The Third International Conference of the IUFRO unit 2.09.02:
Somatic Embryogenesis and Other Vegetative Propagation Technologies

Proceedings

Woody Plant Production Integrating Genetic and Vegetative Propagation Technologies

September 8-12, 2014
Vitoria-Gasteiz, Spain

Proceedings

of the IUFRO Unit 2.09.02 Conference on

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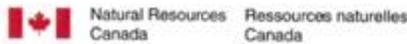Propagation Technologies"

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Preface

Kaixo !

We have had another successful international conference, this time in the beautiful city of Vitoria-Gasteiz, Spain, in the heart of Basque country. The "Vitoria 2014" conference was co-hosted by NeikerTecnalia, a prestigious institution for Agricultural Research and Development operated by the Basque Government and by the Korea Forest Research Institute (KFRI) in the Ministry of Forestry of the Republic of Korea. We are so fortunate and grateful that we have such strong supporters of our work. Thank you, Mr. Josu Ezcurdia, Director of Neiker Tecnalia and Dr. Young-Kyoon Yoon, former Director General of KFRI.

The Vitoria 2014 conference was attended by 120 participants from around the world and was organized into four thematic areas: Application of biotechnologies in tree breeding and deployment; Physiological genetics and epi-genetics of somatic embryogenesis (SE) and other vegetative propagation techniques (VP); Development and application of complementary technologies based on SE and VP; and Development of SE and VP technologies and their scale-up applications. A total of 46 oral presentations (15 invited and 31 voluntary) were made. Equally important, 49 posters were presented. These scientific sessions provided ample opportunities to discuss the newest developments and to network with one another.

The local committee (NEIKER -Tecnalia) executed flawless scientific sessions at historic "Palacio de Villa Suso" and arranged a fantastic social program and field trips: Welcome Reception, Rioja winery visit and dinner, Museum visit and Gala dinner, Old city visit and Pintxo-Pote, and Urdaibai Biosphere visit and farewell dinner. Thank you, Paloma, Iranzu, Itziar, Olatz, Yolanda, Alex and Pablo !

To promote the future scientific endeavors of this group, we held the first "Student Scientific Award" competition. Alexandre Morel (France) received the award, and the runners-up were Azahara Barra Jiménez (Spain), Johanna Carlsson (Sweden), Biljana Đorđević (Czech Republic), and Juliane Raschke (Germany). Also, Drs. Krystyna Klimaszewska, Heung-Kyu Moon, and David Thompson were recognized for their contributions to SE and VP. Dr. Yill-Sung Park, founder and first coordinator of this IUFRO unit, was recognized by all his colleagues for his scientific career and enthusiastic promotion of the working group. In anticipation of Yill-Sung's retirement, Jean-François Trontin assumed the responsibility of the coordinator for this IUFRO unit. Finally, the unit was proud to recognize the contribution of André Franclet† to VP and rejuvenation of trees.

These proceedings contain the papers and abstracts that were submitted. Abstracts that were not submitted for editing are found in their original format in the "Book of Abstracts" published during the conference (and in the usb drive). These are also available from the IUFRO website: http://www.iufro.org/science/divisions/division-2/20000/20900/20902/publications/

Fredericton, Canada
Yill-Sung Park and Jan Bonga

Contents

Full Article

Extended Abstracts

Short Abstracts

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Photo Gallery

Participants

Proceedings of the Third International Conference of the IUFRO unit 2.09.02 on
"Woody Plant Production Integrating Genetic and Vegetative Propagation Technologies"
September 8-12, 2014 ▪ Vitoria-Gasteiz, Spain

IUFRO

Obituary:
André Franclet (1929-2014)

Awarded in 1984 (gold medal) by the Agricultural Academy of France for his significant contribution to vegetative multiplication of woody plants

An overview of André Franclet's career and scientific endeavors in vegetative propagation and rejuvenation of forest trees

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Keywords: tree breeding, clonal forestry, serial grafting and cutting, micropropagation, micrografting, nursery management.

The initial, academic period (1950-1953)

Born in October 17th, 1929 at Le Chesne (Ardennes, France), André Franclet was very early interested in agronomy, forestry and geology. Accordingly, he successfully applied in 1950 to the French National Agronomical Institute (INA, Paris, France) and also to the National High School for Geology and Mining Prospecting (ENSGPM, Nancy, France). He brilliantly graduated as an INA Engineer in 1952. During this training period he already contracted with the Forest Research Service of the Water & Forest Administration in Morocco in 1953 and specialized in Genetics at both INA and the University of Paris (High Study Certificate). His career started in 1953 as a young research scientist (trainee) at the Forest Genetics Laboratory, National School of Water Resources and Forestry (ENEF, Nancy, France).

The Moroccan period (1954-1962): first achievements in vegetative propagation of eucalypts

After his military service for 12 months (Feb. 1953 to Feb. 1954) as Second-Lieutenant of Artillery (Châlons, France), André came back to forest science at the Laboratory of Genetics of the Forest Research Station located in Sidi Amira, Morocco (Nov. 1954 to June 1957). He contributed to the setting up of the lab and was involved in tree breeding and selection for various species of eucalypts, poplars and argans. He pioneered vegetative propagation of *Eucalyptus* spp. and authored a first paper in 1956 *(1)*. Concomitantly, he developed multiple projects in forest ecology and pedology. Unfortunately, the forest research developments initiated by André in Morocco were interrupted in July 1955 by the Algerian war (1954-1962). During this confused period he contributed to public order maintenance in Morocco (Middle Atlas region) especially during the 2nd Rif war (1955). After he returned to his original army corps (Aerial Group for Artillery Observation), he was involved until 1957 in aircraft support at advanced forest observation posts near Marrakech (Aguedal station).

In June 1957 and in accordance with his high initial interest in applied geology, André decided to apply for a job as Research Engineer at the Research Bureau and Mining Participation group (BRPM, Morocco) where he further developed high skills in geochemistry (1958). He was initially involved in developing techniques for measurement of micronutrients content in plants and soil, especially towards accurate detection of valuable minerals and hydrocarbons. These methods were subsequently largely transferred to various labs with expertise in mineral analysis. At this time he became assistant manager of the BRPM laboratories established in Morocco with both

administrative and technical responsibilities. He was in charge of the laboratory dedicated to mineral treatment and enrichment and metal geochemistry. Until 1962 he was organizing a service for geochemistry-based petroleum prospecting in Morocco involving different technical sections for analyzing gasses (by gaseous phase chromatography of extracted gas from soil), bitumology and microbiology. He also contributed to mineral analytic chemistry and developed various colorimetric methods for detection of metals.

The Tunisian experience (1962-1972): towards implementation of clonal propagation in eucalypts commercial forestry and early interest in vegetative propagation of conifers

In 1962, André pursued forest genetics activities in the North Africa context as Director of the French Assistance Mission to the Tunisian forest research (SCET) established at the Institute for Reforestation in Tunis, Tunisia. He contributed to a large collection campaign (5 months) in Australia of *Eucalyptus camaldulensis* provenances (including Lake Albacutya) as well as of several other plant species with putative interest in North Africa. During 7 years (1965 to 1972), he continued to develop the eucalyptus research initiated in Morocco for the FAO under the supervision of Jacques Marion, especially vegetative propagation of *E. camaldulensis (2)*, *(3)*. He obtained the first rooted cuttings for this species but the Tunisian afforestation program was stopped following the appearance of *Phoracantha semipunctata* (eucalyptus longhorned borer) at Cap Bon in 1962. The rooting techniques of young eucalypt cuttings initially developed by André were later used by Bernard Martin (after visiting the Institute for Reforestation in 1970) for other *Eucalyptus* species including 12 ABL and PF1 adult interspecific hybrids and this resulted in a plantation of ca. 50 000 ha around Pointe Noire (Congo Republic). Later (1975) similar techniques were implemented in commercial forests (hundred thousand ha) in Brazil by Yara K. Ikemori and Edgard Campinhos (Aracruz) who received the Veiletzer (Marcus Wallenberg) Prize in 1984.

Concomitantly to eucalyptus breeding André was already interested in vegetative multiplication of conifers *(4)*, plant production and intensive nursery management *(5)*, *(6)*, *(7)*, and *Cupressus* spp. grafting *(8)*, *(9)*. He was also involved in various large plant inventories *(10)*, *(11)*.

The AFOCEL period (1972-1989): from classical breeding to rejuvenation and biotechnology inputs in various hardwoods and conifers

André returned to France in 1972 and following a short experience at INRA (National Agronomical Research Institute, France), he joined AFOCEL (Forest-Cellulose Association, Nangis, France). He was mandated by Georges Touzet (General Director of AFOCEL until 1987) to create the Forest Physiology Laboratory in 1973 that became later (1980) the Biotechnology Lab. André managed this lab for 11 years (1972-1983). At the end of his career at AFOCEL until he retired (1983-1989), André worked as scientific adviser in tree breeding and deployment of new varieties to the Director of Research at AFOCEL (D.-X. Destremau 1975-1988, and then C. Hubert) and to consecutive Biotechnology lab managers (M. Boulay 1983-1986, J. Bercetche 1986-1988, M. Pâques 1988-2002).

André initiated or reactivated most AFOCEL selection programs for both conventional breeding and clonal propagation of eucalypts *(12)*, Douglas-fir *(13)*, *(14)*, Maritime pine *(15)*, hybrid walnut *(16)*, *(17)*, *(18)*, *(19)*, Poplar *(20)*, *Sequoia sempervirens (21,* Fig. 1*)*, *Sequoiadendron giganteum (22)*, *Calocedrus decurrens (22)*, *(23)*, wild cherry *(24)*, Norway spruce *(25)*, and *Platanus acerifolia (21)*. As many other researchers in the world, André was particularly encouraged to invest time in developing cutting technologies in conifers, following pioneering achievements by Henri Chaperon in Congo, from young *Pinus caribaea* trees (results presented in Canberra, Australia, FAO consultation 1977).

Concomitantly, he was actively involved in most AFOCEL large collection campaigns of genetic resources from the natural area of *S. sempervirens* and *S. giganteum* (California, USA), Douglas-fir, *Picea sitchensis*, *C. decurrens*, and *Pinus contorta* (Oregon/Washington, USA), *Juglans* spp. (Iowa & Michigan, USA) and *Pinus sylvestris* (Riga pine, Estonia). He also performed various consulting missions on genetics, micropropagation and nurseries techniques in Brazil. Following these campaigns, multiple provenance and clonal tests as well as gene pools were field-established by AFOCEL in the 80s. André contributed in particular to the first international field evaluation of range-wide provenance tests of *S. sempervirens* (180 clones, 90 provenances from the whole natural area) initiated by J. Kuser (Rutgers University, USA) in 1983 and planted at 2 sites in France *(26)*, one site being the AFOCEL station in Nangis (Fig. 1C). He also established a walnut germplasm composed of 90 families sampled in progenies tests established by USFS (Iowa & Michigan) following collection campaigns from Texas to Dakota and from California to Québec.

André did pioneering work in the field of rejuvenation of mature, selected trees through conventional vegetative propagation methods (serial grafting and cuttings) *(27)*, *(28)*, (Fig. 1A, B, D), and/or tissue culture techniques *(29)*, *(30)*, *(31)*. He communicated at the international level on the rejuvenation theory and practical experience in clonal propagation *(32)*, reiteration and vegetative propagation *(33)*, biotechnology and the genetic

Figure 1. *Sequoia sempervirens, a case study species during the career of André Franclet.* **A.** *André in his garden showing a young rejuvenated S. sempervirens. Photo courtesy of R. Franclet (May 2008).* **B.** *André in a recently established field test of clonally propagated S. sempervirens, in Yunnan, China. Photo courtesy of R. Franclet (April 2000).* **C.** *A view of the "J. Kuser" provenance test planted at the AFOCEL/FCBA station in Nangis, France. André contributed to both establishment and evaluation of this test (180 clones from 90 provenances). Photo credit: FCBA (April 2010).* **D.** *André next to an impressive rejuvenated cutting of S. sempervirens (clone Arc154) in his garden and in company of his faithful doggy Ulysse. Photo courtesy of O. Monteuuis (December 2005).*

improvement of trees *(34)*, or biotechnology and rejuvenation, especially micropropagation of difficult woody species *(35)*. He contributed to a reference book on tissue culture and forestry edited in 1987 by Jan Bonga (Canadian Forest Service, Canada) and Don Durzan (University of California, USA) by co-authoring a significant paper on tree rejuvenation *(36)*.

At the apogee of his 40-year-long professional career (1950-1989), André contributed to the development of tissue culture methods for vegetative propagation and rejuvenation of several major tree species including redwood, giant sequoia, eucalypts, Douglas-fir and maritime pine. He proposed *S. sempervirens* as a model species to provide adequate material for progressing in the understanding of the concept of rejuvenation *(37)*. He was interested in micropropagation *(38)*, *(39)*, and evaluation tools of rejuvenation in this species at both morphological and biochemical levels *(40)*, *(41)*, *(42)*. He had also a high interest in *S. giganteum* as a valuable forest genetic resource for timber production in France and was involved in the refinement of micropropagation methods from juvenile and mature clones *(43)*.

He developed the vegetative propagation of Eucalyptus *(12)*, especially the micropropagation of frost-tolerant eucalypt clones of *E. gunnii* and *E. gunnii x dalrympleana (44)*, *(45)*, including *in vitro* mycorrhizal symbiosis attempts *(46)*. *In vitro* rejuvenation was considered by André as a prerequisite for efficient clonal forestry applied to eucalypts and to other short-rotation forest species *(47)*, *(48)*. Micropropagation of cold-hardy eucalypts has been commercially implemented in France by FCBA (formerly AFOCEL) since the late 90s to produce stoolbeds for conventional cuttings production. André was also concerned about pests and diseases that could affect productivity in multivarietal forestry *(47)*.

Because of various morphological problems, detected after vegetative propagation of Douglas-fir through rooted cuttings *(49)*, André initiated research for developing *in vitro* propagation techniques. He contributed to the development of micropropagation methods based on axillary budding from juvenile bud explants (*14*) and meristem culture from both juvenile and mature trees *(50)*, *(51)*, *(52)*.

In maritime pine (*Pinus pinaster*), he reported on the first morphological evidence of rejuvenation of mature clones from the shoot apical meristem *(53)* and contributed to the development of meristem micrografting onto young seedlings cultivated *in vitro (54)*. This work provided an efficient research tool to study the rejuvenation process in this species as juvenile characteristics can be partially restored. The method has been refined during the last decade and proved to be useful at FCBA for various research applications in the field of rejuvenation.

As reported previously, André was convinced very early that suitable management of both *in vitro* and nursery techniques is critical to produce high-quality plants for forest plantations *(5)*, *(6)*, *(7)*. In 1981 he reported about a system for improved culture of propagules in greenhouse and nurseries: the Melfert plugs *(55)*. This technology (the "cultivation balls") was then patented in both France (1981) and USA (1983) by Franclet and Favereau *(56)*.

The "retirement" period (1989-2014): still an indelible interest in propagation of trees, conservation of genetic resources and management of forests

André retired from AFOCEL in 1989 but remained very active in the field of clonal propagation of trees. He contributed until 1995 to various papers and reviews on tree biotechnology and rejuvenation *(35)*, rejuvenation *(41)* and field-testing in *S. sempervirens (26)*. He set up a home-made biotechnology laboratory at his personal "retirement" address dedicated to research in agroforestry (the Institute of Agronomical & Forest Research) and to the refinement of efficient micropropagation as well as cutting production methods. He was also still collecting and archiving seeds and other tree propagules from various areas worldwide (Yunnan in China, Argentina, USA) from remarkable trees (Fig. 2).

Figure 2. André Franclet in his garden next to a Bristlecone pine seedling (Pinus aristata/longaeva) obtained from one of the oldest living trees on earth (Methuselah clone, tree ortet on the upper left, more than 4800 years old) located in the White Mountains, California, USA. Photo courtesy of R. Franclet (seedlings: September 2004; ortet: December 2008).

André also practiced "tree farming" on his private land based on plantation of experimental plots (e.g. poplar, walnut, sequoia) and sheep breeding. Strikingly André wrote in 2007 (April 7th, translated quote) that "*finally I tried to spend the last years of my life at establishing genetic resources that my successors should be happy to find for mitigating the effects of global warming*". André's field archives are now entering their adult reproductive phase and they received much interest in recent years (e.g. the "Kuser" collection of *S. sempervirens*) as part of the large FCBA testing network (about 1000 ha).

André was often visiting the AFOCEL/FCBA Biotechnology lab during the past decade until the lab moved to Bordeaux-Pierroton, France in 2011. He was still highly interested in the new developments of clonal propagation and in keeping a look at the field plots of redwoods and rejuvenated giant sequoia. He always encouraged the AFOCEL/FCBA team to further develop the vegetative propagation of selected, mature trees and was particularly interested by the new rejuvenation and clonal propagation issues offered by somatic embryogenesis, especially in conifers (at this time AFOCEL/FCBA was developing somatic embryogenesis in Norway spruce and maritime pine).

Recently (2013) André was still planning to contribute to an inventory and study of both native resources and clonal field archives of *Cupressus dupreziana*, one of the most endangered tree species worldwide (only 231 trees remaining at natural spots in 2001). This conifer species is known to have developed a male apomixic reproductive system requiring the closely related *C. sempervirens* as "surrogate mother". André initiated and contributed during epic dromedary rides (1972-1973) to sampling of thousand-year-old trees discovered in 1925 in the Tassili region of Tunisia (Sahara). Thirty-two of these trees have been established in clonal field archives by AFOCEL in the 80s (now part of the FCBA testing network) based on an efficient grafting method of *C. dupreziana* on *C. sempervirens* developed by André more than 45 years ago *(8)*. André suggested to urgently rescue all survival trees from the Tassili region and to study the extent of the "surrogate mother" phenomenon.

André passed away on Monday 3rd February 2014. Only a few days earlier he was still taking care of his (cloned/rejuvenated) trees and was very busy with sheep lambing in his farm. He was buried in the family vault in Soorts Hossegor, the heart of the famous Landes forest (Aquitania, France).

Researchers and other forest actors who met him always depicted André as a very dynamic and enthusiast scientist with impassioned appeals for planted forest development, rescue and valorization of forest resources worldwide. He had real and concrete experience and indelible interest in trees and forests during his remarkable career and more generally during his entire life.

Our IUFRO unit is very proud to recognize the pioneering work and great impact of more than 40 years of research in tree genetics and vegetative propagation by André Franclet (Fig. 3).

Figure 3. The homage plaque presented to André Franclet from the IUFRO unit 2.09.02 during the Third International Conference in Vitoria-Gasteiz, Spain, September 2014 recognizing his Scientific Endeavors to Vegetative Propagation and Rejuvenation of Forest trees.

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"André Franclet will no more walk in his favorites woods, will no longer have a thousand ideas to develop, but the trees he planted still will talk about him." André Franclet's Family (Donnemarie-Dontilly, France), February 4th 2014

"I admired his extremely creative mind, always embedded in concrete. André was a very genuine and hands-on person which did not burden himself with principles to solve problems. André was my mentor and I became his friend." Bernard Martin (Dommartemont, France), February 6th 2014

"W*hat struck me was the great passion I saw in that man, a classical forester, a person very close to the field work and with a great power of observation and interpretation of natural phenomena. Certainly he was one of the pioneers in the field of vegetative propagation and rejuvenation of forest species.*" Mariano Toribio (IMIDRA, Spain), February 9th 2014

"André was so dynamic even towards the end, and so much into the current time ... More operational and concrete than mystic… He was a passionate man driven by a strong motivation, always entirely into it." Olivier Monteuuis (CIRAD, France), February 21th 2014

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Full Article

Formation of embryogenic-like tissues from mature zygotic embryos of stone pine

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Abstract

The stone pine (*Pinus pinea*) is an economically important forest species of the Mediterranean region, considered as a "fruit tree" for edible pine-kernel production. Besides its agronomical value, it is also used for ecological (forestation of coastal and continental dunes) and ornamental purposes. Spain's breeding program is mainly focused on improving seed yield in grafted orchards. Therefore, the production of clonal rootstocks is desired. The induction of somatic embryogenesis (SE) in several *Pinus* species has been investigated dealing with such factors as the choice of the initial explant, basal medium components, growth regulator additions and environmental conditions. The main limitations when immature zygotic embryos are used as explants are the low initiation frequency, the restriction to a short time window for initiation, the impossibility of long-term storage of immature seeds, and the genetic specificity of explants. Some of these problems can be solved and the availability of suitable explants can be increased if mature zygotic embryos (ZE) are used for inducing SE. Embryogenic tissue was obtained from mature ZE in some *Pinus* spp but at too low a frequency for large-scale production. Although the induction of embryogenic lines from immature zygotic embryos of stone pine has previously been reported, this is the first study on induction of SE from mature zygotic embryos. The effects of family, culture medium and collection data on the cell mass induction and proliferation were investigated. Cones from selected trees were collected in two clonal banks during a three year period. Several developmental stages of ZE enclosed in the megagametophytes were observed at the same collection date, that consisted of the dominant (cotyledonary) and subordinate embryos (both cotyledonary and aborted). Isolated mature ZE were cultured on several nutrient medium supplemented with different PGRs (NAA, 2,4-D, BA, TDZ, Brassinolide) in darkness. Responsive tissues were produced by both cotyledons and hypocotyls of the ZE, and also in remains of the suspensor cells. The best response was obtained on medium with both TDZ and Brassinolide. Although induction of embryogenic-like cultures from mature ZE was obtained, the cell masses failed to grow further by continuous subcultures and the proembryogenic structures could not be maintained. These results provide the first information on embryogenic potential of mature embryos and may be useful for the optimization of the somatic embryogenesis protocol in *Pinus pinea*.

Keywords: Agroforestry, embryogenic cultures, forest biotechnology, *Pinus pinea*, somatic embryogenesis.

Introduction

Stone pine (*Pinus pinea* L.) is one of the economically important forest species in the Mediterranean region; it is considered as a "fruit tree" for edible pine-kernel production. Besides this agronomical use, it is also used for ecological (forestation of coast and continental dunes) and ornamental purposes. Several studies reported that stone pine shows a low genetic diversity but broad geographic distribution and a high level of phenotypic plasticity (Mutke et al. 2005, Vendramin et al. 2008). Since there is large variability in seed production, the Spanish breeding program

is mainly focused on improving seed yield by planting selected individuals in grafted orchards (Mutke et al. 2000). Clonal propagation by somatic embryogenesis (SE) is currently applicable to coniferous species, and has become a powerful biotechnological tool for application in tree improvement programs (Merkle and Dean 2000, Park 2002, Zoglauer et al. 2003). The implementation of tested high-value tree varieties in plantations offers a new paradigm in tree breeding and deployment that is more flexible than the current seed orchard system (Park 2014). SE is widely available in many coniferous species, although there are still varying degrees of difficulty in obtaining SE. However, SE is sufficiently refined to the point that it can be implemented in the industrial production of several economically important conifers (Park 2014). Recent reviews have covered improvements in plant regeneration by SE from zygotic embryos (ZE) in *Pinus* species (Stasolla 2003, Zoglauer et al. 2003, Klimaszewska et al. 2007) and the impact of this biotechnology in plantation forestry (Park 2002, Sutton 2002, Nehra et al. 2005).

Improving induction rates has been a major area of SE research; it is influenced by several factors, such as basal medium components, growth regulator additions and exposure time, environmental conditions of cultures, stage of maturity of ZE, and genetic influence (Tautorus et al. 1991, Gupta and Grob 1995, Klimaszewska and Cyr 2002, Park 2002, von Arnold et al. 2002). As described by Filonova et al. (2000), SE is a multi-step regeneration process starting with proembryogenic masses (PEM) that progress to the next stages maintaining the bipolar pattern. Auxins and cytokinins are necessary to maintain PEM proliferation, whereas embryo formation is triggered by the withdrawal of PGRs. The further development of early somatic embryos to mature ones requires abscisic acid. It has been well established that somatic embryogenesis initiation is under the control of strong genetic additive effects (Park et al 1993). The strong interaction between families and procedures clearly indicates that different culture media may be better suited for different genotypes (Mackay et al. 2006). A major bottleneck in initiating embryogenic cultures of *Pinus* species has been the identification of the correct developmental stage of the zygotic embryo ("window") for SE induction. The careful monitoring of the ZE developmental stage may result in higher initiation rates (Park 2002). However, the availability of immature ZEs at the most responsive stage is often limited. To extend the availability of these ZEs, cones may be stored in a refrigerator, although it can be expected that the percentage of initiation declines as the length of the cold storage period increases. However, in some cases, refrigeration even stimulated SE induction (Park 2002).

Modification and refinement of initiation media continues to be an important aspect of SE research, because efficient SE protocols are not yet available for many semi-recalcitrant and recalcitrant conifer species. Manipulations of culture media formulation such as salt concentration, organic and inorganic nitrogen level, type of plant growth regulators and their levels, and application of the more recently discovered growth regulators such as brassinosteroids can be used to overcome recalcitrance (Bonga et al. 2010, Park et al. 2006, Pullman and Bucalo 2011)

An important issue when using megagametophytes enclosing immature ZE as initial explants is that multiple archegonia may be fertilized by different pollen resulting in multiple, genetically different zygotes. Cleavage polyembryony also occurs in *Pinus* species, where the embryos from each fertilization event cleave into four genetically identical embryos (MacKay et al. 2001). Clearly, additional work is needed to determine the effect that initiating from polyembryonic immature explants has on the SE process in *Pinus* species (Bozhkov et al. 1997). Park (2002) indicated that the genetic integrity of clonal lines developed by somatic embryogenesis in pines needs attention, because there is a possibility to obtain embryogenic tissue with mixed genotypes.

Initiation of SE from single mature ZE can solve this problem giving pure clone lines. The use of mature ZE is also more advantageous because it allows independence from the restricted competence window and mature seeds can be cold stored, increasing the availability of starting plant material. SE has been induced in mature ZE of a few conifers, including some species of the genus *Pinus*. Although not completely mature (early cotyledonary) embryos are more responsive than their full mature counterparts, it has been indicated that embryogenic tissue formation in mature embryos could be initiated from the upper hypocotyls (Elhiti and Stasolla 2011).

Plant regeneration by SE from immature ZE was achieved in stone pine (Carneros et al. 2009), but initiation of SE was at very low frequency, with an overall mean around 0.5%. In spite of the low genetic variability that has been reported for this species, variability in initiation frequencies was recorded for several open-pollinated families of stone pine, as in other conifers (Carneros et al. 2009). This research deals with the study of conditions for the initiation and maintenance of embryogenic cultures from mature ZE of stone pine. The effects of seed family, developmental stage of ZE enclosed in the megagametophyte and culture medium on the capacity to undergo SE were investigated. The initiation of embryogenic-like cultures in mature zygotic embryos from selected trees is reported.

Materials and Methods

Plant material

Cultures were initiated in mature ZE from open-pollinated seed families, in three separate experiments. In the experiment started in 2003, cones were collected September 10th from several ramets of five selected clones (F6039, F6047, F6059, F6061 and F6062) in a stone pine clonal bank established at the National Forest Breeding Centre "Puerta de Hierro" (Madrid, Spanish Ministry of Environment). Cones were cold-stored at 4ºC in plastic bags for 7 months until the time of dissection. In the experiment started in 2004, cones were collected August 25th from several ramets of seven selected clones (F6010, F6011, F6047, F6070, F6075, F6087 and F6088) in the same clonal bank and cold-stored at 4ºC in plastic bags for 7 months until the time of dissection. In the experiment started in 2012, cones were collected October 27th from several ramets of seven selected clones (F6011, F6015, F6047, F6053, F6054, F6070 and F6088) in a stone pine clonal bank established at the National Forest Breeding Centre "El Serranillo" (Guadalajara, Spanish Ministry of Environment). Cones were cold-stored at 4ºC in plastic bags for 4 months until the time of dissection.

Before cold storage, whole cones were thoroughly washed by immersion in 70% (v/v) ethanol for 3 min and then treated with a fungicide solution (0.1% captan and 0.1% benomyl). After storage, seeds were removed and surface disinfected by shaking in 75% (v/v) ethanol for 2 min, followed by shaking in a solution of 10% (v/v) commercial bleach (4% active chlorine) plus two drops of Tween 20 for 10 min, followed by three rinses in sterile distilled water in the laminar flow bench. ZE removed from the megagametophyte were used as explants. Their developmental stage was recorded using a stereomicroscope.

Initiation of SE

In the experiment carried out in 2003, two initiation media were tested. The culture medium labelled as NAA-BA-LP consisted of LP nutrient medium (Lepoivre modified by Aitken-Christie, 1988) with half-strength macroelements and supplemented with 10 μM Naphtalenacetic acid (NAA) and 4 μM Benzyladenine (BA). Sucrose was added at 30 gl^{-1}. The culture medium labelled as TDZ-PJ consisted of 505 nutrient medium (Pullman and Johnson, 2002) which is a modified P6 medium (Teasdale et al. 1986) supplemented with 18 μM Thidiazuron (TDZ). Maltose at 15 gl^{-1}, 0.05 gl^{-1} activated charcoal (AC) and 0.5 gl^{-1} casein hydrolysate were added. The medium was solidified with 2.5 gl^{-1} Gelrite®. Overall, a minimum of 390 explants were cultured (five half-sib families x two culture media x 3 ZE per plate x 13 plates per treatment). In the experiment carried out in 2004, two different initiation media were tested. The culture medium labeled as NAA-BA-PJ consisted of the already mentioned 505 medium supplemented with 10 μM NAA acid and 4 μM BA, 15 gl^{-1} maltose, 0.05 gl^{-1} AC and 0.5 gl^{-1} casein hydrolysate. The medium was solidified with 2.5 gl^{-1} Gelrite®. The second medium labelled as 2,4-D-BA-mLV$_2$, consisted of a modified Litvay's nutrient medium (Litvay et al. 1985) with half-strength macroelements, and full-strength Fe-EDTA and vitamins. Microelements were modified as follow: boric acid and manganese and zinc salts were at full strength; molybdenum, copper, and iodine salts were at half strength; cobalt salt was reduced to 0.01 mgl^{-1}, and 0.72 mgl^{-1} NiCl$_2$ was added (Park et al. 2006). This medium included 2.4 μM 2,4-dichlorophenoxyacetic (2,4-D), 2.2 μM BA, 10 gl^{-1} sucrose and 1gl^{-1} casein hydrolysate. The medium was solidified with 4 gl^{-1} Gelrite®. Overall, 252 explants were cultured (seven half-sib families x two culture media x 3 ZE per plate x 6 plates per treatment). In both experiments, three embryos were placed per Petri dish (60x15 mm) with 10 ml of medium and sealed with Parafilm® and then incubated in darkness at 23 ± 1ºC. The explants were subcultured every two weeks during initiation. In the experiment carried out in 2012, four initiation media were tested. The basal medium was the above described as mLV$_2$, and four combinations of growth regulators were tested. The medium labelled as M-mLV$_2$ included 9 μM 2,4-D and 4.5 μM BA. The medium labelled as B-M-mLV$_2$ included M-mLV$_2$ medium plus 0.1μM brassinolide (24-epibrassinolide, Sigma E1641). The medium labelled as HB-M-mLV$_2$ included M-mLV$_2$ medium plus 10 μM brassinolide. The medium labelled as TDZ-mLV$_2$ included mLV$_2$ medium plus 18 μM TDZ. 10 gl^{-1} sucrose and 1 gl^{-1} casein hydrolysate were added. The media were solidified with 4 gl^{-1} Gelrite®. Overall, a minimum of 1120 explants were cultured (seven half-sib families x four culture media x 5 ZE per plate x 8 plates per treatment). Five embryos were placed per Petri dish (90x16 mm) with 25 ml of medium, sealed with Parafilm® and cultured in darkness at 23±1ºC. The explants were subcultured every three weeks during initiation. All media were supplemented with a filter-sterilized solution of 0.5 gl^{-1} L-glutamine added to the cooled medium. The pH was adjusted to 5.8.

The presence of embryonal masses was macroscopically recorded and slide samples were microscopically examined after staining with 1% (w/v) acetocarmine.

Maintenance of cell lines

In the experiments carried out in 2003 and 2004, proliferating cell masses were separated from the explants after 4-8 weeks of culture on initiation media. Then, they were transferred onto fresh medium of the same composition as the one used for the initiation, but with the PGR concentration reduced to one tenth. The proliferating cell masses were subcultured at four-week intervals in darkness at $23\pm1°C$. In the experiment carried out in 2012, the proliferating cell masses were separated from the explants after 3-4 weeks of culture on initiation media. Then, they were transferred onto the maintenance medium described as M-mLV (Carneros et al. 2009) and subcultured at three-week intervals. This medium consisted of a modified Litvay's nutrient medium (Litvay et al. 1985) with half-strength macroelements, and full-strength microelements, Fe-EDTA and vitamins, and supplemented with 9 μM 2,4-D and 4.5μM BA. All media were supplemented with $1gl^{-1}$ casein hydrolysate, 0.5 gl^{-1} L-glutamine, 20 gl^{-1} sucrose, and solidified with 4 gl^{-1} Gelrite®.

Frequency data were analysed through contingency tables and the Fisher's exact test (GraphPad Software, http://graphpad.com/quickcalcs/contingency1.cfm).

Results

Variations in the developmental stage of ZE enclosed in megagametophytes were observed among the collection dates. Mature embryos collected in September-2003 (Fig. 1A) were slightly more advanced in development than those collected in August-2004 (Fig. 2A). At both dates, completely developed dominant embryos with defined cotyledons were observed but most embryos of August-2004 showed remaining suspensor cells. In the seeds of the later collection date, 8 % of the 333 megagametophytes enclosed cotyledonary embryos that maintained subordinated embryos (polyembryony), ranging among families from 0 to 22 % (Table 1). In the October-2012 collection, megagametophytes enclosed ZE at several developmental stages (Fig. 3A-D). For all families, about 86% of the 969 megagametophytes enclosed one cotyledonary embryo, and some of them showed remaining suspensor cells (Fig. 3A). Polyembryony was observed in 14% of the seeds, ranging among families from 7 to 24% (Table 2). This whole frequency of polyembryony could be partitioned into three main classes. Around 5% of the megagametophytes had a dominant cotyledonary embryo and one subsidiary cotyledonary embryo (Fig. 3B), with frequencies among families ranging from 1 to 10%. Megagametophytes enclosing a dominant cotyledonary embryo joined to other embryos at different development states were also observed, representing about 7% of the samples. Differences among families ranged from 1 to 14%. Megagametophytes enclosing numerous aborted embryos were also detected (Fig. 3D), representing 2% of the samples.

Figure 1*. Mature zygotic embryos of Pinus pinea collected in September-2003, and cold-stored for 7 months: (A) Cotyledonary zygotic embryo enclosed in the megagametophyte; (B) Translucent tissue protruding from hypocotyl on TDZ-PJ initiation medium after 4 weeks; (C) Filamentous proliferating cell mass on TDZ-PJ maintenance medium; (D) Initial stage of somatic embryos after 8 weeks; (E) Acetocarmine-stained somatic embryos consisting of densely cytoplasmic cells subtended by suspensor cells.*

Table 1. *Effect of family and initiation medium on the frequency (%) of mature zygotic embryos of stone pine that showed translucent cell mass proliferation after 8 weeks of culture on initiation media. Cones were collected in August-2004 and cold-stored for 7 months. Number of embryos showing proliferation / total number of uncontaminated embryos is provided. Data on the frequency of polyembryony (%) showed by seeds within families is also displayed.*

		Initiation medium		
Family	Polyembryony	NAA-BA-PJ	2,4-D-BA-mLV$_2$	Total
F6010	2/39 (5)	5/6 (83)	3/6 (50)	8/12 (67)
F6011	5/52 (10)	7/10 (70)	6/12 (50)	13/22 (59)
F6047	5/60 (8)	9/12 (75)	8/12 (67)	17/24 (71)
F6070	10/46 (22)	6/8 (75)	6/10 (60)	12/18 (67)
F6075	5/40 (13)	4/6 (67)	5/8 (63)	9/14 (64)
F6087	0/52 (0)	10/14 (71)	4/10 (40)	14/24 (58)
F6088	0/44 (0)	8/10 (80)	5/8 (63)	13/18 (72)
Total	27/333 (8)	49/66 (74)	37/66 (56)	86/132(65)

Table 2. *Effect of family and initiation medium on the frequency (%) of mature zygotic embryos of stone pine that showed translucent cell mass proliferation after 4 weeks of culture on initiation media. Cones were collected in Octob-2012 and cold-stored for 4 months. Number of embryos showing proliferation / total number of uncontaminated embryos is provided. Data on the frequency of polyembryony (%) showed by seeds within families is also displayed.*

		Initiation medium				
Family	Polyembryony	M-mLV$_2$	B-M-mLV$_2$	HB-M-mLV$_2$	TDZ-mLV$_2$	Mean
F6011	34/144 (24)	7/34 (21)	5/35 (14)	4/34 (12)	8/34 (24)	24/137 (18)
F6015	26/145 (18)	9/35 (26)	6/35 (17)	9/35 (26)	0/40 (0)	24/145 (17)
F6047	15/145 (10)	5/32 (16)	8/40 (20)	4/37 (11)	8/36 (22)	25/145 (17)
F6053	9/135 (8)	13/32 (41)	29/33 (88)	17/32 (53)	26/38 (69)	85/135 (63)
F6054	13/113 (12)	8/18 (44)	8/18 (44)	30/42 (70)	25/35 (71)	71/113 (63)
F6070	31/150 (21)	11/42 (26)	8/35 (23)	4/42 (10)	13/31 (42)	36/150 (24)
F6088	10/137 (7)	3/34 (9)	3/34 (9)	8/35 (23)	5/33 (15)	19/136 (14)
Mean	138/969 (14)	56/227 (25)	67/230 (29)	76/257 (30)	85/247 (34)	284/961 (30)

In the 2003 experiment, mature ZE from all families showed translucent cell masses arising from both cotyledon and hypocotyl region of embryos after 4-8 weeks of culture on initiation media. The two tested medium formulations produced significantly different frequencies of tissue proliferation (two-tailed P < 0.0001). Around 84% of the cultured explants protruded cell masses on the ANA-BA-LP medium and 61% of them on the TDZ-PJ medium (Table 3). Putative embryogenic masses induced in hypocotyls and root caps on TDZ-PJ medium began to grow (Fig. 1B), showing filamentous structures on TDZ-PJ maintenance medium (Fig. 1C). Although frequencies of cell mass formation varied with families in a range from 64 to 83% (Table 3), only a few of those tissues could be maintained in successive subcultures. Three out of the 215 tested mature ZE that were initiated and maintained in TDZ containing medium (about 1%) continued growing. They showed embryogenic structures at early developmental stage (Fig. 1D-E). These embryogenic lines arose at 2% frequency from families F6047, F6059 and F6062. However, growth of these proembryogenic structures on maintenance medium declined with subcultures and further development of early somatic embryos did not happen.

Table 3. *Effect of family and initiation medium on the frequency (%) of mature zygotic embryos of stone pine that showed translucent cell mass proliferation after 8 weeks of culture on initiation media. Cones were collected in September-2003 and cold-stored for 7 months. Number of embryos showing proliferation / total number of uncontaminated embryos is provided.*

| Family | Initiation medium | | Total |
	NAA-BA-LP	TDZ-PJ	
F6039	35/46 (76)	25/48 (52)	60/94 (64)
F6047	18/20 (90)	32/49 (65)	50/69 (73)
F6059	39/49 (80)	25/40 (63)	64/89 (72)
F6061	23/27 (85)	16/33 (49)	49/60 (82)
F6062	43/45 (96)	32/45 (71)	75/90 (83)
Total	158/187 (84)	130/215 (61)	288/402 (72)

In the 2004 experiment, translucent cell masses were also induced predominantly in the cotyledons of ZE (Fig. 2B). Significant differences in the frequencies of initiation were recorded between the two medium formulations (two-tailed $P = 0.0439$). Nearly 74% of the explants cultured on the ANA-BA-PJ medium and 56% of those cultured on the 2.4-D-BA-mLV$_2$ medium produced this kind of tissue after eight weeks of culture (Table 1). However, proliferation of most masses failed to grow further by continuous subculture on maintenance medium. Only three proliferating culture lines from one of the seven families initiated on ANA-BA-PJ medium were maintained (4% of the total ZE cultured on that medium), showing a spiky morphotype after 8 weeks on initiation medium (Fig. 2C). This tissue grew as a mixture of both brown-yellow and translucent mucilaginous cell masses after 12 weeks on maintenance medium (Fig. 2D). Somatic embryos at the initial developmental stage showing a polarized appearance was observed (Fig. 2E-F), induced from one of the eight ZE tested from family F6070. However proliferation and further development failed after several subcultures.
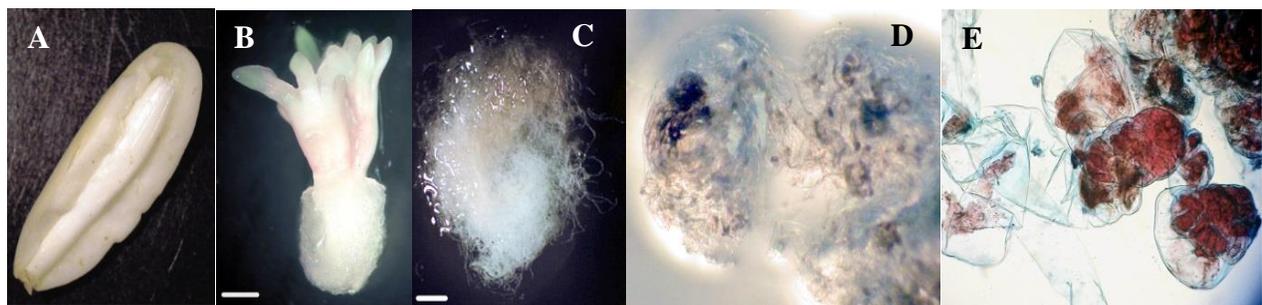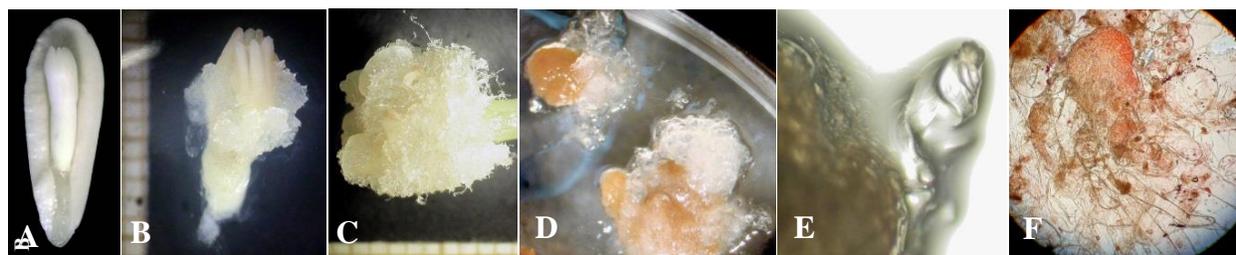

Figure 2. *Mature zygotic embryos of Pinus pinea collected in August-2004, and cold-stored for 7 months: (A) Cotyledonary zygotic embryo enclosed in the megagametophyte; (B) Translucent tissue protruding from cotyledons on ANA-BA-PJ initiation medium after 4 weeks; (C) Spiky proliferating cell mass after 8 weeks; (D) Proliferating cell masses on ANA-BA-PJ maintenance medium after 12 weeks; (E) Initial stage of somatic embryos showing a polarized appearance; (F) Acetocarmine-stained somatic embryos with densely embryonic region associated with some suspensor cells.*

In the 2012 experiment, translucent cell masses were also induced in remains of suspensor cells (Fig. 3A), in hypocotyls (Fig. 3B), in cotyledons (Fig. 3C), and in groupings of aborted embryos (Fig. 3D). Initial tissue growth in hypocotyl and root cap occurred mainly on TDZ-mLV$_2$ medium, while culture on the other media promoted initial growth in the whole embryo. But in these cases, tissue formed from hypocotyls quickly turned brown an only tissue from cotyledons remained alive. Out of 961 mature zygotic embryos tested, 284 of them protruded cell masses (30%) (Table 2). The induction frequency among families showed significant differences after 4 weeks on culture; it ranged from 14% of family F6088 to 63% of families F6053 and F6054 (two-tailed $P < 0.0001$). Among culture media, it

ranged from 25% on M-mLV$_2$ to 34% on TDZ-mLV$_2$ medium (two-tailed P = 0.0211). However, from the initially protruded tissue masses only 114 (13%) from the seven families tested (ranging from 4 to 21%, two-tailed P < 0.0001) proliferated on maintenance medium after 12 weeks of culture (Table 4). In this case, no significant differences were detected among culture media, maintaining growth 12-14% of the lines in all medium formulations (Table 4). The proliferating cell masses induced from cotyledons on HB-M-mLV$_2$ grew quickly after one week of culture and the embryogenic-like structure could be maintained for several subcultures on maintenance medium (Fig. 3E). Morphologically similar cell masses were obtained on TDZ-mLV$_2$ medium (Fig. 3F)
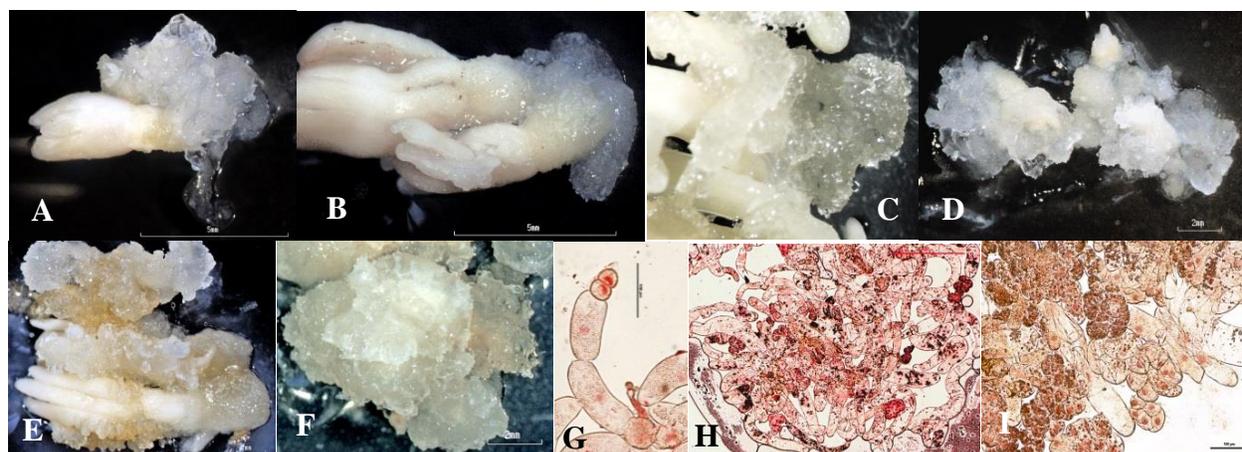
Figure 3*. Mature zygotic embryos of Pinus pinea collected in October-2012 and cold-stored for 4 months. (A) Dominant cotyledonary embryo excised from the megagametophyte and cultured on B-mLV$_2$ initiation medium for 1 week, showing translucent tissue formation from remaining suspensor cells; (B) Dominant cotyledonary embryo with subsidiary cotyledonary embryo on TDZ-mLV$_2$ initiation medium; (C) Translucent tissue induced from cotyledons on B-M-mLV$_2$ initiation medium; (D) Grouping of aborted embryos on M-mLV$_2$ initiation medium; (E) Translucent tissue induced on HB-M-mLV$_2$ after 8 weeks of culture; (F) Embryogenic-like tissue induced on TDZ-mLV$_2$ and cultured on M-mLV maintenance medium for 8 weeks; (G) Proembryogenic structure from embryogenic-like tissue induced on TDZ-mLV$_2$; (H) Small aggregates of meristematic cells with some elongated cells on M-mLV maintenance medium; (I) Dense cell clumps after several subcultures on maintenance medium.*

Table 4*. Effect of family and initiation medium on the frequency (%) of embryogenic-like tissues from the zygotic embryos collected in Octob-2012 that continued growing on M-mLV maintenance medium, after 12 weeks on culture. Number of explants showing proliferation / total number of uncontaminated embryos is provided.*

Family	Initiation medium				Mean
	M-mLV$_2$	B-M-mLV$_2$	HB-M-mLV$_2$	TDZ-mLV$_2$	
F6011	3/34 (9)	5/35 (14)	3/34 (9)	6/34 (18)	17/137 (12)
F6015	0/35(0)	6/33 (18)	9/35 (26)	0/29 (0)	15/132 (11)
F6047	0/32(0)	6/35 (17)	0/37 (0)	0/36 (0)	6/140 (4)
F6053	2/32 (6)	4/33 (12)	5/29 (17)	4/38 (11)	15/132 (11)
F6054	8/28 (29)	5/18 (28)	3/31 (10)	3/27 (11)	19/104 (18)
F6070	11/42 (26)	0/33 (0)	3/27 (11)	13/29 (45)	27/131 (21)
F6088	8/34 (24)	4/34 (12)	3/35 (9)	0 /33 (0)	15/136 (11)
Mean	32/237 (14)	30/221 (14)	26/228 (12)	26/226 (12)	114/912 (13)

Although induction of embryogenic-like cultures from mature ZE could be obtained, some cell masses failed to grow further after several subcultures. In other cell masses further proliferation was maintained and proembryogenic structures were detected (Fig. 3G). Although embryogenic-like cultures grew slowly, small aggregates of meristematic cells with some elongated cells were observed after several subcultures on maintenance medium (Fig. 3H). Old brown cell masses were removed at subculturing, and dense cell clumps were observed in the remaining translucent masses as the cultures ages (Fig. 3I).

Discussion

Although regeneration of plants through somatic embryogenesis was obtained in stone pine (Carneros et al. 2009), several experimental approaches were carried out to overcome the recalcitrance of this species. The initiation frequency from immature zygotic embryos of this species is too low and therefore restricted to a few genotypes, thus limiting selection possibilities and precluding commercial use (Bonga et al. 2010). Several attempts to obtain SE in explants from mature trees as shoot apex and expanding needles were performed, but although translucent callus resembling in appearance embryogenic tissue was induced, further multiplication and somatic embryo production failed (Carneros 2009).

Mature seeds of stone pine are amenable for storage for extended periods. Therefore they are a good source of plant material throughout the year and from year to year. This fact allows having seeds available independent of flowering and seasonal constraints. If SE could be induced using these seeds the need to collect immature cones during the restricted competence window of the immature embryos will be avoided. Furthermore, the use of excised ZE from mature seeds excludes the risk of having multiple genotypes in specific embryogenic lines (Garin et al. 1998). Since the development of SE protocols using mature seeds provide those advantages over immature zygotic embryos, the initiation of embryogenic cultures from mature ZE of stone pine was attempted.

Most reports on SE induction in conifers are in immature ZE at the cleavage stage, which involves a process of continued cleavage as the basis of the formation of embryogenic masses. However the induction of SE in mature embryos requires the dedifferentiation of cells and reprogramming to initiate the embryogenic process (Find et al. 2014). Both processes are different and this may the reason that up to date SE induction in mature ZE has been reported for a few *Pinus* species (Pullman & Bucalo 2011). A main issue when using embryos as initial explants for SE induction is the presence of polyembryony. Some authors recommend studying the effect that initiating from polyembryonic explants has on the SE process in *Pinus* species (Garin et al. 1998). During zygotic embryogenesis cleavage polyembryony may occur not only when a single even of fertilization happened but even also when embryos from multiple fertilization events took place, giving rise to many subordinate embryos (MacKay et al. 2001). These embryos are progressively eliminated by programmed cell death, and usually only one dominant embryo matures. The abortion of remaining subsidiary embryos is slow, asynchronous, and sometimes is delayed until seed germination (Filonova et al. 2003). There is evidence that some subordinate zygotic embryos survive to seed maturity (MacKay et al. 2001).

In stone pine variations in the frequency of seeds that showed polyembryony were observed in both 2004 and 2012 collection dates. Between 8% (ranging among families from 0 to 22%) and 14% (ranging among families from 7 to 24%) of the seeds enclosed cotyledonary embryos that maintained subordinated embryos. In *Pinus taeda*, the observed total number of zygotic embryos per immature seed ranged from 5 to 12 among five different control-crossed families (MacKay et al. 2001). In *Pinus sylvestris*, about 20% of the seeds contained subordinate embryos of great size (Filonova et al. 2003). In *Pinus koraiensis* about 13% of mature seeds contained small subordinate embryos attached to the suspensor of the dominant embryos that varied in size and developmental stage and in some seeds two completely developed embryos (Bozhkov et al. 1997). In *Pinus taeda* embryogenic cultures were initiated from subordinate zygotic embryos that may be genetically different than the dominant ZE (Becwar et al. 1991). Some studies claim that there is a relationship between the number of immature ZE per seed and the initiation of SE (MacKay et al. 2001). These authors indicated that the number of zygotic embryos per seed may be a driver of initiation and therefore could be used as a marker of initiation potential. In stone pine translucent cell masses were induced not only in hypocotyls and cotyledons of mature ZE, but also in the remains of suspensor cells in and in groupings of aborted embryos at different developmental stages. However a relation between frequencies of polyembryony and the initiation of embryogenic tissue was not recorded.

Garin et al. (1998) reviewed the origin of embryogenic tissue in conifers. In pines it usually appears in the suspensor region while in spruces the preferential zone is the hypocotyl-cotyledon. However, in *Pinus strobus* a soft translucent tissue was observed on the surface of the callus that developed in the hypocotyl-cotyledon region of the mature ZE, similarly to *Pinus massoniana*. In *Pinus koraiensis* two alternative pathways of somatic embryo origin were revealed, one in the hypocotyl-cotyledon region and another in the suspensor region of the dominant ZE (Bozhkov et al. 1997). These pathways required different culture media and differ both in the cell types and in the developmental steps. However after several subcultures the translucent and mucilaginous tissue from the suspensor region ceased growing, but the translucent and friable embryogenic tissue from the hypocotyl-cotyledon maintained the proliferation. Similarly in stone pine the growth of proliferating cell masses that originated from the hypocotyl-cotyledon continued longer than the tissue produced from the remaining suspensor cells.

As indicated by Bonga et al. (2010) recalcitrance to SE initiation starts early for many conifers, because only embryos at the polyembryonic cleavage stage will respond while mature zygotic embryos are non-responsive. These authors suggested that determining what external factors, for example stress, will activate embryogenic gene expression could help in overcoming recalcitrance. Results in stone pine indicated that culture medium type influenced the induction of translucent cell masses. Early somatic embryos were obtained from cell masses induced in hypocotyls on thidiazuron containing media, and in cotyledons with all the tested PGR formulations. It has been reported that TDZ has morphogenetic effects that are similar to those induced by stress (Von Aderkas and Bonga 2000). Induction of embryogenesis in mature zygotic embryos of *Abies fraseri* was obtained on medium containing 10 μM thidiazuron, and proliferation of embryo suspensor masses occurred on medium supplemented with cytokinin (Guevin and Kirby 1997). Increased embryogenic tissue initiation when media was supplemented with brassinolide was reported (Pullman et al. 2003). The addition of 0.1 μM brassinolide improved initiation percentages in loblolly pine and stimulated initiation in the more recalcitrant families of loblolly pine and Douglas-fir (Pullman et al. 2003). The induction of embryogenic tissue from ZE usually requires high levels of auxins and cytokinins. Often the maintenance step also requires auxins or cytokinins at lower concentrations than those used for the induction step (Elhiti and Stasolla 2011). Accordingly, in stone pine the maintenance medium had the same composition as the initiation medium but with the PGR concentration reduced to one tenth. Also a maintenance medium different to the one used for initiation was also tested. In spite of these cultural conditions embryogenic tissues could not be maintained.

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Can we induce tolerance to stress in *Pinus radiata* somatic trees?

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Abstract

Plant response to stress has clearly defined metabolic pathways. However, epigenetic variation can also contribute to the phenotypic plasticity of plants, which can be especially important for the adaptation of forest trees to changing environmental conditions. During the last few years, our research team has obtained high numbers of somatic embryos from low amounts of embryogenic tissue in most of the embryogenic lines tested to date. Furthermore, we have developed somatic embryogenesis in other *Pinus* species, and combined this propagation technology with organogenesis. Nowadays, this knowledge provides us with a biotechnological tool to manipulate the physical and chemical conditions at different stages of the somatic embryogenesis process. As a result, an *ex vitro* analysis of clonal material can be carried out to assess what level of stress tolerance they have acquired during the first phases of their development. Furthermore, the analysis of physiological parameters in plants with different tolerance to abiotic stress could give valuable information about the mechanisms used by plants to survive under adverse environmental conditions.

Keywords: conifer, drought, genetic improvement program, micropropagation, radiata pine, somatic embryogenesis

Introduction

In the current context of climate change, drought and increased temperature are the most important consequences that will affect plant growth and distribution. The prediction of the impact of climate change on plant performance and survival is a major challenge facing plant science (Grierson et al. 2011). However, there remain fundamental gaps in our knowledge of which traits can be used to improve ecological drought tolerance (Bartlett et al. 2012).

Pinus spp. are frequently used in reforestation programs, and *Pinus radiata* D. Don is one of the most cultivated species in the north of Spain, especially in the Basque Country. Because of its fast growth and wood production, this species has been studied to find elite plants with tolerance to drought (De Diego et al. 2012). In most cases, the plant's response to stress has clearly defined metabolic pathways. Furthermore, epigenetic variation can also contribute to the phenotypic plasticity of plants, which can be particularly important for the adaptation of forest trees to changing environmental conditions. In this sense, somatic plantlets can offer an added value, such as tolerance to stressful conditions with no detriment in the growth rate. Phenotypic plasticity is considered the ability of a genotype to express different phenotypes in different environments (Pigliucci, 2005). This ability is very important for woody plants to adjust to spatial environmental changes (van Kleunen and Fischer, 2005). Many kinds of plant phenotypic plasticity such us morphological and physiological responses to drought (Heschel et al. 2004) are considered to be adaptive and active reactions to the environment (Dorn et al. 2000). Some studies suggest that the potential for evolution of plasticity can also be created by epigenetic variation (Zhang et al. 2013).

Over the last few years, our research team has optimized different stages of the somatic embryogenesis process (SE) in radiata pine such as initiation, proliferation (Montalbán et al. 2012) and maturation (Montalbán et al. 2010). Thus we have obtained large numbers of somatic embryos from small embryonal masses (EMs) in most of the embryogenic lines that we have tested so far. Furthermore, we have obtained SE in other *Pinus* species (Montalbán et al. 2013), and combined this propagation technology with organogenesis (Montalbán et al. 2011). The knowledge obtained provides us with a biotechnological tool to manipulate the physical and chemical conditions at different stages of the SE process. As a result, it is now possible to obtain a great amount of clonal material grown under different environmental conditions. The analysis of physiological parameters in plants with different tolerance to abiotic stress could give valuable information about the mechanisms used by plants to survive under adverse environmental conditions. For this reason, the objective of this work was to analyse the effect of different environmental conditions during the SE initiation phase on the subsequent drought stress tolerance of the plants produced.

Material and methods

Plant material

One-year-old green female cones, enclosing immature seeds of *Pinus radiata* D. Don, were collected from open-pollinated trees in a seed orchard established by Neiker-Tecnalia in Deba (Spain) (latitude: 43º16'59''N, longitude: 2º17'59''W, elevation: 50 m). In 2012 four open-pollinated families were selected, and ten cones from each tree (12, 14, 42 and 67) were collected in June, when the development stage of zygotic embryos showed a dominant embryo (Montalbán et al. 2012). The cones were stored in paper bags at 4ºC until use for a maximum of 10 days. Intact cones were sprayed with 70% (v/v) ethanol, split into quarters and all immature seeds extracted. Then, immature seeds were surface sterilized in H_2O_2 10% (v/v) plus two drops of Tween 20® for 8 min and then rinsed three times with sterile distilled H_2O under sterile conditions in the laminar flow unit. Seed coats were removed and whole megagametophytes containing immature embryos were excised aseptically and placed horizontally onto the culture medium.

Initiation of EMs

The initiation medium was embryo development medium (EDM) (Walter et al. 2005) with 30 gL^{-1} sucrose and a combination of 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 µM benzyladenine (BA). Before autoclaving, the pH of the medium was adjusted to 5.7. The medium was autoclaved at 121ºC for 20 min. Different concentrations of gellan gum were added to increase or reduce water availability of the medium (2, 3 or 4 gL^{-1} Gelrite®). After autoclaving, filter-sterilized solutions with the pH adjusted to 5.7 containing 550 mgL^{-1}, L-glutamine, 525 mgL^{-1} asparagine, 175 mgL^{-1} arginine, 19.75 mgL^{-1} L-citrulline, 19 mgL^{-1} L-ornithine, 13.75 mgL^{-1} L-lysine, 10 mgL^{-1} L-alanine and 8.75 mgL^{-1} L-proline were added to the partially cooled medium prior to dispensing into Petri dishes (90 × 20 mm). The explants were stored at three different temperatures: 18, 23 or 28ºC (Fig. 1). The experiment involved 9 different treatments and 4 seed families with 8 megagametophytes per Petri dish and 10 Petri dishes per seed family and treatment.

Proliferation of EMs

After 4-8 weeks on initiation medium, proliferating EMs with a size of around 3-5 mm in diameter were separated from the megagametophytes and subcultured onto maintenance medium every 2 weeks. Maintenance medium had the same composition as the initiation medium, but at this stage of the process the gellan gum concentration and temperature were constant for all cultures. Gelrite concentration was set at 4.5 gL^{-1} and temperature was maintained at 23ºC. Following four subculture periods, actively growing EMs were recorded as established cell lines (ECLs), and the percentage of EMs initiated that had proliferated was calculated.

Maturation of somatic embryos

Maturation of somatic embryos was carried out according to Montalbán et al. (2010). Briefly, after 4 subculture periods on maintenance medium, EMs were suspended in liquid growth regulators-free EDM medium in 50 mL centrifuge tubes, and was vigorously shaken by hand for a few seconds. Then, a 4 mL aliquot containing 80-90 mg fresh mass of suspended EM was poured onto a filter paper disc (Whatman no.2) in a Büchner funnel. A vacuum pulse was applied for 10 s, and the filter paper with the attached EM was transferred to maturation medium. Maturation medium had the salt formulation of EDM, but contained a higher concentration of gellan gum, 9 gL^{-1}.

It was supplemented with 60 µM abscisic acid, 60 gL^{-1} sucrose and the amino acid mixture used for initiation and maintenance of the EMs. ECLs were cultured in darkness at $23 \pm 1°C$.

Germination and acclimatization of somatic embryos

Somatic embryos were transferred to germination medium. This medium was LP (Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988) with half strength macronutrients, 2 gL^{-1} activated charcoal (AC) and 7 gL^{-1} gellan gum. One to four Petri dishes per each ECL and 10 somatic embryos per Petri dish were used, with in the latter case the embryonic root caps pointing downwards at an angle of approximately 45º by tilting of the plates. All plates were placed under dim light for 7 days (Klimaszewska et al. 2001). Afterwards, the cultures were maintained at $23 \pm 1°C$ under a 16 h photoperiod at 100 $µmolm^{-2}s^{-1}$ provided by cool white fluorescent tubes (TFL 58 W/33; Philips, France).

Plantlets were subcultured onto fresh medium of the same composition every 6 weeks. After 14-16 weeks on germination medium, the plantlets were transferred to sterile peat:perlite (2:1) and acclimatized in a greenhouse, progressively decreasing the humidity from 90 to 70 %. Survival percentage was recorded after 31 days in ex vitro conditions.

Experimental design

One-year-old plantlets were analyzed in July 2014. Of the 9 treatments applied during the initiation stage, only three were selected for physiological studies; plants taken from ECLs initiated at 18ºC and 2 gL^{-1} gellan gum in the culture medium (18-2), from ECLs initiated at 23ºC and 4 gL^{-1} gellan gum (23-4) and from ECLs initiated at 28ºC and 4 gL^{-1} gellan gum (28-4). Ten plants per treatment were used; half of them were randomly selected for water stress treatment by withholding water (Water-stressed, D), and the remaining plantlets were supplied with water to field capacity three times a week (Controls, W). Drought conditions were maintained for 3 weeks.

Water potential determination

The water potential (Ψ, MPa) of samples from all treatments was measured at predawn (from 6:00 to 8:00 a.m.) (Ψ_{pd}) using a Scholander chamber (Skye SKPM 1400) and the pressure-equilibration technique (Scholander et al. 1965). Measurements were done at the beginning of the experiment and when plants started showing stress symptoms (13, 14 and 21 days after the beginning of the experiment).

Gas exchange parameters

To measure gas exchange parameters, a portable infrared gas analyser (LI-6400 system, LI-COR Inc., Lincoln, NB, USA) was used. Stomatal conductance (g_s, mmol H_2O $m^{-2}s^{-1}$), instant leaf transpiration (E, mmol H_2O $m^{-2}s^{-1}$) and instant net photosynthesis (An, µmol CO_2 m^{-1} s^{-1}) were measured at the beginning of the experiment and after 14 and 21 days. Water-use efficiency (WUE) was calculated as the photosynthetic rate (µmol CO_2 m^{-1} s^{-1}) relative to the transpiration rate (mmol H_2O $m^{-2}s^{-1}$).

Results and discussion

Initiation percentages in explants cultured at 28ºC were significantly lower (4%) than in those cultured at 18ºC or 23ºC (<18%). The best results were obtained by decreasing the usual initiation temperature from 23 to 18ºC. Some authors have pointed out that lowering or increasing temperatures may improve initiation and proliferation stages as they act as a mild stress that favours cell reprogramming (Bonga et al., 2010). On the other hand, the increase of the gellam gum concentration in the culture media increased significantly the success of the initiation stage. At the proliferation stage, the best results were obtained in EMs derived from megagametophytes cultured at 28ºC during the initiation stage. According to the temperature of initiation, explants cultured initially at 28ºC produced a significantly higher number of somatic embryos. Similarly, Li and Wolyn (1996) observed the highest production of somatic embryos when incubating the calluses at 27ºC, and they postulated that high temperature may have influenced the production of storage reserves in the developing somatic embryos. The gellan gum concentration at the initiation stage did not have a significant effect on the number of somatic embryos produced, which disagrees with other studies in which a high gellan gum concentration in the culture medium provoked an increase of somatic embryos production (Choudury et al. 2008). The highest survival rate after one month in *ex vitro* conditions was observed in plants from EMs generated at 18ºC (Table 1).

Figure 1. *Leaf water potential (Ψ$_{pd}$) in Pinus radiata somatic plants from embryogenic cell lines generated at different temperatures (18, 23 and 28ºC) exposed to irrigation (control) or no irrigation conditions during 25 days, M±SE.*

On the contrary, Kvaalen and Johnsen (2008) observed the lowest survival success in *Picea* plants from tissue proliferated and matured at this temperature.

The most evident external symptoms of *P. radiata* plantlets subjected to drought were needle epinasty and apical curvature. These symptoms showed high variability among plants obtained by different treatments. Hubbard et al. (2001) indicated that Ψ_{pd} was a good indicator of soil water status, pointing out that samplings were subjected to the same soil moisture. When Ψ_{pd} was analysed, somatic plantlets obtained from ECLs generated at 23ºC were the first ones showing epinasty and reaching the turgor lost point (TLP), previously described by our research group in -2 MPa for *P. radiata* (De Diego et al. 2012) (Figure 1). After two weeks of drought, the least sensitive plants were those that had been obtained from ECLs generated at the highest temperature (28ºC). However, one week later these plants showed the lowest leaf water potential. It is remarkable that these plants maintained a constant photosynthesis activity during the first two weeks of drought although by the third week it had decreased (Figure 2).
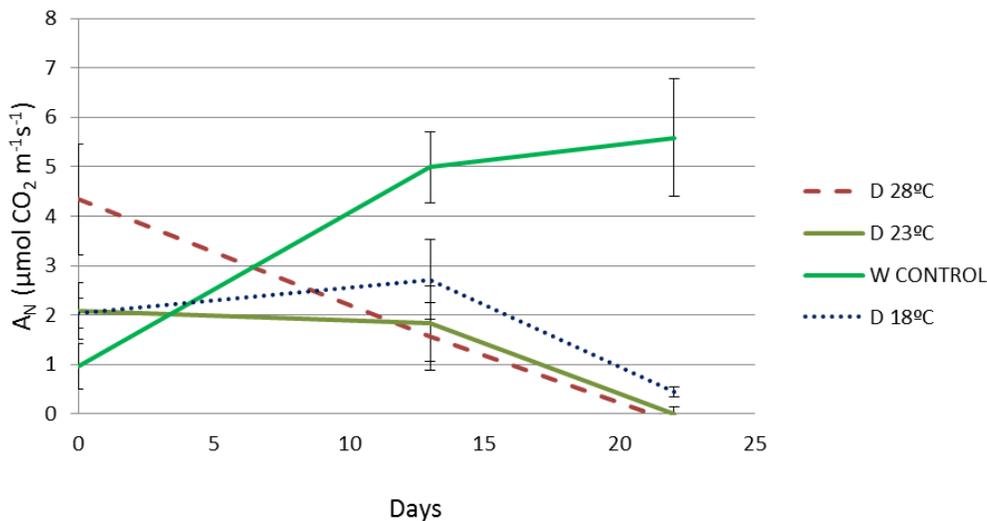
Figure 2. *Instantaneous net photosynthesis (A$_N$) in Pinus radiata somatic plants from embryogenic cell lines generated at different temperatures (18, 23 and 28ºC) exposed to irrigation (control) or no irrigation conditions during 22 days, M±SE.*

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At this point, plants exposed to drought and having been obtained from ECLs developed at 18ºC maintained their Ψ_{pd} potentials although their A_N levels

decreased (Figure 2). That strong gas exchange regulation is characteristic of isohydric species (McDowell et al. 2008; Quero et al. 2011). Plants that display isohydric characteristics have tight and continuous water potential homeostasis through stomatal control; this means they constantly regulate their water loss within a certain range to avoid damaging water deficits occurring within the plant (Buckley 2005). E (Figure 3) and g_s (Figure 4) decreased parallel in all the somatic plants evaluated in agreement with other previous studies
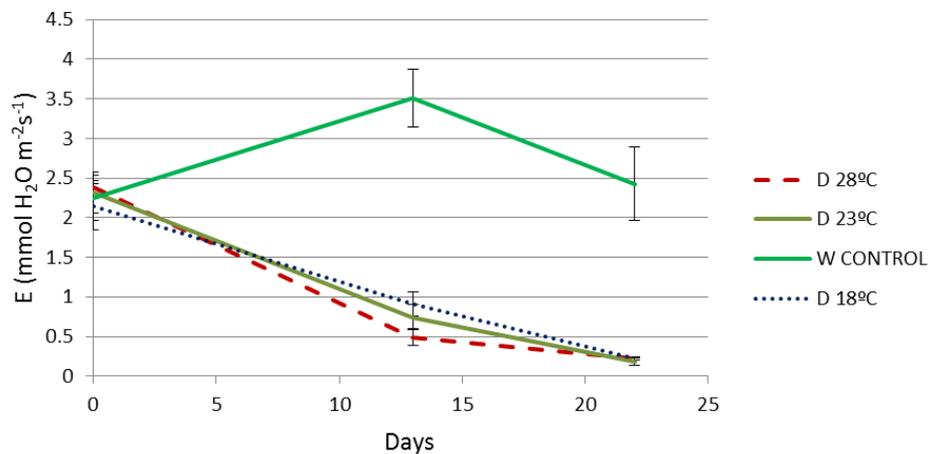
Figure 3.-Transpiration (E) in Pinus radiata somatic plants from embryogenic cell lines generated at different temperatures (18, 23 and 28ºC) exposed to irrigation (control) or no irrigation conditions during 22 days, M±SE.
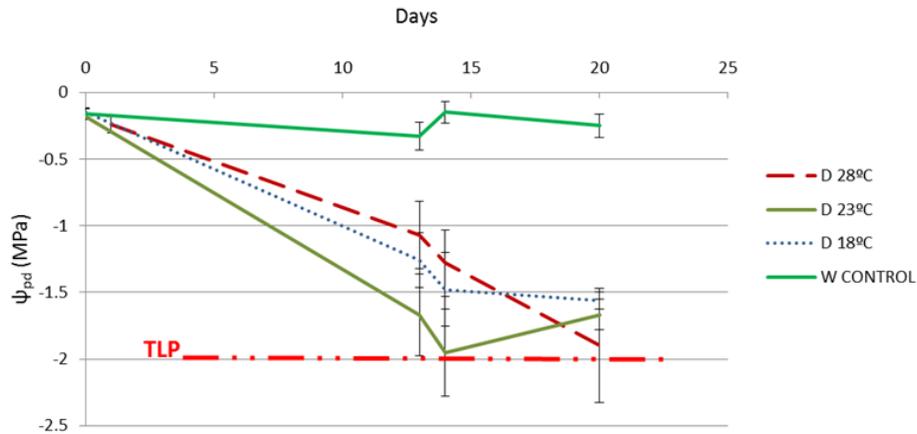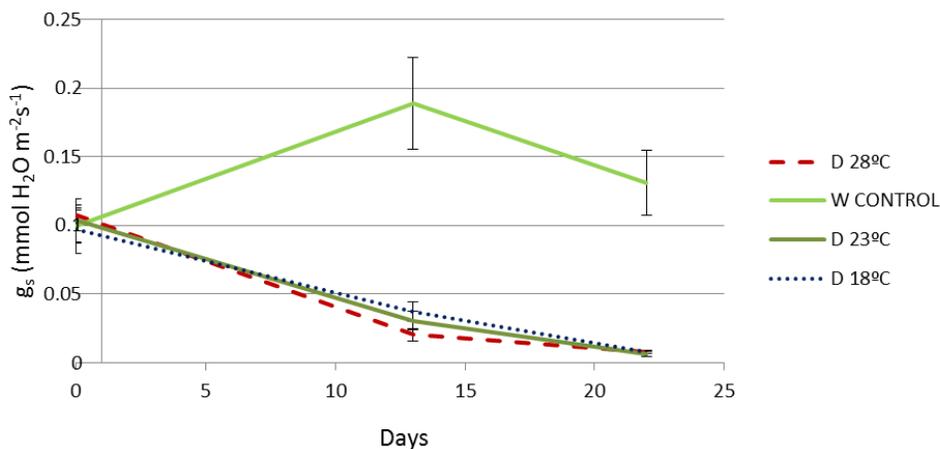

Figure 4.- Stomatal conductance (g_s) in Pinus radiata somatic plants from embryogenic cell lines generated at different temperatures (18, 23 and 28ºC) exposed to irrigation (control) or no irrigation conditions during 22 days, M±SE.

carried out by our research group (De Diego et al. 2014). It seems that stomatal control function was the same in all the plants independent of the temperature applied during the initiation of the SE process. On the contrary, another factor that is important to consider in woody plants under water stress is WUE, which is defined as photosynthetic rate relative to the transpiration rate. Along the first 14 days in drought conditions,

WUE was higher under water stress conditions in *Pinus radiata* somatic plants than in non-stressed ones (Figure 5). It was similar for plants derived from ECLs initiated at 18ºC and 28ºC after 14 days under drought conditions and these levels were higher than those obtained in the control plants. At the end of the experiment (after 3 weeks), only plants exposed to the 18ºC treatment maintained the WUE and presented the highest
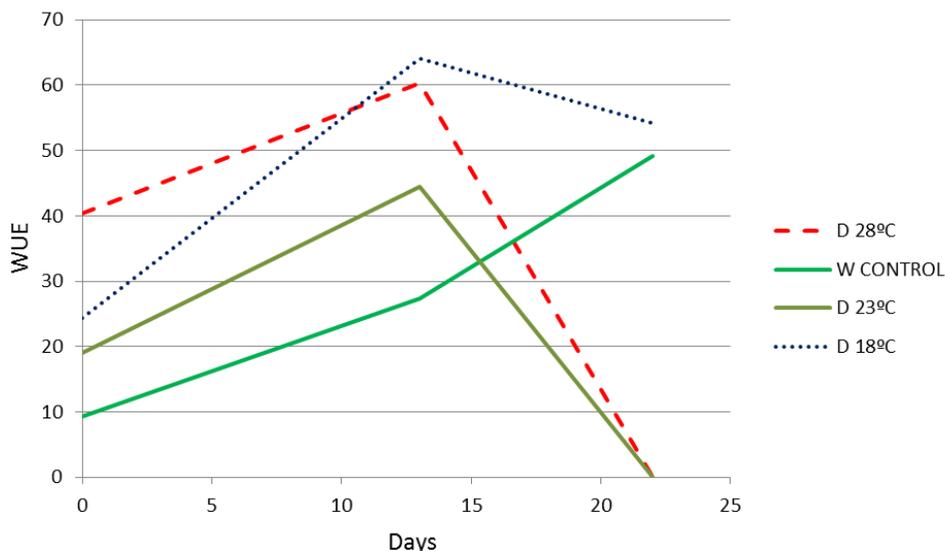
Figure 5.- Water use efficiency (WUE) in Pinus radiata somatic plants from embryogenic cell lines generated at different temperatures (18, 23 and 28ºC) exposed to irrigation (control) or no irrigation conditions during 22 days, M±SE.

levels for this parameter (Figure 5). Therefore, plants obtained from ECLs initiated at 18ºC showed similar efficiency in the use of water as plants provided with weekly irrigation (Figure 5). Our results seem to indicate that our plantlets showed a special memory able to give a differential drought tolerance when exposed to greenhouse conditions. Memory has previously been described in plants produced through zygotic embryogenesis (Johnsen et al. 2005) but now we see the same phenomenon in plants produced through SE. Our results are in agreement with Kvaalen and Johnsen (2008) where they postulated that a mechanism exists in the developing embryo that senses important environmental signals such as temperature, which in turn influences adaptive traits.

The fact that temperature during SE initiation changes water use efficiency in one-year old plants has implications for the future advances of forest plant production.

Summarizing, it seems that the presence of "stress memory" keeps plants prepared for upcoming stresses. Up to now many genetic pathways and regulatory mechanisms have been elucidated, but obviously, further studies are required for a full understanding of stress acclimation mechanisms in plants.

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Storage of plant material at low non-freezing temperature improves somatic embryogenesis in *Pinus radiata*

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Abstract

Many efforts have been carried out to improve the success of somatic embryogenesis (SE) in conifers, but little attention has been paid to the influence of the storage conditions of plant material on the subsequent phases of somatic embryogenesis. Therefore, our objective was to determine if storing of *Pinus radiata* plant material at 4ºC would improve initiation, proliferation and maturation during SE. Cold storage of one to three months enhanced the initiation rates and the number of mature somatic embryos obtained. These results demonstrate the beneficial effect of cold storage and suggest application of a cold treatment of plant material as a good alternative to increase the success of somatic embryogenesis in *Pinus radiata*.

Keywords: conifer, in vitro, micropropagation, radiata pine, somatic embryo.

Introduction

Pinus radiata, a species native to California, is well known as an exotic conifer species deployed in forest plantations, predominantly in New Zealand, Australia, Chile and Spain. Due to its high productivity, it is a highly valued resource for construction timber, furniture, heating, pulp and paper (Charity et al. 2005).

To meet the world's future wood demand, the use of vegetative propagation is the fastest, the most flexible and effective way to produce enough genetically improved material (Lelu-Walter et al. 2013). Organogenesis has been used untill recently (Hargreaves et al. 2005; Montalbán et al. 2011a; Montalbán et al. 2013) but due to its high labour cost it is not easy to use it at a commercial scale. Therefore, somatic embryogenesis (SE) is the only cost effective technology for vegetative propagation of radiata pine and it is being currently used by many companies to produce planting stock of this species industrially.

Since the first reports of SE in *P. radiata* SE (Smith 1997), improvements have been made to the different stages of the SE process, such as initiation (Hargreaves et al. 2009; Montalbán et al. 2012) or maturation (Montalbán et al. 2010; Montalbán et al. 2011b). But still, a drawback of this technique is that, in radiata pine, it can only be accomplished by using immature zygotic embryos as initial explants. Using this type of material implies a narrow competence window for SE initiation (MacKay et al. 2006; Montalbán et al. 2012) and, as a consequence, intensive manual labour is needed during a short time span to obtain the explants required for SE initiation. To try to lengthen the time span during which immature cones are available for explant extraction , cones are usually stored at 4ºC for one week (Salajová and Salaj 2005) to four weeks (Yildirim et al. 2006) before processing them. Longer storage periods are required to increase efficiency of the SE process.

Cold storage has been tested to promote SE in several angiosperm species (Janeiro et al. 1995; Aslam et al. 2011). With *Pinus* species, neither Häggman et al. (1999) for *P. sylvestris,* nor Park (2002) for *P. strobus* found a positive effect of a cold treatment. The aim of our study was to analyse if it is possible to store the initial explants at 4ºC without a detrimental effect on the initiation percentages and number of plantlets produced. Therefore, the effect of cold storage on the initiation, proliferation and maturation stages of SE was assessed.

Material and Methods

Plant Material

One-year-old green female cones, enclosing immature zygotic embryos of *Pinus radiata* D. Don, were collected from open-pollinated trees in a clonal seed orchard established by NEIKER-TECNALIA in Deba-Spain (latitude: 43º16´59´´N, longitude: 2º17´59´´W, elevation: 50 m). All cones were collected from two trees (12 and 14) when the stage of zygotic embryo development was between 2 and 4 (as defined by Montalbán et al. 2012). Intact cones were sprayed with 70% (v/v) ethanol, wrapped in filter paper and stored at 4ºC inside expanded polystyrene boxes on a layer of silica gel. Cones were used for SE initiation at the day of collection (0 days), after 2 weeks, 1 month, 2 months, 3 months and 4 months at 4ºC. At each sampling date and from each mother tree, two to four cones were split into quarters, immature seeds dissected and sterilized following Montalbán et al. (2012). Finally, seed coats were removed and whole megagametophytes containing immature embryos were excised aseptically and placed horizontally onto initiation medium.

Initiation and proliferation

Initiation of embryogenic tissue (ET) was carried out following Montalbán et al. (2012). Briefly, initiation was carried out on EDM culture medium (Walter et al. 2005) with 30 g L^{-1} sucrose and a combination of 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 µM benzyladenine (BA). Filter-sterilized solutions with the pH adjusted to 5.7 containing 550 mg L^{-1} L-glutamine, 525 mg L^{-1} asparagine, 175 mg L^{-1} arginine, 19.75 mg L^{-1} L-citrulline, 19 mg L^{-1} L-ornithine, 13.75 mg L^{-1} L-lysine, 10 mg L^{-1} L-alanine and 8.75 mg L^{-1} L-proline were added to the medium after autoclaving and before it was dispensed into Petri dishes (90 x 15 mm). Eight megagametophytes per Petri dish and a total of 10 Petri dishes per mother tree and cold storage period were cultured, for a total of 960 megagametophytes.
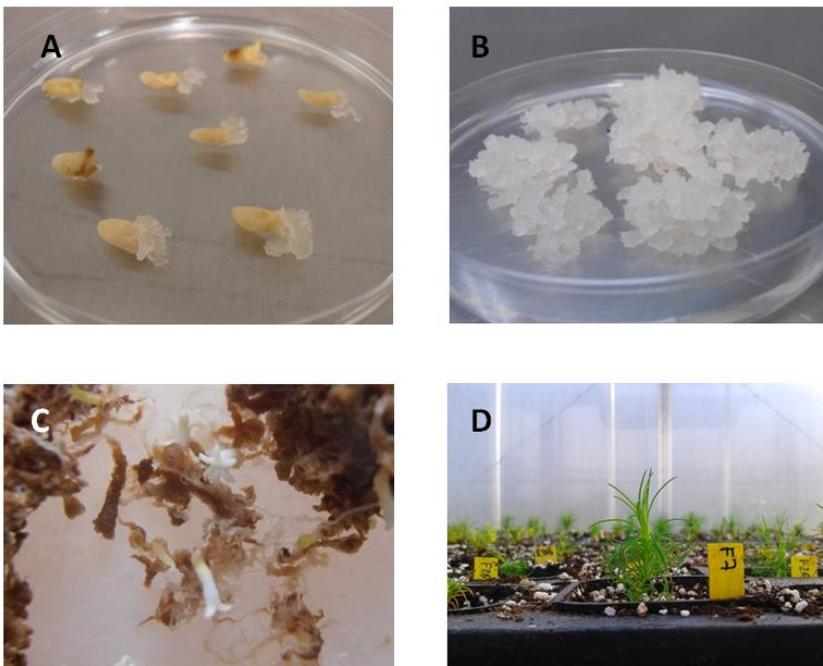
Figure 1. *(a) Initiation of embryonal masses (EM) in P. radiata megagamethophytes cultured on EDM medium; (b) proliferation of EM of P. radiata cultured on EDM medium; (c) Somatic embryos obtained from a ECL from a cone stored at 4ºC for 2 months; (d) Somatic plantlet growing in the greenhouse obtained from a cone stored at 4ºC for 1 month*

After 4–8 weeks from the start of the experiment, proliferating ET was separated from the megagametophytes (Fig. 1 A). ET was subcultured to maintenance medium every 2 weeks; maintenance medium had the EDM composition previously described, but contained a higher concentration of Gelrite® (4.5 g L^{-1}). After 6 to 8 subcultures (Fig. 1 B), proliferating embryogenic cell lines (ECLs) were subjected to maturation treatment.

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Maturation and conversion

Maturation was carried out following the protocol described by Montalbán et al. (2010). Maturation medium had the salt formulation of EDM medium but a higher concentration of Gelrite® (9 g L^{-1}) and was supplemented with 60 μM abscisic acid (ABA), 60 g L^{-1} sucrose, and the amino acid mixture used for initiation and maintenance of ET. Three to five ECLs per mother tree and cold storage period were chosen for the maturation experiments. Four replicates per ECL were kept in darkness at 21 ± 1°C. After 15 weeks on maturation medium, 30 to 40 mature somatic embryos per ECL were selected, isolated from ET (Fig. 1C) and germinated on half-strength macronutrients and complete micronutrients and vitamins of LP medium (Quoirin and Lepoivre 1977, modified by Aitken-Christie et al. 1988) (1/2 LP), supplemented with 30 g L^{-1} sucrose, 2 g L^{-1} activated charcoal and 7 g L^{-1} Gelrite®. Cultures were maintained at 21 ± 1°C under a 16 h photoperiod at 80 μmol m^{-2} s^{-1}. After 6 to 8 weeks, plantlets were subcultured once onto fresh medium of the same composition. After 14-16 weeks on germination medium, plantlets were transferred to sterile peat:perlite (3:2, v/v) and acclimatized in a greenhouse under controlled conditions at 21 ± 2°C and progressively decreasing humidity (Fig. 1 D).

Data collection and statistical analysis

Eight weeks after each sampling date, the number of proliferating ETs per Petri dish was recorded and initiation percentages per mother tree and cold storage period were calculated. Following four subculture periods, actively growing ETs were recorded as ECLs, and the percentage of initiated lines that had proliferated successfully was calculated. To evaluate the effect of the mother tree and cold storage period on the initiation and proliferation of ET, a logistic model and its corresponding analysis of variance (ANOVA) was carried out. To assess multiple comparisons among the different levels of mother trees and cold storage periods, estimable linear functions of model coefficients were computed (McCulloch and Searle 2001) and p-values were conveniently adjusted, following the Benjamini-Yekutieli method (Benjamini and Yekutieli 2001).

After 15 weeks on maturation medium, the number of ECLs subjected to maturation that had produced mature somatic embryos was recorded and the number of mature somatic embryos per gram was registered. A logistic model and its corresponding ANOVA was carried out to evaluate the effect the cold storage period on the proportion of ECLs that had produced somatic embryos. Among the ECLs that had produced somatic embryos, a non-parametric test was performed (Kruskal-Wallis test) to assess the effect of the cold storage period on the number of somatic embryos per gram of ET. Multiple comparisons are based on rank differences (Conover 1999). After 16 weeks on germination medium the % of the total number of somatic embryos produced that had properly germinated (conversion, %) was recorded.

Results

Statistical analysis of initiation percentages showed a significant effect of cold storage period, mother tree and an interaction between cold storage period and mother tree. No cold storage (0 days) or 2 weeks storage period at 4°C provoked SE initiation percentages of 25% (Fig. 2).
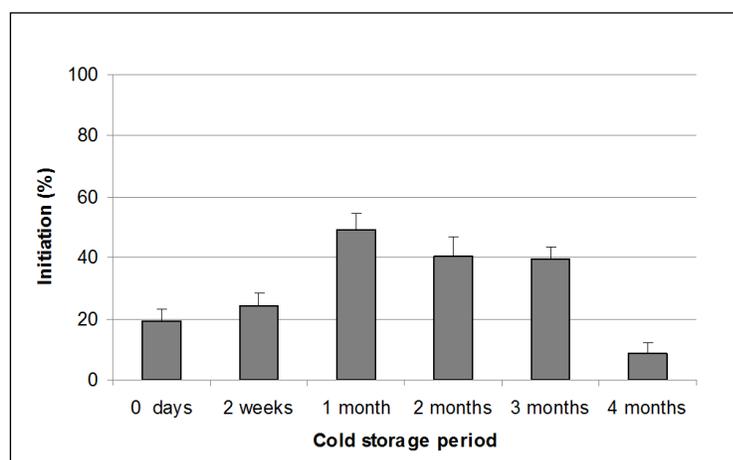
Figure 2. *Embryogenic tissue initiation (%) in Pinus radiata somatic embryogenesis after different periods of storage at 4°C of the green cones used for initiation (M ± S.E.)*

Megagametophytes from cones stored at 4ºC for 1, 2 and 3 months showed the highest initiation percentages (from 39.4% to 49.4%). The lowest initiation percentage was 8.8% and it was observed in explants from cones stored for 4 months. The effect of cold storage in each mother tree is shown in Fig. 3 and Fig. 4.
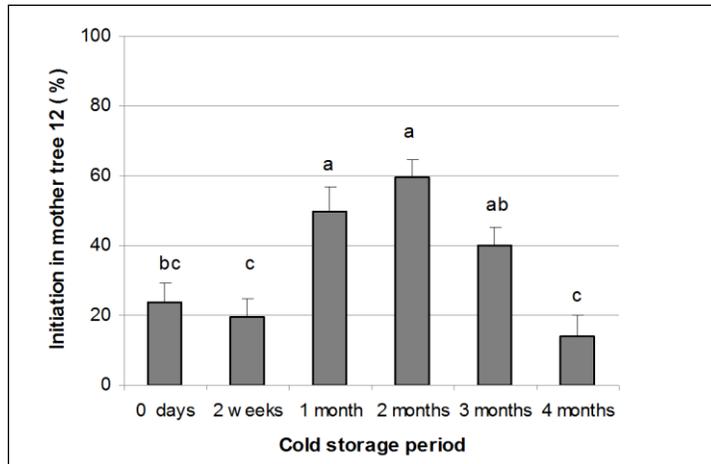
Figure 3. *Embryogenic tissue initiation (%) in Pinus radiata somatic embryogenesis of mother tree 12, after different periods of storage at 4ºC of the green cones used for initiation (M ± S.E.). Different letters show significant differences at p<0.05, p-values have been adjusted by the Benjamini-Yekutieli method*
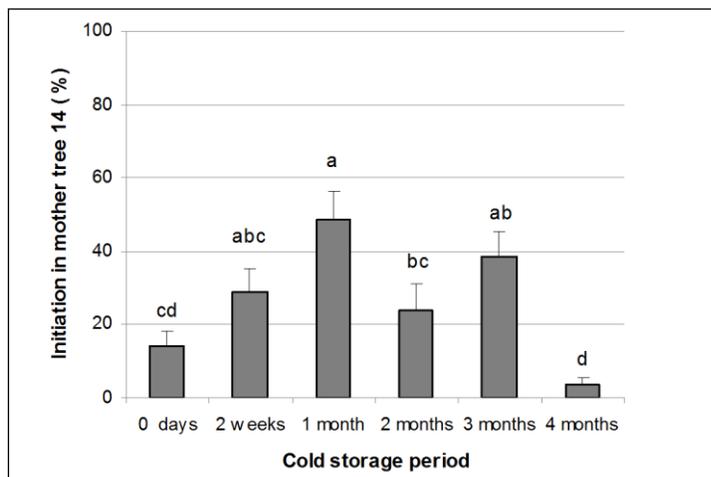
Figure 4. *Embryogenic tissue initiation (%) in Pinus radiata somatic embryogenesis of mother tree14, after different periods of storage at 4ºC of the green cones used for initiation (M ± S.E.). Different letters show significant differences at p<0.05, p-values have been adjusted by the Benjamini-Yekutieli method*

The highest number of ECLs was achieved when cones were stored for one month. After a storage period of two and three months a similar percentage of ECLs was obtained (42% and 41% respectively), while shorter storage periods (0 days or two weeks) led to a lower percentage of ECLs (Fig. 5).

Regarding the percentage of proliferating ECLs, no effect of cold storage period, or mother tree, or an interaction between both factors was observed (Fig. 6). The overall proliferation percentage was 74% and the values for different storage periods assayed ranged from 67% (3 months of cold storage) to 84% (1 month of cold storage) (Fig.6).

The percentage of ECLs that produced somatic embryos was not affected by the cold storage period. However, the ECLs from cones not stored at 4ºC showed the lowest maturation percentage (75%) (Fig. 7).

In those ECLs that produced somatic embryos, the number of embryos per gram of ET was significantly affected by the cold storage duration of cones (Fig. 8) (Montalbán et al. 2014). The ECLs from cones cold stored for 1 to 4 months showed a significantly higher number of somatic embryos (ranging from 302 embryos g^{-1} ET to 405 embryos g^{-1} ET) than those stored for 0 or 2 weeks (112 and 89 embryos g^{-1} ET, respectively). The conversion rate of somatic embryos was 70% and somatic seedlings were successfully acclimatized in the greenhouse (Figure 1 f).
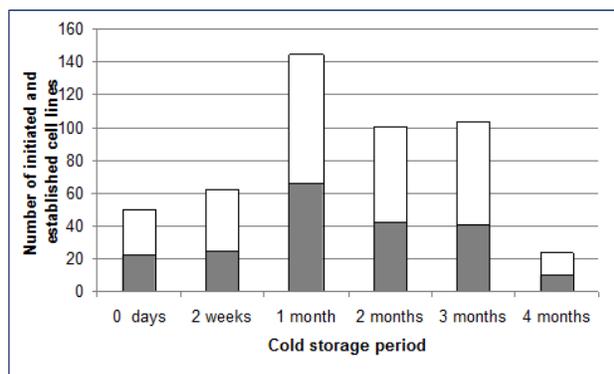
Fiugre 5. Total number of embryogenic cell lines proliferating (grey) of the total embryogenic lines initiated (white), in Pinus radiata somatic embryogenesis after different periods of storage at 4ºC of the green cones used for initiation
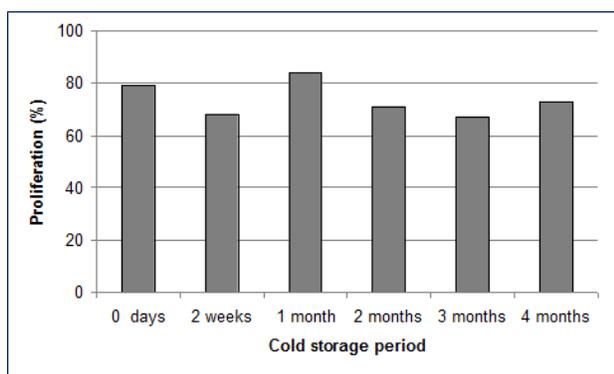
Figure 6. Embryogenic cell lines proliferating (%) of the total embryogenic lines initiated, in Pinus radiata somatic embryogenesis after different periods of storage at 4ºC of the green cones used for initiation
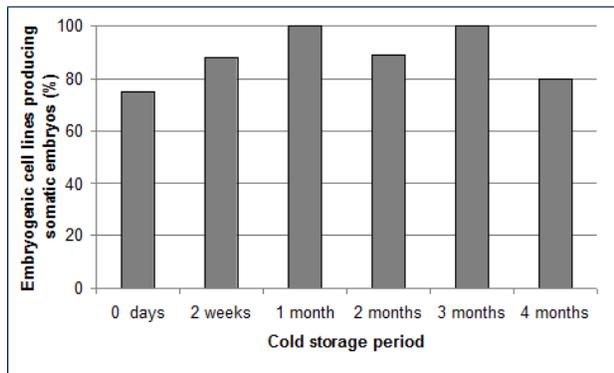
Figure 7. Embryogenic cell lines producing mature somatic embryos (%) in Pinus radiata somatic embryogenesis after different periods of storage at 4ºC of the green cones used for initiation
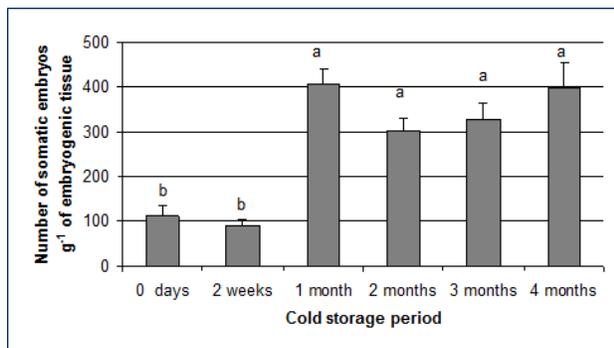
Figure 8. Number of mature somatic embryos per gram of embryogenic tissue in Pinus radiata somatic embryogenesis after different 4ºC storage periods of the green cones used for initiation. M±S.E. Different letters show significant differences at $p<0.05$. Multiple comparisons are based on differences on ranks, p-values are finally adjusted by the Benjamini-Yekutieli method (from Montalbán et al. 2014).

Discussion

Cold storage of cones for 1 to 2 months increased SE initiation rates when compared with those of cones not stored at 4ºC. Our results are opposite to those found by Park (2002) for *P. strobus* where a strong decline in initiation frequencies was observed when the cold storage duration increased. Although the mother tree had a significant effect, we can conclude that the best results for SE initiation were obtained after 1 to 3 months of cold storage of cones. A cold storage period longer than 3 months led to the lowest initiation rates; this could be due to decay and fungal colonization of cones as they were stored at 4ºC without any antifungal treatment. For this reason, application of different antifungal treatments will be carried out in future experiments.

We used megagametophytes as initial explant, with this kind of explant it is possible to achieve SE initiation without a cold preconditioning period, but in agreement with Tomaszewski et al. (1994) working with *Dactylis glomerata* or Luo et al. (2003) who studied *Astragalus adsurgens*, initiation rates were enhanced by a short cold stress as some authors pointed out (Krul 1993; Bonga 1996).

As reviewed by Zavattieri et al. (2010), two categories of inductive conditions allowing differentiated cells to develop into competent dedifferentiated cells have been recognized: plant growth regulators (internal and/or external cellular levels) and stress factors (osmotic shock, culture medium dehydration, water stress, heat or cool shock treatments, etc.). It has been postulated that knowing the endogenous hormone contents and their relation to the embryogenic competence of the explants would permit the induction and expression of SE in recalcitrant genotypes (Merkle et al. 1995; Jiménez and Thomas 2005). Several studies of the hormonal status in responsive genotypes and embryogenic cultures have been published (Pullman et al. 2014; Jiménez and Thomas 2005), but the internal hormone levels are extremely variable in tissues of different genotypes and species (Thomas and Jiménez 2005; Zavattieri et al. 2010). As reviewed by Feher et al. (2003), a higher endogenous IAA concentration has been associated with an increased embryogenic response in various species. Guo et al. (2013) found that shoot organogenesis in *Saussurea involucrata* significantly increased when the leaf explants were incubated at 4ºC for 5 days. As a result of this cold treatment the ratio of zeatin to indole-3-acetic acid (IAA) significantly increased in the explants in comparison to the levels observed in the controls. In a previous study, we analysed the endogenous cytokinins and IAA in bud explants from adult *P. radiata* trees subjected to different organogenic treatments, and we found that the buds with the highest cytokinin bases:IAA ratio were the most productive explants (Montalbán et al. 2013). In white spruce, Kong et al. (1997) found endogenous contents of ABA and IAA were involved in embryogenesis. Similarly, the presence of ABA throughout *P. taeda* embryo development also suggests that ABA may improve culture initiation (Pullman and Bucalo 2014).

In another line of research, Szechynska-Hebda et al. (2012) concluded that mild oxidative stress and hydrogen peroxide-dependent metabolic pathways were crucial for *in vitro* shoot formation via SE and organogenesis. They applied a cold pretreatment to mature and immature explants of *Triticum aestivum* and *Vicia faba*, but they were not able to regenerate plantlets from adult tissues. Nevertheless, cold pretreatment greatly improved the regeneration efficiency in immature explants. These authors observed a higher cellular H_2O_2 content in the immature embryos of both species, moreover production of hydrogen peroxide and enzyme activity were all greatly enhanced during the first week after a 4ºC pre-treatment, suggesting that the factor responsible for maintaining the regeneration potential in cells could be their oxidative/antioxidative capacity. In keeping with these authors, Guo et al. (2013) observed an accumulation of O_2^- rapidly increased in response to a cold treatment and then decreased as superoxide dismutase activity catalyzed the dismutation of O_2^- to molecular oxygen and H_2O_2, resulting in a significant increase in H_2O_2 concentrations in cold-treated explants. Recently other studies supporting the hypothesis that SE results from a specific form of stress related to an adaptation process have been published (Zavattieri et al 2010). In this sense, Ganesan and Jayabalan (2004) showed that addition of haemoglobin to the culture medium increased SE efficiency in cotton by increasing the oxygen level and stress in growing tissues.

Proliferation of ET was not significantly affected by the mother tree or cold storage period tested. This result is encouraging as the highest initiation rates did not drop in the next phase of SE. It could be interesting for a further study to asses the effect of a cold treatment at the proliferation stage, since there are studies showing its positive effect for the subsequent phases of the process (Aslam et al. 2011). The percentage of ECLs that gave somatic embryos ranged from 75% to 100% and was not significantly affected by cold storage period (Montalbán et al. 2014). However, ECLs from plant material stored for 1 month or more at 4ºC produced a significantly higher number of somatic embryos per gram of tissue. This beneficial effect of a cold preconditioning on the number of

somatic embryos obtained has been observed in angiosperm species (Krul 1993). Some authors have reported higher conversion rates to plantlets when ETs or somatic embryos were maintained at low temperatures (Janeiro et al. 1995; Corredoira et al. 2003). Thus, it seems cold storage or cold culture periods enhance later stages of SE by means of promoting maturity of somatic embryos.

The somatic embryos obtained from ECLs exposed to different cold storage periods showed a similar morphology and germination rates. Umetsu et al. (1995) observed that after a cold treatment somatic embryos revealed a different esterase isozyme pattern when compared with somatic embryos from ECLs without a cold treatment. As reviewed by Neilson et al. (2010), it is clear that temperature stress causes molecular responses in plant tissues; low temperature stress response is characterized by significant effects on chloroplast components, ROS detoxification, and energy production. In this sense, proteomic studies could provide suitable candidates for selective breeding programs aimed at enhancing stress tolerance in ecologically and economically important plant species (Neilson et al. 2010). In another study, Kvaalen and Johnsen (2007) showed that a mechanism exists in *Picea abies* that operates during embryo development and adjusts the timing of bud set in accordance with the temperature conditions in which the mother tree lives. As reviewed by Achrem et al. (2012) low temperature stress induces temporary and stable (epigenetic) changes in several species. The presence of "stress memory" keeps plants prepared for upcoming stresses. In keeping with these reports, it would be interesting for a future research project to analyse the stress tolerance to temperature of the plantlets obtained. In summary, we conclude that it is possible to store plant material of *P. radiata* for over one month and that this increases SE initiation rates and production of somatic embryos. Moreover, cold treatment does not affect either proliferation or maturation rates. These findings are important from a practical point of view and it should be established whether they apply to other *Pinus* species as well. It offers the possibility of processing plant material for a longer period of time, it reduces manual labour at the initiation stage and permits the introduction of a higher number of initial explants while also increasing the success rates in the SE process.

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Germination and plant regeneration from somatic embryos of Japanese black pine (Pinus thunbergii Parl.) after maturation in a medium containing polyethylene glycol or a high concentration of gellan gum

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Abstract

Somatic embryogenesis of Pinus thunbergii was initiated from megagametophytes containing immature zygotic embryos. Embryogenic cultures were maintained and proliferated in a medium supplemented with 3 μM 2,4-dichlorophenoxyacetic acid, 1 μM 6-benzylaminopurine, 30 g l−1 sucrose, and 1.5 g l−1 L-glutamine. Somatic embryo maturation experiments were performed in darkness at 25°C. Embryogenic tissues were cultured on maturation media containing 50 g l−1 maltose, 2 g l−1 activated charcoal, 100 μM abscisic acid, and either 100 g l−1 polyethylene glycol or a high concentration of gellan gum (Gelrite 10 g l−1 without polyethylene glycol). After maturation, the number of somatic embryos that germinated and developed into plantlets was recorded after 6 and 12 weeks, respectively. Low germination and conversion frequencies (16% and 12% of the total tested, respectively) were achieved from somatic embryos after maturation on the medium supplemented with polyethylene glycol. In contrast, when somatic embryos were matured on the medium containing a high concentration of gellan gum without polyethylene glycol, the germination frequency recorded was 80%, among which 78% of somatic embryos developed into plantlets. Somatic embryos matured with polyethylene glycol were desiccated to improve both germination and plant conversion frequencies. Desiccation of somatic embryos at a high relative humidity resulted not only in a marked increment in germination frequency (from 16% to 81%) but also a subsequent improvement in plant conversion rate (from 12% to 78%). Somatic plants were acclimatized, and their growth was monitored in the field.

Abbreviations: ABA, abscisic acid; AC, activated charcoal; BA, benzyladenine; 2,4-D, 2,4-dichorophenoxyacetic acid; EM, embryo maturation; ET, embryogenic tissues; PEG, polyethylene glycol; PGR, plant growth regulators; SE, somatic embryogenesis

Introduction

Japanese black pine (*Pinus thunbergii* Parl.) is an important forest tree species used in reforestation and landscaping in Japan. However, Japanese black pine populations have dramatically decreased as a result of pine wilt disease, caused by the pinewood nematode (*Bursaphelenchus xylophilus*). Therefore, the development of an efficient and stable plant regeneration system is essential for the large-scale propagation of resistant clones. Somatic embryogenesis (SE) is the most promising technique for mass propagation of clones and for plant regeneration using genetic transformation. However, for many species, plant conversion efficiency has been one of the limiting factors in using SE for widespread commercial use. Similarly, for Japanese black pine, the plant conversion of mature somatic embryos is limited by the low frequency of root emergence.

Previously, we reported SE and plant regeneration in the Japanese black pine (Maruyama et al. 2005a). In that study, high maturation frequencies of zygote-like cotyledonary somatic embryos on maturation media containing polyethylene glycol (as osmotic agent to decrease the medium water potential) were described; nevertheless, the subsequent germination average rates fluctuated around only 30%. Later, we reported an improved somatic embryo germination protocol for Japanese pines based on the desiccation of somatic embryos after the maturation step (Maruyama and Hosoi 2012). This post-maturation treatment markedly increased germination frequencies and considerably improved synchronization of the germination period. Similarly, in order to improve the somatic embryo germination protocols, post-maturation treatments based on the desiccation of somatic embryos have also been reported to successfully improve germination frequencies in conifers (Hay and Charest 1999, Klimaszewska and Cyr 2002, Stasolla and Yeung 2003). On the other hand, methods involving reduction in water availability to the culture cells by increasing the medium gel strength with a high gelling agent concentration have been reported to efficiently improve somatic embryo germination in several species of pine, spruce, and larch, without any post-maturation treatments (Klimaszewska and Cyr 2002, Klimaszewska et al. 2007).

In the present study, we compare the two methods that are being mostly used for SE in pine species. The results on germination and plant regeneration from somatic embryos of Japanese black pine after maturation on medium with polyethylene glycol or a high concentration of gellan gum are described. The beneficial effect of somatic embryo desiccation after polyethylene glycol–mediated maturation is also reported.

Materials and Methods

Source of plant material and embryogenic culture

Embryogenic tissues (ET) were induced from immature seeds collected from open-pollinated sources of Japanese black pine (14 cell lines) as described by Maruyama et al. (2005a). Excised megagametophytes were cultured on initiation medium that was the same as the embryo maturation (EM) medium (Maruyama et al. 2000), but modified as follows: salts, vitamins, and myo-inositol were reduced to half the standard concentration, and we added 0.5 g l−1 casein hydrolysate, 1 g l−1 L-glutamine, 10 g l−1 sucrose, 10 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 5 µM 6-benzylaminopurine (BA), and 3 g l−1 gellan gum (Gelrite®; Wako Pure Chemical, Osaka, Japan). The pH of the medium was adjusted to 5.8 before sterilization. To promote proliferation, the ET were transferred into the proliferation medium (initiation medium supplemented with 30 g l−1 sucrose, 3 µM 2,4-D, 1µM BA, and 1.5 g l−1 glutamine, and without casein hydrolysate). About 5–10 pieces of ET per Petri dish (90 $\square$ 20 mm) were maintained and proliferated by subculturing at 2- to 3-week intervals. The cultures were kept in the dark at approximately 25°C.

Maturation of somatic embryos

The ET, about 2 weeks after subculture, were used for the maturation of somatic embryos. About 500 mg of freshly weighed ET was suspended in about 3 ml of liquid proliferation medium without plant growth regulators (PGR) and was poured over a 90 × 20 mm Petri dish containing 30–40 ml of semisolid maturation medium. The maturation medium contained salts and vitamins from the original EM medium, 50 g l−1 maltose, 2 g l−1 activated charcoal (AC), 100 µM abscisic acid (ABA), amino acids (Smith 1996) (in g l−1: glutamine 7.3, asparagine 2.1, arginine 0.7, citrulline 0.079, ornithine 0.076, lysine 0.055, alanine 0.04, and proline 0.035), and 100 g l−1 polyethylene glycol 4000 (PEG) (Av. Mol. Wt.: 3000; Wako Pure Chemical, Osaka, Japan) or a high concentration of gellan gum (Gelrite 10 g l−1 without polyethylene glycol). The Petri dishes were sealed with Parafilm and kept in darkness at approximately 25°C for 8–12 weeks.

Desiccation treatment of somatic embryos after maturation with PEG

For desiccation treatment, cotyledonary somatic embryos from PEG-maturation medium were placed over 30-mm-diameter filter paper disks and added into two (central) wells of a six-well multiplate (Iwaki, AGC Techno Glass Co., Ltd., Chiba, Japan), and the remaining four (side) wells of the multiplate were filled with 5–6 ml of sterile water (Fig. 1a), tightly sealed with Parafilm, and kept in the dark at 25°C for 3 weeks. Under these conditions, the generated relative humidity registered with a thermo-hygrometer recorder (RS-10, ESPEC MIC Corporation, Aichi, Japan) was approximately 98%.

Germination of somatic embryos and plant conversion

Mature somatic embryos were transferred into germination medium containing basal salts at concentrations similar to those used for maintenance and proliferation, but without PGR, and supplemented with 30 g l−1 glucose, 2 g l−1 AC, 0.4 g l−1 glutamine, 0.25 g l−1 arginine, and 0.1 g l−1 proline, and solidified with 6 g l−1 gellan gum. Cultures were kept at 25°C under a photon flux density of about 65 µmol m−2 s−1 provided by cool, white fluorescent lamps (100 V, 40 W; Toshiba, Tokyo, Japan) for 16 h. The number of somatic embryos that germinated (root emergence) and converted into plantlets (development of epicotyls from germinated embryos) was recorded after 6 and 12 weeks, respectively.

In vitro growth and acclimatization of somatic plants

Regenerated plantlets were transferred into 300-ml flasks containing 100 ml of fresh germination medium with no amino acids and containing 30 g l−1 sucrose, 5 g l−1 AC, and 12 g l−1 agar (Wako Pure Chemical Industries, Osaka, Japan) or into Magenta® vessels (Sigma, St. Louis, USA) containing Florialite® (Nisshinbo Industries, Inc., Tokyo, Japan) irrigated with a plant food solution modified from Nagao (1983) (containing in mg l−1: NH4NO3 143, NaH2PO4・2H2O 55.1, KCl 47.1, CaCl2・2H2O 52.5, MgSO4・7H2O 61, Fe-III EDTA 25, Cu EDTA 0.1, Mn EDTA 0.1, Zn EDTA 0.1, H3BO3 1.5, KI 0.01, CoCl2・6H2O 0.005, and MoO3 0.005) and kept under the same conditions as described above for 16–20 weeks prior to ex vitro acclimatization. The developed plants were transplanted into plastic pots filled with Kanuma soil and acclimatized in a growth cabinet as described by Maruyama et al. (2002).

Results

Germination of somatic embryos and plant conversion

As shown in Table 1, when somatic embryos matured with PEG were placed directly on the germination medium, the root emergence of embryos and the subsequent plant conversion occurred at a low frequency (an average of 16% and 12%, respectively). In contrast, when the somatic embryos were matured on medium containing a high concentration of gellan gum without polyethylene glycol, the germination frequency recorded was 80%, and then 78% of somatic embryos developed into plantlets. The start of germination (root emergence) was observed 1–2 weeks after transfer into the germination medium, and the embryos subsequently converted into plantlets (emergence of both root and epicotyl) after 4–8 weeks of culture (Fig. 1b).

Table 1. Germination and plant regeneration from somatic embryos of Japanese black pine (Pinus thunbergii Parl.) after maturation with polyethylene glycol or a high concentration of gellan gum

Cell line	Germination frequency（%）			Conversion frequency（%）		
	PEG	PEG + Desiccation	Gellan gum	PEG	PEG + Desiccation	Gellan gum
T216-2-1	21 (109/530)	71 (556/784)	68 (967/1422)	20 (104/530)	70 (547/784)	67 (946/1422)
T205-3-3	60 (150/250)	96 (346/361)	70 (171/245)	51 (128/250)	91 (330/361)	65 (159/245)
T205-3-6	47 (47/100)	70 (70/100)	83 (132/160)	38 (38/100)	61 (61/100)	80 (128/160)
T216-4-1	2 (4/200)	85 (170/201)	92 (194/210)	1 (2/200)	80 (160/201)	90 (190/210)
T205-4-1	0 (0/100)	79 (79/100)	94 (94/100)	0 (0/100)	78 (78/100)	93 (93/100)
T205-4-2	7 (7/100)	70 (105/151)	95 (295/310)	3 (3/100)	67 (100/151)	93 (288/310)
T205-4-3	9 (9/100)	55 (55/100)	91 (100/110)	5 (5/100)	50 (50/100)	91 (100/110)
Sm64-6-1	6 (12/200)	93 (296/320)	95 (572/600)	1 (2/200)	90 (288/320)	93 (555/600)
Tn54-8-5	0 (0/100)	84 (240/287)	66 (119/180)	0 (0/100)	75 (215/287)	64 (115/180)
Tn54-8-8	1 (1/102)	82 (82/100)	84 (84/100)	0 (0/102)	78 (78/100)	75 (75/100)
Tn54-8-17	2 (2/100)	67 (80/120)	80 (120/150)	0 (0/100)	65 (78/120)	79 (118/150)
Tn54-8-20	4 (10/245)	88 (408/466)	77 (154/200)	0 (3/245)	85 (395/466)	70 (140/200)
Ms90-9-1	5 (5/100)	94 (188/200)	90 (358/400)	1 (1/100)	88 (176/200)	89 (354/400)
Mn9037-9-1	8 (8/100)	77 (289/374)	81 (323/400)	2 (2/100)	76 (285/374)	80 (318/400)
Total	**15.6 (364/2,327)**	**80.9 (2,964/3,664)**	**80.3 (3,683/4,587)**	**12.4 (288/2,327)**	**77.6 (2,841/3,664)**	**78.0 (3,579/4,587)**
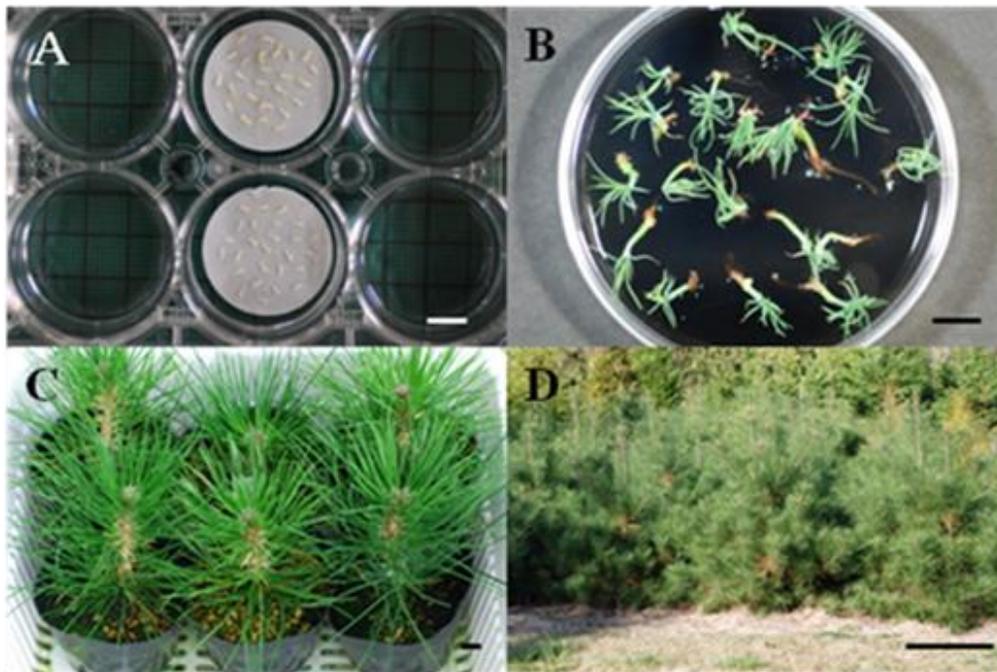
Figure 1. *Germination and plant regeneration from somatic embryos of Japanese black pine (Pinus thunbergii Parl.).* ***A****: Desiccation treatment of somatic embryos after maturation with PEG.* ***B****: Germination and plant conversion from somatic embryos after maturation with gellan gum.* ***C****: Acclimatized plants.* ***D****: Somatic plants growing in the field.* ***Bars****: 1 cm (A, B, C), 1 m (D)*

Effect of desiccation treatment on the germination frequency of somatic embryos matured with PEG

Somatic embryos matured with polyethylene glycol were desiccated to improve germination frequencies. Desiccation of somatic embryos resulted not only in a marked increment in the germination frequencies but also in subsequent improvement of plant conversion rates in all the 14 cell lines tested. The average germination and conversion frequency was improved by more than five-fold (16%−81%) and more than six-fold (12%−78%), respectively, compared with the frequencies obtained by somatic embryos that were not desiccated (Table 1).

Plant regeneration and establishment in the field

Somatic plants were successfully acclimatized in a growth chamber at 25°C and 80% relative humidity. Subsequently, the acclimatized plants (Fig. 1c) were transferred to a greenhouse and grown for about 1 year before transplanting to the field. The somatic plants showed 100% survival after being transplanted to the field and their growth is currently being monitored (Fig. 1d).

Discussion

The SE technology is the most recent vegetative propagation system to be implemented on an operational scale (Grossnicle 2011). In addition, regarding the cloning of conifers by SE, the most important advantage is that the tissue can be cryopreserved without altering its genetic makeup or loss of juvenility (Park et al. 1998). Hence, vegetative propagation by SE on an industrial scale has now been developed for several conifers (Jain et al. 1995, Klimaszewska and Cyr 2002, Stasolla and Yeung 2003, Jain et al. 2005). However, in a number of cases, such as in Japanese pines, the low germination rate hampers efficient large-scale production (Maruyama et al. 2005a, Maruyama et al. 2005b, Maruyama et al. 2007). The production of high quality somatic embryos that permit a high plant conversion frequency is essential for using SE protocols in commercial mass production, breeding programs, and genetic engineering.

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In order to improve plant conversion protocols, the desiccation of somatic embryos after PEG-mediated maturation has been recommended for conifer species. For most species, desiccation presumably acts to terminate developmental processes and to initiate those metabolic processes necessary to prepare the seeds for germination and growth (Kermode and Bewley 1985). Despite reports that desiccation of somatic embryos after the maturation process is beneficial in improving the quality of germinants, the germination frequencies vary according to the rate of desiccation in each condition and the desiccation tolerance of mature embryos. Desiccation tolerance in conifer somatic embryos generally decreased with increasing rapidity of desiccation (Bomal and Tremblay 1999). Relative humidities of 81% and lower were lethal to the somatic embryos of interior spruce, whereas germination was enhanced following treatments at humidities greater than 95% relative to the untreated controls (Roberts et al. 1990). Similarly, the results of post-maturation treatments in somatic embryos of Japanese black pine indicated that the desiccation of somatic embryos at high relative humidity was most effective in promoting germination (Maruyama and Hosoi 2012). These results suggest that desiccation at high relative humidity causes germination-promoting physiological changes in somatic embryos. Although the specific changes were not explored in this study, the improved performance of somatic embryos after desiccation treatment can be attributed to a change in endogenous hormone levels and accumulation of storage reserves (Ackerson 1984, Dronne et al. 1997, Kong and Yeung 1992, Find 1997, Stasolla et al. 2001, Klimaszewska et al. 2004). The beneficial effect of desiccation treatment after maturation with PEG, improving the germination frequencies and decreasing the time required for germination, was also reported for somatic embryos of interior spruce (Roberts et al. 1990), sitka spruce (Roberts et al. 1991), white spruce (Kong and Yeung 1992, Kong and Yeung 1995, Attree et al. 1995), hybrid larch (Lelu et al. 1995, Dronne et al. 1997), patula pine (Jones and van Staden 2001), and Ryukyu pine (Hosoi and Maruyama 2012).

In recent years, somatic embryo maturation methods, involving reduction in water availability to the cultured cells by increasing the medium gel strength (with a high concentration of gellan gum) to produce mature somatic embryos with low water content, have been reported in several improved protocols for pine species. Restricting water availability has resulted in high germination rates and subsequent high plant conversion frequencies in P. radiata (Smith 1996), P. strobus (Klimaszewska and Smith 1997, Klimaszewska et al. 2001), P. sylvestris (Lelu et al. 1999), P. monticola (Percy et al. 2000), P. pinaster (Lelu et al. 1999, Lelu et al. 2006), and P. halepensis (Montalban et al. 2013). With this method, besides the fact that no pretreatment is required for germination, the ET may remain in the same medium throughout the entire maturation period, which lasts up to 12 weeks (Klimaszewska et al. 2007).

In conclusion, based on our results, the production of somatic embryos and the subsequent plant conversion was feasible by both maturation methods for the efficient propagation of Japanese black pine. Although this improvement represents a promising perspective for an efficient mass propagation of this species, further studies are required to establish an optimal protocol for the commercial production of high quality somatic plants. Evidence that culture conditions during somatic embryo development may affect the quality and the growth performance of somatic plants has been reported for several conifers (Grossnickle et al. 1994, Bozhkov and von Arnold 1998, Hogberg et al. 2001). Therefore, the growth characteristics of somatic embryo–derived plants obtained by both methods will be monitored in the field.

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Megagametophyte *in vitro* tissue culture of *Pinus sibirica* and *Larix sibirica* and somaclonal variation

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Abstract

The main objectives of the study were to: 1) develop a procedure for obtaining a haploid embryogenic callus in a megagametophyte *in vitro* tissue culture of Siberian pine and Siberian larch; 2) investigate the characteristic features of embryogenesis in these cultures (morhogenesis and cytological analysis; 3) genotype these cultures using microsatellite (SSR) markers, and 4) conduct molecular genetic studies of gynogenic cultures (DNA analysis). The objectives 3) and 4) were completed only for Siberian larch. Megagametophytes of *P. sibirica* and *L. sibirica* were cultured *in vitro* on 1/2 LV and AI medium (patented), supplemented with sucrose (30 gl^{-1}), L-glutamine (1 gl^{-1}), casein hydrolysate (0.5 gl^{-1}), mesoinositol (1 gl^{-1}), and ascorbic acid (0.3 gl^{-1}). 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (6-BAP) were at concentrations of 2 and 1 mgl^{-1}, respectively. Callus formation began on the cut surface of megagametophytes on the 7th day of cultivation. By the 14th day, callus growth was observed in the apical region of the megagametophytes. The first stage of embryogenic competence of explants in the megagametophyte culture consisted of the formation of long vacuolated cells (300-500 μm long). Then, the formation of a nuclear coenocyte was observed. Free nuclei moved to either cell pole to form embryoids. In the proliferating megagametophyte culture embryogenic cells (initials) experienced multiple divisions and formed embryo globules, at the distal ends of which embryonic tubes were developed and formed the suspensor. Embryoid formation was exhibited at this stage. Eleven nuclear simple sequence repeat (SSR) microsatellite markers were genotyped in calli obtained from *L. sibirica* megagametophytes to check the calli's stability. Microsatellite genotypes demonstrated high frequency of mutations in the obtained megagametophyte-derived callus cultures. All cultures contained new mutations in one or more microsatellite loci. Four lines were haploid and can potentially be used for whole genome sequencing.

Introduction

Siberian pine (*Pinus sibirica* Du Tour) and Siberian larch (*Larix sibirica* Ledeb.) are the most widespread woody plant species in the Siberian forest. Siberian pine is hard to regenerate naturally, because it needs vernalization and has a longer (27 months) generation cycle as compared to other pine species. It is also characterized by a high polyembryony (there could be up to 16 embryos per seed). Moreover, natural Siberian pine stands contain a small proportion (0.2%) of unique genotypes with a highly accelerated generation cycle of only two months between pollination and fertilization instead of one year normally. However, no embryos were found in the seeds from these trees. It is of great interest to study trees with these unique genotypes, but it is impossible to propagate them by seeds (Tretyakova 1990). We hope that a megagamethophyte *in vitro* tissue culture can be used to

generate somatic embryos for further propagation and study of these trees.

Siberian larch possesses an extremely high plasticity in their vegetative and generative organs, which makes larches different from other representatives of the family Pinaceae. At the same time, Siberian larch is characterized by a rather irregular yield in a long term cycle and a very low seed quality. In addition, this species is severely affected by a larch bud gall midge that negatively influences the yield of larch forests. Trees that are resistant to this pest only rarely occur in larch forests (Tretyakova et al. 2006). Therefore, it is tempting to investigate if *in vitro* tissue culture can be be used to generate somatic embryos for further propagation and study of these trees.

In order to solve the problem of plant regeneration in Siberian pine and Siberian larch the best approach is to develop programs based on modern biotechnological methods of microclonal propagation, such as somatic embryogenesis. Somatic embryogenesis is one of the most promising tools in forest biotechnology, which is widely used in many countries in implementing the Multi-Varietal Forestry (MVF) programs (Park 2002; Park et al. 2010). Usually mature and immature zygotic embryos (Nagmani and Bonga 1985) and, more rarely, megagametophytes (Klimaszewska and Cyr 2002) or fragments of vegetative shoots (Malabadi and Van Staden 2005) are used as explants.

In vitro haploid tissue or cell cultures are of particular interest for studying developmental processes and for other molecular genetics experiments. Recently, haploid cultures have become of great interest due to their potential for use in whole genome *de novo* sequencing of conifers, such as pine (Arrillaga et al. 2014) and larch (Krutovsky et al. 2014a). These conifers are characterized by huge genome sizes (14–30 Gb), which are several times larger than the human genome (3.2 Gb; Krutovsky et al. 2012a,b,c; Krutovsky 2014, Krutovsky et al. 2014b). Haploid cultures have only one set of chromosomes, which can greatly facilitate the assembly of a genome sequence due to the lack of allelic variation (Krutovsky et al. 2012c).

However, a high somaclonal variation, which is typical for tissue cultures (Bnojwani and Dantu 2013), can potentially complicate their use. Therefore, tissue cultures should be tested for somaclonal variation before their use, and tissue culture methods should be optimized to prevent or minimize somaclonal variation, especially chromosomal aberrations. Nuclear microsatellite markers or simple sequence repeats (SSR) are widely used to study somaclonal variation (Isoda and Watanabe 2006; Khasa et al. 2000, 2006). Nuclear SSR markers were used to study the genetic stability in embryogenic cell cultures in maritime pine (*Pinus pinaster*; Marum et al. 2009; Arrillaga et al. 2014), Scots pine (*Pinus sylvestris*; Burg et al. 2007), cork oak (*Quercus suber*; Lopes et al. 2006), pedunculate oak (*Quercus robur*; Endemann et al. 2001; Wilhelm et al. 2005), quaking or trembling aspen (*Populus tremuloides*; Rahman and Rajora 2001), Arabian coffee (*Coffea arabica*; Etienne and Bertrand 2003), pepper (*Capsicum annuum* L.; Ryu et al. 2007), and many other plants. Similar to traditional diploid *in vitro* tissue cultures, haploid cultures are also characterized by genetic instability, leading to increased frequency of aneuploidy and mutations (von Aderkas and Anderson 1993; von Aderkas et al. 2003), although spontaneous diploidization of haploid cultures can lead to genetic stabilization (Pattanavibool et al. 1995).

It is worth noting that somaclonal variation was not detected in several studies in spruce (*Picea abies*, *P. glauca*, *P. mariana P. glauca*, Mo et al. 1989; Eastman et al. 1991; Fourre et al. 1997; Tremblay et al. 1999; Harvengt et al. 2001; Helmersson et al. 2004) and pine (*Pinus pinaster*; Arrillaga et al., 2014), and was low in *Coffea arabica*, which could be explained by culture conditions that allow moderate cell proliferation (Landey et al. 2013).

The main objectives of the study were to: 1) develop a procedure for obtaining a haploid embryogenic callus from a megagametophyte *in vitro* tissue culture of Siberian pine and Siberian larch; 2) investigate the characteristic features of embryogenesis in these cultures (morhogenesis and cytological analysis); 3) genotype these cultures using microsatellite (SSR) markers, and 4) conduct the molecular genetic studies of gynogenic culture (DNA analysis). The objectives 3) and 4) were completed only for Siberian larch.

Material and methods

The plant material was obtained from a grafted clonal plantation of Siberian pine in the Western Sajan Mountains and from Siberian larch trees growing in the Arboretum of the Institute of Forestry, Siberian Branch of Russian Academy of Sciences, Krasnoyarsk. Explants for callus induction were obtained from megagametophytes of 20 trees representing five clones of Siberian pine and 18 trees of Siberian larch. The latter ones were ranked as resistant (R) or susceptible (S) to the larch bud gall midge (*Dasyneura laricis* F. Lw.). To generate the megagametophyte haploid cultures, megagametophyte tissues from megastrobili were isolated at the following different developmental stages: 1) coenocytic or free-nuclear (Siberian pine – 15.05.2011 & 2012, Siberian larch –

10.06.2012); 2) fertilization (Siberian pine – 10.06.2012 & 2013, Siberian larch – 25.06.2012); 3) cleavage polyembryony (Siberian pine – 25.06.2012 & 2013, Siberian larch – 25.06-10.07.2012); 4) globular embryo (Siberian pine – 1-10.07.2012 & 2013, Siberian larch – 25.06-10.07.2012); and 5) initiation of the embryo cotyledons (Siberian pine – 10-15.07.2012 & 2013, Siberian larch – 15.07.- 20.07.2012).

Seeds that were used to isolate megagametophytes for *in vitro* culturing (stages 1-5) were pretreated with an aqueous potassium permanganate solution, then washed in running water, and separated from the integument. The extracted megagametophytes were sterilized with sodium hypochlorite for 10 min, washed three times in sterile distilled water, and incubated in 10% hydrogen peroxide for 5 min. Then, each megagametophyte was aseptically splitinto two parts or fragments, the embryo was removed, and the explants were placed on a medium, six or seven pieces per plate.

Megagametophytes of Siberian pine were cultured *in vitro* on 1/2 LV (Litvae et al. 1985), Siberian larch on MSC (Becwar et al. 1990) and AI medium (the RF patent number 2456344; http://www.freepatent.ru/images/patents/ 5/2456344/patent-2456344.pdf), supplemented with sucrose (30 g/L), L-glutamine (1 g/L), casein hydrolysate (0.5 g/L), mesoinositol (1 g/L), and ascorbic acid (0.3 g/L). 2,4-Dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (6-BAP) were at concentrations of 2 and 1 mg/L, respectively.

Cytological and cytogenetic analysis. For cytological analysis, squashed cell preparations were stained with an aqueous safranin ("Chmapol", Praga, Czechoslovakia) solution (2 mg/mL). For cytogenetic analysis, preparations were stained with acetic hematoxylin (1 g of hematoxylin per 100 mL 45% acetic acid). The preparations and cell structures were visualized and measured using the MBI-6 microscope (LOMO, Russia). Statistical analysis and single factor ANOVA were conducted following standard procedures in Microsoft Excel. Morphological changes were recorded using a digital camera Fujifilm FinePix S7000 (Japan).

Microsatellite genotyping. It was done only for the larch callus. DNA was isolated from needles of the maternal larch tree № 30 resistant to the larch bud gall midges and from the 5-week old callus of six cell culture lines (10, 12, 13, 15, 16, 18) derived from the megagametophytes of this tree, and were used for microsatellite genotyping. Total DNA was extracted using the modified CTAB method (Devey et al. 1996). The isolated DNA was amplified using PCR with 11 pairs of oligonucleotide primers designed previously for microsatellite genotyping of different larch species (Isolda and Watanabe 2006; Khasa et al. 2000, 2006; Chen et al. 2009). Microsatellite loci and PCR conditions used in this study are described in Table 1.
The GenePak® PCR Core kits (IsoGene Laboratory Ltd, Moscow, Russia) were used for PCR. Amplification products were separated by electrophoresis in 6% (*w/v*) polyacrylamide gels using vertical electrophoresis chambers with Tris-Borate-EDTA (TBE) electrode buffer and were visualized using a gel documentation system. The molecular weight of the fragments was determined by comparison with DNA size standards (plasmid DNA pBR322 digested by the *Hpa*II restriction enzyme) using the PhotoCapt program.

Table 1. *Microsatellite loci and the PCR conditions used for genotyping of in vitro haploid cell lines of Siberian larch*

Microsatellite locus	Repeat	Number of alleles*	Fragment size (bp)
bcLK056	$(AG)_{20}$	12	174-200
bcLK066	$(TG)_{12}$	6	155-172
bcLK189	$(AG)_{17}AT(AG)_6$	20	162-196
bcLK194	$(AG)_{17}$	9	116-136
bcLK224	$(AG)_{17}$	5	152-168
bcLK225	$(GA)_{20}$	18	180-213
bcLK232	$(AG)_{19}$	5	142-178
bcLK260	$(TG)_{14}(AG)_9$	7	115-126
bcLK235	$(TC)_9(AC)_2AG(AC)_{14}$	19	177-220
UBCLXtet_1-22	$(TATC)_9(TA)_{12}$	6	175-250
UAKLly6	$(GT)_{17}$	20	214-264

* From published data. Annealing temperature was 63–-53ºÑ (touchdown) for bcLK056, bcLK066, bcLK189, bcLK194, bcLK224, bcLK225, bcLK232, bcLK260, bcLK235 (Isoda and Watanabe 2006), and 58ºÑ for UBCLXtet_1-22 (Chen et al. 2009) and UAKLly6 (Khasa et al. 2000, 2006).

Results

Siberian pine

The successful induction of callus formation from Siberian pine megagametophyte cells depended on the developmental stage of the explant. There was no callus formation when using megagametophytes from all developmental stages except the stage of globular embryo and initiation development of the zygotic embryo cotyledons (1-15.07.2012&2013).

The Siberian pine explants formed callus after 7–10 days *in vitro* in the corrosive cavity along the longitudinal axis of a dissected megagametophyte placed on the medium with a cut side up. The callus mass was white and had a dense or loose structure. After a month of culturing, the callus covered the entire cut surface in the vast majority of explants (Fig. 1). By the end of the initiation stage (30 days), the callus weight ranged from 0.052 ± 0.001 g to 0.088 ± 0.001 g.

When calli were transferred to the proliferation medium with a reduced content of sucrose and 6 - BAP, the embryogenic callus was formed in only two clones (nos. 283/26 and 153/13). In these clones, the number of explants that reached the proliferation stage was 68.7 and 66.7%, respectively. After 120 days of culturing, the weight of proliferating calli was 0.077 ± 0.001 g and 0.098 ± 0.001 g for two clones, respectively (Fig. 2). After 4 months of culturing, the calli became completely necrotic.

Cytological and cytogenetic analysis. After 7–10 days of megagametophyte culturing, stretching of cells along the longitudinal axis of the cut was observed. By the end of the first month of culturing in the initiation medium, the formed callus consisted of elongated vacuolated cells whose length reached, on average, 495 ± 24.2 μm. The majority of these cells did not complete cytokinesis; as a result, two nuclei in one cell formed (Fig. 2A), which were divided and formed a four-nuclear coenocyte. These four-nuclei migrated to one of the poles of the long cell (Fig. 2B), where the formation of cell walls was observed, and four small cells with a dark cytoplasm (embryoid initials) were formed. The basal end of these cells adjoined to the long anuclear cells, which then probably died off (Fig. 2C). The initial cells of embryoids continued to divide and formed globules after 2 months of culturing. On the proliferation medium, the cells of the lower part of the globule (which adjoined the long degraded vacuolated cell) began to elongate and formed a suspensor (Figs.2D, 2E) consisting of six to seven long, linearly arranged vacuolated cells. A somatic embryo was formed, which consisted of an embryonic globule and a suspensor (Fig. 2F).
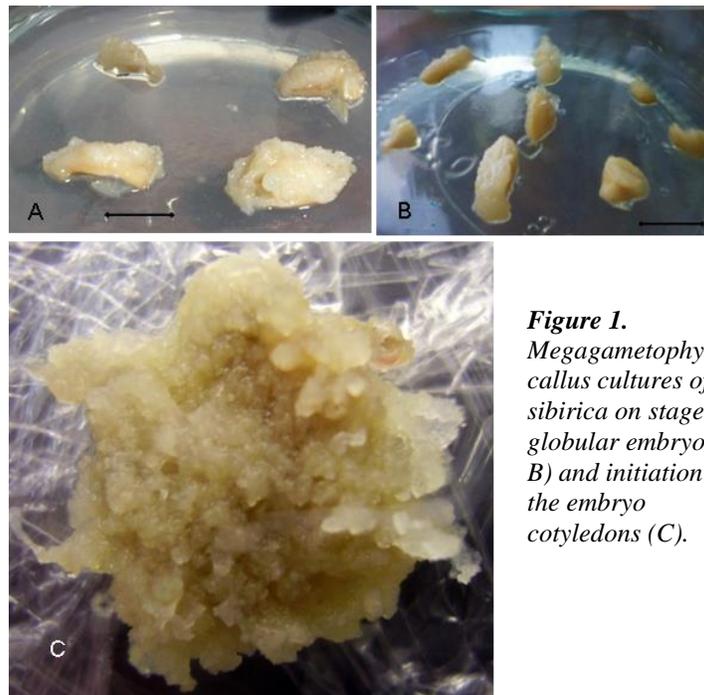
Figure 1. Megagametophyte callus cultures of P. sibirica on stage globular embryo (A, B) and initiation of the embryo cotyledons (C).

An active formation of the embryonal suspensor mass (ESM) in the callus was observed (Fig. 2G). In the outermost suspensorcells, the formation of several nuclei was observed, which migrated to the distal end (Fig. 2H) and formed a four-nuclear coenocyte. Then, the four-cells yielded aggregates and, finally, new globular somatic embryos formed (Fig.2I). Thus, for the first time we have derived the embryogenic cell cultures from Siberian pine megagametophytes, which were capable of self-maintenance and production of embryoids.
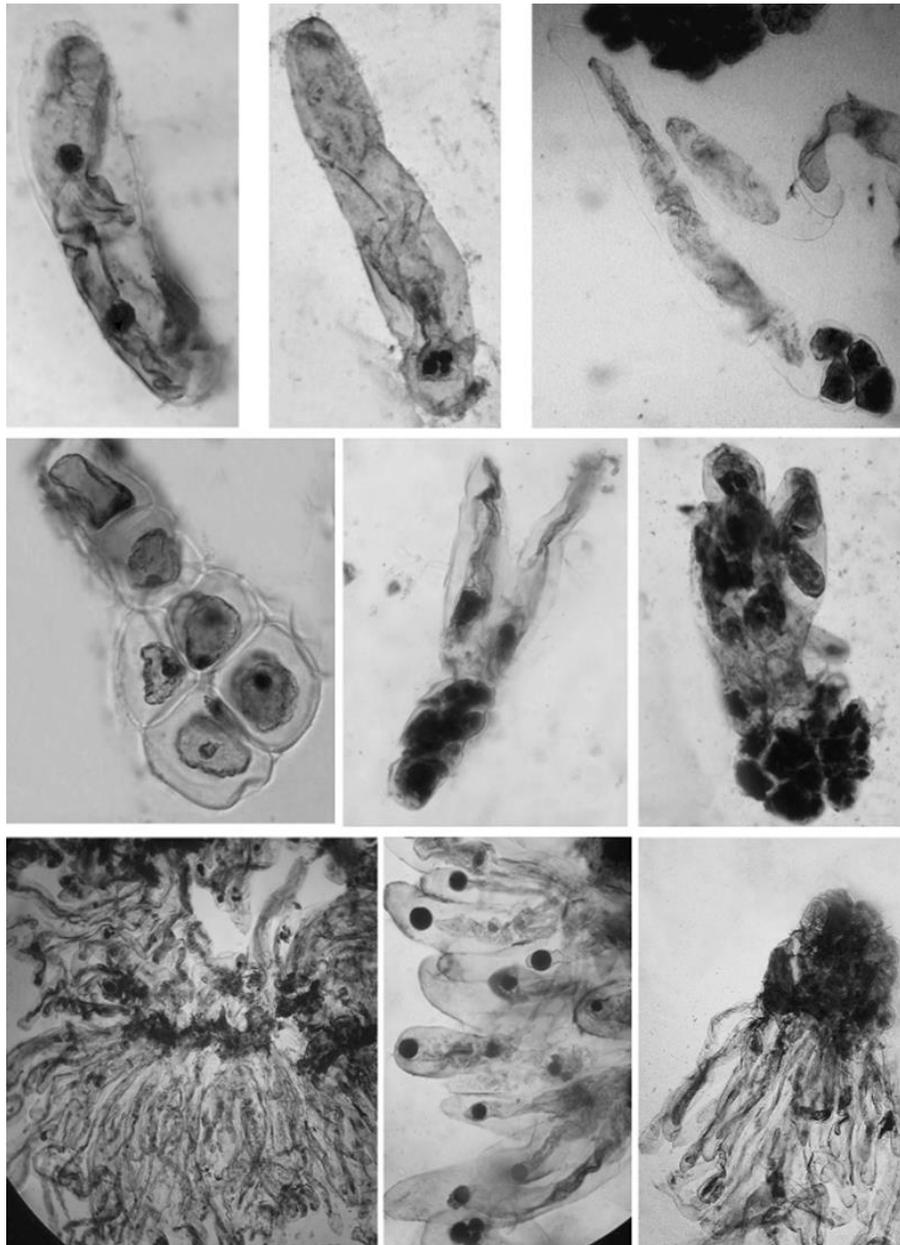
Figure 2. Formation of embryoids in the Siberian pine megagametophyte culture (A) formation of a two-nuclear coenocyte; (B) migration to four nuclei of the coenocyte to the long cell pole; (C) formation of four initial cells and a nuclear-free cell tube; (D) formation of a suspensor cell; (E) formation of two cells of the suspensor; (F) globular embryoid with the suspensor; (G) group of embryos; (H) migration of nuclei in the suspensor cells to the distal end; (I) formed embryoid

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Siberian larch

Callus formation from Siberian larch megagametophyte cells was succeeded using initiation and development of the zygotic embryo cotyledons stages (10-15.07.2012). Callus formation was detected only in explants from eight out of 18 trees using AI and MSG media. An intensive callus formation (91%) was observed on the AI media for tree № 30, which is resistant to the larch bud gall midges (Table.2).

Table 2. *Percent of callus derived from megagametophyte explants of the Siberian larch trees after 30 d cultivation in different media*

Tree^	Medium*	
	MSG	AI
1 R	10.00 ± 0.21	0
2 R	9.00 ± 0.15	7.00 ± 0.22
30 R	0	90.91 ± 0.52
4 S	10.00 ± 0.21	7.00 ± 0.22
5 S	0	14.29 ± 0.24
6 S	0	0
7 S	30.00 ± 0.27	0
8 S	0	0
9 S	0	0
10 S	11.00 ± 0.28	0

^ Trees were either resistant (R) or susceptible (S) to the bud gall midge (Dasyneura laricis F. Lw.)
* See Materials and Methods for medium description

Callus formation began on the cut surface of megagametophytes on the 7th day of culturing (Fig. 3*A,B*). By the 14th day, callus growth was observed in the apical region of the megagametophytes (Fig. 3C,D).
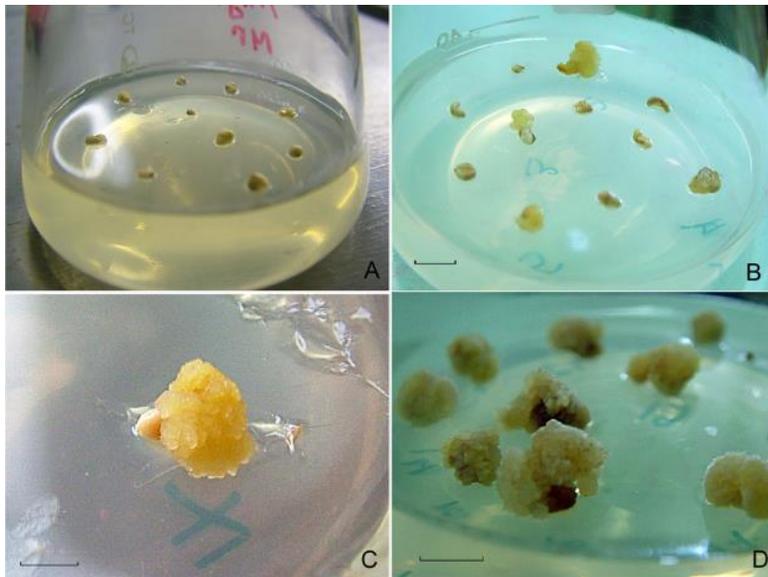
Figure 3. Initiation and development of callus generated from Siberian larch megagametophytes on the 7th (A), 14th (B), and 36th (C, D) days of cultivation.

The growth dynamics of callus cultures derived from the four genotypes was recorded for two months after planting (Fig. 4). Fresh callus weight ranged from 0.26 to 2.0 g among these genotypes. Eighteen viable cell lines (CLs) were obtained from trees № 1, 5, 7, and 30. CL 1 (derived from the maternal tree № 1) and CLs 2–7 (from tree № 7) had solid yellow-white calli and rounded cells on the MSG medium. CL 8 derived from the maternal tree № 5 had a friable yellow-white callus and elongated cells on the AI medium. CLs 10–19 were derived from the maternal tree №

30 on the AI medium: CL 10 had a loose white-yellow callus and elongated cells, but CLs 11–19 had thick white-yellow calli and round cells. Over time, the calli turned brown in color, and growth stopped after 30–40 wk.
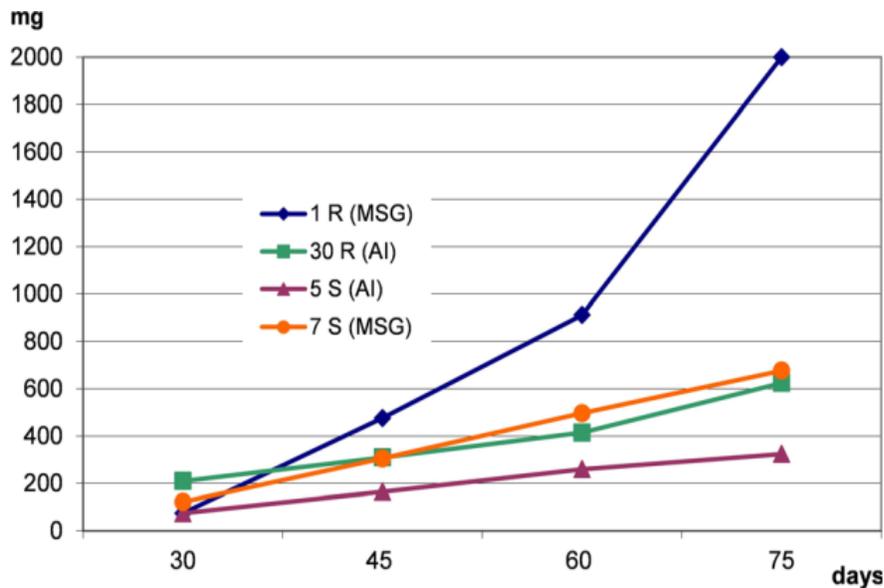
Figure 4. Weight of fresh callus (mg) in the megagametophyte explant cultures that formed on the MSG and AI media and obtained from Siberian larch trees, two of which (no. 1 and no. 30) were resistant (R) and two (no. 5 and no. 7) were susceptible (S) to the larch bud gall midge (Dasyneura laricis F. Lw.).

Cytological and cytogenetic analysis. Cytological analysis demonstrated that calli from individual CLs differed from each other by the cell morphology. Calli from CLs 8 and 10 consisted of elongated cells of 200–250 microns, i.e., cells acquired characteristics of embryogenic cell competence – a transition of somatic cells towards embryogenesis (Fig. 5*A*, *B*). All other CLs had rounded cells, a characteristic for non-embryogenic callus (Fig.5C,D).
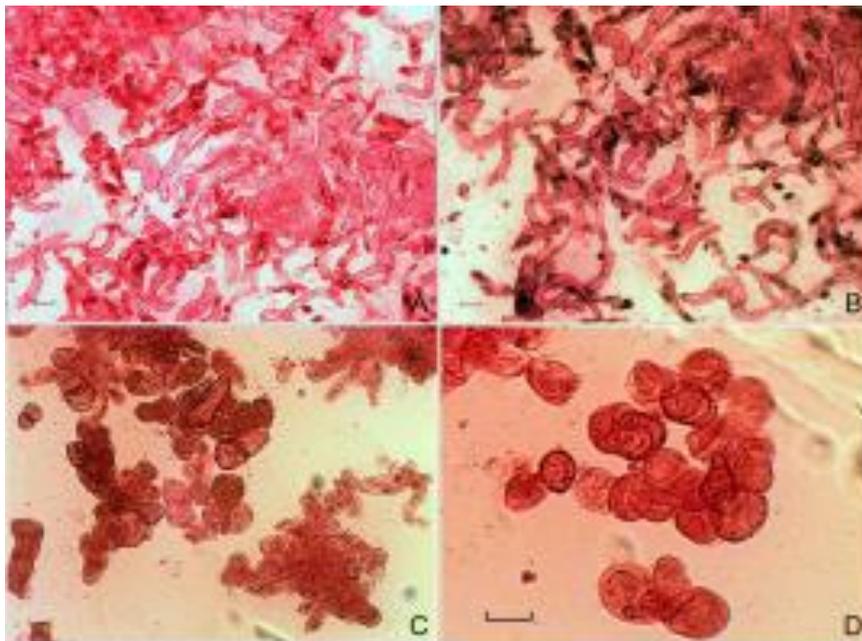
Figure 5. Cell line 10 from tree no. 30 after 40 d of cultivation (A, B) and cell line 2 from tree no. 7 after 34 d of cultivation (C, D).

Cytogenetic studies demonstrated that CLs 1–8, 14, 17, 19 contained diploid chromosomes (Fig. 6*A,B,C,E*). Haploidy was suspected for four CLs 10, 15, 16, and 18 derived from the maternal tree № 30 (Fig. 6*D,F*).
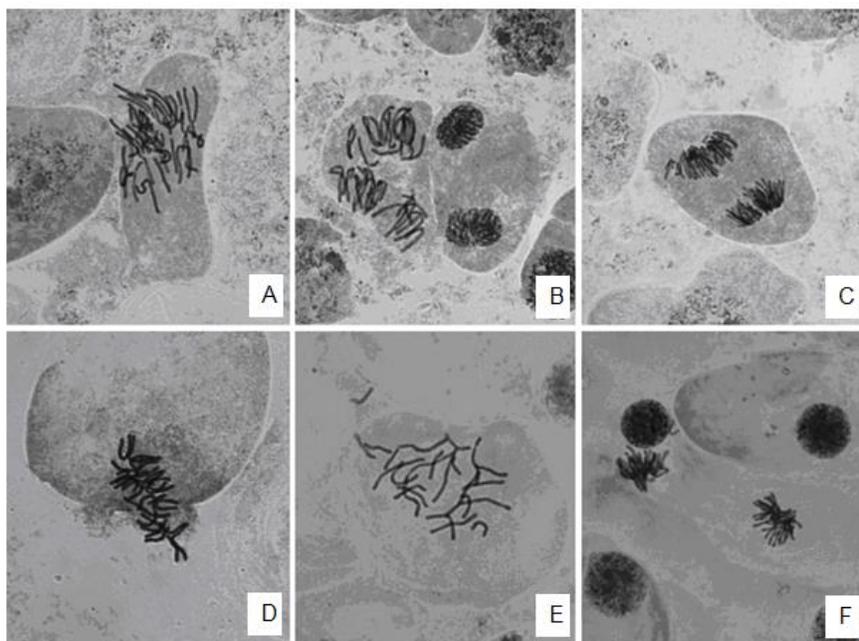
Figure 6. *Mitosis in cell cultures generated from Siberian larch megagametophytes. A–C, diploid cell line 1; D and E, haploid cell line 10; and F, haploid cell line 16*

Microsatellite genotyping of the cell lines. CLs 10, 12, 13, 15, 16, and 18 were used for microsatellite analysis at the age of two to five months. DNA isolated from needles of the maternal tree № 30 was used as a control. The study found that only one locus (*UAKLly6*) was heterozygous for the maternal tree № 30, and therefore could serve as a diagnostic locus for haploid CLs (Table 3). At this locus, none of the CLs contained both maternal alleles, so presumably they were all haploid. However, all CLs contained unique alleles at 3–7 loci, which were absent in the maternal tree, and were likely to have a mutational origin as a result of a very high rate of somaclonal variation. Not a single CL of Siberian larch, obtained in the *in vitro* culture, was matching the maternal tree alleles in all 11 nuclear microsatellite loci studied. Practically, all CLs were chimeric, showing mutational allelic variation in addition to the maternal allele. Moreover, the CL 10 did not have any of the two maternal alleles at the locus *UAKLly6*. CLs 15, 16, and 18 also had none of the maternal alleles for locus *bcLK260* (Table 3; Fig. 7).

Table 3. *The microsatellite alleles (bp) and genotypes of the maternal tree [1] 30 and six cell lines 10, 12, 13, 15, 16 and 18 obtained from this tree^*

Microsatellite locus	[1] 30	10	12	13	15	16	18
bcLK056*	139/139	139/173	139/171	139/145	139	139/145	139/145
bcLK066	151/151	151	151/147	151	151/147	151	151/147
bcLK189*	168/168	168	168/162	168/160	168	168	168
bcLK194*	107/107	107/111	107	107	107/105	107	107/113
bcLK224	130/130	130	130/128	130/128	130	130	130/128
bcLK225	166/166	166	166	166/198	166	166	166
bcLK232	134/134	134	134/134	134/134	134/134	134/134	134/134
bcLK235	204/204	204	204/208	204	204/208	204/208	204/208
bcLK260*	102/102	102	102	102	104	104	106
UBCLXtet_1-22	180/180	180	180	180	180	180	180
UAKLly6*	232/252	248	232	232	232/248	232/248	232/248

^ *Alleles from the mother tree is shown in bold font.*
* *Alleles of these five loci are also presented in Fig. 5. In bold are mutant alleles detected in cell lines, but not in the mother tree*
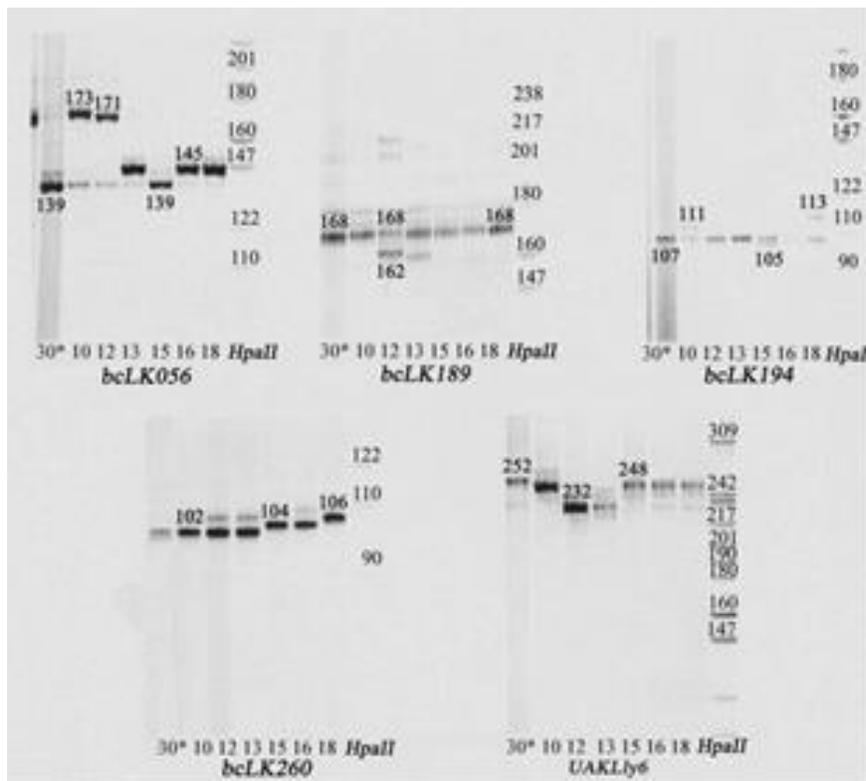
Figure 7. Polyacrylamide gel electrophoregrams of nuclear microsatellite loci bcLK056, bcLK189, bcLK194, bcLK260, and UAKLly6. Numbers immediately adjacent to the bands indicate the amplified microsatellite allele lengths (bp). Lane numbers at the bottom of the image correspond to cell lines derived from the mother tree no. 30. In the leftmost lanes are microsatellite loci amplified from the DNA isolated from the mother tree no. 30 needles (asterisk). In the rightmost lanes are standard size marker fragments derived from plasmid pBR322, digested by the restriction endonuclease ere susceptible (S) to the bud gall midge (Dasyneura laricis F. Lw.).

Discussion

Callus induction of Siberian pine and Siberian larch significantly depended on the development stage of the megagametophyte. There was no callus formation when using megagametophytes at the free nuclear stage, fertilization stage and cleavage stages. The intensive callus mass of Siberian pine and Siberian larch was developed using the globular embryo and at the initiation of the zygotic embryo cotyledons stages. The study by Arrillaga et al. (2014) demonstrated that callus culture of immature megagamethophytes of *Pinus pinaster* did not proliferate. Only mature megagamethophytes isolated from cones before seed dispersal were able to develop haploid callus (Arrillaga et al. 2014).

In Siberian larch and Siberian pine, the first stage of embryogenic competence of explants in megagamethophyte culture consisted in the formation of long vacuolated cells. The cells elongated first and then formed coenocytes with four nuclei. This process was accompanied by the migration of the nuclei to one of the poles, where four cells were formed. These cells actively divided and formed a somatic embryo globule, at whose distal end suspensor cells were developed. Therefore, the first stage of somatic embryogenesis in the cultures of megagametophytes of Siberian pine and Siberian larch is the acquisition of embryogenic competence which manifests itself in the elongation and polarization of cells.

The embryogenic callus formation in a megagametophyte culture was described for European larch (*L. decidua*), whose explant cells produced long (suspensor) cells with a multinuclear structure. The nuclei migrated to one of the poles, where cell walls formed around them, which led to the formation embryoids (von Aderkas and Bonga 1988; von Aderkas et al. 1991). In the Siberian pine and Siberian larch, the formation of embryos in the megagametophyte culture was similar to that in the European larch. This suggests that haploid embryogenesis in coniferous species is a well-organized system that can serve as a model system to study the molecular and genetic events at the very early stages of plant development. An intensive callus formation (91%) was observed from megagametophyte tissues of Siberian larch on the AI media. (Table.2). Of the six CLs derived from haploid tissue of the female gametophyte of tree № 30, which were studied cytogenetically and genotyped for 11 microsatellite loci, four lines were haploid and can potentially be used for the whole genome sequencing, which would greatly simplify

de novo genome assembly. A total of sixteen haploid callus lines from megagametophytes of *Pinus pinaster* were also studied by microsatellite analysis. One of these lines is being used as a source of DNA for massive sequencing of the maritime pine genome (Arrillaga et al. 2014).

Our results of cytogenetic and microsatellite analyses demonstrated the genetic instability of *in vitro* CLs derived from Siberian larch megagametophytes. From analysis of 11 nuclear microsatellite loci, none of the resulting CLs retained the original maternal haplotype. Practically, all CLs were chimeric and contained mutational allelic variation in addition to the original maternal allele at one or more loci. It is interesting to note that the CL 10 did not have any of the two maternal alleles at the locus *UAKLly6*. CLs 15, 16, and 18 also had none of the maternal alleles for locus *bcLK260* (Table 3; Fig. 7). The observed mutations in these loci most likely occurred at the early stage of development of these lines, and the mutated cells either completely overgrew and replaced the original (unmutated) cells or were singularly selected during subculturing.

Genetic instability is typical for cells of proliferating embryogenic callus maintained for a long time. For instance, mutations were detected in 7 microsatellite loci in the callus of several CLs of *Pinus pinaster* after 6 mo of culturing, and then in 9.6% of the regenerated lines (Marum et al. 2009; Miguel and Marum 2011). A high mutation rate for microsatellite sequences was detected in embryogenic CLs of *Pinus sylvestris* (Burg et al. 2007). The frequency of polyploidy (mostly tetraploidy) reached 8% in seven-year-old embryogenic CLs of *Quercus robur* (Enderman et al. 2001). The mutation rate during somatic embryogenesis in this species was 29.2%−62.5% depending on the donor tree genotype (Lopes et al. 2006). Significant somaclonal variation was detected in the 12-month-old embryogenic cultures derived from different genotypes of *Coffea arabica* (Etienne and Bertrand 2003). Somatic embryogenesis led to the appearance of tetraploid cells in *Picea abies* (Lelu 1987), polyploidy in *Pinus nigra* (Salajova and Salaj 1992), and trisomy in *Larix marschlinsii* (Nkongolo and Klimaszewska 1995) and *Pinus radiata* (O'Brien et al. 1996). Variation of endogenous hormones, polyamines, and DNA methylation was found in embryogenic cultures of different age in *Pinus pinaster* (Klimaszewska et al. 2009). In general, the longer the culture was maintained *in vitro*, the more changes appeared at the genetic level. As a result, heterogenous cells appeared inside the callus with genotypes quite distinct from the parental tree, which was also observed in our CLs derived from Siberian larch megagametophytes.

There is also a study reporting that the callus culture of haploid megagametophytes from *Larix decidua* generated by Nagmani and Bonga in 1985 and maintained for two years in a medium without hormones did not result in changes to the ploidy, i.e., callus cells remained haploid (von Aderkas and Bonga 1988). Later studies showed that among these CLs of *L. decidua* only one of seven CLs was a stable haploid line, while other CLs contained aneuploid cells (chromosome number varied from 11 to 27), as well as diploid and tetraploid cells (von Aderkas and Anderson 1993). After eight years or more of *in vitro* culture all CLs of *L. decidua* became diploid, and chromosome numbers varied from 24 to 31 in different CLs (von Aderkas et al. 2003). One of the possible reasons for changes in ploidy in cultured cells could be a fusion of the nuclei, which was proposed by Mazia (1961). This phenomenon was observed in *Larix decidua* (von Aderkas and Bonga 1988), *L. eurolepis* and *L. leptolepis* (von Aderkas et al. 1990). Multinucleated cells were found in the megagametophyte culture of Siberian pine during initiation of somatic embryogenesis (Trety'akova and Voroshiliva 2014).

However, several studies did not detect somaclonal variation during somatic embryogenesis of *in vitro* culture of several gymnosperms, such as *Picea abies* (Mo et al. 1989; Fourre et al. 1997; Harvengt et al. 2001; Helmersson et al. 2004), *P. glauca* and *P. mariana* (Eastman et al. 1991; Tremblay et al. 1999), *Pinus pinaster* (Arrillaga et al. 2014). It is assumed that the cells with changes in the chromosome number and/or DNA rearrangements observed at the callogenesis were eliminated during long culture period and following formation of somatic embryos that were identical to the original explants (Helmesson et al. 2004; Klimaszewska et al. 2009).

Stability of three nuclear microsatellite loci was detected in *Picea glauca* during somatic embryogenesis and in the regenerated lines; mature somatic embryos and regenerated plants were identical to the original explants (Helmersson et al. 2004). Chromosome count, flow cytometry and seven polymorphic microsatellite have been used to confirm the haploid status of megagametophyte-derived callus lines of *P. pinasres* (Arrillaga et al. 2014).

It is interesting to note that the new mutations often resulted in the same allele length in different lines of L, sibirica (Table 3). This can possibly be explained by the canalized mode of mutations in the simple sequence repeat regions that are expected to follow the stepwise mutation model. For instance, 128 bp and 132 bp size mutations are expected to be the two mostly likely mutations for the original allele size of 130 bp in the *bcLK224* locus with the AG dinucleotide repetitive motif. Therefore, it is not surprising that the original allele in the *bcLK224* locus mutated to a 128 bp mutant allele in three CLs (12, 13 and 18) among the total six CLs genotyped. Similar explanations can be applied to all other loci with the same or similar allele length mutations in multiple lines. Nevertheless, there

seem to be a bias toward some mutations that occurred more often than others. However, more studies are needed to determine whether it was plant growth regulator treatments that promoted particular mutations or whether some other factors responsible.

Thus microsatellite analysis conducted revealed a high frequency of somatic mutagenesis and genetic heterogeneity in the obtained gynogenic cultures after two months of culturing that can potentially complicate *de novo* genome assembly.

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Induction of somatic embryogenesis in leaf and shoot apex explants derived from red oak trees. Effects of explant type, silver thiosulphate and activated charcoal on the embryogenic system

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Abstract

Among North American oak species, *Q. rubra* (red oak) is of particular ecological and economic importance, and the development of a procedure for inducing somatic embryogenesis (SE) would be of great interest for accelerating red oak improvement programs. The objectives of this study were to induce SE in explants from young trees of *Q. rubra* and to establish the culture conditions for reliable embryo proliferation of embryogenic lines by secondary embryogenesis in order to obtain suitable material for plant regeneration. Axillary shoot proliferation cultures, established from three 7- year-old *Q. rubra* trees, were used as a source of both leaf explants and shoot apex explants (1.5-2.0 mm long). Induction of somatic embryos was achieved by a culture procedure including the following steps: a) successive culture of explants in Murashige and Skoog medium supplemented with naphthalenacetic acid (21.48 μM, NAA) and 6-benzyladenine (2.22 μM, BA) for 8weeks; b) transfer onto fresh medium of the same composition but with lower concentrations of NAA and BA (0.54 μM and 0.44 μM, respectively) for a further 4 weeks; and c) culture of explants in plant growth regulator-free medium for another 8 weeks. SE was successfully induced in all three red oak genotypes, with frequencies ranging from 2.0% to 3.3%, and embryogenic lines were established and maintained by repetitive embryogenesis. To promote embryo proliferation and development of secondary embryos up to the cotyledonary stage, nodular embryogenic structures (0.2 mg fresh weight) and torpedo-stage embryos (0.8 mg fresh weight) were used as embryogenic explants and were subcultured on a different embryo proliferation medium containing 20 μM silver thiosulphate (STS). Secondary embryogenesis was promoted by use of nodular structures as embryogenic explants and culture in medium containing 0.4% charcoal, yielding an induction rate of 79.2%, in contrast to the 48.8% achieved with torpedo-embryo explants. Embryo production was also improved with the proliferation medium containing 3% sucrose, in which well-formed and easily detachable cotyledonary-shaped embryos were formed. Somatic embryos were germinated on medium supplemented with 0.44 μM BA and 20 μM STS, yielding 40% embryo germination and 11% plantlet conversion.

Keywords: nodular embryogenic structures, *Quercus rubra*, secondary embryogenesis, STS, sucrose, young trees.

Introduction

The genus *Quercus* includes important hardwood species native to the Northern Hemisphere. Among North American oak species, *Q. rubra* (red oak) is of great ecological and economic importance and is widely distributed throughout much of the eastern United States and southeastern Canada (Steiner 1993). The wood is commonly used to make furniture, cabinets, veneer, flooring, fence posts, caskets and interior trim. Oaks are generally difficult to breed, and improvement programs are constrained by limited knowledge about the genetic variation, the long period to reach reproductive maturity, production of seeds that are recalcitrant to storage by conventional procedures, and difficulties in vegetative propagation (Vieitez et al. 2012). The application of biotechnological tools such as somatic embryogenesis (SE) would be of great interest for accelerating red oak improvement programs (Vieitez et al. 2012).

Unfortunately, the few studies that have investigated SE in red oak were only successful in initiating somatic embryos from very juvenile material such as immature zygotic embryos (Gingas and Lineberger 1989; Vengadesan and Pijut 2009) and leaf discs from young seedlings (Rancillac et al. 1996). These authors did not maintain red oak embryogenic lines by repetitive embryogenesis. Induction of SE in tissues from adult trees is highly desirable as clonal tests can be established with genotypes in which phenotypic selection has been reliably performed (Barra-Jiménez et al. 2014). Important advances in cloning selected adult trees by SE have been made in developing embryogenic systems for European oaks, including *Q. suber* (cork oak) and *Q. robur* (pedunculate oak). Thus, initiation of embryogenic cultures from mature trees has been achieved by using leaf explants excised from epicormic shoots forced from branch segments (Hernández et al. 2003; Toribio et al. 2004) and from shoot apex explants and leaf explants excised from shoot cultures derived from oak trees (San-José et al. 2010). The aims of the present study were as follows: 1) to induce SE from material other than zygotic embryos by using leaf and shoot apex explants from young *Q. rubra* trees; 2) to establish embryogenic lines of this species and to define the culture conditions required for reliable embryo proliferation by secondary embryogenesis in order to yield suitable material for plant regeneration.

Material and Methods

Plant material and culture conditions

Axillary shoot proliferation cultures were established in vitro from shoot tips and nodal explants excised from forced shoots in branch segments of three 7-year-old *Q. rubra* trees designated ROQ-8, ROQ-10, and ROQ-11. Initiation of shoot cultures and proliferation of clonal axillary shoots were achieved following previously described procedures (Vieitez et al. 2009). Briefly, forced shoots were stripped of leaves and surface sterilized by successive immersion in 70% ethanol (20 s) followed by immersion for 8 min in a 0.6% solution of free chlorine (Millipore® chlorine tablets); the shoots were then rinsed three times in sterile distilled water. Shoot tips and nodal explants (1 cm long) were cut from the disinfected shoots and used to initiate shoot cultures on medium consisting of WPM (Woody Plant Medium, Lloyd and McCown 1981) supplemented with 2.22 µM benzyladenine (BA), 3% (w/v) sucrose and 0.65% (w/v) Vitroagar (Hispanlab S.A.). After 6-8 weeks, new shoots longer than 1 cm, from which the apices (2 mm) had been removed, were placed horizontally on shoot proliferation medium consisting of shoot initiation medium containing 3 mg/L $AgNO_3$ and with the concentration of BA reduced to 0.88-0.44 µM. Shoot explants were transferred onto fresh medium after 2 weeks, during the 6-week multiplication cycle. The stock shoot proliferation cultures were used as a source of explants for induction of somatic embryogenesis.

All culture media (pH 5.7) were autoclaved at 115 ºC for 20 min prior to use. Unless otherwise indicated, all cultures were incubated in a growth chamber with a 16-h photoperiod provided by cool-white fluorescent lamps (photon flux density of 50-60 µmol m^{-2} s^{-1}) at 25 ºC light/20 ºC dark (standard culture conditions).

Induction of somatic embryogenesis

The shoot apex (1.5-2.0 mm long, comprising the apical meristem and 2-3 pairs of leaf primordia) and the two most apical expanding leaves in the apical region of the shoots were excised from stock shoot cultures and used as explants for initiation of SE. Somatic embryos were induced using a procedure similar to that reported for *Q. alba* (Corredoira et al. 2012) and *Q. bicolor* (Mallón et al. 2013). Briefly, the explants were cultured horizontally (shoot apex) and abaxial side down (leaves) on induction medium (M1) consisting of MS (Murashige and Skoog 1962) mineral salts and vitamins, 500 mg/L casein hydrolysate, 3% sucrose and 0.6% Vitroagar (basal medium) supplemented with 21.48 µM naphthaleneacetic acid (NAA) and 2.22 µM BA. After 8 weeks of culture in darkness at 25 ºC, the explants were transferred to fresh medium of similar composition, but with lower concentrations of NAA and BA, of 0.54 µM and 0.44 µM respectively (M2 medium), and were cultured under standard conditions for a further 4 weeks. The explants were transferred to a third medium consisting of M2 medium without PGRs (expression medium M3) and were maintained under standard conditions for 8 weeks (i.e. overall for 20 weeks after the start of culture). Ten shoot apices and ten leaf explants were placed in 90 mm Petri dishes containing 25 ml of medium. At least 60-70 explants were used for each genotype and explant type, and the experiments were repeated three times. The embryogenic response was determined after successive culture of explants in M1, M2 and M3 media, by recording the percentage of explants forming callus and the percentage of explants forming somatic embryos in each genotype.

Somatic embryo proliferation

Primary somatic embryos were isolated from original explants of the three red oak genotypes at the end of the 8-week culture period on M3 expression medium and were transferred to Petri dishes on basal medium enriched with 0.44 μM BA with or without 0.27 μM NAA. The embryos were then proliferated by secondary somatic embryogenesis following the protocol described by Mallón et al. (2012) for *Q. robur*. To determine the culture conditions for optimizing secondary somatic embryogenesis in red oak, nodular embryogenic structures (0.8-1.0 mm and average fresh weight, 0.20 mg) and torpedo-stage embryos (2.0-2.5 mm and average fresh weight, 0.80 mg) derived from the ROQ-8 embryogenic line were used as embryogenic explants. The explants were subcultured individually in Petri dishes on three different types of embryo proliferation medium enriched with 20 μM silver thiosulphate (STS): 1) STS-basal medium supplemented with 0.44 μM BA; 2) STS-basal medium including 0.4% (w/v) activated charcoal (AC); and 3) STS-basal medium without 0.4% AC. Each proliferation medium was also prepared with 3% and 6% sucrose. Each treatment was applied to four replicate Petri dishes each containing 10 explants (nodular structures or torpedo embryos). A total of 40 embryogenic explants were cultured per explant type, proliferation medium and sucrose concentration, and the experiment was repeated twice. After culturing the explants for 5 weeks, the proliferation ability was calculated as the percentage of explants that generated secondary somatic embryos (SSEs). Embryo production was assessed by recording the number of secondary nodular embryogenic structures and the number of secondary somatic embryos at different developmental stages (bipolar embryos) produced per replicate Petri dish in each treatment.

Germination of somatic embryos

Before germination, cotyledonary-stage embryos developed on STS proliferation medium containing AC were matured on MS basal medium (half-strength macronutrients) devoid of casein hydrolysate and supplemented with 6% (w/v) sorbitol. The plates were stored at 4ºC for 2 months. Somatic embryos (6-8 mm) were then transferred to 500 ml glass jars containing 70 ml of germination medium consisting of GD medium (Gresshoff and Doy1972) supplemented with 0.44 μM BA, 0.29 μM gibberellic acid (GA$_3$), 3% sucrose and 0.6% Vitroagar. The effect of inclusion of 20 μM STS in the germination medium was also evaluated. An alternative procedure was also evaluated by placing somatic embryos (developed on proliferation medium) directly in empty Petri dishes and storing at 4ºC for two months prior to culture in germination medium. Each germination treatment was evaluated using six replicate jars, each containing six mature somatic embryos (36 embryos per treatment), and the experiments were repeated at least twice. The germination response was determined after 8 weeks by recording the number of embryos with roots only and the number of embryos that converted into plants (both epicotyl development and root development ≥ 5 mm). The percentage of explants exhibiting these responses provides an estimate of the total germination capacity of red oak somatic embryos.

Statistical analysis

A two-way ANOVA test (factorial design) was used to examine the influence of embryo proliferation medium and sucrose concentration and their possible interaction on the secondary embryogenesis ability of both explant types used. Differences in effects were considered significant at P≤0.05. The arcsine square root transformation was applied to proportional data prior to analysis, and non-transformed data are shown in figures. All statistical tests were carried out using SPSS for Windows (version 19.0, Chicago, USA).

Results

Induction of somatic embryogenesis

After culture of shoot apex and leaf explants in M1 induction medium, small compact callus tissue was mainly observed on the surface of cut ends of explants. The average frequencies of callus formation were 25%, 56%, and 39% for ROQ-8, ROQ-10 and ROQ-11, respectively. In some of these explants, somatic embryos and nodular embryogenic structures began to arise from the callus tissue during the last week of culture in M2 medium and the first 4 weeks of culture in M3 medium (Fig. 1A-C). SE was induced in all three genotypes at low and similar frequencies (Fig. 2). The percentage of leaf explants in which SE was induced was 2.3%, 3.3%, and 2.0% for ROQ-8, ROQ-10, and ROQ-11, respectively. Similar responses were obtained in ROQ-8 (2.3%) and ROQ-10 (2.2%) shoot tip explants, while no somatic embryos were initiated in ROQ-11 shoot tips (Fig. 2). Somatic embryos were isolated from original explants and multiplied by repetitive embryogenesis to establish embryogenic lines of all three genotypes.
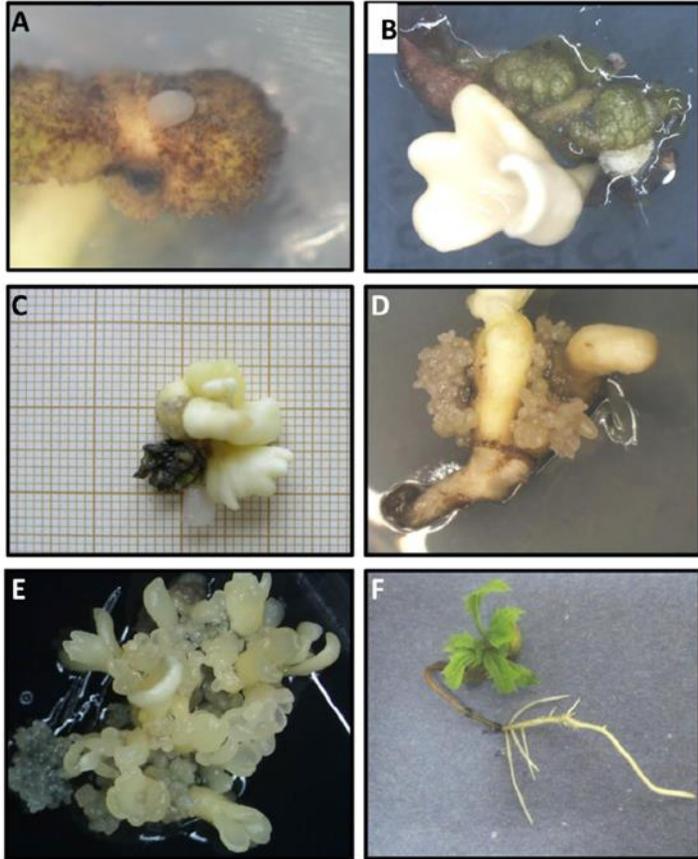
Figure 1. *Somatic embryogenesis in explants derived from stock shoot cultures of Q. rubra. A, B Somatic embryos initiated from leaf explants of ROQ-8 (A) and ROQ-10 (B) genotypes. C Somatic embryos generated from a shoot apex explant of ROQ-10 genotype. D, E Repetitive embryogenesis in ROQ-8 embryogenic line following 5-week culture on basal medium supplemented with 0.44 μM BA and 0.27 μM NAA (D), and on basal medium supplemented with 20 μM STS, 0.4% activated charcoal and 3% sucrose (E). Note the absence (D) and presence (E) of new individual cotyledonary-stage embryos. F Plant regeneration from a somatic embryo after 8 weeks of culture in germination medium.*
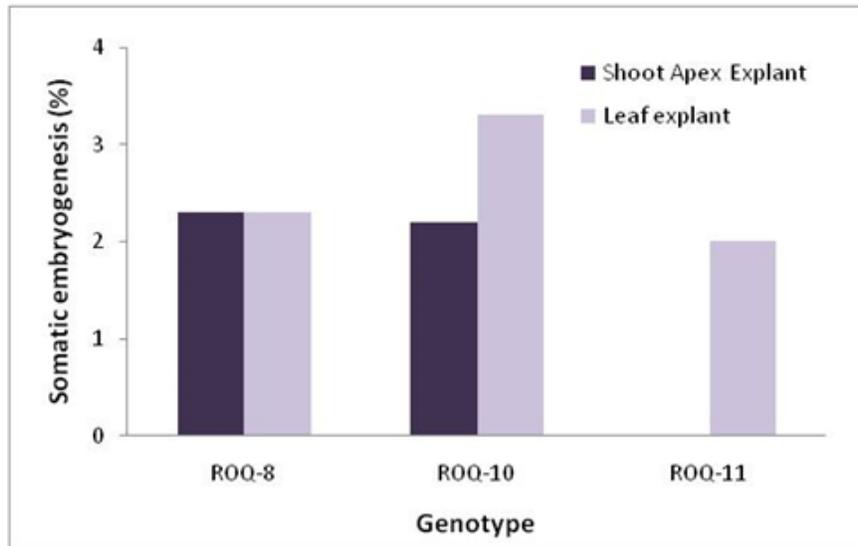
Figure 2. Effect of the genotype and explant type on the frequency of induction of somatic embryogenesis in Q. rubra.

Initially, somatic embryos isolated from original explants were cultured on basal medium containing 0.44 µM BA with or without 0.27 µM NAA. The embryos proliferated by secondary embryogenesis, generating creamy translucent nodular embryogenic structures with the occasional emergence of new secondary embryos (Fig. 1D). However, maintenance of embryogenic lines was problematic as very low embryo proliferation rates were obtained for all three red oak genotypes. To address this problem, we evaluated the suitability of nodular structures and torpedo-stage embryos for secondary embryogenesis following culture in different STS-basal proliferation media. Since very similar responses were obtained with all three embryogenic lines, only the quantitative results for the ROQ-8 line are presented.

The frequency of embryogenic explants in which secondary embryogenesis occurred was significantly affected by the type of explant ($P \leq 0.01$) and proliferation medium ($P \leq 0.001$), and a significant interaction between these factors was also observed ($P \leq 0.05$). The sucrose concentration did not significantly influence this parameter. Nodular structures yielded a better response than torpedo explants, and the latter were less affected by medium treatment than nodular explants. Thus, culture of nodular structures in charcoal-containing medium was the most efficient combination, yielding a higher rate (79.2%) of secondary embryogenesis than with torpedo explants (48.8%).

When nodular structures were used as embryogenic explants (Fig. 3A), the number of new secondary nodular structures and the number of SSEs produced per Petri dish were both significantly ($P \leq 0.001$) influenced by the proliferation medium, whereas the sucrose concentration only affected ($P \leq 0.05$) the number of SSEs. No significant interaction between these factors was observed. The yield of SSE was higher in media containing AC, and more embryos were produced by addition of 6% sucrose. Considering the different developmental stages of the SSEs generated (torpedo-, cotyledonary- ≤ 5 mm, and cotyledonary-stage>5 mm), the medium containing AC and 3% sucrose favoured the development of white-opaque cotyledonary embryos >5 mm (Fig. 2E). Moreover, the large embryos developed on AC medium supplemented with 3% sucrose were relatively longer, especially along the embryonic axis, than those developed on AC medium supplemented with 6% sucrose, with the latter also showing smaller and thicker cotyledons.

When torpedo-stage embryos were used as embryogenic explants (Fig. 3B), the number of nodular structures and the number of SSEs produced per Petri dish were stimulated by culture in AC-proliferation medium ($P \leq 0.01$). In contrast to results obtained with nodular structure explants, the sucrose concentration did not have a significant influence on the parameters evaluated, except for the development of large cotyledonary embryos (>5 mm), which was favoured by inclusion of 3% sucrose in the medium. However, embryo yield and the proportion of large cotyledonary embryos obtained from torpedo explants cultured in AC-media were both lower than those obtained with nodular structure explants (Fig. 3A and 3B).
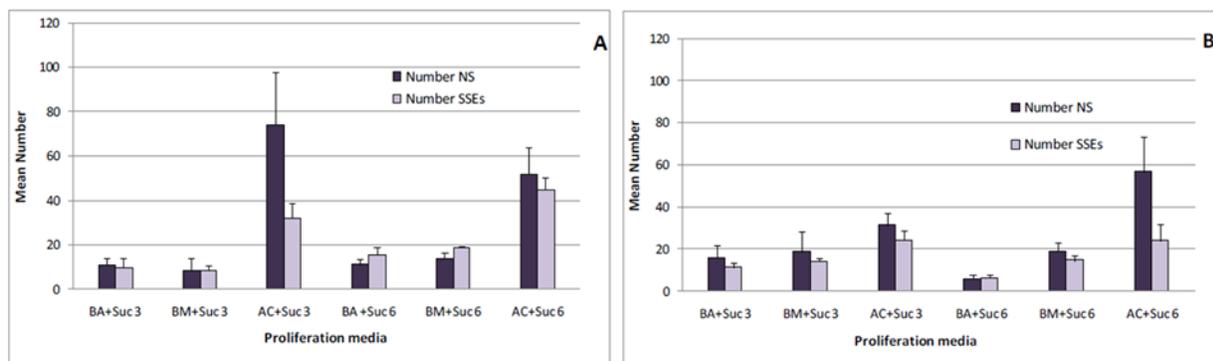
Figure 3. *Effect of sucrose concentration (3% or 6%) and embryo proliferation medium on the mean number of nodular embryogenic structures (NS) and bipolar secondary somatic embryos (SSEs) generated by nodular structures (A) or torpedo-stage embryos (B) used as subcultured explants to maintain secondary embryogenesis in Q. rubra. The proliferation medium consisted of STS-basal medium (BM) with or without activated charcoal (AC) or supplemented with 0.44 µM BA. Each column represents the mean value of four replicates and scale bars indicate the standard errors of the mean values. The statistical analysis of the data is presented in the text.*

Cultures grown in the presence of AC proliferated as bipolar embryos with clearly distinguishable and easily detachable nodular structures, in contrast to the compact appearance caused by fused embryos grown in the absence of AC. In conclusion, proliferation medium containing STS, AC and 3% sucrose was the most appropriate for production of nodular structures and bipolar embryos for maintenance of embryogenic lines and development of cotyledonary embryos for use in maturation and germination experiments with *Q. rubra*.

Germination of somatic embryos

After desiccation with sorbitol and chilling treatment, the somatic embryos germinated exhibiting a considerable increase in the size of cotyledons (which became reddish in colour), hypocotyl elongation and root growth. The germination response was promoted by incorporation of STS in the germination medium. Some germinating embryos in which roots but no shoots developed were obtained in media with and without STS (at frequencies of 40% and 23% respectively). Germinated embryos with longer roots were also observed in medium containing STS (63 mm vs. 20 mm root length). However, conversion into plantlets (both shoot and root development) was only achieved in 11% of embryos (Fig. 3F). Similar germination and conversion rates were observed in somatic embryos stored in empty Petri dishes during the two-month chilling treatment.

Discussion

Induction of SE from immature zygotic embryos and young seedling explants of *Q. rubra* has been reported (Gingas and Lineberger 1989; Rancillac et al. 1996; Vengadesan and Pijut 2009). However, here we describe, for the first time, a reproducible protocol for SE induction and maintenance of embryogenic lines from tissues derived from 7-year-old *Q. rubra* trees. The present study also provides new evidence for the suitability of shoot tip and leaf explants (excised from shoot cultures) for the induction of SE in recalcitrant *Quercus* species (Vieitez et al. 2012; Corredoira et al. 2014). Despite the low embryo induction frequencies, embryogenic lines were established and maintained in all three genotypes. Vengadesan and Pijut (2009) reported higher induction frequencies for immature cotyledon explants of red oak than those reported in the present study. However, the difference in embryogenic response may be attributed to the age of the donor plant and cell differentiation in the explant tissues (immature cotyledons vs. juvenile leaves).

Given the general difficulty in regenerating plants by SE in species of the genus *Quercus*, optimisation of the protocols requires full control of all steps. The data presented here showed that secondary embryo proliferation with development of cotyledonary-shaped embryos in *Q. rubra* was significantly improved by the addition of STS and AC to the basal proliferation medium. High concentrations of ethylene in proliferating cultures modify histodifferentiation and progression of developing embryos up to the cotyledonary-stage or to mature embryos (Kumar et al. 1998). The positive effect of STS (an inhibitor of ethylene action) is consistent with the high ethylene production associated with the process of secondary embryogenesis of holm oak (Mauri and Manzanera 2011). Charcoal affects the differentiation of somatic embryos, especially promoting maturation of cotyledon-shaped embryos and later germination (Merkle et al. 1995). In other *Quercus* species, charcoal (1%) has been used to promote maturation of 3–4 mm cotyledonary-stage embryos of cork oak (Hernández et al. 2009; Pintos et al. 2010) and holm oak (Barra-Jiménez et al. 2014), with a significant increase in both embryo size and fresh weight and prevention of repetitive embryogenesis during maturation. However, the results of the present study indicate that charcoal may be used prior to the embryo maturation step, as small embryogenic nodular structures subcultured in proliferation medium containing AC produce well-developed and singularised SSEs. This avoids further damage caused by the laborious separation of individual embryos generated in media without AC, which represents a serious hindrance to production of SSEs.

Sucrose has been used as a carbon and energy source, and at high concentrations it enhances somatic embryo maturation by causing osmotic stress (Sánchez et al. 2003). Accumulation of storage products usually converts translucent embryos into white-opaque embryos. This change has been used as an indicator of maturity in several species, including cork oak (Pintos et al. 2010), olive (Cerezo et al. 2011) and avocado (Palomo-Ríos et al. 2013). The present findings indicate that sucrose concentration was another important factor determining SSE development in Q. *rubra*, with the production of white opaque embryos >5 mm being enhanced by inclusion of 3% sucrose in the medium. This suggests an adverse effect of osmotic stress caused by the higher level of sucrose on embryo size.

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The present findings illustrate the importance of the type of embryogenic explant used for subculture as this had a notable influence on the efficiency of secondary embryogenesis. Nodular structures and torpedo-stage embryos, which correspond to early developmental stages of somatic embryos, comprise tissues with a low level of histodifferentiation, which possibly is the cause of their high proliferation potential, especially in the case of nodular structures. Morphological and histological observations (unpublished results) indicate that nodular structures resembling proembryogenic masses are similar in appearance to those described during induction of SE in *Q. robur* and *Q. bicolor*, although nodular structures were not used to maintain embryogenic lines of these species (Corredoira et al. 2006; Mallón et al. 2013).

Problems associated with induction of SE in *Quercus* species include low frequencies of embryo conversion into viable plants (Corredoira et al. 2014). Although improvement of secondary embryo quality has been achieved in *Q. rubra* embryogenic lines, plant recovery occurred at low rates. Further optimisation of the post-maturation treatments and germination conditions that determine shoot development and plant recovery is necessary for high plant production.

In conclusion, induction of somatic embryogenesis was achieved on tissue explants derived from *Q. rubra* trees, and a protocol for reliable maintenance of embryogenic lines by secondary embryogenesis was established. High yields of embryos were also obtained from isolated nodular embryogenic structures similar to proembryogenic masses (PEMs).

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Induction of somatic embryogenesis in leaf and shoot apex explants from adult trees of the genus *Eucalyptus*

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Abstract

This is the first report of the successful induction of somatic embryogenesis from shoot apex and leaf explants derived from mature trees of the species *E. globulus* and the hybrid species *E. saligna* x *E. maidenii*. Embryogenic response was significantly affected by the genotype, auxin and explant type. Picloram was more efficient than naphthaleneacetic acid (NAA) for embryo induction. The highest induction frequencies were obtained in medium containing 40 µM picloram and 40 mg/L arabic gum, in which 51.1%-64.0% of the shoot apex explants yielded somatic embryos. The embryogenic cultures initiated on picloram containing medium consisted of nodular embryogenic structures surrounded by a mucilaginous coating layer that emerged from a watery callus tissue developed from the initial explants. Cotyledonary somatic embryos were differentiated after subculture of these nodular embryogenic structures on basal medium without plant growth regulators. The embryogenic lines were maintained by repetitive somatic embryogenesis by subculturing embryo clusters, at 4-5-week intervals, on proliferation medium supplemented with 16.11 µM NAA. Plantlet conversion occurred when somatic embryos were transferred to liquid germination medium.

Keywords: arabic gum*, Eucalyptus globulus*, *Eucalyptus saligna* x *E. maidenii*, NAA, picloram.

Introduction

Species of the genus *Eucalyptus* are the most widely planted hardwood trees in the tropical and subtropical world (Grattapaglia and Kirst 2008). The genus is native to Australia and some islands to the north, and it was initially introduced to Southern Europe and Northern Africa in the mid 1800s. The importance of eucalypts has since increased and plantations can be found in more than 90 countries, with the largest plantations in Brazil, India and China. The estimated area planted with eucalypts worldwide is around 20 million hectares (GIT Forestry 2009). Of the 894 taxa of the genus, including subspecies and natural hybrids, the predominant species cultivated in commercial plantations are *E. grandis*, *E. globulus, E. camaldulensis, E. urophylla* and their hybrids (Potts and Dungey 2004). The following factors have contributed to the trees of the genus being the most widely planted hardwood crops: their fast growth rate, large biomass, the content of essential oils, the ability to grow in a wide range of environments and soils, the good wood quality for solid wood products, and the short cellulose fiber, which is suitable for pulp production, particularly for paper and tissue (Labate et al. 2008). From an industrial perspective, *E. globulus* is highly appreciated for its excellent fiber morphology for pulp production and because it displays several advantages over other *Eucalyptus* species, such as the need for smaller amounts of chemicals to obtain the bleached pulp due to a lower lignin content (Patt et al. 2006).

Improving *Eucalyptus* tree species through conventional breeding is constrained by long generation times and high genetic load (Chauhan et al. 2014). Biotechnological approaches such as gene cloning, plant tissue culture and genetic transformation offer the possibility of tree improvement within a limited time frame. Theoretically, somatic embryogenesis (SE) is the most efficient in vitro procedure for mass propagation and it has a great potential

for improvement of forest trees (Lelu-Walter et al. 2013). This procedure has several advantages over other micropropagation procedures, including rapid large scale clonal propagation, cryopreservation of valuable germplasm, and genetic transformation. Despite these important advantages, induction of SE in *Eucalyptus* has been reported in very few species of the genus (Pinto et al. 2013; Chauhan et al. 2014). In most of these embryogenic systems, cultures are initiated from zygotic embryos and/or young seedlings (1-4 weeks old). To our knowledge, induction of somatic embryogenesis in explants derived from mature trees has only been mentioned in three eucalypt species. Production of embryo like structures has been described in *E. grandis* (Qin and Kirby 1990), and information on *E. dunni* and *E. saligna* is not readily available because the somatic embryogenesis protocol is under patent (Termignoni et al. 1998; Patent No. PI 9801485-4 INPI). Specifically in *E. globulus*, SE has only been induced from immature and mature zygotic embryos (Nugent et al. 2001; Pinto et al. 2002, 2008a).

Effective procedures for the induction of SE from mature trees permit more rapid propagation of elite trees, thus increasing genetic gains in each breeding generation (Correia et al. 2011). Unfortunately, most woody species undergo a marked phase change that leads to a decrease in their ability for micropropagation, in particular for SE, as their age increases. Despite these difficulties, a number of reports describing SE initiation in explants derived from adult trees have been published in recent years (Hernández et al. 2003; Stefanello et al. 2005; San José et al. 2010; Klimaszewska et al. 2011; Correia et al. 2011; Corredoira et al. 2012; Mallón et al. 2013; Blasco et al. 2013).

In addition to the type and ontogenetic stage of the explant, the type and concentration of auxin are also important factors in SE induction. Naphthaleneacetic acid (NAA) is the most commonly used auxin for SE induction in eucalypt species (Pinto et al. 2013). Picloram, which is known for its auxin-like activity, may be more effective than other auxins for inducing SE (Steinmacher et al. 2011). Although picloram has scarcely been tested in *Eucalyptus* species, it has been used to stimulate somatic embryogenesis in material derived from adult trees (Karun et al. 2004; Stefanello et al. 2005; Steinmacher et al. 2007).

The main objective of the present study was to develop a reliable procedure for induction of somatic embryogenesis in mature eucalypt trees. For this purpose, the following studies were carried out: (1) Research on the potential of both leaf and shoot tip explants excised from axillary shoot proliferation cultures established from mature trees of the species *Eucalyptus globulus* and hybrid *E. saligna* x *E. maideii* to induce somatic embryogenesis and (2) Evaluation of the effects of NAA and picloram on induction and maintenance of the embryogenic response.

Materials and Methods

Plant material

Clonal axillary shoot proliferation cultures, previously established from two trees of the species *E. globulus* and one hybrid of *E. saligna* x *E. maidenii* (all 12-year-old elite trees), were used as sources of initial explants for induction of SE (Fig. 1A). The clones established were denominated 41-1-AC and 22-6-RP (*E. globulus*) and Sal-May (the hybrid tree). Shoot proliferation was achieved by subculturing shoots (1 cm long) every 3-4 weeks on basal shoot proliferation medium consisting of MS (Murashige and Skoog 1962) mineral salts and vitamins (Duchefa Biochemie, The Netherlands), supplemented with 10 mg/L ascorbic acid, 10 mg/L citric acid, 1 mg/L folic acid, 0.054 µM NAA and 7 g/L Vitroagar. This basal medium was supplemented with 0.41 µM meta-topolin (mT) and 20 g/L glucose for clone 22-6-RP, whereas 0.44 µM 6-benzylaminopurine (BA) and 20 g/L sucrose were included in the shoot proliferation medium used for clones 41-1-AC and Sal-May. The pH of the culture media was adjusted to pH 5.6–5.7 before they were autoclaved at 121ºC for 20 min. Stock shoot cultures of the three genotypes were maintained by periodical subculture for two years before being used in SE induction experiments. Stock shoot cultures were incubated in a growth chamber with a 16-h photoperiod (provided by cool-white fluorescent lamps at a photon flux density of 50–60 µmol m^{-2} s^{-1}) at 25ºC light/20ºC dark (i.e. standard culture conditions).

Induction of somatic embryogenesis

Shoot apex explants (1-2 mm long; Fig. 1B) and leaf explants (the two most apical expanding leaves from the apex; Fig. 1B) were cultured on basal induction medium consisting of MS mineral salts and vitamins, 500 mg/L casein hydrolysate, 30 g/L sucrose and 6 g/L Vitroagar. In the first experiment, shoot apex and leaf (1 and 2) explants from the three eucalypt genotypes were cultured on basal induction medium supplemented with 16.11 µM NAA plus 40 mg/L arabic gum from acacia trees (AG, Sigma, USA, G-9752). In a second series of experiments, shoot apex and leaf 1 explants of 41-1-AC genotype were cultured on basal induction medium supplemented with different concentrations of picloram (20, 30, and 40 µM) and 40 mg/L AG. In a further experiment, induction
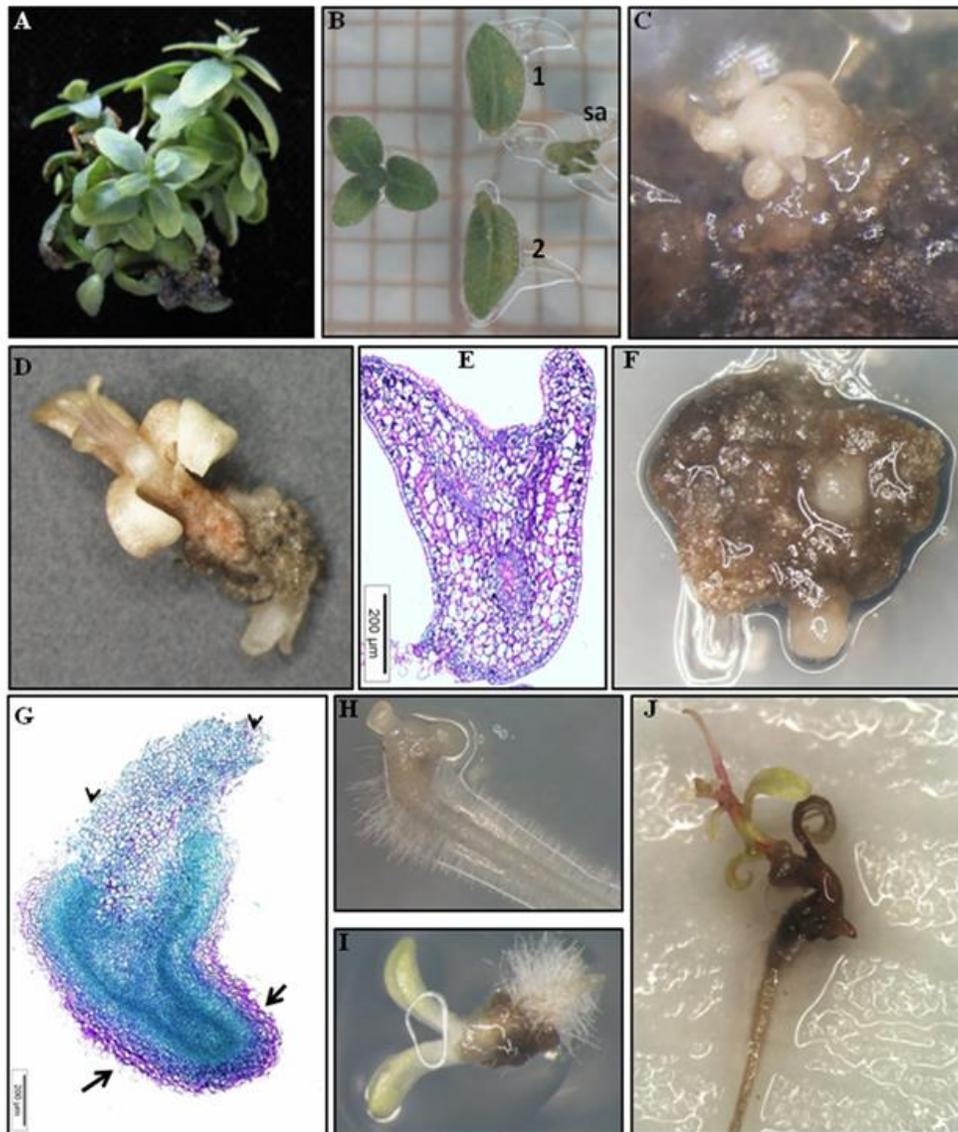
Figure 1. *Somatic embryogenesis in leaf and shoot explants derived from axillary shoot cultures of adult trees of the species E. globulus and the hybrid species E. saligna x E. maidenii.* ***A*** *Shoot proliferation cultures used as source of initial explants.* ***B*** *Shoot apex (sa), leaf at position 1 and leaf at position 2 used as initial explants.* ***C, D*** *Somatic embryos initiated in a leaf explant of 41-1-AC genotype (**C**) and a shoot apex explant of Sal-May genotype (**D**) after 8 weeks of culture in NAA induction medium.* ***E*** *Cotyledonary-stage somatic embryo with developing root meristem and poor development of shoot apical meristem.* ***F*** *Nodular embryogenic structures with a white-translucent and shiny appearance arising from a watery callus generated in a leaf explant of 41-1-AC genotype.* ***G*** *Section of the nodular embryogenic structure arising from the disaggregated callus tissue (arrow head). Note the presence of a stratified coating (arrow) surrounding the nodular embryogenic structure.* ***H*** *Secondary embryos developing from the cotyledonary region of a primary somatic embryo.* ***I*** *Secondary somatic embryo with precocious germination.* ***J*** *Plant regeneration from a somatic embryo after 6 weeks of culture in liquid germination medium*

medium containing 40 µM picloram and 40 mg/L AG was selected to test the embryogenic ability of two types of explants (leaf 1 and shoot apex) from the three eucalypt genotypes. The AG was filter sterilized and added to the autoclaved induction media. Selection of this concentration of AG was based on its positive effect reported in previous studies on somatic embryo induction in *Quercus* species (Corredoira et al. 2014).

Ten shoot apices (horizontally orientated) and ten leaf explants (abaxial side down) were placed in 9 cm Petri dishes containing 25 ml of induction medium. For each genotype and explant type, at least 60-100 explants were subjected to each auxin treatment.

In all SE induction experiments, the cultures were incubated in darkness at 25 ºC for 8 weeks. After this period, the following data were recorded: the percentage of explants forming callus, percentage of explants forming adventitious roots, the relative number of explants showing an embryogenic response and the percentage of explants in which an embryogenic response was observed (formation of bipolar embryos and/or embryogenic structures). The morphogenic response was analyzed under a stereomicroscope (Olympus SZX9, Japan) and photographed with an Olympus DP10 digital camera (Japan).

Maintenance of the embryogenic capacity

Somatic embryos initiated from the original explants in NAA or picloram induction media were isolated and subcultured for secondary embryo production, giving rise to clonal embryogenic lines. For proliferation of embryos, isolated cotyledonary-stage embryos or small clusters of globular and torpedo-stage embryos were used as explants. These were cultured in Petri dishes containing 25 mL proliferation medium consisting of MS mineral salts and vitamins, 500 mg/L casein hydrolysate, 30 g/L sucrose, 6 g/L Vitroagar, 20 μM silver thiosulfate (STS) and 16.11 μM NAA. STS was filter sterilized and added to the autoclaved proliferation medium. Embryogenic lines were maintained by secondary embryogenesis with sequential subculture at 4-5-week intervals, and the cultures were incubated in darkness at 25ºC.

Histological study

Nodular structures and somatic embryos were fixed in a mixture of formalin, glacial acetic acid and 50% ethanol [1:1:18 (v/v/v)], dehydrated through a graded n-butanol series and embedded in paraffin wax. Sections (8 μm) were cut and stained with periodic acid-Schiff (PAS)-naphthol blue-black to detect starch and other insoluble polysaccharides and total proteins, respectively (Feder and O'Brien 1968).

Statistical analysis

The influence of the main experimental factors (the explant type and species genotype) on the percentage of root formation (Fig. 2) and on the percentage of embryogenic response (Figs. 2 and 3) was evaluated by the Chi-squared test ($p \leq 0.05$). The interaction between both factors was analyzed by a log-linear model ($p \leq 0.05$). SPSS for Windows (version 19.0, Chicago, USA) was used for statistical analysis.

Results and Discussion

Effect of NAA on somatic embryo initiation

Several factors, namely explant type, auxin and species/genotype, have been shown to affect SE initiation. Explant source and its developmental stage are considered key elements in SE (Fehér 2006). The use of cultured shoots maintained in vitro as a source of explants for SE induction is an important step in improving control of somatic embryogenesis. This type of material offers many advantages regarding the induction of somatic embryos: it is easy to handle in vitro as it does not need to be sterilized, and experiments can be carried out at any time of the year and are not determined by seasonal growth of trees or by climatic and edaphic factors. This approach was applied to eucalypt species of the present study, in which initiation of SE was obtained with both shoot apex and leaf explants obtained from axillary cultures during active shoot growth.

In a preliminary experiment, the effects of the auxin NAA and of the explant type (leaf or shoot apex) on somatic embryo induction were evaluated. This plant growth regulator was selected because it was found to be effective for somatic embryo induction in zygotic embryos of *E. globulus* (Pinto et al. 2002). Leaf (1 and 2) and shoot apex explants isolated from Sal-May and 41-1-AC genotypes were cultured with three concentrations of NAA (10.74, 16.11, and 21.48 μM). In both clones, most of the explants responded by forming callus tissue and adventitious roots. The values of both parameters were higher for apices than for leaves at the three concentrations of NAA tested. The best embryogenic response, defined as the presence of embryos and/or embryogenic structures in the initial explants, was observed when shoot apex explants from the hybrid genotype were treated with 16.11 μM NAA (data not shown). A concentration of 16.11 μM NAA was therefore selected for the next experiment, in which

the effect of the explant type and the species/genotype was tested (Fig. 2). Root formation was significantly affected by species/genotype (p≤0.001) and explant type (p≤0.001), and there was a significant interaction (p≤0.001) between these two factors. The explants of three genotypes responded by forming callus and adventitious roots, with the highest rate of root formation in apex explants from the hybrid genotype (Fig. 2A). SE induction was also significantly influenced by the species/genotype (p≤0.001) and explant type (p≤0.001), and again a significant interaction (p≤0.01) between both factors was observed. An embryogenic response was observed in the three genotypes, and the best results were obtained with the hybrid genotype: 14.3% induction for shoot apex explants and 7.5% for leaf 1 explants (Fig. 2B). The embryogenic capacity of *Eucalyptus globulus* was significantly lower than that of the hybrid material, specifically in shoot apex explants.
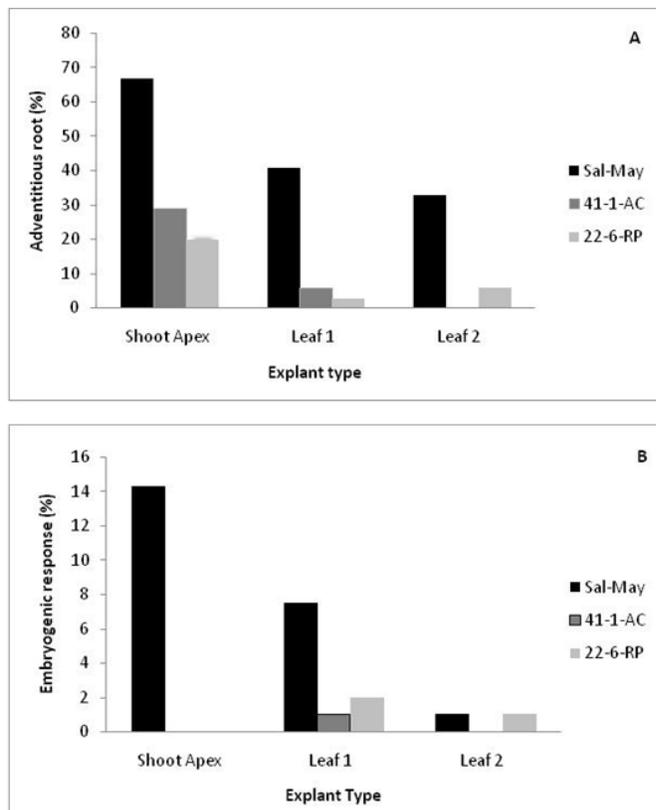
*Figure 2. Adventitious root formation (**A**) and somatic embryogenic response (**B**) observed in leaf 1, leaf 2 and shoot apex explants after 8 weeks of culture on induction medium supplemented with 16.11 μM NAA and 40 mg/L arabic gum. Explants were excised from axillary shoot cultures of adult trees of the hybrid species E. saligna x E. maidenii (Sal-May genotype) and the species E. globulus (41-1-AC and 22-6-RP genotypes). Statistical analysis of the data is summarized in the text.*

Simultaneous regeneration of somatic embryos and roots was also observed to occur in other embryogenic systems with leaf explants including *Q. rubra* (Rancillac et al. 1996), *E. globulus* (Pinto et al. 2002), and *Petiveria alliacea* (Webster et al. 2008). Isolated or combined use of NAA with other plant growth regulators induced somatic embryogenesis in leaf explants of several woody plants including chestnut (Corredoira et al. 2006) and oak species (Corredoira et al. 2014). This auxin has also been reported to induce somatic embryogenesis in zygotic embryos of *Eucalyptus* species such as *E. citriodora* (Muralidharan et al. 1989; Muralidharan and Mascarenhas 1995)*, E. dunnii* (Termignoni et al. 1996), *E. tereticornis* (Prakash and Gurumurthi 2005) and *E. camaldulensis* (Prakash and Gurumurthi 2010).

The embryogenic structures and/or somatic embryos were induced from the callus generated in the initial explant (Fig. 1C and 1D), although direct formation of embryos has also been observed. In some initial explants, somatic embryos exhibiting precocious germination were also observed. Somatic embryos were clearly bipolar, demonstrating the development of root and shoot poles and two small cotyledons of a scale-leafy appearance. They generally appeared well separated and detached readily from the callus. At the histological level, somatic embryos exhibited bipolar organization with root and shoot apex meristems and closed vascular tissue bifurcating into cotyledons (Fig. 1E). The primary root pole appears more developed than the shoot pole, which is formed by a few meristematic cell layers.

Effect of picloram on somatic embryo initiation

To improve SE induction in *Eucalyptus globulus* genotypes, the effect of picloram on SE initiation was evaluated. Shoot apex and leaf 1 explants of 41-1-AC genotype were cultured with three different concentrations of picloram and 40 mg/L AG. In all treatments, almost 100% of initial explants formed callus (Table 1), but unlike

Table 1. Morphogenic response of leaf 1 and shoot apex explants excised from shoot cultures of E. globulus (41-1-AC genotype) after 8 weeks of culture in medium supplemented with different picloram concentrations and 40 mg/L arabic gum.

Picloram (µM)	Watery callus (%)		Embryogenic response[1]	
	Leaf 1	Apex	Leaf 1	Apex
20	98.3 (59/60)	100.0 (60/60)	No	Yes (+)
30	100.0 (100/100)	98.0 (98/100)	Yes (+)	Yes (++)
40	98.8 (79/80)	100.0 (100/100)	Yes (+)	Yes (+++)

[1]*Characterization of embryogenic response: No, not embryogenic response; Yes, presence of nodular embryogenic structures: + poor, ++ good and +++ best.*

induction with NAA, no adventitious roots were obtained. SE induction was observed with all three concentrations of picloram, although the best results were obtained with shoot apex explants cultured on medium containing 40 µM picloram (Table 1). Embryogenic explants consisted of nodular embryogenic structures that arise from the watery callus (Fig. 1F). This callus was initially soft and yellow in color, and it later became brown and necrosed. Embryogenic structures were white-translucent, shiny and of variable shape; although most were nodular-ovoid, cup-shaped and curved structures were also observed. These embryogenic structures were surrounded by a mucilaginous coating, which at histological level is made up of stratified cells with high levels of starch grains (Fig. 1G). A similar layer has also been described in somatic embryos induced from zygotic embryos of *E. nitens* (Bandyopadhyay and Hamill 2000). In these embryogenic systems, somatic embryos appear completely enclosed in a thin, stratified and translucent coating. The authors suggested that this layer may have a protective role against the high levels of phenolic compounds observed in the callus tissue. The presence of a mucilaginous coating surrounding the embryogenic structures was not observed in the embryogenic cultures induced in medium containing NAA in the present study or in somatic embryos generated from zygotic embryos of *E. globulus* (Pinto et at. 2002; 2008a).

On the basis of the above results, we selected the medium supplemented with 40 µM picloram and 40 mg/L AG for evaluating the embryogenic ability of the three clones. SE was achieved in both types of explants from the three genotypes, with percentages ranging between 13.8% and 68.8% (Fig. 3). The frequency of explants developing embryogenic structures was significantly influenced by the species/genotype (p≤0.001) and the explant type (p≤0.001), with a significant interaction (p≤0.001) between these two factors. The most responsive material was the Sal-May hybrid, in which a similar embryogenic capacity was observed for both type of explants. In contrast, in the *E. globulus* genotypes, the embryogenic ability of leaf explants was significantly lower than that of the shoot tip explants. Although the highest embryogenic response was obtained in Sal-May, the embryogenic response in *E. globulus* was considerably higher than that obtained with NAA. Picloram is considered as a potent plant growth regulator capable of stimulating the embryogenic process; however, this auxin has scarcely been used for SE induction in the *Eucalyptus* species. Exceptionally, Nugent et al. (2001) obtained somatic embryos from cotyledon of *E. globulus* zygotic embryos cultured on medium containing 50 µM picloram or 100 µM IBA, although the embryogenic response was very low (1.3%).

Histological analysis revealed that the nodular embryogenic structures generated on picloram medium comprised cells of embryogenic appearance (Fig. 1G). These cells showed a dense protein-rich cytoplasm, small vacuoles, a high nucleoplasmic ratio and starch grains, which were especially abundant in tightly packed cells of external layers. Small, undifferentiated and more vacuolated cells were also evident in the central area of the nodular structures. Somatic embryos at different stages of development (including globular-, torpedo- and cotyledonary-stage embryos) were also observed. Somatic embryos were clearly bipolar, with a highly differential primary root pole,

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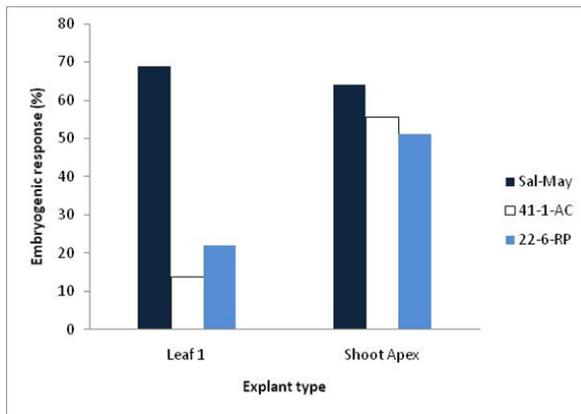whereas the apical pole was less well developed and was sometimes even blocked or absent.

Figure 3. *Somatic embryogenic response obtained in leaf 1 and shoot apex explants excised from shoot cultures derived from three Eucalyptus genotypes, following culture for 8 weeks in induction medium supplemented with 40 µM picloram and 40 mg/L arabic gum. Statistical analysis of the data is summarized in the text.*

After 8 weeks of culture in the medium containing picloram, isolated embryogenic structures were subcultured for 6 weeks into a secondary medium devoid of plant growth regulators (PGR), in which embryo histodifferentiation and production of somatic embryos at globular-, torpedo-, cotyledonary-stage and even pre-germinated embryos were enhanced. When the secondary medium was supplemented with NAA (16.11 µM), with or without addition of mT (0.41 µM), embryo differentiation was less effective, whereas embryogenic structures became necrotic following culture on PGR-free medium containing 0.4% activated charcoal.

Somatic embryogenesis maintenance and plant regeneration

Once somatic embryos were initiated, the next step was to maintain their embryogenic capacity. Induction of repetitive embryogenesis and maintenance of the embryogenic capacity in woody plants appear to be dependent on the species, genotype and explant type (Watt et al. 1999). In *Eucalyptus* species, problems in maintaining the embryogenic capacity by secondary embryogenesis have been reported, and such problems appear to be inherent in eucalypt embryogenic systems (Pinto et al. 2013). Secondary somatic embryos have been reported for *E. gunnii* (Boulay 1987), *E. citriodora* (Muralidharan et al. 1989; Muralidharan and Mascarenhas 1995) and *E. globulus* (Pinto et al. 2004, 2008b), but only Muralidharan and Mascarenhas (1995) described an effective protocol for the induction of repetitive SE, enabling large-scale propagation of *E. citriodora*. In the present study, cotyledonary-stage embryos or small clusters of globular- and torpedo-stage embryos were isolated from initial explants and transferred to medium containing NAA (16.11 µM), in which new cycles of secondary somatic embryos occurred. These secondary somatic embryos often formed in the cotyledonary region (Fig. 1H). Moreover, secondary somatic embryos showing precocious germination were also found on proliferation medium (Fig 1I). So far, we have maintained the embryogenic lines for more than three years under these conditions.

Regeneration of plantlets from somatic embryos is a frequent problem in many species, including eucalypt species. With the exception of successful SE reported for *E. citriodora* (Muralidharan and Mascarenhas 1995) and *E. dunni* (Termignoni et al. 1998), high frequencies of plantlet regeneration by somatic embryo conversion have not generally been reported in *Eucalyptus* species. In the present study, somatic embryos underwent rapid drying and browning when they were cultured on semisolid germination medium. Use of liquid germination medium in the somatic embryo germination was evaluated as a possible means of preventing this problem. Cotyledonary-stage embryos were isolated from embryogenic clusters and placed on two filter paper discs (Whatman grade 181) in Petri dishes containing 10 mL of liquid germination medium consisting of MS medium supplemented with 30 g/L sucrose, 6 g/L Vitroagar, 0.44 µM BA and 1.44 µM gibberellic acid. Although embryo conversion into plantlets was observed under these conditions (Fig. 1J), very few regenerated plantlets were obtained. To control the maturation and germination of somatic embryos, further research is therefore needed.

The findings reported in this research demonstrate for the first time the successful regeneration of somatic embryos from adult trees of the species *E. globulus* and the hybrid species *E. saligna* x *E. maidenii*, opening the possibility of mass multiplication of material that has been genetically improved by breeding.

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Nestlé Cocoa plan: Cocoa propagation by somatic embryogenesis

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Abstract

Cocoa production is threatened by many factors such as aging trees and increasing exposure to biotic and abiotic stresses. As a consequence the world's average yields are very low (about 400kg of dry beans/ha) and the economic situation of farmers is difficult. While cocoa production has remained stable, the world's consumption is increasing, raising the issue of long term supply security.

To tackle this situation, Nestlé launched in 2009 The Nestlé Cocoa Plan (NCP), which aims to enable farmers to run profitable farms, to improve social conditions in cocoa communities and to support the production of sustainable, good quality cocoa.

Today the NCP is deployed in the main cocoa producing countries. The distribution of high-yielding trees is a key part of enabling farmers within the NCP. To support tree distribution, Nestlé is promoting, among different approaches, the propagation of clonal material via somatic embryogenesis and orthotropic rooted cuttings.

The Nestlé R&D Center in Tours has developed expertise in cocoa somatic embryogenesis over the last 20 years. We will describe the propagation process that we have implemented. It is mainly based on the multiplication of embryogenic callus developing from secondary embryos and on the use of liquid medium for the production of the embryos.

Nevertheless, the response to somatic embryogenesis remains variable (genotype and experiment dependent) resulting in relatively unpredictable and low conversion rates (5 to 20% of embryos give plants). As an example of optimization trials that we conducted, we show results of our study dealing with visual criteria for the selection of SEs able to regenerate plantlets, particularly the criteria of embryo opacity.

Despite the challenges, we have developed a unique collection of SE trees that is maintained in the field in Ecuador and in the greenhouse. It has around 140 accessions, many of which are also cryopreserved.

Keywords: *Theobroma cacao*, vegetative propagation, TIS, orthotropic rooted cuttings

Introduction

Theobroma cacao L. trees are grown in the humid tropics to produce cocoa beans. Four main countries produce 75% of the total cocoa beans: Ivory Coast, Indonesia, Ghana and Nigeria (FOA stats). In producing countries, it is considered as a cash crop but it is also a crucial crop for the confectionary business as it provides the key raw material for chocolate based products. Nestlé is transforming about 12% of the world's cocoa production.

The world's average yield of 400kg of cocoa beans/ha/year is very low, far from the estimated potential of the tree (which should be around between 1 and 2 t/ha depending on the variety). This situation can be explained by the accumulation of the following factors:

-   Age of the trees. In most of the producing countries orchards are getting old and farmers cannot afford to replace the old trees by planting newly selected varieties,

- Biotic stresses. Following ICCO (The International Cocoa Organization) estimates, between 30 and 40% of the world production is lost by pest and fungal attacks,
- Inappropriate crop management. Almost no fertilization is applied nor phytosanitary treatment,
- Migration of people from the country-side. Farmers are looking for better social conditions, causing a diminution of the rural population and, therefore, the available labor supply,
- Climate change. Causing drought in some of the cocoa producing areas making them unsuitable for cocoa cultivation.

As a consequence, cocoa orchards are challenged by other crops like rubber and palm oil. These crops need less inputs and labor management while yielding higher income to the farmers.

This situation is raising the issue of long term supply for the confectionary business as the production is stagnating while the consumption is increasing because of the new consumption habits in countries like China and India.

To tackle this situation, Nestlé launched in 2009 The Nestlé Cocoa Plan (NCP) which has 3 pillars (Fig. 1).

Figure 1: Nestlé Cocoa plan's organization

Cocoa Propagation

Cocoa is mainly allogamous and propagated from cross-pollinated seeds leading to orchards planted with unselected seedlings resulting in a great heterogeneity.

Vegetative propagation of selected genotypes would be desirable, but conventional methods such as cuttings or grafting often present abnormalities. In such clonal plantations, cocoa trees present a plagiotropic shape with some branches being close above the soil. Pruning is needed in order to correct and lift the crown of such trees.

In most of the producing area, like in West Africa, the current orchards consist of trees having orthotropic branches. Consequently, in the frame of the NCP which aims to renew old plantations, Nestlé made the choice to promote clonal propagation methods that keep the plant architecture to which the farmer is familiar. For that reason, selected clones are propagated by somatic embryogenesis. The somatic plantlets are directly planted in the field or used to establish clonal gardens. R&D-Tours has developed during the last 3 years a cutting protocol, named

orthotropic rooted cuttings, to propagate selected SE trees.

The advantages of combining SE and orthotropic rooted cuttings process are:
- To facilitate international exchange of planting material,
- To speed up the diffusion of selected clones (higher yield, disease resistance,...),
- To keep the plant habit prefered in most of the producing countries,
- To limit the dissemination of diseases which can be transmitted by conventional cuttings and grafting (by appropriate crop management of the clonal gardens).

Somatic embryogenesis at Nestle R&D Tours

The process described in the figure 2 is based on indirect SE, using embryogenic calli that developed from secondary embryos (Fontanel A. et al. 2002, Li Z. et al. 1998). It starts off with the induction of primary embryos from immature floral explants. They are cut to obtain and to multiply embryogenic calli. The expression step is achieved on solid medium or in liquid medium. In liquid medium, the expression step is managed by using a Continuous (CIS) or Temporary (TIS) Immersion System. CIS is done in flasks. It requires many manipulations as embryos have to be harvested several times. TIS is conducted in a 2-L glass bioreactor and is fully automatized but requires a more sophisticated infrastructure. When harvested, embryos are transferred onto a solid maturation medium for 3 weeks. Rooting, the last in vitro step induces the development of complete plantlets from the embryos. They have been sent to producing countries, mainly Ecuador and Ivory Cost.
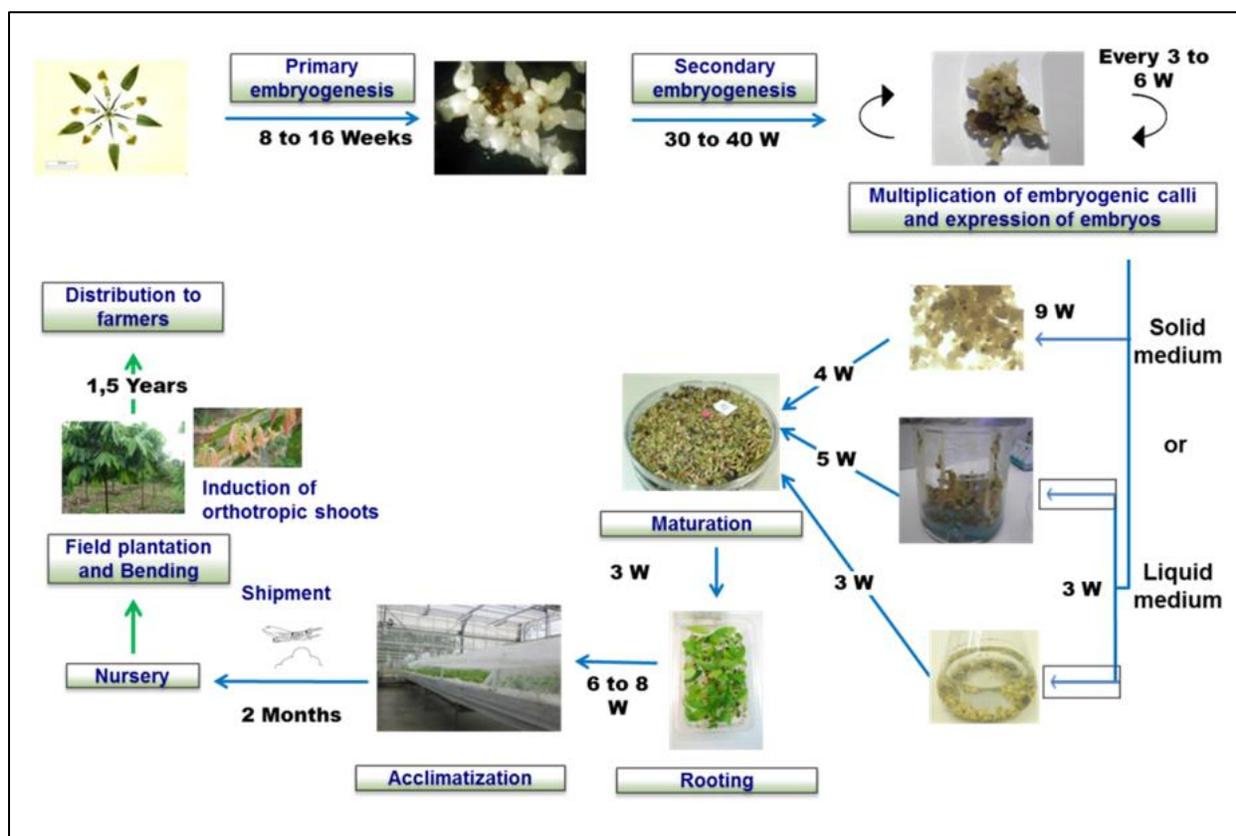

Figure 2: *Cocoa propagation by Somatic Embryogenesis at Nestlé R&D-Tours. From embryogenic callus to transplanted plantlet one person can obtain 9 000 plants / year in solid medium and 12 000 plants / year in liquid medium. Between 2009 and 2012, we regenerated around 260,000 acclimatized somatic plantlets.*

The somatic plantlets have been distributed to farmers or could be used to establish clonal gardens to obtain orthotropic rooted cuttings (Figure 3).
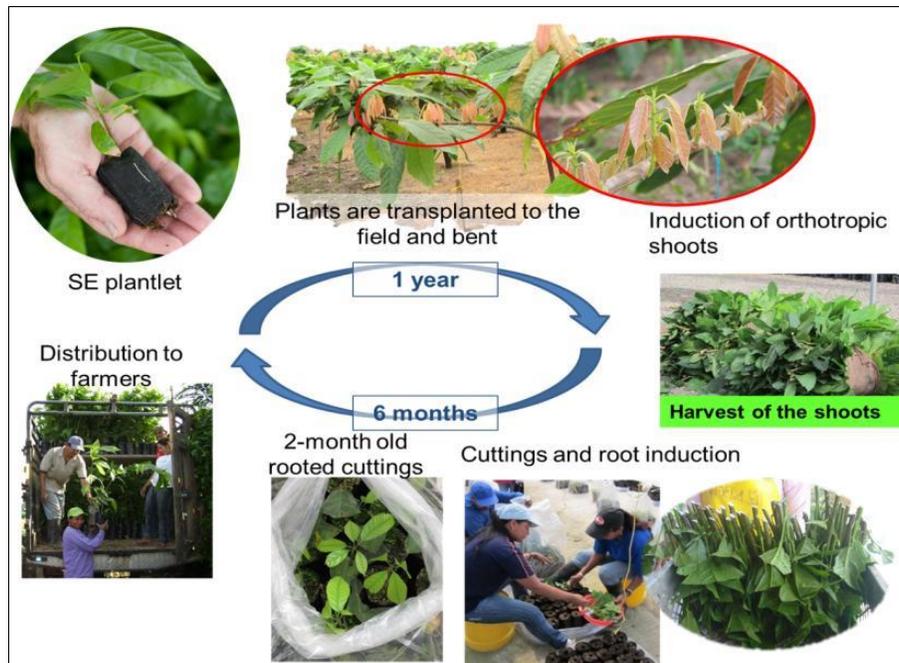

Figure 3: Multiplication of somatic plantlets by the production of orthotropic shoots in clonal gardens followed by their rooting.

By preparing orthotropic rooted cuttings, one person can produce 25 000 plants by year. One plant can produce more than 100 cuttings / plant / year and the rooting rate is around 60-70 %. The experimental farm in Chollo can distribute up to 200 000 orthotropic plants/year.

Limits of somatic embryogenesis

Firstly, the efficiency of SE is hampered by a genotype-dependent response in obtaining primary SE induction (Masseret B. et al. 2008). Figure 4 shows the variability of response for 114 genotypes:
-   18% of the clones reacted positively to primary SE >30% ,
-   26 % gave a low response between 0 to 5%.

It is important to underline that the genotypes responsive to primary SE will produce secondary SE for the induction of embryogenic calli at a rate of 95%.

Secondly, as in many crops, the main limitation of this process is the aptitude of the embryos to regenerate a plantlet. For example, we show the embryo-to-plantlet conversion rates for 3 Ecuadorian genotypes (Figure 5). The embryos were produced by TIS between 2008 and 2013. On average, only 7% of the embryos regenerated a plantlet. However, we noted a large lot-to-lot variability as the conversion rates ranged from 0.5% to 25%. For some batches, we had a 60% conversion rate. Different reasons can be advanced to explain this lot-to-lot variability. The main one is the duration of the multiplication step in liquid medium: the calli become less embryogenic with the age of the cell lines and the frequency of abnormal plantlets tends to increase. It is necessary to create new cell lines at least once a year.

Thirdly, several authors reported that the opacity of the embryos was a relevant marker to anticipate the aptitude of the embryos to regenerate plantlets. We have indeed observed that white embryos have a better histological organization with higher concentrations in proteins and starch granules than in the translucent embryos (Figure 6). We checked this assumption with a large quantity of embryos produced by TIS by selecting them at the

transfer time onto the maturation medium. White embryos have a better conversion rate than the translucent ones, but the difference is not statistically significant (Figure 7). So, we confirmed only partially that a white aspect can be a marker for embryo quality. On the other hand, a selection according the size is not effective.
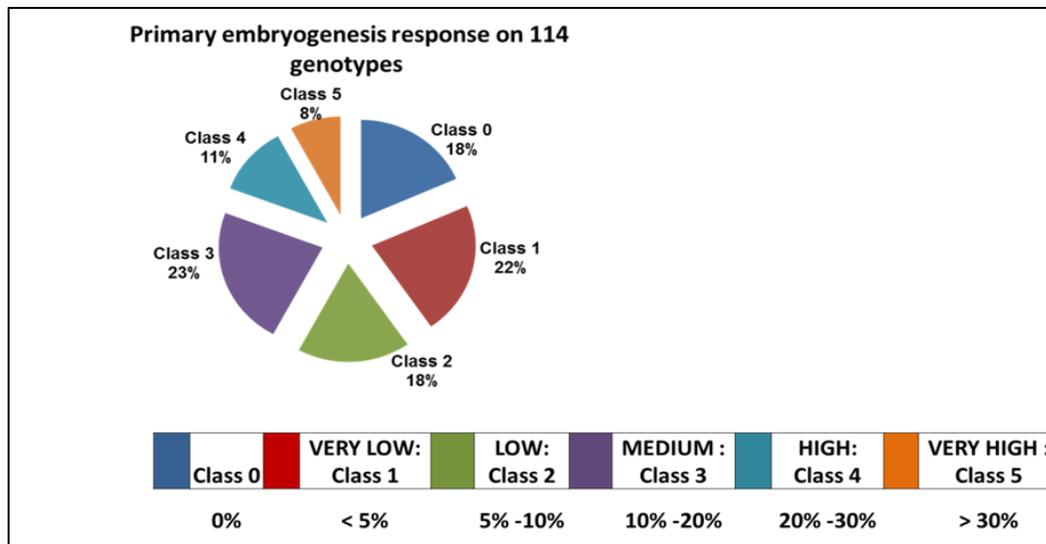

Figure 4: Primary somatic embryogenesis response (percentage of explant giving at least 1 embryo)
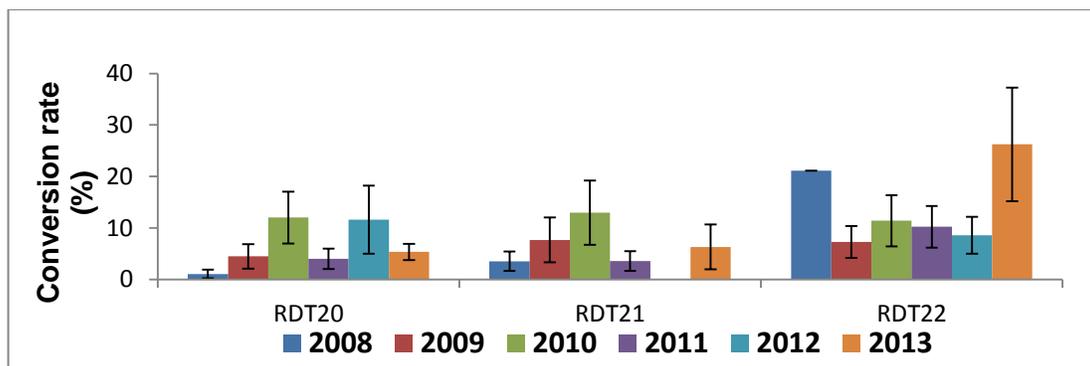

Figure 5: Embryo-to-plantlet conversion rates for 3 Ecuadorian genotypes (Production values are obtained from 4 000 000 embryos produced out of 900 production batches)
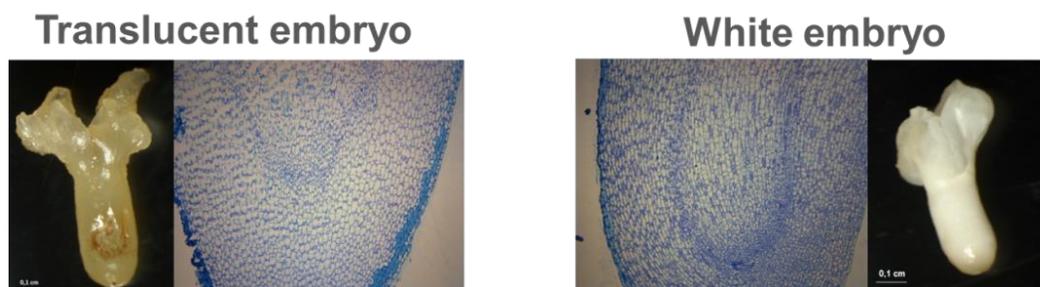

Figure 6: Aspect of translucent and white cocoa embryos at the end of the maturation step
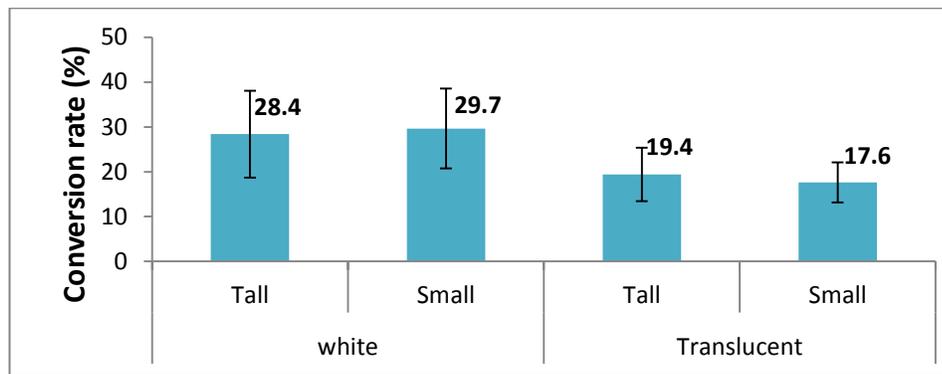
Figure 7: Conversion rate of the embryos according their aspect (white or translucent) and their size (small <0.5cm or tall >0.5 cm) at the time of the transfer onto the rooting medium. Data are the average of all genotypes obtained with 20,000 embryos in total.

Applications

One of the objectives of the Tours organization is to establish a core collection of plants produced by SE in Ecuador and Ivory Coast. It has around 140 different genotypes in the field and cell lines in liquid nitrogen. The aim is to classify the genotypes and identify trees resistant to diseases or producing a good chocolate quality. Biochemical, biomolecular and panel analysis will help us for this project.

Conclusion

Genotype plays a preponderant role in somatic embryogenesis response and in the conversion rate of embryos. All tests (morphological aspects, cocoa quality, yielding etc.) over a 12 year period have indicated that SE trees are true to type, (Masseret B. et al. 2005). Production by somatic embryogenesis is possible but we have to improve the process and obtain a better conversion rate and understand how to select the "good embryo". The Nestlé Cocoa Plan wishes to improve access to farmers to good planting material through propagation, distribution and technology transfer. The technology of SE and Cuttings was transferred to the Abidjan R&D Center and also to our experimental farm in Chollo in Ecuador. Each year Nestlé Tours is training scientists from producing countries like Malaysia, Mexico, Indonesia and Costa Rica…

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Challenges for the large-scale propagation of forest trees by somatic embryogenesis - a review

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Abstract

The large number of plants produced by somatic embryogenesis could potentially be the most effective way to produce large numbers of vegetatively propagated forest trees for commercial use. Or is it? In reality the number of forest trees actually produced by somatic embryogenesis is relatively small. This is mainly due to problems in the effectiveness, efficiency and thus the economics of current somatic embryogenesis production protocols. These present the greatest challenge to the large-scale use of this technology. Imaginative production and deployment strategies including the use of bioreactors, automation and synthetic seed have been proposed to make SE propagation more cost competitive. However, all of these approaches would greatly benefit from research to improve the quality of the somatic embryos produced and thus improve the economics of the SE process. Improving the quality of somatic embryos should be the major area for future work.

KeyWords: somatic embryogenesis, commercial-scale propagation, hybrid propagation systems, bioreactors, automation, artificial seed.

Introduction

Somatic embryogenesis (SE) of forest trees offers a potential method for the large-scale propagation of material on a scale not previously possible with conventional vegetative propagation methods, mainly rooted cuttings. It was assumed that because SE involves the formation of a complete plant in one step that the production costs will be less than those associated with organogenesis methods that require several different steps to produce a complete plant. Unfortunately this assumption has not proven to be correct.

Although a great deal of work has gone into the development of SE methods, only a few species have provided production cost data. This information suggests that the production costs of most SE plants are currently uneconomic. Most of these costs are due to inefficiencies in the current SE production protocols. These costs could be reduced, but this depends on the production of large numbers of high quality somatic embryos with high maturation, germination, conversion and acclimatisation rates. This will require work to improve the quality of the somatic embryos currently being produced.

The objective of this paper is to review the current challenges that face the commercial use of large-scale production of forest trees by SE and to suggest possible ways to overcome these challenges.

Challenges for Scale-up of SE

The development of a large-scale production process based on publications of laboratory-based research-scale trials suffers from many problems. Many publications are based on experiments done once, or experiments based only on the use of one very specifically selected cell line. Some publications simply do not provide sufficient details to allow replication of the work. Many publications do not provide details on the survival rates at the various steps of the process including greenhouse and nursery survival rates and their performance. Indeed, many publications end after a small number of "germinated emblings" (plants produced by somatic embryogenesis) in Petri dishes are produced. There is also a large amount of knowledge, which due to restrictions on "proprietary information" (e.g. available only in either patent applications or in conference presentations), provides few if any details of how these results were achieved. All of these factors limit the scale-up of SE.

History Could Repeat Itself?

Large-scale vegetative propagation of forest tree species is not a new idea. In the 1970s work on organogenesis in forest trees, specifically conifer species, attracted a great deal of attention both in academia and industry. Plants were produced and field trials were established, however by the mid-1980s this technology was on the decline and today is not widely used. One reason was that the cost of these plants were somewhere between 2.4 and 10 times the cost of conventional seedlings (Hasnain et al. 1986; Smith, 1986). A second reason was the fact that even though the plants originated from juvenile tissues (excised zygotic embryo explants) many of the plants (especially in *Pinus* species) showed advanced maturation effects (slow growth, mature morphology, poor root systems) that caused interest in this technology to decline. A third reason was that the production of conifers by SE offered the possibility of reduced production costs.

After 25 years the reason for the advanced maturity of the plantlets can be explained. A study by Hargreaves et al. (2005) found that plants of Radiata pine (*Pinus radiata*) propagated by adventitious axillary shoots from cotyledons showed a higher degree of maturation (slower growth, poorer root systems and a general increase in physiological age) than adventitious axillary shoots from epicotyls of the same zygotic embryos. This demonstrates that dramatic differences in performance can result from shoots derived from different tissues of the same zygotic embryo.

The experience with organogenesis demonstrates that even though a technology looks attractive and resources are invested in it, if problems develop, costs are too high and alternatives are available, a technology can very quickly be replaced or abandoned.

Somatic Embryogenesis

In order for a propagation method to be commercially successful it must meet certain criteria which include:

Effectiveness- the ability to propagate a wide range of genotypes;

Efficiency- the ability to produce a large number of easily established plants;

Economic- the ability to compete in price with comparable planting stock.

Effectiveness and efficiency interact to determine the economics of the process. If the process works with only a small number of genotypes it will be necessary to initiate a large number of cell lines to have enough plants available for field planting which will increase production costs. Similarly if only a very limited number of emblings can be produced by each cell line then more cultures of each cell line must be grown to produce the required number of emblings which will also increase production costs.

Effectiveness

Genetics plays a major role in the effectiveness of the SE process. Park et al. (1998) have shown that initiation rate, and to a lesser extent maturation and germination rates, are under genetic control. As a result, some families are easier or more difficult to propagate by SE. Cyr (2000), working with SE in interior spruce (*Picea glauca X Picea engelmanni*) reported that ”…only 20% of the half-sib and 44% of full-sib crosses produced stable cultures using mature seed…”. This illustrates problems of the effectiveness of initiation.

Some papers have suggested that perhaps families should be "bred" specifically to be highly embryogenic. While this might assist in advancing knowledge of the basic SE process, it would not be useful in advancing the practical application of SE. This is because material for large-scale commercial propagation will come from tree breeding programmes which have been selected for increased productivity and not SE ability.

Genetic variation in effectiveness (and efficiency) is not only available at the family level. Variation occurs between different genera (e.g. *Pinus* compared with *Picea*), between different species (e.g. Norway spruce [*Picea abies*] compared to Sitka spruce [*Picea sitchensis*]) as well as at the half-sib (open pollinated), full-sib (controlled pollination) and even at the individual level. There may be as much genetic variation between individuals within the same family (a cross made between two parents) as there is between crosses made between different parents. An example of the variation in field performance between different cell lines (clones) resulting from a cross between the same two parents is shown in Table 1. Significant differences in height growth between clones are clear and not all clones are superior even though they had the same parents. For this reason it is necessary to initiate as many cells lines from a family as possible to ensure that some of the cell lines are superior clones. If the effectiveness of initiation is low this will mean either more work to initiate more cell lines or that superior clones may not be established in culture.

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Table 1. *Height growth differences between clones (cell lines) of the same full-sib cross after 3 years in the field*
(average of 25 trees (Sitka spruce) per clone). (Thompson, unpublished)

Clone	Height (cm)	Percent Increase relative to clonal average
13.1	145.0	+6.4
13.2	131.0	-3.9
13.4	135.0	-0.9
13.6	151.4	+11.1
13.8	141.1	+3.5
13.9	112.9	-17.6
13.10	168.1	+23.3
13.11	125.2	-0.8
13.17	117.3	-14.1
Clonal Average	136.3	

Efficiency

The efficiency of the SE process is the cumulative result of the efficiency of each step in the process. However, in most research publications the efficiency of each step let alone the overall efficiency of the SE process are rarely if ever discussed. Timmis (1998) observed that "Low yield of product at any step may be due to unsuitable conditions for that step, poor quality (incompetence for further development) of incoming product or both,". He goes on to say that "Thus, there may be unrecognised problems with a much earlier step".

There may also be genetic effects on the efficiency of the process. Table 2 is an example of the variation in the number of germinated somatic embryos in 9 cell lines resulting from the same full-sib cross. Some cell lines are very productive while others are not, demonstrating the importance of genetic differences between individuals (cell lines) of the same parents.

Together the effectiveness of initiation (Table 1) and the efficiency of propagation (Table 2) determine whether a cell line commercially useful and valuable or not.

Table 2. *Variation in the number of germinated emblings produced in the laboratory from 9 different cell lines of the same full-sib cross (Sitka spruce) .(Thompson, unpublished)*

Cell Line	Number of Emblings
23.1	217
23.2	1,182
23.3	154
23.4	3,424
23.6	56
23.7	290
23.8	806
23.9	106
23.10	27

In some cases generalised statements have been made about the efficiency of a particular SE protocol which makes the process seem to be more effective than it may in fact be. It is perhaps somewhat misleading to make general statements such as "…about 60% of the seeds will produce embryogenic cultures and of these about 80% will form clonal plants." What this really probably means is that across a range of genotypes the initiation rates are on average about 30% (assuming 60% was the maximum) and maturation/conversion rates average about 40%. The figures presented this way do not suggest a very efficient propagation method.

In perhaps the only published detailed study of the efficiency of the SE process Hogberg et al. (1998) reported that the average recovery rates at different steps in the SE process in a Norway spruce were as follows: initiation rate 50%, selection of fast growing cell lines 50% of the preceding step, regeneration of plants 50% of the preceding step, and acclimatisation of plants 25% of the preceding step. Hogberg concluded that overall a 50% yield

at each step in the SE process was what could be expected with the then current protocol. Table 3 is an example of how the cumulative effect of the different efficiencies at each step in the SE process affects the overall efficiency of the SE process. As a result, starting with 500 immature zygotic embryos only 31 good quality embryogenic cell lines were produced for an overall 6.2% efficiency rate. This would clearly not be a very commercial process in its current state.

Table 3. *The effect of different rates of efficiency at each step in the SE process on the overall number of quality embryogenic cell lines starting with 500 immature zygotic embryos in spruce (Picea). After Hogberg et al. 1998.*

Step in SE process	Efficiency	Result
Initiation	50%	500 immature zygotic embryos produce 250 embryogenic cell lines
Established Cell Lines	50%	250 embryogenic cell lines decreases to 125 growing cell lines
Good SE Producing Cell Lines	50%	125 growing cell lines decreases to 62 good embryogenic cell lines
Maturation	50%	62 good embryogenic cell lines decreases to 31 producing matured emblings
Germination	50%	31 cell lines decreases to 15 that produce growing emblings

In addition Hogberg et al. reported than on average only 50% of the cell lines placed in cryostorage could be recovered. Recovery rates from cryopreservation protocols are rarely if ever reported. If the losses due to cryogenic storage are as high as Hogberg reported, then this is another area that requires further attention to increase the efficiency of the SE process.

Based on these results it is clear that the initiation rate is not the most critical step. As will be discussed later, high maturation/germination/conversion/acclimatisation rates are more important. Thus work to increase initiation rates alone is probably not warranted. It is easier to initiate more cell lines, but if they cannot be efficiently converted into mature plants then the SE process will not be commercial. The physiological quality of the somatic embryos being produced is critical.

Understanding of the Basic SE Process

After many years of research on SE our understanding of the basic process is still very limited in many areas. We have a basic understanding of how the embryogenic and suspensor cells interact in the development of somatic embryo plants (emblings), we know something about the general conditions necessary to produce emblings and we are attempting to understand the genes involved in this process.

However, what we do not understand is why certain cells can become embryogenic and what factors trigger them to do so. Once we understand this it will help us to increase the efficiency and effectiveness of the initiation step and may also help in the initiation of SE in mature tissues.

A recent review of SE in conifers (Teixeira da Silva and Malabadi, 2012) discusses the triggering of the SE process in conifers. Several types of stress (wounding, high salt concentrations, heavy metal ions, osmotic stress and high levels of plant growth regulators) have been suggested as the main "induction" factor in somatic embryogenesis in plants (Feher et al., 2003). In fact Feher (2008) has suggested that somatic embryogenesis may be a process that is normally suppressed in plants and that our "induction" treatments are actually de-repressing the SE process.

In embryogenic cell lines the specific cells that are "competent" to form somatic embryos capable of producing viable emblings make up only a small proportion of the total population of cells. Part of the problem is that we do not know how to identify these competent cells. Namasivayam (2007) commented in a review of the acquisition of embryogenic competence that "All these studies showed that competent cells have a variable appearance that prevents their identification on the basis of morphology".

We also do not know how to identify which somatic embryos will go on to produce acclimatised plants. Timmis (1998) commented that in work with Douglas-fir (*Pseudotsuga menziesii*) embryogenic cell lines that "Embryo yield was not reliably predictable from any features of liquid culture morphology, except in specific circumstances. Such as a particular genotype, large treatment contrasts or obviously nonembryogeneic culture."

Timmis commented that sometimes somatic embryos that look like zygotic embryos often fail to germinate whereas poorly formed somatic embryos may germinate remarkably well. The selection of somatic embryos with distinct epicotyl shoot and root growth has proven to provide a good morphological predictor in the selection of somatic embryos that will acclimatize and grow, but this is too late in the process to identify good embryogenic cells at an earlier stage.

While zygotic embryos might provide a general model for somatic embryogenesis, differences in the way somatic embryos are cultured (on nutrient media with high levels of exogenous growth regulators) and develop (naked and not enclosed in a protective and nutritive female gametophyte [in gymnosperms] or endosperm [in angiosperms]) as compared to zygotic embryo development may in fact be very different and may not have all that much in common with each other. Probably comparisons between somatic and zygotic need to be made carefully.

Within genotype fluctuations in embryo production (embryo number and quality) and morphology have been observed (Timmis, 1998 figure 6), but the reasons for these fluctuations have never been fully examined and are rarely discussed in spite of the fact that they are commonly observed in most laboratories.

Thus, while we know some of the basic conditions necessary to produce somatic embryogenesis in certain tissues at certain stages in their development, we know very little about the physiology of the induction and development of somatic embryos. We should pay more attention to the factors involved in the competence of cells to respond and the triggers involved in the SE induction process. This could help in the initiation of SE from more mature explants. If we could identify competent cells from the start of the process this might help to identify high quality somatic embryos later on in the SE process that might help make the SE process more efficient.

Economic

The importance of the economics in making the SE process practical has been mentioned many times, but the actual details of the costs to produce SE plants have rarely been discussed. Cost will depend to a great extent on the effectiveness and efficiency of SE in a given species, but from the limited data available they all are currently too high to be commercial.

Some have argued that the high costs of vegetatively propagated material (including SE) can be justified by the increased productivity and uniformity of the resulting crop. While this sounds plausible, it is not always **how much** of a return on investment will be achieved, but more importantly **how soon** it will be realised. In forestry this is a particularly important consideration especially with species that require long times to reach full rotation of the crop. The difference between having to wait 25, 40, 60 or even 100 years, depending on the species, to capture the full return on the original investment is very important. The less that has to paid and the sooner the returns will be realised, the better.

Thus the question is actually not "…**how much could you afford to invest** for a future return of a certain rate.." but rather "…**how little do you need to invest (and how long will you have to wait)** for a future return of a certain rate".

In the literature over the past 20 years several general comments have been made about the cost of SE which includes:

"…the cost of SE is about twice that of a seedling…" (1993)

"I estimate the cost of an acclimatised somatic embryo plant to be 25 to 50 and perhaps 100 times greater than that of a seedling." (2002)

"…the cost of SE will be less than that of a rooted cutting…" (2003)

"…the benefits of biotechnology will occur only when…clonal seedlings are produced at a cost approaching that of seed orchard seedlings…" (2007).

Of all these statements, the last is perhaps the most correct. At first it seems to be a very difficult target to achieve, but in reality it is where the costs need to be in order for SE to be commercial. As an example, rooted cuttings of conifers, which typically cost at least twice (or more) the cost of a comparable seedling, in most cases these are deemed to be too expensive for normal large-scale planting in spite of the fact that the cuttings may be improved planting stock. The closer to the cost of a seed orchard derived plant, the more likely a market for SE planting stock will develop.

What does SE Produced Planting Stock Cost?

The first question that needs to be answered regarding the cost of SE planting stock is where do the costs arise? The results of a detailed study of the origin of the costs of SE in *Picea* were published by Cervelli and Senaratna in 1995 and are summarised in Table 4.

Table 4. *Origin of costs in SE propagation of Spruce (Cervelli and Seneratna, 1995).*

Step	Percentage of Total Cost
Bulk-up	2
Maturation	3
Desiccation	30
Conversion	45
Planting	20
Total	100

From this table it can be seen that the majority of the costs are associated with the later stages of the process mainly desiccation, conversion and planting. This is because in these steps individual plants must the handled rather than masses of cell or even developing somatic embryos in the earlier steps (bulk up and maturation). The majority of the costs are due to the labour associated with these steps. Timmis (1998) made the point that "Since the unit cost at a particular point in the process is inversely proportional to the yield of the last step, then the larger and later these losses, the more serious their effect on the product cost."

The second question is how much does a SE plant cost? There are two published papers which provide information on the selling price of loblolly pine (*Pinus taeda*) SE plants in the south-eastern US (Sorensson, 2006; Bettinger et al. 2009) which are summarised in Table 5. The conclusion is that the "varietal" (SE produced) plants cost between 7.5 to 10 times the cost of a conventionally produced seedling. This makes these plants a very difficult product to market.

Table 5. *Costs of different sources of loblolly pine (Pinus taeda) planting stock.*

Sorensson (2006)	Cost/Plant
1.5 Generation seed orchard seedling	$0.042
2.0 Generation seed orchard seedling	$0.046
2.0 Generation elite seedling	$0.054
"Varietal" seedling (emblings)	$0.36 to 0.40
Bettinger et al. (2008)	
Bare root seedling	$0.05
Containerised seedling	$0.10
"Cloned tree seedling" (emblings)	$ 0.30 to 0.40
Emblings cost between 7.5 to 10 times the cost of a seedling.	

In an effort to make these costs more acceptable several imaginative marketing schemes have been developed. These include the "FlexStand" concept promoted by Arborgen in which rows of improved "varietal" (SE plants) material destined for the saw log market is planted with alternating rows of unimproved material. This is a variation of the "MOCAS" (**M**ixture **O**f **C**lones **A**nd **S**eedlings) as originally proposed by Park (2002). CellFor had promoted the idea of "value based pricing" in which the embling costs is based on a proportion of the future value of the crop. If production costs of emblings cannot be reduced additional imaginative marketing schemes such as these will be needed.

In the horticultural industry micropropagation using organogenesis is mainly used only to propagate new varieties that can command a higher price in the market. Once the market is adequately supplied with these new varieties, the price drops and as a result it is uneconomic to produce them by micropropagation. The same will probably be true for forest tree crops. The newest material from breeding programmes, rare or endangered material and special material for research purposes would command high prices. In special crops such as clonal Christmas trees, where certain traits and uniformity in the crop is critical, higher prices are possible.

It is clear that the current production costs for SE forest tree species need to be reduced, approaching that of seed orchard seed, if SE planting stock is to become commercial. A later section of this review will provide some suggestions on how this may be achieved.

Other Aspects of SE to Consider Regarding Scale-Up

Two additional points, epigenetic changes and public acceptance of SE are topics that need to be mentioned because they could have an effect on the commercial production of planting stock using SE.

Epigenetic Changes

Epigenetic changes are changes in gene expression which are also known as "environmental pre-conditioning" because such changes in gene expression may be influenced by environmental conditions including stress. They were first observed in plants in the 1970s (Cullis, 1977) but were largely ignored until very recently.

Among forest tree species epigenetic changes were first observed in a decrease in the cold-hardiness of seedlings of northern sources of Norway spruce in seed which was produced in seed orchards in southern locations when the resulting seedlings were planted back in northern locations (Johnson et al., 2005). It is hypothesized that the environmental conditions during zygotic embryo development in the warmer southern seed orchard promoted the development of less cold-hardy progeny.

This work was later extended to the effect of the culture temperature used in the SE process (Kvaalen and Johnson, 2008). Norway spruce emblings from cell lines grown at 28 ^{O}C grew longer into the autumn than the same cell line grown at 18 ^{O}C. The result is that emblings grown at the warmer culture room temperature were less frost hardy against an early autumn frost in the same way the seed orchard seed was.

This observation raises the question of whether other epigenetic changes are happening that have not yet manifested themselves. It also suggests that the effect of changing the culture room temperature and the effect of other environmental changes to production protocols should be thoroughly investigated, even in the performance of the trees in the field for several years, before production protocol are changed.

Public Acceptance

In general, the Public do not understand and appreciate many of the modern forestry methods currently known as "intensive forest management practices" and much prefer a more "natural forestry". For example, a recent study in Sweden (Hemstrom et al., 2014), where forests account for about 80% of the land area, found that of three "intensive forest practices" including fertilisation, the use of exotic species and the use of clonal planting stock that clonal planting stock was the least acceptable to the public. . It is important to appreciate that the general public do not distinguish between "vegetative" and "clonal" propagation. To them it is all the same.

The main concern of the public is that clonal planting stock will reduce the genetic diversity of the forest thus reducing the resilience of the forest to deal with threats from environmental changes, insects and diseases. If these concerns are not addressed it is possible that public pressure could result in local, national, or regional (e.g. European Union) regulations as well as possible restrictions imposed by forest certification organisations.

Currently forest certification as "sustainably managed forests" is provided by several organisations. Under the Forestry Stewardship Council (FSC- managed by Greenpeace) certification of the use of any genetically modified trees is prohibited, but interestingly the FSC does not currently object to the planting of clonal material. In Europe there are no overall European Union regulations on the use of clonal trees, but several European countries have different national regulations ranging from no restrictions to very precise regulations (see Lelu et al., 2013 for a summary of current regulations in Europe). If sufficient public pressure were applied to any of these organisations, the use of vegetatively propagated forest planting stock could be affected.

This is an area that requires attention because if it is not addressed the planting of clonal material might not be permitted and the use of SE technology for the production of this material would not be necessary. More work needs to be done to explain to the public the benefits and the liabilities of vegetative and clonal planting stock as well as to explain that these liabilities are recognised and are being addressed.

Possible Solutions to the "Challenges" discussed above.

The word "challenges" has been deliberately used in this paper rather than the term "problems" or "obstacles" because the points discussed above are things that can be addressed and resolved. The remainder of this paper will attempt to provide possible solutions to make SE of forest trees more commercially acceptable.

"Hybrid" SE/rooted cutting propagation systems

The use of a combined, two-step propagation process where SE is used to produce stock plants which produce cuttings for rooting is currently used in Ireland (Coillte Teoranta) with Sitka spruce and in New Zealand (Forest Genetics Ltd.) with radiata pine.

In Ireland seed orchards have been established but are not yet in production. Full-sib crosses are made between progeny tested parents and immature zygotic embryos are used to establish cell lines. Emblings are estimated to cost between 2 and 3 Euro each as compared to 0.25 Euro for a seedling transplant. As a result emblings cost is about 10 times that of a seedling, which is similar to the costs for loblolly pine in Table 4. If however the emblings are grown in nursery beds to produce cuttings for rooting, over a 5 year period each stock plant will produce between 200 and 250 cuttings, the cost of the embling disappears.

Sitka spruce rooted cuttings cost about twice the cost of a seedling (due to the labour required to collect and insert cuttings for rooting and the overall rooting percentage). Planting a hectare of pure rooted cuttings is not economic but planting a 50:50 mixture of unimproved seedlings and improved rooted cuttings ensures that enough improved material will be available for the final crop trees while reducing the per hectare plant costs.

The cost of the rooted cuttings, due to the additional labour required for the collection and rooting of the cutting as well as the yields, makes marketing of this material a challenge. The advantage of the use of the hybrid SE/rooted cutting system is that the number of stock plants produced is greater and faster than by any other propagation method.

In the future when seed orchards come into production, provided that the breeding programme continues to be active, further improved material will be available and the SE/rooted cutting process will be the fastest way to get the newest improved material into production as quickly as possible.

Bioreactors

The main idea to use bioreactors in SE was initially seen as a way to produce large volumes of embryogenic tissue quickly. However, as the analysis of SE of Cervelli and Seneratna (1995) has shown, producing embryogenic tissue is not the most labour intensive and thus costly step in the process. More recently the use of Temporary Immersion Bioreactors (TIB) has been used in the final steps of embling production. It has been shown to increase the number and the quality of the SE produced in them. Many examples of the use of TIB in the production of SE plants and the benefits of such a system have been summarised by (Watt, 2012). One report claimed a 6 to 16 fold increase in the number of somatic embryos produced in a TIB system compared to semi-solid media (Ibraheem et al., 2013). Another example is the production of coffee emblings where before TIB was used SE plants cost US$ 1.52 each to produce compared to a seedling cost of US$ 0.25 to 0.35 each (Etienne et al. 2010). The production of pre-germinated emblings in a TIB and sowing them directly on horticultural substrate reduced the cost to US$ 0.50 each. About 90% of the savings was due to the reduced manpower needed for the subculturing of the emblings, but in addition the quality and quantity of the emblings was also increased (H. Etienne, personal communication 2014).

Automation

Automation is often seen as the most effective way to reduce the production cost of micropropagated plants because it directly addresses the most expensive item, labour costs. However, while automation can reduce costs, if the biological aspect of the production system does not produce large numbers of high quality emblings (high maturation/germination/conversion/acclimatisation rates) then the automated production system will not be as cost effective as it could be. Automation will not make up for poor quality biological material. Automation works best with systems that produce large volumes of a single product in a year-round production system. The production of forest tree planting stock will involve the production of many individual cell lines (20 to 40) mainly at one specific time of the year which will present new challenges.

Several automated systems for the production of plants by organogenesis have been developed (Tisserat and Vandercook, 1985; Aitken-Christie, 1991). Automation has also been used in the handling and sorting of somatic embryos (Zhang et al, 1999) however, both high quality somatic embryos and the ability to recognise them are necessary to make such systems effective.

Because the majority of the costs occur in the handling of individual plants at the last stages of the SE production process (Cervelli and Seneratna, 1995) two main approaches have emerged; 1) the fully automated planting using robotic devices for the handling, sorting and planting of somatic embryos (Aidun and Egertsdotter, 2012; Find and Krogstrup, 2013) and 2) a semi-automated approach using a human operated machine to do the planting (Roberts et al. 1995).

Unfortunately due to the proprietary nature of this work much of the details of the efficiency, capital costs and the reduction in production costs are not currently available. As a result the practicality of this technology and its ability to significantly reduce costs has yet to be demonstrated.

Synthetic Seed

The use of SE to produce "synthetic", "artificial" or "manufactured" seed" is an idea that dates back more than 30 years. In 1991 a claim was made that in forestry "In the future production facilities producing SE as an artificial seed may replace production in seed orchards." While this still may happen "in the future", it has not happened in the 23 years since this prediction was made.

Nevertheless, synthetic seeds offer a potentially very efficient delivery system for SE, however, it is not as easy as it looks and still has major problems that need to be resolved (Carlson and Hartle, 1995; Sharma et al.,2013). Once again the quality of the somatic embryos is a major limiting factor.

Several types of synthetic seeds exist including desiccated and undesiccated as well as coated or encapsulated or naked synthetic seed. There are problems in the ability to store such artificial seeds for extended periods of time as can be done with zygotic seeds.

Of all the SE delivery systems the synthetic seed is the most demanding in terms of the quality of the SE material producing the synthetic seeds. For such a system to be practical in a container growing situation SE germination rates would need to be at least 85% to be effective (based on results with sowing seed in container systems). In most cases this level of SE germination has not been achieved.

If such synthetic seeds could be developed it would be the most efficient way to handle emblings. Unfortunately once again due to the proprietary nature of this work, information on the effectiveness, the reduction of production costs and the fact that no system has been demonstrated makes the evaluation of the current utility of this technology not possible.

Conclusions

This review began with the question of whether SE could be an effective way for the large-scale commercial propagation of forest trees. While theoretically and technically possible, the current high cost to produce SE planting stock makes this technology currently uneconomic. The main reason is the high production costs of emblings due to the inefficiency of current propagation protocols. However, with additional work to improve the quantity and quality of somatic embryos large-scale commercial propagation using SE could become practical.

It has become a bit of a cliché to end a research paper with the sentence, "...further research is needed...", but that is exactly what is needed. Further research is specifically needed to improve the quality of the somatic embryos that are produced. Increasing the effectiveness (across a wide range of genotypes) and the efficiency (consistently producing large numbers of emblings) are key to this process being economic. In addition increasing the quality of emblings (high maturation/ germination/ conversion/ acclimatisation rates) is essential for the process to become economic. Evidence suggests that use of bioreactors may be useful in accomplishing this. Automated propagation systems and synthetic seed may help reduce the costs, but they would greatly benefit from higher quality somatic embryos.

SE plant costs need to be brought as close to the cost of seed orchard produced planting stock as possible. Convincing forest companies that own and manage forest land to plant vegetatively propagated stock is difficult when it ranges in price from two times for rooted cuttings to six to ten times for emblings compared to conventional seedling planting stock. Convincing private land owners to make such an investment is even more difficult.

Recovery rates from cryogenic storage need to be documented and if found to be a significant bottleneck, they need to be addressed.

We also need a much better understanding of the basic process of somatic embryogenesis. Identifying the genes involved in SE is important but we lack a good understanding of the embryogenic competence of cells, the triggers of embryogenesis and the physiology of developing somatic embryos. We need to have a better defined, reliable system both for further research into the process of SE but also to make the process commercially practical. This knowledge may also help work on the initiation of SE in mature tissues.

The importance of environmentally induced epigenetic changes and the public acceptance of vegetatively propagated trees needs to be more widely understood and appreciated. The potential problem of narrowing the genetic base of forestry crops and its implications on the public acceptance of vegetatively propagated forest trees needs to be discussed with the public to prevent restrictions on the use of this technology.

Cost is not everything, but if the cost of a technology is too high no matter much benefit the technology provides, it will not be used commercially. SE does offer the best option for the large-scale vegetative propagation of forest trees provided that production costs can be reduced. If they cannot, then SE will probably remain as a research tool to help in the understanding of sexual propagation of trees. We would do well to remember what happened with organogenesis as a method to propagate forest trees.

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Extended Abstracts

Integration of tree biotechnologies into multi-varietal forestry

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In the past 50 years, tree breeding programs around the world have contributed greatly to increased forest productivity. Traditional tree breeding typically employs a form of a recurrent selection system, and the production of genetically improved material is delivered by wind pollinated seed orchards. While the use of seed orchards remains the main method of obtaining improved seed, new biotechnologies such as somatic embryogenesis (SE) and genomics technology could provide more effective and alternative delivery of genetic improvement. We examined the issues and outlook for integrating SE and other biotechnologies into commercial Multi-Varietal Forestry (MVF). MVF is defined as the use of a range of genetically tested tree varieties in commercial plantation forestry. It is also known as "Clonal Forestry", but the term is not precise because a clone usually refers to any genotype and its ramets, while a variety is a clone selected for specific purposes or attributes.

SE is the most important biotechnology in conifers for the development and production of tree varieties with desirable traits, and thus enabling MVF. Seedling production by the SE process in conifers involves the initiation, proliferation, maturation, germination, and greenhouse culture with varying degrees of success at each step. While obtaining SE for several commercially important tree species may still be difficult, the SE system for several conifers has advanced to the stage where it can be implemented in large-scale commercial production, especially for spruce species and a few pine species. SE is not a tree breeding method, but it is a required biotechnology when tree breeding aims at the development of tree varieties and their deployment. The most important advantage of conifer production by SE is that the embryogenic cell lines can be cryogenically stored in a juvenile state indefinitely, which was not possible with other vegetative propagation techniques for trees. This allows for the long-term field testing and for the subsequent selection and retrieval of the tested cell lines for mass propagation. This led to the commercial implementation of MVF in eastern Canada by JD Irving Limited. Thus, our discussion is based on the integrated breeding and SE efforts of white spruce (*Picea glauca*) in eastern Canada.

The traditional seed orchard based breeding process involves the formation of multiple breeding populations, controlled pollinations among parents within the breeding population, establishing, maintaining, and evaluating progeny tests at multiple sites. Subsequently clonal seed orchards are established for the production of improved seed, while the selected parents form a new breeding population for the next cycle of breeding. Selection of parents is usually based on the high estimated breeding values of individual trees through their performances in the field test using phenotypic data and pedigree information. Therefore, tree breeding programs require extensive resources and an extended period of time. Also, the establishment of the land-based seed orchards is expensive and they remain fixed and inflexible until the establishment of the new next generation of orchards. A typical breeding cycle, using a subset of a breeding population for white spruce, is illustrated in Figure 1 and takes about 17 years to complete and 19 years to produce improved seeds. This seed orchard-based tree breeding scheme typically produces about 10% volume increase per generation, and this is expected to be the primary means of obtaining genetic improvement in conifers in the near future.
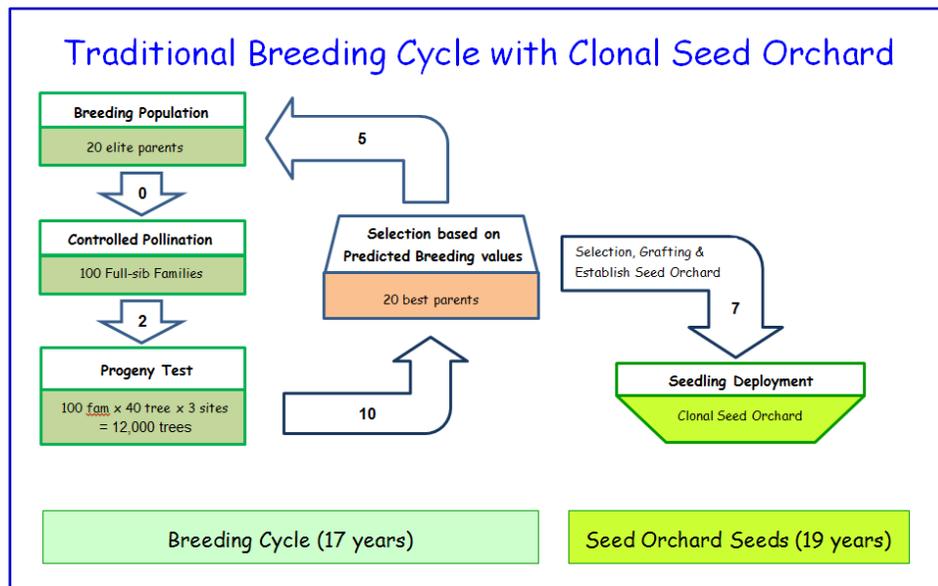
Figure1. *Current tree breeding and seed orchard production cycle for white spruce in eastern Canada. The numbers in the arrow indicate the number of years to accomplish that step*

During the past 20 years, there has been a rapid development in marker technology, and the availability of inexpensive molecular markers offer a possibility of using them to improve the efficiency of tree breeding. The use of molecular markers in breeding was primarily focused on marker-aided selection (MAS). The concept of MAS is that if the QTL is identified with corresponding molecular markers, they could be used to select superior genotypes in the breeding population. However, the QTL mapping and candidate gene approach in forest trees has not been used widely, primarily due to the fact that most of the important traits are controlled by the many QTLs, each with only a small effect on the trait. Genomic selection (GS) or genome-wide selection is a form of MAS. However, it is distinctly different from the traditional MAS based on QTL, because the markers are used to elucidate the genomic relationships among individuals in the breeding population instead of relating the markers with specific traits. In GS, a large numbers (thousands) of Single Nucleotide Polymorphism (SNP) makers are used for genotyping. Using a training population, a genetic model is developed to predict a genomic estimated breeding value (GEBV). For example, an individual's phenotype is the sum of all SNP marker effects. Once a model equation is developed, GEBV is predicted in the breeding population using only genotype (marker) data, and GEBV is used to select next-generation parents for the formation of new breeding population (Fig 2).
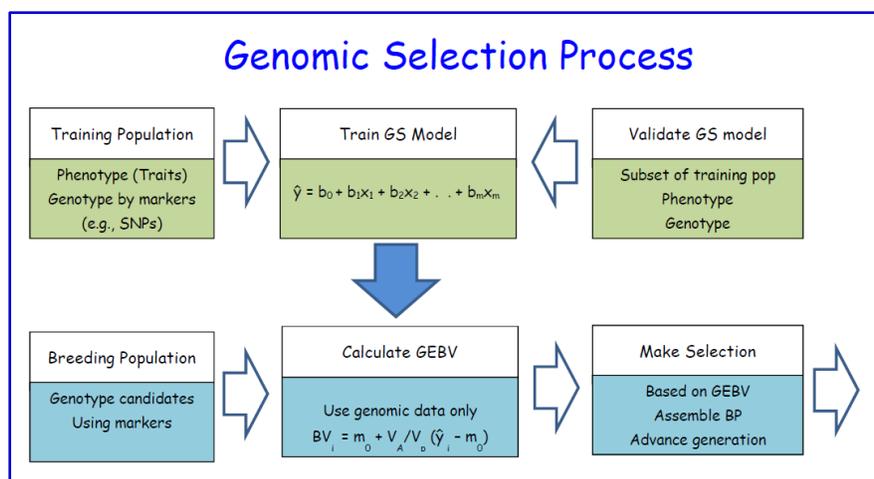
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Figure 2. Schematic presentation of genomic selection. The upper boxes indicate the GS model development (training phase), while the lower boxes indicate the selection phase using the model developed in the training phase

The integration of SE and GS in MVF is illustrated in Fig 3. As in traditional breeding, controlled crosses are made and the resulting offspring is used for the development of SE lines. After a short cryopreservation, a part of the lines are thawed, genotyped, and the GEBV of each line is calculated. The best GEBV individuals are used for next generation parents as well as for the current generation's deployment. Again considering the flowering maturity, it take 19 years to complete the breeding cycle with SE and GS; however, for MVF, the gnomically selected lines are available for vegetative deployment within 4 years instead of the 19 years required by traditional breeding, resulting in realization of a gain in time of 15 years.
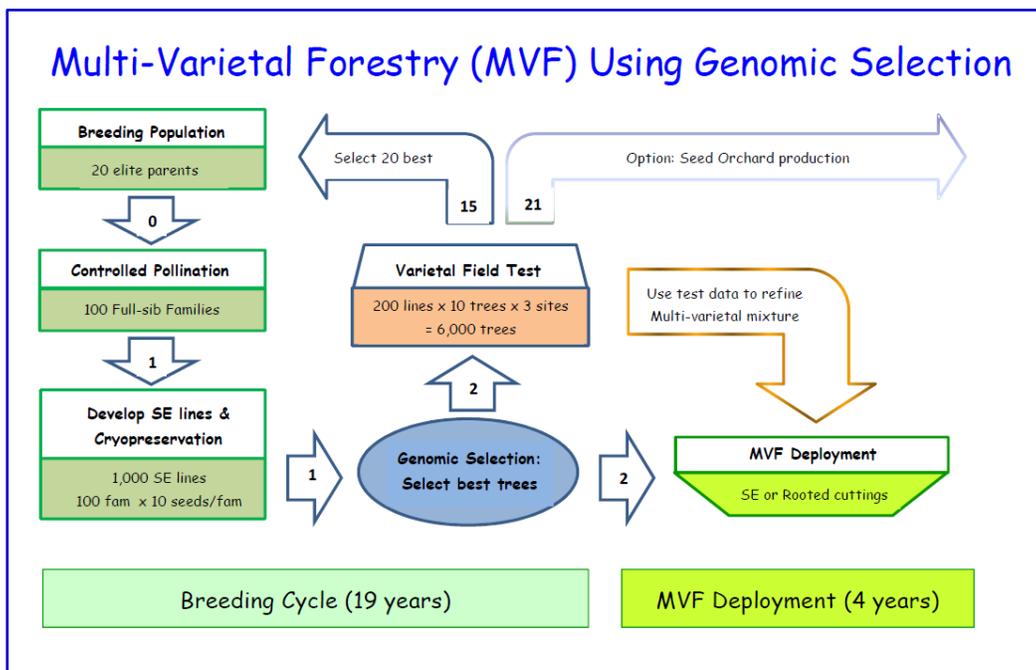

Figure 3. Multi-Varetal Forestry integrating SE and genomic selection. The breeding cycle is delayed by two years due to the SE and GS steps; however, time for deployment is reduced by 15 years through vegetative deployment instead of seed orchard production

There are many benefits of MVF: obtaining much greater genetic gain than is obtained by traditional seed orchard breeding; production of uniform, high quality wood; flexibility to rapidly adapt to changing climate, breeding goals, and insect and disease conditions; improved ability to manage plantation diversity. With the integration of GS and vegetative deployment by SE, genetic improvement may be realized as much as 15 years sooner.

Genomic Selection and Clonal Forestry Revival

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Summary: The recent developments in both genomics (Next Generation Sequencing) and quantitative genetics (methods that incorporate dense sequencing information in evaluation and selection (i.e., genomic selection)) have created a paradigm shift where the long-term phenotype-dependent selection that was practised in the past is to be replaced by genotype-dependent selection, thus reducing the time for economic traits' genetic evaluation to virtually days or weeks. Shortening economic and complex traits' long-term genetic evaluation will result in: 1) speeding breeding generations' turnover and 2) considerable increase in the selection differential, thus yielding substantial genetic gain increase and programs' flexibility for coping with unperceived contingencies (e.g., climate change or resistance to pests). The combined effect of early selection and greater selection differential calls for appropriate tree improvement delivery systems that by-pass sexual reproduction and maintain the genetic integrity of the selected elite genotypes in congealed state. Vegetative propagation and somatic embryogenesis, in particular, offers unprecedented opportunity to the delivery of the genetic gain attained through conventional and marker-based selection programs. In this presentation, an overview of genomic selection covering various pedigree scenarios and the integration of cloning with modern selection methods will be presented.

Keywords: Tree breeding, quantitative genomics, somatic embryogenesis, genetic gain

Traditional tree breeding programs follow a common path characterized by repeated rounds of selection, breeding and testing (a.k.a., recurrent selection (Allard 1999)), each with incremental improvement of the target traits' output. Substantial gains have been attained through this process (McKeand et al. 2003); with sexual reproduction as the main mode of gain delivery from seed orchards (Figure 1). While seed orchards represent the most economic vehicle for genetic gain delivery, they have proven to fall short of their theoretical expectations, mainly due to issues associated with fertility and reproductive phenology variation and pollen contamination (El-Kassaby 1995), and the often ignored negative genetic correlation between fertility and vegetative growth (El-Kassaby and Barclay 1992). Furthermore, the genetic diversity and gain of seedling crops are different from their seed crops as nursery culture practices such as thinning and culling causes substantial changes (El-Kassaby and Thomson 1996, El-Kassaby 2000). These drawbacks forced forestry managers to adopt alternative gain delivery methods such as controlled mass pollination (Bridgwater et al. 1998) followed by vegetative propagation to overcome the high cost of seed production (Gupta et al. 1987; Greenwood et. al. 1991). Somatic embryogenesis (SE) is, by far, the most promising technology for bulking-up elite genotypes for maximizing genetic gain delivery (Sutton et al. 2004) and the technique's incorporation in tree breeding program has been effectively developed (Klimaszewska et al. 2007).

The recent development of DNA sequencing technologies such as Next-Generation Sequencing (NGS) have revolutionized biological research through the generation of massive single nucleotide polymorphism (SNP) data sets (Cheng et al. 2013). The most important feature of these sequencing technologies is their ability to produce extensive DNA sequences for non-model species including those with large, complex, and unreferenced genomes, such as conifers, thus paving the road for genomic data use in these species (Elshire et al. 2011). The development of NGS technologies and the utilization of sequence data have literally revolutionized quantitative genetics and novel application became possible, such as: Genomic Best Linear Unbiased Predictor (GBLUP) (VanRaden 2008), the unified single-step evaluation approach (a.k.a., HBLUP) (Misztal et al. 2009), and creative tree breeding

methods such as Breeding without Breeding (BwB) (El-Kassaby and 2009; El-Kassaby et al. 2011). The most prominent application among these new methods is Genomic Selection (GS) (Meuwissen et al. 2009). GS collectively uses the genome-wide marker data in predicting the phenotype by estimating individuals' genomic breeding values. GS does not require the identification of the QTLs or linked markers with the target traits as all marker effects are estimated simultaneously and used to develop the prediction model for estimating individuals' Genomic Estimated Breeding Values (GEBV). Therefore, this method is particularly suitable for selection of complex traits such as those following Fisher's infinitesimal model (Fisher 1918). In a forest tree breeding context, GS has the ability to predict the phenotype for selecting elite genotypes at an early developmental stage, thus substantially shortening the breeding cycle and increasing the selection differential, ultimately maximizing the genetic gain per unit time (Resende et. 2012a,b; Zapata-Valenzuela et al. 2013; Beaulieu et al. 2014).
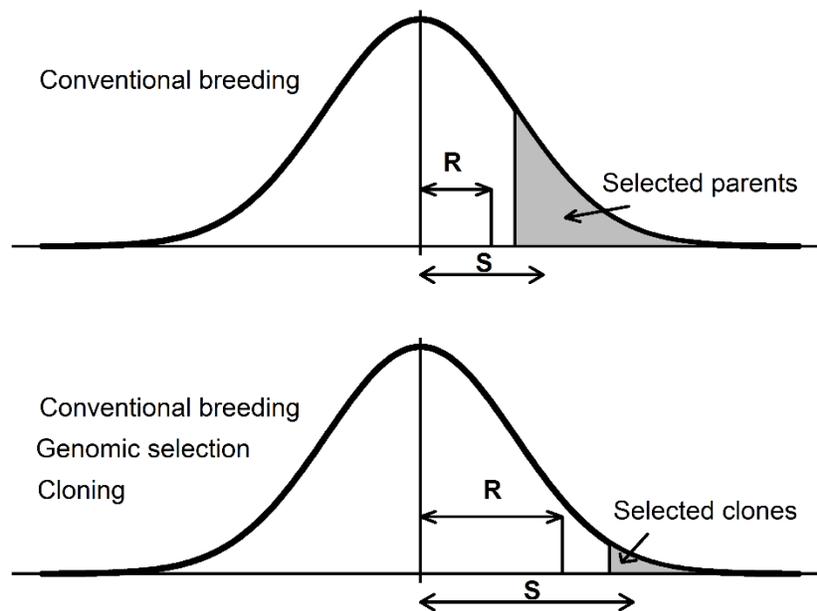

Figure 1. Schematic diagram illustrating the difference between: a) conventional sexual reproductions through seed orchard and b) the combined genomic selection and somatic embryogenesis multiplication method.

A possible synergy is the combination between GS and SE whereby GS identifies elite genotypes at high selection intensity and early age and SE amplifies these elite genotypes for reforestation programs (Figure 1), thus creating opportunities for increasing clonal forestry adoption. A major differences between the SE and seed orchard delivery systems is that the formers' main multiplication mode is mitosis (i.e., maintaining genotypes in congealing state due to the lack of recombination) while the later produces offspring through meiosis yielding a genic combination that is different from that of its parents.

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Pine tree biotechnology for high value forestry

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Keywords: Pinus, micropropagation, somatic embryogenesis, cryopreservation, genotyping.

Nowadays, a growing demand for high quality forestry products reinforces the need to establish new methodologies for genotyping, clonal propagation and preservation of high value genotypes that retain traits of interest. In this context, Klón - Innovative Technologies from Cloning invests in research activities related to molecular marker-assisted selection and mass propagation techniques, via somatic embryogenesis and micropropagation of mature trees. This allows to obtain Pinus spp clones from elite trees with industrial interest providing higher frequencies of homogeneous plants and enabling a large genetic gain. Micropropagation is one the most suitable methods for large-scale production of superior genotypes in tissue culture. Our research team has been dedicated to the multiplication of elite tropical Pinus trees by application of meristem isolation techniques from mature tree shoots (Figure 1). We have developed a reproducible tissue culture procedure by introducing variations in culture media composition and conditions during induction, elongation and rooting phases. Different basal macro and micronutrients and concentrations of cytokinins, auxins and carbohydrates have been tested resulting in continuous increments in the multiplication rate (Bronson and Dixon 1991; Prehn 2003; Tang and Newton 2007; Cortizo et al. 2009; De Diego et al. 2010; Zhu et al. 2010).

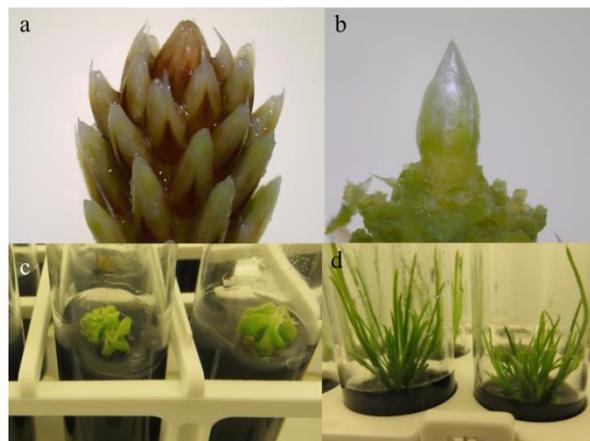**Figure 1**: *Micropropagation of an elite tropical Pinus tree (Pinus elliottii var. elliottii x Pinus caribaea var. hondurensis) by meristem isolation. (a) Mature tree shoot, (b) Apical meristem with needle primordia, (c) Meristems developing into shoots after seven weeks of culture, (d) Propagated shoots developing from an in vitro cultured meristem after 10 months of subculture.*

Somatic embryogenesis (SE) is one of the most promising techniques for large-scale propagation of elite genotypes. However, few publications have dealt with tropical and subtropical pine species (David et al. 1995; Liao and Amerson 1995; Newton et al. 1995; Newton and Jain 2005). Somatic embryogenesis in hybrid pine has been one of the procedures we followed to propagate elite plants. Initiation and maturation of embryogenic tissue can be critical steps for the commercial application of SE in Pinus species. The effect of collection date or developmental stage of the zygotic embryos has been studied simultaneously with the application of different basal media and plant growth regulators (PGR) combinations to overcome bottlenecks. A successful protocol of somatic embryogenesis for elite tropical hybrid pine has been established, following the subsequent stages of initiation and maturation of somatic tissue and conversions to plants (Figure 2).
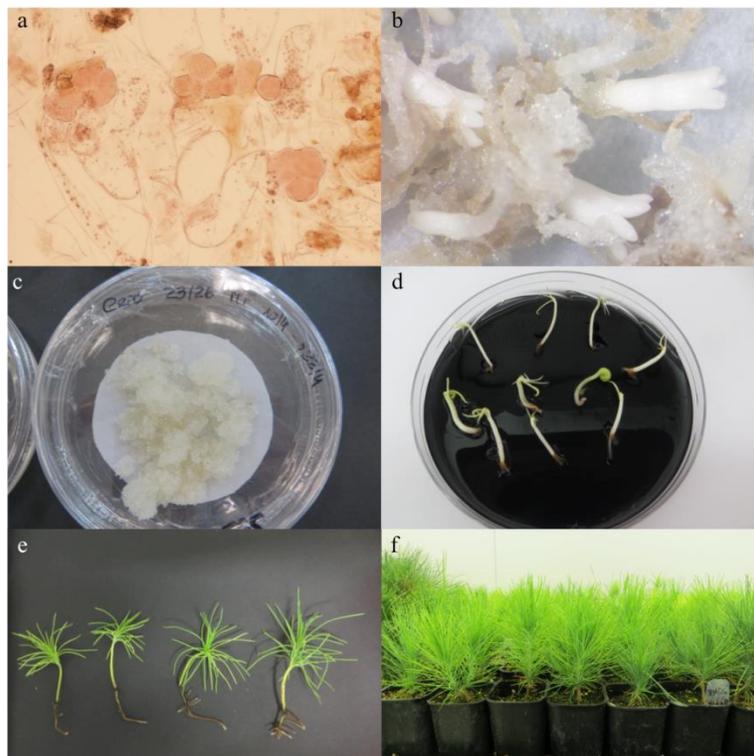

Figure 2: Elite hybrid tropical pine (Pinus elliottii var. elliottii x Pinus caribaea var. hondurensis) regenerated by somatic embryogenesis. (a) Somatic embryogenic cell aggregates derived from immature zygotic embryos, (b) mature somatic embryos, (c) embryogenic mass recovered from cryopreservation, (d) somatic embryos germinated, (e) somatic plants to acclimatization, (f) somatic plants after 2 months in the greenhouse.

Preservation of selected plant material with important forestry features is also one of Klón's objectives. We have been developing systems for long term storage of plant material at low temperature in liquid nitrogen for cryopreservation of somatic embryogenesis tissue, ensuring the availability of juvenile material, avoiding losses of embryogenic capacity and providing biological material that is genetically stable. Protocols of cryopreservation were developed successfully for selected genotypes of tropical pines from a breeding program established in 1991 (Figure 2-c). Klón has the structural capacity to cryopreserve about 35 800 samples of high value germplasm.

In long-lived species such as forest trees, the conventional breeding programs can take a long time to provide returns. Hence, nowadays, molecular marker-assisted selection is a powerful tool to accelerate selection and make breeding programs more efficient and productive. Therefore, Klón has been investing in the identification and selection of new molecular markers for high throughput genotyping of pine trees with valuable economic traits. The research has been focused mainly on two types of molecular markers, namely, Single Sequence Repeats (SSRs) and Single Nucleotide Polymorphism (SNP). A set of 9 SSRs markers (Echt et al. 1999; Elsik et al. 2000; Shepherd et al.

2002), previously described for Pinus spp., is being validated in populations of tropical Pinus tree species. In parallel, based on sequencing data generated by transcriptomic analysis of selected pine trees, we are also developing new SNPs and SSRs markers associated with genes that control traits with high economic interest (resin and wood production).
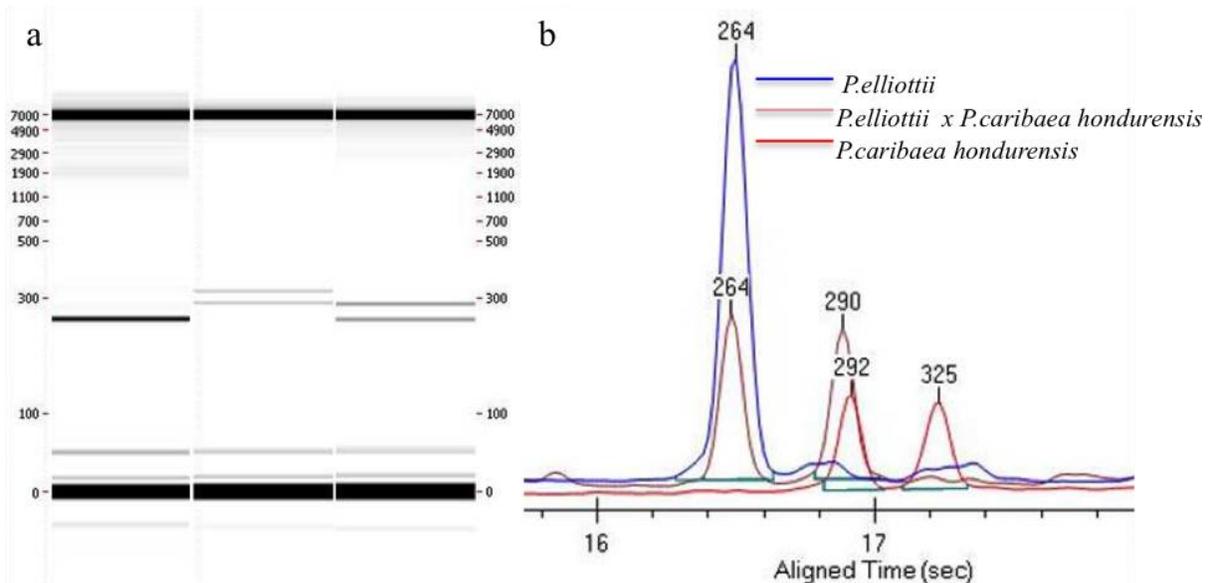

Figure 3: Fragment analysis of a SSR marker in the hybrid Pinus elliottii var. elliottii x Pinus caribaea var. hondurensis and in the species Pinus elliottii var. elliottii and Pinus caribaea var. hondurensis, using an automated electrophoresis system. (a) Example of a virtual gel image and (b) an electropherogram of the three biological samples.

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An overview of the current achievements in Holm oak (*Quercus ilex* L.) somatic embryogenesis

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Keywords: plant regeneration, secondary embryogenesis, liquid medium, cryopreservation.

Holm oak (*Quercus ilex* L.) is one of the main evergreen species of the Mediterranean forests in the Iberian Peninsula. It is commonly found with the cork oak in the *dehesas* or *montados*, which have historical importance in social and rural development. Besides its ecological importance, the main economic interest relies on its acorn production to feed livestock and on the establishment of symbiotic relationships with several edible fungi, the most profitable species being the black truffle (*Tuber melanosporum*). However, the survival of holm oak is threatened by variable climatic conditions, wildfires, the oak decline syndrome and a low natural regeneration. Genetic amelioration and breeding programs can improve heritable factors for diseases resistance and production.

Oaks species are considered recalcitrant species in terms of seed conservation and their organogenic capacities are limited up to a few months after germination, which complicates the development of genetic conservation and improvement programs. Among the vegetative propagation techniques, somatic embryogenesis (SE) enables mass propagation and the implementation of multivarietal forestry. SE has been defined before as a good method for clonal regeneration of adult trees in other *Quercus* species (Corredoira et al. 2014), which has allowed the establishment of highly efficient clonal tests of selected phenotypes.

Contrary to other oak species, the induction of SE from leaves of mature holm oak trees was unsuccessful, but catkins and the integuments of developing acorns were found to be suitable explants depending on their developmental degree (Blasco et al. 2013; Barra-Jiménez et al. 2014).

In the case of the maternal ovule integuments, several media compositions were tested for SE induction. The macronutrients of Gamborg (Gamborg 1966, PRL-4-C) and SH (Schenk and Hildebrandt 1972) were tested, supplemented with micronutrients and vitamins from MS (Murashige and Skoog 1962) and 30 g l^{-1} sucrose and 6 g l^{-1} agar. Also, the effect of the presence *vs.* the absence of PGRs and the influence of arabinogalactan proteins (AGPs), either from *Larix* or from *Acacia* were studied.

For the presence *vs.* absence of PGRs, the explants were cultured on basal medium supplemented with 10 μM 1-naphthaleneacetic acid (NAA) and 10 μM 6-benzyladenine (BA) in darkness at 25 ºC for 30 days. Then, they were transferred to the same medium with the same plant growth regulators (PGRs) but reduced to 0.5 μM and finally, the explants were cultured on proliferating medium without PGRs. Each of the last two steps was performed under a 16-h photoperiod (120-180 μmol m^{-2} s^{-1}) at 25 ºC for 30 days.

Because of the low SE induction frequencies (1-2.3%), the effect of genotype and culture media formulation on the embryogenic response remained unclear. Nevertheless, SH macronutrients and the lack of PGRs or AGPs were the most effective conditions in the induction of SE.

Several embryogenic lines were established by secondary SE and cotyledonary embryos differentiated spontaneously in the proliferation medium. However, the differentiation capacity of some genotypes declined over time. Maturation, germination and conversion of those embryos were successful (Barra-Jiménez et al. 2014) (Fig.1).
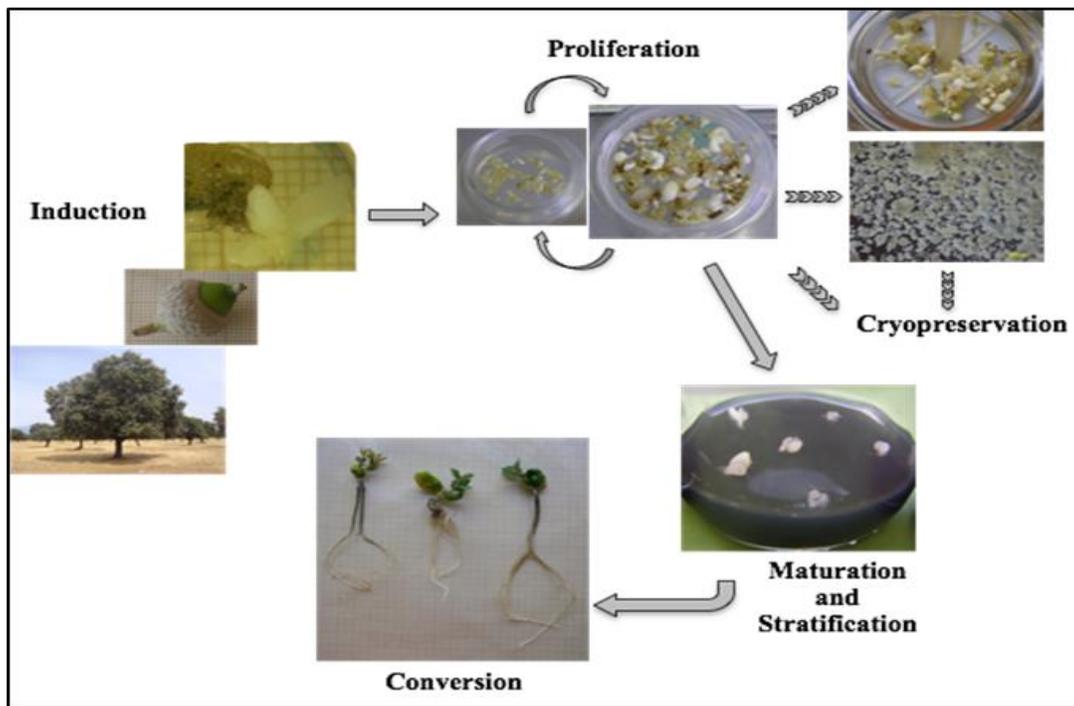
Figure 1. *Complete scheme of the regeneration protocol of mature holm oak trees achieved by somatic embryogenesis.*

Throughout the regeneration process, it was confirmed that genotype had an influence over the proliferation capacity and the plant conversion of somatic embryos (11.3-30.6%). The presence of 0.25 μM indole-3-butyric acid (IBA) and 0.11 μM (BA) favoured the conversion rate of the somatic embryos (11%), but it did not affect the germination capacity (Barra-Jiménez et al. 2014).

In order to improve efficiency and quality of the embryogenic cultures, the influence of light and the presence of PGRs were evaluated during the secondary embryogenesis and it was concluded that both factors reduced the quality of the cultures, but had no clear effect on the productivity. The use of SH liquid medium was also studied. A temporary immersion system (RITA®) increased the growth depending on genotype, when compared to semisolid cultures. However, it did not improve the differentiation of single cotyledonary embryos under the conditions tested for *Quercus robur (Mallón et al. 2012).*

The initiation and maintenance of suspension cultures of holm oak was achieved following the cork oak´s method (Barra-Jiménez et al. 2013). The addition of glutamine to these proliferation cultures significantly improved the growth but morphological differences were observed.

In order to prevent the loss of embryogenic competence or contamination problems, and also to decrease the cost of maintenance of the lines, some of the holm oak embryogenic lines were cryopreserved using the vitrification protocol developed for cork oak (Valladares et al. 2004; Barra-Jiménez et al. 2013). The influence of high sucrose concentration (0.3 M) and PVS2 on the growth and differentiation capacities before and after liquid nitrogen immersion (LN) were determined for semisolid and suspension cultures. It was concluded that both responses were independent from each other and also that they were under the influence of genotype. The proliferation capacity was recovered in all the cryopreserved embryogenic lines, contrary to the differentiation capacity, which depended on genotype and cryopreserved material. The combination of 0.3 M sucrose and PVS2 prior to LN immersion provided higher recovery rates (Jiménez et al. 2013). When the embryogenic lines were cryopreserved for 30 days, none was able to differentiate.

The analysis of eight SSR markers disclosed the occurrence genetic variation in the case of the short-term cryopreserved material when they were compared with the non-cryopreserved tissues. The SSR loci examination indicated that genetic stability was genotype-dependant and that suspension cultures were probably stable since no genetic variation was detected after cryopreservation. Nonetheless, the existence of a relationship between the

genetic instability and the loss of the differentiation capacity remained unclear.

As a conclusion, the complete regeneration of mature holm oak trees was successfully achieved by SE (Fig. 1), although there are some bottlenecks to be improved in order to secure a high productivity of the process.

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Differentiation of cork oak somatic embryos in liquid medium

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Keywords: Forest biotechnology, micropropagation, *Quercus suber*, secondary embryogenesis, somatic embryogenesis

Cultural and genetic improvement practises have to be applied to increase yield in intensively managed forest plantations. A powerful tool that has been used in genetic improvement of many woody crops is vegetative propagation. Clonal plant regeneration by somatic embryogenesis (SE) is the way in which forest biotechnology may obtain vegetative propagules in large numbers at a reduced cost. But forest biotechnology must be coupled with classical breeding schemes in order to obtain its full advantage. The strategy of multi-varietal forestry (Park 2014), which is defined as the use of genetically tested tree varieties in plantation forestry balancing genetic gain with diversity, is beginning to be implemented due to the development of embryogenic systems. Clonal propagation by SE can quickly capture the benefits of breeding and is expected to play an important role in increasing productivity, sustainability and uniformity of forest plantations. Most protocols to clone tree species by SE use semisolid medium culture, although there are some notable exceptions (Merkle and Nairn 2005). However, cultures in liquid medium need to be developed for economically viable mass propagation (Ibaraki and Kurata 2001).

The cork oak (*Quercus suber* L.) is one of the most important broadleaf species of the Mediterranean region. Cork is the renewable raw material produced by this species that is used for relevant industrial applications. Many studies have been carried out to define protocols for inducing SE in cork oak, including those for cloning adult trees (Hernández et al. 2003, 2011). Also conditions for culturing embryogenic tissues in liquid medium and for establishing suspensions have been reported (Jiménez et al. 2011, 2013).

Proliferation of embryogenic tissues in liquid medium has received much attention, but studies dealing with the differentiation of somatic embryos in liquid cultures are scarce. Three ways of differentiation of somatic embryos were observed in liquid cultures of cork oak embryogenic tissues (Alegre et al. 2010). In the first way, embryos developed from embryogenic tissues following the pattern usually observed in cultures on semisolid medium. Secondary embryos developed, beginning with the formation of a shoot apex and cotyledons while the embryos maintained a broad attachment with the original tissue, resulting in clusters of embryos (Fig. 1A).

Figure 1: *Different ways of differentiation of secondary somatic embryos of cork oak in liquid medium. **A**, secondary embryos developing cotyledons and maintaining a broad attachment with the original tissue; **B**, secondary embryos arising as globular embryos detaching from the surface of embryogenic tissues; **C**, globular embryo formed from a small cell aggregate in suspension culture.*

In the second way, which was observed in differentiated embryos or in organized structures such as embryogenic clumps (EC), new secondary embryos differentiated as globular embryos that detached from the surface of embryogenic tissues (Fig. 2B). They usually developed into free and well-formed cotyledonary embryos. In the third way, small non-organized aggregates of a few embryogenic cells in suspension cultures were able to develop globular embryos (Fig. 1C).

The two later ways of differentiation are desired because single somatic embryos are produced. It was observed that when embryogenic clumps or single embryos were cultured in liquid medium at low inoculation density and low speed, the second way of differentiation was preferentially expressed. The main purpose of this study was to evaluate and optimize the production of single secondary somatic embryos by the second way of differentiation.

Embryogenic lines obtained from six adult cork oak trees that were induced on semisolid medium and then proliferated in liquid medium and were the source of explants for this study. They were maintained in 125 ml Erlenmeyer flasks filled with 50 ml of liquid medium composed of macronutrients of Schenk and Hildebrandt's (SH) medium (Schenk and Hildebrandt 1972), and micronutrients, cofactors and Fe-EDTA of MS medium (Murashige and Skoog 1962), 30 g l^{-1} sucrose and without plant growth regulators (PGR). Cultures were placed in a growth chamber at 25 ± 2 °C and a 16-h photoperiod (180 μmol m^{-2} s^{-1}) on orbital shakers at 110 rpm, and were subcultured monthly.

The initial experiments designed to test the effect of several cultural factors on the production of single differentiated embryos were carried out with one genotype, the embryogenic line ALM80. The factors assayed were i) the of type of initial explant, single somatic embryos and embryogenic clumps (EC); ii) the agitation, stationary culture *vs* shaken at 40 rpm; iii) the culture under light or in darkness; iv) the number of subcultures; v) the density of inoculation, 2, 3, 4 or 5 embryos/vessel; and vi) the volume of the culture vessel, 100 and 150 ml. Then the optimal culture conditions were used to evaluate the effect of genotype with five additional embryogenic lines. In all experiments the number of single cotyledonary embryos produced after 60 days in culture was recorded. Data were analysed with ANOVA (GLM procedure, SPSS v.13.0 software for Windows).

When single cotyledonary embryos or EC were cultured in liquid medium both stationary and at low shaking speed, secondary globular embryos were observed on the surface of initial explants within two weeks of culture (Fig. 2A). After four week many globular embryos and some heart-shaped embryos were covering the hypocotyl and root cap of cotyledonary embryos and most of the surface of the EC (Fig. 2B). After six weeks newly formed embryos at the cotyledonary stage were free in the medium (Fig. 2C).

Figure 2: Differentiation of cork oak somatic embryos following the second way of differentiation. A, embryogenic clump after 2 week of culture sowing some globular embryos on its surface. B, many globular and heart-shaped embryos formed after 4 weeks in culture. C, after six weeks of culture some cotyledonary embryos are free in the liquid medium.

The first experiment assayed the effect of the type of initial explant and the effect of shaking. Just one single somatic embryo and one EC were removed from proliferation medium and cultured in Erlenmeyer flasks of 150ml. There were no significant differences in the number of embryos produced by the two types of explants at 0 or 40 rpm after 60days of culture. Cotyledonary embryos produced 18 ± 3 embryos/embryo and EC 15 ± 3 embryos/EC.

Culture under light or in darkness did not affect the production of new embryos significantly. There was an important effect of the number of subcultures. Culturing four embryos per vessel under light, the production of the first embryos picked from proliferation cultures was 30 ± 16 embryos/embryo. These embryos (second generation) were cultured again and produced 14 ± 2 embryos/embryo, and those of the third generation 5 ± 1 embryos/embryo. Production was dramatically reduced after the third subculture to 1 ± 1 embryos/embryo (Fig. 3A).

There was a negative effect of the density of inoculation. When 2, 3, 4 or 5 embryos of the third generation were cultured under light the production of new embryos decreases (Fig. 3B). The best density of inoculation was two embryos per flask that produced 8 ± 2 embryos/embryo. Increasing densities reduced the differentiation ability.

The volume of the culture vessel had a significant effect on the differentiation of secondary embryos. Using first generation embryos as explants, the production in 150 ml Erlenmeyer was almost double that in 100 ml. This happened in all tested densities of inoculation (Fig. 3C). In the absence of agitation and using the same volume of liquid medium in vessels of different volume, the thickness of the layer of liquid medium over the embryogenic tissues had an important effect on the differentiation ability.

This way of embryo differentiation was tested with five additional genotypes. There was a significant effect of genotype and high heterogeneity among replicates (Fig. 3D). All of them produced embryos but some genotypes maintained more broad attachment with the original tissue. Counting only the number of isolated and well-developed somatic embryos there were significant differences among genotypes.
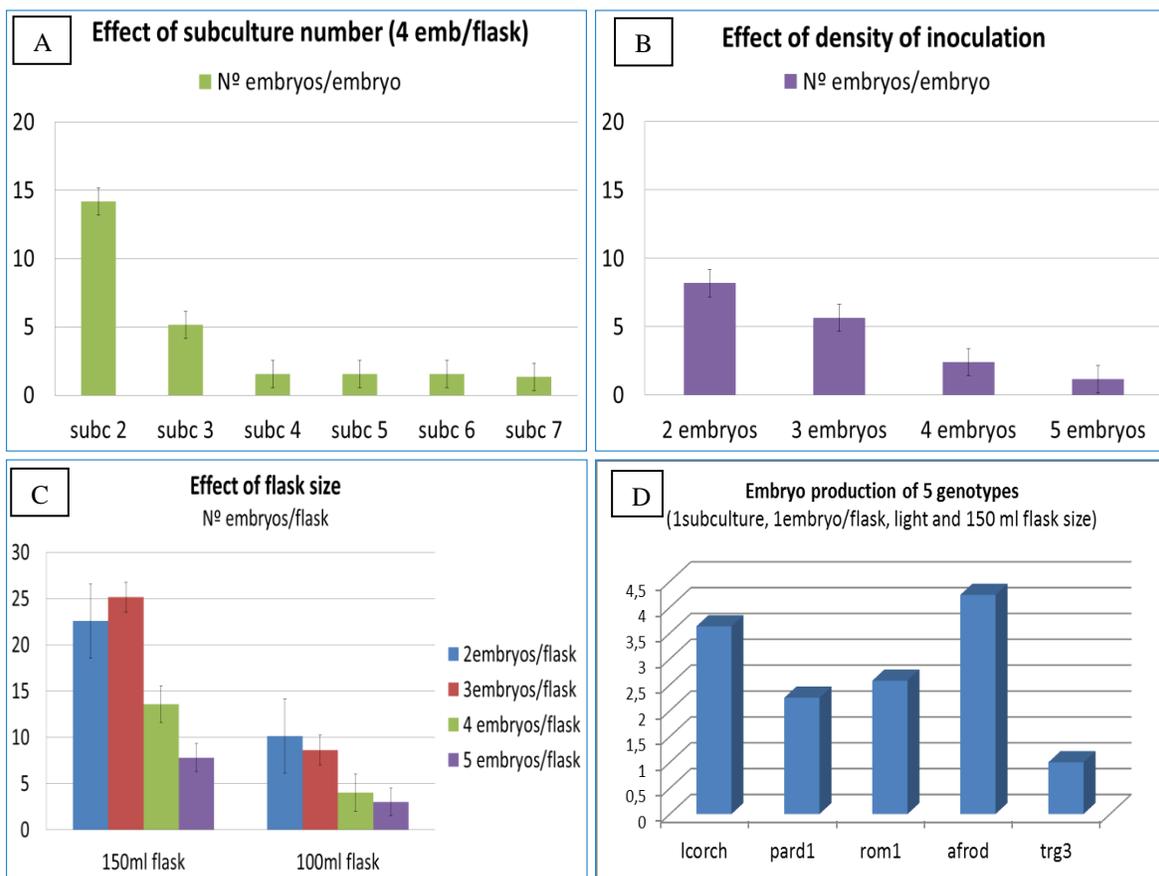

Figure 3: *Effect of different culture conditions on the number of cork oak secondary embryos (genotype ALM80) produced in liquid medium after 60 days of culture. **A**, Effect of the number of subcultures; **B**, Effect of density of inoculation; **C**, Effect of vessel volume; **D**, Production of single cotyledonary somatic embryos of different genotypes.*

This study confirms the possibility of producing single and well-developed somatic embryos up to the cotyledonary stage. The best conditions are the culture of two embryos in liquid medium in 150 ml Erlenmeyer flasks, both static and shaken at 40 rpm. The embryo production may be from 1 to 25 embryos per cultured embryo depending on genotype, but this production capacity is lost after several subcultures in liquid medium.

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Use of a Continuous Immersion System (CIS) for micropropagation of chestnut in photoautotrophic and photomixotrophic conditions

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Keywords: bioreactors, CO_2, LEDs, liquid culture, sucrose

Introduction

Protocols for conventional micropropagation of chestnut are well established (Vieitez et al. 2007) but these methods are expensive and labour-intensive. The use of liquid culture systems in bioreactors may reduce labour costs and improve proliferation rates in chestnut propagation (Vidal et al. 2015). In this study, a continuous immersion system (CIS) for micropropagation of axillary shoots of European chestnut and hybrids of European and Asiatic chestnut was evaluated, in order to improve productivity and allow large-scale plant propagation. Also, different growth conditions (photoautotrophic or photomixotrophic) were applied by varying sucrose, light intensity and CO_2 levels inside the bioreactors.

Materials and methods

Explants from six chestnut genotypes were cultured in different bioreactors with Murashige and Skoog medium with half strength nitrates ($MS^{N½}$) supplemented with 0.05 mg/L N^6-benzyladenine (BA) and 30 g/L sucrose. Parameters such as the vessel size (1, 6, 8 or 10 L), volume of medium per explant, explant type (apical or basal explants attached to the basal callus), explant size (1-1.5 or 2-3 cm), supporting material (rockwool cubes, perlite, glass beads, vermiculite) and air exchange were evaluated on the basis of shoot quality and proliferation rates after 5 weeks of culture. For mixotrophic conditions, explants were cultured in medium with 30 g/L sucrose. Cultures were incubated under a 16-h photoperiod provided by cool-white fluorescent lamps (50–60 µmol m^{-2} s^{-1}) and a 25 °C light/ 20 °C dark temperature regime. An air pump was used to apply 0.2 µm filtered air for 2 min each hour.

In order to propagate European chestnut in photoautothropic conditions, an experimental unit was used, equipped with light-emitting diodes (LEDs) of different wavelengths, as well as with a forced ventilation unit for supplying CO_2-enriched air. Explants were cultured in 10 L bioreactors with $MS^{N½}$ medium supplemented with 0.05 mg/L BA, and rockwool cubes were used as inert supporting material. Two levels of sucrose (10 and 30 g/L) and photosynthetic photon flux densities (PPF) of 50 and 150 µmol m^2 s^{-1} were tested. CO_2 levels in the culture vessels were manipulated by applying forced ventilation with CO_2-enriched air or the vessels were used without.

The variables used for evaluation were: 1) the quality of the new shoots, calculated as the percentage of non-hyperhydric shoots (% NHS), 2) nº of shoots/explant (NS), 3) multiplication coefficient (MC), calculated as the nº of new segments obtained per initial explant, 4) the length of the longest shoot per explant (SL), 5) the nº of rootable shoots per explant (RS; those shoots longer than 3-4 cm with an actively growing apex), 6) the length (LL) and the width (LW) of the largest leaf per explant.

Results and Discussion

The first experiments were performed in photomixotrophic conditions. The results obtained indicate that the use of inert supporting materials such as rockwool cubes, together with increasing air exchange by applying forced aerations, prevented hyperhydricity, a disorder that can be considered one of the major problems of axillary culture in liquid medium. Basal explants rendered higher proliferation rates than apical explants, but the latter could be propagated successfully when initial explant size was increased to 2-3 cm (instead of 1-1.5 cm used in semisolid cultures). Explants cultured in vessels of 6, 8 or 10 L showed longer shoots and higher proliferation rates than those cultured in 1 L vessels. After several experiments, a tentative protocol was applied to six chestnut clones. In this protocol, explants consisting of 1.5 cm basal segments attached to the basal callus were cultured in 10 L containers with 1900 ml of liquid medium, using rockwool cubes as supporting material. The results, shown in **Fig 1A, B** and **Fig 2**, indicate that successful propagation in CIS was achieved for all clones. Genotypical differences were detected, suggesting that the protocol should be further adapted for some of them.

Figure 1. A) *Apical and basal explants of clone 90025 growing in CIS in photomixotrophic conditions with different supporting materials (perlite, vermiculite and glass beads), B) Basal explants of clone CO63 growing in CIS in photomixotrophic conditions with rockwool cubes as supporting material, C) Experimental unit used for photoautotrophic culture of chestnut, D) Apical and basal explants of clone PO43 growing with 1% sucrose and CO_2-enriched air.*
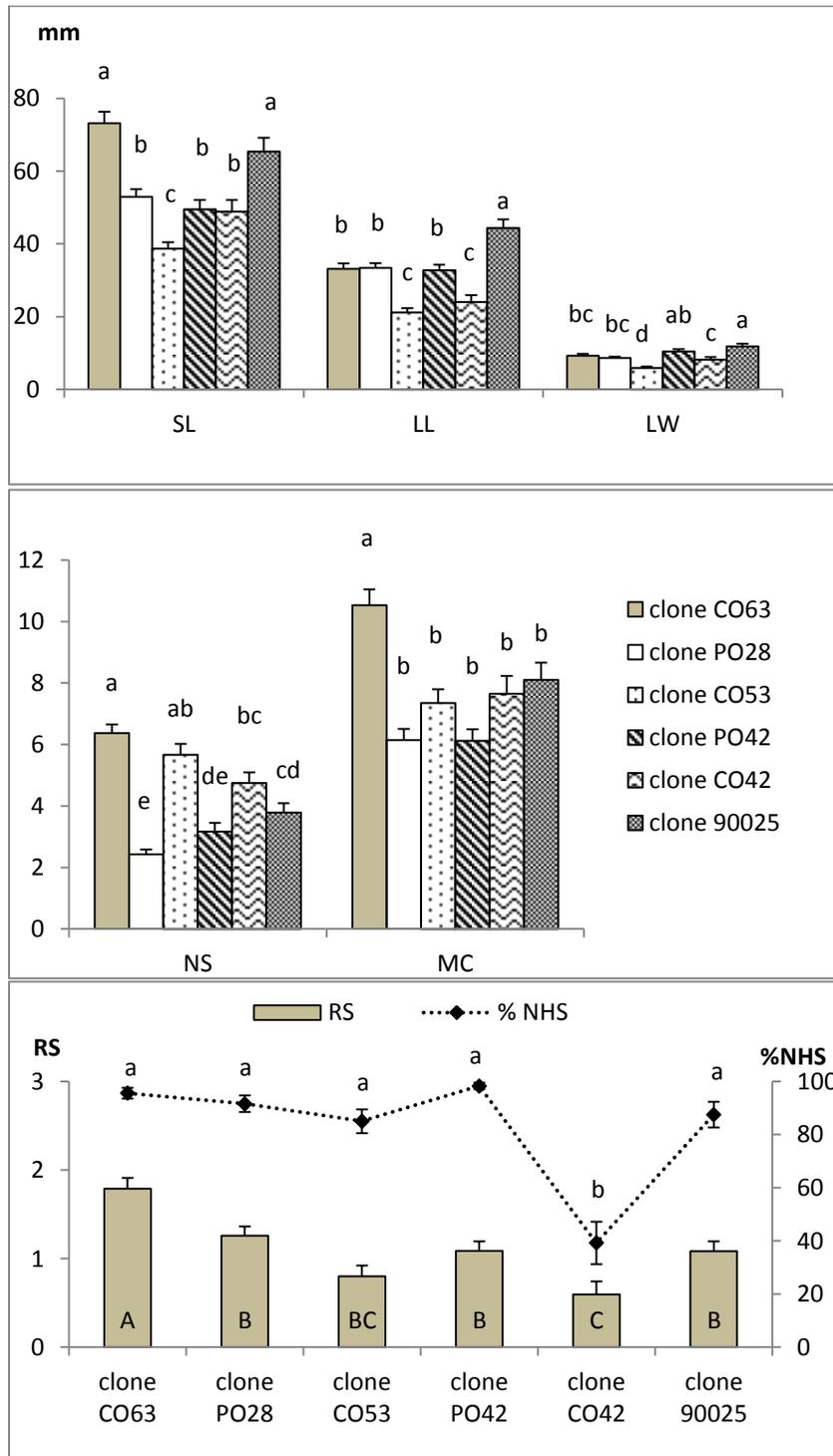
Figure 2. *Shoot quality and proliferation rates of basal explants of 6 chestnut genotypes cultured in CIS in photomixotrophic conditions. NS, nº of shoots/explant; MC, multiplication coefficient; SL, length of the longest shoot; LL, leaf length; LW, leaf width; RS, nº of rootable shoots per explant; %NHS, percentage of non-hyperhydric shoots. For each variable, different letters mean significant differences at P= 0.05 level (F- test).*

Also, the feasibility of culturing apical and basal chestnut explants in photoautothropic conditions was explored. The preliminary results of culturing basal explants (3 cm) of chestnut clone PO43 with two levels of sucrose (10 and 30 g/L) under a PPF of 150 μmol m^2 s^{-1} with and without CO_2 application are shown in **Fig 1D** and **Fig 3**. The results indicate that lowering sucrose levels causes a decrease in the proliferation rates of chestnut shoots, but this decrease can be reverted if CO_2-enriched air is applied to the bioreactors suggesting that a certain level of photoautotrophy has been attained (Xiao et al. 2011).
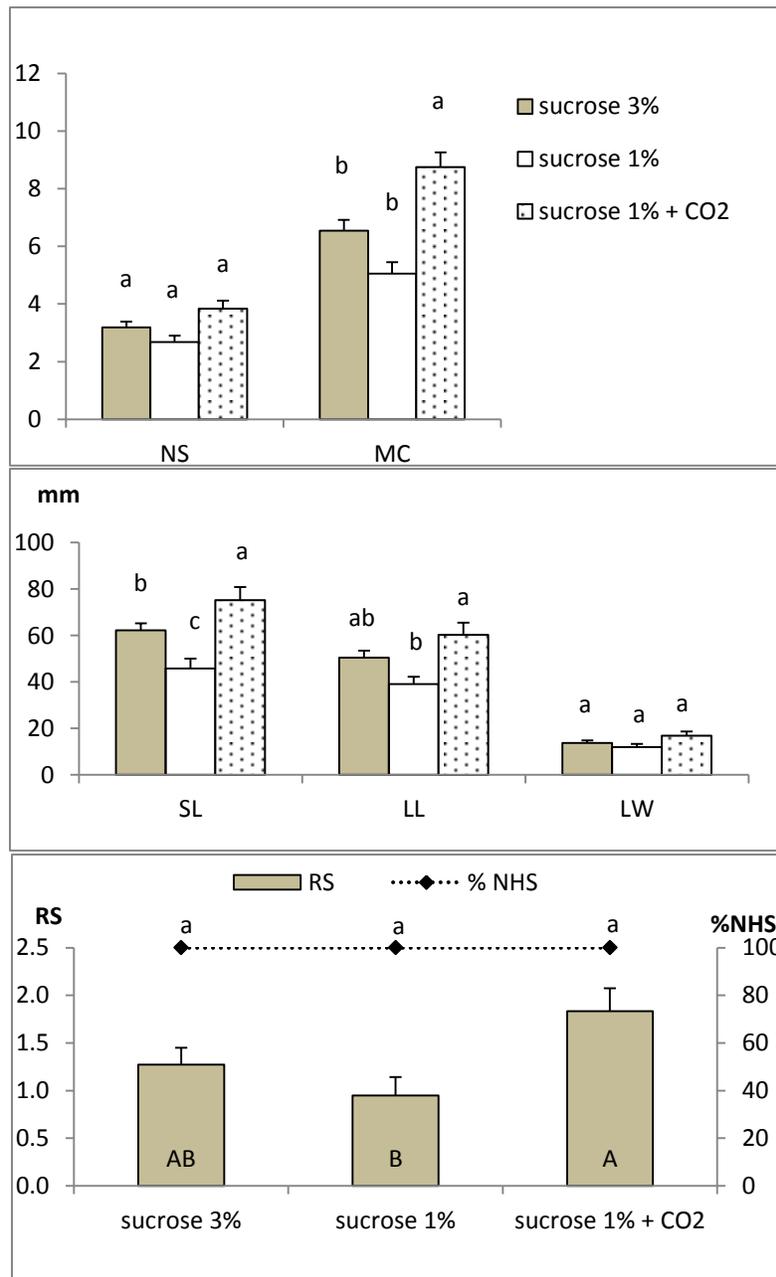
Fig 3. *Shoot quality and proliferation rates of basal explants of chestnut clone PO43 cultured in CIS in photomixotrophic and photoautotrophic conditions. NS, nº of shoots/explant; MC, multiplication coefficient; SL, length of the longest shoot; LL, leaf length; LW, leaf width; RS, nº of rootable shoots per explant; %NHS, percentage of non-hyperhydric shoots. For each variable, different letters mean significant differences at P= 0.05 level (F- test).*

Conclusions

The present results show the feasibility of culturing axillary shoots of chestnut in liquid medium by a continuous immersion system. This system makes it possible to manipulate medium composition and the aerial environment inside the bioreactors to increase the photoautotrophic behavior of the chestnut explants. In next experiments photoautotrophic conditions will be applied to more chestnut genotypes, and biochemical parameters to test the attained level of photoautotrophy will be measured.

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Characterization and expression analysis of *QsTCTP* and *QsADF* during *Quercus suber* somatic embryogenesis

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Key words: Actin-depolymerizing factor, cork oak, gene expression, somatic embryos, translationally controlled tumor protein

Introduction

Somatic embryos develop through a sequence of stages associated with morphological and biochemical changes related to genomic activity. The developmental transitions during somatic embryogenesis are controlled by the temporal expression of specific genes. The identification and characterization of these genes will provide a better understanding of the molecular mechanisms underlying somatic embryogenesis.

Translationally controlled tumor protein (TCTP) is a highly conserved protein that is widely expressed in all eukaryotic organisms including plants, yeast and mammals, and its expression is controlled post transcriptionally. The actin-depolymerizing factors (ADFs) are ubiquitous low-molecular-mass actin-binding proteins that are important for actin filament assembly in eukaryotes facilitating dynamic remodeling of the actin cytoskeleton (Chen et al., 2002). They are both known to be involved in different developmental processes such as morphogenesis, plant cell division and growth.

The objectives of this work were 1) to isolate and identify homologous genes to TCTP and ADF in Quercus suber and to study their expression patterns during the development and histodifferentiation of cork oak somatic embryos by quantitative real-time PCR (qPCR) and in situ hybridization.

Materials and methods

Quercus suber somatic embryos were initiated from expanding leaves of epicormic shoots forced to flush in branch segments of a centenarian tree according to the protocol described by Hernández et al. (2003). Embryos were subcultured monthly by secondary embryogenesis in RITA® vessels with MS medium (Murashige and Skoog, 1962) supplemented with 0.1 mg L^{-1} benzyladenine, 0.05 mg L^{-1} naphthalene-1-acetic acid and 3% sucrose. Somatic embryos at globular, torpedo and cotyledonary stages were collected from proliferating cultures and used for RNA isolation and gene expression analysis.

To identify homologous genes to TCTP and ADF total RNA was isolated from *Q. suber* somatic embryos and specific primers were designed from EST database sequences. Reverse transcription polymerase chain reaction (RT-PCR) and 3´-5´ Rapid Amplification of cDNA Ends (RACE) were performed with the SMART-RACE cDNA amplification kit (Clontech) following the manufacturer´s instructions and PCR products were cloned and sequenced. Expression analyses of the genes used in this study was performed by qPCR and in situ hybridization during somatic embryo development. Quantitative real-time PCR was performed in an optical 48-well plate with a Step One Real Time PCR System (Applied Biosystems, CA, USA). The expression data of each gene was normalized against the expression of three reference genes, Actin (Soler et al., 2008), Tubulin (Porth et al., 2005) and Ubiquitin, previously selected based on their stability during embryo development. In situ hybridization experiments were performed as described by Vielba et al. (2011).

Results and discussion

Analysis of sequences reveals that QsADF and QsTCP encoded proteins of 143 and 168 aa, respectively, that belong to the ADF/cofilin and TCTP families, respectively. Two characteristic domains of the TCTP family (TCTP1 and TCTP2) and the ADF-H1 domain were identified in the QsTCTP and QSADF proteins, respectively, (Figures 1, 2) using the PROSITE search. A microtubule-binding sequence was also found in the TCTP protein, which can be related to its role in the cell cycle by controlling cell proliferation (Figure 1). A conserved serine residue was also detected in the putative QsADF protein (Figure 2), which seems to regulate its activity through phosphorylation-dephosphorylation mechanisms as has been reported in other systems (Chen et al., 2002).

Expression analysis of both QsTCTP and QsADF genes in cork oak somatic embryos showed an increase in the relative abundance of transcripts during the development of somatic embryos, with the highest levels at the cotyledonary stage embryos. These results suggest that both genes may be involved in embryo histodifferentiation.
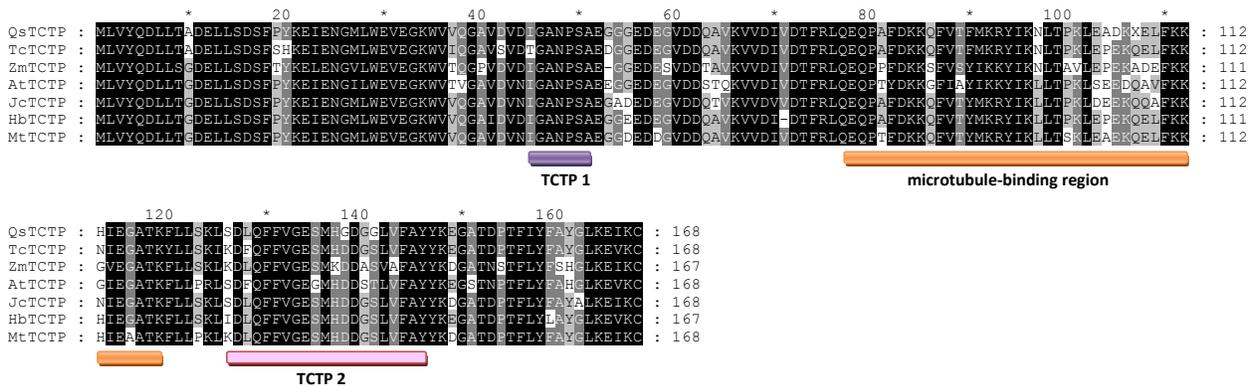
Figure 1. *Multiple sequence alignments of the deduced aminoacid sequence of QsADF deduced protein from Quercus suber with that of other TCTPs. All other entries are derived from National Center for Biotechnologie Information database (NCBI; http://www.ncbi.nlm.nih.gov/).* **Qs**: *Quercus suber;* **Tc**: *Theobroma cacao, Q9ZSK2;* **Zm**: *Zea mays, P46251.1;* **At**: *Arabidopsis thaliana, Q39250.1;* **Jc**: *Jatropha curcas, KDP26678.1;* **Hb**: *Hevea brasiliensis, ADI78873.1;* **Mt**: *Medicago trunculata, XP_003591150.1.The TCTP1, TCTP2 domains and microtubule-binding region are indicated.*
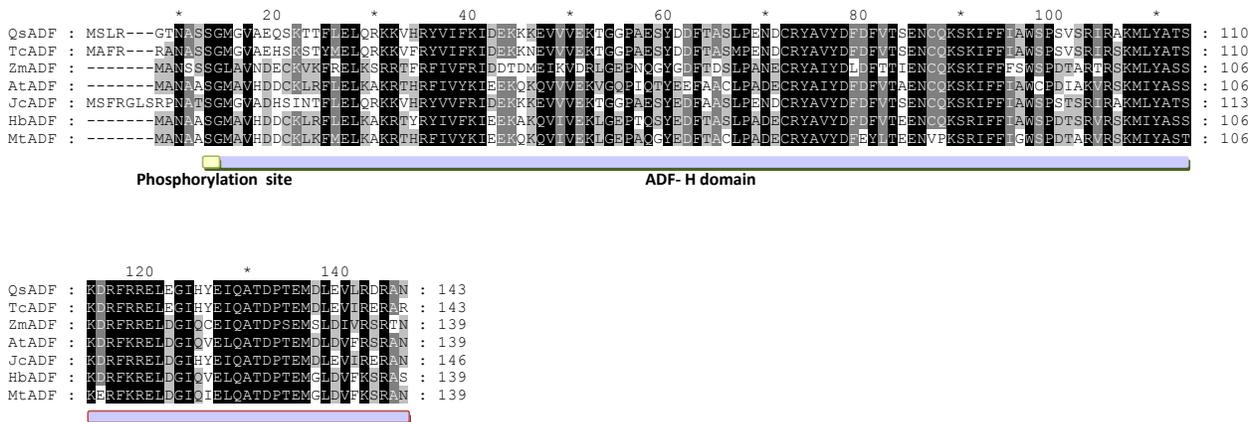
Figure 2. *Multiple sequence alignments of the deduced aminoacid sequence of QsADF deduced protein from Quercus suber with that of other TCTPs. All other entries are derived from National Center for Biotechnology Information database (NCBI; http://www.ncbi.nlm.nih.gov/).* **Qs**: *Quercus suber;* **Tc**: *Theobroma cacao EOY11510.1;* **Zm**: *Zea mays, Q8H6A5;* **At**: *Arabidopsis thaliana, P31265;* **Jc**: *Jatropha curcas, A9LC95;* **Hb**: *Hevea brasiliensis, AEQ20639.1;* **Mt**: *Medicago trunculata, XP_003591150.1. The phosphorylation site and ADF-H domain are indicated.*

In situ localization revealed that QsTCTP mRNA was slightly and uniformly distributed in all cells of globular embryos. As the embryo developed to more advanced stages, TCTP transcripts were localized in the shoot apical meristem and radicular pole of cotyledonary embryos. TCTP genes have been reported to be highly expressed in actively dividing and differentiating cell types (Berkowitz et al., 2008). Furthermore, the expression pattern of the LaTCTP gene during somatic embryogenesis of Larix leptolepis indicated that it might participate in the regulation of somatic embryo development (Zang et al., 2013).

The QsADF expression signal was high in the shoot apical meristem of cotyledonary embryos as well as in the procambium. The QsADF riboprobe also showed a strong specific signal in the cortex/endodermis initial cells of the root pole, suggesting that the gene could be involved in the radial patterning of the root. One member of the ADF family, ADF9, is also expressed in the shoot apical meristem and root sub-apical region of Arabidopsis (Burgos-Rivera et al. 2008).

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Gene expression analysis during in vitro shoot development and root morphogenesis in chestnut

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Keywords: adventitious roots, axillary bud, micropropagation, root primordia

Introduction

The axillary bud micropropagation system is one of the simplest and most applied methods for true-to-type in vitro propagation of elite tree genotypes. Protocols for *in vitro* cloning and production of mature trees of chestnut via axillary shoots have been defined when physiologically juvenile-like material is used as initial explants. Shoot proliferation and adventitious root formation are hampered by the low morphogenetic capacity of many forest species and also by the loss of morphogenetic competence associated with maturation. The identification of molecular players involved in these developmental processes and their regulatory mechanisms will help in the practical improvement of the in vitro production of important genotypes. Two experimental systems, chestnut shoot cultures and leaves excised from microshoots were used to study genes involved in shoot development as well as to investigate how differentiated cells acquire the developmental plasticity to form a new root. The aim of this study was to analyze the expression of four genes, *CsERF1* (*Ethylene-responsive factor*), *CsGH3* (*auxin-inducible GH3-like*), *CsLRR-RLK* (*leucin-rich repeat receptor-like kinase*) and *CsSCL1* (*Scarecrow-like-1*), in different organs from chestnut microshoots as well as during the induction of adventitious roots. Furthermore, we also evaluated the effect of NPA (N-1-naphthyphthalamic acid) on the relative expression levels of *CsGH3* and *CsSCL1* genes in IBA-treated leaves during the early steps of adventitious rooting.

Materials and methods

Stock shoot cultures of chestnut initiated from basal shoots of an adult chestnut tree were used in this study. A detailed description of protocols for chestnut shoot proliferation as well as for the induction of adventitious roots has been previously reported (Sanchez and Vieitez, 1991; Ballester et al., 1999). Organs were excised from microshoots at the end of the proliferation cycle: shoot apex, axillary buds, internodes and leaves. Adventitious roots developed from indol-3-butyric acid (IBA)-treated shoots were also harvested and divided in three different zones designated: apical, middle and basal (Figure 1). The apical part includes the division and elongation zones of the root and the middle part corresponds mainly to the maturation area.

Adventitious roots were also induced on the tree youngest and expanded leaves that were excised from the apical part of microshoots. Leaves were placed on GD medium (Gresshoff and Doy, 1972) with 1/3-strengh macronutrients supplemented with 0 (control) or 25 µM IBA for 5 days under dark conditions and then transferred to IBA-free medium and maintained under a 16h photoperiod. To study the effect of NPA on the rooting response of leaves, they were treated simultaneously with IBA (25 µM) and NPA (500 µM) for 5 days and subsequently transferred to NPA- and IBA- free medium.

Expression analyses of the genes used in this study were performed in different organs as well as during the adventitious root formation by qPCR and in situ hybridization. Three reference genes, *Actin*, *Tubulin* and *Polyubiquitin*, were selected for qPCR analysis and used in our study as internal controls to normalize all data.

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In situ hybridization experiments were carried out as described elsewhere (Valladares et al., 2013).

Figure 1: *Adventitious root developed from chestnut microshoots treated with 4.9 mM of IBA for 1 min and subsequently transferred to IBA-free medium. **a**: apical part of the root. **b**: middle zone of the root. **c**: basal zone of the root.*

Results and discussion

The expression analysis performed in different organs showed that *CsLR-RLK* is highly expressed in the shoot apex and axillary buds. In the shoot apex, the *CsLR-LRK* message localizes in the outer layer as well as in the axils of the youngest leaf primordia. Although the lowest levels of *CsLR-RLK* were detected in roots, the transcripts are localized in the outermost layers of the root primordia, lateral root primordia and axillary meristems. These data suggest that *CsLR-RLK* could be involved in the regulation of meristem growth or meristem activity. The *Clavata1* gene encoding a leucine-rich repeat receptor-like kinase has been suggested to promote the progression of meristem cells toward organ initiation (Clark et al., 1997; Schoof et al., 2000).

The highest transcript levels of *CsERF1*, which encodes a putative transcription factor of the AP2/ERF family, were found in axillary buds, and its localization was confined to the central zone of the axillary bud as well as to the apical growth zones of leaf primordia, suggesting that the gene participates in the maintenance of the stem cell niche and in regulating the axillary bud outgrowth. The *DNR* gene, encoding an AP2/ERF factor, is also expressed in the meristem central zone of shoot apical meristem (SAM), and is likely to be a factor that regulates the cellular organization of the SAM (Carles and Fletcher, 2003). The *Apetala 2* gene has also been implicated in the stem cell maintenance in SAM by modifying the *WUS-CLV3* feedback loop (Würschum et al., 2006).

Transcripts of *CsERF1*, *CsGH3* and *CsSCL1* accumulated to relative high levels in roots compared to leaves or internodes (Figure 2). Among the different parts of the root, the highest mRNA levels were detected in the middle part of the root (Figure 2), which corresponds to the maturation zone where lateral root primordia are formed. In line with these results, the data obtained from in situ hybridization experiments also revealed a specific hybridization signal in lateral root primordia as well as in adventitious root primordia. Therefore, the expression pattern of these three genes, *CsERF1*, *CsGH3*, *CsSCL1*, provides evidence for an important role of these genes in the root meristem initiation or maintenance.

The analysis carried out during the induction of adventitious roots on detached leaves revealed that *CsGH3* and *CsSCL1* genes are inducible by auxin during the early steps of root initiation. Transcripts of *CsSCL1* were maintained at low and constant levels in control leaves during the first 24h of the experiment. In contrast, IBA treatment lead to a three-fold increase in levels of *CsSCL1* mRNA in leaves harvested 24h after treatment, a time when cell reorganization takes place during the induction of adventitious roots. Our results are in agreement with those previously obtained during the induction of adventitious roots in pine hypocotyls and chestnut microshoots (Sánchez et al., 2007; Vielba et al., 2011). The expression of *CsGH3* was also analyzed during the first 24h of the root induction process. *CsGH3* mRNA levels were induced by auxin as early as 6 h and high levels were maintained during the 24 h of treatment. In IBA-treated leaves, *CsGH3* mRNA levels increased to values about 30-fold greater than in control leaves. Ahkami et al. (2013) have also reported an increase of *GH3* transcript levels during the adventitious root formation in the stem base of *Petunia hybrida* shoot tip cuttings.

Furthermore, we found that rooting rates of leaves were negatively affected by the NPA, an inhibitor of the auxin polar transport. Expression analysis of *CsSCL1* and *CsGH3* also showed that NPA treatment reduced the

expression levels of both genes in IBA-treated leaves. Therefore, there is a direct correlation between adventitious root inhibition by NPA treatment and expression of both genes (*CsSCL1* and *CsGH3*). These data together with the specific location of transcripts in the root primordia of lateral and adventitious roots indicate that *CsGH3* and *CsSCL1* are involved in root meristem initiation.
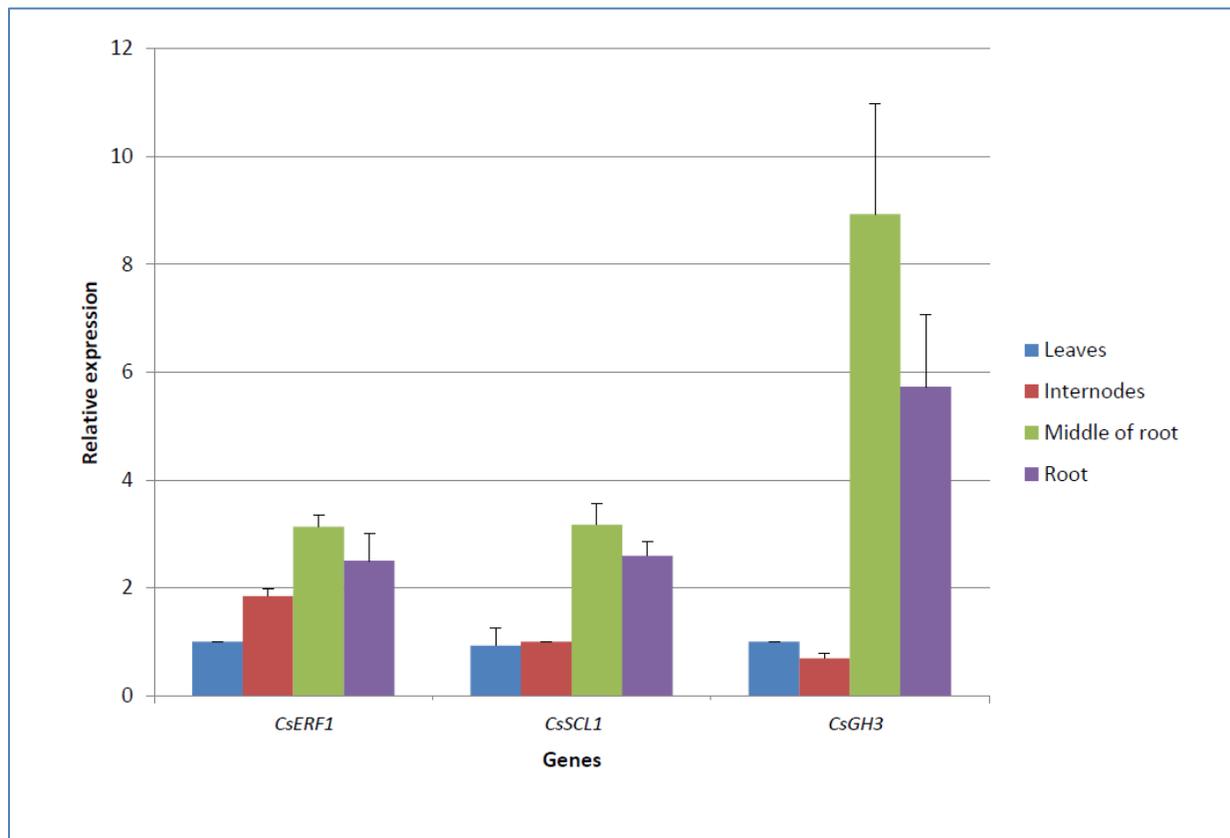

Figure 2: *Expression analysis of CsERF1, CsSCL1 and CsGH3 genes in different organs of chestnut microshoots: leaves, internodes and roots. Relative amount of transcripts were normalized to the levels of actin, tubulin and polyubiquitin. Results are expressed as mean values±SE relative to leaves (CsERF1 and CsGH3) or internodes (CsSCL1).*

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Short Abstracts

Timing as critical factor in cryopreservation of *in vivo*-buds of hybrid aspen

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Keywords: cryopreservation, dormant bud, micropropagation, *Populus*, timing

Hybrid aspen is an artificial cross between European aspen (*Populus tremula* L.) and American aspen (*Populus tremuloides* Minchx.). Most of the aspen cultivation in Finland has been with hybrid aspen; due to heterosis the growth rate of the best hybrids has been 2- to 3-fold that of domestic aspen, with the best volume growth reaching 24 m3/year/ha. For the conservation of hybrid aspen germplasm, cryostorage of dormant in vivo buds is a convenient back-up method for field collections. When needed, the stored material is regenerated using micropropagation. In practice in Finland, bud collection for cryopreservation has been performed from February to March, as a compromise between the still remaining cold hardiness and the increasing bud burst ability. The aim of this study was to assess how this time schedule can be extended without compromising regeneration. In addition, an easily measurable marker for successful cryopreservation was examined.

Timing of cryopreservation by a slow-cooling method was tested from August to February, using dormant buds from both outdoor and indoor plants. To find a marker, water content and gene expression (dehydrin, *dhn*) of hybrid aspens, as well as environmental factors such as temperature, development of temperature sum (degree days, d.d., i.e. the temperature sum with a threshold of 5°C), and light period were followed.

Cryopreservation was successful from October to February, when, on an average, at least 75% of the buds regenerated through micropropagation, and there was no difference to non-frozen controls. Significant genotypic variation was observed in October and February, with regeneration rates of 61-100% and 37-98%, respectively. The overall contamination rate of the present material was relatively low: 4.6 ±0.6% had a fungal contamination and 6.8 ±0.8% a bacterial contamination in micropropagation. No marker for successful cryopreservation was found among the studied factors. The results provide flexibility for the undertaking of practical work, with a recommendation that cryopreservation can be carried out from November to January – earlier than the current practice.

Reference: Aronen & Ryynänen 2014. Cryopreservation of dormant *in vivo*-buds of hybrid aspen: Timing as critical factor. Cryoletters 35 (5): 385-394.

Embryo maturation ability is subjected to line ageing – a way to assure the quality of somatic embryos of Nordmann fir

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Keywords: somatic embryogenesis, clonal varieties, cryopreservation, maturation

Nordmann fir (*Abies nordmanniana*) has an enormous commercial importance for Christmas tree production in Europe. It is exclusively grown from seeds harvested from natural populations in the Caucasian mountains. Clonal varieties would help to considerably improve the quality of trees and the cultivation characteristics. Methods based on somatic embryogenesis are expected to become a realistic possibility to solve these problems in the near future.

The biotechnical procedure for clonal mass propagation of Nordmann fir has been studied for more than 10 years, yet critical steps remain that need further improvement. One of the main obstacles is the long-term propagation of embryogenic cultures. Long term culture frequently results in varying developmental behaviour within clones – a problem that is manifested in declining numbers of normally developed mature embryos (which can be used for germination) per gram fresh weight.

This loss of maturation ability after long propagation times is counteracted by early cryopreservation which allows the production of stable quantities of somatic embryos over time. To this end essential aspects of the preservation protocol have been determined and a user-optimized procedure to manage a high number of clones has been established. Accordingly we were able to reduce the embryo production time (starting from the induction of somatic embryos until their cryopreservation) by securing a large number of potentially superior clones while assessing their quality simultaneously.

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Vegetative propagation of *Pinus maximinoi* H. E. Moore with different sizes of cuttings and substrates in South of Brazil

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Keywords: rooting, size of cuttings, substrates, pine

The species *Pinus maximinoi* is a pine tree from Central America that shows good growth in some parts of Brazil, especially in the north of Parana State, a transition zone between subtropical to tropical clime, where it is growing, in some trials, up to 20% more in volume than *Pinus taeda*, the most planted pine in Brazil. The breeding of this species is in an initial stage, so the availability of good genetic seeds is very low, therefore the propagation by cutting of progenies with good performance is an alternative to produce plants on a large scale. The aim of this work was to test the influence of three sizes of cuttings and four different substrates in *Pinus maximinoi* rooting. Cutting sizes tested: 3 cm, 5 cm and 7 cm. Substrates tested: (a) 100% composted pine bark; (b) 50% composted pine bark, 50% carbonized rice bark; (c) 25% composted pine bark, 25% carbonized rice bark and 50% coconut fiber; (d) 25% vermiculite, 25% carbonized rice bark and 50% coconut fiber. The genetic material used was a bulk of five elite progeny planted in hydroponic sand beds. The cuttings were collected, planted in 55 cm³ containers with the respective substrate treatments, placed in a greenhouse with approximately 85% relativity humidity and 25°C for 60 days. After this period the plants were reallocated to a shadow house. The statistic design used was randomized complete block with 24 plants per plot and 6 replications. The location of the trial was in Telêmaco Borba City, Paraná State, south of Brazil. After 150 days of the planting the percentage of cuttings with roots, the diameter and height of the cuttings, weight of the roots and weight of shoots were measured. The data were analyzed with the ANOVA and Tukey tests in the SAS program. Statistically there were no differences between any treatments and any parameter analyzed. The parameters general means were: 90% of rooting, 15.6 cm of height, 3.4 mm of diameter, 0.9 g of shoots and 0.3 g of the roots. This work shows that is possible to produce plants by rooting cutting of young *Pinus maximinoi* progenies with relatively facility using different sizes of cuttings and different kinds of substrates.

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Agrobacterium-mediated co-transformation of European chestnut somatic embryos with genes encoding for a chitinase and a thaumatin-like protein

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Keywords: blight disease, *Castanea sativa*, cisgenic, gfp, ink disease, pathogenesis-related proteins

Castanea sativa Mill. (sweet chestnut, European chestnut) is a widely distributed and economically important tree species in Europe, where it covers an area of more than 2 million hectares (Conedera et al. 2004). Ink disease (caused by *Phytophthora* spp.) and chestnut blight (caused by *Cryphonectria parasitica*) are the two most important diseases that affect European chestnut. Genetic transformation of the disease-prone *Castanea* species with antifungal or antimicrobial genes in order to increase disease resistance or tolerance may be the first step towards a reliable, and complementary biotechnological alternative to conventional breeding efforts. The most widely used approach is the overexpression of pathogenesis-related (PR) proteins such as chitinases and thaumatin-like proteins. PR proteins are defined as host plant encoded proteins induced under pathological or related conditions (Veluthakkal and Dasgupta 2010). The objective of the present study was to obtain chestnut somatic embryos and plants that simultaneously overexpress chestnut chitinase (*CsCh3*) and thaumatin-like protein (*CsTL1*) genes.

Explants, consisting of small clumps of two to three somatic embryos at globular or early-torpedo stages isolated from two chestnut embryogenic lines, were co-cultured for 5 days (Corredoira et al. 2015) with *Agrobacterium tumefaciens* strain EHA105 harbouring pK7WG2-CsCh3 or pK7WG2D-CsTL1 binary vectors. Both plasmids contain the neomycin phosphotransferase (*nptII*) selective gene, whereas the plasmid pK7WG2D-CsTL1 also contains the green fluorescent protein (*egfp*) reporter gene. Vectors were inserted separately in EHA105 so that each *Agrobacterium* clone contained only one vector. Co-transformation was performed by mixing equal concentrations of two *Agrobacterium* clones transformed with each vector. The fluorescent protein simplified and improved the evaluation of transformation events relative to the GUS assay used in previous protocols in chestnut transformation.

The transformation efficiency, determined on the basis of the fluorescence of surviving explants, was clearly genotype-dependent, with rates of 33% obtained for line CI-9 and 1% for line CI-3. A total of 34 independent transformed lines were obtained. The presence of both *CsTL1* and *CsCh3* genes in genomic DNA was determined by PCR. The results reported in the present work on the overexpression of two native genes in chestnut somatic embryos can be considered as a first step towards the production of fungal-disease tolerant cisgenic chestnut plants.

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Differential protein profile in *Pinus radiata* somatic embryos from cell lines with differences in initiation success

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Keywords: conifer, micropropagation, radiata pine, somatic embryogenesis, tissue culture, protein profile

Improving the initiation rate of somatic embryogenesis is critical for large-scale commercial use of this propagation tool. Our aim was to analyse the effect of physical and chemical environments on the initiation stage of radiata pine somatic embryogenesis. Therefore, the first objective was to assess if different temperatures and/or water availability during initiation of embryogenic tissue affected the success rate of the process. Our second objective was to test if embryogenic lines, exposed to different environmental conditions that lead to either high or low initiation rates, produced somatic embryos with different protein profiles. Moreover, we wanted to correlate the success of initation stage with the expression of certain proteins in the somatic embryos obtained. Some treatments (18ºC, 4 gL^{-1} gellan gum) produced high initiation percentages and others produced low initiation percentages (28ºC, 2 gL^{-1} gellan gum). Somatic embryos obtained by these treatments underwent 2-D electrophoresis to obtain their proteomic profiles. Our results showed differences between the profiles of the groups tested. A total of 139 differentially expressed proteins were found between the high initiation and low initiation group samples, when compared to the control group (23ºC, 2 gL^{-1}). Of these, 26 proteins were significantly overexpressed ($p < 0,05$) in all the high initiation groups and 10 others in the low initiation groups. An approach based on liquid chromatography together with tandem mass spectrometry (LC-MS/MS) will allow quantifying and identifying differentially expressed proteins.

Effect of temperature on growth and maturation of stone pine embryogenic lines

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Keywords: Agroforestry, embryogenic cultures, forest biotechnology, *Pinus pinea*, somatic embryogenesis.

The temperature condition in which seeds develop was reported to influence height growth and bud phenology of plants (Johnsen et al. 2005). *Picea abies* plants regenerated from somatic embryos were also influenced by temperature during somatic embryo development (Kvaalen and Johnsen 2008). Regeneration of plants by somatic embryogenesis was achieved in Stone pine (*Pinus pinea*) (Carneros et al 2009). In that study, proliferation and maturation of somatic embryos was carried out at 23 ± 1 ºC. In the present study the effect of colder (18 ºC) and warmer (28 ºC) temperatures, besides the usual temperature (23 ºC), during the proliferation of embryonal masses (EM) and embryo maturation steps on the production of somatic embryos and conversion was assessed. Growth was measured by weighing (fresh weight and dry weight) of individual clumps from EM of four genotypes at the

beginning and at the end of a period of 10 weeks in culture. Eight individual clumps (60 mg fresh weight) per treatment were biweekly subcultured on proliferation medium (M-mLV) at the tested temperatures (P18, P23 and P28) in darkness. After each subculture, the individual clumps were divided into two to four pieces, depending on the size achieved, and placed again onto fresh medium. The experiment was performed twice. Before maturation, individual clumps (100 mg fresh weight) collected at the EM periphery from the tested embryogenic lines, were preconditioned by biweekly subculturing on the same proliferation medium but with reduced nutrient and PGR concentrations (UL-½ mLV) and cultured at each tested temperature. After 4 weeks, samples of the EM periphery (50 mg fresh weight) were dispersed and poured onto filter paper disks, and then placed on maturation medium supplemented with a high concentration of ABA, maltose and Gelrite (mLV2). EM from cultures at each of the proliferation temperatures were incubated at 18, 23 and 28 ºC for differentiation-maturation. The number of aberrant and normal cotyledonary somatic embryos was recorded for each of the nine temperature combination treatments (e.g. P18M18, P18M23, P18M28) after 4 months in culture. Normal somatic embryos produced were germinated according to Carneros et al. (2009).

The growth of embryogenic lines was less at 18 and 28 ºC than at 23 ºC. Proliferation of EM at 28 ºC was less than at 18 ºC, and most samples showed necrosis. Although early somatic embryos were formed, the developmental embryo patterning was not completed in embryos of three of the embryogenic lines. Normal cotyledonary somatic embryos (20 embryos per gram) were obtained in one of the embryogenic lines. Production of normal embryos was promoted by increasing temperatures. These results are in accordance with those reported in *Picea abies* (Kvaalen and Johnsen 2008). The best results were obtained when temperatures during maturation were higher than those during proliferation (e.g. P18M23, P18M28, P23M28). Some of the normal embryos germinated.

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Applying SE-technology for mass-propagation of high-quality Norway spruce in Finland

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Keywords: vegetative propagation, somatic embryogenesis, *Picea abies*

Irregular flowering of seed orchards and problems caused by pests have caused periodic shortages in availability of genetically improved Norway spruce (Picea abies (L.) Karst.) seed over the past decade in Finland. Vegetative propagation based on somatic embryogenesis (SE) is expected to become a remedy to the supply problems and an effective way to produce high-quality clonal material for forest reforestation. The SE laboratory of the Punkaharju unit, the Finnish Forest Research Institute, is currently making rapid progress in producing SE clones of Norway spruce for genetic field testing.

Seed embryos used as a starting material in SE originate from controlled crosses between superior plus trees. The embryos are excised from immature seeds and used as explants for producing the SE clones. Suitability of the SE clones for mass-propagation is evaluated in multiple-stage tests carried out in the laboratory: SE clones viable after cryopreservation and with the highest embryo producing capacity will be selected for clonal field testing.

The first field trials with SE clones are planted in 2014-15. Two-year-old emblings are used for the trials. The trees will be evaluated, e.g., for growth, phenology, lammas growth and resistance to various pathogens.

Each set of clones will be tested in at least four sites with ten emblings per clone and site. Following the completion of field testing, the selected clones will be taken from cryopreservation and delivered to commercial plant producers for large-scale multiplication.

In the future, emblings may be utilized in reforestation either as mixtures of tested clones or as untested full-sib families. However, dissemination of the new material requires implementation of the SE-technology in commercial plant production. Transferring the know-how and technology from research to practice has already begun in a pilot project with Norway spruce special forms selected for ornamental use.

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Possibilities of somatic embryogenesis for production of Scots pine trees with improved heartwood quality

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Keywords: *Pinus sylvestris*, stilbenes, vegetative propagation

Stilbenes, phenolic compounds rich in Scots pine (*Pinus sylvestris* L.) heartwood, have strong fungicidal properties. Accumulation of stilbenes in the stem is correlated with wood decay resistance. Concentration of the stilbenes varies among the trees and is highly inheritable. Hence, breeding the resistant material from selected mother trees could be possible for future use. Vegetative propagation of Scots pine provides a way to produce the plant material with certain characters; in this study we studied the potential of somatic embryogenesis (SE) for production of Scots pine trees with improved heartwood quality.

Our aim was to use donor trees having a high stilbene concentration in their heartwood to initiate embryogenic cultures, and screen for progenies with high stilbene content. Effect of stilbene concentration in the donor tree on the somatic embryogenesis in Scots pine is not known. Hence, six mother trees with high or low phenolic content were selected to produce embryogenic cultures from open pollinated and control-pollinated immature cones during 2011-2013. In 2011, immature cones were picked 4 times from the open-pollinated donor trees. 1800 megagametophytes with zygotic embryos were used for initiation. In total, 67 lines survived. In 2012 and 2013, SE was initiated from explants from the control-pollinated donor trees. All together 3000 megagametophytes were used and 125 lines initiated. Phenolic concentration has been analyzed by HPLA/DAD from the donor trees, responding and non-responding megagametophytes, embryogenic lines with higher embryo production capacity, and germinated emblings.

Results showed that embryogenic lines having high phenolic content could be initiated from all the donor trees (both the high and the low phenolic containing mother trees) following both open-pollination and controlled-crossings. Phenolic compounds in megagametophytes did not show an inhibitory effect on the initiation rate. Stilbene concentration in the proliferating embryogenic cultures, without any induction treatment, varied from 0.002-1.5 mg/g, which is rather low in comparison with the donor tree range from 5-23 mg/g in heartwood. Response of the embryogenic cultures having either the high or low stilbene concentration to UV irradiation and fungal induction was also studied. The cultures with low stilbene content showed a stronger response to these stresses. The result indicated that SE provides an option for clonal testing and accelerated production of genetically improved materials in Scots pine.

Multivariate analysis of selected factors contributing to successful induction of somatic embryogenesis in *Larix x eurolepis* – Establishing a tool to evaluate large data sets

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Keywords: plant growth regulators, genetic background, developmental stage

The hybrid larch (*Larix x eurolepis*) has shown potential to fit the demands of large scale biomass production for applied forestry sciences as it is a fast growing and therefor economically important conifer species. Unfortunately it suffers from infrequent and unreliable mast (seed yield). Somatic embryogenesis is a useful tool to generate material from seed obtained by directed crossing and continuously provides clonal plantlets in large quantities. There are several protocols for the induction of somatic embryogenesis which we used in order to establish a clone collection. We studied the influence of several selected factors (different plant growth regulators (PGR), genetic backgrounds and developmental stages of the primary material) to improve induction rates and we gathered extensive data. We conducted a multivariate-analysis of variance to find correlations between the respective factors and induction rates by using a generalized linear model with Poisson distribution: Preliminary data confirmed the influence of PGR, parental background and age of the zygotic material as well as coactions within these parameters.
Our work is an approach to assess a complex data set and delivers a tool for multi-layered analyses that can be extended and transferred to similar study designs. The gained information on induction success will help to select potent parental genotypes in combination with their specific requirements. These new insights will help to increase the number of superior clones, save time and resources.

Synthetic Seeds for the production of disease-free cassava plants

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Keywords: clonal propagation, artificial seeds, synseeds, certified planting material, artificial endosperm.

Cassava (*Manihot esculenta* Crantz) is a multipurpose crop important for food security in developing countries. Its heterozygosity is the main reason for vegetative propagation, which promotes the spread of systemic phytopathogens such as ACMV (African cassava mosaic virus) and CBSV (cassava brown streak virus) causing considerable yield losses. As an alternative way of propagating disease-free planting material, we present a protocol for the production of synthetic seeds of cassava genotype 60444 by encapsulating somatic embryos.

First, the rate of somatic embryo production was determined from 34 mg of Friable Embryogenic Callus (FEC) every ten days until the 100th day. Histology was used to confirm differences between shapes of mature somatic embryos that were classified as normal, with two cotyledons, or abnormal, with fused, trumpet-shaped

cotyledons or one cotyledon only. The effect of morphology on embryo-to-plant conversion was determined by pre-germinating somatic embryos on MS2 solid medium in the dark, supplemented with NAA, and then germinating them on solid and liquid media. Plants grown on solid medium were transferred to the greenhouse for two months, survival rates were scored and the plants were then moved to the field. Between 703 and 2152 matured somatic embryos appeared in 100 days. Abnormal embryos showed at higher frequency (61.5 ± 4.1 %), while the frequency of normal embryos was 38.5 ± 4.1 %. Histological observations showed the development of an apex in normal embryos while it was absent in abnormal embryos. Therefore, morphology determined whether the somatic embryos were suitable for the conversion to plant, independently of type of medium, although conversion in liquid medium was faster, less than two weeks, while on solid medium it lasted three weeks. The plant survival rate reached 86% in the greenhouse, and establishment in the field was 100%. Plants in the field showed an apparently normal phenotype.

Encapsulation of normal somatic embryos did not affect plant conversion significantly ($65 \pm 7.1\%$) when compared to non-encapsulated embryos ($65.3 \pm 3.8\%$), neither did storing encapsulated embryos for 15 days at 10°C ($63 \pm 3.2\%$), although plants developed from encapsulated embryos were more vigorous than those from non-encapsulated ones.

The use of FEC as source of somatic embryos allowed the development of a preliminary protocol to produce synthetic seeds of cassava. However, this protocol is subject to improvement before it can be applied to more genotypes, especially commercial ones with resistance to viruses.

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Control of shoot necrosis during micropropagation of *Platanus* x *hispanica*

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Keywords: Forest biotechnology, London plane, organogenesis, vegetative propagation

London plane trees are hybrids of *Platanus orientalis* and *Platanus occidentalis*. The most planted plane tree in Spain is *P. acerifolia* (Ait.) Willd, also known as *P. hispanica*. Currently many Spanish plane trees suffer of anthracnose, a disease caused by the fungus *Apiognomonia veneta* (Tello et al., 2000; Alfonso Corzo et al., 2009). The severity of the disease under common environmental conditions is influenced by the genotype (Tello et al., 2000), which allows to select the most tolerant individuals. A micropropagation protocol was started in order to clone some individuals of *P. hispanica* tolerant to anthracnose using the culture medium described by Liu and Bao (1993) supplemented with 1.33 µM BAP and 0.27 µM NAA. Three genotypes potentially tolerant to anthracnose (A, B and D), and one of high ornamental value (S), were successfully established in culture (Alegre et al., 2013). However, high endophyte proliferation and shoot necrosis occurred during multiplication restricting the protocol's applicability. This work is focused on alleviating shoot leaf necrosis from established organogenic cultures of these four genotypes.

Shoots from established organogenic cultures of the four genotypes were cultured on the multiplication medium described above. The effect of several factors on the percentage of shoots showing necrosis, on multiplication rates and other parameters was tested. The factors were: decrease of the concentration of ammonium nitrate of MS medium to half (LB_{AL}); use of glutamine as a reduced nitrogen source instead of ammonium nitrate (LB_{GL}); use of Silver Thiosulfate (STS) as ethylene inhibitor (LB_{STS}); use of two different types of antibiotics (Augmentine® and streptomicyn sulfate at different concentrations) to reduce the endophyte proliferation (LB_{A50}, LB_{A150}, $LB_{S50,}$ and LB_{S150}); and culture on medium with higher calcium content (DKW). Explants were subcultured to fresh medium after 4 weeks, and data were recorded after 8 weeks.

Differences in the frequencies of necrosis among media were observed. Culture on medium with reduced ammonium nitrate content or with increased calcium proved effective in reducing shoot necrosis. In contrast, the use

of antibiotics did not solve the problem of necrosis, and shoot multiplication was negatively influenced. There were also differences in survival rate. The use of glutamine and STS caused the death of most explants. While survival rate, biomass and axillary multiplication rate were higher when the ammonium nitrate reduced medium was used, shoot leaf necrosis although alleviated remained in the cultures.

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The effect of tissue culture and cryopreservation on tree telomeres

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Keywords: *Betula pendula*, genotypic variation, *Picea abies,* telomeric repeats, tissue culture

Eukaryotic chromosomes are formed by a single DNA molecule, which terminates in specialized heterochromatin called telomeres. The function of telomeres is to protect chromosomes from degradation and fusion during DNA replication. Shortening of telomeres has been connected with ageing and loss of cell replication or regeneration capacity. The information on the role of telomeres in ageing of plants is limited, and telomeres of long-living trees have only been studied in a few species. In addition, biotechnological methods are applied more and more in tree species. Transgenic trees are studied, while clonal propagation by tissue culture is already a standard practice in many species, and clones can be maintained under tissue culture conditions or cryopreserved for a long time. There is, however, no data on the effects of tissue culture and its duration, or stress factors involved in biotechnologies, on telomere length in long-living trees.

The aim of the present study was to examine the potential effect of tissue culture and cryopreservation on tree telomeres. Tissue culture effects were studied in silver birch (*Betula pendula*) using clonal materials consisting of different-aged outdoor trees and tissue cultures of seven genotypes. In addition, the effect of cryopreservation and long-term culture was examined by comparing 12 embryogenic cultures of Norway spruce (*Picea abies*) prior to and following cryostorage.

In silver birch, no correlation of ageing and the length of telomeric repeats was found when germinated seeds, and leaf and cambium samples from 15- and 80-year-old trees were compared. Positional variation in the telomere length was, however, observed in the cambium of mature trees, the stem base having longer repeats than the upper parts of the tree. Tissue cultures were found to have shorter telomeres than outdoor trees: prolonged culture, callus culture and stressful conditions were all observed to shorten telomeric repeats and should thus be avoided in birch micropropagation. There were significant differences among the studied silver birch genotypes in their telomere length, and these differences were consistent over the sample types.

In Norway spruce, successful cryopreservation did not affect the length of telomeres in the embryogenic cultures. In non-regenerating cultures, however, telomeric DNA was observed to be severely damaged. Significant genotypic differences among the SE lines in their telomere length were found also in Norway spruce.

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Factors affecting somatic embryogenesis of Norway spruce - endophytes and cryopreservation

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Keywords: *Picea abies*, vegetative propagation

In several countries including Finland, somatic embryogenesis (SE) systems are currently developed for vegetative propagation of selected spruce materials for future reforestation. From a sustainability and biological diversity point of view, it is important to know the properties of SE materials well prior to applications. In practical work with Norway spruce (*Picea abies*), the cryopreservation step has proved to be a bottleneck for the application of SE. Thus, several pretreatment and slow-cooling methods are being tested to enhance the survival of cell lines stored in liquid nitrogen.

Endophytes are micro-organisms that live inside plant tissue or cells at least part of their life cycle without causing any harm to the host plant, but may also be beneficial for the host. The most common microbes occurring as endophytes are bacteria and fungi, but also mycoplasmas, rickettsia and archebacteria have been found. In Norway spruce, numerous endophytes have been found, both in the needles of mature trees, but also in the seed embryos. The aim of the study was to determine if endophytes are present in embryogenic cultures of Norway spruce, and if so - what kind of endophytes they are, and where do they come from. For detection of endophytes, molecular methods were used. For molecular screening, five PCR primer pairs specific for known endophyte groups were used for embryogenic cultures, mature somatic embryos, regenerated emblings, seeds used as explants for the cultures, and pollen used in the controlled crossings from which the seed originated.

Primer pairs specific towards fungi and *Mycobacteria* were able to amplify right-sized fragments from the samples. Sequencing and subsequent BLAST analyses of the obtained PCR-products identified several different fungi belonging to two out of the five fungal phyla, *Ascomycota* and *Basidiomycota*. This study indicates that endophytes, especially fungal endophytes, are present in embryogenic cultures of Norway spruce, and that they are transferred through the tissue culture process to regenerated emblings. It also seems that fungal endophytes could be transmitted to embryogenic cultures from seed explants used for initiation of cultures.

Time is of the essence - Cotyledon formation in somatic embryos of *Larix decidua* requires proper auxin efflux over a right period and at the right point in time

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Keywords: Conifer*,* Embryogenesis, Auxin inhibition, NPA, Cotyledon formation

The development of a proper mature embryo is closely linked to the formation of a fully functional shoot apical meristem (SAM). This process largely depends on a precisely timed interaction of plant growth regulators (PGR) such as auxin with genetic regulators. The auxin level and distribution during embryogenesis is a key factor to trigger cell differentiation and thus initiate cotyledon development and separation. Essential studies on the transferability of functions known from angiosperms to conifers have been initiated for *Picea abies*. Further analyses are necessary for detailed comparisons regarding conserved developmental strategies.

We used somatic embryos (sE) of *Larix decidua* as an experimental system, because these reach maturity within a month and yield up to 100 sE per 0.1 g FW of embryogenic tissue. It has been our objective to investigate the effect of an inhibited auxin flux on meristem and cotyledon development. To this end the polar auxin transport (PAT) has successively been disrupted or was restored with 1-N-naphthylphthalamic acid (NPA) during embryogenesis. Thus a time frame which is crucial for correct auxin transport was defined at which sE are less affected by auxin flow disruption. In order to find a way to measure the effect of NPA, the embryos were grouped into several categories regarding cotyledon morphology using light and RE microscopy techniques:

NPA treatment caused two major types of embryos with distinct morphological aberrations - either forming embryos with a closed cotyledon ring – "Cups", or a "Cucumber"-like embryo, with a pin-formed apex. Yet the later NPA was applied to the embryos, the lesser was the effect on its morphology. Embryos are to some extend always affected by an NPA application: the staggered application/reduction of NPA lastingly impaired cotyledon development and resulted in varying degrees of cotyledon fusion.

Subsequent analyses were conducted to demonstrate the effect of NPA on the auxin distribution. Therefore, the activity of the reporter gene GUS, which was controlled by an auxin inducible promoter (GH3::GUS) was studied in mature sE, which were continuously treated with NPA. The results indicated that NPA reduces the auxin concentration. Furthermore changes in expression levels in embryos were analysed with the intention to correlate auxin efflux to genes with relevance to embryogenesis and patterning [*PINFORMED (PIN)* and *SHOOT MERISTEMLESS (STM)*]. First results suggest an effect of auxin inhibition on the gene regulation.

We have demonstrated that the formation of a proper embryo apex depends on a defined auxin flux and the interplay with genetic regulators. This fine tuning seems most important before cotyledon appearance.

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Gene Expression in relation to growth traits in *Pinus pinaster* AIT.

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Keywords: qPCR, relative expression

Selection of elite tree individuals for superior biomass production is a labour- and time-consuming slow process. Phenotypic traits such as plant diameter and height are regulated by a number of genes that constrained the application of biotechnological tools to the selection of elite genotypes. Understanding the processes of plant growth through the study of the underlying gene expression patterns can contribute to the improvement of plant production.
The goal of this study was the identification of candidate genes in relation to phenotypes of differential growth in diameter and height in *Pinus pinaster* Ait. To achieve this goal, we analyzed the relative expression levels of 12 genes potentially related to growth in height and diameter using quantitative PCR (qPCR). These genes were previously selected after comparative transcriptomic analysis among individuals that showed contrasting phenotypes. Total RNA was extracted from needles of pines vegetatively propagated by rooting of cuttings from progenies of superior pine trees obtained by Tragsa. According to the phenotypes of the selected individuals, five groups were defined as a function of the diameter and height of the plants after one year of growth: Group 1, plants with high diameter and high height; Group 2, plants with high diameter and low height; Group 3, plants with low diameter and high height; Group 4, plants with low diameter and low height; and a Control Group, plants with intermediate values for the traits examined. The relative expression of the candidate genes in the different phenotypic groups with respect to the control group was analyzed, and the results were normalized using two reference genes (Actin and Ubiquitin). In addition, the relative expression of each individual sample was also calculated, and the obtained data were correlated with those of growth in diameter and height of each plant.
From the 12 analyzed genes, 10 showed significant differences in expression in some of the phenotypic groups in comparison to the control one. From all analyzed genes, only prokaryotic-AAT and DAHP showed a linear expression pattern among the different phenotypic groups, having groups 1 and 4 showing antagonistic expression, whereas the groups 2 and 3 showed intermediate expression. These results suggest that the prediction of elite individuals (both in diameter and height) would be possible by using these genes.
Correlation analyses showed that the expression of 6 genes was correlated with growth in diameter, whereas the expression of the other 2 was correlated with growth in height. None of the analyzed genes showed a statistically significant correlation for both traits at the same time. The results of the correlation analyses allowed us to complement the data of comparison among phenotypic groups for gene expression, helping us to understand them and showing synergistic and antagonistic features between both traits of diameter and height of the plants.

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Regulators of gene expression in pine embryogenesis

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Key words: Conifer, epigenetics, *Pinus pinaster*, small RNAs, transcription factor, transcriptomics

The elucidation of the molecular mechanisms governing conifer embryogenesis may contribute valuable information to understand the basis for the distinct features observed during embryo development in angiosperms and gymnosperms. Additional insight into the molecular regulation of embryo development in conifers will have great utility for the improvement of these species and their vegetative propagation through somatic embryogenesis.

We used a transcriptomic approach to identify mechanisms of epigenetic and transcriptional control by transcription factors of the gene expression program during pine embryogenesis. Microarray analysis of *P. pinaster* zygotic embryos at five stages of development showed that most changes in transcript levels occurred in the transitions from early to pre-cotyledonary embryo and cotyledonary to mature embryo (de Vega-Bartol *et al*., 2013). Several epigenetic regulation mechanisms were highlighted by the analysis of functional categories of the differentially expressed genes throughout embryogenesis. In early embryogenesis several putative orthologs of transcripts associated with mechanisms that target transposable elements and repetitive sequences were strongly expressed while PRC2-mediated repression of genes seemed more relevant during late embryogenesis. Interestingly, functions related to sRNA pathways appeared differentially regulated across all stages of embryo development with a prevalence of miRNA functions in mid to late embryogenesis. Transcriptional control by transcription factors putatively related to auxin transport and response appeared critical during early to mid stages of embryogenesis and are probably related embryo patterning establishment. Later in development, transcripts with homology to genes acting on modulation of auxin flow and determination of adaxial-abaxial polarity were up-regulated, as were putative orthologs of genes required for meristem formation and function as well as establishment of organ boundaries.

The sequencing of sRNA libraries prepared from embryos at the same stages of development as used for microarray analysis allowed the identification of several conserved miRNA families putatively involved in the regulation of embryo development. The most expressed one was the MIR166, known to be involved in organ polarity and vascular development through HD-ZIPIII regulation. A search for the sRNA candidates regulating the differentially transcribed genes implicated in epigenetic regulation (de Vega-Bartol *et al*., 2013) showed evidences of sRNAs involvement in the regulation of other epigenetic players during the zygotic embryo development. Further analysis of the sRNA transcriptome of *P. pinaster* developing embryos is ongoing and, together with the information on the coding transcriptome, is expected to help elucidating the regulatory network involved in the coordination of gene expression during pine embryogenesis.

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Molecular phenotyping of Maritime pine somatic plants transformed with an RNAi construct targeting cinnamyl alcohol dehydrogenase (*CAD*)

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Keywords: Transgenic plant, CAD gene, silencing, transcriptomics, proteomics, off target effect

As a major conifer species for genomic research with great ecological and socio-economical interests in Southern Europe, various biotechnological approaches are concurrently being developed for *Pinus pinaster* towards enhanced selection efficiency and deployment of improved varieties. Recent accumulation of information about expressed genes (e.g. reference transcriptome), genomic areas, and gene pathways controlling breeding traits paved the way for designing practical, marker-based strategies to improve biomass and wood productivity. Functional genomics revealed a wealth of candidate genes to be validated at the population scale (allelic variation, expression and association studies) or more readily by establishing a direct association between genes and adaptively significant phenotypes through loss-of-function techniques such as RNAi. For this specific purpose we developed a post-transcriptional gene silencing method based on stable constitutive expression of intron-spliced hairpin RNA constructs in embryogenic tissue and somatic plants derived thereof. To circumvent the lack of a full genome sequence, we elaborated a strategy based on 4 constructs per gene involving gene sequence tags (GSTs) designed to address RNAi efficiency vs. specificity. Three independent, tandemly arranged GSTs (a, b, c) and one overlapping GST (f) were selected in the variable EST regions of 21 candidate genes involved in wood formation. We specifically report here on data obtained with cinnamyl alcohol dehydrogenase (*CAD*), a gene involved in the last step of monolignols biosynthesis whose silencing is expected to result in decreased lignin content, increased free phenolic groups and pulping efficiency. RT-qPCR analysis of CAD expression in needles of up to 16-month-old somatic plants regenerated from 7 transgenic lines revealed a strong GST effect. CAD expression was significantly downregulated only in independent lines (CADf5, CADfG) transformed with the GSTf-derived construct. Additional analyses of 2.5-year-old transgenic plants from these lines and corresponding non-transgenic controls grown in the greenhouse confirmed the strong reduction in *CAD* expression with concomitant decreased level of CAD protein and activity in the stem. The same samples were used to explore putative genome-wide effects of *CAD* downregulation at both transcriptomic and proteomic levels. Numerous deregulated transcripts and/or proteins were detected in somatic plants from downregulated lines. Differences between stems and needles suggested a large off target, pleiotropic effect of constitutive *CAD* RNAi. Variation between independent lines could also be indicative of a strong positional effect of the CAD transgene. We concluded that genome profiling is a useful molecular tool to identify transgenic lines with minimal genome-wide effect (e.g. CADf5). It is also a prerequisite for accurate interpretation of the wood phenotype in transgenic plants.

Influence of ethylene modulators on the expression of *QsERF1* during secondary embryogenesis of *Quercus suber*

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Keywords: ACC, cork oak, *ethylene response factor 1*, qPCR, somatic embryos, STS

Ethylene production and signaling play an important role in somatic embryogenesis, especially for species that are recalcitrant to *in vitro* culture. Precursors to ethylene, like 1-Amino-1-cyclohexane carboxylic acid (ACC), and inhibitors of ethylene synthesis or perception like silver thiosulphate (STS), have been reported to have different effects on the induction and development of somatic embryos depending on the species and on their developmental stages. In the present study we investigated the effect of different concentrations of ACC and STS on the morphological features and the proliferation levels of *Quercus suber* somatic embryos. We also studied the transcript accumulation of *QsERF1* using qPCR in embryos collected at different times after ACC and STS treatments.

QsERF1 (*Q. suber ethylene response factor 1*), isolated by our research group, encodes a member of the ethylene response factor (ERF) transcription factors. ERFs belong to the APETALA2/Ethylene Responsive Factor (AP2/ERF) family, whose members are conservatively widespread in the plant kingdom. *QsERF1* is related to *Medicago truncatula somatic embryo-related factor1* (*MtSERF1*), which has been shown to be required for somatic embryogenesis.

Quercus suber somatic embryos were initiated from expanding leaves of epicormic shoots forced to flush in branch segments of a centenarian tree according to the protocol described by Hernández et al. (2003). Embryos were subcultured monthly by secondary embryogenesis in RITA$^{®}$ vessels with Murashige and Skoog medium supplemented with 0.1 mg L^{-1} benzyladenine, 0.05 mg L^{-1} naphthalene-1-acetic acid and 3% sucrose. For ACC and STS experiments, embryos were cultured in Petri dishes in the same medium described above but gelled with 0.6% Vitroagar. This medium was supplemented with 0, 10, 50 or 100 µM of ACC or STS. Two types of explants were used: 1) embryo clumps at globular/torpedo stages, and 2) isolated cotyledonary embryos (6-10 mm). The effect of ACC and STS on the morphological features of *Q. suber* embryos was monitored weekly and the proliferation levels were recorded after 4 weeks of culture. Both embryo clumps and isolated cotyledonary embryos treated with ACC showed good performance without significant differences with the control treatment. However, the ability of the cork oak embryogenic cultures to produce secondary embryos was severely affected by the presence of 50–100 µM STS, and a significant increase in percentage necrosis was also observed. The relative transcript abundance of *QsERF1* was analyzed during somatic embryogenesis by qPCR in samples of embryo clumps of immature stages, as well as in isolated cotyledonary embryos that were collected at 0, 2, 4, 7, 14, 21 and 28 days after plating.

Photo Gallery

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