotea catharinensis.* Plant Cell Tiss Organ Cult 90:93-101

Shimizu JY, Oliveira YMM (1981) Distribuição, variação e usos dos recursos genéticos da araucária no sul do Brasil. EMBRAPA-URPFCS, Curitiba, pp 9

Silveira V, Santa-Catarina C, Tun NN, Scherer GFE, Handro W, Guerra MP, Floh EIS (2006) Polyamine effects on the endogenous polyamine contents, nitric oxide release, growth and differentiation of embryogenic suspension cultures of *Araucaria angustifolia* (Bert.) O. Ktze.. Plant Sci 171:91-98

Stasolla C, Yeung EC (2003) Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. Plant Cell Tiss Organ Cult 74:15-35

Stasolla C (2010) Glutathione redox regulation of in vitro embryogenesis. Plant Physiol Biochem 48(5):319-27

Steiner N, Santa-Catarina C, Silveira V, Floh EIS, Guerra MP (2007) Polyamine effects on growth and endogenous hormones levels in *Araucaria angustifolia* embryogenic cultures. Plant Cell Tiss Organ Cult 89:55-62

Steiner N, Santa-Catarina C, Andrade JBR, Balbuena TS, Guerra MP, Handro W, Floh EIS, Silveira V (2008) *Araucaria angustifolia* Biotechnology. Funct Plant Sci Biotechnol 2:20-28

Steiner N, Santa-Catarina C, Guerra MP, Cutri L, Dornelas MC, Floh EIS (2012) A gymnosperm homolog of S OMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE-1 (SERK1) is expressed during somatic embryogenesis. Plant Cell Tiss Organ Cult 109:41-50

Stefenon VM, Steiner N, Guerra MP, Nodari RO (2009) Integrating approaches towards the conservation of forest genetic resources: a case study of *Araucaria angustifolia*. Biodivers Conserv 18(9):2433-2448

Svobodová H, Albrechtova J, Kumstyrova L, Lipavská H, Vagner M, Vondrakova Z (1999) Somatic embryogenesis in Norway spruce: anatomical study of embryo development and influence of polyethylene glycol on maturation process. Plant Physiol Biochem 37:209–221

Tun NN, Santa-Catarina C, Begum T, Silveira V, Handro W, Floh EIS, Scherer GFE (2006) Polyamines induce rapid biosynthesis of nitric oxide (NO) in *Arabidopsis thaliana* seedlings. Plant Cell Physiol 3(47):346-354

Vieira LN, Santa-Catarina C, Fraga HPF, dos Santos ALW, Steinmacher DA, Schlogl PS, Silveira V, Steiner N, Floh EIS, Guerra MP (2012) Glutathione improves early somatic embryogenesis in *Araucaria angustifolia* (Bert)

O. Kuntze by alteration in nitric oxide emission. Plant Sci 195:80-87

Zhang SG, Han SY, Yang WH, Hua-Li W, Zhang M, Qi LW (2010) Changes in H_2O_2 content and antioxidant enzyme gene expression during the somatic embryogenesis of *Larix leptolepis*. Plant Cell Tiss Organ Cult 100:21-29

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The prospects for using somatic embryogenesis to propagate Sitka spruce in the UK

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Introduction

Sitka spruce (*Picea sitchensis* (Bong.) Carr.) is native to the west coast of North America (Fig. 1.) and is closely related to the white spruce (*P. glauca* (Moench) Voss.), but over the past 80 years it has become the UK's predominant commercial forest tree species, due to its fast growth rate and its ability to thrive in a mild damp climate (Samuel et al., 2007). Sitka spruce now accounts for 26% of the forested area in Great Britain or 49% of all conifers, and over 60% of the UK's production of timber (Ward, 2012).

Forest Research (FR) is the research agency of UK Forestry Commission (FC), and maintains an active breeding programme for this species and provides seed lots from controlled crosses (family forestry) for supply to the UK forest nursery industry (Lee, 2006; Lee and Connolly, 2010), which are in turn used to produce large numbers of rooted cuttings for deployment (Lee and Watt, 2012). These efforts have not so far included the use of tissue culture approaches at any stage of the process, however.

In order to maximise the breeding gains available to the UK forest sector, specific efforts are now being made to replicate the tissue culture methods and multi-varietal forestry (MVF) approaches that have been successfully applied in recent years to white spruce by the Canadian Forest Service (Park, 2002; Klimaszewska et al., 2011). This article describes the progress that has been achieved at FR for initiating and proliferating embryogenic cell cultures of Sitka spruce from immature and mature seeds; the production of somatic embryos (SE) and plants from these cultures; and the cryo-preservation of selected cell lines.

These methods have become sufficiently reliable that MVF is now becoming a viable prospect for Sitka spruce in the UK. The likely contribution and value of this technology to the UK forest sector is discussed.

Why use Sitka spruce in the UK?

By 1905 agricultural, industrial and urban expansion had caused the UK's forested area to decline to 4.7% (Ward, 2012), which led to severe timber shortages during and immediately after the First World War (Pringle, 1994; Foot, 2012).

In an effort to remedy this, the FC was created in 1919 (Pringle, 1994; Foot, 2012), which ultimately led to the decision to concentrate efforts on Sitka spruce, especially from the 1960s onwards, as it thrives in mild wet climates and is very exposure resistant, but is still capable of producing mean maximum increments in the range of 16-20 $m^3.ha^{-1}.yr^{-1}$, even on relatively poor soils that are unsuitable for modern agriculture (Faulkner and Wood, 1957).

In short, Sitka spruce is an ideal tree for many of the wetter upland areas of the northern and western parts of the country UK (Samuel et al., 2007; Miller, 2009).

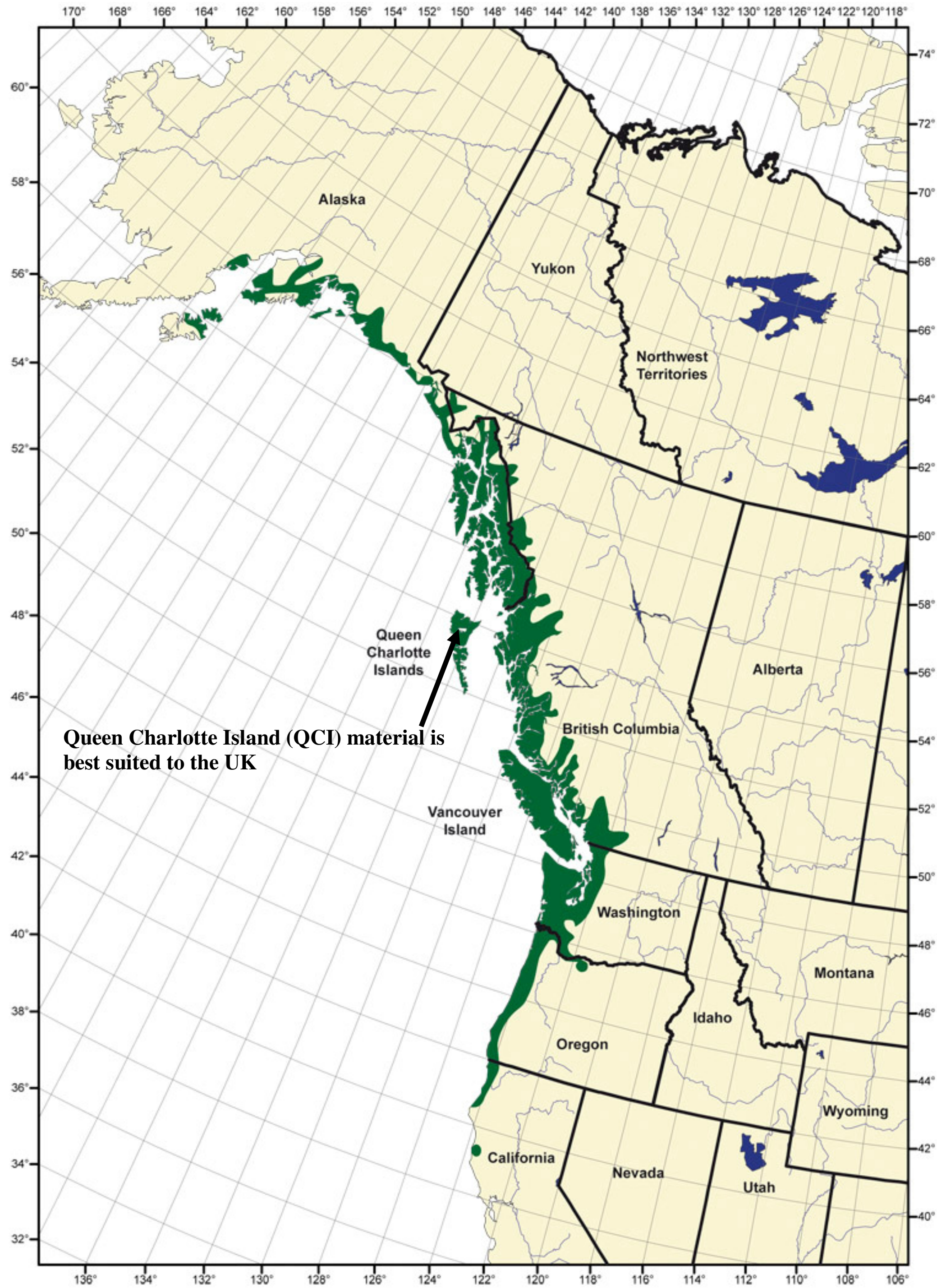


Figure 1. *The native range of the Sitka spruce (Picea sitchensis) shown in green, covers 24 degrees of latitude from Southern Alaska to Northern California, and includes enormous genetic diversity. The provenances that are best suited to UK conditions originate for the most part from Queen Charlotte Island, and are referred to as "QCI material" (Samuel et al., 2007; Lee and Watt, 2012).*

The huge native range of Sitka spruce means that there is a great diversity of material to choose from to suit a wide variety of maritime climates (Fig. 1.), although material from the correct latitude needs to be chosen. For instance, QCI material suits UK conditions because its photoperiod adaptation is the same as for the UK, while material from further south would be at risk from frost damage in the UK, while those from further north would cease growing too soon to take full advantage of the growing season (Lee, pers. comm.; Faulkner and Wood, 1957).

***Figure 2**. Left: Sitka spruce extraction from its native range in Oregon, early 1900s. Top right: Sitka spruce operations in the UK today favour upland locations on low grade land that is unsuitable for modern agriculture. Rotation is commonly around 40 years. Bottom right: forest management in the UK is now highly mechanised, with planting and harvest operations optimised by complex decision support tools, as well as breeding research and development, in order to meet production and environmental objectives (Pringle, 1994; Read et al., 2009; Foot, 2010).*

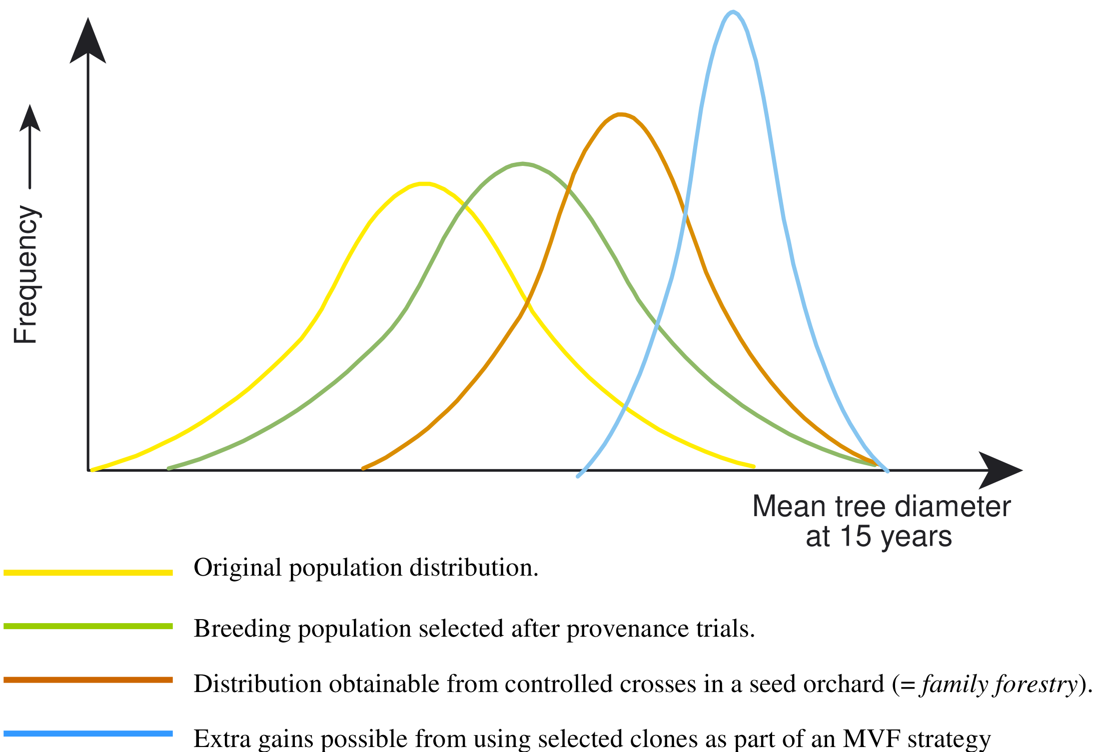
Since 1905, the forested land area of the UK has increased to nearly 13% today, or just over 3 million hectares (Ward, 2012). In the UK mainland, there are 3 million hectares of woodland, of which just over half is conifers, 49% of which is Sitka (or 26% of the total, or 692,000 ha). However, conifers account for 95% of the UK timber harvest or nearly 10 million green tonnes in 2011-12, of which ~67% was Sitka spruce (Ward, 2012; Ditchburn, pers. comm.).

From this it can be estimated that Sitka spruce forests are nearly 6 times as productive as the other UK forests in terms of the wood they produce. However, if the best planting material currently available from the FR breeding programme is used in conjunction with the best planting and management practices (Fig. 2.), then the potential advantage to the UKs commercial forest sector of using Sitka spruce is probably even greater than these figures suggest.

Breeding with Sitka spruce in the UK

A breeding programme was begun in the UK for Sitka spruce in 1963, starting from ~1800 ‘plus trees’ which had performed well in British plantation forests, and which mainly consisted of Queen Charlotte Island (QCI)

material (Samuel et al., 2007). The end of rotation genetic gains currently being achieved for improved planting stock varies from a 21-29% increase in wood volume for family mixtures on average (including the use of seed orchards), and a 29% height advantage over unimproved material after 10 years (Lee and Matthews, 2004; Lee, 2006; Lee and Watt, 2012) (Fig. 3.).



Original population distribution.

Breeding population selected after provenance trials.

Distribution obtainable from controlled crosses in a seed orchard (= *family forestry*).

Extra gains possible from using selected clones as part of an MVF strategy

Figure 3. *A diagrammatic representation of the gains in tree diameter that can be achieved by various tree improvement strategies. Multi-varietal forestry (MVF, shown in blue) can produce the greatest breeding gains, as it enables the best performing clones to be selected for use from a more diverse sub-population. Note that this approach can also be used for many other traits of interest, and not only for maximising the gains in mean tree diameter.*

The improved material is propagated from cuttings taken from stock hedges grown from full-sib seed lots, which are then distributed to foresters (5-7 million annually) by FC and private nurseries (Lee and Watt, 2012). To date though, no tissue culture procedures have been used at any stage of the production or breeding process, partly due to cost and the uncertainty over the extra gains that might be achieved, and partly also due to difficulties that were encountered in pilot scale operations. However, after the MVF approach was successfully demonstrated with white spruce by the Canadian Forest Service and the private company J.D. Irving Limited in New Brunswick, Canada (Park, 2002), the possibility for using this approach in the UK with Sitka spruce was re-considered.

In brief, the MVF approach involves establishing embryogenic cell lines from zygotic embryos, extracted from seeds obtained from the breeding population of the tree species of interest and determining their performance first in tissue culture and then later with field trials of the clonal plants derived from them (Fig. 4.). In this way the performance characteristics of the tissue cultures and the clonal plants derived from them can be accurately determined; the tested and selected clones thereafter being referred to as *varieties*.

A critically important issue for successfully implementing MVF is to cryo-preserve the tissue cultures (genotypes) as they are produced, so that they can be stored in their highly embryogenic state without change in genetic make-up or loss of juvenility. In this way, once the best performing clones have been identified by field

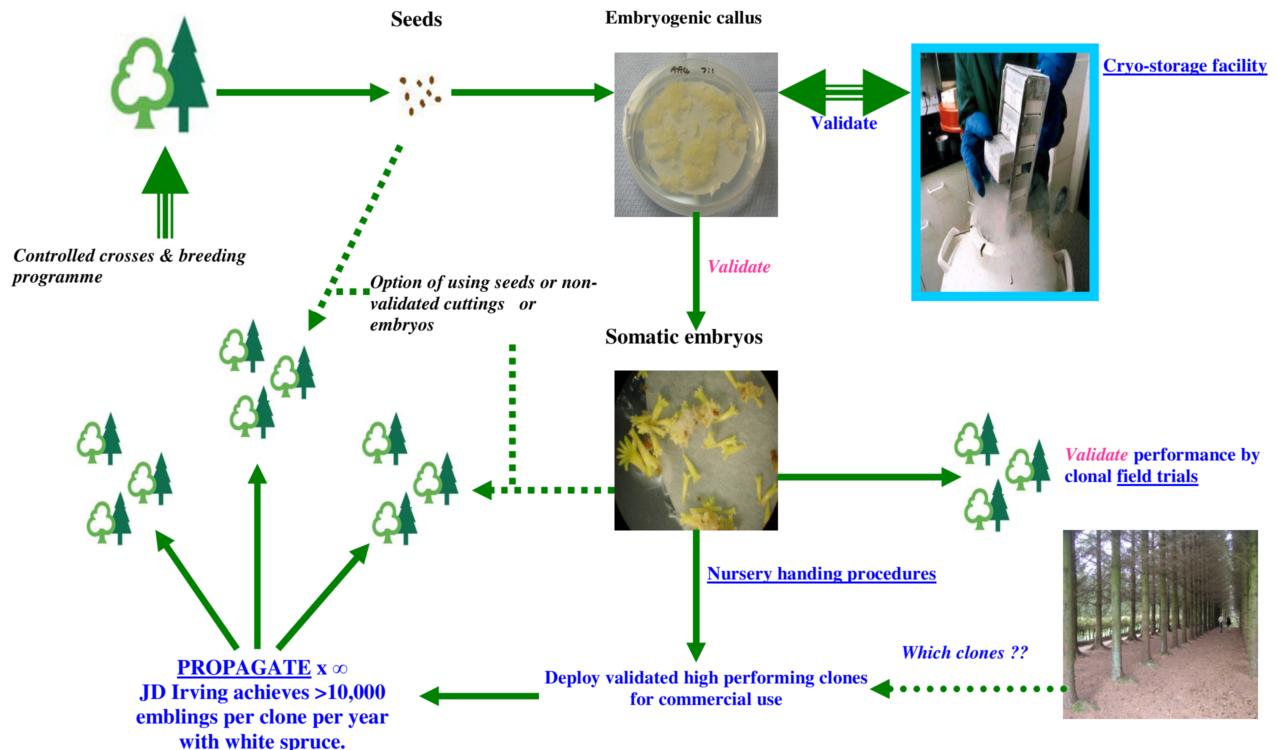


Figure 4. *A diagrammatic representation of the MVF approach to propagating and deploying SE derived clonal conifers. Note that the protocol would break down if it were not for the use of cryo-preservation, as it would no longer be possible to propagate the desired clones by the time they were required, as the cultures would have lost their embryogenic potential by the time the field trials of the plants derived from them had been completed, which require 5-10 years or more. In collaboration with the Canadian Forest Service, the company JD Irving Limited have successfully adopted this approach and are now deploying large numbers of P. glauca SE plants into the forests of eastern Canada and the US every year. The dotted arrows indicate the options which may be employed for the production of nursery stock at various stages when developing an MVF system from scratch. Clonal varieties are regarded as having been 'validated' once the data about their performance from the field trial data is available, such that their behaviour in subsequent commercial plantings can be reliably predicted.*

trials, the corresponding embryogenic cultures can then be re-propagated, possibly many years after they were first generated, and thereafter as many normal healthy plants can be produced from these cultures as are required (Fig. 4.).

Tissue culture of Sitka spruce at Forest Research

Due to the success of the Canadian Forest Service with MVF and white spruce in Eastern Canada (Park, 2002), FR decided to re-evaluate the potential of such methods for Sitka spruce in the UK. Although there were various reports in the literature of assorted tissue culture methods being applied to Sitka spruce, including for SE and cryo-preservation purposes (Moorhouse et al., 1996; Drake et al 1997; Find et al., 1998; Gleeson et al., 2004; Gale et al., 2007), it was decided to copy as closely as possible the methods and approach of the Canadian Forest Service with white spruce (Park, 2002; Klimaszewska et al., 2011). This was because the two species are closely related and the protocols developed for white spruce by the Canadian Forest Service had shown themselves to be amenable to being scaled up to support an active breeding and deployment programme (Park, 2002; Klimaszewska et al., 2011),

which is also required for Sitka spruce in the UK context.

This report summarises our findings for implementing this scheme for Sitka spruce at FR, especially those points that may diverge somewhat from the procedures utilised by the Canadian Forest Service for white spruce, whether due to differences between the species or differences in laboratory organisation.

Protocol Development and Testing

1. Testing the establishment and proliferation phases – general :

- Immature zygotic embryos extracted from cones harvested in late July – early August give generally high levels of initiation and establishment (in the range of 35-80%, but ~70% on average), after 1-3 months on a modified Litvay medium at 25 ^{o}C, as described by Park (2002) for *P. glauca* (Figs. 4. and 5.).
- Only slightly lower levels of success were obtained with zygotic embryos extracted from mature seeds (~50%), provided that they had not been in storage for more than a few months.
- It was possible to establish cell lines from zygotic embryos extracted from mature seeds that have been in storage for some years, albeit with lower levels of success (4-44%) and much more work.
- Initiation was higher on average (~70%) when 6 g.L^{-1} Bacto agar was used as a gelling agent, as opposed to when Phytagel (3-5 g.L^{-1} tested) was used (~30%).
- Embryogenic callus proliferated faster when 6 g.L^{-1} Bacto agar was used as a gelling agent, as opposed to when Phytagel was used, but the callus was very wet and did not perform well in this condition for cryo-preservation or somatic embryo production purposes (Figs. 5. and 6.).

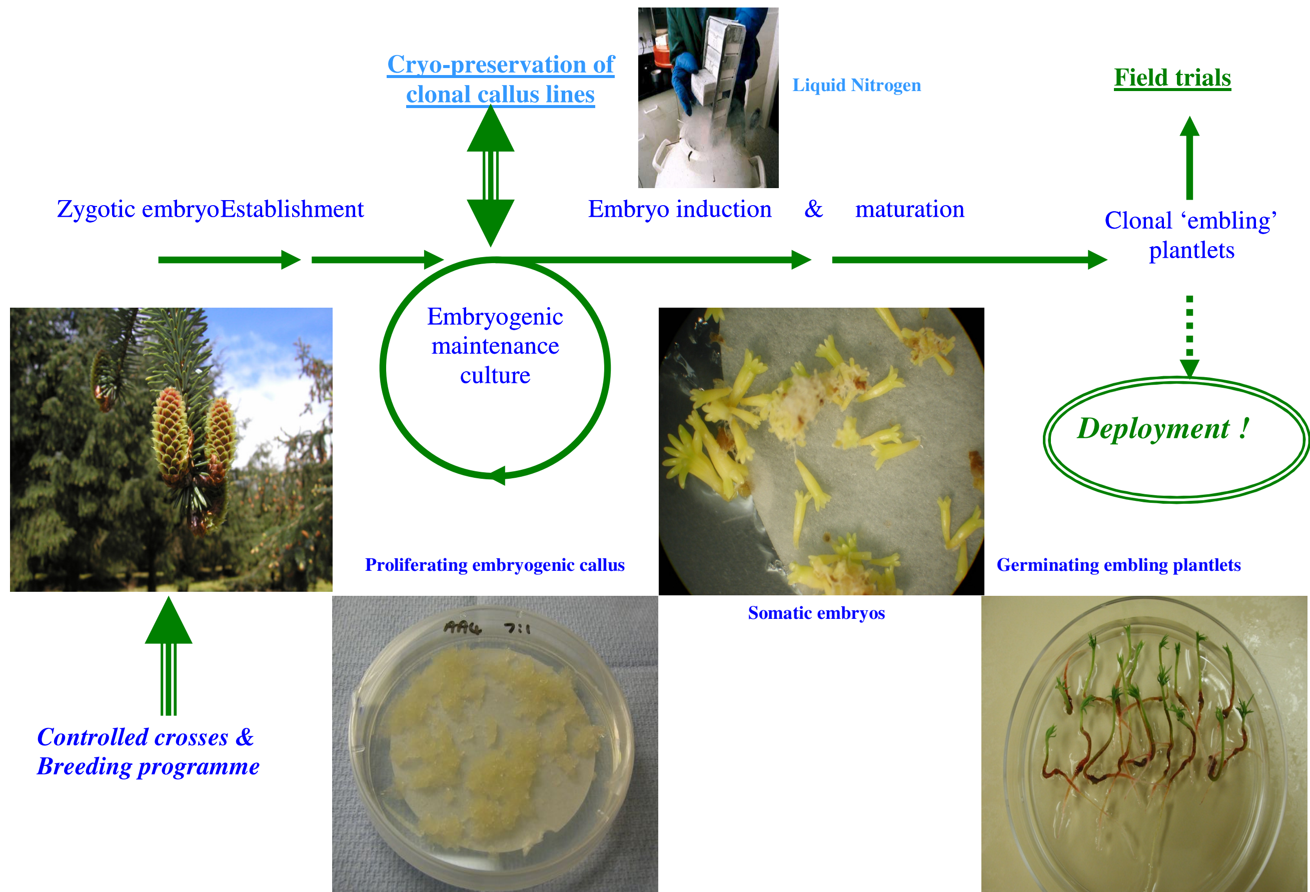


Figure 5. *A more detailed schematic of how Sitka spruce can be mass propagated by SE, for the purposes of MVF. Note that the somatic embryos need to be germinated in vitro (as shown in the Petri dish on the photo on the lower right), before being weaned off for normal growth in a nursery, as they would not survive otherwise.*

2. Testing the cryo-preservation protocols – general :

- The cell lines being prepared for cryo-storage are subcultured 4x bi-weekly and maintained at 25 ^{o}C on a modified Litvay medium solidified with 5 g.L^{-1} Phytagel prior to cryo-preservation, and using small pieces (~5mm) of rapidly proliferating Sitka callus material, preferably not more than 12 months after being established as a culture, as adapted from Park (2002) for *P. glauca*.
- Callus samples are then shaken overnight in 100 ml flasks, in a liquid version of the standard proliferation medium as above except for the addition of 0.5m sorbitol and 2% sucrose, with 3.5g callus used in 14 ml of medium.
- The cryo-preservation medium is also the same as the above medium, except for the addition of DMSO to a final concentration of 10%, and keeping the time that the samples are exposed to the DMSO mix before freezing to <30 minutes.
- 1.8 ml internally threaded Nunc™ cryo tubes are used for cryo-storing the prepared samples, with 1.5 ml of sample per tube.
- Mr Frosty™ cooling boxes are used for the cooling the samples (pre-cooled to 4 ^{o}C), by placing them in the -80 ^{o}C freezer for 90 minutes, before transferring the samples to the liquid N_2 cryo-storage system.
- The samples are defrosted in sterile water at 40 ^{o}C for ~2 minutes and then poured off onto a piece of Nylon mesh over pads of paper discs for drawing off the moisture, rather than draining off the sample directly onto a paper disc.
- Standard proliferation medium is used for recovering samples, transferring the nylon discs with the ex-cryo samples on to new medium after 24 hours and 7 days only.
- Recovery is usually observed after 1-2 weeks, and up to 70% of cell lines can be recovered this way, and 90-100% of their samples. Cell lines that do not produce good numbers of somatic embryos are excluded from this system, however, and since only ~40% of the available cell lines that have been tested so far fulfil this requirement, only a minority of the cell lines and the breeding lines they represent will be available for use with this MVF programme with existing methods.

The alternative approach (as utilised for *P. glauca* in Canada) is to cryo-preserve all of the cell lines as they are produced, and later discard those that have either not survived the cryo-preservation procedures or cannot be induced to produce somatic embryos at all.

This is the basis of the methodology that is now being used to place validated cell lines into a long term cryo-storage facility, established from seeds that were taken from controlled crosses within FR's breeding programme for Sitka spruce. Work is still ongoing to optimise the somatic embryo production, germination and weaning off procedures, as described in the next section and Fig. 6 below.

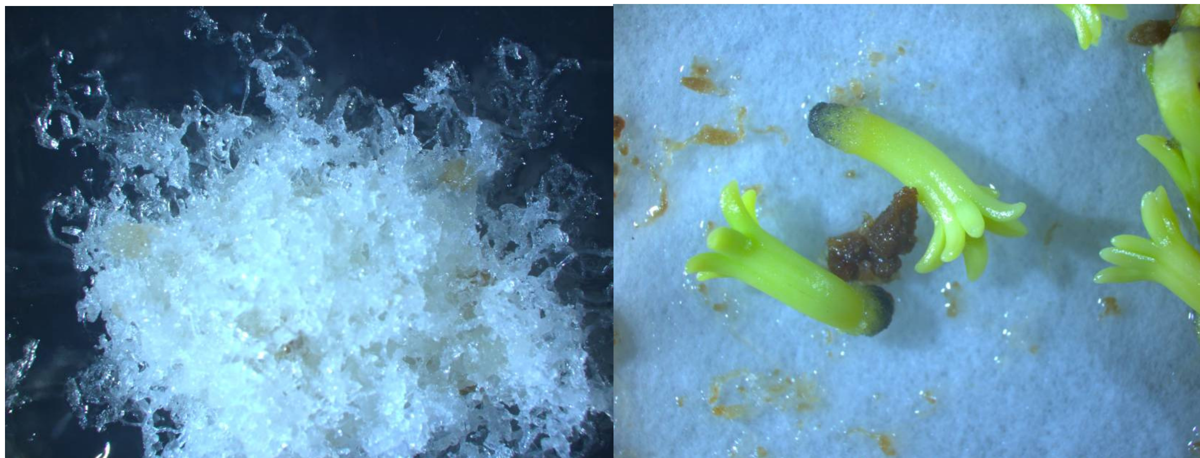


Figure 6. *Top: Sitka spruce embryogenic callus (~15 mm across), proliferating under similar conditions to those described by Park (2002) and Klimaszewska et al., (2011) for white spruce. Note the presence of very large suspensor cells which are clearly visible to the naked eye. Bottom: fully formed mature somatic embryos (~5mm long), also produced largely according to the same conditions as utilised for white spruce.*

3. Germination and nursery procedures – general :

- 2011 Germination: 76% of emblings had roots, 24% did not, from 19 clones and 1318 plants.
- 2012 Germination: ~25% of emblings had roots and ~75% did not, from ~4000 plants.

It was not initially clear what had caused this major discrepancy in the germination rates between the two years, which was obviously a matter of concern.

2011-12 nursery data

i.e. using the emblings from 2011 for growing on in the nursery in the spring of 2012.

- 65% (+/- 3.9 Standard Error) of the emblings that had roots prior to beginning the weaning procedures, were still alive after 3 months in the nursery across 19 clones.
- Only (24% +/- 5.3 SE) of the emblings that did not have roots prior to beginning the weaning procedures, were still alive after 3 months in the nursery across 16 clones.
- 88.2% (+/- 4.3 SE) of the emblings that had roots prior to beginning the weaning procedures and were produced from embryos *that had not been subjected to controlled drying* (but instead were simply stored on the maturation medium at 4 ^{o}C for 3 months prior to germination), were still alive after 3 months in the nursery across 4 clones. 72.3% (+/- 6.5 SE) of the same 4 clones that were subjected to the controlled drying procedures survived over the same time period, however.
- The time of weaning off and planting into the nursery from April to June appeared to have no significant effect on the emblings survival.

From this, it is clear that there is a need to identify the causes of the intermittent poor germination and rooting *in vitro*. The nursery procedures also need to be optimised so as to minimise losses and maximise subsequent plant growth, including identifying the best rooting substrate and watering regime for the young emblings.

The advantages and disadvantages of adopting MVF approaches for Sitka spruce

Advantages

- The best clones can be 'captured' within the breeding programme, for later use or deployment according to need.
- Enables the identification and capture of the best clones with multiple desirable traits.
- The best clones can be multiplied up almost infinitely.
- Greater genetic gain should be possible than from controlled crosses in a seed orchard alone, as has already been demonstrated with *P. glauca* in Canada.
- Flexibility to deploy different varieties into plantations in order to cope with new challenges as they arise, such as climate change or diseases.
- Increased predictability, uniformity and repeatability of the plant material.

Disadvantages

- The cost base will probably be higher than when using cuttings based systems alone, potentially resulting in more expensive plants, although this probably will also be affected by how the work is organised and the numbers of SE plants produced.
- Customers may be unwilling to pay more for the MVF derived plant material, unless they either understand the benefits on offer and believe these to be worthwhile for them, or if the extra costs associated with the improved planting stock are subsidised in some way, to encourage the use of the superior material generated.
- The benefits of MVF will accrue mainly to the forester and/or the customer of the forest products, but the extra costs will mainly be borne by the breeder, necessitating some form of levy or subsidy regime to support the extra work required.
- Long term field trials are needed to assess the performance of the MVF clones.
- These field trials will need to be retained, in case the objectives of the breeding or planting programmes change, and alternative clones need to be selected.
- An ever expanding cryo-storage system is needed for retaining the tissue cultures, which adds to the cost of the MVF operation.

Future work and Conclusions

The tissue culture and cryo-preservation procedures that were developed for white spruce by the Canadian Forest Service have proved to be sufficiently robust to enable the successful development of MVF for commercial use, including the large scale production of embling plantlets derived from somatic embryos for use in production forestry settings (Park, 2002; Klimaszewska et al., 2011).

These procedures have now been successfully adapted and applied to the pilot scale projects already underway at FR for the production of SE Sitka spruce plantlets in the UK, including for the initiation and proliferation of embryogenic cell lines established from zygotic embryos, which were derived from controlled crosses within FRs breeding programme for the species. In addition, extensive modifications and tests of the cryo-preservation protocol have demonstrated that it is now sufficiently reliable to begin placing embryogenic cell cultures of Sitka spruce into long term storage, which is a necessary first step to be able to deploy defined clonal varieties when and as required in future.

It is also clear that further work is needed to optimise the production and germination of the somatic embryos from Sitka spruce *in vitro*, and to maximise their survival in the first weeks after their transfer to independent growth in nursery conditions. However, this need not be an obstacle to planning and developing the necessary long term field trials before FR can offer validated clonal varieties of Sitka spruce to the UK forest industry, as part of a fully functional and effective MVF strategy.

The current problem with the erratic germination and nursery survival rates of the plantlets, will need to be resolved before the large scale commercial production of plants by these methods can be offered to UK forest industry (Lee and Watt, 2012). As soon as this issue is resolved, however, there is no reason to suppose that similar levels of success to that observed with MVF in Eastern Canada cannot be enjoyed by the UK forest sector with Sitka spruce.

The development of an MVF scheme as described here for Sitka spruce will not only enable improvements in the productivity and the quality of the timber produced by the UK's commercial forest sector, but will also increase the flexibility of the sector to cope with the twin challenges of climate change and the increasing number of pest and disease outbreaks that are affecting the UK's forests today (Brasier, 2008; Read et al. 2009). This is because different clones can be selected from the cryo-store, either as objectives change over time (Foot, 2010), or as data become available from the field trials, indicating that certain clones are better able to meet the requirements than others.

From this, it can be seen that developing MVF approaches for Sitka spruce in the UK will not only improve the productivity of the UK's commercial forest sector over time by much more than is likely to be achieved otherwise, but will also increase its resilience to many of the biological and physical threats that are likely to be encountered in future. In summary, the MVF approaches which are being developed for Sitka spruce will go a long way to 'future proof' the UK forest sector, and will in all likelihood more than justify the required investment.

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References

Brasier, C.M. (2008). The biosecurity threat to the UK and global environment from international trade in plants. *Plant Pathology* **57** (5), 792-808.

Ditchburn, B. *Personal communication*: National Forest Inventory Programme Leader, Forestry Commission, Silvan House, Edinburgh, UK.

Drake, P.M.W., John, A., Power, J.B. and Davey, M.R. (1997). Expression of the *gus A* gene in embryogenic cell lines of Sitka spruce following *Agrobacterium*-mediated transformation. *Journal of Experimental Botany* **48** (306), 151-155.

Faulkner, R. and Wood, R.F. (1957). *Picea sitchensis* (Bongard) Carrière. Forestry Commission Bulletin No. 30.

Exotic Trees in Great Britain. pp.85-91.
Find, J.I., Kristensen, M.M.H., Nørgaard, J.V. and Krogstrup, P. (1998). Effect of culture period and cell density on regrowth following cryopreservation of embryogenic suspension cultures of Norway Spruce and Sitka spruce. *Plant Cell, Tissue and Organ Culture* **53** (1), 27–33.
Foot, D. (2010). Woods and People: Putting Forests on the Map. The History Press, Stroud, Gloucestershire, UK.
Gale, S., John, A. and Benson, E.E. (2007). Cryopreservation of *Picea sitchensis* (Sitka spruce) embryogenic suspensor masses. *CryoLetters* **28** (4), 225-240.
Gleeson, D., Lelu-Walter, M.A., and Parkinson, M. (2004). Influence of exogenous L-proline on embryogenic cultures of larch (*Larix leptoeuropaea* Dengler), sitka spruce (*Picea sitchensis* (Bong.) Carr.) and oak (*Quercus robur* L.) subjected to cold and salt stress. *Annals of Forest Science* **61** (2), 125–128.
Klimaszewska, K., Park, Y.S. and Bonga, J.M. (2011). Tissue culture research at the CFS: Its history, current status and potential benefits. Canadian Forest Service Publications, Catalog ID: 32295: http://cfs.nrcan.gc.ca/publications?id=32295
Lee, S. and Matthews, R. (2004). An indication of the likely volume gains from improved Sitka spruce planting stock. The Forestry Commission, Silvan House, 231 Corstorphine Road, Edinburgh, Scotland, UK, EH12 7AT : http://www.forestry.gov.uk/PDF/fcin055.pdf/$FILE/fcin055.pdf
Lee, S.J. (2006). It's a family affair. 'Full-sib' families are a new generation of improved Sitka spruce planting stock. *Forestry and British Timber* **35** (12), 14-16.
Lee, S., and Connolly, T. (2010). Finalizing the selection of parents for the Sitka spruce (*Picea sitchensis* (Bong.) Carr) breeding population in Britain using Mixed Model Analysis. *Forestry* **83** (4), 423-431.
Lee, S. and Watt, G. (2012). Improved Sitka spruce planting stock; seedlings from clonal seed orchard or cuttings from full-sibling families? *Scottish Forestry* **66** (1), 18-25.
Lee, S. *Personal communication*: Senior tree breeder and Programme Group Manager for Tree Improvement, Northern Research Station, Forest research, UK.
Miller, J. (2009). The Foresters. The story of Scotland's forests. Birlinn Limited, West Newington House, Edinburgh, Scotland, UK.
Moorhouse, S.D., Wilson, G., Hennerty, M.J., Selby, C. and MacAntSaoir, S. (1996). A plant cell bioreactor with medium-perfusion for control of somatic embryogenesis in liquid cell suspensions. *Plant Growth Regulation* **20** (1), 53-56.
Park, Y.S. (2002). Implementation of conifer somatic embryogenesis in clonal forestry: technical requirements and deployment considerations. *Annals of Forest Science* **59** (5-6), 651-656.
Pringle, D. (1994). The Forestry Commission – The First 75 Years. The Forestry Commission, Silvan House, 231 Corstorphine Road, Edinburgh, Scotland, UK, EH12 7AT.
Read, D.J., Freer-Smith, P.H., Morison, J.I.L., Hanley, N., West, C.C. and Snowdon, P. (eds). 2009. Combating Climate Change - a role for UK forests: An Assessment of The Potential of The UK's Trees and Woodlands to Mitigate and Adapt to Climate Change. The Stationery Office Limited, 26 Rutland Square, Edinburgh EH1 2BW : http://www.forestry.gov.uk/forestry/infd-7y4gn9
Samuel, C.J.A., Fletcher, A.M. and Lines, R. (2007). Choice of Sitka spruce seed origins for use in British forests. Forestry Commission Bulletin 127. The Forestry Commission, Silvan House, 231 Corstorphine Road, Edinburgh, Scotland, UK, EH12 7AT.
Ward, S. (2012) Forestry Statistics 2012 : http://www.forestry.gov.uk/forestry/infd-8xfcwy

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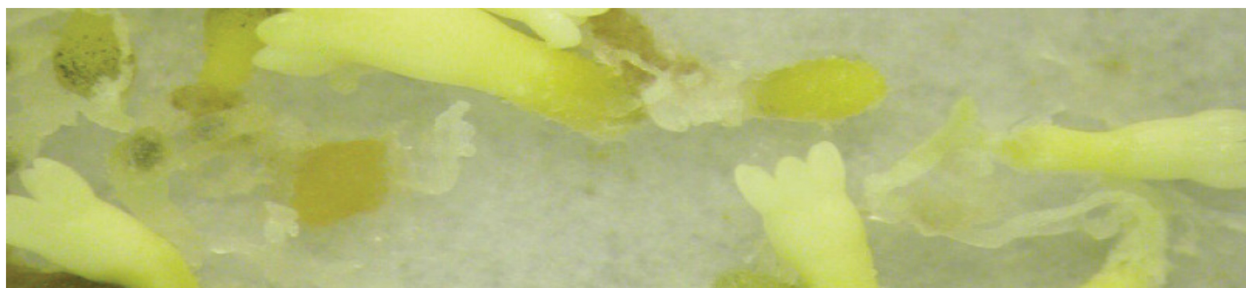
The GB Forestry Commission : http://www.forestry.gov.uk/
Forest Research, home page : http://www.forestry.gov.uk/forestresearch
Forest Research, "50 years of tree breeding in Britain" : http://www.forestry.gov.uk/fr/INFD-5WNJBW
Breeding and production of conifers in the UK : http://www.forestry.gov.uk/FR/INFD-65FBR9
JD Irving, Limited : http://www.jdirving.com/

Extended Abstract



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"Integrating vegetative propagation, biotechnologies and genetic improvement for tree production and sustainable forest management"
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Patterning during somatic embryogenesis in conifers

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Keywords: *Picea abies,* early embryo differentiation, gene expression, somatic embryogenesis

Improvement of forest trees by breeding is a slow process in which economically significant characteristics are continuously improved. The improvement is obtained by testing and selection. Each breeding cycle for Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) takes in Sweden 20 to 25 years. The selected trees are used as parents in the next breeding cycle. The selected genotypes are also used for mass-propagation. This is done by establishing seed orchards which start to produce seeds after 15 to 20 years. Consequently, seed orchards are 20 years behind the current breeding front. Somatic embryogenesis is an attractive method to combine breeding and propagation programmes. We believe that somatic embryos will replace at least some seed orchards in the future.

Embryogenic cultures of Norway spruce can be established from mature zygotic embryos, however, the embryogenic potential decreases during germination. In Arabidopsis, *LEAFY COTYLEDON* (*LEC*) genes are expressed during the embryonic stage, and must be repressed to allow germination. Treatment with the histone deacetylase inhibitor trichostatin A (TSA) causes de-repression of *LEC* genes. *ABSCISIC ACID3* (*ABI3*) and its *Zea mays* ortolog *VIVIPAROUS1* (*VP1*) act together with the *LEC* genes to promote embryo maturation. We have isolated two LEC1-type *HAP3* genes, *PaHAP3A* and *PaHAP3B*, from Norway spruce (Uddenberg et al. 2011). A comparative phylogenetic analysis of plant *HAP3* genes suggsts that *PaHAP3A* and *PaHAP3B* are paralogous genes originating from a duplication event in the conifer lineage. The expression of *PaHAP3A* is high, both in zygotic and somatic embryos, during early embryo development, but decreases during late embryogeny. In contrast, the expression of *VP1* is initially low but increases during late embryogeny. After exposure to TSA, germinating somatic embryos of *P. abies* maintain the competence to differentiate embryogenic tissue while the germination progression is partially inhibited. Furthermore, when embryogenic cultures of *P. abies* are exposed to TSA during maturation, the maturation process is arrested and the expression levels of *PaHAP3A* and *PaVP1* are maintained. This suggests a possible link between chromatin structure and expression of embryogenesis-related genes in conifers.

We are using somatic embryogenesis in Norway spruce as a model system for studying embryology in conifers (von Arnold and Clapham 2008). The model system includes a well-characterized sequence of developmental stages, resembling zygotic embryogeny, which can be synchronized by specific treatments, making it possible to collect a large number of somatic embryos at specific developmental stages. The process from differentiation of early embryos to production of mature cotyledonary embryos occurs within 5 to 7 weeks.

Polar auxin transport (PAT) is essential for the correct patterning of both the apical and basal parts of conifer embryos. PAT is inhibited when embryogenic cultures and developing embryos are treated with the PAT inhibitor NPA. Blocked PAT causes fused cotyledons, aborted embryonal shoot apical meristem (SAM) and an irregular root meristem (Larsson et al. 2007). During embryo development in most conifers the establishment of the SAM occurs concomitantly with the formation of a crown of cotyledons surrounding the SAM.

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. Four *KNOX1* genes have been identified in Norway spruce, *HBK1*, *HBK2*, *HBK3* and *HBK4*. During embryo development the *HBK2* and *HBK4* genes are significantly up-regulated concomitantly with the formation of an embryonic SAM, the up-regulation is delayed in NPA- treated

embryos lacking a SAM (Larsson et al. 2012). 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 HBK2 and HBK4 are essential for somatic embryogenesis and the formation of SAM.

In Arabidopsis the establishment of cotyledonary boundaries is dependent on PAT and the expression of *CUP-SHAPED COTYLEDON* (*CUC*) genes, which belong to the large *NAC* gene family. The CUC proteins presumably block cell proliferation at the cotyledon margins, thereby stimulating cotyledon separation and SAM formation. We have isolated two CUC-like genes, *PaNAC01* and *PaNAC02* from Norway spruce (Larsson et al. 2012). PaNAC01, but not PaNAC02, harbors previously characterized functional motifs in CUC1 and CUC2 from Arabidopsis. Furthermore, *PaNAC01* can functionally substitute for *CUC2* in the Arabidopsis *cuc1cuc2* double mutant. The expression profile of *PaNAC01* during embryo development shows that its expression is regulated by PAT and associated with formation of cotyledons and differentiation of SAM.

Taken together our results show that the central parts of the regulatory network for embryo development are conserved between angiosperms and gymnosperms, despite their separation 300 million years ago.

References

Larsson E, Sitbon F, Ljung K, von Arnold S (2008) Inhibited polar auxin transport results in aberrant embryo development in Norway spruce. New Phytol 177:356-366

Emma Larsson, Folke Sitbon, Sara von Arnold (2012) Differential regulation of *Knotted1-like* genes during establishment of the shoot apical meristem in Norway spruce (*Picea abies*). Plant Cell Rep DOI 10.1007/s00299-011-1224-6

Emma Larsson, Jens Sundström, Sitbon, Sara von Arnold (2012) Expression of *PaNAC01*, a *Picea abies CUP-SHAPED COTYLEDON* orthologue, is regulated by polar auxin transport and associated with differentiation of the shoot apical meristem and formation of separated cotyledons. Ann Bot (in press)

Daniel Uddenberg, Silvia Valladares, Malin Abrahamsson, Jens Sundström, Annika Sundås-Larsson, Sara von Arnold (2011) Embryogenic potential and expression of embryogenesis-related genes in conifers are affected by treatment with a histone deacetylase inhibitor. Planta 234:527-539

Sara von Arnold, David Clapham (2008) Spruce embryogenesis. In: Suarez MF, Bozhkov PV (eds) Plant embryogenesis Methods in molecular biology 427. Humana Totowa, pp 31-47

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Somatic embryogenesis induction on male catkins of Holm oak (*Quercus ilex* L.)

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Keywords: adult trees, catkin, genotype, oak, *Quercus*, somatic embryogenesis

Abstract: Both individual male flowers and catkins appear to be a suitable explant source to establish embryogenic lines from adult holm oak. SE induction is strongly dependent on the genotype. Under our experimental conditions, only one out of the six genotypes tested showed embryogenic competence.

Introduction

Quercus ilex L (holm oak) is the commonest evergreen oak species in the Mediterranean forests. Traditionally the species has been used as a source of acorns for animal feeding and lately as one of the top trees for the establishment of truffle orchards. Therefore, the species has become of great economical importance and could contribute to rural development in depressed areas. Traditional breeding of holm oak is hampered due to the low survival of the seeds, poor growth in plantations, and their recalcitrance to vegetative propagation. During the last few years our group has been working on the development of a suitable somatic embryogenesis (SE) protocol to propagate adult *Q. ilex*. Here we present our first results on the effect of male catkins developmental stage on somatic embryogenesis induction.

Materials and methods

Male catkins of *Q. ilex* were sampled at different developmental stages in five locations of the Valencia area (Spain): Ayora, Hunde, Portera, Remedio and Villar del Arzobispo (hereafter named as genotypes). Three different male catkins developmental stages were defined (Figure 1): Stage A: floral buds up to 2-4 mm with a varying number of meristematic structures developing at the same point; Stage B: catkin starting to develop with a short bare pedicel with distinguishable flowers (catkin size no larger than 1 cm); Stage C: elongated catkin (up to 2 cm in length) with erect catkins and closed flowers distinguishable along the axis.Catkins and isolated flowers from stages B and C were first cultured for 10 days in darkness on preconditioning semisolid medium (Gamborg's nutrients without growth regulators) (Gamborg et al., 1968).

For somatic embryogenesis induction, the explants were first cultured for 20 days in the dark on a modified solid MS (Murashige and Skoog, 1962) medium supplemented with 10µM BA and 50µM NAA; subsequently, the explants were transferred to the same medium with 5µM BA and 25µM NAA and maintained under dim light. After 60 days, explants were subcultured on manifestation medium (solidified MS medium with ammonium nitrate reduced to a half of its standard concentration, 0.5 µM BA and 0.5 µM NAA).

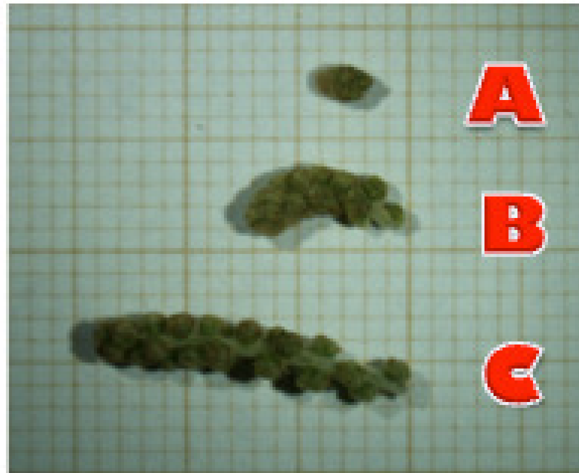

Fig. 1. Developmental stages of the male catkins used as primary explants

Results and conclusions

No explant contamination was observed during the preconditioning phase. After the first month on induction medium, and irrespective of the catkin developmental stage, callus formation was observed in the material sampled from the five genotypes.

Somatic embryogenesis induction was dependent on the genotype. Somatic embryos (SE) were only formed on brown callus with white-yellowish nodules (Figure 2) from the Hunde genotype sampled at stage C (6.7% and 1.5% of explants producing SE from catkins and isolated flowers, respectively). After 6 months, 8 SEs were obtained: 6 from individual flowers and 2 from catkins. Embryogenic lines are maintained through secondary embryogenesis (Figure 3). Ploidy level of somatic embryos will be determined.

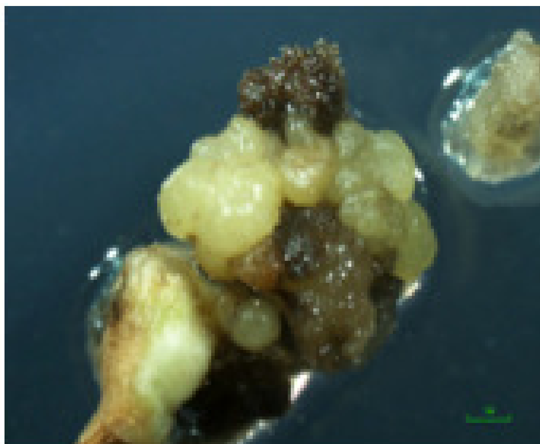

Fig. 2. Brown callus with white-yellowish nodules resembling globular embryos,.

Fig. 3. Embryogenic line established through secondary embryogenesis in holm oak cultures

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References

Gamborg O, Miller R, Ojima K (1968). Nutrient requirement suspensions cultures of soybean root cells. Exp Cell Res 50:151-158.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15: 473-497

IUFRO WORKING PARTY 2.09.02 SOMATIC EMBRYOGENESIS AND OTHER VEGETATIVE PROPAGATION TECHNOLOGIES

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Scaled-up egg suspension cultures of *Taxus brevifolia* biosynthesize taxol in an artificial sporangium

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Eggs rescued and scaled up in a 1-liter aqueous artificial sporangium synthesize the anti-cancer drug, paclitaxel, commonly known as taxol (A). Specific antibodies, which detect the ring or side-chain structure of taxol, demonstrated that the taxane ring derives its carbon from amyloplasts. The side chain was added to the taxane ring during the passage of precursors through the cytoplasm and endoplasmic reticulum (B). Taxol and its precursors where protected from binding to microtubules as components passed along actin fiber as vesicles and became bound to xyloglucan membranes (C). Membranes with taxol, its side chains were distributed outside the egg as membranes. This enabled the use of different magnetic antibodies to recover taxol, its side chain and taxane rings for studies aimed at increasing the recovery of this valuable drug (D). A project, involving the Eureca satellite (Daimler-Benz Aerospace) and the space shuttle (Boeing Aerospace), and my lab at the University of California was initiated to demonstrate if taxol production could be achieved in the Biotechnology Facility of the International Space Station. Space Hardware Optimization Technology (SHOT) developed an instrument which would enable the retrieval of the magnetic antibodies bound to taxol and its precursors. In the meantime, Weyerhaeuser cloned *Taxus brevifolia* for biomass production under field conditions. Extraction of this biomass offered a source of genotypes having high taxol content ultimately for use of taxol in treating ovarian and other cancers which were difficult to treat.

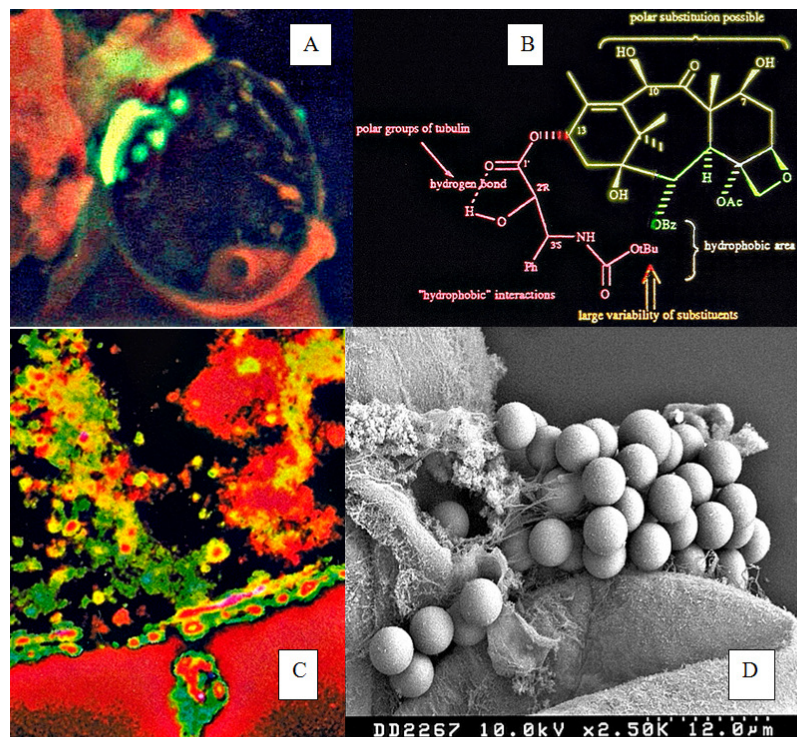

***Fig 1**. A. Taxus egg (ca. 150 µm dia), stained with fluorescent antibodies, produces taxol (yellow) from a taxane ring (green) and a side-chain (red). Both remain membrane-bound and are released into the culture medium. B. Taxol side-chain and ring structure. C. Egg cytoplasm releases bound taxanes into the culture medium (bottom). Artificial fluorescence. D. Magnetic antibodies are used to collect bound taxol and taxanes which are recovered by a magnetic separator (SHOT).*

US Patents

Durzan DJ, Ventimiglia FV
(1966) Taxane production in haploid-derived cell cultures **5,547,866**
(1997) Recovery of taxanes from conifers **5,670,633**
(1999) Recovery of taxanes from conifers **5,955,621**
(1999) Recovery of taxanes from plant material **5,981,777**

References

Durzan DJ, Ventimiglia FV (1994) Free taxanes and the release of bound compounds having taxane antibody reactivity by xylanase in female, haploid-derived cell suspension cultures of *Taxus brevifolia.* In Vitro Plant Cellular and Developmental Biology 30P: 219-227

Durzan DJ, Ventimiglia FV, Havel L (1998) Taxane recovery from cells of *Taxus* in micro- and hypergravity. Acta Astronautica 42: 455-463

Durzan DJ (1999) Gravisensing, apoptosis, and drug recovery in *Taxus* cell suspensions. Gravitation and Space Biology Bulletin 12: 47-55

Pedroso MC, Magalhaes JR, Durzan DJ (2000) Nitric oxide induces cell death in *Taxus* cells. Plant Science 157: 173-181

Durzan (2000) Metabolic engineering plant cells in a space environment. Biotechnology and Genetic Engineering Revs 17: 349-383

Choi H-K, Kim S-I, Song J-Y, Son J-S, Hong S-S, Durzan DJ, Lee H-J. (2001) Localization of paclitaxel in suspension cultures of *Taxus chinensis*. Journal of Applied Microbiology and Biotechnology 11: 458-462

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Towards mass-propagation of Norway spruce in Finland

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Abstract: A new project "Vegetative propagation – knowhow and technology for enhancing bioeconomy" has been launched in Finnish Forest Research Institute (Metla), Finland. The aim of the project is to deepen knowhow and develop technology for vegetative propagation of forest trees in Finland, and to enhance collaboration among research institutions and practical plant producers.

Vegetative mass-propagation could serve as a solution to the lack of high-quality seed of Norway spruce

In Finland, there is a lack of high-quality Norway spruce seed for forest regeneration. The area under seed orchards, established for production of genetically superior seed is only less than 300 ha. Recently, these orchards have been suffering from irregular flowering of the species, as well as problems caused by plant pathogens and pests. To meet the demand for genetically superior seed, the seed production should radically be enhanced by increasing the area under seed orchards**,** or an alternative solution partly replacing the seed production should be invented.

Establishment of new seed orchards is, however, a long –lasting and costly action. Thus, a tissue culture approach using somatic embryogenesis (SE), and potentially combined with cutting technology, is developed and applied in this newly launched project. The primary goal of the project is to develop a vegetative mass propagation method, applicable for commercial production of Norway spruce. Also possibilities for applying the methods for commercial mass-propagation of the selected clones or bred families originating in the national tree breeding programme, will be evaluated.

Careful multi-step selection of the plant material ensures the high quality of the emblings propagated for forest cultivation

Controlled crosses among the top trees of the Norway spruce breeding programme are used as a source of explants for somatic embryogenesis in order to get high-quality forest regeneration material. Both immature and mature seed embryos are tested as explants and embryogenic lines obtained from the initiations, are tested for their capacity to produce embryos. The lines with the highest embryo producing capacity are selected for field testing and cryopreservation in liquid nitrogen.The lines expressing rapid growth combined with high resistance and adaptation in the field tests, are selected for mass-propagation. To ensure genetic diversity broad enough for forest cultivation, the material will be delivered as deliberated combinations of tested SE-lines with utilisation area defined. After several years of field testing in Metla, the selected lines with the high quality can be taken out of cryopreservation and delivered to commercial plant producers for mass-propagation. Also combinations of SE-lines that have not been field-tested, but originate in the top crosses can be mass-propagated, with certain restrictions of their production numbers. This "multiplied families" -option will be utilised e.g. for piloting of mass-propagation.

Cooperation and effective communication between research and commercial plant production is a prerequisite for applying the new production method in the practise

Concurrently, with the propagation material (SE lines) being produced and tested, methods for mass-propagation of the SE-lines included in the "multiplied families" are tested in cooperation with commercial plant producer. Based on the experience gathered, a service model for the future dissemination of these materials for commercial use is created.

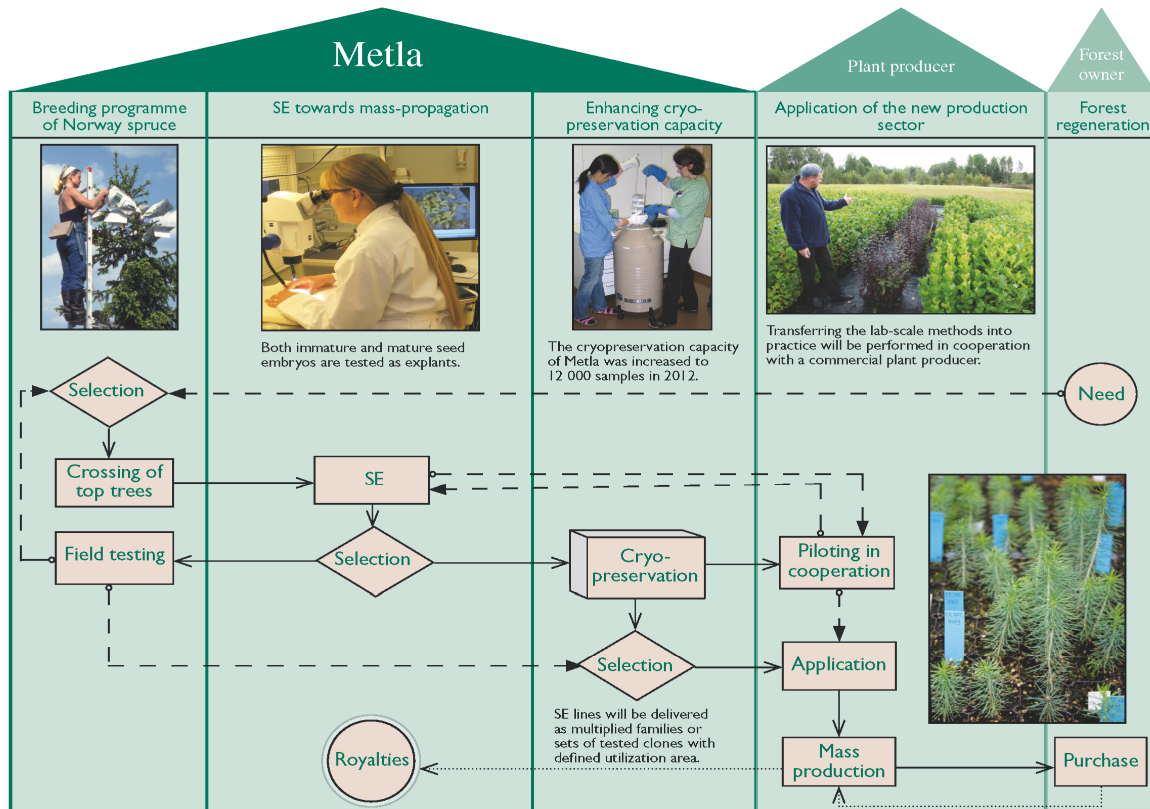

Fig. 1 Outline of the process to be developed for future dissemination of SE material for commercial mass-propagation of Norway spruce. Solid arrows indicate the flow of plant material and dashed arrows indicate information flow. Dotted arrows show money flow.

In addition, the earnings principle of the developed service is fine-tuned. The idea is to describe and rationalize the government funded parts of the process that are performed by Metla, as well as user rights and possible royalties paid by commercial producers. It is suggested that creation, testing, and storage of the propagation materials, i.e. controlled crossings, SE initiations, clone selection, and cryopreservation would be carried out by Metla, in order to ensure material being equally available for all the plant producers in Finland. To support these actions, the commercial producers are suggested to pay small royalties based on the amount of emblings sold on the market.

The first year of the project resulted in circa 200 cryopreserved lines

During the first year of the project, 12 Finnish full-sib families were tested for SE using 3175 immature embryo explants placed on either LM-medium (Litvay et al. 1985, as modified by Klimaszewska et al. 2001) or on LP-medium (von Arnold and Eriksson 1981, as modified by Högberg et al. 1998).

The average SE initiation rate among these families was 62 % on LM-based medium and 17 % on LP-medium (Fig. 2). Alltogether, ca. 900 embryogenic lines were tested for their embryo production, with the best lines showing the capacity to produce 600-700 embryos per g fresh weight (FW). Approximately 22% of the tested lines showed both the high germinability of 65% or more and the capacity to produce at least 75 embryos / g FW, and were thus selected for cryopreservation.

In the near future, the project continues by evaluation of the regenerated emblings, new SE initiations, and further development of the technology, such as cold storage of mature embryos, and finally, by a commercial pilot with the company partner.

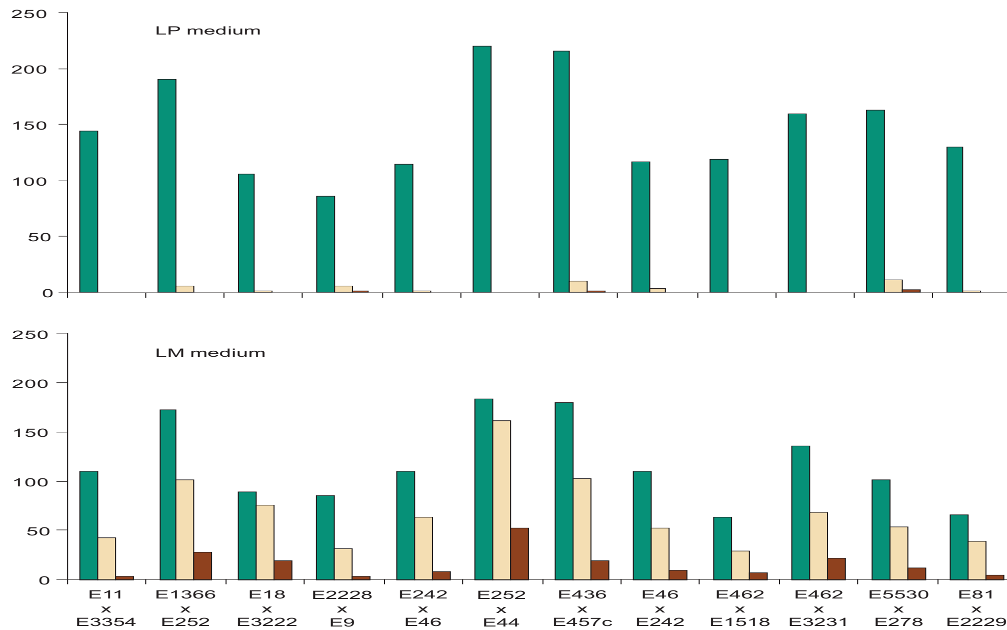

Fig. 2 *The number of initiations 2011 (green bars) performed on LP-based and LM-based media using 12 full-sib families. Yellow bars indicate the number of maturated lines and brown bars show the number of the lines selected for cryopreservation based on their high germinability and capacity to produce embryos*

Acknowledgements: The project is realized by Metla with the University of Eastern Finland and a commercial company Taimityllilä Ltd as partners. The project is funded with 600 000 € for the years 2011 to 2014 by the European Regional Development Fund of EU.

References

von Arnold S, Eriksson T (1981) *In vitro* studies of adventitious shoot formation in *Pinus contorta*. Can. J. Bot. 59:870-874

Högberg K-A, Ekberg I, Norell L, von Arnold S (1998) Integration of somatic embryogenesis in a tree breeding programme: a case study with *Picea abies*. Canadian Journal of Forest Research 28: 1536-1545

Klimaszewska K, Lachance D, Pelletier G, Lelu A-M, Seguin A (2001) Regeneration of transgenic *Picea galuca, P. mariana,* and *P. abies* after cocultivation of embryogenic tissue with *Agrobacterium tumefaciens*. In Vitro Cell. Dev. Biol. –Plant 37:748-755

Litvay JD, Verma DC, Johnson MA (1985) Influence of loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucus carota* L.) Plant Cell Rep. 4:325-328

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SE propagation and genetic diversity – example from a practical case

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Keywords: family forestry, Norway spruce, status number, vegetative propagation

Family forestry is a concept where the very best parents in a breeding population are crossed and the resulting progeny is mass propagated. For some species, e.g. Norway spruce, flowering is uneven and erratic, which makes vegetative mass propagation an attractive option. Propagation by rooted cuttings is already used for this, but has a limitation due to the low multiplication rate. From this point of view propagation via somatic embryogenesis (SE) has a greater potential. However, SE is notorious for the uneven distribution of cell lines among families and uneven distribution of plants per cell line. The uneven structure is normally generated already at initiation and continues throughout the propagation. This unevenness will decrease the genetic diversity from what is entered in the beginning of the propagation procedure. A practical case with Norway spruce (*Picea abies* L. Karst.) elucidates this process.

Status number (Ns) was used as a measure of genetic diversity. Ns is defined as half the inverse of group co-ancestry and corresponds to the census number of a population consisting of non-related and non-inbred individuals. Mathematically, the formula $Ns = 1/2\Theta$ is applied, where Θ = average relatedness coefficient in the population.

In a practical propagation situation, 1 927 zygotic embryos from 37 full-sib families, generated by 35 parents, entered SE initiation. After initiation, 1 206 cell lines from 34 families remained to the proliferation step. The number of cell lines per family varied considerably (Fig. 1).

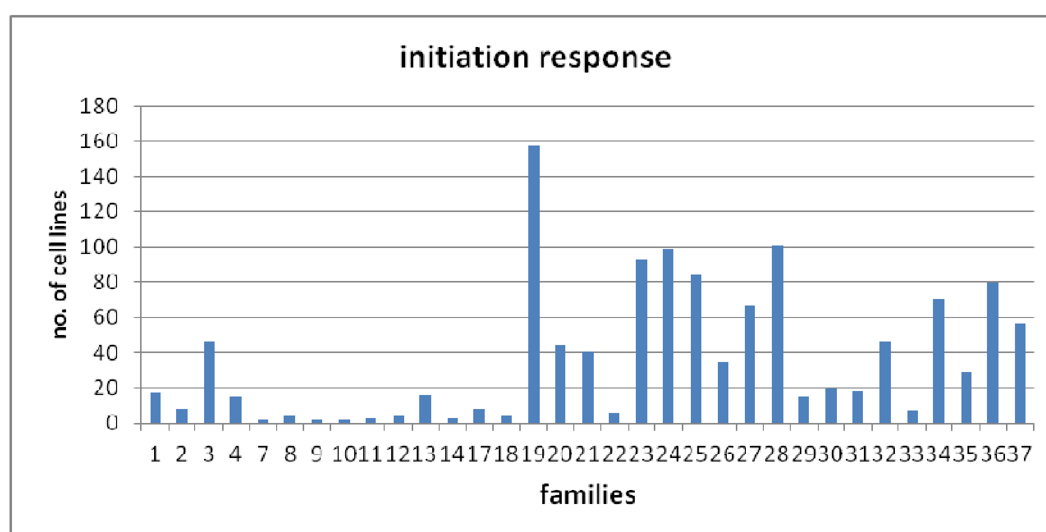

Fig. 1. *Number of cell lines per family with a positive initiation response.*

During the following propagation steps, including cryopreservation and recovery, cell lines were lost successively and the genetic diversity decreased. All cell lines were treated in the same way according to a standard protocol. At the stage where plantlets were acclimatised to nursery conditions, 154 cell lines distributed on 28 families remained (Fig. 2).

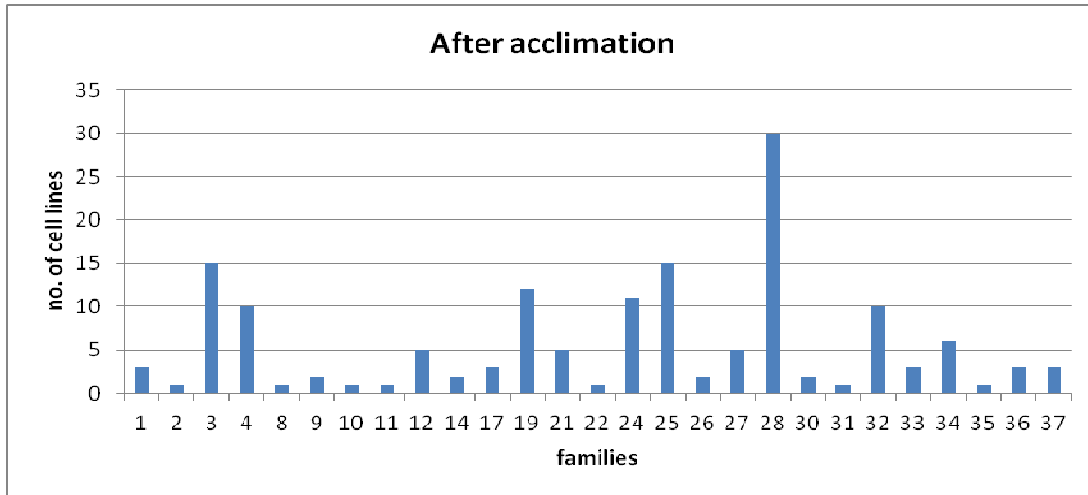

Fig. 2. *Number of cell lines per family that passed the acclimation stage successfully.*

The uneven representation among families, and relatedness among families, gave a Ns of 15.2, assuming that the number of plants per cell line was equal. In practice, this was not the case, and after taking into account also the uneven distribution of plants among cell lines (Fig. 3), Ns increased slightly to 16.

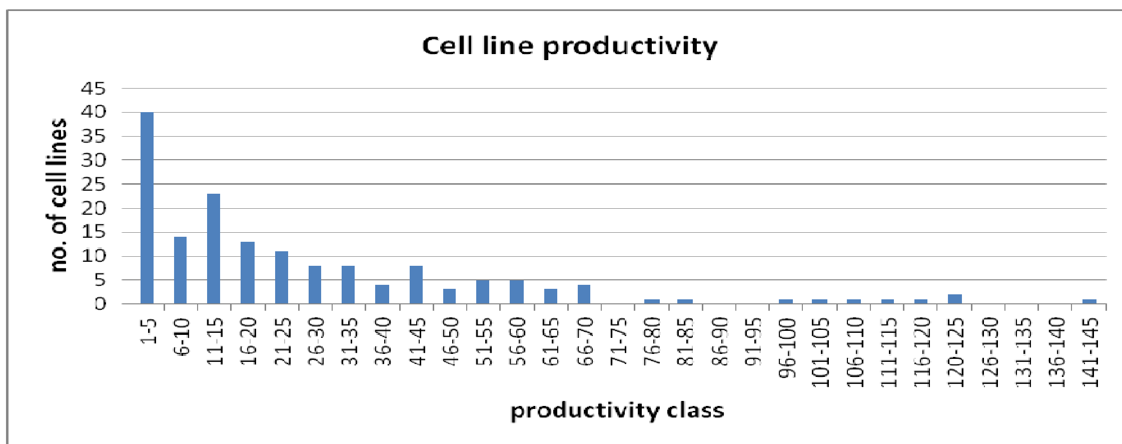

Fig. 3. *Cell lines remaining after acclimation stage, distributed on plant productivity classes. The class limits are determined by the number of plants produced by each cell line and reflects the plant production capacity.*

Cell lines differ considerably in plant production capacity (productivity) and in a practical situation the best plant producing cell lines will be economically beneficial. If only the 16 most productive cell lines were included in the final propagation, the status number dropped to 8.1, mostly due to reduction of number of families and to a lesser extent relatedness and uneven cell line representation among families.

In a practical production of untested clones following the family forestry concept, losses underway and uneven family and clone representation can lead to considerably lower genetic diversity than the material entering the propagation indicates. Furthermore, if the mass propagation is restricted to cell lines that are high-productive plant producers the genetic diversity will drop significantly.

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In vitro propagation of *Uncaria rhynchophylla* – a medicinal woody plant

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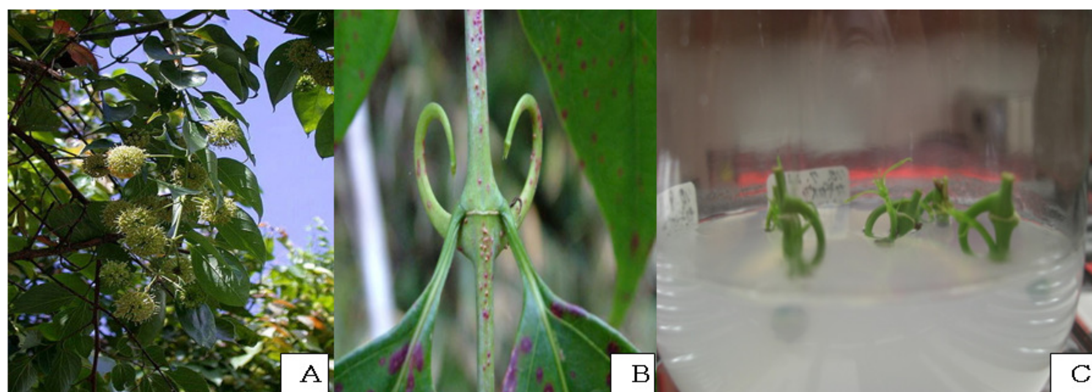
Keywords: tissue culture, *Uncaria, rhynchophylline*

Uncaria rhynchophylla (Kagikazura or the cat's claw herb) is a plant species used in traditional Chinese medicine and is a woody plant found widely in Japan and China. It contains alkaloids (rhynchophylline, iso-rhynchophylline, hirstine and so on (Shi et al. 2003)) which are good for remedy of high blood pressure and dementia. (+)-Catechin and (-)-epicatechin are also found in the plant (Hou et al. 2005). It is also used in Kampo medicine which is the Japanese study and adaptation of traditional Chinese medicine. It is in 4 of the 148 Kampo medicine formulae. Kampo does not incorporate any human body parts nor animal parts, thus avoiding issues with animal cruelty prevalent in traditional Chinese medicine. Kampo herbal medicines are regulated as pharmaceutical preparations and their ingredients are exactly measured and standardized. Access to Kampo herbal medicines is guaranteed as part of Japan's national health plan for each of its citizens. For the purpose of in vitro propagation and development of a basis for useful substance production by breeding and cell culture, a tissue culture procedure was developed for this species.

Shoots were induced from stem spines (thorns) of kagikazura in the 1/2MS medium containing BAP or Zeatin. Callus induced around the stem segments were continuously subcultured in fresh 1/2LP medium containing 0.5 µM BAP and 1 µM 2,4-D. These cell lines can be used for the possible secondary metabolite production and for chemical breeding by somaclonal variation or molecular genetics technology. Regenerated plants were obtained by rooting of these shoots on 1/2MS medium containing 1 µM IBA. Rooted plantlets were cultured in giffy 7® with 60 ml of 0.1 % Hyponex® medium in plant boxes (65 x 65 x 100 mm). Each plant box contained 1 regenerated plantlet. Culture condition was at 25 ℃ constant temperature under a 16 h photoperiod of 70 $\mu Mm^{-2}s^{-1}$ by cold fluorescent lamps. Then after 2 months, they were habituated under a nursery terrace® system (MKB Dream Co., Japan) which provides 100 % humidity and automatic watering for 1 month, then grown in greenhouse for 6 months. Field plantation was successful. Selection of clones with higher chemical content is planned.



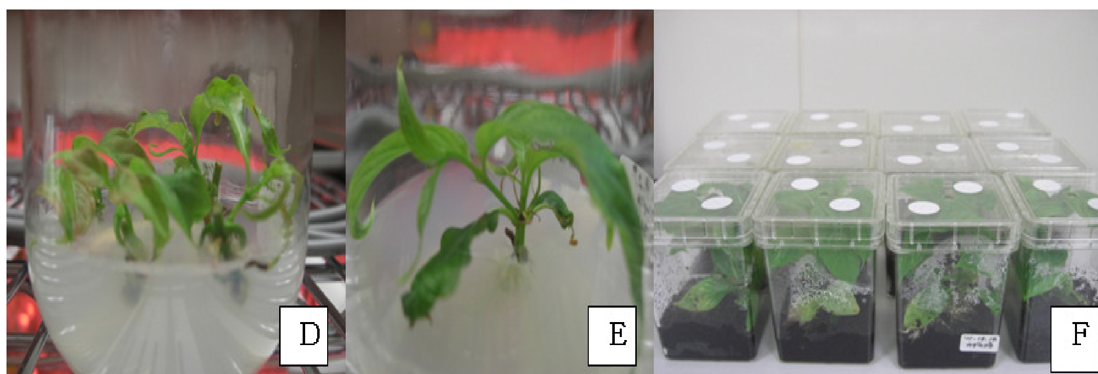


Fig. 1 *Process of in vitro propagation of Uncaria rhynchophylla. A. Uncaria rhynchopylla, B. stem spine, C. Shoot induction from spine, D. Shoot elongation, E. Rooting, F. Regenerated in vitro plantlets in plant boxes.*

References

Shi, JS; Yu, JX; Chen, XP; Xu, RX (2003). "Pharmacological actions of Uncaria alkaloids, rhynchophylline and isorhynchophylline". Acta pharmacologica Sinica 24 (2): 97–101.

Hou WC, Lin RD, Chen CT, Lee MH (August 2005). "Monoamine oxidase B (MAO-B) inhibition by active principles from Uncaria rhynchophylla". J Ethnopharmacol 100 (1–2): 216–20.

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Somatic embryogenesis of black saxaul (*Haloxylon aphyllum*), a plant for saline soil reclamation of the dry aral seabed

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Keywords: *Haloxylon aphyllum,* , somatic embryogenesis, embryogenic callus, phytohormones

Drying of the Aral Sea is a problem that has already been in the center of research attention for 30 years. As a result of the irrational economic activity in the Aral Sea basin environmental problems have arisen that require a national priority level. One solution to the problems of the Aral Sea is phytoremediation, which invloves the use of salt-tolerant plants - halophytes to improve environmental remediation, retention of soil, and overall reduction of wind erosion. Huge experimental plantings with plots up to 200 ha have shown (Wucherer and Breckle 2005), that only very few species are suitable for this purpose: *Haloxylon aphyllum* (Fig. 1) and to a lesser extent *Halocnemum strobilaceum* are such species. There is a need to study the biology and propagation of the former for reclamation of saline soils.

Figure 1. Black saxaul (Haloxylon aphyllum)

Haloxylon aphyllum is of great value and importance in protecting and supporting sensitive ecosystems like the desert. It is effective as a live windbreaker, it prevents erosion of soil and improves the level of organic materials of the soil in the long term, it improves the structure of the soil and increases the plant enrichment of the area under coverage. In addition for those who live in desert, it is an important source for the provision of fuel and fodder for cattle. *Haloxylon aphyllum* is a small tree belonging to the *Amaranthaceae*. It is found in continental deserts of Middle and Central Asia, including northern China, where it is a dominant plant along with the related white saxaul. The black saxaul ranges between 4 and 10 meters in height and lacks large foliage-type leaves. It has a deep pivotal root system allowing it to thrive in dry, saline, and sandy environments.
At present the majority of plantings of black saxaul are affected by various injurious organisms (pests) and diseases (Fig. 2). High contamination by injurious pests and diseases (various cecidomyids (gall-gnats)) interfere with the establishment of forests and production of viable seeds. Selection and *in vitro* propagation of *Haloxylon aphyllum* will allow production of improved genetically homogeneous planting material.

The objective of the present investigation was to achieve suitable culture conditions for induction of callus on top parts of seedlings and regeneration of plantlets.

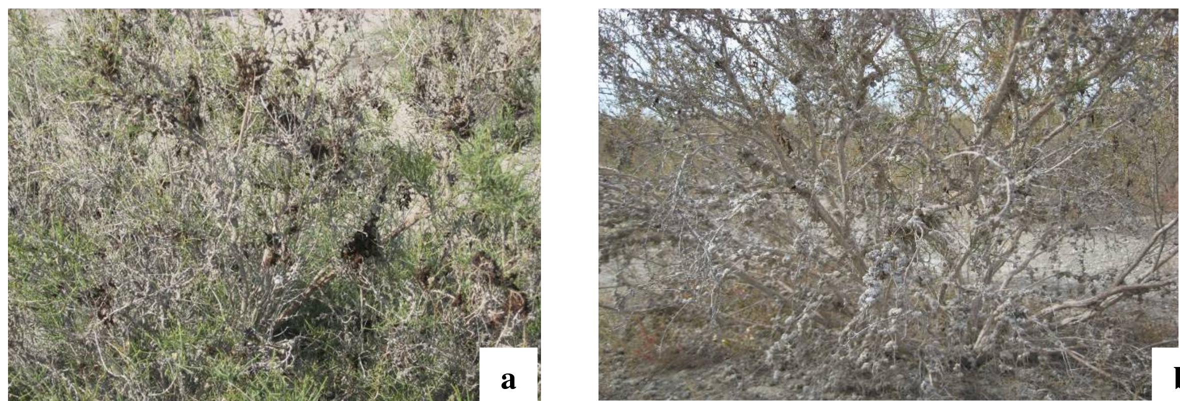

Figure 2. *Haloxylon aphyllum, affected by gall-gnats of Asiodiplosis noxia Marik **(a)** and Asiodiplosis stackelbergi Marik **(b)***

Somatic embryogenesis has been a great resource for cloning trees. Scientists go out into the woods and find trees that show strong traits such as weather, insect and wind resistance etc. Once they find these trees they collect the cones and remove the bracts to get out the seeds. A few years ago a mountain pine beetle that was destroying the western coast made it over the Rocky Mountains and started destroying trees in the forest towards New Brunswick, Canada. This was a terrifying outbreak. Along lakes where these beetles would drown there were stacks of dead ones up to a foot high. These beetles were everywhere. This is why Somatic Embryogenesis is important. We can take a seed of one tree and produce hundreds of the same trees. If we find a tree that is resistant to the beetle we can clone it to help save/rebuild the destroyed forest (http://www.greencareersguide. com/Somatic-Embryogenesis-and-Our-Plants.html).

Tissue culture methods have been evaluated for black saxaul by several research groups. Callus from mature zygotic embryos of *Haloxylon aphyllum* was induced on Burnouf-Radosevich-Paupardin (BRP) medium supplemented with different concentrations (0.1, 1.0, 5.0 mg/l) of 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) alone or combined with 0.1 mg/l 6-benzyladenine (BA) or kinetin (Kin). The highest frequency of callus induction and greatest amount of callus proliferation occurred on BRP medium supplemented with 1.0 mg/l 2,4-D combined with 0.1 mg/l BA. Following transfer to BRP medium without growth regulators, cell clusters proliferated on the surface of the callus and groups of elongated suspensor cells developed from the clusters. The morphology of the cell clusters and the suspensor-like cells that developed from them support the hypothesis that the tissue is embryogenic callus (Ramirez and Birnbaum 2001).

Chinese scientists studied *in vitro* propagation of wild *Haloxylon ammodendron. Haloxylon ammodendron* is a subspecies of *Haloxylon aphyllum*. The regeneration system of wild *Haloxylon ammodendron in vitro* was studied with stem shoots as explants to investigate the effects of different plant growth regulators, $AgNO_3$ and sucrose etc. on H. *ammodendron* stem shoots callus induction, bud differentiation and rooting of explants. Results showed that the optimal medium for callus induction was MS + 2,4-D 2.0 mg/l + 6-BA 0.5 mg/l + sucrose 30 g/l + agar 6.0 g/l ,and the induction frequency was 100%. The best bud differentiation medium was MS + 0.5 mg/l NAA + 1.0 mg/l TDZ +3.5 mg/l $AgNO_3$ + 26 g/l sucrose + 6.5 g/l agar , and the bud differentiation rate was 97.8%. The major factors affecting bud differentiation and bud multiplication of *H. ammodendron* plants were NAA and TDZ in the first place and $AgNO_3$,and sucrose in the next place (Du Min-hua *et al.* 2007).

Seeds (Fig. 3a) of *Haloxylon aphyllum* were collected from plantings on the Dry Aral Seabed. They were sterilized for 40-50 min with a 0.5% solution of $KMnO_4$. Top parts of seedlings (Fig. 3b) as explants were rinsed in a strong solution of soap, and were sterilized for 15 min with commercial bleach (sodium hypochlorite, 3.5% active chlorine) diluted with water (3:10 v/v) and rinsed in running water. In sterile conditions top parts of seedlings were sterilized for 10 min with a 0.025% solution of thimerosal (merthiolat) in combination with commercial bleach and washed in sterile double-distilled water (20 min).

Sterilized top parts of seedlings inoculated into Murashige and Skoog (MS) media (Murashige and Skoog 1962.), supplemented with 20 g/l sucrose, 6 g/l Difco Bacto-agar, 100 mg/l myoinositol, 25 mg/l glutamine. For callus induction the medium was supplemented with phytohormones at concentrations 0.5, 1.0, 1.5 mg/l of BAP (6-benzylaminopurine), Kin (kinetin), GA_3 (gibberellic acid), 2,4-D (2.4- dichlorophenoxyacetic acid) alone. The pH of the media was adjusted to 5.6-5.8 before autoclaving. Cultures were incubated at 26±2°C with a 16 hour illumination.

MS medium, supplemented with 1.0 mg/l BAP, was found to be the most suitable for induction of embryogenic callus (Fig. 3c-d) from top parts of seedlings of *H. aphyllum* in comparison with Kin, GA_3, 2,4-D (Table). Induction of callus began 3-4 weeks after inoculation of explants. Growth of callus was observed during 2-3 weeks. The callus has bright green in color. Shoot induction occurs from embryogenic callus (organogenesis) (Fig. 3e), then shoot elongation (Fig. 3f). Shoots were separated and transferred to hormone-free MS media for further shoot elongation (Fig. 3g-i). Elongated shoots were transferred to MS media plus various auxins (IAA, IBA or NAA) for rooting (Fig. 3j). Somatic embryogenesis can produce from one seed of *H. aphyllum* hundreds of plantlets.

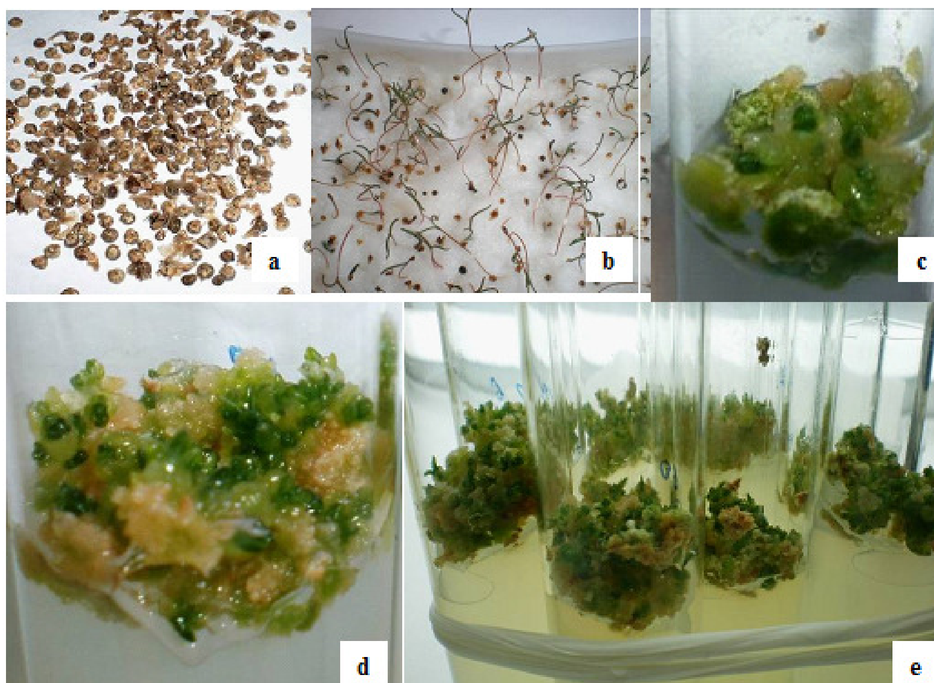

Figure 3. *Somatic Embryogenesis of Haloxylon aphyllum on MS medium with 1.0 mg/l BAP:* ***a*** *– Seeds,* ***b****- Seedlings,* ***c-d*** *– Embryogenic callus around top parts of seedling,* ***e*** *- Shoot induction from embryogenic callus (organogenesis),* ***f*** *- Shoot elongation,* ***g-i*** *– Adventitious shoots,* ***j****- Root development*

Table 1. *Callus induction on top parts of seedlings of H. aphyllum cultured on MS medium; influence of BAP, Kin, 2,4-D and GA_3. Data were recorded 3-4 weeks after inoculation*

phytohormone	**concentration (mg/l)**	**% of explants producing callus**	**relative degree of callusing**
BAP	0.5	80	high callus
	1.0	100	high callus
	1.5	80	high callus
Kin	0.5	75	moderate callus
	1.0	80	moderate callus
	1.5	70	moderate callus
2,4-D	0.5	70	moderate callus
	1.0	80	moderate callus
	1.5	70	moderate callus
GA_3	0.5	20	minimal callus
	1.0	25	minimal callus
	1.5	20	minimal callus

References

Du Min-hua, Hui Feng-li, Liu Zheng, Chai Chun-yue 2007. Plant regeneration *in vitro* from stem shoots explants of wild *Haloxylon ammodendron*. Journal of Northwest A&F University (Nat. Sci. Ed.). 35:164-168.

Murashige, T., Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissues cultures. Physiol Plant. 15:473-497.

Ramirez, A.E., Birnbaum, E.H. 2001. Induction and characterization of callus in *Haloxylon aphyllum* (Minkw.) Iljin. Acta Hort. (ISHS). 560:465-468.

Wucherer, W, Breckle, S.W. 2005. Desertifikationsbekämpfung und Sanierung der Salzwüsten am Aralsee. Sukzession und Phytomelioration, Naturschutz und nachhlatige Entwicklung. Bielefelder Ökologische Beiträge. 19:94.

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Secondary phenolic compounds in somatic embryogenesis of *Pinus sylvestris* L. : a preliminary study

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Background and aims

The increasing concern about the ecological impacts of wood preservation chemicals has raised interest in the natural durability of Scots pine (*Pinus sylvestris* L.) heartwood. Phenolic compounds such as stilbenes as well as monoterpenes have been found to inhibit fungal growth and thus make the wood more resistant to decay (Venäläinen et al. 2003). There is a strong genetic component in the decay resistance of the heartwood in Scots pine (Harju and Venäläinen 2002), and a positive genetic correlation has been found between the concentration of constitutive stilbenes in the heartwood of mother trees and in their seedling progenies (Harju et al. 2009).

Vegetative propagation of Scots pine individuals with high content of phenolics could provide a better way to produce more durable timber than propagation of those with low phenolics. A new EU (ERDF)-funded project "Vegetative propagation – knowhow and technology for enhancing bioeconomy" was launched in Finland in 2011. It is carried out at Finnish Forest Research Institute in cooperation with the University of Eastern Finland and a commercial company Taimityllilä Ltd. One of the project aims is to study potentials of vegetative propagation in improving the heartwood quality in Scots pine. This is done by comparing success of somatic embryogenesis (SE) in Scots pine families producing a high concentration of phenolics with those producing small amounts of these compounds. Furthermore, analyses of secondary phenolic compounds induced in embryogenic cultures will be carried out and their suitability for *in vitro* selection will be studied.

Material and methods

Three donor trees with either high or low content of phenolic compounds were chosen in 2010 according to a previous study (Harju *et al.* 2009). Open-pollinated immature cones were collected in 2011 at five different degree days for testing the success of SE initiation. Somatic embryogenesis was performed according to the methods and media modifications developed by Aronen and co-workers (2009) and Lelu-Walter and co-workers (2006). Two basal media were used for comparing the success of initiation. They were DCR-based medium (Gupta & Durzan, 1985; Becwar et al., 1990) and LM-based (Litvay *et al*. 1985) medium.

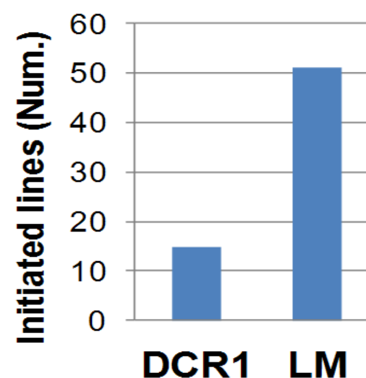

Fig. 1 *Effect of media on the success of initiation.*

The concentrations of secondary phenolic compounds were analyzed by HPLC/DAD according to the published method (Julkunen-Tiitto and Sorsa 2001). Explants both responding (able to initiate embryogenic cultures, ECs) and non-responding (not able to initiate ECs), as well as the obtained ECs were used for this analysis. The phenolics were methanol extracted from the

freeze-dried tissues (Julkunen-Tiitto and Sorsa 2001) and quantified by HPLC using a high performance series 1100 LC /DAD from Agilent Technologies (Palo Alto, CA). The samples were re-dissolved in 400 µl methanol : water (1 : 1) and eluted (flow rate 2 ml min^{-1}) using a methanol : water gradient. Phenolic metabolites were identified by comparing their retention times and UV spectra with those of standards, and by means of HPLC atmospheric pressure ionization – electronspray (API-ES) mass spectrometry.

Results

Success of initiation: After ten weeks of initiation, 4.7 % of the explants in total produced embryogenic cultures on both media. The LM-based initiation medium proved to be better than the DCR-based one (Fig. 1). Most lines were initiated from the explants collected between 526 and 576 dd. The initiation rate achieved at these two time points was 33%, while at the other time points it was remarkably lower. The effect of genotype on the initiation rate was significant (Fig. 2). The seed parents having a high content of phenolics had lower initiation rates than those having a lower content of phenolics, but it was possible to obtain embryogenic lines from both types of donor trees. As seen in the figure 2, the donor trees K803 and K836 had about the same stilbene concentration in the explants, but differed in the number of the embryogenic lines produced.

Stilbenes in explants and their effects on SE initiation: Effects of phenolics on the initiation of ECs has been investigated in the explants of each donor tree at different maturation stages. Seventeen different phenolic compounds were detected according to their retention time and UV- spectra. Most of the compounds were found to be more abundant in the non-responding explants, including stilbenes. Of these compounds, we first focused on stilbenes including pinosylvins, pinosylvin monomethyl ether and stilbene derivatives, as they are rich in Scots pine heartwood. The results showed that the stilbene concentration in responding and non-responding explants in the donors had no clear effect on the SE initiation frequency (Fig. 2). Neither was there a difference between the explants of the donors having either high or low content of the phenolic compounds.

Stilbenes in embryogenic cultures: There was a huge variation in the concentration of stilbenes in all obtained ECs

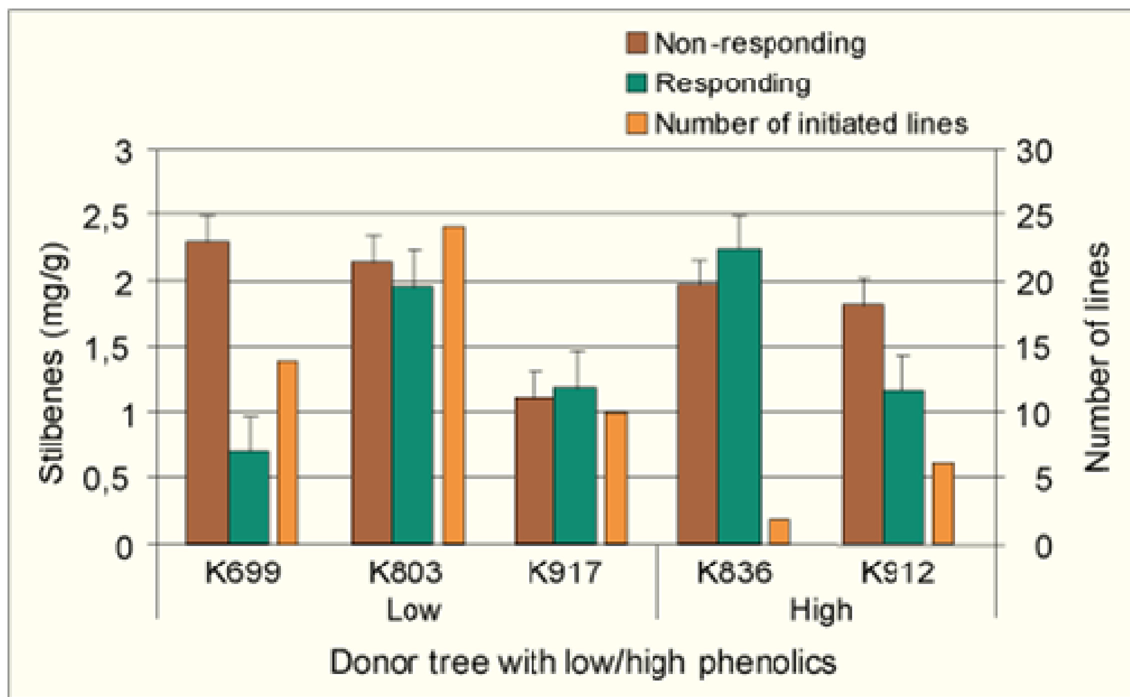

Fig. 2 *The concentration of stilbenes in responding and non-responding explants (left axis) from the donors with a low or high content of phenolics (x-axis) and the number of initiated lines from each donor (right axis).*

from both the high and the low phenolics containing donors. Generally, the SE lines with a high concentration of stilbenes produced less mature somatic embryos, whereas the embryo conversion rate did not vary depending on the stilbene concentration of the line.

Conclusions

According to this preliminary study, we could conclude that ECs could be initiated from both donors with high and low phenolic contents on the selected medium. There was no correlation between the stilbene concentration of the explants and the success of SE initiation. Concentration of phenolic compunds was observed to vary remarkably in the ECs initiated, and mature somatic embryos could be produced both from the ECs having a high and low stilbene content. However, the high stilbene concentration of the ECs may inhibit embryo production. To confirm these observations, experiments will be repeated with the explants originating from the controlled crossings in 2012.

References

Aronen T, Pehkonen T, Ryynänen L (2009) Enhancement of somatic embryogenesis from immature zygotic embryos of *Pinus sylvestris*. Scand J Forest Res 24:372–383

Becwar MR, Nagmani R, Wann SR (1990) Initiation of embryogenic cultures and somatic development in loblolly pine (*Pinus taeda*). Can J Forest Res 20:810–817

Gupta PK, Durzan DJ (1985) Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugarpine (*Pinus lambertiana*). Plant Cell Rep 4:177–179

Harju AM, Venäläinen M, Laaksi T, Saranpää P (2009) Wounding response in xylem of Scots pine seedlings shows wide genetic variation and connection with the constitutive defence of heart wood. Tree Physio 29:19–25

Harju AM, Venäläinen M, (2002) Genetic parameters regarding the resistance of *Pinus sylvestris* heartwood to decay caused by *Coniophora puteana*. Scan J Forest Res 17:199–205

Julkunen-Tiitto R, Sorsa S (2001) Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. J Chem Ecol 27:779–789

Lelu-Walter MA, Bernier-Cardou M, Klimaszewska K (2006) Simplified and improved somatic embryogenesis for clonal propagation of *Pinus pinaster* (Ait.). Plant Cell Rep 25:767–776

Litvay JD, Verma DC, Johnson MA (1985) Influence of loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucus carota* L.). Plant Cell Rep 4:325–328

Venäläinen M, Harju AM, Kainunen P, Viitanen H, Nikulainen H (2003) Variation in the decay resistance and its relationship with other wood characteristics in old Scots pine. Ann For Sci 60:409–417

Somatic embryogenesis from Spanish provenances of maritime pine

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Key words: epibrassinolide, *Pinus pinaster*, provenances, somatic embryogenesis
Abstract: Induction and establishment of SE from megagametophytes of maritime pine was genotype dependent and was stimulated by epibrassinolide.

Introduction

Maritime pine (*Pinus pinaster* Ait.) covers more than 4 million hectares in southwestern Europe (Spain, Portugal, Italy and France). In Spain, maritime pines are distributed in twenty provenance regions that belong to three geographical groups: The Atlantic, the Continental and the Mediterranean. This work examines the capacity of megagametophytes from four of these provenances for the induction and establishment of somatic embryogenesis (SE). The effect of 24-epibrassinolide (EBR) on SE response is also assayed.

Material and methods

Cones were sampled in early-mid July from open pollinated plus mother trees belonging to the following Spanish provenances (P) of the species (Fig. 1): The "Noroeste interior" (Anllo, Lugo); the "Soria-Burgos" (Valsain, Segovia); The "Serrania de Cuenca" (Sinarcas, Valencia) and the "Levante" (Sierra Calderona, Valencia). Anllo and Valsain cones belong to the Atlantic and Continental groups, respectively, whereas Sinarcas and Calderona cones belong to the Mediterranean group.

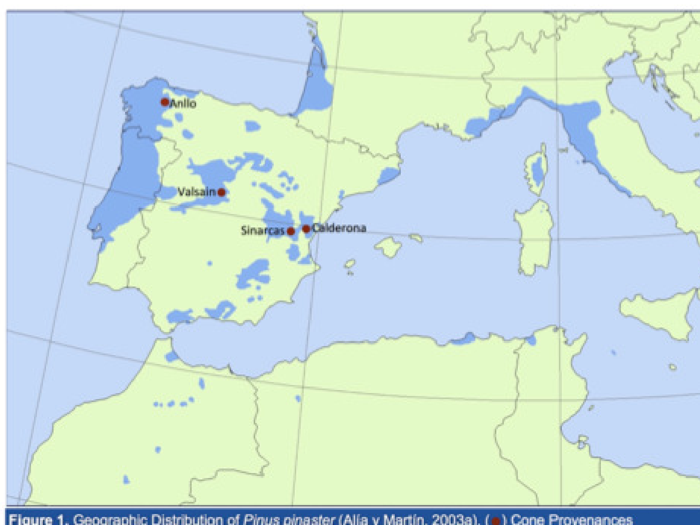

Figure 1. Geographic Distribution of *Pinus pinaster* (Alía y Martín, 2003a). (●) Cone Provenances

Results and conclusions

After sterilization, isolated megagametophytes containing the immature zygotic embryos at the precotyledonary stage (Fig. 2) were cultured on a modified Litvay's medium (mLV) as described by Lelu et al. (2006) that includes 2,4-D and BA. Alternatively, medium was supplemented with 0.1 µM EBR as the only plant growth regulator (mLV+EBR). The frequencies of somatic embryogenesis initiation (SEI) and establishment (SEE) were recorded after 45 and 120 days, respectively.

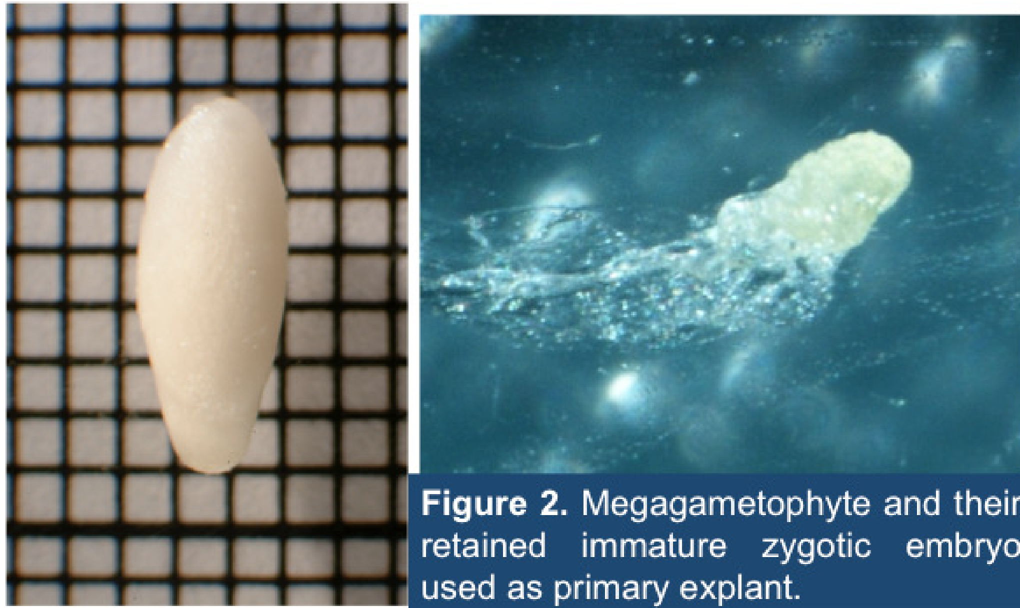

Figure 2. Megagametophyte and their retained immature zygotic embryo used as primary explant.

The provenance, the culture medium and the mother tree affected the embryogenic response of the megagametophytes. Those from the Atlantic and Continental groups (Anllo and Valsain) showed a higher capacity for SEI and SEE than those from the Mediterranean group (Sinarcas and Calderona; $p < 0.05$) (Table 2). Irrespective of the provenance, the brassinolide EBR was superior to the 2,4-D/BA combination for SEI (percentages of 21.1 vs 26.6 % for mLV and mLV+EBR, respectively; $p < 0.05$) and SEE (percentages of 13.4 and 18.4, for mLV and mLV+EBR, respectively; $p < 0.05$). An important effect of the mother tree on SE response was also observed. Maturation, carried out following standard protocols (Lelu et al. 2006), followed by germination, and plantlet production are in course (Figure 3).

Table 2. Effect of provenance on SE induction (SEI) and establishment (SEE) from megagametophytes of maritime pine. For each entry, mean separation by Tukey´s test.

Provenance	SEI (%)	SEE (%)
Calderona	22.2b	12.6b
Sinarcas	16.5b	9.2b
Valsaín	30.0a	20.8a
Anllo	32.1a	24.4a

Figure 3. Maritime pine SEs on maturation medium

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References

Alía, R. and S. Martín. 2003. EUFORGEN Technical Guidelines for genetic conservation and use for Maritime pine (*Pinus pinaster*). International Plant Genetic Resources Institute, Rome, Italy

Lelu-Walter M. A.; Bernier-Cardou M.; Klimaszewska K. 2006. Simplified and improved somatic embryogenesis for clonal propagation of Pinus pinaster (Ait.). Plant Cell Rep 25: 767–77

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Somatic embryo maturation in maritime pine (*Pinus pinaster*): contribution of a 2-DE proteomic analysis for a better understanding

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Keywords: maritime pine, somatic embryogenesis, proteomic, maturation

Abstract: Somatic embryogenesis has reached application stage for *Picea* and *Larix* species; however it remains to be optimized for pine species. Thus maritime pine maturation needs improvement. To optimize the maturation step, it is necessary to develop markers that can be used to verify or monitor the quality of somatic embryos (SE). Therefore, a proteomic study was performed of two maturation stages to identify protein markers. There were about 100 significantly and differentially expressed proteins (Student's test, $p<0.001$). They were mainly involved in carbohydrate or lipid metabolism and genetic information processing. In addition, many storage proteins were identified (vicillin-like, legumin-like, LEA proteins); some of them were, surprisingly, observed from the beginning of maturation. Our ultimate goal is to have a better understanding of SE maturation in *Pinus pinaster*. It is expected that this information will help to optimize the process and *in vitro* plant production.

Introduction

Maritime pine is extensively used in south-western Europe for lignocellulose production. Increasing demand for wood from this pine species requires efficient production of improved varieties and this is currently achieved only through seed orchard management. As in most long-lived tree species, variety design and deployment would greatly benefit from setting up an efficient vegetative propagation system of selected seed resources. Somatic embryogenesis is a promising technology for most conifers (Klimaszewska et al., 2007) and significant progress has been made in recent years for maritime pine (Lelu-Walter et al., 2006). However, whether « true » maturity of harvested cotyledonary SE has been achieved is still a debatable question. Cotyledonary SE are currently selected at the end of the maturation phase based on morphological criteria. Embryo maturation is a crucial step, giving rise to the establishment of reserve compounds including specific storage proteins that will ensure optimal germination and subsequent *in situ* plantlet development. A 2D-PAGE proteomic approach has been developed at INRA of Orléans (Teyssier et al., 2011) to define the protein status of developing SE. We aim to achieve a better understanding of the processes acting during the maturation phase of SE.

As a first step towards this goal this work presents our first results in proteomic analysis during SE development. This new knowledge is expected to refine our current methodology for practical application of somatic embryogenesis in maritime pine.

Materials and methods

Experiments were conducted with the embryogenic line AAY06006 initiated in July 2006 by FCBA from immature zygotic embryos harvested from mother clone G1.2631 (Landes origin). Trees were pollinated with a mixture of pollen from Morocco genotypes. Maturation was performed according to Lelu-Walter et al., 2006 with

the following modifications. Basal maturation medium consisted in mLV basal medium (Litvay et al., 1985) that contained 9 gl^{-1} gellan gum (Phytagel™, SIGMA), 80 µM *cis-trans* (±) abscisic acid (ABA), and 0.2M sucrose. Cotyledonary SE were harvested after 1 (immature stage) or 12 weeks maturation (cotyledonary stage) for 2DE-proteomic analysis according to Teyssier et al. (2011). Briefly, total soluble proteins were extracted in liquid nitrogen from 400 mg ES (fresh mass) and precipitated with phenol. In the first dimensional separation (IEF), samples containing 450 µg protein were loaded and equilibrated onto 24-cm IPG strips, pH 4–7 (Protean IEF Cell system, Biorad, Marnes-La-Coquette, France). The second dimensional separation was performed in 2-D PAGE. Five biological replicates were analyzed for each sample. Gels were stained with colloidal CBB-G according to Gion et al., (2005), then images were scanned and analyzed using Progenesis software (Nonlinear Dynamics, Newcastle upon Tyne, United Kingdom). The volume of each spot detected was normalized relative to the total volume of the spots on the gel. Every spot automatically detected was manually checked. Selected spots were analyzed for protein identification by in-gel proteolysis followed by peptide extraction and nanoLC-MS/MS (nano high performance liquid chromatography on Dionex Ultimate coupled to tandem mass spectrometry on a Bruker Esquire HCT Ion Trap) and interrogation of *Pinus* TC databases.

Results and discussion

Statistical analysis and mass spectrometry identification

More than 1000 spots were reproducibly defined from 2D gels. Student's t test was performed on each of the normalized volume spots and more than 10% of all spots have shown a significant volume difference between the 2 tested stages ($P < 0.001$). Figure 1 shows their location on a 2D gel sample after 12 weeks of maturation. The proteins inside spots are defined according to their pI and MW.

The proteins were identified by in-gel proteolysis followed by nanoLC-MS/MS, using current TC DNA sequence databases. Whenever possible, the identified proteins were classified according to their biological function.

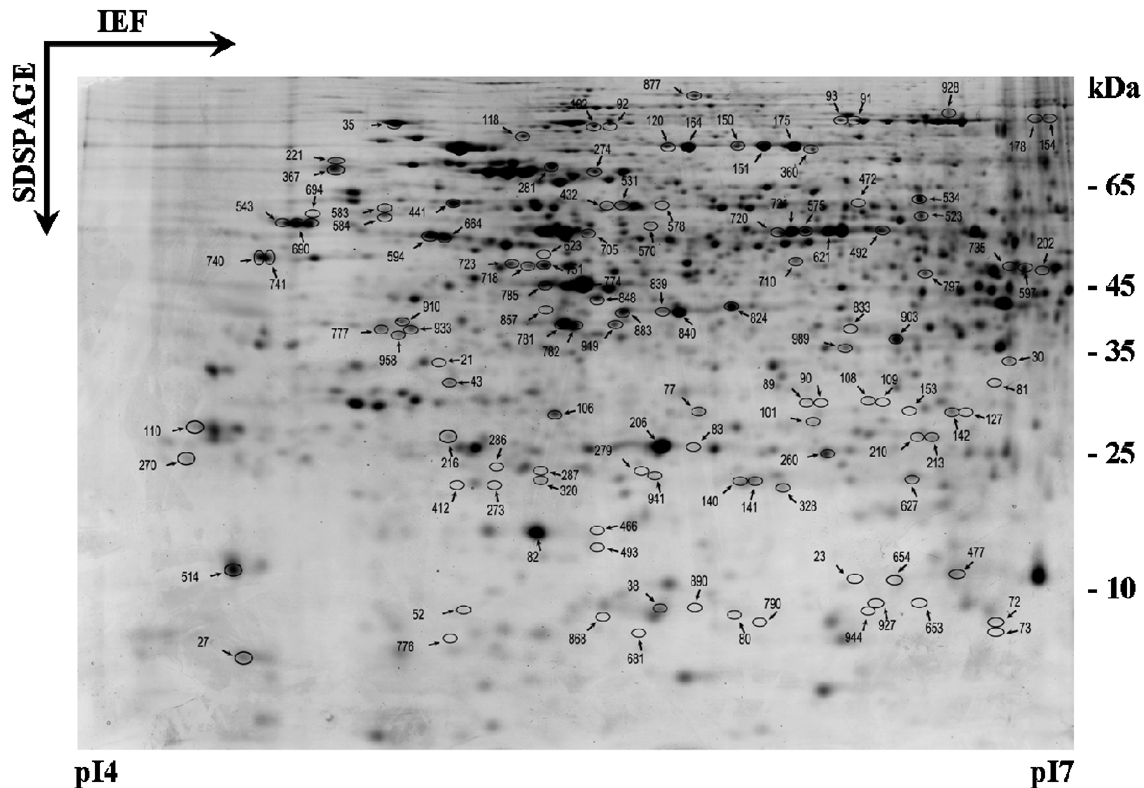

Figure 1. *Representative 2-DE map obtained for maritime pine SE after 12 weeks of maturation. Marked spots displayed significant differences in their abundance (P < 0.001) between 1 week (immature SE) and 12 weeks of maturation (cotyledonary SE).*

Biological interpretation

Based on the comparison of major functional classes (Fig. 2), we found that more proteins involved in metabolism are overexpressed in cotyledonary SE (12 weeks) than in immature ones (50% vs. 41% of total protein extract, respectively). Overexpressed proteins in cotyledonary SE are involved in carbon metabolism (20%), lipid synthesis (15%) and energy metabolism (15%); considering immature SE, over-expressed proteins are involved in

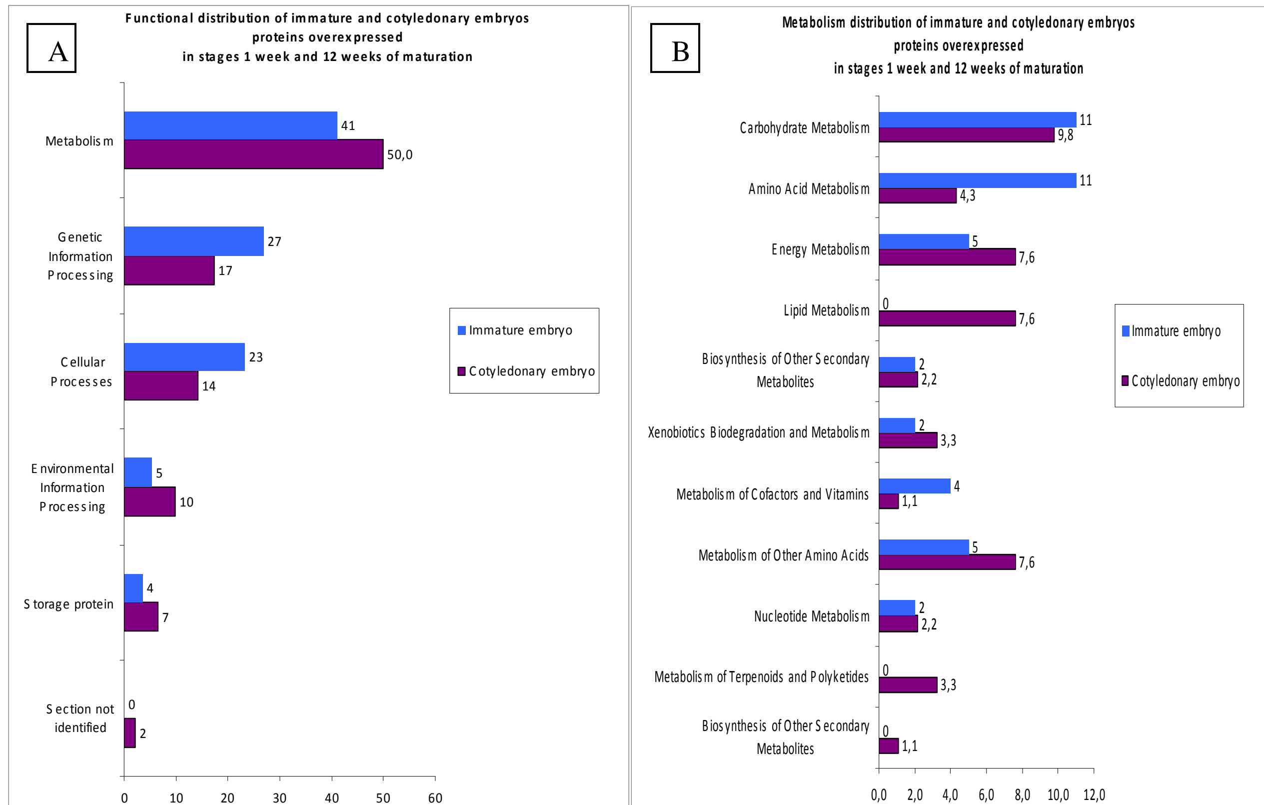

Figure 2. *Distributions of the identified putative proteins among functional class (A) and metabolism class (B) according to two culture stages (immature and cotyledonary SE). Functional classification catalog Kegg: Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/. Numbers represent percentage of total proteins extracted for a stage of culture. Proteins involved in more than one process were assigned to more than one categorical group. Hence the sum of proteins in the categories exceeds the total number of proteins.*

amino acid synthesis (26%) and carbon metabolism (25%). Among the protein enzyme group, we found that ascorbate peroxidase involved in amino acid metabolism was significantly overexpressed (x 2.1) in immature SE when compared to cotyledonary SE. A similar observation was reported for Chinese fir (Shi et al., 2010) and soybean (Bailly et al., 2001) for zygotic embryos. Thus, ascorbate peroxidase could characterize the immature state of maritime pine SE. On the opposite, glutathione peroxidase, an enzyme involved in lipid metabolism, was 5.1 times more expressed in cotyledonary SE than in immature SE. An increased expression of this enzyme has been shown during SE development of *Eleutherococcus senticosus* (Shohael et al., 2007). We concluded that glutathione peroxidase is a good candidate marker of the cotyledonary state of maritime pine SE.

Conclusion and Perspectives

This preliminary work allowed us to identify putative protein markers involved in the main metabolic pathways such as energy and lipid metabolism. Metabolism of the amino acids seemed to be low in cotyledonary SE. Even if some markers have been identified, they could be related to embryogenesis and not to maturity. To answer this question it would be necessary to study the mature zygotic embryo by proteomic.

References

Bailly C, Audigier C, Ladonne F, Wagner MH, Coste F, Corbineau F, Côme D (2001). Changes in oligosaccharide content and antioxidant enzyme activities in developing bean seeds as related to acquisition of drying tolerance and seed quality. J Exp Bot 52: 701-708.

Gion, JM, Lalanne, C, Le Provost, G, Ferry-Dumazet, H, Paiva, J, Chaumeil, P, Frigerio, JM, Brach, J, Barré, A, de Daruvar, A, Claverol, S, Bonneu, M, Sommerer, N, Negroni, L, Plomion, C (2005). The proteome of maritime pine wood forming tissue. PROTEOMICS 5: 3731-3751.

Klimaszewska K, Trontin JF, Becwar M, Devillard C, Park YS, Lelu-Walter M-A (2007). Recent progress on somatic embryogenesis of four *Pinus* spp. Tree For Sci Biotech 1: 11-25.

Litvay JD, Verma DC, Johnson MA (1985). Influence of a loblolly pine (*Pinus taeda* L.). Culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucus carota* L.). Plant Cell Rep 4: 325-328.

Lelu-Walter M-A, Bernier-Cardou M, Klimaszewska K (2006). Simplified and improved somatic embryogenesis for clonal propagation of *Pinus pinaster* (Ait.). Plant Cell Rep 25: 767-76.

Shi J, Zhen Y, Zheng R-H (2010). Proteome profiling of early seed development in *Cunninghamia lanceolata* (Lamb.) Hook. J Exp Bot 61: 2367-2381.

Shohael A, Ali M, Hahn E, Paek K (2007). Glutathione metabolism and antioxidant responses during *Eleutherococcus senticosus* somatic embryo development in a bioreactor. Plant Cell Tissue Organ Cult 89: 121-129.

Teyssier C, Grondin C, Bonhomme L, Lomenech A-M, Vallance M, Morabito D, Label P, Lelu-Walter M-A (2011). Increased gelling agent concentration promotes somatic embryo maturation in hybrid larch (*Larix*×*eurolepis*): a 2-DE proteomic analysis. Physiol Plant 141: 152-165.

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Native state of extracellular matrix of early conifer embryogenic tissue imaged by environmental scanning electron microscope

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Keywords: ESEM, detectors, methodology, in situ study, plant biology

An environmental scanning electron microscopy is described that by using our newly introduced methodology allows observation of conifer somatic embryos in their native state. This research method prevents morphological changes during sample dehydration and avoids appearance of the artifacts that are commonly created by traditional preparation techniques needed for the study of biological samples in classical scanning electron microscopy.

Environmental scanning electron microscopy (ESEM) opens up a wide range of new applications in the field of electron microscopy. Some of these biological applications rely on us being able to image samples in, or close to, their native state [1, 2]. In ESEM the specimens can be observed in a wide range of pressure, from 0,001 Pa (comparable with the one used in SEM) to over a thousand Pa in the specimen chamber [3]. In high pressure conditions very wet non-conductive samples can be observed free of artifacts caused by charging and without a conductive coating covering their surface. If the gas pressure is sufficiently increased or the sample's temperature reduced, its natural and fully hydrated surface structure is preserved [4, 5, 6].This study is focused on introduction of methodology enabling the creation of suitable conditions for the study of ECM *in situ*. Early somatic embryogenic tissues of selected conifers (*Abies alba, Abies numidica* and *Pinus sylvestris*) were observed free of sputter coating with an electrically conductive layer, without use of any chemical fixation or preparation technique which means the material was in a really native state. Our method was the following: our samples were placed on a cooled Peltier stage with a temperature range from -18 °C to -22 °C with the specimen chamber of the ESEM under high pressure (550 Pa). To observe the natural surface of early conifer somatic embryogenic tissues a specially designed ionization detector of secondary electrons and an yttrium aluminum garnet activated with a trivalent cerium (YAG: Ce^{3+}) detector of backscattered electrons were used. Fig. 1. (a-f) shows native early embryogenic tissue of *Abies alba*, *Abies numidica* and *Pinus sylvestris* covered by a network of fibrillar material forming ECM layer.

We found that an environmental scanning electron microscope that uses the above introduced methodology is useful and very convenient for observing the native state of plant tissue, even though we suppose that this method is unsuitable for observing animal tissue without freezing damages. Additionally, plant tissue free of chemical fixation procedures allows observing the extracellular matrix in its native state. This method is fast and simple and, moreover, relatively inexpensive. We expect that it will be a generally applicable tool in the field of plant research.

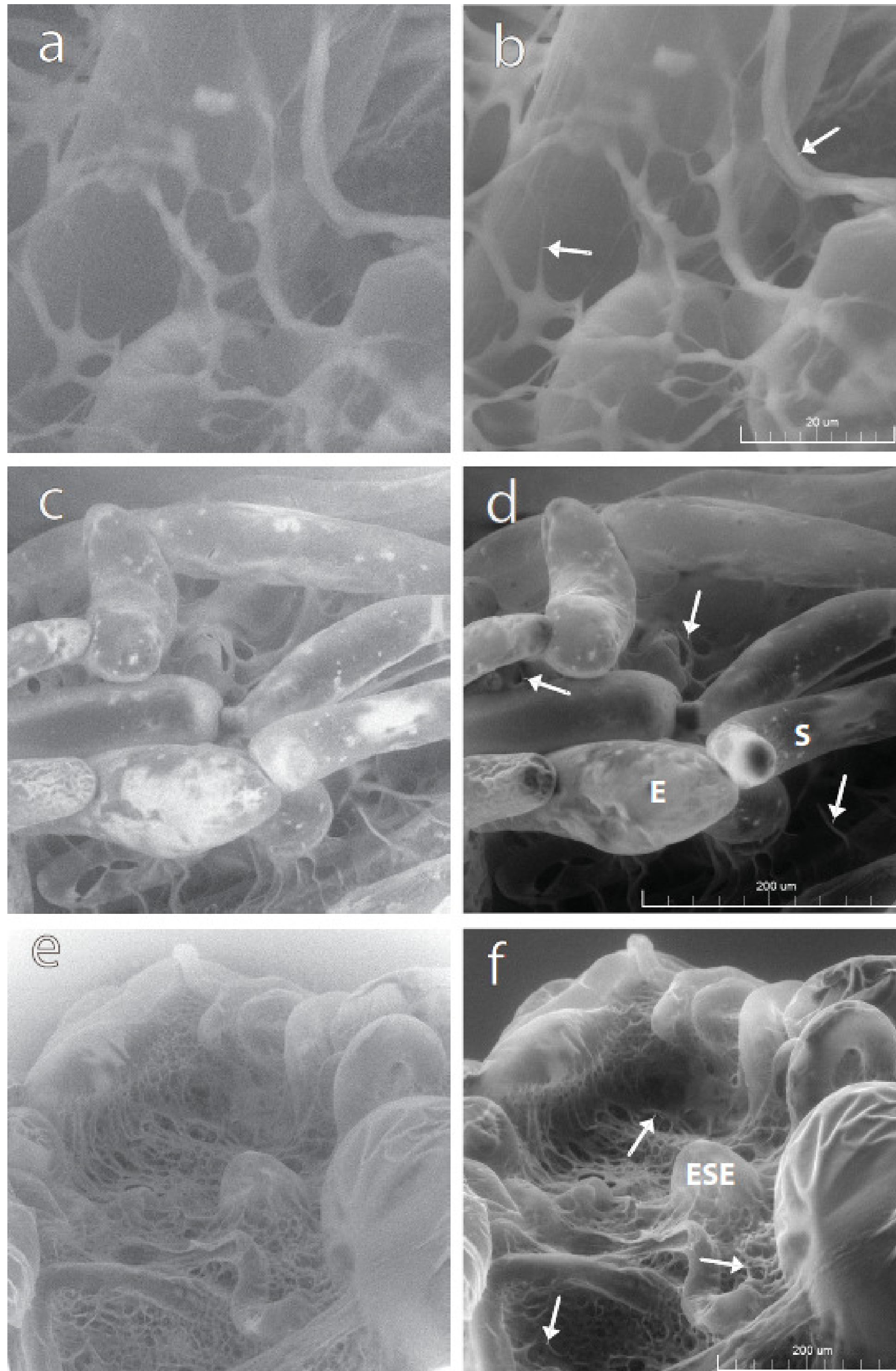

Fig. 1. *Comparison of ESEM observation with two detectors of embryogenic tissue of conifers. Abies alba (a, b); Abies numidica (c, d) and Pinus sylvestris(e, f). On the left side of the figure is the ionization detector and on the right side is the BSE YAG detector (the presence of ECM is indicated by arrows; E-embryonic part; ESE-early somatic embryo; S-suspensor cell).*

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References:

[1] Danilatos, G.D.: Journal of Microscopy 121: 235-238, 1980
[2] Kirk, S.E., Skepper, J.N. and Donald, A. M.: Journal of Microscopy 233: 205-224, 2009
[3].Danilatos, G.D.: Adv. Electron Phys. 71: 109-250, 1988
[4] Stokes, D.J.: Investigating biological ultrastructure using environmental scanning electron microscopy (ESEM). - In: Mendez-Vilas, A. (ed.): Science, Technology and Education of Microscopy: An Overview, Trans. Tech. Publications LTD. Badajoz Spain 2003, pp. 564-570
[5] Neděla, V.: Journal of Microscopy 237: 7-11, 2010
[6] Neděla, V.: Microscope Res. Tech. 70: 95-100, 2007

Incorporating wood quality traits in multi-varietal forestry of white spruce

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Keywords: genetic correlation**,** heritability, wood quality, multi-vatietal forestry

Genetic effects of wood quality traits (latewood proportion (LWP), wood density (WD), fibre length (FL) and microfibril angle (MFA)) and their relationship with growth, i.e., 14 year height (HT14) and volume (VOL14), were studied using thirty clone lines from a clonally replicated genetic test of white spruce at age 19 years. In order to sample genotypes across the range of growth, six families, two each from fast-, moderate-, and slow-growing families, were sampled. Within each family, five clones were selected randomly.

Both growth and wood quality traits appear to be under moderate genetic control. The main contributor of variation in growth traits was variation due to clones within family. For wood quality traits (LWP, WD, and FL), variation due to families was greater than the clonal variation (Table 1).

Table 1. *Estimated genetic parameter of growth and wood quality traits of plantation grown white spruce*

Parameter	HT14 (cm)	VOL14 (dm^3)	LWP (%)	WD (kg/m^3)	FL (mm)	MFA (º)
Mean	502	15.4	18.1	391.2	1.9	16.4
σ^2_{family}	0.0	0.0	25.3**	17.9*	21.3**	4.8
σ^2_{clone}	34.6**	19.5**	10.4*	6.4	9.9*	3.9
$\sigma^2_{site\ x\ family}$	7.5	16.5*	0.1	3.3	4.0	1.1
$\sigma^2_{site\ x\ clone}$	0.0	1.4	6.0	2.6	1.9	14.2*
σ^2_{Error}	57.9	62.6	58.2	69.8	62.9	76.0
$H^2_{Individual}$	0.35	0.20	0.36	0.24	0.31	0.10
$H^2_{Clone-mean}$	0.83	0.70	0.77	0.71	0.77	0.34

. , significant at p<0.05 and **, significant at p<0.01*

Generally, faster growth resulted in a significantly lower overall wood density but no significant correlations were found with FL (Table 2).

Table 2. *Genetic correlation among growth and wood quality traits.*

	VOL14	LWP	WD	FL	MFA
HT14	0.94**	-0.49*	-0.34	0.30	-0.31
VOL14		-0.94**	-0.75**	0.31	-0.09
LWP			0.97**	-0.13	0.12
WD				0.18	-0.21
FL					-0.43

** , significant at p<0.05 and **, significant at p<0.01*

This study demonstrated that fast growth owing to better site quality and genetic selection for growth traits may have negative effects on WD, especially at the family level (Figure 1a), whereas such effects are negligible for FL and MFA. Despite the negative correlation between growth and wood quality, clones that break such correlations may be found through multi-varietal forestry aimed at improving growth without compromising WD (Figure 1b).

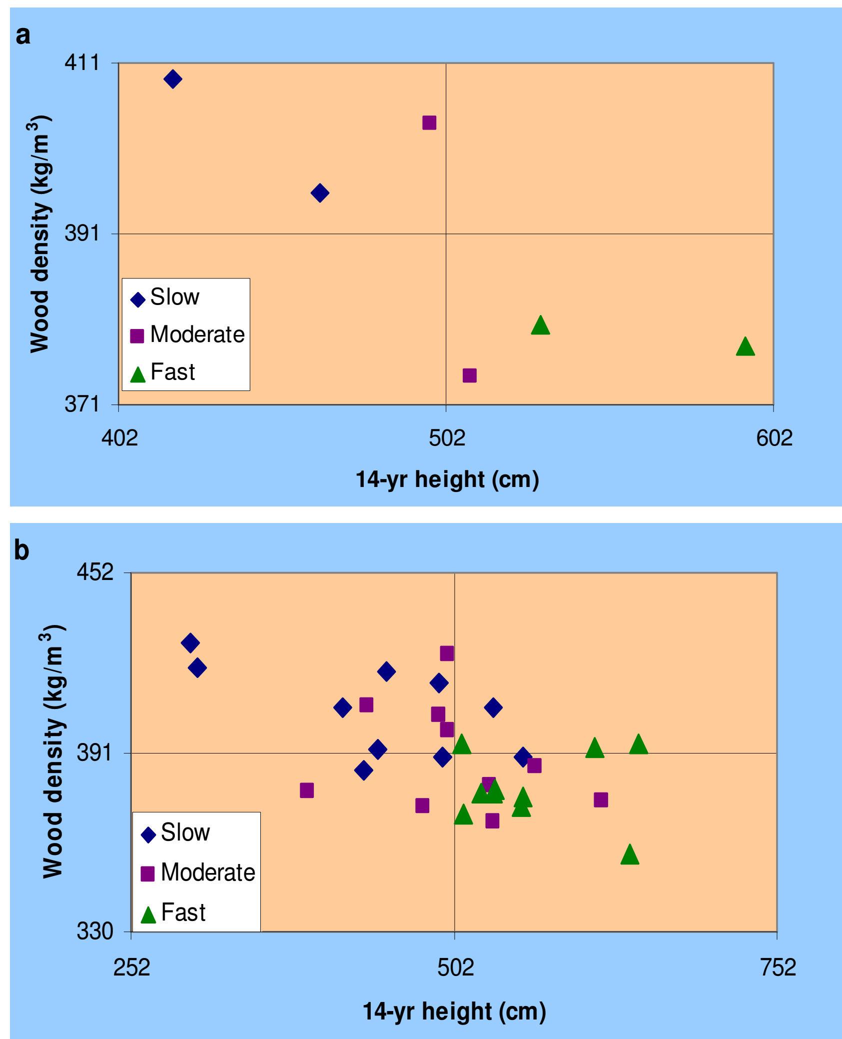

Figure 1. *Relationship between wood density and 14 year height of growth categories of families (a) and clones (b). Note that the 2 fast growing clones also maintain average density.*

The biochemical characteristics of the physiological activity of beech and spruce embryos

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Key words: abscisic acid, indolyl-3-acetic acid, fumarase, somatic embryogenesis, seeds quality

Introduction

It is necessary to find convenient and measurable criteria for the evaluation of seeds/embryos quality before germination because of a long time interval between the preparation of seeds or embryos (before germination) and obtaining the seedlings or emblings. The success of all treatments before germination is assessed by the quality of the newly formed plants only. The aim of our experiments is to estimate the potential of seeds harvested from different sources and/or of the somatic embryos developed in different embryogenic cultures just before germination, to develop into high quality plants. During the search of a suitable method we selected 3 substances to measure: 1) ABA (abscisic acid) as the phytohormone which controls the dormancy in seeds and which regulates the maturation of somatic embryos; 2) IAA (indolyl-3-acetic acid) as the phytohormone which regulates the growth and the development of embryos and whole seedlings and 3) fumarase as the enzyme which is often correlated with the dormancy and the germination of seeds. Fumarase is the key enzyme in mitochondrial metabolism. Its activity indicates the respiration rate and the ability of seeds to use stored reserves.

Methods

ABA and IAA

The weighted plant material (around 0.1 g FW) was milled on a DNA mill and extracted in a modified Bieleski solution. The extract was centrifuged and dried on a rotary vacuum concentrator at room temperature.
Dried samples were dissolved in a 15 vol % solution of acetonitrile in water, injected into HPLC and precleaned on C-18 with gradient elution and fractionation on a fraction collector. The fraction at time 23.05 min was collected for 1 min and dried. After drying the collected fraction was derivatized by diazomethane solution in ether, dried, and dissolved in 100 µl of acetone. Of the redissolved sample 8 µl was injected into GC-MS/MS and analyzed by Ion trap in MS/MS scan mode (MS1: full scan 50-300 amu; MS2 IAA: precursor 130.1 amu, product full scan 65-200 amu; MS2 labelled IAA: precursor 136.1 amu, product full scan 70-200 amu; MS2 ABA: precursor 190.2 amu, product full scan 65-200 amu; MS2 labelled ABA: precursor 194.2 amu, product full scan 70-200 amu) (Kosova et al 2012).

Fumarase

The seeds stored at -80ºC were used. The weighed 5 pieces of embryonal axis (or an adequate amount of somatic embryos) was milled on a DNA mill and extracted by extraction buffer. The solution was centrifuged and the pure extract was filtered by a 0.2 µm microfilter. A quantity of 150 µl of filtered extract was added to a reaction mixture (HEPES buffer, malate dehydrogenase, NADP+, $MgCl_2$, KH_2PO_4). After 3, 6, 9 and 12 minutes of reaction the increase of absorbance at 340 nm (NADPH formation) is measured in a 50 mm cuvette. It is proportional to the amount of fumarase in the sample (method adapted according Hatch 1978 and Shen and Oden 2000).

The optimization of the enzymatic reaction was realized in two steps: 1) The timing of the reaction using pig fumarase from Sigma and the optimum for measurement were assessed to be 340nm during 1 – 18

minutes of reaction; 2) The effect of fumarase concentration on absorbance. The maximum of the sensitivity measurement is between 0 and 0.25 IU of fumarase.

Material

Beech seeds and spruce somatic embryos were used for verification of these three methods. We compared the quality of dormant and non-dormant beech seeds and the success of the maturation process of spruce somatic embryos, i.e. we tried to define the different quality of somatic embryos at the end of maturation.

Results

The endogenous level of ABA

The ABA content in dormant and non-dormant beech embryos_was measured in the embryonal axis, cotyledons and in the whole embryo from the freshly harvested dormant seeds and from the stored seeds just before germination. We found relatively high endogenous ABA in beech embryos; the highest level of ABA was detected in the embryonal axis. The difference between the ABA content in dormant and non-dormant embryos is more than 50%. The endogenous ABA decreases during the whole process of seeds stratification (13 weeks), but the ABA level decreases more rapidly at the beginning of stratification (Fig. 1).

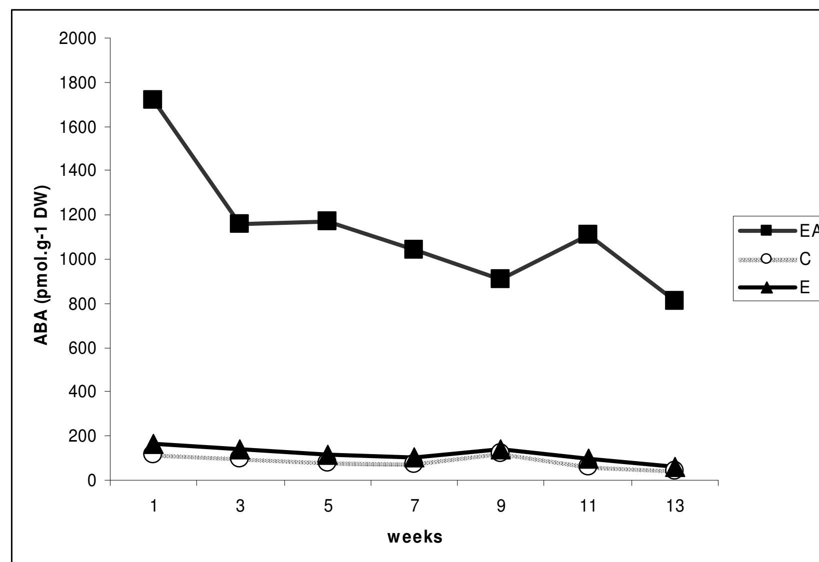

***Fig. 1**: The changes in endogenous ABA (in pmols of ABA in 1 g of dry weight of beech embryos) during 13 weeks of stratification (EA = embryonal axis; C = cotyledons; E = whole embryos)*

The endogenous ABA in spruce somatic embryos increases during 4 weeks of embryos maturation (Vágner et al 1999). (Maturation is carried out on the medium supplemented by ABA.) During the last two weeks of maturation and during the whole desiccation the ABA level decreases. The somatic embryos are able to germinate when the ABA content is low.

The endogenous level of IAA

The IAA content in dormant and non-dormant beech embryos_was measured in the same samples as ABA. We found extremely low IAA levels in beech embryos; the highest level was in the embryonal axis. The endogenous IAA fluctuates during the whole process of stratification. No differences between the level of IAA at the start and after 13 weeks of stratification were found. No difference between the IAA content in dormant and non-dormant embryos was detected (Fig. 2).

The endogenous IAA in spruce somatic embryos_increases during the 2nd and in the 3rd week of maturation when the root and shoot pole of embryos are formed. (Vágner et al 1999) The 2nd maximum of IAA

content is found at the end of desiccation just before germination. We can speculate that the transient increase of IAA together with low ABA content is necessary for successful germination of spruce somatic embryos.

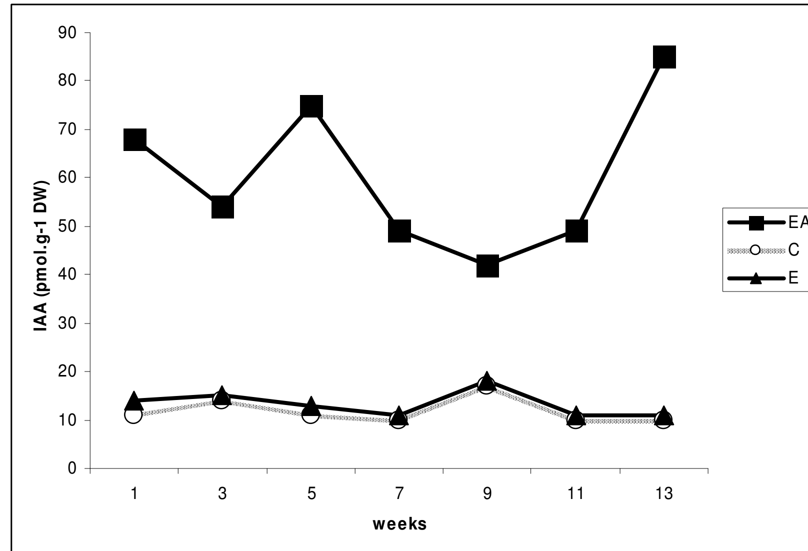

Fig. 2: *The changes in endogenous IAA (in pmols of ABA in 1 g of dry weight of beech embryos) during 13 weeks of stratification (EA = embryonal axis; C = cotyledons; E = the whole embryos)*

The fumarase activity

In the first step the method was verified using embryonal axis from stored seeds and from dead seeds of beech. The enzyme fumarase is active in living cells only, i.e. the activity measured in dead embryonal axis represents the base level for measurement. It is possible to differentiate the living and dead embryos using this method.

The fumarase activity in embryonal axis from dormant and non-dormant beech seeds_differs during the whole measurement (3, 6, 9, 12 min. of reaction). The lower fumarase activity is observed in embryonal axis from dormant seeds (Fig. 3).

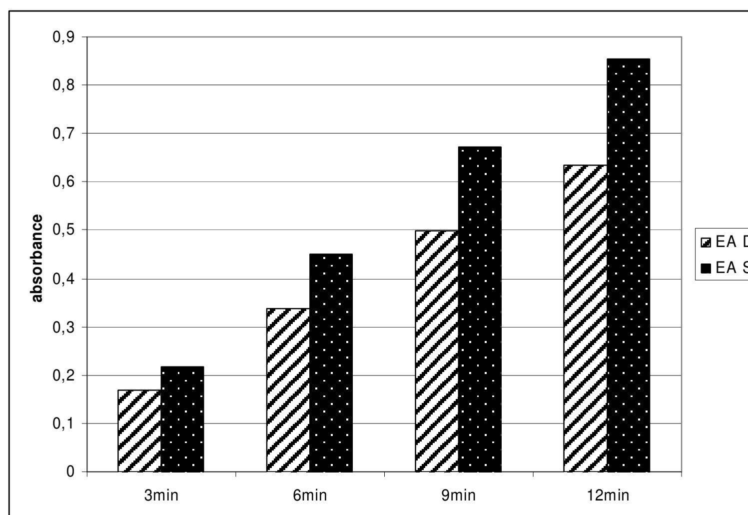

Fig. 3*: The fumarase activity in embryonal axis from dormant beech seeds and from seeds after 15 weeks of stratification (EA D = embryonal axis from dormant seeds; EA S = embryonal axis from non-dormant seeds, after 15 weeks of stratification)*

The fumarase activity in matured somatic embryos of spruce_was measured in the embryos of different quality and in the rest of the embryonic suspensor mass (consisting of dead cells and embryos). We did not find any activity of fumarase in ESM. The differences in fumarase activity among other somatic embryos are rather small. The higher fumarase activities were found in growing somatic embryos; ie. in developing globular embryos and in malformed embryos, where callogenesis starts. The lower fumarase activity was found in somatic embryos at the end of successful maturation (Fig. 4).

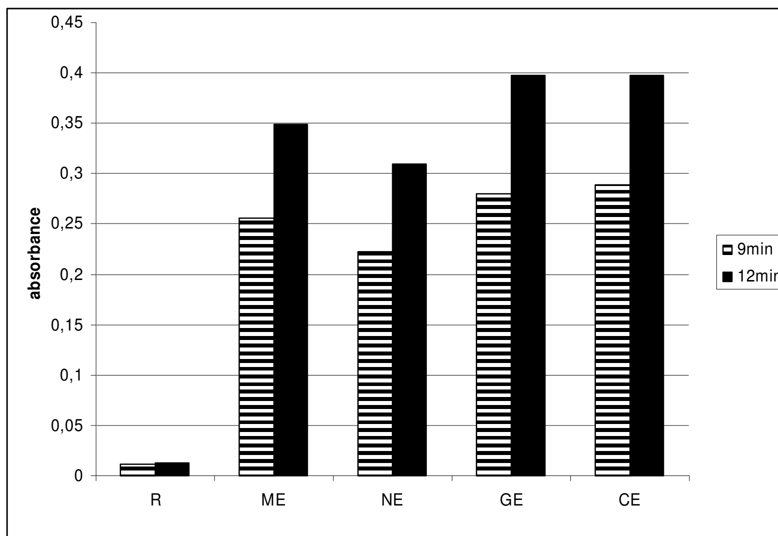

Fig. 4: *The fumarase activity in different maturated somatic embryos after 9 and 12 min. of reaction (R = rest of embryonic suspensor mass; ME = maturated embryos; NE = non-maturated embryos; GE = globular embryos; CE = malformed embryos with callus formation)*

Conclusions

1) The endogenous level of ABA can indicate the depth of dormancy and the effect of stratification on beech embryos. ABA can also characterize the ability of somatic embryos to germinate. This accurate method of ABA determination could be successfully used for embryo evaluation.

2) The endogenous level of IAA does not correlate with the dormancy of beech embryos and/or stratification. The endogenous level of IAA increases during polarization of spruce somatic embryos. The increase of the IAA level correlates with the start of somatic embryo germination. The IAA content can characterize the specific steps in the embryo development, but it is not the marker of deep of dormancy.

3) The fumarase activity can clearly distinguish the living and dead cells or tissue. After the rigorous optimization of the method (it is very sensitive to external conditions) it could be a promising method for testing dormancy. The fumarase activity is not a suitable criterion for the evaluation of spruce somatic embryos quality.

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References

Kosova K, Prasil IT, Vitamvas P, Dobrev P, Motyka V, Flokova K, Novak O, Turetkova V, Rolcik J, Pesek B, Travnickova A, Gaudinova A, Galiba G, Janda T, Vlasakova E, Prasilova P, Vankova R (2012) Complex phytohormone responses during the cold acclimation of two wheat cultivars differing in cold tolerance, winter Samanta and spring Sandra. J Plant Physiol 169:567-576

Hatch MD (1978) A simple spectrophotometric asssay for fumarate hydratase in crude tissue extracts. Analytical Biochem 85:271-275

Shen TY, Oden PC (2000) Fumarase activity as a quick vigour test for Scots pine (*Pinus sylvestris* L.) seeds. Seed Sci Technol 28(3):825-835

Vágner M, Vondráková Z, Špačková J, Cvikrová M, Eder J, Lipavská H, Albrechtová J, Svobodová H, Macháčková I (1999) Norway spruce somatic embryogenesis: endogenous levels of phytohormones during somatic embryo development. In: Altman A, Ziv M, Izhar S (eds.) Plant Biotechnology and In Vitro Biology in the 21st Century, Kluwer Academic Publishers, Netherlands, pp. 93-96

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Micropropagation of a recalcitrant pine (*Pinus pinea* L.): An overview of the effects of ectomycorrhizal inoculation

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Stone pine (*Pinus pinea* L.) is an economically important forest species in some regions of the Iberian Peninsula. Portugal and Spain have nearly 500,000 ha of stone pine stands, representing 85% of the worldwide distribution. The trees are exploited mainly for their wood, resin and pinion. The first two products have diminished in importance over the years but there is an increased demand for the pinion in the food industry. In addition to its enormous profitability as a seed producer, stone pine plays a positive role in soil protection, dune fixation and also as a pioneer species particularly in cork and holm oak degraded ecosystems. At present, the stone pine plantations are a major source of income for forestry holdings. The investments and research programs have targeted breeding, reforestation, forest management and harvesting. In 1988, the Portuguese National Forestry Station initiated a breeding program whose main objective was to genetically improve the quality and quantity of pinion (Barreira and Alpuim, 1988). Since then, various actions were initiated within integrated R&D projects such as PAMAF 2090 "Improvement of *Pinus pinea* L. for the production of edible seeds in Southern Portugal" and PIDDAC 212 "Improvement of *Pinus pinea* L. for pinion production". At an early stage of implementation of these breeding programs Provenance Regions were first delimited (Carneiro et al., 1998). These regions served for the basic identification and selection of reproductive material in accordance with seed certification guidelines. Simultaneously, selected stands of good producers of pinion were subsequently registered in the National Catalogue of base Materials (CNMB: http://www.dgrf.min-agricultura. pt).

The maternal inheritance of desirable characteristics such as cone weight, number of seeds per cone and seed length is considerably high in stone pine, thus encouraging the selection of seeds from selected trees (Alpuim, 1994; Carneiro, 2002; Evaristo et al., 2008). Two different approaches were studied under the PAMAF 2090 project for multiplication of the selected material: grafting and micropropagation. Grafting is arduous and generates high variability due to scion-rootstock interaction that varies production levels and, therefore, is not suitable for large-scale multiplication of elite cultivars. Micropropagation has been shown to be feasible in other conifer species and therefore in 1997 micropropagation studies funded by PAMAF 2090 began in The Breeding and Biotechnology Laboratory of ICAAM (LMBT), University of Évora (partner in the project). For a decade, the research group at ICAAM tried to enhance all phases of the stone pine micropropagation especially adventitious rooting of *in vitro* produced shoots and acclimatization. During this time, continuous increments in the multiplication rate and rooting frequency were achieved by varying the culture medium composition and physical conditions. Significant advance

was finally obtained by testing different combinations of carbon source, light and temperature during the induction and expression phases of the adventitious root formation (Zavattieri et al., 2009). With the new combination of chemical and physical factors it was possible to increase the rooting percentage from 30 to over 75 in several tested clones. Despite these promising results, the growth of adventitious roots could not be sustained rendering the acclimatization of plants either difficult or impossible. In a parallel research project, the group at the LMBT demonstrated that some fungi were beneficial in overcoming the root growth cessation when co-cultured *in vitro*. From a random sample of 12 fungi derived from soil samples of a pine stand from Mata de Valverde (Alcácer do Sal, Southern Portugal) at least nine were repeatedly capable of enhancing root growth (Oliveira et al., 2003). These results prompted a new interesting study on the effect of beneficial microorganisms in overcoming the problems with adventitious rooting and acclimatization in stone pine.

In 2007 the Portuguese Science and Technology Foundation approved and financed the project PTDC/AGR-CFL/71437/2006 "***Analysis and mastering*** of root growth signalling by ectomycorrhizal fungi in *Pinus pinea* L." coordinated by the LMBV and with the scientific advice of Dr. Krystyna Klimaszewska (Canadian Forest Service) to advance the biotization research between stone pine and ectomycorrhizal fungi. First results of *in vitro* biotization showed improvement in various root parameters during *in vitro* co-culture of *Pinus pinea* with *Pisolithus arhizus* before physical contact occurred (Fig. 1). Significant differences were found in the number of branches, in the number of roots plus branches, in total length of roots, in total length of roots plus branches, in average root length and in the length of the longest root in inoculated plants compared with non-inoculated plants (Table 1). The roots of inoculated plants also grew better in vermiculite and during acclimatization in mixed substrates compared with control plants resulting in the development of a vigorous root system. Overall, mycorrhizal inoculation increased the survival rate of the regenerated plantlets during acclimatization (Ragonezi et al., 2012). To confirm that the plants inoculated *in vitro* were actually mycorrhized; histological and microscopic observations of the roots were carried out after one month of acclimatization in sterile vermiculite (Fig. 2).

Table 1. *Means ± standard errors of variables with significant differences between control and inoculated plants (Ragonezi et al., 2012). Differences were investigated separately for each stage by exact or approximate Student's t tests after checking for homocedasticity and at the probability level P=0.05. Sample size in control was n=10 except in vitro where n=13; in inoculated plants always n=13.*

Growth stage	Variable	Control	Inoculated	P
In vitro	Change in number of branches	0 ± 0	1.4 ± 0.6	0.018
	Change in number of roots plus branches	0 ± 0	1.4 ± 0.6	0.018
Vermiculite	Change in total length of roots (mm)	3.8 ± 3.1	29.2 ± 7.2	0.003
	Change in total length of roots plus branches (mm)	2.8 ± 4.2	32.3 ± 7.1	0.002
	Change in average root length (mm)	0.9 ± 1.2	9.7 ± 3.4	0.014
	Change in length of the longest root (mm)	1.0 ± 2.0	14.7 ± 3.7	0.002
Mixed substrate, 2nd measurement	Change in number of roots	-1.0 ± 0.3	-0.1 ± 0.1	0.001
	Change in number of roots plus branches	-1.0 ± 0.3	-0.1 ± 0.1	0.001
	Change in total length of roots (mm)	-4.2 ± 8.8	17.4 ± 7.6	0.038

Fig. 1 *In vitro biotization in double phase medium composed of two solid phases (upper thin solid phase for fungi; lower solid phase modified WPM for root growth development) during in vitro co-culture of Pinus pinea with Pisolithus arhizus (Ragonezi et al., 2012).*

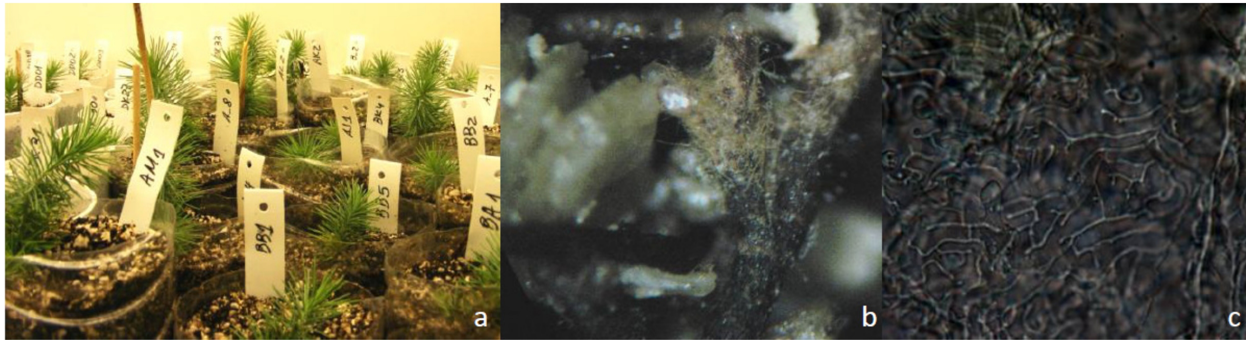

Fig. 2. *2a Inoculated Pinus pinea plants during acclimatization in mixed substrates; 2b Ectomycorrhiza derived from inoculated plants with Pisolithus arhizus, covered with brownish mycelia; 2c Transversal section of the root showing the Hartig net, optical microscopy observation 1250X magnification.*

To better understand the positive effects of fungal inoculation on root growth parameters before the establishment of physical contact between these partners, the biochemical compounds released by the roots were analysed. The analysis was facilitated by developing a co-culture system of stone pine plantlets and the fungus in a two-phase semi-solid/liquid medium (now under provisional patent No. 105239 of the National Institute of Industrial Property, INPI (Fig. 3).

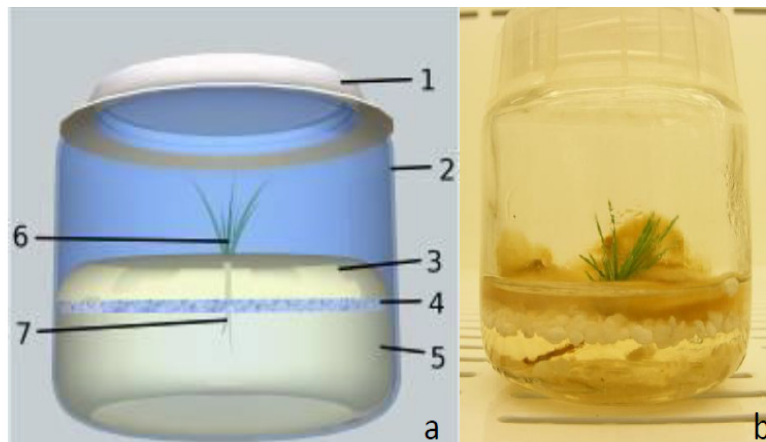

Fig. 3 *3a 1 - Cover, 2 - Flask, 3 and 4 - WPMS (Woody Plant Medium - Lloyd and McCown, 1981 - solid phase - with perlite facing down - 4), 5 - WPML (liquid phase), 6 - Microshoot, 7 - Root system (Provisional Patent INPI Nº 105239). 3b Double phase semi-solid/liquid medium co-culture system between Pinus pinea plantlets and the ectomycorrhizal fungus Pisolithus arhizus (Ragonezi et al. submitted).*

References

Alpuim M, Rocha ME (1994) Breve estudo biométrico sobre as características da pinha e semente de *Pinus pinea* L. In *Actas do III Congresso Florestal Nacional*, Figueira da Foz.

Barreira LL, Alpuim M (1988) Contribuição para um programa de melhoramento de pinheiro manso. *In Actas do "Encontro sobre Pinheiro manso"*. SPCF, Alcácer do Sal, 25-26 de Novembro.

Carneiro MM, Alpuim M, Rocha ME, Carvalho MAV (1998) Delimitação e caracterização de regiões de proveniência de *Pinus pinea* L. Silva Lusitana 6(2): 129-263.

Carneiro A (2002) Avaliação da variabilidade e controlo genético de características biométricas das pinhas e da semente da *Pinus pinea* L. Relatório de fim de curso de Engenharia Florestal. Universidade Técnica de Lisboa - Instituto Superior de Agronomia, p. 58.

Evaristo I, Tenreiro R, Costa R (2008) Characterisation of Biometric Parameters and Fatty Acids Content of *Pinus pinea* L. Pine Nuts of Portuguese Populations. Silva Lusitana [online]. 16(1):. 1-19.

Lloyd G, McCown, B (1981) Commercially feasible micropropagation of mountain laurel, Kalmia latifolia, by the use of shoot tip culture. Proc Plant Prop Soc 30:421-427.

Oliveira P, Barriga J, Cavaleiro C, Peixe A, Zavattieri Potes A (2003) Sustained *in vitro* root development obtained in *Pinus pinea* inoculated with ectomycorrhizal fungi. Forestry 76: 579-587.

Ragonezi C, Caldeira AT, Martins MR, Dias LS, Santos-silva C, Ganhão E, Miralto O, Pereira I, Louro R, Klimaszewska K, Zavattieri MA (2012) *Pisolithus arhizus* (Scop.) Rauschert improves growth of adventitious roots and acclimatization on *in vitro* regenerated plantlets of *Pinus pinea*. Prop. Orn. Plants 12:139-147.

Ragonezi C, Teixeira D, Caldeira AT, Martins MR, Santos-Silva C, Ganhão E, Klimaszewska K, Zavattieri A (2012) O-coumaric acid ester, a potential signaling molecule detected during early *in vitro* co-culture between *Pinus pinea* plantlets and the ectomycorrhizal fungus *Pisolithus arhizus*. Symbiosis (submitted).

Zavattieri A, Lima M, Sobral V, Oliveira P, Costa A (2009) Effects of carbon source, carbon concentration and culture conditions on in vitro rooting of *Pinus pinea* L. microshoots. Acta Hortic. 812: 173-180.

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Somatic embryogenesis as an effective regeneration support for reverse genetics in maritime pine: the Sustainpine collaborative project as a case study

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Keywords: maritime pine, somatic embryogenesis, transgenesis, reverse genetics, functional gene validation

Background

Reverse genetics, defined as ectopic candidate gene expression or silencing, has become an indispensable tool for functional dissection of traits of interest in forest trees. In maritime pine as in other conifers, long generation time and long life span, high genetic loads as well as high genetic redundancy are major obstacles to perform standard genetic approaches including association genetics. Validating marker associations with specific properties before transferring into breeding selection models is still challenging. Stable *Agrobacterium*-mediated genetic transformation of maritime pine was first reported by Trontin *et al.* (2002) and developed in both France (FCBA, INRA) and Portugal (IBET) with sufficient refinement (reviewed in Trontin *et al.* 2007) to envisage practical application in reverse genetics as an attractive alternative to association studies. As a result, the technology has been recently implemented in French (GenoQB, 2006-2009) and transnational (Sustainpine, 2010-2013) or European initiatives (ProCoGen 2012-2016). Somatic embryogenesis (SE) was revealed as a key tissue culture system for achieving genetic transformation, easy cryopreservation of transgenic tissue and efficient transgenic plant regeneration in maritime pine. Much consideration is given in France, Portugal and Spain to apply SE as a clonal propagation system for maritime pine improvement and deployment strategies in multivarietal forestry (MVF, Park YS, reviewed in Klimaszewska *et al.* 2007). By using this technology it is expected that we will obtain greater genetic gain, high flexibility in deployment of tested embryogenic varieties and easy balancing of genetic gain and diversity in plantations. Both SE and *Agrobacterium*-mediated transformation methods developed at FCBA, INRA or IBET are being successfully transferred to different partners through running the Sustainpine project (http://www.scbi.uma.es/sustainpine/).

SE in model line PN519: effective transfer at different labs

Using published protocols from FCBA, INRA and IBET (Klimaszewska *et al.* 2007), most SE steps could be

completed by Sustainpine partners for the selected embryogenic line provided by FCBA (PN519). A 100% recovery and multiplication of embryogenic tissue was achieved from a shared FCBA cryopreserved stock. PN519 line maturation required only minor adaptations at different labs with yields established in the range 60-221 embryos per g fresh mass (f.m.) tissue (FCBA/INRA reference yield: 85 ± 16 embryos g^{-1} f.m.). Progressive loss of embryogenic ability as a function of line ageing (usually within 6 months propagation) could explain the lower yields obtained in some cases (< 20 embryos g^{-1} f.m.). Efficient handling of embryogenic line post-reactivation using a combination of adapted culture practices is a key point for successful regeneration of transgenic plants. Cotyledonary embryos could be converted into acclimatized plantlets at a rate of 31-60% (INRA/FCBA reference data: 35-45%).

Genetic transformation of PN519 with reference, control binary vector

pCbar, a binary vector provided by FCBA and derived from pCambia1301 (Roberts *et al.* 1998) and pAHC20 (Christensen and Quail 1996), was used as a reference to test for genetic transformation of PN519 at different labs. Considering published papers (reviewed in Trontin *et al.* 2007) and unpublished data, a common Sustainpine protocol based on phosphinothricin (PPT) selection was established by FCBA and INRA. Considering FCBA/INRA reference data for this line (89.6 ± 18.4 PCR-positive, PPT-resistant lines g^{-1} f.m), protocol transfer was considered effective for most partners with a transformation rate established in the range of 19.0-78.4 PCR-positive, PPT-resistant lines g^{-1} f.m. However it is still a tricky step that required technical adaptations at some labs. A key consideration for successful transgenic plant regeneration was to avoid PN519 line ageing and associated decrease in both transformation rate and embryogenic ability.

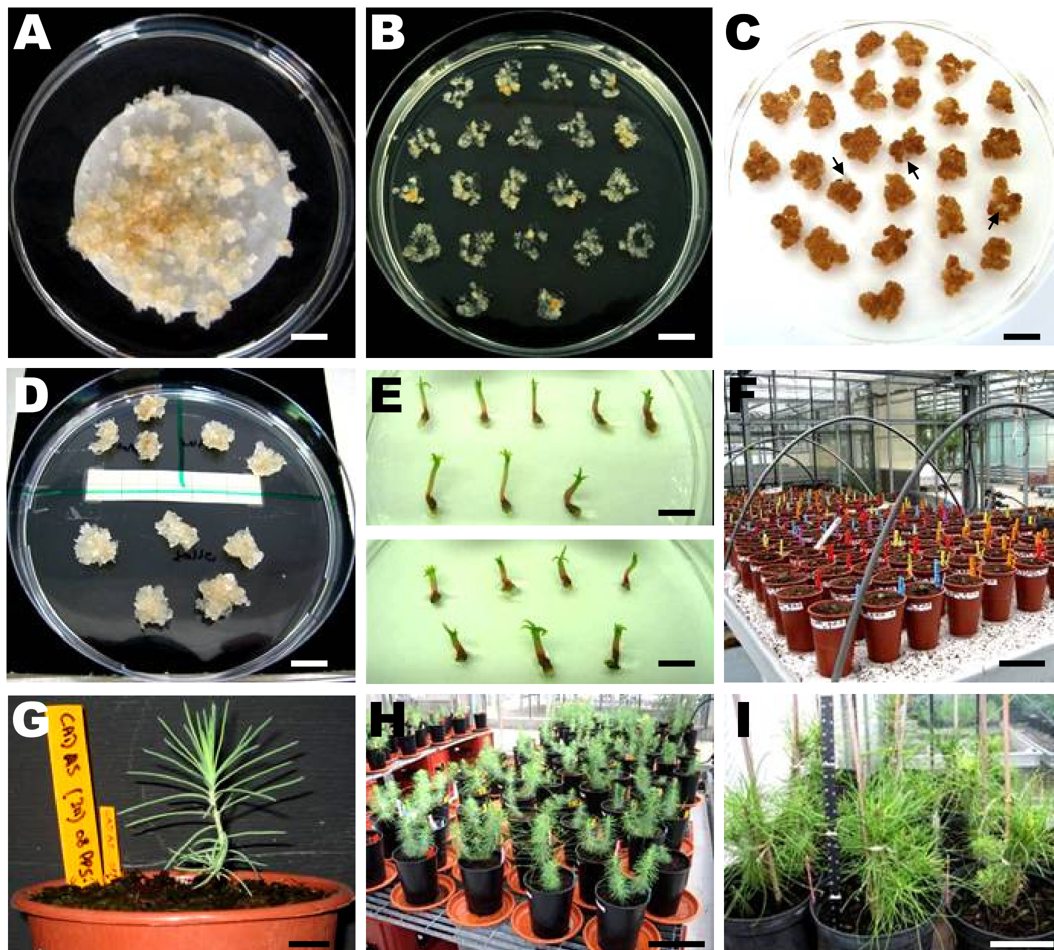

Fig. 1 *Agrobacterium-mediated genetic transformation and plant regeneration through somatic embryogenesis in maritime pine.* ***a*** *Cocultivated embryogenic tissue from line PN519 (1 week after cocultivation with Agrobacterium tumefaciens).****b*** *Small embryogenic cell clumps on selective medium (phosphinothricin) for 1 week.* ***c*** *Culture behaviour after selection for 7 weeks. Note the discreet phosphinothricin-resistant embryogenic whitish tissue (arrows) escaping from the surrounding, browning non-transformed cells.* ***d*** *Phosphinothricin-resistant embryogenic lines propagated on selection medium.* ***e*** *Germinating cotyledonary somatic embryos from transgenic (upper photo) and non-transformed control (lower photo).* ***f*** *Acclimatized plantlets in the greenhouse.* ***g*** *Young plantlet (2 months old post acclimatization) expressing an ihpRNA targeting the CAD gene.* ***h*** *One-year-old transgenic plants expressing ihpRNA constructs targeting the CAD, GRP, KOR or MYB14 gene.* ***i*** *Two-year-old transgenic plants overexpressing the GS1a gene. Bars = 1cm (a-e, g) or 10 cm (f, h, i). Picture origin: Sustainpine project (a-e), GenoQB project (f-h), FCBA/Univ. Málaga collaboration (i)*

Transformation rate of PN519 with selected overexpression and RNAi vectors

Ubiquitin promoter-based binary vectors developed by PSB/Gent University (Karimi *et al.* 2002) were selected for constitutive overexpression (OE, pMBb7Fm21GW-UBIL) or downregulation of gene expression through RNAi (ihpRNA: intron-spliced hairpin RNA strategy, pBb7GW-I-WG-UBIL). Standard protocols for maritime pine transgenesis using PPT as selective agent resulted in a quite low transformation rate (PCR-positive, PPT-resistant lines g^{-1} f.m.) for both the OE (4.0-8.0) and RNAi vector (3.0-8.7) as compared with reference pCbar. The number of transgenic lines produced in standard transformation experiment is sufficient to fulfil the requirement for biological repeats, i.e. 3-10 independent transgenic lines per construct. However validation of alternative, improved selection schemes is underway.

Conclusion

Previous expertise in maritime pine SE and genetic transformation (FCBA, INRA Orléans, IBET) has been transferred and is currently jointly developed with 3 other partners (Univ. of Alcalá, Univ. of València, Humboldt Univ. of Berlin). This transnational task force developed in the frame of Sustainpine (workpackage 3) is supporting one of the greatest efforts in conifers worldwide towards reverse genetics for functional candidate gene analysis (39 genes investigated). Biological outcomes in maritime pine will be facilitated by integrated analysis (collaboration between 9 Sustainpine partners) of new and previously created transgenic resources (cryopreserved embryogenic lines and somatic plants up to several years old).

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References

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high-level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res. 5:213–218

Karimi M, Inzé D, Depicker A (2002) GATEWAY vectors for *Agrobacterium*-mediated plant transformation. Trends in Plant Science 7:193–195

Klimaszewska K, Trontin JF, Becwar M, Devillard C, ParYS, Lelu-Walter MA (2007) Recent progress on somatic embryogenesis of four *Pinus* spp. Tree and Forest Science and Biotechnology 1:11–25 (review).

Roberts CS, Rajagopal S, Smith LA, Nguyen TA, Yang W, Nugroho S, Ravi KS, Cao ML, Vijayachandra K, Patell V, Harcourt RL, Dransfield L, Desamero N, Slamet I, Keese P, Kilian A, Jefferson RA (1998) A comprehensive

set of modular vectors for advanced manipulations and efficient transformation of plants by both *Agrobacterium* and direct DNA uptake methods. Vector release man. v3.05, CAMBIA, Canberra 6 pp.

Trontin JF, Harvengt L, Garin E, Lopez-Vernaza M, Arancio L, Hoebeke J, Canlet F, Pâques M (2002) Towards genetic engineering of maritime pine (*Pinus pinaster* Ait.). Annals of Forest Science 59:687–697

Trontin JF, Walter C, Klimaszewska K, Park YS, Lelu-Walter MA (2007) Recent progress on genetic transformation of four *Pinus* spp. Transgenic Plant Journal 1:314–329

Short Abstract

Culturing nucellus as a way to produce proteins involved in conifer reproduction

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Key words: suspension culture, *Pseudotsuga menziesii*, conifers, callus, proteomics

The nucellus has many roles during conifer ovule development. Initially, it is a sporogenous tissue that undergoes meiosis to produce a megagametophyte and its eggs. Next, during pollination it produces a pollination drop that traps pollen. Afterwards, it interacts with pollen tubes. In a mature seed, the nucellus dies, leaving only a wax-rich hydrophobic layer. We sampled Douglas-fir (*Pseudotsuga menziesii*) nucellus at the time of pollination drop secretion. Nucellar cultures were easily induced on different media, then multiplied in suspension. Proteins that were secreted into the growth medium were harvested and analyzed by mass spectrometry. We were able to confirm that many of these proteins were the same as those produced by ovular secretions during reproduction. Nucellar culture represents a significant advance in the study of the genesis of pollination drop constituents. Nucellar tissue culture not only overcomes the restrictions imposed by *in situ* studies such as short collecting seasons and low volumes, but is potentially a tractable experimental system for studying pollen-ovule interactions in controlled conditions.

Somatic embryogenesis from clonal sources of *E. camaldulensis*

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Keywords: Somatic Embryogenesis, *Eucalyptus camaldulensis*, Clone Propagation

Somatic embryogenesis in eucalyptus has been studied since the early 1980s. Embryogenesis has been reported in various eucalyptus species including *E. citriodora*, *E. nitens*, *E. dunnii*, *E. grandis*, *E. tereticornis*, *E. camaldulensis* and *E. globulus*. All these studies have been done using mature seeds as the source of explants, which reduces the homogeneity of the material and manifests vast variation. For commercial use, clonal genetic material would be the preferred source for mass propagation of elite lines. Reproduction of clones by somatic embryogenesis could also be significant for overcoming rooting problems of recalcitrant species, and may facilitate the cryopreservation of desired lines. Additionally, embryogenic callus can be used to introduce new traits into elite clones via transformation, for the development of yet further improved transgenic clones.

We report here embryogenesis from a clonal source of *E. camaldulensis*. By using leaf explants from an *E. camaldulensis* clone, we have established a protocol to obtain embryogenic callus. Different stages of embryos could be observed on the callus and we have succeeded in germinating the embryos. We are currently planning further work to optimize the procedure.

Analysis of different promoters and reporter genes in somatic embryos of *Pinus pinaster* Ait. and *Larix decidua* Mill.

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Keywords: Reporter Genes, Promoter Analysis, *Pinus pinaster*, *Larix decidua*

Reporter gene systems play a key role in many gene expression studies and in detection or localization of genes of interest. We analyzed reporter gene expression in somatic embryos of *Pinus pinaster* and *Larix decidua* to develop a screening strategy for accurate T-DNA integration into the plant genome and to identify false positive lines which frequently occur during the transformation process of somatic embryos from conifers. Also, the right choice of the promoter that drives the expression of either a foreign gene, a reporter gene and/or the selectable marker gene is of importance considering the temporal or spatial level of expression, not least in regard to overexpression studies. Hence, β-*GLUCURONIDASE (GUS)* expression regulated by either the cauliflower mosaic virus *(CaMV) 35S* or the maize-derived *UBIQUITIN 1 (UBI-1)* promoter during somatic embryogenesis of *L. decidua* was analyzed quantitatively and qualitatively. Genetic transformation was conducted with the plasmids pCAMBIA1305.2 (*35S::GUSPlus*), pGH217 (*35S::GUSA*), pSB241 (*UBI-1::GUSA*), modified pCAMBIA1301 (*35S::GUSA*), pLH6000 (*UBI-1::sGFP*) and mt-rb CD3-992 (*35S::mCherry*) comprising the respective reporter gene and the selectable marker genes *HYGROMYCIN PHOSPHOTRANSFERASE (HPT)* and/or *PHOSPHINOTRICIN ACETYL TRANSFERASE (PPT)*. For *Agrobacterium*-mediated transformation of embryogenic tissue of *P. pinaster* and *L. decidua* the 'droplet method' was used. The integration of the T-DNA into plant genomes was confirmed by polymerase chain reaction (PCR). The transgenic lines harboring green or red fluorescent proteins (sGFP or mCherry) were tested for reporter gene activity by fluorescence microscopy. The activity of the reporter gene *GUS* was observed by quantitative (fluorometric assay) and qualitative (X-gluc staining) detection during development of somatic embryos. The promoter analysis demonstrated that expression of *GUS* regulated by the *UBI-1* promoter is tenfold higher than driven by the *CaMV 35S* promoter during embryogenesis of *L. decidua*. The GUS assay showed high enzyme activity in the proliferation stage of somatic embryos in *P. pinaster* and *L. decidua*. Then the GUS enzyme activity decreased significantly in early stage of embryo maturation and increased again after 20 days of embryogenesis in both species. However, in general, the *GUS* expression regulated by *CaMV 35S* was much higher in *P. pinaster* compared to *L. decidua*. These results indicate that the *CaMV 35S* promoter activity depends on species and the respective developmental stage. The GFP and mCherry fluorescent signals were detectable in somatic embryos of *P. pinaster* that were tested positively by PCR. However, these fluorescent signals were not detectable in the whole embryogenic tissue of the same transgenic line. Also, a clear assignment of GFP fluorescent signals was impaired by the low intensity in comparison to that exhibited by model plants and the considerably high background fluorescence of wild type tissue.So far fluorescence screening by itself is unreliable for gene expression studies in conifers because of difficulties in the identification of proper signals. The improvement of expression efficiency and fluorescent intensity has to be further investigated by using other GFP variants including e.g. an intron and/or an endogenous promoter. It has been shown that the *GUS* reporter gene system was more efficient to monitor gene activity in somatic embryos compared to the *GFP* and *mCherry* genes systems. However, further studies on expression of different fluorescent reporter genes as vital markers in conifers are necessary.

From angiosperm models to forest trees: A study on expression behavior of *Arabidopsis* homologous genes during somatic embryogenesis of *Larix decidua*

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Keywords: *WUSCHEL-related HOMEOBOX2 (WOX2), PIN-FORMED (PIN), SHOOTMERISTEMLESS (STM), LEAFY COTYLEDON1 (LEC1), BABYBOOM (BBM), SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE (SERK),* inhibition of polar auxin transport

The organization of a plant embryo is defined by the first division of the zygote. In *Arabidopsis*, members of the *WUSCHEL-related HOMEOBOX* family (*WOX*) determine the apical and basal part of the developing embryo. Auxin transport and organogenesis is mediated by the family of PIN-FORMED (PIN) auxin efflux carriers. After the shoot and root meristems are established, the transcription factor *SHOOTMERISTEMLESS (STM)* is required. *LEAFY COTYLEDON1 (LEC1) is* necessary to prevent an early maturation. *BABYBOOM (BBM)* and *SOMATIC EMBRYOGENESIS RECEPTOR-like KINASE (SERK)* are known factors to carry embryogenic potential as their overexpression leads to the formation of somatic embryos.

It was our intention to study genes in *Larix decidua* that are homologous to the above mentioned factors to embryogenesis in *Arabidopsis.* Furthermore, we were interested in the correlation between these genes and the hormonal control executed by the master regulator auxin. Accordingly, we analyzed expression patterns of selected genes depending on auxin distribution within the embryo. To alter auxin availability during maturation we supplemented maturation media with the inhibitor of polar auxin transport **N**-1-naphthylphthalamic acid (NPA; 20 µM).

Full length or at least partial length sequences of homologous genes were identified beforehand, screening a cDNA library as well as using PCR-based methods. For expression studies we used somatic embryos of an established line that was found easy to maintain, mature, convert and transform, taking advantage of readily available and accessible starting material as well as of the controllability of the *in vitro* system itself. Relying on these benefits we analyzed expression sites of several genes of interest in mature somatic embryos by the means of in-situ-hybridization.

Further we aimed to assess tissue-specificity of the genes of interest in order to establish an appropriate indicator of early differentiation between embryogenic and non-embryogenic tissue (callus). Thereby successful induction of somatic embryogenesis will be simplified. So far confirmation of positive induction events has been done by microscopic analysis of cultures of a certain age (several weeks): callus is characterized by a loose structure and globular cells, whereas somatic embryos are subdivided in suspensor and embryo head. The use of expression

markers is necessary to prove the embryogenic competence as soon as cells are reprogrammed. According to this we analyzed the expression of several genes in different tissue types. This led to the conclusion that specific transcription patterns of particular genes enable an early distinction.

Identification and expression studies point towards a relevant function of the genes of our interest (*LdBBM, LdLEC1, LdPIN, LdSERK, LdSTM, LdWOX2*) during somatic embryogenesis of *Larix decidua* and the results further suggest a conserved role of principal regulators during land plant embryogenesis. An interaction of these genes and endogenous hormones can be derived from the NPA assay. Functional studies are necessary to confirm the assumed roles for the individual genes. Embryo-specificity could be demonstrated for several genes, thus possibly introducing a potent tool to improve the induction of somatic embryogenesis.

Propagation of American chestnut using non-germinable somatic embryos

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Keywords: *Castanea dentata*, adventitious buds, genetic transformation, plant regeneration

In our efforts to produce blight-resistant American chestnuts (*Castanea dentata*) using genomics, in vitro propagation and gene transfer, embryogenic cultures have been proven to be good targets for genetic transformation. However, constitutive expression of some transgenes may disturb somatic embryo development, resulting in a high percentage of abnormal embryos, such as segmented embryos or embryos with single or fused cotyledons. Plant regeneration through germination of such abnormal embryos is either impossible or occurs only at very low frequencies. In this study, a micropropagation method was developed to regenerate plants from non-germinable transgenic embryos. Multiple adventitious buds were induced from the apical meristem areas of the abnormal embryos. Buds could be induced from these embryos at early developmental stages, although a better induction rate was obtained with more mature embryos. Bud induction success varied with genotype and concentration of 6-benzylaminopurine (BAP). After bud induction and multiplication, individual buds were removed and cultured on shoot elongation medium with reduced BAP for 2 weeks, then transferred to root induction medium containing 5 mg l^{-1} indole butyric acid for one day before culturing on root development medium with no plant growth regulators. All cultures were maintained under light (16/8 h). Rooted plants were obtained from non-germinable transgenic embryos of American chestnut genotype WB484-3 engineered with constructs carrying a GUSi-YFP gene fusion or a Chinese chestnut deoxy-arabino-heptulosonate phosphate synthase gene. This study demonstrates a novel system, through which plant regeneration was enhanced, for producing phenotypically normal plants from genetically engineered tissue.

Climatic adaptation in Norway spruce - Molecular dissection of a novel epigenetic memory mechanism

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Key words: *Picea abies,* micro RNA analysis, temperature adaptation, transcriptome, somatic embryogenesis

Forests are ecologically and economically important in our ecosystem. Upon world climate changes, it is worthy to understand climatic adaptation of trees. The temperature level experienced during zygotic embryogenesis and seed maturation in Norway spruce (*Picea abies*) affects an epigenetic memory and vital phenological traits in the progeny. Timing of bud burst as well as growth cessation and bud set occurs early if the embryo temperature is low whilst late if the temperature is high. We intend to identify and characterise genes involved in this epigenetic memory of temperature, as well as of traits where the epigenetic memory is expressed in plants and embryos. To recognize molecular mechanisms of epigenetic phenomena, we isolated micro RNA from seedlings and embryos that showed distinct differences in epigenetic phenotypes. In seedlings, four selected genes *PaLPT4*, *PaGaMYB, PaMYB10* and *PaSPB13* regulated by miRNAs may be involved in epigenetic memory regulation. Although the functions of these genes are not elucidated yet, these findings imply these miRNAs may involve or at least affect the molecular mechanisms underlying the temperature sensitive epigenetic memory in Norway spruce. Using the

Illumina based MACE (massive analysis of cDNA ends) approach transcriptome changes were monitored in *in vitro* propagated somatic embryos from two full-sib genotypes during the early maturation stage at two different temperatures (18° *vs.* 30°C). MACE results were validated for a variety of candidate genes using qRT-PCR. We revealed striking differences in transcriptomes between genetically identical embryogenic tissues grown under different temperatures as well as between genotypes originating from crossings of the same parental trees under cold (outdoors) and warm (greenhouse) conditions. Also, a common pattern was observed for both genotypes in common temperature conditions. A large number of candidate genes thought to be involved in epigenetic regulation was found. Formation of different epitypes under warm and cold conditions during embryogenesis is linked to expression of different sets of genes, which provide candidate genes fit for further studies of the epigenetic memory initiation.

The role of polyamines in the embryogenic and organogenic capacity of yellow polar (*Liriodendron tulipifera*)

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Keywords: embryogenic cell, putrescine, spermidine, recalcitrant species, genotype

In plants, polyamines (PAs) have been implicated in the regulation of the developmental processes; cell division, proliferation, morphogenesis, growth and environmental stress responses in in vitro and ex vitro plants. In addition to a multitude of potential functions of polyamines in plants, it has been suggested that polyamines play a critical role in morphogenesis in plant cell and tissue cultures, especially somatic embryogenesis and organogenesis. In woody plants, recalcitrance of in vitro culture has often been considered as a species-specific characteristic because of the difficulty to identify the reasons for the problem as it is a consequence of the complex interplay of several physiological factors and regulation at the transcription level. To approach this notorious characteristic, we studied metabolites, mainly PAs, to investigate their role in the embryogenic and organogenic capacity of yellow poplar (*Liriodendron tulipifera*). Embryogenic (EC) and non-embryogenic cells (NEC) of yellow poplar were investigated for their metabolic compositions including PAs. The PA contents were remarkably different between the cell types, the highest levels occurring in the NEC on proliferation medium, when putrescine and spermidine were most abundant. However, the putrescine/spermidine (Put/Spd) ratio was higher in EC of yellow poplar. A comparison of metabolic compositions of NEC and EC using GC/MS identified around 50 compounds, partly displaying significant changes in metabolite levels, e.g., highly elevated levels of xanthosine and methyloxazole in EC compared to in NEC. From this analysis, we have identified numerous compounds including PAs involved with the embryogenic state. For organogenesis of yellow poplar (YP) the recalcitrant genotypes (YP1 & YP2 – recalcitrant & less recalcitrant) and of Italy poplar (IP) the non-recalcitrant genotypes (IP1 & IP2 – less & high regeneration ability) were used. Stem discs cultured on regeneration medium were sampled and the contents of three PAs were analyzed during the entire culture period. Total PA contents were always higher in the recalcitrant species (YP) and more recalcitrant genotype within the species, and the tendency was similar with the spermidine/spermine (Spd/Spm) ratio. Interestingly the Put/Spd ratio was higher in non-recalcitrant species (IP) and genotype (IP2) than that of YP. These results showed a close relationship between cellular PA levels and their Put/Spd ratio with the in vitro regeneration capacity in yellow poplar and suggest that the cellular PAs and Put/Spd ratios are important indicators of the regeneration ability in yellow poplar, and morphogenetically poor and recalcitrant species/genotype could be found by investigation of the PAs analysis.

Somatic embryogenesis in *Abies nordmanniana*: present status and future application

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Keywords: Nordmann fir, clonal mass propagation, somatic embryos, embryo maturation, acclimatization, somatic seedlings

The biotechnical procedure for clonal mass propagation of Nordmann fir (*Abies nordmanniana*) has been studied for more than 10 years and has been improved with regard to the critical steps of long-term propagation of embryogenic cultures, loss of maturation ability, storage of mature somatic embryos, conversion, acclimatisation and field establishment.

At present, 80 to 90 % of the embryos with normal morphology can be converted into plantlets, independently of their genotype. After approx.16 days of conversion, seedlings are ready for acclimatisation. Various substrates, potting systems, humidification systems, plant protection etc. have been compared resulting in a survival of 90 % of the plants under appropriate conditions. In contrast to other genera, *Abies* seedlings go into dormancy in the cotyledonary stage even under long-day conditions. However, 2 to 3 months after acclimatisation dormancy can be broken using an artificial cold treatment for 6 weeks minimum at 5° C or lower. This treatment is followed by a second flush in the same season. Although this treatment may speed up their growth, somatic seedlings develop slower and loose up to 1 year compared to plantlets from seeds. Therefore, a focus of the future research will be on development of optimal culture conditions to overcome the disadvantages of the somatic seedlings in the first year compared to plantlets from seeds.

Mass clonal micropropagation of teak Plus trees for high yield and superior quality plantations

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Keywords: Axillary budding, *In vitro* culture, Mass production, Mature, Meristem culture, Propagation by cuttings, Rejuvenation, *Tectona grandis,* Tree improvement, True-to type cloning

Abstract: The dramatic reduction of high value timber supplies from natural stands and an increasing worldwide demand has accounted for a greater interest in teak (*Tectona grandis*) plantation establishment. Production of a high yield of top quality teak wood in short rotations is now becoming a priority for a lot of land owners and investors in many humid tropical countries. This trend warrants the current attractiveness of superior teak clones that can be planted either as monocultures or in combination with other crops. During the early 1990's in Sabah (East Malaysia), the company Yayasan Sabah Group Biotech ("YSG Biotech"), jointly with CIRAD-Forêt (France), had

developed a very efficient method for mass cloning superior teak trees of any age by nursery or in vitro methods. Physiological rejuvenation can be gradually achieved by successive cycles of propagation by rooted cuttings or by *in vitro* subcultures of microshoots, or more readily by shoot apical meristem culture. The outstanding field behavior of the first clonal offspring produced from the mature teak ”Plus” trees locally selected has led to their rapid *in vitro* mass production by axillary budding to meet local and international demands. To date, several millions of these clonal offspring have been produced worldwide and the demand keeps increasing due to the superiority of these materials. Meanwhile, efforts aiming at enriching the YSG Biotech teak genetic base have been pursued, and today the company owns the world widest teak gene pool. This richness is essential for genetic improvement through wisely established breeding populations, from which advanced generations of new clones can be produced by resorting to non destructive wood analyses methods and DNA markers for upgrading the clonal selections. The advantages of the micropropagation technique developed for utilizing at best these valuable genetic resources, including the true-to-type mass cloning of any mature selected teak individual, and for exporting these clones all around the world bypassing even very stringent phytosanitary requirements, are presented.

References

Chaix G, Monteuuis O, Goh DKS, Bailleres H, Boutahar N (2008) Quality control and mass production of teak clones for tropical plantations. In: Proc. of the international symposium held in 2007 on “ Processing and marketing of Teak wood products of planted forests” (KM Bhat, M Balasundaran, KV Bhat, EM Muralidharan and PK Thulasidas eds), Kerala Forest Research Institute, India and International Tropical Timber Organization, Japan. 146-157.

Chaix G, Monteuuis O, Garcia C, Alloysius D, Gidiman J, Bacilieri R, Goh DKS (2011) Genetic variation in major phenotypic traits among diverse genetic origins of teak (*Tectona grandis* L.f.) planted in Taliwas, Sabah, East Malaysia. Ann For Sci 68:1015–1026

Goh D, Monteuuis O (1997) Vegetative propagation of teak. ITTO Tropical Forest Update, 7 (2), 13

Goh D, Monteuuis O (2001) Production of tissue-cultured teak: the Plant Biotechnology Laboratory experience. In: Proc. of the Third Regional Seminar on Teak: “Potential and opportunities in marketing and trade of plantation Teak: Challenge for the New Millenium”. July 31 – Aug. 4, 2000, Yogyakarta, Indonesia, 237-247

Goh DKS, Alloysius D, Gidiman J, Chan HH, Mallet B, Monteuuis O (2005) Selection and propagation of superior teak for quality improvement in plantations: case study of the ICSB/Cirad-Forêt joint project. In: Proc. of the international symposium held in 2003 on “ Quality Timber Products of Teak from Sustainable Forest Management” (KM Bhat, KKN Nair, KV Bhat, EM Muralidharan and JK Sharma eds), Kerala Forest Research Institute, India and International Tropical Timber Organization, Japan. 390-399

Goh DKS, Monteuuis O (2005) Rationale for developing intensive teak clonal plantations, with special reference to Sabah. Bois et Forêts des Tropiques 285:5-15

Goh DKS, Chaix G, Bailleres H, Monteuuis O (2007) Mass production and quality control of teak clones for tropical plantations: The Yayasan Sabah Group and Forestry Department of Cirad Joint Project as a case study. Bois et Forêts des Tropiques 293: 65-77

Goh DKS, Monteuuis O (2009) Status of the “YSG BIOTECH” program of building teak genetic resources in Sabah. Bois et Forêts des Tropiques 301:33-49

Goh DKS, Monteuuis O (2012) Behaviour of the “YSG BIOTECH TG1-8” teak clones under various site conditions: first observations. Bois et Forêts des Tropiques 311:5-19

Monteuuis O, Bon M-C, Goh DKS (1998) Teak propagation by *in vitro* culture. Bois et Forêts des Tropiques, 256:43-53

Monteuuis O, Goh D (1999) About the use of clones in teak. Bois et Forêts des Tropiques 261: 28-38

Monteuuis O (2000) Propagating teak by cuttings and microcuttings. In: Proc. of the international seminar “Site, technology and productivity of teak plantations” FORSPA Publication N°24/2000, Teaknet Publication N°3, 209-222

Monteuuis O, Maître HF (2007) Advances in teak cloning. ITTO Tropical Forest Update, 17 (3): 13-15

Monteuuis O, Goh DKS, Garcia C, Alloysius D, Gidiman J, Bacilieri R, Chaix G (2011) Genetic variation of growth and tree quality traits among 42 diverse genetic origins of *Tectona grandis* planted under humid tropical conditions in Sabah, East Malaysia. Tree Genetics and Genomes 7:1263-1275

Effect of genotype and culture conditions on somatic embryogenesis and plantlet regeneration of a rare and endangered tree species *Oplopanax elatus*

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Keywords: somatic embryogenesis, *Oplopanax elatus,* genotype, sucrose, conversion

Oplopanax elatus, a rare and endangered species, is an important medicinal tree recommended for research as a ginseng-type cultivation source. Because of its medicinal properties against such maladies as asthenia, depression, and hypertension, demand for the species has increased, but its distribution in Korea is quite limited, occurring only on several high mountains and areas where access is difficult. In an attempt to establish a mass production system of *O. elatus* by somatic embryogenesis (SE), various factors affecting SE was studied. Embryogenic cell lines were induced from 13 mature embryos collected from 4 different mountains, and these cultures were used for Simple Sequence Repeat (SSR) analysis to investigate the genetic diversity and genotype effect. Embryogenic capacity was affected by genotype but had little correlation with the location where seeds were collected. Root segments of *in vitro* plantlets were the best explants to induce embryogenic cultures, producing a higher embryogenic culture initiation than petiole and leaf segment explants. Among carbon sources, 5% sucrose effectively produced somatic embryos from embryogenic cultures (around 180 embryos per Petri dish), while glucose and maltose produced less than 20 per Petri dish. Sorbitol and mannitol inhibited embryo formation. Culture density was important in promoting somatic embryo formation when cells were cultured at 10~50mg per Petri dish, with 50mg/plate of culture density actually inhibiting it. Over 50% of somatic embryos were converted into plantlets on MS medium solidified with 0.8% agar or agar and gelrite mixture (0.6% gelrite + 0.4% agar). Medium containing 0.2 mg/L of GA_3 enhanced the conversion rate up to 80%. About five hundred somatic plantlets were transferred to an artificial soil mixture and cultivated in a greenhouse. After two months, approximately 80% of transplanted plantlets were successfully acclimatized.

References

Moon HK, Kim JA, Park SY, Kim YW , Kang HD. 2006. Somatic embryogenesis and plantlet formation from a rare and endangered tree species, *Oplopanax elatus*. Journal of Plant Biology, 49(4): 320-325

Park SY, Moon HK, Murthy HN, Kim YW. 2011. Improved growth and acclimatization of somatic embryo-derived *Oplopanax elatus* plantlets by ventilated photoautotrophic cultures. Biologia Plantarum 55(3): 559-562

Figure 1. *Somatic embryo germination medium containing 0.2mg/L GA3*

Figure 2. *7-year-old Oplopanax elatus derived from SE*

Induction of somatic embryogenesis in developing ovules of *Quercus ilex* L.

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Key words: Adult trees, Arabinogalactan protein, Holm oak, *Quercus ilex*, somatic embryogenesis.

The implementation of multivarietal forestry as part of breeding strategies is expected to provide more productive forest plantations. In order to achieve this, a reliable and effective method of mass production of clonal plants is needed. Somatic embryogenesis is considered as a suitable way of vegetative propagation for this purpose. Moreover, techniques able to clone adult trees are desired because they will improve these strategies by maximizing the performance of clonal tests and excluding the need of cryopreservation while tests are in progress. The holm oak (*Quercus ilex* L.) is a Mediterranean evergreen tree with economic interests because of the acorn production and edible fungi mycorrhization. The aims of this work were to obtain embryogenic lines from teguments of ovules from mature trees of this species, assessing the influence of the explant's developmental degree along with genotype and media composition, and evaluate the effect of arabinogalactan proteins (AGP), on the induction rate of somatic embryogenesis. Developing flowers of four trees (genotypes E00, E0, E2, E4) were collected in El Encín (Madrid) at three developmental points: containing six ovules of the same size (putatively unfertilized); one of the ovules predominates in size (putatively fertilized); the predominant ovule reaching around 3 mm width and 5 mm length. After surface sterilization, ovules were removed from ovaries (zygote embryos were excised from the larger ovules and discarded) and were cultured on G-macronutrients medium lacking plant growth regulators (PGRs). Teguments from ovules of genotypes E00, E0, and E4 at the third stage of development were also cultured on SH-macronutrients medium without PGRs. They were maintained for 5 months at 25 ± 2 ºC in darkness with monthly subcultures. To test the influence of arabinogalactans and their interaction with plant growth regulators a mix of immature acorns from seven trees were collected in Sotolargo (Guadalajara). Ovules ranging 3-5 mm width and 5-10 mm length (without zygotic embryos) were cultured on SH-macronutrients media containing either 0, 2, 6 mgl^{-1} of *Larix*-AGP or 0, 10, 30 mgl^{-1} of *Acacia*-AGP, both groups of treatments carried out in a factorial combination with 10 µM of NAA plus 10 µM of BAP or without PGRs. After 30 days at 25 ± 2 ºC in darkness, they were subcultured to the same media, but with reduced PGR concentrations of 0.5 µM of both NAA and BAP. They were placed in a 16-hour photoperiod growth chamber and after 30 days transferred to media lacking PGRs. No embryogenic response was observed in explants collected at the first and second developmental points. However somatic embryos developed from explants of the third developmental point. Two out of the four genotypes were captured when G medium was used, while all three tested genotypes formed somatic embryos when SH medium was used. Induction frequencies ranged between 1.2 and 3.2 %. Somatic embryos were obtained with the *Larix*-AGP treatments only when no PGRs were supplied, although induction frequencies were not improved. No response was observed when the effect of AGP from the *Acacia* genus was tested.

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Effect of size of inoculum, density of inoculation and shaking on growth and differentiation of *Quercus suber* embryogenic suspensions

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Key words: Cork oak, embryogenic cultures, inoculum density, *Quercus suber*, suspension culture.

The cork oak (*Quercus suber* L.) is one of the most important forest species of the Mediterranean ecosystem. This evergreen tree forms along with other *Quercus* species the Iberian “dehesas” and “montados”, and its main products are cork, which is used for several industrial applications, and acorns, to feed the Iberian race of pigs. Somatic embryogenesis is considered the most suitable way of vegetative propagation for implementing multivarietal forestry. Mass production of cloned plants at profitable cost is needed and, therefore, protocols for culturing embryogenic tissues in liquid medium are required. The induction of SE in mature cork oak trees and the establishment of embryogenic suspension cultures has been achieved. In this study the effect of density of inoculation and shaking of two size fractionated inoculums on proliferation and somatic embryo differentiation was evaluated. These fractions represented two gross developmental stages: the smallest one was comprised of isolated cells and unstructured aggregates (PEMs) and the largest one contained mainly structured embryogenic clumps. Suspension cultures were initiated from embryogenic cultures of ALM80 and TRG3 lines, which were maintained on semi-solid SH medium without plant growth regulators (PGRs) for more than 4 years retaining embryogenic ability, in the same liquid medium. Two fractions were collected, 41-180 µm (S) and 180-800 µm (L) from this suspension. The S-fraction was inoculated into 250 ml baffled Erlenmeyer flasks with 50 ml of fresh medium at 0.5 and 1 gl^{-1}, while the L-fraction was inoculated at 0.5, 1, 2, 4 and 6 gl^{-1}. All these suspension cultures were maintained static or agitated on an orbital shaker at 110 rpm, in a growth chamber at 25 ±2 ºC under a 16 h light photoperiod (180 $\mu E\ m^{-2}\ s^{-1}$) for 30 days. Growth was determined by fresh weight quantification of fractions of 41-180, 180-800 and larger than 800 µm, and the total biomass. Cultures were qualitatively assessed by photographic patterns and a scale to score different features: necrosis, proportion of structured *vs* unstructured aggregates, morphology of structures, and presence of cotyledonary embryos. Regardless of genotype, the size of inoculum had a dramatic effect on growth. When the S-fraction was inoculated at 0.5 gl^{-1} the initial weight multiplied by 20 when it was shaken and reduced to half when the culture remained static. However, the L-fraction inoculated at the same density showed a 70-fold increase when the culture was agitated and a 24-fold increase occurred when it maintained static. For the L-fraction a clear inverse relationship was observed between density of inoculation and multiplication rate, both in shaking culture and static. The structures contained in the S-fraction showed a limited development. Few structures larger than 800 µm were observed, slightly more when the lower density of inoculation was used. The L-fraction mainly produced structured and compact embryogenic clumps, and some polar forms were detected. Genotype, density of inoculation and shaking showed some minor differences in qualitative characteristics. Genotype ALM80 exhibited a higher frequency of structures with confined necrosis, and lower ability to disaggregate than genotype TRG3. Cotyledonary embryos were not observed in any treatment.

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Effect of shaking on growth, morphology and maturation ability of embryogenic suspension cultures of *Pinus pinea* L.

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Plant regeneration by somatic embryogenesis in Stone pine (*Pinus pinea* L.) has the potential to enable mass propagation of the best performing genotypes for pine nut production or suitable rootstocks in agroforest plantations. The large scale production of somatic embryos requires the establishment of an efficient system for growth and plant formation in liquid culture medium. Liquid culture conditions may support growth of cell clusters and early stage embryos at several stages of development. Patterns of the number and sizes of clusters in the suspension cultures could be associated with the prospective development of somatic embryos. Several physical factors of the culture that affect oxygen transfer and hydrodynamic stress may influence these developmental patterns. This study reports on the orbiting speed effects on somatic embryo development, which were evaluated by growth parameters in a liquid culture system as well as qualitative differences among cultures. Relation between the morphology of suspensions and their maturation ability was also examined. Suspension cultures were initiated from proliferating embryogenic tissue of Stone pine lines 2F47, 1F11 and 7F11, by transferring 500 mg of embryonal-suspensor masses (ESM) to 200 ml Erlenmeyer flasks containing 25 ml of M-mLV liquid medium. They were cultured on an orbital shaker at 50, 100 and 150 rpm in the dark at 23 ± 1ºC, for three weeks. The growth of embryogenic lines was determined by measuring the settled cell volume (SCV), and the fresh (FW) and dry weight (DW) after filtration. Qualitative characteristics of embryogenic tissue were determined by microscope examination of samples of 1 ml of suspension placed on Petri dishes. Growth parameters were significantly affected by the orbiting speed. The highest SCV rate was obtained at the lower speed. However higher orbiting speeds decreased biomass production (FW and DW). Significant effects of line and their interaction with orbiting speed for growth parameters were found. Line 2F47 showed the maximum increase of SCV; however line 1F11 produced higher FW. No significant differences among embryogenic lines were recorded for DW. Cultures at 50 rpm most commonly grew as an accumulation of clusters of embryonal cell masses, whereas at 150 rpm they showed a higher frequency of freely suspended cell structures. Moreover, the larger clumps were disaggregated into smaller ones at 100 rpm. Somatic embryo proliferation of line 1F11 was characterized by more organized embryonal-suspensor structures. This organization was reduced at the highest orbiting speed, showing less organized bipolar structures from suspension culture with a higher number of visible elongated single cells and proembryonal heads. Cotyledonary mature somatic embryos were recovered from the three embryogenic lines on ABA-containing semi-solid medium. Production of somatic embryos depended on the orbiting speed. When 100 rpm was applied, 54 mature embryos were obtained per gram of FW from all embryogenic lines, while 34 mature embryos were obtained under 50 rpm. Lines 2F47, 7F11 and 1F11 produced 78, 41 and 6 mature embryos per gram of FW, respectively.

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