An overview of current achievements and shortcomings in developing Maritime pine somatic embryogenesis and enabling technologies in France

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IUFRO Working Party 2.09.02
2011 Somatic Embryogenesis of Forest Trees Conference

PROCEEDINGS





Advances in Somatic embryogenesis of Trees and Its Application for the Future Forests and Plantations

KOREA FOREST RESEARCH INSTITUTE

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Welcome Address

On behalf of the organizing committee, I would like to welcome you to the inaugural conference of the Working Party. For a personal reason, I am particularly pleased to welcome you here in Suwon because this is where I went undergraduate school. Although I left Korea 40 years ago, I always kept fond memories of my "Suwon" days. This is the place where my passion for forestry and genetics were seeded. In fact, Suwon is the cradle of forest tree breeding dating back to 1922, but most tree breeding research was started in 1953 after the Korean War. By 1964, Institute of Forest Genetics was fully in operation contributing to massive reforestation efforts of the devastated land during the two wars.

In the past 40 years, tree improvement research flourished. More recently, a lot of efforts were directed to various tree biotechnology researches. However, in my mind, Somatic Embryogenesis is the leading technology that is ready to be applied in the intensive forest management system. In fact, commercial implementation of SE has started, particularly for spruce and some pine species. SE offers a new paradigm in tree breeding and deployment.

However, although SE available for many tree species, they are at the different stages of development: For some species, it is well refined and ready for commercial implementation but for some it is extremely difficult to obtain SE. This is the reason why we have organized a working party and having this inaugural conference. The aim is to progress together by sharing success stories, exchanging knowledge, collaborating and networking.

We have a collection of expertise in this conference, including tree breeder, quantitative geneticist, molecular biologist, tissue culturist, physiologists, biotechnologists, etc. We hope this is an excellent opportunity to connect various disciplines. So, we wish you a productive conference and happy networking.

Since this is our inaugural conference, I would like to take this opportunity to introduce Deputy Coordinators of this working party: They are Dr. Heung-Kyu Moon of KFRI, Korea, Dr. Mariano Toribio of IMIDRA, Spain and Dr. Jean-Francois Trontin, FCBA, France.

Also, on behalf of organizing committee, I would like to thank Dr. Wan-Yong Choi, Director General of KFRI, for supporting and hosting this conference. Without his support, having this conference is not possible. Thank you.

Once again, welcome and have a great conference.

Dr. Yill-Sung Park
Coordinator
IUFRO working party 2.09.02

Message from General Director of KFRI

It is my great honor to open The “Conference on advantages in somatic embryogenesis of trees and its application for future forests and plantations”. Let me also show my sincere appreciation and gratitude to Drs. Yill-Sung Park and Eul-Sun Baik, the cohosts of IUFRO Working Party 2.09.02 for their excellent preparation of the conference.

This conference is held as a preconference before IUFRO World Congress in Seoul next week. In the congress, we are going to discuss how we could contribute to the fights against climate change and ever decreasing forest resources. I am sure forestry could play an important role in the fight against such global disasters.

We know that one way of lessening the impact of global warming is to increase forest productivity. By increasing plantation productivity, we can help save natural forests. There are many ways by which we improve forest productivity. We can develop rapidly growing trees to produce more wood in less land area. Or we may develop trees with stress tolerance thereby reducing losses from various stresses. I am a tree breeder and have been working on a number of tree breeding projects for about 30 years. Although our tree breeding programs produced large genetic gains in many species, it has not been very effective to realize the gain. It just takes too long to fix the gains since tree breeding involves repeated crossings and selections. With efficient vegetative propagation tools, we can achieve this in much less time with less cost.

Tissue culture has been considered as an efficient tool for vegetative propagation. For this reason, we established a tissue culture lab at the Institute of Forest Genetics back in 1980. The lab has been expanded to become a division under KFRI now. Since then, a number of research projects have been carried out to develop such tools. We have been quite successful with poplars and birches. However, we do not plant those trees in a large scale any more. Social demands for wood change over time. For example, pitch pine was good at covering naked land in Korea after Korean War. It adapted well to naked infertile soil then. However, it is now not popular for its poor quality wood. Thus, it is high time that we renewed pitch pine stands. And this time, we have to make sure to use good quality planting materials. Yellow poplar is one of the most promising candidates here. It has been introduced and planted across this country since 1950’s. It adapted well to the climatic conditions of Korea. However, to replace the pitch pine stands, we need more than 840 million seedlings in 10 planned years. Thus, we badly need an efficient vegetative propagation tools for elite trees. To be successful, the technology should be in the right time and in the right place.

I think somatic embryogenesis is the right technology for this purpose. Therefore, with a great deal of expectation, we are trying to apply somatic embryogenesis system to the realization of clonal forestry. It may be the first example of biotechnology application in the field in Korea. However, I know that the system is not perfect yet. We are still struggling to find a way to regenerate somatic embryos from mature tissues.

Today, many distinguished scientists in the field of somatic embryogenesis of trees came here to present, discuss and answer their findings and questions. I am sure that this conference will provide us with very practical ideas that will strengthen and widen our knowledge on the somatic embryogenesis process. I hope all the participants will benefit from attending this conference.

Thank you for inviting me to speak to you and I look forward to continuing our excellent working relationship into the future.

Dr. Choi, Wan-Yong
The Director General
Korea Forest Research Institute

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Application of Somatic Embryogenesis in Forest Management and Research

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Abstract

Since the first report in 1985 of successfully obtaining somatic embryogenesis (SE) in conifers, impressive advances have been made. SE is now available for many economically and ecologically important species and is already applied in forest management systems. Among its many applications, the most important current one is implementation of multi-varietal forestry (MVF), which is the deployment of tested tree varieties in plantation forestry. In conjunction with cryopreservation, SE offers an efficient production of the same tested genotypes consistently over time and thus enables the development of high-value tree varieties equivalent to agronomic varieties. This has led to commercial-scale deployment of varieties of spruces and pines, offering a new paradigm in tree improvement. Furthermore, SE is the primary enabling technology for many tree biotechnology procedures including genetic transformation and subsequent mass propagation. The application of SE in the preservation of ecologically important and threatened species opens a new dimension for genetic resource conservation and species restoration; efforts are already underway to preserve threatened and disease-prone species such as white-bark and limber pines in western Canada by means of SE. Furthermore, there are many as-yet unexploited possibilities for SE in tree biology research, including genetics, physiology, disease resistance, genomics, metabolomics, proteomics, development, epigenetics, etc. The uniform and precise sampling properties offered by clonal replicates produced by SE will be an important benefit for such studies.

Key words: Multi-varietal forestry, cryopreservation, tree breeding, genetic resource conservation, embryo recue, epigenetic memory

Introduction

In the past 40 years, tree improvement programs around the world have contributed greatly to the productivity and wood quality attributes of plantation forestry. The delivery of such improvement has been through seed orchards and this will continue to be the primary means of providing genetic improvement. These programs typically produce 3-5% gain in height when evaluated at about 10 years of age. However, seed orchards are often associated with several inefficiencies such as pollen contamination and unequal parental contributions. In addition, conventional breeding programs are usually long-term efforts, involving multi-generation recurrent selection and testing that are generally expensive and time consuming. Furthermore, we are faced with new challenges: (1) productivity of plantation needs to be pushed to a higher level, prompting discussion on prime-site intensive silviculture: (2) product goals are changing as we search for new products; (3) climate change casts uncertainty on adaptation of trees; (4) pest resistance will continue to be an issue and (5) we are expected to contribute to conservation and restoration of threatened tree species. To meet these challenges, we need to develop a "flexible" breeding and deployment system Thus, it is necessary to modify the current breeding strategy to meet these challenges. With recent developments and refinements in tree biotechnology, complementary and/or alternative breeding, testing and deployment strategies to meet these challenges have become available for several conifer species. Somatic embryogenesis (SE), cryopreservation, and use of molecular genetic markers are such a technology that can be used for implementing complementary MVF, simplifying breeding and selection schemes, studying pollen dynamics of seed orchards, providing an additional dimension for species conservation and restoration, and in research elucidating genetic response to biotic and abiotic factors. The purpose of this paper is to promote the application of SE in various aspects of forest research and management; however, our discussion is primarily focussed on the SE of conifers.

Brief review of conifer somatic embryogenesis

The development of SE in forest trees started in 1985 when SE was reported in Norway spruce (Hakman *et al*. 1985, Chalupa 1985) and in European larch haploid culture (Nagamani and Bonga 1985). In the following year, SE was reported in sugar pine (Gupta and Durzan 1986). Today, SE is widely available for may tree species although there are still varying difficulties in obtaining SE in many instances.

The initiation of embryogenic culture masses is the first stage in conifer propagation by SE and generally requires the use of plant growth regulators (PGR), typically 2,4-D and BA. The required level of PGR is variable, depending on the species, and this offers an opportunity to improve initiation media formula. For example, working with eastern white pine (*Pinus strobus*), Klimaszewska *et al.* (2001) developed an optimized

initiation medium by manipulating the levels of PGR. Similar success was also obtained from other pine species (Park *et al*. 2006). Initiation of SE is also affected by the developmental stage of zygotic embryos, particularly for pines. For example, for eastern white pine the imitation rate was less than 10% when we used explants collected in late June , but, a week later, the initiation peaked at 60%, and then subsequently declined. Thus, it was revealed that the most responsive developmental stage for initiation was soon after fertilization around the time of cleavage polyembryony (Klimaszewska *et al*. 2001). The maturation of somatic embryos is often the most difficult step in the SE process. For maturation, abscic acid (ABA) is generally required and the amount being optimal is quite variable depending on the species. The quality of the somatic embryos depends not only on the PGR type and concentration but is also affected by the gelling agent, light, and temperature used (Lelu-Walter *et al*. 2006). For example, Harrison and von Aderkas (2004) showed that the cotyledon number is affected by the PGR type used, showing that BA produced generally a lower number of cotyledons than ABA. It is also known that SE is under genetic control. Particularly, the initiation rate is under strong additive genetic control, indicating that initiation can be manipulated by breeding. The genetic control declines at the subsequent proliferation, maturation, and germination phases. Furthermore, we found no genetic correlation among different phases of SE (Park *et al*. 1993, 1994). This led to breeding for improvement of SE initiation percentages (MacKay *et al.* 2006). Owing to these advances, SE for several conifer species, particularly for spruce and several pine species, is sufficiently refined that it can be implemented commercially (Table 1). In addition, it has been shown that

Table 1. *SE initiation and maturation percentages of conifers obtained at Canadian Forest Service and collaborating laboratories.*

Species	Zygotic embryo explant types		Maturation
	Immature	Mature	
Picea glauca	68	20	89
P. mariana	65	21	85
P. abies	75	29	80
P. sitchensis	35	-	80
Pinus strobus	61	2	76
P. taeda	36	0	66
P. pinaster	76	0	58
P. sylvestris	20	0	85
P. albicaulis	15	-	75
P. flexilis	15	-	-
P. patula	13	-	-
P. monticola	6	0	75
P. banksiana	4	0	90

- Not attempted

embryogenic tissue will survive freezing in liquid nitrogen and subsequent thawing. Genetic stability during cryopreservation has been demonstrated and somaclonal variation is not an issue (Park *et al*. 1998, de Verno *et al.* 1998). SE trees are morphologically normal and grow well in plantations. This gives us a green light to go ahead with industrial application.

Multi-varietal forestry

Among many important applications of SE, the current most important one is its application in commercial MVF, which is defined as the use of tested tree varieties in plantation forestry. The advantages of MVF include: capture of much greater genetic gain than is possible by seed orchard breeding by exploiting both additive and non-additive genetic variation; flexibility to rapidly deploy suitable varieties with changing breeding goals, climate, or environments; and ability to design genetic gain and diversity. In addition to SE, the key technology for implementing MVF is cryopreservation, which is the storage of embryogenic tissue in liquid nitrogen (–196 ℃) indefinitely without changing the genetic makeup or viability. This gives us a unique opportunity to propagate the same genotypes consistently over time, which is analogous to producing agricultural or horticultural varieties. In other words, we now have the ability to produce conifer varieties that were not available with sexual reproduction (seed orchards).

Multi-varietal forestry is a new paradigm in tree breeding and deployment. However, MVF is likely to be used only in high-value prime-site intensive forestry while the genetically improved seed orchard seeds will still be used extensively in other forms of silviculture. The complementary function of MVF in connection with traditional seed orchard breeding is schematically illustrated in Figure 1. Typical clonal seed orchard (CSO) breeding uses some form of recurrent selection and maintains a breeding population (BP) consisting of genetically selected individuals. Grafts of these are planted in seed orchards for the production of improved seeds. To obtain genetic improvement for the next generation, the individuals in BP are mated, often by positive assortative mating (PAM), to produce material for next generation selection and genetic testing. Based on the genetic testing, the initial CSO can be rogued for further improvement, leaving the best progenies as the next generation BP for recurrent selection. Seeds from the seed orchards are genetically improved as they capture the additive genetic variability among the parents and are well suited for extensive reforestation. The complementary MVF may begin with the elite individuals selected from the BP. The elite parents can be controlled-pollinated (CP), open-pollinated (OP), or supplementary mass pollinated (SMP) to produce an offspring population. The resulting seeds from these crosses are subjected to SE initiation and, once SE is initiated, the embryogenic tissue (ET) lines are cryogenically stored. Parts of the ET from each line are then thawed, propagated and planted in clonally replicated varietal test (CRT). Based on the CRT, high-value embryogenic varieties are retrieved from the cryo-storage and mass propagated for planting on the productive sites. The deployment of tested varieties offer

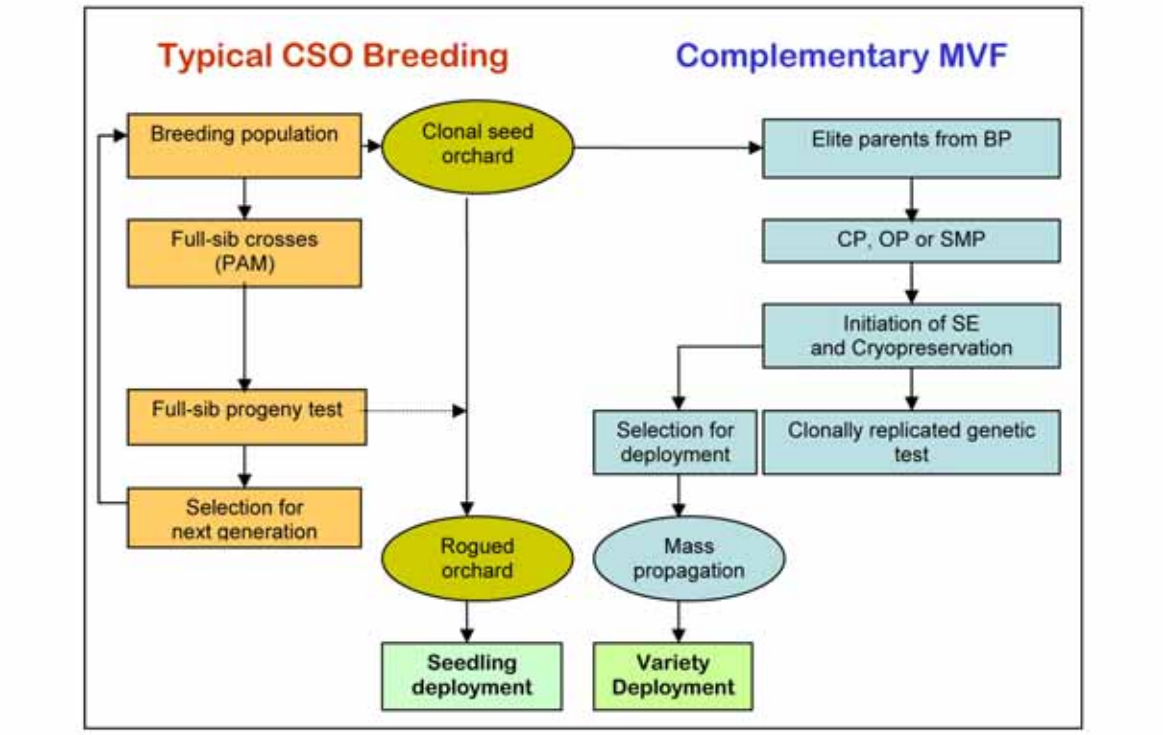

***Figure 1**. A complementary MVF strategy in comparison with typical clonal seed orchard breeding*

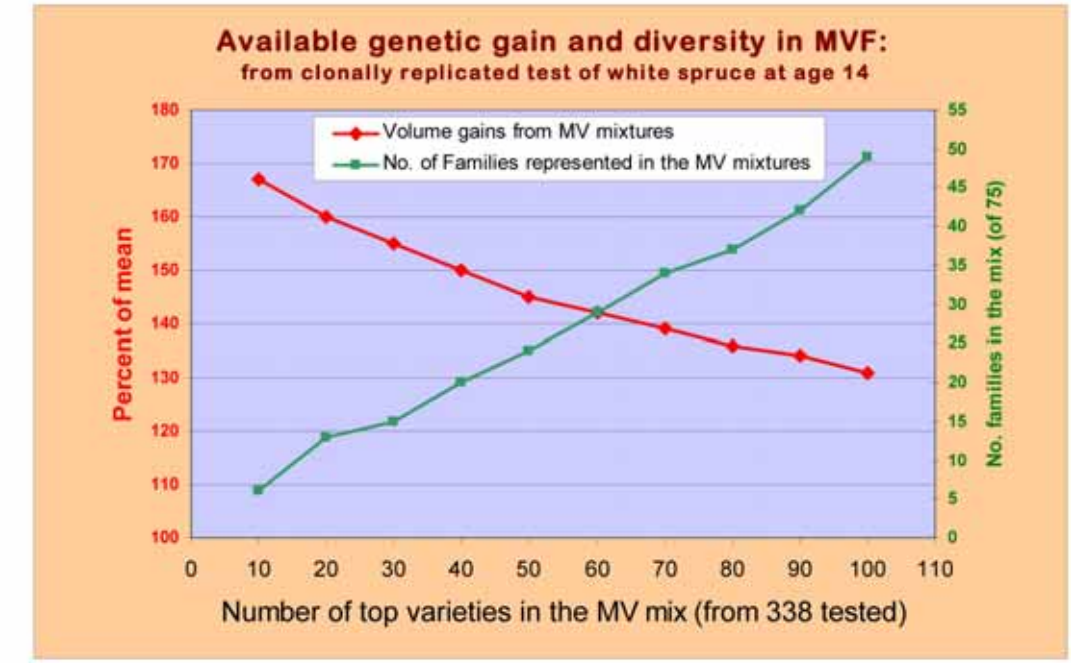

***Figure 2.** Available genetic gain and diversity for MVF of white spruce evaluated at age 14.*

much greater genetic gain than the CSO seeds as it captures both additive and non-additive genetic variation. Furthermore, it offers flexibility to modify the deployment with changing conditions.

The potential genetic gain and management of plantation diversity is illustrated in Figure 2, where the data shown were obtained from a 14-year old CRT of white spruce (*Picea glauca*) that included 338 candidate varieties derived from 75 full-sib crosses. In the graph, if we take top 10 varieties of the 338 in the test, the genetic gain for volume is 68% better than the average of all varieties in the test. Even when we take the 100 best, the genetic gain is 30% better than the average.

This is still a huge genetic gain when compared to that obtained by seed orchard breeding. Incidentally, the average (100%) represents idealized seed orchard gain. The number of family representation in a varietal mix may be considered as an indicator of diversity. If we take the 10 best varieties, this only represents 7 of the 75 full-sib families in the test. If we take the top 100, i.e., taking 30% above the seed orchard gain, the varietal mix is represented by 48 of 75. In other words, we can balance a desired level of genetic gain and diversity based on the test results. This strategy is termed the "desired gain and diversity" approach. Obviously, as the CRT gets older, the reliability of data and efficiency of managing MVF will increase.

Clonally replicated genetic testing

Clonally replicated genetic testing, i.e., by making use of the cloned ramets from the genetic entries in the test, is an important element of MVF because it will provide appropriate data for varietal selection and management of plantation diversity. The flexibility of MVF is primarily derived from the CRT, because it is expected that CRT plantation will continuously provide relevant data throughout the rotation age and beyond. For example, the final assessment of growth, quality, and pest resistance traits of candidate varieties in the field may take a long time. However, whenever the updated data becomes available, the breeders can quickly adapt to the changes by simply thawing and deploying appropriate varieties from cryopreservation. Thus, it is generally accepted that a large number of varieties be included in the variety mix when the field test is young, but when the tests are sufficiently old, breeders can deploy a small number of well-characterized elite varieties with confidence. Therefore, it is important to characterize the candidate varieties in the test regularly and whenever genetic variability is observed such as insect and disease resistance and environmental tolerance incidence.

In addition to identifying suitable varieties for MVF deployment, CRT has been used in tree breeding experiments to partition non-additive genetic variances in open-pollinated families (Foster 1985, Park and Fower1987). Using controlled-pollinated families, Mullin and Park (1991) further demonstrated the partitioning of epistatic variance. Thus, CRT provides additional genetic information for breeding experiments and offers efficiency and precision of parameter estimation for quantitative traits (Foster and Shaw 1988, Shaw and Hood 1985).

The use of clonal replicates is essential for the disease and insect resistance screening experiments, especially when the objective of the experiment is developing resistant varieties. Challenge tests, based on a family or provenace level, lack the precision of identifying resistant individual genotypes because these tests include several different individuals comprising family or provenance, and thus, they only provide the resistance levels at the family or provenance average. The removal of the "genetic" effect, which is achieved by the use of clonal replicates that provide genetic uniformity of candidate varieties in the challenge test, is essential for capturing individual genotypes that are truly resistant.

Hybrid varieties

In agriculture, hybridization usually refers to crossing of different strains (or homozygous lines) within a species, but in forestry, it refers to crossing between different species or distinctly different races within a species. The benefits of hybridization in forestry include the capture of hybrid vigor in growth traits and combination of desirable traits. An example of hybrid vigor is demonstrated by the interspecific crosses between Japanese and European larch, while the Pitch and Loblolly pine hybrids are successfully used in Korea by combining the cold tolerance and fast growth in the respective species. Despite the huge potential benefits, hybridization in conifers was rarely used as a modern breeding method, partly due to labor intensiveness of hybrid seed production by controlled pollination or inefficiencies of seed production in a bi-species orchard. SE is an ideal tool for developing hybrid varieties as it can be used for mass production of hybrid seedlings. Also, the use of SE enables the selection of elite individuals within the crosses. The development of blister rust resistant white pine varieties through interspecific hybridization between *P. strobus* and *P. wallichiana* (and backcrossing) is underway in Ontario, Canada, which adopted the use of SE (Lu 2008).

Species conservation and restoration

Somatic embryogenesis combined with cryopreservation can have an important impact on species conservation and restoration. For example, whitebark pine (*P. albicaulis*) is a keystone species growing in subalpine regions of Alberta and BC and is threatened in its natural range. No seeds are available for reforestation as they are a food source for birds and animals and its wingless seed is dispersed only by birds. The serious threats come from white pine blister rust (*Cronartium ribicola*), white pine weevil (*Pissodes strobi*), attack by mountain pine beetle (*Dendroctonus ponderosae*), prolonged fire suppression preventing natural regeneration, and projected climate change. In this case, SE can be used as an alternative means of producing planting stock as the seed availability is very limited. Through SE, valuable genotypes can be cryogenically stored long-term, and restoration of the sites with better adapted and pest resistant genotypes can be accomplished at a later date. This is a strategy

adopted by the Alberta Sustainable Resource Development in Canada (Park *et al.* 2010). Similar efforts are also in progress for limber pine (*P. flexilis*).

Recalcitrance and SE from adult trees

Obtaining SE from a wide range of species is important as it offers new possibilities for improvement and conservation. However, despite the significant advances in SE technology, obtaining SE is still difficult or not possible for many important tree species. Most of SE in conifers has been obtained from zygotic embryo explants, which are juvenile explants, and is generally easier achieved with immature than mature zygotic embryos. The degree of recalcitrance varies among species. In general, for spruce and some pine species it is relatively easy to obtain SE, but for many conifers, for example, jack pine (*P. banksiana*), lodgepole pine (*P. contorta*), and yellow-cedar (*Chamaecyparis nootkatensis*) it is still difficult (Table 1). Bonga *et al.* (2010) suggest some approaches to overcome recalcitrance; however, it remains a major obstacle especially when adult trees are used. The benefits of obtaining SE from mature adult trees with proven trait quality, including growth and pest resistance, are obvious. Recently, Klimaszewska *et al.* (2010) achieved SE from 10-year old white spruce trees that had originally been obtained by SE from zygotic embryos. SE from these 10-year-old trees occurred in primordial shoot cultures. This is the most successful plant regeneration by SE from explants other than zygotic embryo. It is interesting to note that SE-derived trees are more amenable to SE initiation than seed propagated trees. This provides an opportunity to assess the current hypothesis that the lack of SE potential in adult is caused by biochemical and molecular modification associated with phase change.

Genetic engineering

Genetic transformation allows integration of valuable genes that are absent in elite genotypes into these genotypes. SE is the primary enabling technology for both transformation and subsequent propagation. Among the available methods, transformation of embryogenic culture is the most common one. It is generally achieved through co-cultivation with *Agrobacterium* carrying the transgene. The advantages of using SE in genetic engineering are: the process can be carried out in a strictly confined environment; the transformed and non-transformed cells can be easily separated by including an antibiotic resistance gene; since SE in most species start from single cells, one can avoid ending up with chimera, i.e., individuals with both transformed and non-transformed cells (Klimaszewska *et al.* 2010). However, the stability and containment of transgenes are important issues that have to be dealt with before genetically modified trees are deployed. Engineering of sexual sterility genes into SEs is a means of achieving containment, but it is not yet widely available. Due to a potentially adverse environmental impact and bio-safety issues, most jurisdictions around the world regulate testing and deployment of transgenic trees (Trontin *et al.* 2007).

Embryo rescue

Somatic embryogenesis can be used in embryo rescue. Crossing between related species can be a useful technique for creating unusual genotypes but such crosses usually result in abortion. Embryo rescue has been carried out with a large number of species, including tree species. A couple of recent examples of the latter are citrus triploid hybrids (Aleza *et al*. 2010) and banana (Uma 2011) but, to our knowledge, has never been attempted with conifers. It is generally believed that embryo abortion is due to a genetic incompatibility between the developing embryo and the maternal tissue surrounding it. Even though embryos may not develop fully within the megagametophyte (seed), these embryos can develop normally if they are removed from seeds before abortion occurs and grown to maturity *in vitro* or immature embryos may be induced to imitate SE. The latter is a potential possibility because SE often initiates during the initial cleavage polyembryony stage of the zygotic embryo. Thus embryo rescue has a capacity to create genotypes that are not possible to obtain through breeding. Subsequent mass propagation of rescued embryo can be highly effective if propagated by SE.

Epigenetic memory

It is presently generally accepted that epigenetics refers to changes in phenotype or gene expression caused by mechanisms other than changes in the underlying DNA sequence (Meehan *et al.* 2005). Epigenetic memory effects in Norway spruce (*Picea abies*) during zygotic embryogenesis and seed maturation have been reported by Johnsen *et al.* (2005). Von Aderkas *et al.* (2007), working with interior spruce (*P. glauca* x *P. engelmannii*) SE, reported that somatic embryos, matured at a lower temperature (5 ºC), showed significantly higher cold tolerance than those matured at 20 ºC. However, it is not known that such epigenetic memory effects will continue during the seedling and adult tree stages. This is an important research area for plant adaptation especially under climate change scenarios. SE can be used for studying epigenetic memory effects as well as embryo development.

Conclusions

Somatic embryogenesis has been the most important development for plant tissue culture, not only for mass propagation but also for enabling the development of related biotechnologies and new products and forest management strategies. The most important current application of SE is in implementing MVF, which is particularly suited for intensively managed, high productivity sites and offers much greater genetic gain and flexibility to adapt to an uncertain future than traditional tree improvement practises. Varietal field testing with cryopreservation is the key strategy for meeting future challenges as it provides continuously updated information offering flexibility to modify or

fine-tune forest management. SE also offers many new possibilities for biological research.

References

Aleza, P., Juarez, J., Cuenca, J., Ollitrault, P., Navarro, L. 2010. Recovery of citrus triploid hybrids by embryo rescue and flow cytometry from 2x x 2x sexual hybridization and its application to extensive breeding programs. Plant Cell Rep. 29:1023-1034.

Bonga, J.M., Klimaszewska, K., von Aderkas, P. 2010. Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tiss. Org. Cult. 100:241-256.

Chalupa, V. 1985. Somatic embryogenesis and plant regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. Commun. Inst. For. Cech., 14:57-63.

DeVerno, L.L., Park, Y.S., Bonga, J.M., Barrett, J.D. 1999. Somaclonal variation in cryopreserved embryogenic clones of white spruce [*Picea glauca* (Moench) Voss]. Plant Cell Rep. 18:948-953.

Foster, G.S., Shaw, D.V. 1988. Using clonal replicates to explore genetic variation in a perennial plant species. Theor. Appl. Genet. 76:788-794.

Gupta, P.K,, Durzan, D. 1986. Somatic embryogenesis from callus of mature sugar pine embryos. Bio/Technology 4:643-645.

Hakman, I., Fowke, L.C., von Arnold, S., Eriksson, T. 1985. The development of somatic embryogenesis in tissue culture initiated from immature embryos of *Picea abies* (Norway spruce). Plant Sci 38:53-59.

Harrison, L.G., von Aderkas P. 2004. Spatially quantitative control of the number of cotyledons in a clonal population of somatic embryos of hybrid larch Larix x leptoeuropaea. Ann. Bot. 93:1-12.

Johnsen, Ø., Fossdal, C.G., Nagy, N.E., Mølmann J., Dæhlen O.G., Skrøppa, T. 2005. Climatic adaptation in *Picea abies* progenies is affected by the temperature during zygotic embryogenesis and seed maturation. Plant, Cell and Environment 28: 1090-1102.

Klimaszewska, K., Park, Y.S., Overton, C., MacEacheron, I., Bonga, J.M. 2001. Optimized somatic embryogenesis in *Pinus strobus* L. In Vitro Cell. Dev. Biol.-Plant 37:392-399.

Klimaszewska, K., Overton, C., Stewart, D., Rutledge, R.G. 2010 Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old somatic white spruce and expression profiles of 11 genes followed during the tissue culture process. Planta 233:635-647.

Lelu-Walter, M.-A., Bernier-Cardou, M., Klimaszewska, K. 2006. Simplified and improved somatic embryogenesis for clonal propagation of *Pinus pinaster*. Plant Cell Rep. 25:767-776.

Lu, P. 2008. Breeding eastern white pine for blister rust resistance through interspecific hybridization and back crossing: a review of progress in Ontario (Extended Abstract). In Proc. Breeding and Genetic Resources of five-needle pines, Yangyang, Korea. 81-82.

MacKay, J.J., Becwar, M.R., Park, Y.S., Corderro, J.P., Pullman, G.S 2006. Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. Tree Genetics & Genomes 2:1-19.

Meehan, R.R., Dunican, D.S., Ruzov, A., Pennings, S. 2005. Epigenetic silencing in embryogenesis. Exp. Cell Res. 309:241-249.

Mullin, T.J., Park, Y.S. 1992. Estimating genetic gains from alternative breeding strategies for clonal forestry. Can. J. For. Res. 22:14-23.

Nagamani, R., Bonga, J.M. 1985. Embryogenesis in subcultured callus *of Larix decidua*. Can. J. For. Res. 15: 1088-1091.

Park, Y.S., Fowler, D.P. 1987. Genetic variances among clonally propagated populations of tamarack and the implications for clonal forestry. Can. J. For. Res. 17:1175-1180.

Park, Y.S., Pond, S.E., Bonga, J.M. 1993. Initiation of somatic embryogenesis in white spruce *(Picea glauca*): genetic control, culture treatment effects, and implication for tree breeding. Theoretical Applied Genetics, 86:427-436.

Park, Y.S., Pond, S.E., Bonga, J.M. 1994. Somatic embryogenesis in white spruce (*Picea glauca*): genetic control in somatic embryoexposed to storage, maturation treatments, germination and cryopreservation. Theoretical and Applied Genetics, 89:742-750.

Park, Y.S., Barrett, J.D., Bonga, J.M. 1998. Application of somatic embryogenesis in high-value clonal forestry: Deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell. Dev. Biol.-Plant 4:231-239.

Park, Y.S., Lelu-Walter, M.-A., Harvengt, L., Trontin, J.-F., MacEacheron, I., Klimaszewska, K., Bonga, J. 2006. Initiation of somatic embryogenesis in *Pinus banksiana, P. strobus, P. pinaster, and P. sylvestris* at three laboratories in Canada and France. Plant Cell. Tiss. Organ Cult. 86:87-101.

Park, Y.S., Barnhardt, L., Klimaszewska, K., Dhir, N., MacEacheron, I. 2010. Somatic embryogenesis in whitebark pine (*Pinus albicaulis*) and its implication for genetic resource conservation and restoration. In Proc. of IUFRO conference on Somatic Embryogenesis of Trees. August 18-21, 2010. Suwon, Korea.

Trontin, J.-F., Walter, C., Klimaszewska, K., Park, Y.S., Lelu-Walter, M.-A. 2007. Recent progress on transformation of four *Pinus* species. Invited Review. Transgenic Plant Journal 1:314-329.

Uma, S., Lakshmi, S., Saraswathi, M.S., Akbar, A. Mustaffa, M.M. 2011 Embryo rescue and plant regeneration in banana (*Musa* spp.) Plant Cell Tissue Organ Cult. 105:105-111.

von Aderkas, P., Kong L., Hawkins B., Rohr R. 2007. Effects of non-freezing lowtemperatures on quality and cold tolerance of mature somatic embryos of interior spruce [*Picea glauca* (Moench) Voss x *P. engelmanni* Parry ex. Engelm.] Propagation of Ornamental Plants 7:112-121.

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Somatic Embryogenesis in Scots Pine (*Pinus sylvestris* L.) – Embryo quality, Long-term Cryo-storage, and Mature Explants as Challenges

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Introduction

Somatic embryogenesis (SE) is considered to be the vegetative propagation method that has the best potential for Scots pine (*Pinus sylvestris* L.). The existing SE protocols for Scots pine are based on the use of immature zygotic embryos as explants for culture initiation (Häggman *et al.* 1999, Lelu-Walter *et al.* 2008). Compared to other pine species, Scots pine appears to be a difficult species to work with (Klimaszewska *et al.* 2007), and enhancement of the different steps along the SE process is still needed in order to fully utilize the potential of the propagation technique. Furthermore, SE initiated from tissues of mature material would be of a great interest because it promises the possibility to propagate selected trees with known characteristics, such as good growth and desired wood quality.

Using juvenile explants, the final performance of the SE clones can be evaluated only by field tests of regenerated plants that in Scots pine take at least 5-10 years. However, because Scots pine SE cultures gradually lose their embryo production capacity within 1-2 years of continuous *in vitro* culture, cryopreservation of SE cultures is a prerequisite for successful clone delivery, cryopreservation maintaining the regeneration ability during field testing. Cryopreservation techniques (Häggman *et al.* 1998, Lelu-Walter *et al.* 2008) have been developed for Scots pine SE cultures, but there are no reports on long-term cryostorage.

In this presentation, experience gained with both juvenile and mature explants in SE initiation, proliferation and maturation, as well as the significance of the embryo quality for embling performance, and the effects of the long-term cryopreservation are discussed.

Materials and methods

As described by Aronen *et al*. (2009), different SE initiation and proliferation techniques, using immature zygotic embryos as explants, were examined. Following proliferation, different maturation techniques were tested for their interaction with the proliferation methods used, and for their effect on the number and quality of the somatic embryos produced, including also conversion into plants and further performance of the emblings both in the greenhouse and under field conditions.

Mature tree explants have also been tested for initiation of SE cultures in Scots pine. Thin slices of surface-sterilized buds or new shoots from 10-15 –year-old trees, collected in the beginning of growing season, were subjected to different pre-treatments and placed onto modified DCR or LM basal medium containing varying amounts of plant growth regulators. Recognizing potentially embryogenic tissue and its separation from the explant was performed at different times during initiation, and was followed by proliferation and maturation experiments. The embryogenicity of the induced tissues was studied by acetocarmine staining and gene expression markers. Altogether over 16 000 explants were treated in years 2008-2010.

Potential effects of the long-term cryopreservation and of different cryoprotectants (PGD mixture 1:1 or 1: 2½, DMSO solely) on the viability, proliferation ability or embryo maturation capacity of Scots pine SE lines was studied using samples of the 112 SE lines initiated from four donor trees and cryo-stored for 2 to 14 years, as described in detail by Latutrie & Aronen (2011).

Results and Discussion

Juvenile explants and SE quality

As presented by Aronen *et al*. (2009), using juvenile explants, i.e. immature zygotic embryos, SE initiation frequencies of 20-30 % can be achieved, but there are also recalcitrant families. In order to obtain high-quality somatic embryos that perform well as emblings in the greenhouse, the SE cultures should be proliferated and matured as a thin layer spread on filter paper and should be exposed to a high (80-90 µM) abscisic acid concentration during maturation. The somatic embryos should be harvested for germination only during a limited period, and specific attention has to be paid to their quality: slim-type embryos have the best germination and greenhouse performance, being superior to intermediate and stub-type embryos. With optimised culture conditions and tight quality control, over 90% of the embryos will germinate and develop into vigorously-growing emblings.

The first results of the field experiment established with emblings regenerated from different types of somatic embryos have recently been obtained: The growth of the emblings raised from the slim-type embryos during the first growing season is better than that of the emblings raised from the stub-type embryos, further confirming the significance of original embryo quality for the true

performance of the emblings (Figure 1.)

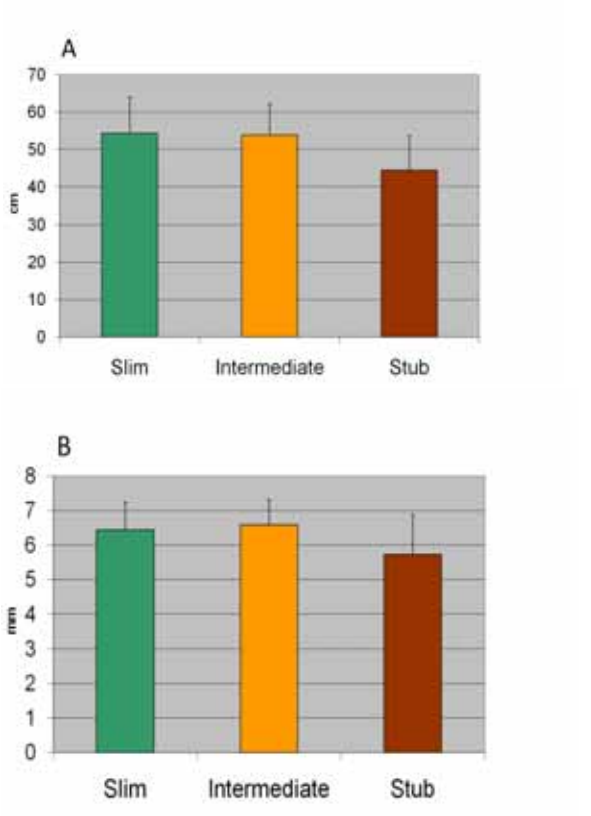

Figure 1. *Field performance of Scots pine emblings that had originated from different types of somatic embryos (slim, intermediate, or stub). A) Plant height shown with the SD after two growing seasons. B) Plant stem base diameter with the SD after one growing season, n= 240.*

Mature explants

Attempts to initiate SE from shoot apex explants from 10- and 15-year-old Scots pines showed repeatedly that the explants are responsive in the beginning of the growing season, and that embryogenic-looking cultures can be raised. Distinguishing and separating potential SE tissue is difficult, and repeated testing by microscopy of acetocarmine stained samples is required. Also endophytic explant contamination poses a serious problem for culture initiation and further development. For the lines of mature tree origin, somatic embryo maturation has proved to be a challenge, and so far none of the embryos produced have germinated and converted into plants.

Long-term cryopreservation

When testing the effects of long-term cryo-storage (Latutrie & Aronen, 2011), 80% or more of the Scots pine SE lines cryo-preserved with PDG mixtures for two to ten years remained viable and started proliferating following thawing. The recovery was significantly lower for the lines cryo-preserved with PGD for 12 years (44%). The lines cryopreserved using DMSO for 14 years did not recover. Cryo-storage time or donor tree did not impact significantly the growth ratio of the recovered SE lines, the increase of tissue fresh weight in a 6-week proliferation test varying from 19x to 24x. The length of cryopreservation significantly affected production of cotyledonary embryos during maturation , the number of embryos being lower in the lines cryo-preserved for 8-12 years (varying from 60± 21 to 68 ±29/gFW) than in the ones cryo-stored for two years (292±72/gFW). The PDG mixture used and donor tree had no effect on embryo production capacity.

Based on the results, the length of cryo-storage has an effect on Scots pine SE cultures. In theory, cryopreservation should store the biological samples unchanged over time due to standstill of all metabolic activity of the cells (Kartha 1985). However, the observed differences in the present material can be partly due to clonal differences in Scots pine SE (Niskanen *et al*. 2004), since the lines cryopreserved for varying times represent different genotypes. In addition, it is probable that the original quality of the oldest cryopreserved SE lines was not as good as that of the latest lot, due to recent developments (Lelu-Walter *et al.* 2008, Aronen *et al*. 2009) in SE technology in Scots pine.

References

Aronen, T., Pehkonen, T., Ryynänen, L. 2009. Enhancement of somatic embryogenesis from immature zygotic embryos of *Pinus sylvestris*. Scan. J. For. Research. 24:372-383.

Häggman, H., Jokela, A., Krajnakowa, J., Kauppi, A., Niemi, K., Aronen, T. 1999. Somatic embryogenesis of Scots pine: cold treatment and characteristics of explants affecting induction. J. Exp. Bot. 50:1769–1778.

Häggman, H., Ryynänen, L., Aronen, T.,Krajnakowa, J. 1998. Cryopreservation of embryogenic cultures of Scots pine. Plant Cell Tissue Org. Cult. 54:45–53.

Kartha, K. 1985.Meristem culture and germplasm preservation. In: Kartha, K.K. (ed.) Cryopreservation of Plant Cells and Organs, pp. 11-134. CRC Press Inc. , Boca Raton, Florida.ISBN 0-8493-6102-8.

Klimaszewska, K., Trontin, J.-F., Becwar, M., Devillard, C., Park, Y.S., Lelu-Walter, M.-A. 2007. Recent progress on somatic embryogenesis in four *Pinus* spp. Tree and Forest Science and Biotechnology. 1: 11-25

Latutrie, M., Aronen, T. 2011. Long-term cryopreservation of embryogenic Scots pine cultures. Manuscript.

Lelu-Walter, M.-A., Bernier-Cardou, M., Klimaszewska, K. 2008. Clonal plant production from self- and cross-pollinated seed families of *Pinus sylvestris* (L.) through somatic embryogenesis. Plant Cell Tissue Org. Cult. 92: 31-45.

Niskanen, A.-M., Lu, J., Seitz, S., Keinonen, K., von Weissenberg, K., Pappinen, A. 2004. Effect of parent genotype on somatic embryogenesis in Scots pine (*Pinus sylvestris*). Tree Physiology 24: 1259–1265.

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Recent Observations Regarding *Pinus radiata* Somatic Embryogenesis using Juvenile and Mature Material

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Abstract

Nowadays, *in vitro* clonal propagation of conifers is only possible for some species. In others, success is obtained at rates too low for commercial use. *In vitro* propagation is often achieved from juvenile material instead of adult material. As we all know, the economic value of a tree can only be assessed after it reaches maturity but, unfortunately, the maturation proccess induces changes in meristem behaviour that reduce the propagation potential of the tree (Von Aderkas and Bonga 2000). Therefore, the challenge is the development of protocols that use tissues of selected and tested adult trees of conifer species that have sofar been recalcitrant. Therefore, during the last few years our research team has focused on the study of *in vitro* organogenesis in adult pines (De Diego *et al*. 2008; Cortizo *et al*. 2009; De Diego *et al*. 2010) and specifically in radiata pine somatic embyogenesis using juvenile (Montalbán *et al*. 2010) and adult plant tissues. Radiata pine somatic embryogenesis was first described by Smith *et al*. (1994). Later on, several studies have been performed in order to understand different aspects of this process (Aquea *et al*. 2008; Hargreaves *et al*. 2009). The main objective of our research with immature zygotic embryos was to improve the maturation and germination steps of the process. Following this task was to obtain somatic embryogenesis from tissues of adult trees. Therefore, experiments with different genotypes, primary explants, culture media, and growth regulators have been carried out over the last two years. Recently, we have used *in vitro* organogenesis to reinvigorate tissues from adult trees and then, we have used the *in vitro* buds obtained to promote somatic embryogenesis in the selected material.

Key words: conifers, mature, organogenesis, radiata pine, rejuvenation, re-invigoration.

Introduction

Clonal propagation of high-value forest trees through somatic embryogenesis has the potential to rapidly capture the benefits of breeding or genetic improvement programs and to increase the uniformity and quality of the nursery stock (Find *et al*. 1993).

Somatic embryogenesis (SE) in *Pinus* genus is usually initiated from immature seeds; either from immature embryos left in the excised megagametophytes (Yildirim *et al*. 2006) or from excised zygotic embryos (Hargreaves *et al*. 2009). However, the competence window for the initiation of the embryogenic mass (EM) is narrow, lasting around 4 weeks (MacKay *et al*. 2006).

For many economically important pine species, the initiation frequency of SE is insufficient for commercial application. The improvement in SE initiation is important for developing varietal lines as well as managing genetic diversity (Park *et al*. 2006).

EM initiation is influenced by the genotype of the plant, the developmental stage of the initial explant and the induction media (Radojevic *et al*. 1999). Some studies have focused on improving initiation and proliferation through testing of different media (Zhang *et al*. 2007), plant growth regulators (Klimaszewska *et al.* 2001; Choudhury *et al*. 2008) sugars (Salajová *et al*. 2005) and/or gelling agent concentrations (Li *et al*. 1998).

If elite mature trees could be cloned 'true-to-type' on a large scale by SE, superior characteristics could be captured immediately without having to depend on the clonal testing that is required when zygotic embryos are used as explants (Bonga 2004). At present, there are no reports describing SE from adult radiata pine. Recently, EM has been obtained in *P. contorta* (Park *et al.* 2010) and *P. sylvestris* (Aronen 2010) but its conversion into plants still presents a challenge.

In conifers, the reduction in morphogenic potential due to the phase change can be reverted *in vitro* with reinvigoration procedures such as culture in presence of cytokinins (Cortizo *et al*. 2009; Zhang *et al*. 2010). The term "reinvigoration" can be defined as a temporary increase in vigour that enables the recovery of some organogenic abilities (Valdés *et al*. 2003). This transient effect has been demonstrated through the analysis of the DNA methylation rate in *Acacia mangium* in relation to their time in *in vitro* culture (Monteuuis *et al.* 2009).

Our research team has developed protocols for organogenesis from tissues of adult *Pinus radiata* (unpublished results), *P. pinaster* (De Diego *et al*. 2008), and *P. sylvestris* (De Diego *et al*. 2010) trees. However, conifer SE has generally been far more productive than propagation by organogenesis. Furthermore, SE has the advantage that both a root and a shoot develop simultaneously, which simplifies the process of plant regeneration (Conde *et al*. 2004). Therefore, trying to obtain propagation of mature conifer trees by SE is worthwhile (Bonga 2004).

When trying to micropropagate adult trees there are some zones within trees that are more morphogenetically competent than other parts of the tree (Bonga *et al*. 2010) such as microcuttings in *Cedrus* (Renau-Morata *et al*. 2005), vegetative buds in *Pinus* (De Diego *et al*.

2010) or basal sprouts in *Sequoia* (Boulay *et al*. 1987). SE has also been achieved from shoot buds of 10-year-old *Picea glauca* somatic trees (Klimaszewska *et al*. 2010). In some studies *in vitro* shoot cultures have been used as a source of explants (Conde *et al*. 2004; San-José *et al*. 2010). These cultures enable better control of the growing conditions of stock material, avoid difficulties associated with possible differences in the physiological state of the explants and guarantee a supply of an unlimited number of explants all year around.

The purpose of our research was to study factors affecting initiation of EM by varying the initial explant, basal media, plant growth regulators and aminoacids in cultures of juvenile and adult material.

Materials and Methods

Juvenile material

In 2008, cones were collected from three open pollinated trees from a seed orchard established by Neiker-Tecnalia in Deba-Spain (latitude: 43º16'59''N, longitude: 2º17'59''W, elevation: 50 m). Three open-pollinated families were processed weekly for four weeks, from mid June to mid July; a total of 768 megagametophytes were tested. Two initiation media were tested: the EDM medium (Walter *et al*. 1998) and CGM medium. CGM medium only differed from EDM medium in the amino acid composition. Its amino acid composition consisted of 1 g L^{-1} casein hydrolysate added to the medium prior to sterilization and 500 mg L^{-1} L-glutamine added to the medium after autoclaving (CG amino acid mixture). EDM medium was supplemented with 1 g L^{-1} inositol, 30 g L^{-1} sucrose, and a combination of 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.7 µM benzylaminopurine (BA). Before autoclaving, the pH of the medium was adjusted to 5.7 and then 3 g L^{-1} Gelrite® were added. Medium was autoclaved at 121ºC for 20 min. After autoclaving, filter-sterilized solutions (pH=5.7) of 550 mg L^{-1} L-glutamine, 525 mg L^{-1} asparagine, 175 mg L^{-1} arginine, 19.75 mg L^{-1} L-citrulline, 19 mg L^{-1} L-ornithine, 13.75 mg L^{-1} L-lysine, 10 mg L^{-1} L-alanine and 8.75 mg L^{-1} L-proline (ED amino acid mixture) were added to the cooled medium .After 4–8 weeks, proliferating EM, with a size around 3-5 mm in diameter was subcultured to proliferation media every 2 weeks. These media had the same composition as the initiation media, but contained a higher concentration of Gelrite®, 5.5 g L^{-1}.

Actively growing EM samples from the two maintenance media were stained with acetocarmine (2% w/v) and Evan´s blue (0.5% w/v) (Gupta and Durzan 1987). Samples were observed with an inverted microscope (LEICA DM4500, Leica Microsystems GmbH using a 40-fold magnification, in order to analyze the cellular organization we especially focused on the proembryo and early embryo development stages in the embryonal masses.

For maturation of the EM, the methodology described by Montalbán *et al*. (2001) was followed. After two months on proliferation media, the number of proliferating cell lines per mother tree was recorded, and one-way analysis of variance (ANOVA) was carried out to determine differences among initiation and proliferation media.

Adult material

Experiment 1

In 2008, seven trees were selected from a seed orchard established by Neiker-Tecnalia in Deba-Spain (latitude: 43º16'59''N, longitude: 2º17'59''W, elevation: 50 m). These trees were 19 years old at the moment of collection. Apical shoot buds (3–5 cm long) were taken from the mid-basal part of the trees. The buds were collected fortnightly from the 18th of February (Figure 2a) to the 29th of April (Figure 2b), wrapped in moist paper to prevent dehydration and stored in polyethylene bags at 4ºC for a maximum of a week. Buds were sprayed with 70% (v/v) ethanol, and then rinsed with sterile distilled H_2O. Afterwards, the buds were submerged in 50% (v/v) commercial bleach (active chloride >5%) plus 2 drops of Tween20® and agitated for 10 min. Finally, they were rinsed three times in sterile distilled H_2O in aseptic conditions. When possible, bud scales were removed, the buds were cut transversely into 1–1.5 mm thick slices with a surgical scalpel blade and were then laid on the culture medium (Figure 2c). At the first and the second collection dates (18th of February and the 3th of March) explants were cultured on two initiation media. The first medium was EDM (Walter *et al.* 1998) and the second one was DCR-I (DCR basal medium, Gupta and Durzan 1985) containing 0.2 g L^{-1} polyvinylpyrolidone-40 (PVP-40), 32.4 g L^{-1} maltose, 1 g L^{-1} inositol and supplemented with 20 µM 2,4-D, 25 µM 1-naphthaleneacetic acid (NAA) and 9 µM BA. Before autoclaving, the pH of the medium was adjusted to 5.7 and then 1.5 g L^{-1} Phytagel was added. After autoclaving, filter-sterilized solutions (pH= 5.7) of 1 g L^{-1} casein hydrolysate and 1 g L^{-1} L-glutamine were added to the cooled medium prior to dispensing into gamma-irradiated Petri dishes (90 x 20 mm).

From the third to the sixth collection date (from the 17th of March to the 29th of April) half of the collected buds were cultured on DCR-I, and the rest were given a cold pre-treatment. This pre-treatment consisted of culturing the buds at 4ºC on full-strength DCR basal medium containing PVP-40 (0.2 g L^{-1}), maltose (32.4 g L^{-1}), 0.3% (w/v) activated charcoal (AC) and Phytagel (1.5 g L^{-1}) (DCR-P); after 3 days, these buds were subcultured on DCR-I.

At each collection date, five bud slices per Petri dish were cultured; seven Petri dishes per treatment were laid out randomly on the shelves of the growth chamber. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks. Then the bud slices and/or the proliferating tissues were transferred to maintenance medium. Maintenance medium for explants cultured on EDM was the same used for initiation; and maintenance medium for explants cultured in DCR had the same basal composition but contained 43.2 g L^{-1} maltose, 1 g L^{-1} inositol and was supplemented with 2 µM 2,4-D, 2.5 µM NAA and 1 µM BA. The amino acid mixture was the same as for

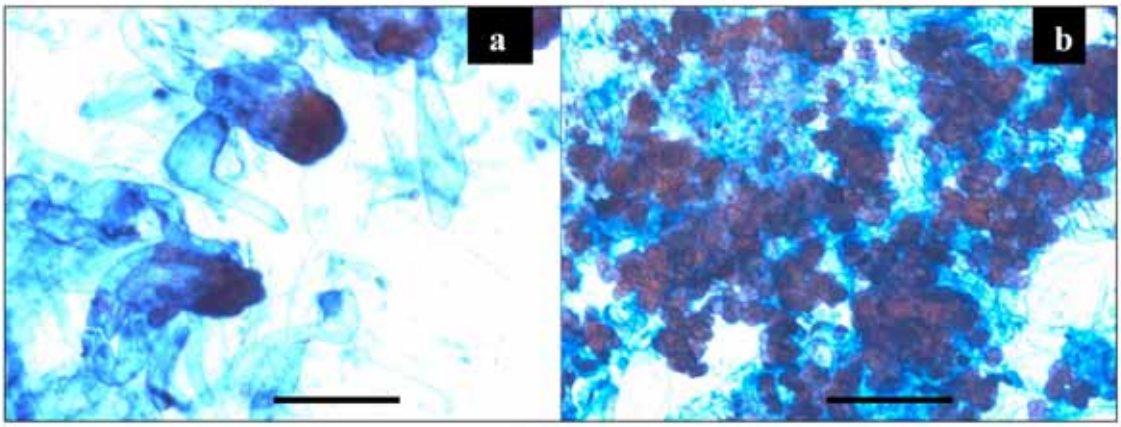

Figure 1 *Microscopic details of Pinus radiata ET stained with 2% acetocarmine and 0.5% Evan´s blue: (a) EM proliferating on EDM medium where proembryos can be observed, bar=100µm; (b) EM proliferating in CGM medium with an unorganized structure, bar =200µm.*

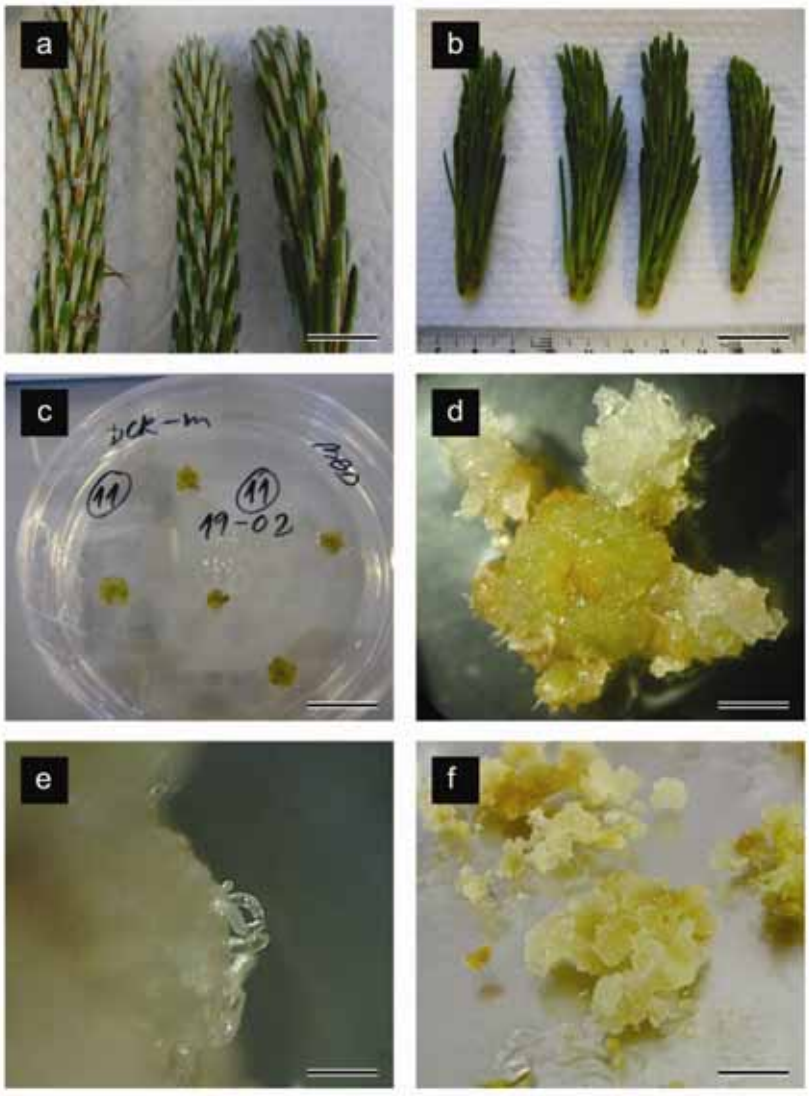

Figure 2 *Experiment 1: (a) shoot buds from the field collected at the end of February, bar=12 mm; (b) shoot buds from the field collected at the start of April, bar=19 mm; (c-f) mid February collection; (c) bud slices cultured on DCRI medium, bar=14 mm; (d) bud slice cultured on DCRI for three weeks, bar=4 mm; (e) elongated cells in the proliferating tissue from the bud slices, bar=1 mm; (f) tissue proliferating on DCRM medium, bar=9 mm.*

initiation. Cultures were maintained in the dark at 21º±1ºC for 4 to 8 weeks on maintenance medium.

Experiment 2

Ten trees over 20 years old were selected from a seed orchard established by Neiker-Tecnalia in Amurrio-Spain (latitude: 43º03'00''N, longitude: 3º01'00''W, elevation: 50 m). Apical shoot buds (3–5 cm long) were taken from the mid-basal part of the trees (Figure 3a). The buds were collected fortnightly from the December of 2009 to January of 2010. The buds were stored and disinfected as described in experiment 1. Buds were cut transversely into 1-1.5 mm and 6-7 mm thick slices. The slices of two different thicknesses were cultured on EDM and LP-I (LP basal medium, Quoirin and Lepoivre 1977, modified by Aitken-Christie *et al*. 1988). This LP-I medium was supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} inositol, 20 µM 2,4-D, 25 µM NAA and 9 µM BA; before autoclaving the pH of the medium was adjusted to 5.8 and 3 g L^{-1} Gelrite® were added. After autoclaving the ED amino acid mixture was added. Cultures were maintained in the dark at 21±1ºC for 4 to 8 weeks.

Another set of buds cut into 6-7 mm slices was cultured on modified LP basal medium to induce organogenesis (LP-O) following the methodology described by De Diego *et al*. (2008). LP-O medium was supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} inositol and 22 µM BA; before autoclaving the pH of the medium was adjusted to 5.8 and 8 g L^{-1} Difco Agar® were added. Cultures were maintained at 21±1°C under a 16 h photoperiod of 120µmol m^{-2} s^{-1} provided by cool white fluorescent tubes (TLD 58 W/33; Philips, France) for 8 weeks.

The bud slices cultured on EDM were transferred to the same medium. The slices cultured on LP-I were subcultured either on LP-I or on EDM. The buds cultured on LP-O were subcultured to LP-I.

Experiment 3

In 2010, *in* vitro adventitious buds were obtained from vegetative buds of ten trees over 20 years old from the same seed orchard mentioned in experiment 2 (Figure 4a). These explants were obtained by culturing the buds from the field on LP-O for 4 weeks to induce organogenesis (De Diego *et al*. 2008), and then by transferring the explants to LP basal media lacking growth regulators and supplemented with 0.2% (w/v) AC (LP-E). The explants were transferred from one medium to another every month. After one year, *in vitro* buds were cut into halves, quarters or slices (Figure 4b) and cultured on EDM or on EDM supplemented with 20 µM 2,4-D, 25 µM NAA and 9 µM BA (EDM-2). After 4 to 8 weeks, when proliferation of the tissue was observed, the explants were transferred to maintenance medium. The maintenance media were those used for initiation. Cultures were maintained in the dark at 21º±1ºC.

Small pieces of proliferating tissue were stained with 2% (w/v) acetocarmine directly on glass slides for 4 min (Gupta and Durzan 1987). Samples were observed with an inverted microscope using a 40-fold magnification.

Results

Juvenile material

The overall initiation rates for the two initiation media assayed did not differ statistically (41.6% for CGM and 37.4% for EDM). The proliferation rates were significantly higher on CGM medium (28.3%) than on EDM medium (20.3%). Squashes of ETs established on EDM medium showed well organized proembryos (Figure 1a), whereas the EMs that had proliferated in CGM medium showed a more unorganized structure and these lines stopped proliferating after 4 months on CGM proliferation medium (Figure 1b). The EMs proliferating on EDM were subjected to maturation treatments. The conversion of the EMs into somatic embryos was optimal on a maturation medium with the ED amino acid mixture, 60 g L^{-1} sucrose and 9 g L^{-1} Gelrite® and 83% of the tested lines produced over 200 somatic embryos per gram of fresh weight of EM (Montalbán *et al*. 2010).

Adult material

Experiment 1

In material from the first and second collection dates and in all the genotypes assayed, the bud slices cultured on EDM developed tissue in the peripheral part of the buds (Figure 2d). The buds cultured on DCR-I showed the same growth of tissue but it grew less and slower than on buds cultured on EDM. These cell proliferations were formed by white-traslucent masses formed by elongated cells (embryogenic-like cells, Figure 2e) and a lower fraction of rounded cells (non-embryogenic like cells). When transferred to maintenance medium, the masses continue proliferating at high rates but the percentage of rounded cells increased and the tissue showed a yellow-brownish colour (Figure 2f). For all genotypes from the third to the sixth collection dates, the growing masses on bud slices cultured on DCR-I showed the same trend mentioned above for the initiation and maintenance. The cold pre-treatment did not have a beneficial effect on the bud slices, half of these slices did not develop any tissue and necrosed. The other buds when transferred to DCR-I formed tissue in the axillary zone of the buds in the needle insertion zone. Although when they proliferated on DCR-M they tended to have a yellowish colour and after one month rounded cells started to appear (Figure 2f). When they started to proliferate these masses were white-traslucent and embryogenic-like cells could be seen (Figure 2e).

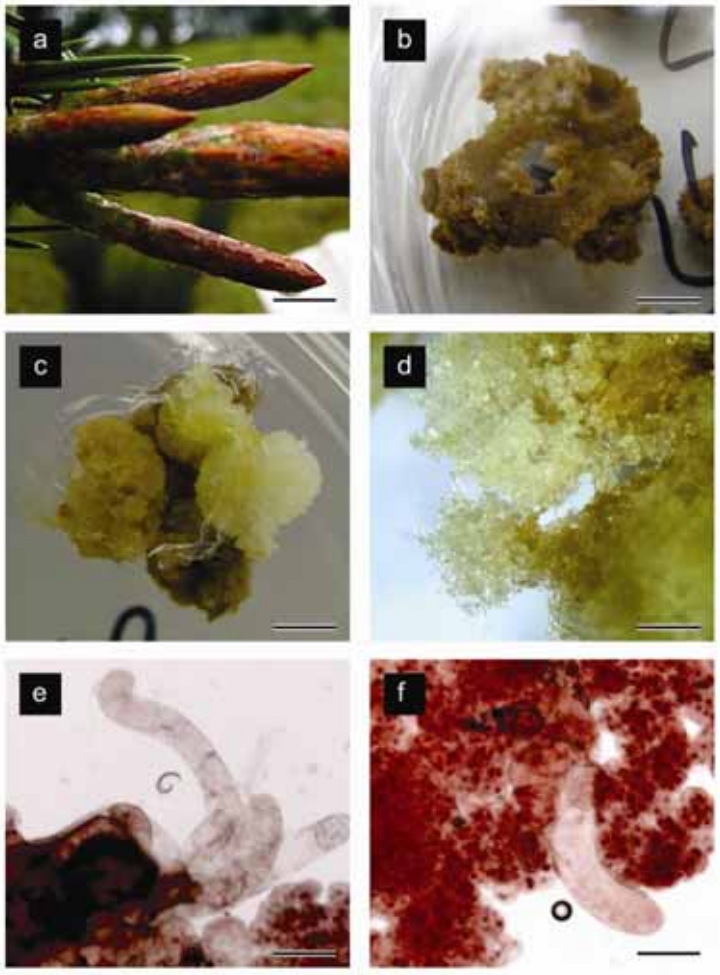

Figure 3 *Experiment 2: (a) shoot buds from the field collected at the end of December, bar=17 mm; (b) 1.5 cm thick bud slice cultured on EDM, bar=4 mm; (c) tissue growing on 7 mm bud slices cultured on LPI medium, bar=4 mm; (d) elongated and rounded cells in the proliferating tissue, bar=3 mm; (e) micro-morphology of elongated cells in the proliferating tissue, bar=0.15 mm; (f) micro-morphology of elongated and rounded cells in the proliferating tissue, bar=0.2 mm*

Experiment 2

The bud slices cut transversely into 1-1.5 mm thick slices necrosed rapidly and did not produce any tissue (Figure 3b). When the initial explants were bud slices cut into 6-7 mm thick slices and cultured on EDM, a developing white-traslucent tissue was observed with embryogenic-like cells (Figure 3e); this tissue was subcultured to the same medium and became yellowish with a higher proportion of rounded cells. On explants cultured and subcultured on LP-I the tissue grew more slowly than on those subcultured on EDM or cultured from the beginning on EDM. However, after 4 to 8 weeks the masses growing on LP-I showed the same macro and micro-morphological features as the ones growing on EDM (Figure 3f). When cultured on LP-O most buds showed a development in the brachyblast meristems, when transferred to LP-I 50% of them necrosed; the others developed globular structures (Figure 3c) that finally burst showing the appearance of the tissue type previously observed on the other assayed culture media (Figure 3d).

Experiment 3

Tissue proliferation was observed in the three types of explants tested (halves, quarters and slices), and this growth was higher in buds cut into quarters (Figure 4c). In the explants cultured on EDM the tissue grew at a higher rate than in the explants cultured on EDM-2 (Figure 4d). As observed in previous experiments, when transferred to maintenance medium the tissue became yellowish (Figure 4f) and the cells showed a rounded morphology in a higher proportion. However, initiatlly, the proliferating tissue was white-translucent and embryogenic-like cells were observed, showing promising

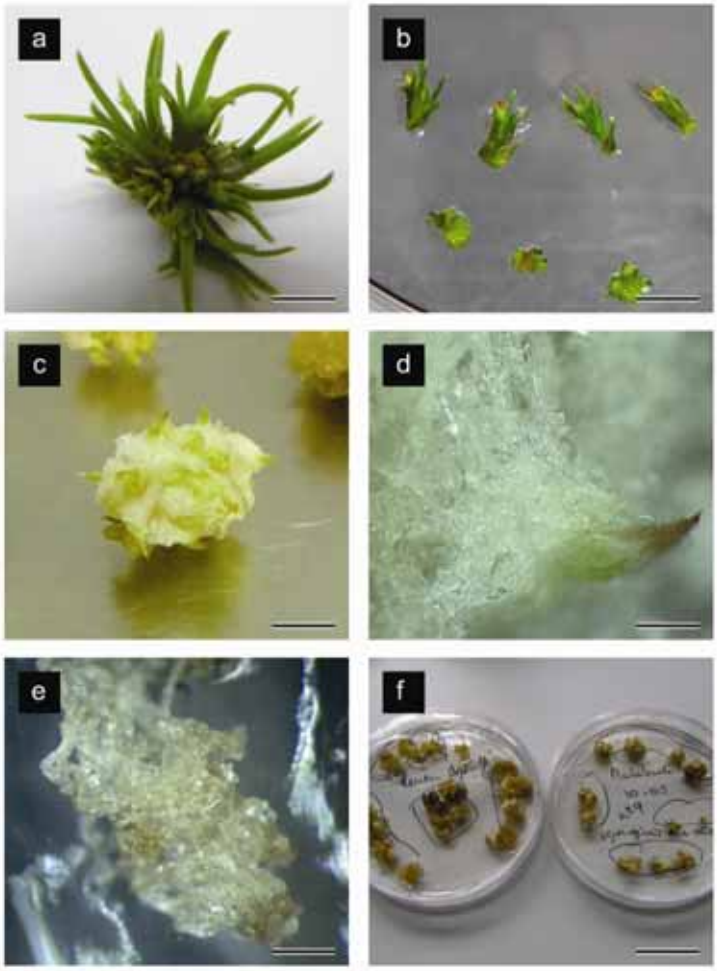

Figure 4 *Experiment 3: (a) in vitro adventitious buds, bar=7 mm; (b) in vitro buds cut into quarters, halves and sections, bar=5 mm; (c) in vitro bud after culture on EDM for 4 weeks, bar=6 mm; (d) elongated cells in proliferating tissue from in vitro buds cultured on EDM for 4 weeks, bar=0.8 mm; (e) elongated cells arranged into embryo-like structures from in vitro buds cultured on EDM for 6 weeks, bar=0.7 mm; (f) tissue growing on EDM on the left, and tissue growing on EDM2 on the right, bar=30 mm.*

macro-morphological features (Figures 4d and 4e).

Discussion

Amino acids have been widely studied in several plants for their importance in SE (Pérez-Rodríguez *et al*. 2006; Robinson *et al*. 2009). We have tested two media in the initiation of EM from megagametophytes with the same basal composition differing only in the amino acid mixture. EDM medium was used for *Pinus radiata* SE (Walter *et al*. 1998) and also for other *Pinus* species such as *P. strobus* (Klimaszewska *et al*. 1997). The amino acid mixture in CGM medium has been employed for several *Pinus* species (Klimaszewska *et al*. 2001; Lelu-Walter *et al*. 2006; Carneros *et al*. 2009). Hargreaves *et al*. (2009) reported better initiation results on Glitz medium which contained the CG amino acid mixture. We obtained higher proliferation rates on CGM medium but these were coupled with negative effects on the organization of the EM at microscopic level. This lack of organization in the tissue has been associated with EM aging and worse maturation yields in *P. pinaster* (Breton *et al*. 2005; Breton *et al*. 2006). The ED amino acid mixture also proved to be more efficient in the maturation process. Some amino acids present in the ED mixture could have played a key role, not only in the number but also in the quality of the somatic embryos obtained (Montalbán *et al*. 2010). Amino acids such as proline in peanut (Murch *et al*. 1999), alanine in carrot (Higashi *et al*. 1996) or arginine in oil palm (Morcillo *et al*. 1999) favoured the quantity and/or the quality of somatic embryos. As we reported previously (Montalbán *et al*. 2010) the ED amino acid mixture also increased significantly the osmolality of the maturation medium when compared with the CG amino acid mixture. This in turn may have had an effect on the maturation

response. Thus, although the results in the initiation experiments were encouraging with regard to the CG amino acid mixture, taking into account the proliferation and maturation results, the ED amino acid mixture was the more efficient for *Pinus radiata* SE from juvenile material. To date, SE in *Pinus radiata* has only been achieved from juvenile material. Mild stresses, such as a cold pretreatment, have been reported to induce changes in the physiological status of plant cells triggering the SE process (Bonga *et al*. 2010). Cold pretreatment has been used to induce SE in different species (Janeiro *et al.* 1995; Luo *et al.* 2003). In our first experiment, buds slices were subjected to a cold pretreatment for 4 days. In contrast with the results obtained in *Larix* (Bonga 1996; 1997), in radiata pine the cold treatment induced embryogenic-like tissue formation but did not have a significant effect on the initiation frequency as observed in *P. sylvestris* (Häggman *et al.* 1999) or *Abies cephalonica* SE (Krajňaková *et al.* 2008). In experiment 1, apart from a cold pretreatment, induction media with different basal compositions were assayed (DCR and EDM) for SE initiation.These media also diferred in the amino acid mixture. Although the tissue had the same appearance growing on both media, when the media was DCR supplemented with glutamine and casein it grew more slowly (Figure 2d). Different performances of EM, depending on the amino acid formulation of the induction medium, have been reported for sorghum (Elkonin *et al.* 1995) and in our initiation experiments with immature seeds from radiata pine. Our results showed that cutting the buds into thin slices (1-1.5 mm) had a negative effect (Figure 2b). This could be due to the fact that *P. radiata* buds were big, and slices thicker than 3 mm were needed to preserve a whole whorl of brachyblast primordia. Thinner slices cut these brachyblast primordia and the explants died shortly after.

Time of harvest and shoot selection are particularly important factors because the various cells of an organ or tissue differ in their ability to respond *in vitro* at different developmental stages (Becwar *et al.* 1990). In experiment 1, we collected the buds from February to April and we observed a similar *in vitro* response over this period for all the tested genotypes. Our results did not show a correlation between the physiological status of the explant and its embryogenic potential. In our experiments with buds from the field as initial explants, the tissue always proliferated from the peripheral part of the bud slices (in the insertion zone of the needles). When we used adventitious buds obtained *in vitro* as initial explants, the tissue arose from the basal part of the shoot primordia. This tissue had again the same external characteristics as those observed in previous experiments (white-translucent tissue formed by embryogenic-like cells). On the contrary, in experiments with adult tissues from *Larix* (Bonga *et al.* 1997; Bonga 2004) the tissue was formed by green nodules which developed into structures that resembled mature embryos. Because *in vitro* adventitious buds are available year around they do not depend on the seasonal restrictions of buds from the field, which is contrary to the observations made by De Diego *et al.* (2008) for *P. pinaster* organogenesis. The buds obtained *in vitro*, when subjected to SE induction treatments, developed tissue at a higher rate than buds from the field (Figure 4c). Moreover, the exogenously applied cytokinins during organogenesis may provoke the acquisition of juvenile characteristics (De Diego *et al.* 2010) such as polyamine levels (Fraga *et al*. 2003). This reinvigoration could make it easier to obtain SE from the newly formed adventitious buds. This approach, using juvenile *in vitro* explants from adult trees developed *in vitro* has been successfully used for *Quercus robur* (San-José *et al.* 2010) and *Ulmus minor* (Conde *et al.* 2004). In our experiments the tissue that proliferated was soft and showed a consistency similar to that of the embryogenic tissue initiated from immature seeds. We cannot establish yet if the tissue initiated was embryogenic or not, especially in the case of the tissue obtained from *in vitro* adventitious buds (experiment 3), because the micro and macro-morphological observations were not conclusive. Aronen (2010) noted the importance of identifying the embryogenic tissue in adult *P. sylvestris* cultures. Once this tissue has been identified as embryogenic it has to be separated carefully and its evolution has to be followed. In order to discern whether the tissue produced at early stages of SE induction is embryogenic or not, some authors have proposed the need for analysing the protein pattern of the tissues (Von Arnold *et al.* 2002, Šamaj *et al.* 2008), the endogenous contents of different growth regulators or the expression of genes known to be involved in SE initiation (Fehér *et al.* 2003; Fehér 2008). Bonga *et al.* (2010) have reviewed different gene families involved in SE induction such as oxidative stress-related genes (Sharma *et al.* 2008; Stasolla *et al.* 2004), molecular markers that identify cells that are competent to initiate SE (SERK gene family) (Rose and Nolan 2006), genes expressed only in embryogenic lines (WUS related WOX genes) (Palovaara and Hakman 2008; Klimaszewska *et al*. 2010), or a transcription factor the ectopic expression of which induces cell proliferation leading to SE in *Arabidopsis* (*Brassica napus* BABY BOOM) (Passarinho *et al.* 2008).

If the tissue initiated is embryogenic, the next step would be to optimize the maintenance medium and culture conditions. In our experiments, the initiated tissue changed its morphology, colour and behaviour when transferred from induction to maintenance medium, but we could not confirm if it changed its potential for SE.

Our next challenge is to choose one of the abovementioned SE indicators and follow its evolution through initiation and maintenance. Moreover, we will focus on the correct isolation of EMs, and once we have established EMs from adult radiata pines we will study their conversion into plantlets.

References

Aitken-Christie, J., Singh, A.P., Davies, H. 1988. Multiplication of meristematic tissue: a new tissue culture system for radiata pine. *In* Hanover, J.W., Keathley, D.E. (eds) Genetic manipulation of woody plants. Plenum Publishing Corp, New York, pp.

413-432.

Aquea, F., Poupin, M.J., Matus, J.T., Gebauer, M., Medina, C., Arce-Johnson, P. 2008. Synthetic seed production from somatic embryos of *Pinus radiata*. Biotechnol. Lett. 30:1847-1852.

Aronen, T. 2010. Somatic embryogenesis in Scots pine (*Pinus sylvestris* L.)- Embryo quality, long-term cryostorage, and mature explants as challenges. Abstract 2010 IUFRO Somatic Embryogenesis and Forest Trees Conference, Suwon, Korea, p. 26.

Becwar, M.R., Nagmani, R., Wann, S.R. 1990. Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Can. J. For. Res. 20:810-817.

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

Bonga, J.M. 1997. The effect of collection date and frozen storage on the formation of embryo-like structures and elongating shorts from explants from mature *Larix decidua* and *L.* x *eurolepis.* Plant Cell Tissue Organ Cult. 51: 195-200.

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

Bonga, J.M., Klimaszewska, K., Von Aderkas, P. 2010. Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tissue Organ Cult.100: 241-254.

Boulay, M. 1987. Conifer micropropagation: applied research and commercial aspects. *In* Bonga, J.M., Durzan, D.J. (eds) Cell and tissue culture in forestry, case histories: gymnosperms, angiosperms and palms, vol 3. Martinus Nijhoff Publishers, Dordrecht, pp. 185-206.

Breton, D., Harvengt. L., Trontin, J.-F., Bouvet, A., Favre, J.M. 2005. High subculture frequency, maltose-based and hormone-free medium sustained early development of somatic embryos in maritime pine. In Vitro Cell. Dev. Biol.-Plant 41:494-504.

Breton, D., Harvengt, L., Trontin, J.-F., Bouvet, A., Favre, J.M. 2006. Long-term subculture randomly affects morphology and subsequent maturation of early somatic embryos in maritime pine. Plant Cell Tissue Organ Cult. 87:95-108.

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

Choudhury, H., Kumaria, S., Tandon, P. 2008. Induction and maturation of somatic embryos from intact megagametophyte explants in Khasi pine (*Pinus kesiya* Royle ex. Gord.). Current Sci. 95:1433-1438.

Conde, P., Loureiro, J., Santos, C. 2004. Somatic embryogenesis and plant regeneration from leaves of *Ulmus minor* Mill. Plant Cell Rep. 22:632-639.

Cortizo, M., de Diego, N., Moncaleán, P., Ordás, R.J. 2009. Micropropagation of adult Stone pine (*Pinus pinea* L.). Trees Struct. Funct. 23:835-842.

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

De Diego, N., Montalbán, I.A., Moncaleán, P. 2010. *In vitro* regeneration of adult *Pinus sylvestris* trees. S Afr. J. Bot. 76:158-162.

Elkonin, L.A., Lopushanskaya, R.F., Pakhomova, N.V. 1995. Initiation and maintenance of friable, embryogenic callus of sorghum (*Shorgum bicolor* (L.) Moench) by amino acids. Maydica 40:153-157.

Fehér, A., Pasternak, T.P., Dudits, D. 2003. Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult. 74:201-228.

Fehér, A. 2008. The initiation phase of somatic embryogenesis: what we know and what we don't. Acta Biol. Szeged 52:53-56.

Find, J.I., Floto, F., Krostrup, P., Møller, J.D., Nørgaard, J.V., Kristensen, M.M.H. 1993. Cryopreservation of an embryogenic suspension culture of *Picea sitchensis* and subsequent plant regeneration. Scand. J. For. Res. 8:156-162.

Fraga, M.F., Rodríguez, R., Canal, M.J. 2003. Reinvigoration of *Pinus radiata* is associated with partial recovery of juvenile-like polyamine concentrations. Tree Physiol. 23:205-209.

Gupta, P.K., Durzan, D.J. 1985. Shoot multiplication from mature trees of Douglas fir and sugar pine. Plant Cell Rep. 4:177-179.

Gupta, P.K., Durzan, D.J. 1987. Biotechnology of somatic polyembryogenesis and plantlet regeneration in loblolly pine. Bio./Technol. 5:147-151.

Häggman, H., Jokela, A., Krajnakova, J., Kauppi, A., Niemi, K., Aronen, T. 1999. Somatic embryogenesis of Scots pine: cold treatment and characteristics of explants affecting induction. J. Exp. Bot. 50: 1769-1778.

Hargreaves, C.L., Reeves, C.B., Find, J.I., Gough, K., Josekutty, P., Skudder, D.B., Van der Maas, S.A., Sigley, M.R., Menzies, M.I., Low, C.B., Mullin, T.J. 2009. Improving initiation, genotype capture, and family representation in somatic embryogenesis of *Pinus radiata* by a combination of zygotic embryo maturity, media, and explant preparation. Can. J. For. Res. 39:1566-1574.

Higashi, K., Kamada, H., Harada, H. 1996. The effects of reduced nitrogenous compounds suggests that glutamine synthetase activity is involved in the development of somatic embryos in carrot. Plant Cell Tissue Organ Cult. 45:109-114.

Janeiro, L.V., Ballester, A., Vieitez, A.M. 1995. Effect of cold storage on somatic embryogenesis systems of *Camellia*. J. Hortic. Sci. 70:665-672.

Klimaszewska, K., Smith, D.R. 1997. Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellam gum. Physiol. Plant. 100:949-957.

Klimaszewska, K., Park, Y.S., Overton, C., Maceacheron, I., Bonga, J.M. 2001. Optimized somatic embryogenesis in *Pinus strobus* L. In Vitro Cell. Dev. Biol.-Plant. 37:392-399.

Klimaszewska, K., Overton, C., Stewart, D., Rutledge R.G. 2010. Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old white spruce and expression profiles of 11 genes followed during the tissue culture process. Planta DOI 10.1007/s00425-010-1325-.

Krajňaková, J., Gömöry, D., Häggman, H. 2008. Somatic embryogenesis in Greek fir. Can. J. For. Res. 38:760-769.

Lelu-Walter, M.A., Bernier-Cardou, M., Klimaszewska, K. 2006. Simplified and improved somatic embryogenesis for clonal propagation of *Pinus pinaster* (Ait.). Plant Cell Rep. 25:767-776.

Li, X.Y., Huang, F.H., Gbur, E.E. 1998. Effect of basal medium, growth regulators and Phytagel concentration on initiation of embryogenic cultures from immature zygotic embryos in loblolly pine (*Pinus taeda* L.). Plant Cell Rep. 17:298-301.

Luo, J.P., Jiang, S.T., Pan, L.J. 2003. Cold-enhanced somatic embryogenesis in cell suspension cultures of *Astragalus adsurgens* Pall.: relationship with exogenous calcium during cold pretreatment. Plant Growth Regul. 40:171-177.

MacKay, J.J., Beckwar, M.R., Park, Y.S., Cordero, J.P., Pullman, G.S. 2006. Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. Tree Genet. Genomes 2: 1-9.

Montalbán, I.A., De Diego, N., Moncaleán, P. 2010. Bottlenecks in *Pinus radiata* somatic embryogenesis: improving maturation and germination. Trees Struct. Funct. 24:1061-1071.

Monteuuis, O., Baurens, F.C., Goh, D.K.S., Quimado, M., Doulbeau, S., Verdeil, J.L. 2009. DNA Methylation in Acacia mangium in vitro and ex-vitro buds, in relation to their within-shoot position, age and leaf morphology of the shoots. Silvae Genet. 58:5-6.

Morcillo, F., Aberlenc-Bertosi, F., Noirot, M., Hamon, S., Duval, Y. 1999. Differential effects of glutamine and arginine on 7S globulin accumulation during the maturation of oil palm somatic embryos. Plant Cell Rep. 18:868-872.

Murch, S.J., Victor, J.M.R., Krishnaraj, S., Saxena, P.K. 1999. The role of proline in thidiazuron-induced somatic embryogenesis of peanut. In Vitro Cell. Dev. Biol.-Plant. 35:102-105.

Palovaara, J., Hakman, I. 2008. Conifer WOX-related homeodomain transcription factors, developmental consideration and expression dynamic of WOX2 during *Picea abies* somatic embryogenesis. Plant Mol. Biol. 66:533-549.

Park, S.Y., Klimaszewska, K., Park J.Y., Mansfield S.D. 2010. Lodgepole pine: the first evidence of seed-based somatic embryogenesis and the expression of embryogenesis marker genes in shoot bud cultures of adult trees. Tree Physiol. 30:1469-1478.

Park, Y.S., Lelu-Walter, M.A., Harvengt, L., Trontin, J.-F., MacEacheron, I., Klimaszewska, K., Bonga, J.M. 2006. Initiation of somatic embryogenesis in *Pinus banksiana*, *P. strobus*, *P. pinaster*, and *P. sylvestris* at three laboratories in Canada and France. Plant Cell Tissue Organ Cult. 86:87-101.

Passarinho, P., Ketelaar, T., Xing, M., Van Arkel, J., Maliepaard, C., Weemen Hendriks, M., Joosen, R., Lammers, M., Herdies, L., Den Boer, B., Van der Geest, L., Boutilier, K. 2008. BABY BOOM target genes provide diverse entry points into cell proliferation and cell growth pathways. Plant Mol. Biol. 68:225-237.

Pérez-Rodríguez, M.J., Suárez, M.F., Heredia, R., Ávila, C., Breton, D., Trontin, J.-F., Filonova, L., Bozhkov, P., Von Arnold, S., Harvengt, L., Cánovas, F.M. 2006. Expression patterns of two glutamine synthetase genes in zygotic and somatic pine embryos support specific roles in nitrogen metabolism during embryogenesis. New Phytol. 169: 35-44.

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

Radojevic, L., Álvarez, C., Fraga, M.F., Rodríguez, R. 1999. Somatic embryogenic tissue establishment from mature *Pinus nigra* Arn. ssp. Salzmannii embryos. In Vitro Cell. Dev. Biol.-Plant 35: 206-209.

Renau-Morata, B., Ollero, J., Arrillaga, I., Segura, J. 2005. Factors influencing axillary shoot proliferation and adventitious budding in cedar. Tree Physiol. 25: 477-486.

Robinson, A.R., Dauwe, R., Ukrainetz, N.K., Cullis, I.F., White, R., Mansfield, S.D. 2009 Predicting the regenerative capacity of conifer somatic embryogenic cultures by metabolomics. Plant Biotechnol. J. 7: 952-963.

Rose, R.J., Nolan, K.E. 2006. Genetic regulation of somatic embryogenesis with particular reference to *Arabidopsis thaliana* and *Medicago truncatula*. In Vitro Cell Dev. Biol.-Plant 42:473-481.

Salajová, T., Salaj, J. 2005. Somatic embryogenesis in *Pinus nigra*: embryogenic tissue initiation, maturation and regeneration ability of established cell lines. Biol. Plant. 49:333-339.

Šamaj, J., Salaj, T., Matúšová, R., Salaj, J., Takáč, T., Šamajová, O., Volkmann, D. 2008. Arabinogalactan-protein epitope Gal4 is differentially regulated and localized in cell lines of hybrid fir (*Abies alba* x *Abies cephalonica*) with different embryogenic and regeneration potential. Plant Cell Rep. 27:221-229.

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

Sharma, S.K., Millam, S., Hedley, P.E., McNicol, J., Bryan, G.J. 2008. Molecular regulation of somatic embryogenesis in potato: an auxin lead perspective. Plant Mol. Biol. 68:185-201.

Smith, D.R., Walter, C., Warr, A.A., Hargreaves, C.L., Grace, L.J. 1994. Somatic embryogenesis joins the plantation forestry revolution in New Zealand. Biological Sciences Symposium, TAPPI Proceedings,

Minneapolis, pp. 19-29.

Stasolla, C., Bozhkov, P.V., Chu, T.M., Van Zyl, L., Egertsdotter, U., Suarez, M.F., Craig, D., Wolfinger, R.D., Von Arnold, S., Sederoff, R.D. 2004. Variation in transcript abundance during somatic embryogenesis in gymnosperms. Tree Physiol. 24: 1073-1085.

Valdés, A.E., Centeno, M.L., Fernández, B. 2003. Changes in the branching pattern of *Pinus radiata* derived from grafting are supported by variations in the hormonal content. Plant Sci. 165:1397-1401.

Von Aderkas, P., Bonga, J.M. 2000. Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment.Tree Physiol. 20: 921-928.

Von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., Filonova, L. 2002. Developmental pathways of somatic embryogenesis. Plant Cell Tissue Organ Cult. 69:233-249.

Walter, C., Find, J.I., Grace, L.J. 1998. Somatic embryogenesis and genetic transformation in *Pinus radiata. In* Jain. S.M., Gupta, P.K., Newton, R.J. (eds) Somatic embryogenesis in woody plants, vol 4. Kluwer Academic Pub, The Netherlands, pp. 491-504.

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

Zhang, C.X., Li, Q., Kong, L. 2007. Induction, development and maturation of somatic embryos in Bunge´s pine (*Pinus bungeana* Zucc. ex Endl.). Plant Cell Tissue Organ Cult. 91: 273-280.

Zhang, H., Horgan, K.J., Reynolds, P.H.S., Jameson, P.E. 2010. 6-Benzyladenine metabolism during reinvigoration of mature *Pinus radiata* buds *in vitro*. Tree Physiol. 30:514-526.

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Somatic Embryogenesis and Phase Change in Trees

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Abstract

The advantages of clonal plantations are obvious for a lot of tree species. Somatic embryogenesis is a clonal propagation method with the greatest potential for achieving this goal, especially if combined to genetic engineering. However, more than for other vegetative propagation techniques, the practical use of somatic embryogenesis remains strongly impeded by the genetic identity and the physiological age of the mature selected trees to be cloned. So far, somatic embryogenesis has been successfully obtained from mature individuals only for a very limited number of broad-leaved or deciduous species using as primary explants leaves in a proper physiological condition and also sporophytic tissues from the reproductive organs. It is currently still limited to the embryonic phase of the ageing process for many evergreen coniferous species of high industrial impact. Shoot apical meristems owing to their key role in phase change warrant special consideration for attempting to clone mature trees by somatic embryogenesis. If direct induction from *in situ* collections is still hazardous in the absence of reliable indicators for the more responsive physiological stage, preconditioning *in vitro* procedures are worth considering when attempting to succeed in somatic embryogenesis from mature trees. These *in vitro* techniques include serial microcutting in subcultures as well as meristem culture and micrografting. With these techniques meaningful results have been obtained for different tree species in terms of rejuvenation. If some are limited to *in vitro* conditions, others are more unequivocal.

Abbreviations: BA: 6-Benzylaminopurine; SAM: Shoot apical meristem; SE: Somatic embryogenesis

Foreword

The meaning of the terms "*phase change*" and "*rejuvenation*" can be controversial and has often been debated (Wareing 1987, Pierik 1990, Jones 1999). They are considered in this paper in their most simple and literal form: *Phase change*: change from juvenile to mature characteristics and *vice-versa*: it includes therefore rejuvenation; various traits can be involved; *Rejuvenation*: recovery, even partially, of juvenile characteristics. The production of leaves exhibiting a juvenile-like morphology by shoot apical meristems (SAMs) from mature trees demonstrates the possibility for such SAMs to recover a certain degree of physiological juvenility. This reversion from mature to juvenile characteristic features must be considered in all objectivity as a demonstration of rejuvenation at the SAM level, even if this recovery is often fugacious. In this respect, the rationale for using heteroblastic species exhibiting a conspicuous foliar dimorphism between the juvenile and the mature phases for studying SAM phase change must be emphasized (Von Passecker 1947, Schaffalitzky de Muckadell 1959). Rejuvenation should be distinguished from reinvigoration (Wareing 1987, Pierik 1990). The latter means literally the regaining of vigor and is restricted to this definition in this paper. The most juvenile plants *i.e.* young germinants, with the highest capacity for adventitious rooting - which remains one of the most commonly acknowledged indicators of juvenility - are indeed not vigorous. Vigor corresponds to the attainment to a certain degree of maturation, or physiological ageing, generally associated to a decline of capacity for adventitious rooting (Borchert 1976): vigorous shoots generally do not root easily. This is why rejuvenation and reinvigoration must be differentiated.

Introduction

The advantages of clonal plantations are obvious for a lot of arborescent species including rubber (Carron *et al* 2009), fruit (Jain and Ishii 2003), ornamental and forest trees (Libby and Rauter 1984, Lindgren 2002). Except in some contexts, where grafted plants may be preferable, somatic embryogenesis (SE) is the clonal propagation method with the greatest potential for achieving this goal (Park *et al.* 1998, Jones 2002, Park 2002). In addition, SE can be combined with genetic engineering (Jones 2002, Malabadi and Nataraja 2007). However, more than for other vegetative propagation techniques used for producing plants with their own roots, the practical outcome of SE remains strongly impeded by the genetic identity and the physiological age of the selected trees to be cloned (Park *et al.* 1998, Bonga *et al.* 2010). So far, SE has been obtained from mature individuals only for a limited number of broad-leaved or deciduous species using as primary explants leaves in a proper physiological condition and also sporophytic tissues from the reproductive organs (Dunstan *et al.* 1995, Von Aderkas and Bonga 2000, Bonga *et al.* 2010). It is currently still limited to the first stages of embryo development for a few evergreen coniferous species of high industrial impact (Park 2010). SAMs, owing to their key role in phase

change and to the juvenile potential of their meristematic cells, warrant special consideration when attempting to clone mature trees by somatic embryogenesis (Monteuuis 1989). Induction could be done directly from SAMs collected *in situ*, or after *ex-vitro* and *in vitro* preconditioning.

Prospects of inducing SE directly from SAMs collected *in situ*

Through their organogenic capacities, SAMs ensure the aerial development of plants according to their ontogenetical program with associated morphological and anatomical characteristics (Sussex 1989, Weigel and Jürgens 2002). SAMs are prone to physiological ageing in relation to growth cycles and distance to the root system (Schaffalitzky de Muckadell 1959, Borchert 1976, Kerstetter and Poethig 1998). Such sequential phase changes at the SAM level are reflected, more or less saliently, according to species by various morphological indicators such as leaf shape or appearance of flowers (Schaffalitzky de Muckadell 1959, Robinson and Wareing 1969, Jones 1999). This has been called cyclophysis and it suggests that shoots can show "windows" of juvenility that are more tip and time restricted as ageing increases during the ontogenetical process (Schaffalitzky de Muckadell 1959, Olesen 1978). These windows of physiological juvenility will become ultimately confined to the SAMs, or even to within SAM zones or cells during the period of higher metabolic activity preceding shoot expansion or flush (Krenke 1940, Monteuuis 1988, 1989), as illustrated in Figures 1. These windows correspond to budbreak in temperate countries. A that time, SAMs from mature and juvenile genotypes demonstrate similarities in many respects, including *in vitro* culture success rates (Monteuuis 1987a).

Figures 1. *Examples of cyclophysis in Callistemon sp (left) and in Sequoia semperviens*

Recent findings have established that there are similarities between SAMs from mature and juvenile trees in relation to particular plastochronic phases (Mankessi *et al.* 2010, Mankessi *et al.* 2011). Another factor to consider for inducing SE from SAMs collected *in situ* is their size, liable to vary a lot from species to species, with the age of the donor tree and the within tree location of the SAMs (Parke 1959, Owston 1969). Too often, shoot apices are confused with true SAMs. These latter consist of the apical dome and emerging leaf initia or primordia (Romberger 1963). SAMs of about 100µm as overall size seem to be the smallest that can be introduced routinely in culture, bearing in mind that SAMs of a lot of species are much tinier than this (Romberger 1963, Mankessi *et al.* 2010). According to Nozeran (1978, 1984) and Nozeran *et al.* (1982), the more mature the tree, the smaller the portion of SAM tissue liable to contain cells that have remained juvenile and that would be responsive after excision and *in vitro* culture. The aim is to prevent the negative ageing influence of the more mature surrounding cells and tissues on the juvenile potential of the SAMs. Rare, but meaningful observations in the field demonstrate the existence of cells within SAMs of mature trees that have remained juvenile and organogenic (Figure 2).

Figure 2*. Demonstration of natural rejuvenation: juvenile-like shoot produced by a SAM from a mature Eucalyptus sp. (courtesy of Franclet).*

Ideally, such juvenile cells or tissues must be excised during the most appropriate time window to be set in culture. This time window has been assumed to be more and more restricted as the age of the donor tree increases (Krenke 1940, Monteuuis 1989). Practically, at present, it is still difficult to identify the more juvenile SAM cells or tissues to be excised in viable condition at the most suitable period from mature *in situ* selected individuals for SE induction. All these constraints plus the stress caused by the excision of the selected tissues make such operations hazardous. The smaller the size of tissue removed for placement in culture, the stronger the negative influence of stress on explant survival and further development (Bonga and Von Aderkas 1992).

Ex-vitro preconditioning procedures aimed at stimulating SE induction capacity

***Figures 3**. a) Shoots from proventitious buds exhibiting a juvenile morphology for the first leaves produced in Acacia mangium and b) stimulation of interfascicular buds giving rise to shoots with euphylls (arrows) after BA sprays on grafted mature Pinus pinaster scions.*

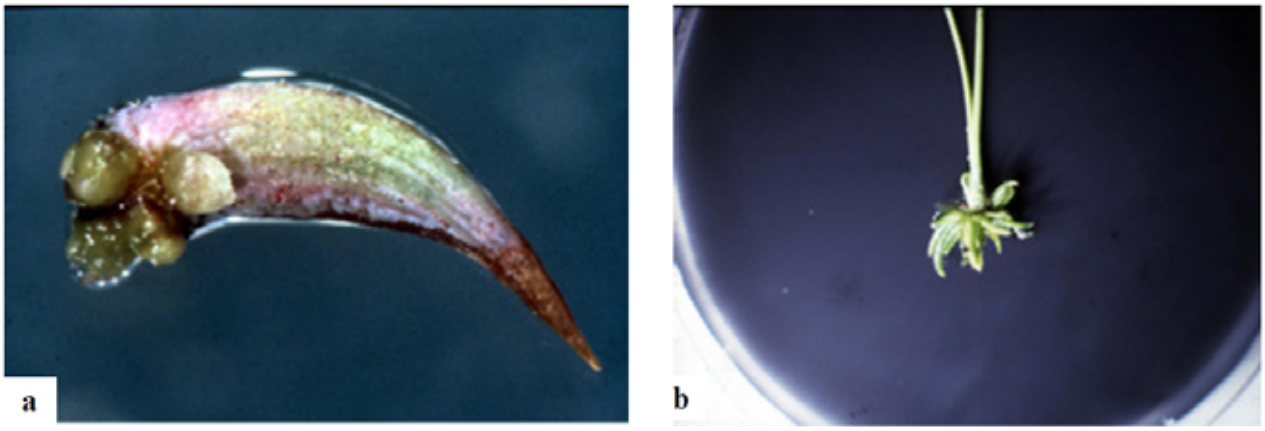
Different treatments have rejuvenated SAMs, resulting in the production of juvenile-like leaves from mature genotypes. These arose from quiescent proventitious buds after the suppression of apical dominance by operations such as pinching, pruning, hedging or even felling. In this latter case, coppicing shoots with juvenile-like traits can be produced from the stumps of the felled trees (Monteuuis *et al.* 1995a). A more conservative and as such a more recommendable method is to cut branches into longitudinal parts with at least one axillary bud, and to place the sticks thus obtained vertically under mist and shade (Monteuuis *et al.* 1995b). The first leaves produced by the very soft shoots starting to elongate usually exhibit a juvenile morphology, as is illustrated in Figure 3a. Another option consists in applying cytokinins, in the form of sprays of BA in aqueous solutions, on grafted scions collected from mature selected ortets (Franclet 1983). It has been very efficient when applied at the end of winter, just before budbreak, to mature *Pinus pinaster* auxiblast shoot tips grafted onto seedling rootstocks (Figure 3b).

Concomitant to the elongation of the terminal bud, the numerous needle fascicle buds of the mature scion started to increase in size to give rise soon to shoots with primary leaves or euphylls (Dumas 1987). This attested that the quiescent interfascicular buds had been rejuvenated by the application at the right time of BA sprays. Instead of only one terminal bud at the top of the mature scion, this treatment induced the formation of plenty of rejuvenated shoots with buds at the tip end of each. The advantage of using primary leaves or euphylls instead of brachyblasts with pseudophylls or needle fascicles that characterize the mature phase for pines is explained hereafter.

Serial or “en cascade” grafting has been reported to induce “rejuvenation”, especially on Douglas fir, as the number of grafting cycles of the selected mature scions onto the young seedling rootstocks increases (Franclet 1983). However, it seems more proper to talk about reinvigoration when a regaining of vigor is the main effect resulting from these serial graftings.

Prospects of serial *in vitro* subcultures for inducing SE

Subculturing, sometimes for several years, of microcuttings on appropriate *in vitro* culture media has induced in different species a degree of rejuvenation, at least for certain traits, in comparison with juvenile controls exposed rigorously to the same experimental conditions. More or less pronounced reversions to juvenile characteristics were observed as the number of subcultures increased with regard to traits such as: leaf morphology, growth, multiplication by axillary budding and adventitious rooting rates, and even the capacity for adventitious budding (Mullins *et al.* 1979, Fouret *et al.* 1986, Walker 1986). Usually the subculture media used contained cytokinins, but this was not always the case. In *in vitro* cultures of giant sequoia, analogies between a 100-yr-old mature clone and the juvenile control became more and more obvious for different traits as the number of microcutting transfers onto a MS-derived medium with activated charcoal and free of cytokinin increased. However, replacing the MS salt composition by a Knop-derived macroelement formulation had a totally opposite effect (Monteuuis 1988). Subculture-induced



Figures 4. *de novo formation of adventitious buds from euphylls (a) and pseudophylls (b) produced by long-time subculture of microcuttings from mature selected Pinus pinaster.*

rejuvenation has been particularly obvious for heteroblastic species like *Acacia mangium* where SAMs from mature trees can produce after several subcultures juvenile-like leaves instead of the phyllodes that characterize the mature phase (Baurens *et al.* 2004, Monteuuis 2004). Meaningful responses (Figures 4) were also observed for *Sequoia sempervirens* and *Pinus pinaster* where *de novo* formation of buds and shoots could be obtained from juvenile-like leaves produced by meristems of microcuttings from mature selected ortets, after that these microcuttings had been for a long time subcultured (Walker 1986, Dumas and Monteuuis 1991). This demonstrates the possibility of using well adapted subculture protocols for physiologically rejuvenating SAMs from mature trees, even if most of such rejuvenation has been limited to the tissue culture episode and has reverted to the mature phase after acclimatization under *ex vitro* conditions (Mullins *et al.* 1979, Fouret *et al.*

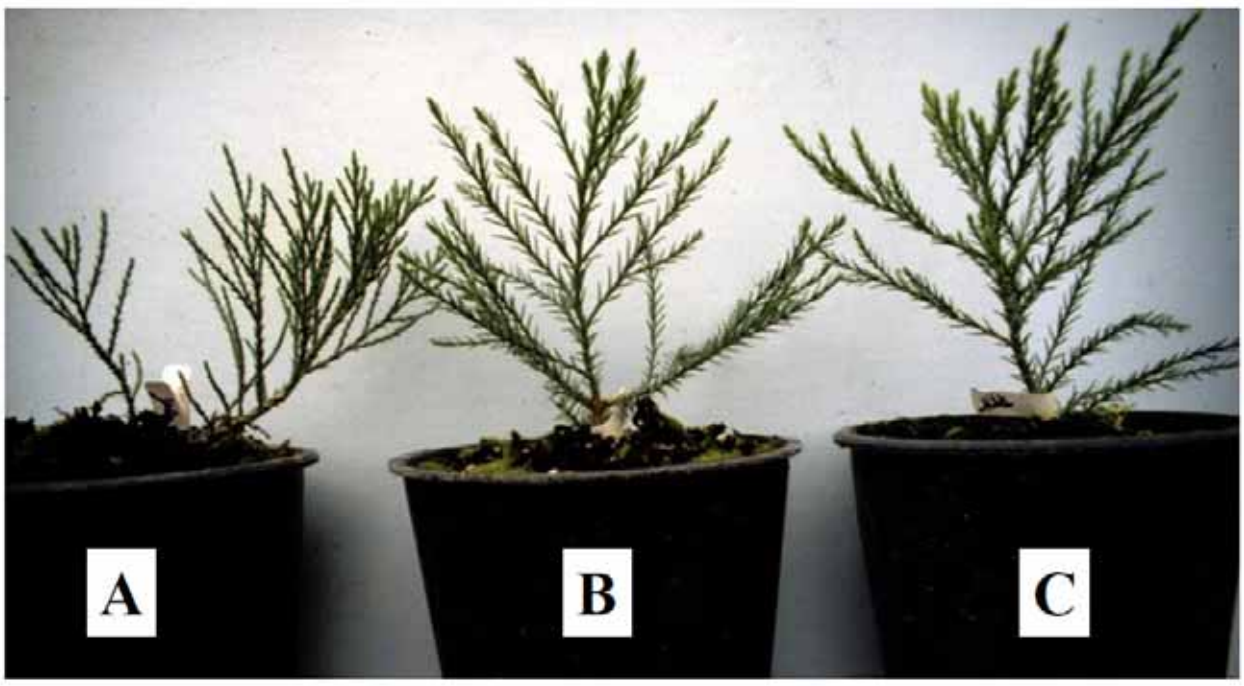
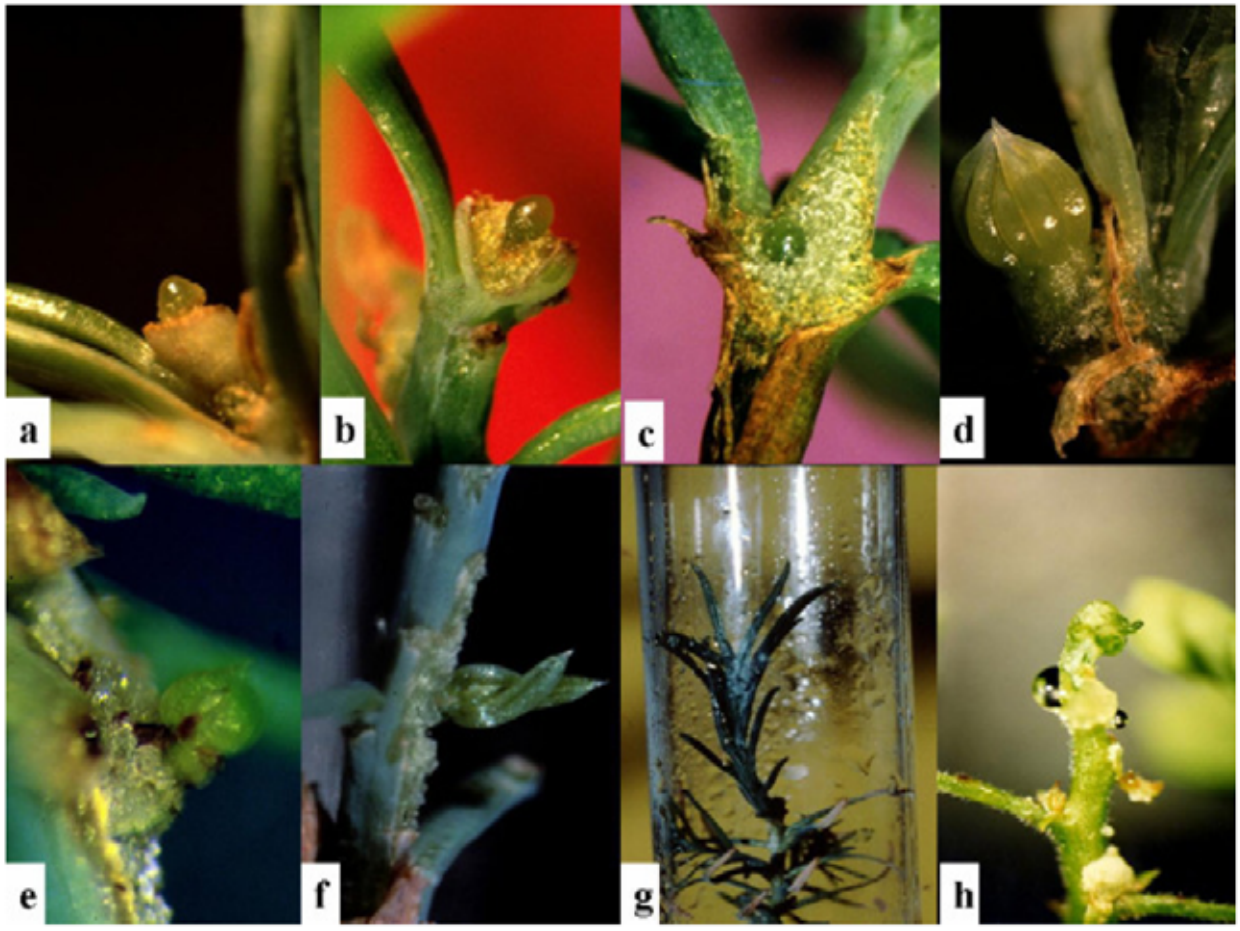


Figure 5. *(A) a 3-yr-old graft from a 100-yr-old Sequoiadendron giganteum ortet; (B) a representative of the rejuvenated line from the same 100-yr-old ortet as in (A), 6 months after acclimatization, self rooted and exhibiting an orthotropic growth pattern and juvenile morphology like the control (C); this rejuvenated line was obtained from a SAM in vitro (see Monteuuis, 1991 for more details).*



***Figures 6**. SAM micrografting of Pseudotsuga menziesii"top-grafting" (a), "cleft-grafting" (b), "side-grafting" (c) and a more advanced stage of development (d), of Pinus pinaster, "side-grafting" (e),and (f) for a more advanced stage of development, of Sequoiadendron giganteum exhibiting a morphologically rejuvenated scion (g), and first stages of development of an Acacia mangium micrografted shoot apex (h).*

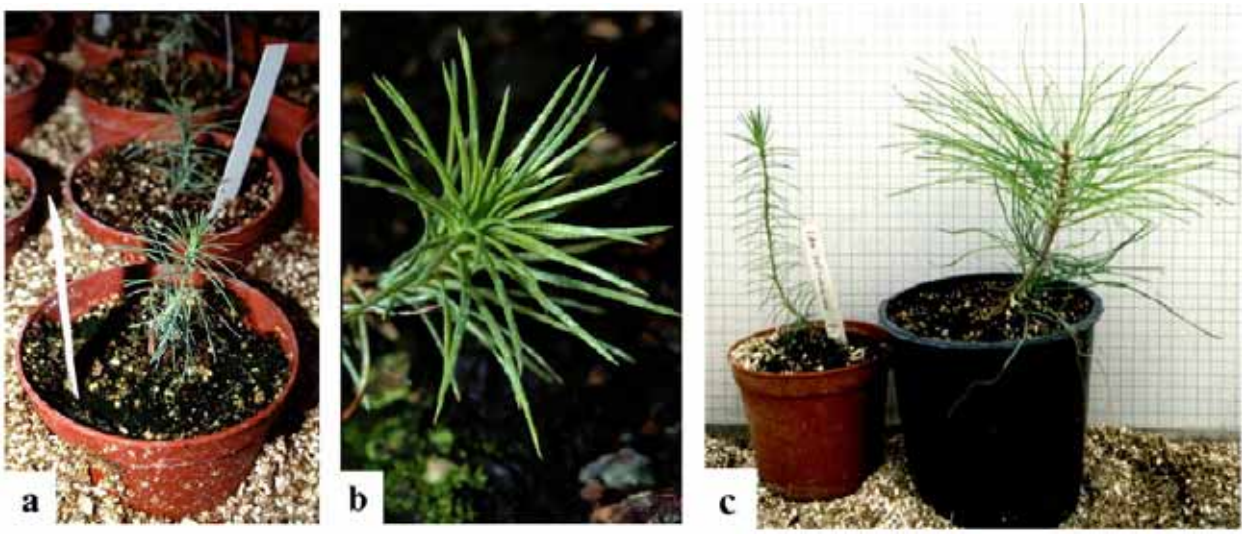
1986).

Prospects of SAM culture and micrografting for inducing SE

In addition to phytosanitary advantages, *in vitro* culture of SAMs can induce physiological rejuvenation (Bonga and Von Aderkas 1992). This is a far more immediate and straightforward strategy than serial grafting or subcultures. These latter involve bigger vegetative structures, with more mature tissues left attached below the SAM, and aim at obtaining increasingly less differentiated cells as the number of successive transfers increases.

In 1986, one SAM out of a few hundreds excised from a 100-yr-old giant sequoia gave rise to a truly rejuvenated line *in vitro* and its juvenile traits persisted after transfer to *ex vitro* (Figure 5). This rejuvenated line has been maintained in culture in a juvenile state ever since (Bon and Monteuuis 1991, Monteuuis 1991, Monteuuis *et al.* 2008). Such rejuvenated materials could profitably be used for SE induction.

SAM micrografting offers all the advantages of SAM culture (Monteuuis 1987b, Monteuuis and Dumas 1990). In addition, it avoids the limitations associated with the composition of the synthetic media used for SAM culture. It should be noted that SAMs become increasingly more medium-sensitive as the donor tree from which they are taken ages (Monteuuis 1987a). A young seedling is undoubtedly a more natural and suitable culture support for excised SAMs than a synthetic medium. Moreover, the hypothetical beneficial influence of rejuvenating substances that may be produced by the juvenile seedlings used as rootstocks on the micrografted SAMs cannot be ruled out (Monteuuis 1987b, Bon 1988, Huang *et al.* 1992). For SAM micrografts the required minimum size of the tissue grafted again appears to be about 100 µm. In addition to size of the graft, success also depends on the species, the age and the organogenic stage of the SAM or shoot apices. Shoot apices include more differentiated cells and tissues and these are liable to have a negative influence on the expected rejuvenation.



Figures 7. *In vivo rejuvenated shoots resulting from SAM micrografting of mature Pinus pinaster ortets (a and b), exhibiting a juvenile morphology compared to a cutting of the same mature ortet that had rooted with difficulty (c).*

SAM micrografting has been successfully applied for initiating tissue culture from mature selected trees of various species (Figures 6) that could not or only hardly be multiplied by SAM culture on synthetic *in vitro* media. These include *Sequoiadendon giganteum* (Monteuuis 1986, 1987b), *Pinus pinaster* (Dumas *et al.* 1989), *Pinus strobus* (Goldfarb *et al.* 1992), *Picea abies* (Monteuuis 1994) and *Pseudotsuga menziesii* (Monteuuis 1995). In contrast, the 200µm long scions used for *Acacia mangium* (Monteuuis 1996) were not SAMs *per se* but shoot apices, the SAMs being too tiny to be micrografted. Different grafting techniques can be used (Figures 6).

Some of the micrografted SAMs developed juvenile-like leaves, which persisted after transfer to *ex-vitro* conditions, attesting that these SAMs had been physiologically rejuvenated by micrografting (Figures 7). Such rejuvenated leaves, or in the case of *Pinus* pinaster, primary leaves or euphylls, produced adventitious shoots that subsequently rooted and were acclimated (Monteuuis and Dumas 1992, Dumas, unpublished results). This raises the prospect that micrografting of SAMs from mature, selected trees could eventually lead to the formation of tissues that could be used for inducing SE from these trees.

Discussion and concluding remarks

SE is the only way of achieving complete ontogenetic rejuvenation as it resets the ontogenetic process to zero through the formation of embryos. The fact that SE-derived vegetative structures at different stages of development *i.e.* newly formed *in vitro* primary somatic embryos or outdoor growing emblings demonstrate an enhanced capacity for (secondary) SE suggests also some positive physiological influence, as discussed for *Hevea brasiliensis* (Lardet *et al.* 2009). Field planted emblings of different species and more specifically of *Coffea spp* have been observed to flower earlier than seedlings of the same chronological age (Berthouly, unpublished observation). The most rational hypothesis that could account for this premature physiological ageing of emblings could be caused by tissue-culture-induced stress due to non-optimal *in vitro* conditions (Dunstan *et al.* 1995, Von Aderkas and Bonga 2000). These could have a strong negative influence on the few unique isolated cells from which the somatic embryos arose (Berthouly and Michaux-Ferrière 1996). One of the causes could be modification of the DNA methylation status with associated risks of somaclonal variations (Jaligot *et al.* 2004, Xu *et al.* 2004, Leljak-Levanić *et al.* 2009).

SE is still strongly hampered by physiological ageing, and the possibility to clone by SE any mature selected Plus tree remains an ultimate challenge. We deliberately chose to focus our considerations on the prospects of using SAMs for succeeding in SE from mature trees due to the particularity of SAMs of any species to retain a certain degree of juvenility, even if this juvenility becomes more and more time and tissues or cells limited with increasing maturation (Nozeran *et al.* 1982, Monteuuis 1989). The possibility of achieving SE from mature trees or palms, using sporophytic tissues from reproductive organs or leaf portions, although proven quite efficient in certain cases, only works for some genotypes or species, and also might be prone to somaclonal variation or mutation risks (Jaligot *et al.* 2004).

As previously noted, SAMs collected *in situ*, with or without preconditioning rejuvenating treatments prior to their excision, can be used for attempting to induce SE. Such direct introduction into *in vitro* culture conditions could be carried out with minimal if not total absence of disinfection measures. It is interesting to note that the

smaller the shoot tips used - SAMs being the tiniest that can be routinely removed by hand for a limited number of species -, the higher the chances of more stable rejuvenation. This accords with the concept of miniaturization (Nozeran 1978). Subculturing (Fouret *et al.* 1986) or serially grafting longer shoot tips (Huang *et al.* 1992) *i.e.* SAMs with a certain quantity of mature tissues underneath give rise most of the times to *in vitro*-restricted rejuvenations which disappear after transfer to *ex-vitro* conditions. It seems however worth using the juvenile-like leaves formed or even the transitory rejuvenated SAMs for SE induction attempts, either directly or via the adventitious buds or dedifferentiated cells that can arise from these *in vitro* rejuvenations.

A lot of efforts have been devoted during the past years to SE cloning of mature trees of species of economic value, pines especially, including resort to highly sophisticated and advanced technologies (Park 2002, 2010). Given this context and based on the literature available, it is surprising that only limited interest has been shown in the use of preconditioning rejuvenating techniques like subcultures or SAM micrografting that are described in this paper. These techniques, developed some 20 years ago, remain easily applicable, are economical and seem promising for enhancing the SE responsiveness of mature selected trees. This is particularly true for pines considering the heteroblastic features of these species, which are indicators of physiological rejuvenation at the SAM level that should be made use of.

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References

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

Berthouly, M., Michaux-Ferrière, N. 1996. High frequency embryogenesis in *Coffea canephora* Robusta: Induction conditions and histological evolution. Plant Cell, Tiss. Org. Cult.. 44:169-176.

Bon, M.C., 1988. J16: an apex protein associated with juvenility of *Sequoiadendron gigantem*. Tree Physiol. 4:381-387.

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

Bonga, J.M., Von Aderkas, P. 1992. In vitro culture of trees. Kluwer Academic Publishers, Dordrecht, Boston, London, 236p.

Bonga, J.M., Klimaszewska K.K., Von Aderkas, P. 2010. Recalcitrance in clonal propagation, in particular of conifers. Plant Cell Tiss Organ Cult 100:241-254

Borchert, R. 1976. The concept of juvenility in woody plants. Acta Hortic 56:21-36.

Carron, M.P., Lardet, L., Lecomte, A., Dea B.G., Kéli, J., Granet, F., Julien, J., Teerawatanasuk, K., Montoro, P**.** 2009. Field trials network emphasizes the improvement of growth and yield through micropropation in rubber tree (*Hevea brasiliensis*, Muëll.-Arg.). *In* : Romano A (Ed) . Proceedings of the 3rd International Symposium on Acclimatization and Esta Micropropagated Plants, Sept. 12-15, 2007, Faro, Portugal . Leuven : ISHS Acta Horticulturae 812: 485-492.

Dumas, E. 1987. Micropropagation d'un clone âgé de pin maritime en vue de l'obtention de pieds-mères. Annales AFOCEL 1986, 96-107.

Dumas, E., Franclet, A., Monteuuis, O. 1989. Microgreffage de méristèmes primaires caulinaires de pins maritimes (*Pinus pinaster* Ait.) âgés sur de jeunes semis cultivés in vitro. C.R. Acad. Sc. Paris, 309 (III), 723-728.

Dumas, E., Monteuuis, O. 1991. Régénération in vitro de pins maritimes âgés par bourgeonnement adventif sur euphylles. Annales AFOCEL 1989-1990:43-58.

Dunstan, D.I., Tautorius, T.E., Thorpe, T.A. 1995. Somatic embryogenesis in woody plants. *In*: Thorpe TA (Ed). In vitro embryogenesis in plants. Kluwer Academic Publishers, Dordrecht, pp 471-537.

Fouret, Y., Arnaud, Y., Larrieu, C., Miginiac, E. 1986. *Sequoia sempervirens* as an *in vitro* rejuvenation model. New Zealand.Journal of Forest Science 16: 319-327.

Franclet, A. 1983. Rejuvenation: Theory and practical experiences in clonal silviculture. *In*: Clonal Forestry: its impact on tree improvement and our future forests. Proceedings of the XIXth meeting of the Canadian Tree Improvement Association, 22-26/8/1983, Toronto, 96-134.

Goldfarb, B., McGill, G.E., Hackett, W.P., Monteuuis, O. 1992. In vitro culture and micrografting of White pine meristems. Proc. Int. Plant Propagators Soc., 42:412-414.

Huang, L.C., Lius, S., Huang, B.L., Murashigen T., Mahdin T., Van Gudyn R. 1992. Rejuvenation of Sequoia sempervirens by repeated grafting of shoot tips onto juvenile rootstocks *in vitro*. Plant Physiol. 98:166-173.

Jain, S.M, Ishii, K. 2003. Micropropagation of woody trees and fruits. Kluwer Academic Publishers, Dordrecht, Boston, Lonfdon, 840 p.

Jaligot, E., Beulé, T., Baurens, F.C., Billotte, N., Rival, A. 2004. Search for methylation-sensitive amplification polymorphism associated with the 'mantled' variant phenotype in oil palm (*Elaeis guineensis* Jacq.). Genome 47:224-228.

Jones, C. 1999. An essay on juvenility, phase change, and heteroblasty in seed plants. Int. J. Plant Sci.160 (6 Suppl.): S105-111.

Jones, N. 2002. Somatic embryogenesis as a tool to

capture genetic gain from tree breeding strategies: risks and benefits. Southern African Forestry Journal, 195: 93-101.

Kerstetter, R.A., Poethig, R.S. 1998. The specification of leaf identity during shoot development. Annual Review of Cell and Developmental. Biology 14: 373-398.

Krenke, N.P. 1940. The theory of the cycle of senescence and rejuvenation of plants and its practical application. Plant Breeding. Abstracs 15:1-135.

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

Leljak-Levanić, D., Mihaljević, S., Jelaska, S. 2009. Variations in DNA methylation *in Picea omorika* (Panc) Purk. embryogenic tissue and the ability for embryo maturation. Propagation of Ornamental Plants. 9:3-9.

Libby, W.J., Rauter, R. 1984. Advantages of clonal forestry. The Forestry Chronicle, June 1984: 145-149.

Lindgren, D. 2002. Advantages of clonal propagation. *In*: Welander M & Zhu L H (Eds). Proceedings of Workshop on high quality birch – clonal propagation and wood properties. August 27-28, 2001. p 98-109.

Malabadi, R.B., Nataraja, K. 2007. Genetic transformation of conifers: Applications in and impact on commercial forestry. Transgenic Plant Journal. 1:289-313.

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

Mankessi, F., Saya, A.R., Montes, F., Lartaud, M., Verdeil, J.L.., Monteuuis, O. 2011. Histocytological characteristics of *Eucalyptus urophylla* x *Eucalyptus grandis* shoot apical meristems of different physiological ages. Trees. 25:415-424.

Monteuuis, O. 1986. Microgreffage de points végétatifs de *Sequoiadendron giganteum* Buchholz séculaires sur de jeunes semis cultivés in vitro. C.R. Acad. Sc. Paris, 302, (III), 223-225.

Monteuuis, O. 1987a. In vitro meristem culture of juvenile and mature *Sequoiadendron giganteum*. Tree Physiol. 3:265-272

Monteuuis, O. 1987b. Microgreffage du séquoia géant. Annales AFOCEL 1986:39-61.

Monteuuis, O. 1988. Aspects du clonage de séquoias géants jeunes et âgés. Ph D thesis, Univ. Blaise Pascal, Clermont-Ferrand, 190p.

Monteuuis, O. 1989. Maturation concept and possible rejuvenation of arborescent species. Limits and promises of shoot apical meristems to ensure successful cloning. *In*: Proceedings of the IUFRO Conference on: "Breeding Tropical Trees: Population Structure and Genetic Improvement Strategies in Clonal and Seedling Forestry". Pattaya, Thailand, 28 Nov.-3 Dec. 1988:106-118.

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

Monteuuis, O. 1994. Effect of technique and darkness on the success of meristem micrografting of *Picea abies*. Silvae Genet. 43:91-95.

Monteuuis, O. 1995. Influence of the grafting technique on meristem micrografting of Douglas-fir. New Forests. 10:267-273.

Monteuuis, O. 1996. *In vitro* shoot apex micrografting of mature *Acacia mangium*. Agroforestry Systems 34: 213-217.

Monteuuis, O. 2004. In vitro rooting of juvenile and mature *Acacia mangium* microcuttings with reference to leaf morphology as a phase change marker. Trees 18:77-82.

Monteuuis, O., Dumas, E. 1990. Microgreffage de méristèmes de conifères âgés: fondements et présentation. *In*: Les Colloques de l'INRA, INRA, Paris, 51:299-301.

Monteuuis, O., Dumas, E. 1992: Morphological features as indicators of maturity in acclimatized *Pinus pinaster* from different in vitro origins. Can. J. For. Res. 22:1417-1421

Monteuuis, O., Vallauri, D., Poupard, C., Chauvière, M. 1995a. Rooting *Acacia mangium* cuttings of different physiological age with reference to leaf morphology as a phase change marker. Silvae Genet. 44:150-154.

Monteuuis, O., Vallauri, D., Poupard, C., Hazard, L., Yusof, Y., Wahap latip, A., Garcia, C., Chauvière, M. 1995b. Propagation clonale de tecks (*Tectona grandis*) matures par bouturage horticole. Bois et Forêts des Tropiques 243:25-39.

Monteuuis, O., Doulbeau, S., Verdeil, J.-L. 2008. DNA methylation in different origin clonal offspring from a mature *Sequoiadendron giganteum* genotype. Trees 22:779-784.

Mullins, M.G., Nair, Y., Sampet, P. 1979. Rejuvenation *in vitro*: induction of juvenile characters in an adult clone of *Vitis vinifera* L. Ann. Bot. 44:623-627.

Nozeran, R. 1978. Polymorphisme des individus issus de la multiplication végétative des végétaux supérieurs, avec conservation du potentiel génétique. Physiol. Vég. 16:177-194.

Nozeran, R. 1984. Integration of organismal development. *In*: Positional controls in plant development, Barlow PW and Carr DJ eds, Cambridge University Press 13:375-401.

Nozeran, R., Ducreux, G., Rossignol-Bancilhon, L. 1982. Réflexions sur les problèmes de rajeunissement chez les végétaux. Ull. Soc. Bot. Fr. 129, Lettres bot. 107-130.

Olesen, P.O. 1978. On cyclophysis and topophysis. Silvae Genet. 27:173-178.

Owston, P.W. 1969. The shoot apex in eastern white pine: its structure, seasonal development, and variation within the crown. Canadian Journal of Botany 47:1181-1188.

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

Park, Y.S. 2010. Achievements in somatic embryogenesis of conifers and its implementation in the management of future forests and plantations. Oral communication presented during the XXIII IUFRO World Congress August 23-28, 2010 Seoul, Korea.

Park, Y.S, Barrett, J.D., Bonga, J.M. 1998. Application of somatic embryogenesis in high-value clonal forestry: Deployment, genetic control, and stability of cryopreserved clones. In Vitro Cell. Dev. Biol.-Plant 34:231-239.

Parke, R.V. 1959. Growth periodicity and the shoot tip of *Abies concolor*. American Journal of Botany 46: 110-118

Pierik, R.L.M. 1990. Rejuvenation and micropropagation. *In*: Nikkamp H.J.J, Van der Plas L.H.W., and Van Aartrijk J., (Eds), Progress in plant cellular and molecular biology. Proceedings of the VIIth international congress on plant tissue and cell culture, Amsterdam, Netherland, Kluwer Academic: 91-101.

Romberger, J.A. 1963. Meristems: growth and development in woody plants. U.S. Dep. Agr. Tech. Bull. Ed.Robinson, L.W., Wareing, P.F. 1969. Experiments on the juvenile-adult phase change in some woody species. New Phytologist 68: 67-78.

Schaffalitzky de Muckadell, M. 1959. Investigations on aging of apical meristems in woody plants and its importance in silviculture. Kandrup and Wunsch's Bogtrykkeri, København, pp 313-346.

Sussex, I.A. 1989. Developmental programming of the shoot meristem. Cell 56:225-229.

Von Aderkas, P., Bonga, J. M. 2000. Influencing micropropagation and somatic embryogenesis in mature trees by manipulation of phase change, stress and culture environment. Tree Physiol. 20: 921-928.

Von Passecker, F. 1947. Entwicklungsphasen und vegetative Vermehrung holziger Gewächse. Centralblatt für das gesamte Forstwesen 70: 270-292.

Walker, N. 1986. *Sequoia sempervirens*: réjuvénilisation et culture de méristèmes en cascade. Annales AFOCEL 1985: 25-47.

Wareing, P.F. 1987. Phase change and vegetative propagation. *In*: Abbott A.J., and Atkin R.K., (Eds), Improving vegetatively propagated crops, Acad. Press, London: 263-270. Weigel, D., Jürgens, G. 2002. Stem cells that make stems. Nature 415: 751-754.

Xu, M., Li, X., Korban, S.S. 2004. DNA-methylation alterations and exchanges during in vitro cellular differentiation in rose (*Rosa hybrida* L.). Theor Appl Genet. 109:899-910.

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Comparison of Morphological and Physiological Characteristics of Emblings Derived by Somatic Embryogenesis, and Seedlings in Yellow-Poplar (*Liriodendron tulipifera* L.)

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Abstract

Field performance of somatic emblings of yellow-poplar (*Liriodendron tulipifera*) produced by somatic embryogenesis was compared with that of seedlings at age 5. No significant differences were seen between the two types of plants in most of the growth and morphological characteristics examined. These include both height (seedlings: emblings, 3.8±0.3m, 3.6±0.4m) and DBH growth (38±4.1㎝, 37.1±5.8㎝) and the length (108.1±8.8mm, 123.5±8.3mm), width (149.1±16.3mm, 168.5±19.9) and fresh weight of leaves (2.1±0.3g, 2.8±0.6). Although the total leaf chlorophyll contents varied with plants, the emblings (916.4±79.2㎍/g) tended to contain a higher level of total chlorophyll than did seedlings (800.1±13.5.1㎍/g). Other histo-physiological parameters including photosynthetic rate (10.7±1.3, 10.1μmol$CO_2$$m^{-2}$$s^{-1}$1.0), transpiration rate (1.7±0.2, 1.7±0.2mmol$H_2O$$m^{-2}$$s^{-1}$), leaf stomatal conductance (0.2±0.02, 0.2±0.04mol$H_2O$$m^{-2}$$s^{-1}$) and leaf anatomy did not reveal any significant difference between the two types of plants either. No changes in either ploidy level or RAPD patterns were observed among the emblings.

Introduction

Clonal propagation of high–value forest trees through somatic embryogenesis (SE) has the potential to rapidly capture the benefits of breeding or genetic engineering programs and to improve the uniformity and quality of the nursery stock (Park 2002). Since first reported for yellow-poplar (Merkle and Sommer, 1986), SE is favored as a promising tool for mass propagation of coniferous trees. Breeding strategies using SE to produce improved clonal stock are being promoted as an alternative to conventional seed-orchard programs. In addition, SE can be used to propagate superior genotypes rapidly and with great efficiency. However, before SE can be introduced into forest regeneration programs, we need to determine the growth pattern and physiological attributes of somatic plants and to asses how these characteristics compare with those of normal zygotic seedlings. In this paper, somatic plants and zygotic plants were compared in terms of morphological, physiological, and histological variables linked to field performance.

Materials and Methods

Plant material, initiation of PEM and culture medium formulation

Open pollinated cones were collected from yellow-poplar trees at the Korea Forest Research Institute located in Suwon, Korea. After disinfection treatment, seeds were extracted from the cones with help of surgical knifes. Seed coats were removed, and the whole remainder was placed on PEM (Pre-embryogenic masses) initiation medium- a modified LM (Litvay *et al*. 1985) that contained full-strength macro- and micro- salts, vitamins, 1 g/L casein hydrolysate, and 87.6 mM sucrose with 2.0 mg/L 2,4-D and 0.25 mg/L BA. The cultures were initially kept in darkness at 24±1℃ for 8 weeks.

Maturation of somatic embryos

The PEM suspensions were homogenized, the dispersed tissue were poured over a filter paper (Whatman #2, 5.5 cm) and placed in a Büchner funnel. After draining the medium with low pressure pulse vacuum, the filter paper with ESM on it was placed on maturation LM medium containing 0.12 M sucrose, 250 mg/L casamino acid, 250 mg/L L-glutamine and 0.4% gellan gum

Plant regeneration

Somatic embryos were cultured on ½LM medium containing 60 mM sucrose and 0.3% gellan gum. The cultures were kept under light (50 μ$Em^{-2}$$s^{-1}$), 16-h photoperiod, 24±1℃. After germination treatment, the somatic plants were transplanted into an artificial soil mixture {perlite: peatmoss: vermiculite (1: 1: 1, v:v:v)} in trays with a transparent lid, and were watered once a day for acclimatization. After acclimatization under green-house condition for one year, the plants were transplanted to an experimental field at the Institute

Morphological variables and growth analysis

At the end of a 5-year growth period, the height, DBH, leaf length, width, fresh weight were measured on three seedlings and 7 somatic plants selected randomly from each family and clone tested.

Total leaf chlorophyll contents

The content of total chlorophylls was measured. Fresh leaves were extracted in 80% acetone and pigments were determined according to the method of Lichtenthaler

(1987) using a UV/VIS spectrometer *Agilent 8453* (Germany).

Histological analysis

The middle portions of leaf were fixed in glutaraldehyde (1.5%) and paraformaldehyde (1.6%) in phosphate buffer (0.05M, pH 6.8) under refrigeration for 3 months. Dehydration was done at room temperature in a series of different concentrations of ethanol, followed by infiltration with Historesin (Technovit 7100, Kluzer, Germany). Serial sections (3μm) were prepared with a rotary microtome with a tungsten-carbide knife. Sections were double stained with Periodic acid-Schiff's (PAS) (0.1%) and Toluidine blue O (0.05%) and observed under a Light microscope (Leica D.M.R., Germany).

Net photosynthesis rate, stomatal conductance and transpiration rate

Net photosynthesis rate (μmol CO_2 $m^{-2}s^{-1}$), stomatal conductance (mol H_2O $m^{-2}s^{-1}$) and transpiration rate (mmol H_2O $m^{-2}s^{-1}$) were measured using a portable photosynthesis measuring system (Li-cor 6400, Li-cor, USA) with inlet CO_2 concentration fixed at 400±10 μmol mol^{-1}.

RAPD

Genomic DNA was extracted by using the MagExtractor Plant Genomic DNA Mini prep kit supplied by TOYOBO (Japan). RAPD amplification was performed using a PCR mixture (RexGene Biotech, SUPER BIO Co. LTD.) (25μl) containing 25 ng of genomic DNA, 10X PCR buffer (25μl), 100 μM dNTPs, 0.5 U of Taq DNA polymerase and 1 μM of each primer (Operon Biotechnologies GmbH, Germany). PCR was performed by 45 cycles of 1 min denaturation at 94℃, 1 min annealing at 36℃, and 2 min extension at 72℃ using a thermal cycler (Biometra, T3 Thermocycler, Germany).

Flow cytometery

Flow cytometric estimations of DNA contents were accomplished by UV-excitation of 4,6-diamidino-2-phenylinolide (DAPI)-stained nuclear DNA which was prepared by usage of the Partec 'Cystain UV Precise P' kit ('nuclei extraction buffer', 'staining buffer', Partec GmbH, Munster, Germany). Sample preparations as well as measurement techniques with the Partec Ploidy Analyser (Partec GmbH) are described in Schmidt *et al*. (2006).

Results and Discussion

Morphological variables and growth analysis

Under similar growth conditions, no significant differences were observed in the height, DBH, leaf length, width, fresh weight between somatic and zygotic plants. These include both height (seedlings: emblings, 3.8±0.3m, 3.6±0.4 m) and DBH growth (38±4.1 ㎝, 37.1±5.8 ㎝) and the length (108.1±8.8 mm, 123.5±8.3 mm), width (149.1±16.3 mm, 168.5±19.9) and fresh weight of leaves (2.1±0.3g, 2.8±0.6) (Fig.1).

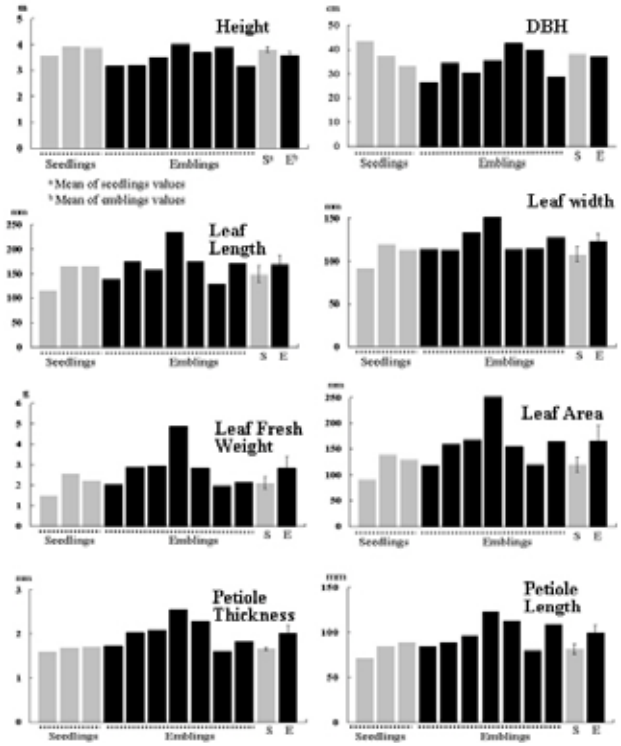

Fig. 1. *Comparison of means for morphological variables between zygotic and somatic seedlings of Liriodendron tulipifera. Bars: Standard deviation*

Total leaf chlorophyll contents

In the comparison of total leaf chlorophyll contents between two groups, although contents varied with plants, the emblings (916.4±79.2㎍/g) tended to contain a higher level of total chlorophyll than did seedlings (800.1±13.5.1㎍/g) (Fig.2).

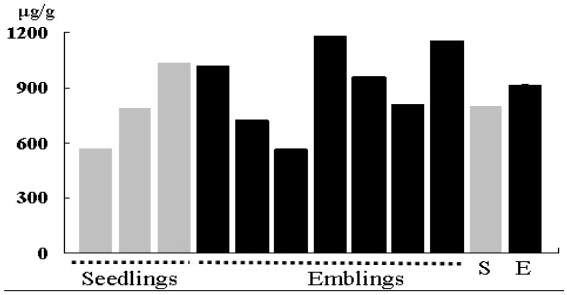

Fig. 2*. Comparison of means for total chlorophyll content of leaf between zygotic and somatic seedlings of Liriodendron tulipifera.*

Net photosynthesis, stomatal conductance and respiration rate

Other histo-physiological parameters including photosynthetic rate (10.7±1.3, 10.1μmol$CO_2$$m^{-2}s^{-1}$1.0), respiration rate (1.7±0.2, 1.7±0.2mmol$H_2O$$m^{-2}s^{-1}$), leaf

stomatal conductance (0.2±0.02, 0.2±0.04mol$H_2Om^{-2}s^{-1}$) and leaf anatomy did not reveal any significant difference between the two types of plants either (Fig.3).

Histological analysis

Cross-sections of the leaf did not reveal any significant difference between the two groups of plants either (Fig.4). The palisade parenchyma was formed by cells that were regular in shape and size between zygotic and somatic plants, respectively.

RAPD

All plants regenerated from SE, when examined by RAPD analyses, did not show any nuclear DNA polymorphism (Fig.5).

Flow cytometery

No changes in ploidy level were observed among the somatic emblings (Fig.6).

Conclusions

We conclude that somatic plant clones of yellow-poplar regenerated from somatic embryos are suitable for reforestation programs. Somatic clones show that growth and morphological characteristics are mainly equal to those of zygotic plants when compared. However, this assumption will have to be validated if the comparisons are conducted with more zygotic and somatic plantations. We are now investigating, with somatic plants recovered from various genotypes, the adaptability of these clones over range of planting sites.

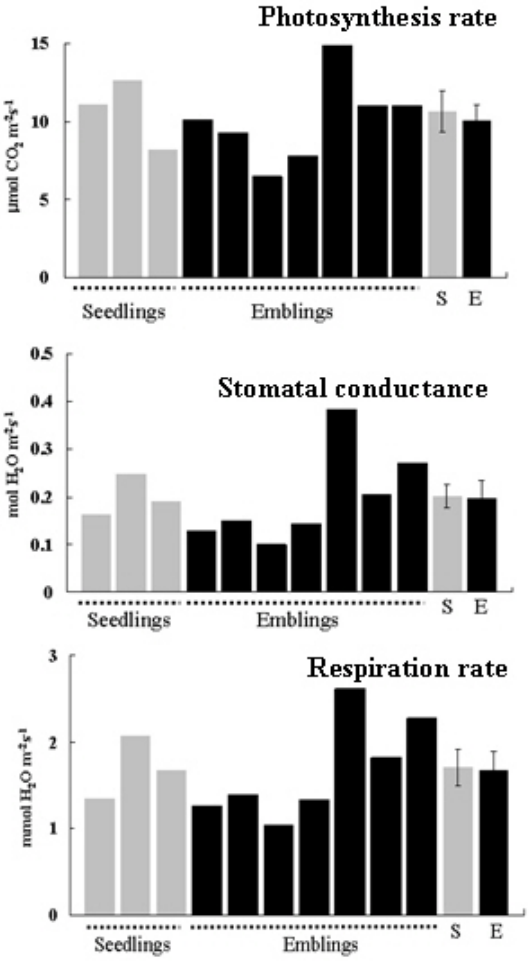

Fig. 3. *Comparison of means for photosynthesis rate, stomatal conductance and respiration rate between zygotic and somatic seedlings of Liriodendron tulipifera. (Bars: Standard deviation)*

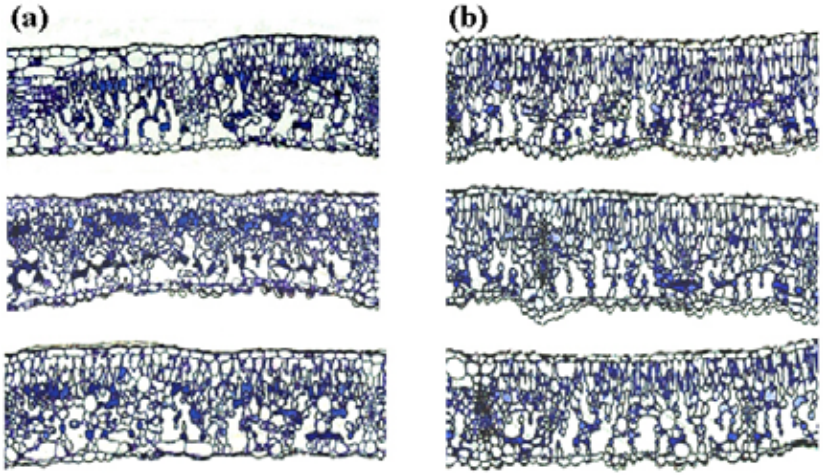

Fig. 4. Comparison of micro-structure from leaf between zygotic (a) and somatic seedlings (b) of *Liriodendrontulipifera.*

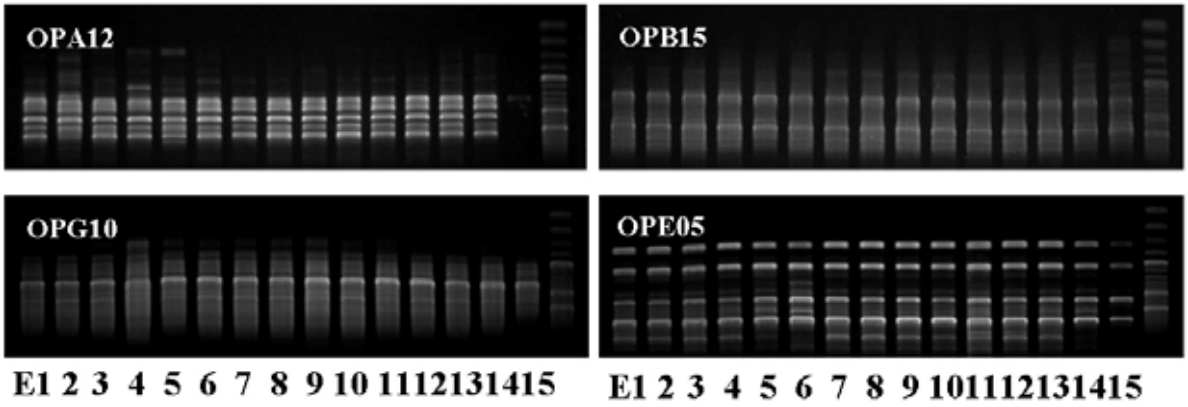

Fig.5. RAPD profiles of the 4 primers for somatic seedlings through somatic embryogenesis.

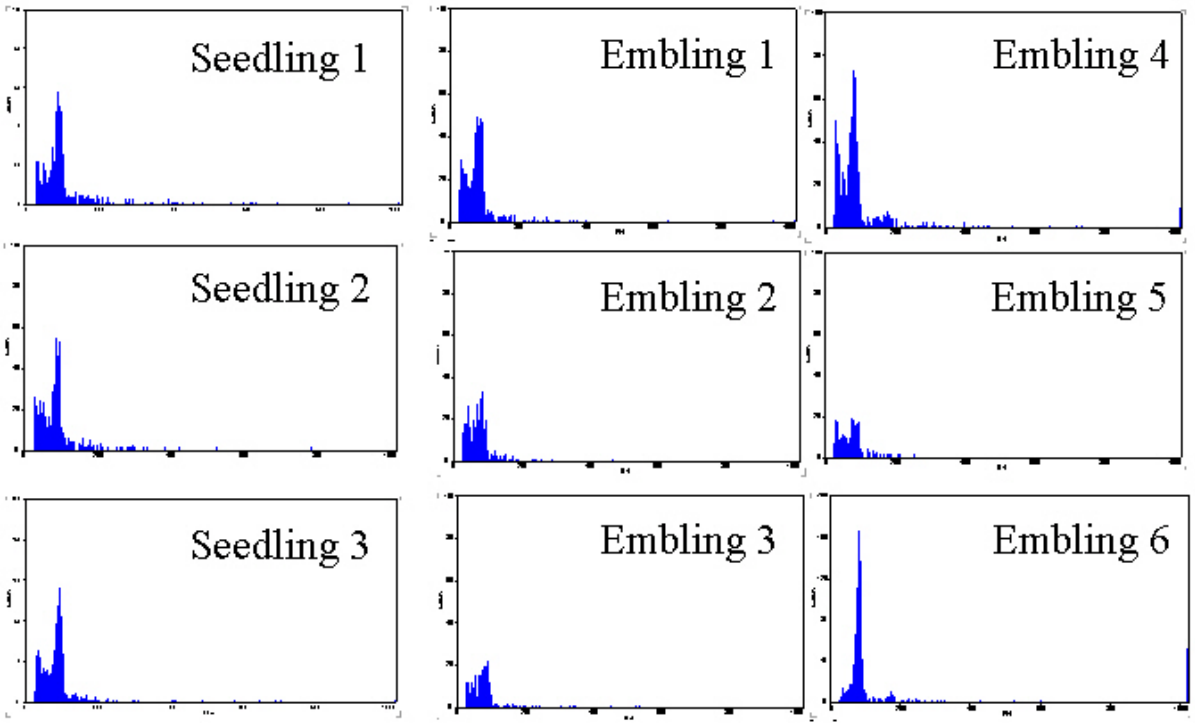

Fig. 6. *Flow cytometric comparison of leaves between zygotic and somatic seedlings of Liriodendron tulipifera*

References

Lichtenthaler, H.K. 1987. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods Enzymol. 148:350-383.

Litvay, J.D., Verma D.C. and Johnson, M.A. 1985. Influence of a loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Darcus carota* L.). Plant Cell Rep. 4:325-328.

Merkle, S.A. and Sommer, H.E. 1986. Somatic embryogenesis in tissue cultures of *Liriodendron tulipifera.* Can. J. For. Res. 16:420-422.

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

Schmidt, T. Ewald, A. Seyring, M. and Hohe, A. 2006. Comparative analysis of cell cycle events in zygotic and somatic embryos of *C. persicum* indicates strong resemblance of somatic embryos to recalcitrant seeds. Plant Cell Rep. 25:643-650.

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A Review of *in vitro* Micropropagation Techniques for *Kalopanax septemlobus* Trees

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Abstract

The *Kalopanax septemlobus* tree is native to Southeast Asia including Korea, Japan and China. Although *in vitro* vegetative propagation techniques have been developed for many different woody species, such technical application to *K. septemlobus* has been very limited. We here review *in vitro* micropropagation studies of the species, which have been mainly conducted by Korea Forest Research Institute (KFRI). Although axillary bud induction, shoot proliferation, root induction and soil acclimation of the plantlets have been conducted using 2-years-old trees for organogenesis, these approaches have not been successful from a practical point of view. On the other hand, *in vitro* micropropagation using somatic embryogenesis (SE) is attractive. In early research of somatic embryo induction, we first developed an optimal SE system using immature zygotic embryos. This resulted in normal plant regeneration, starting with embryogenic cell formation and ending with plant conversion. Later, the same system was applied to mature trees using grafting as a rejuvenation method. This finally established a protocol of mass propagation of *K. septemlobus*. In order to further improve the SE system, cell suspension culture was attempted. Artificial seed production, using algenic acid and seed germination on various artificial soil matrix, were also evaluated.

Key words: woody species, somatic embryogenesis, grafting rejuvenation

Introduction

Kalopanax septemlobus Koizi (Syn. *Kolopanax pictus*) is a perennial woody tree, which belongs to the *Araliaceae* family. This species has been used for various purposes such as timber production, traditional medicines and as a fresh vegetable source. In recent years, as many more people are starting to prefer "health" foods, the demand for this species is growing significantly. Although *K. septemlobus* produces abundant seeds, the germination of its seeds requires at least 2 years under natural conditions due to extreme seed dormancy. Rooted cuttings are inappropriate material for mass propagation (Yeoung *et al*. 2001). In vitro propagation has been used as an alternative for conventional propagation techniques. Commercial scale propagation and practical application using SE has recently been applied to many important species such as coffee, Norway spruce and loblolly pine (Ducos *et al*. 2003; Sutton 2002). In this review we discuss research aimed at *in vitro* propagation of *K. septemlobus*, mainly focusing on somatic embryogenesis.

Organogenesis

To induce efficient organogenesis, axillary shoot proliferation and rooting were tested with 2-year-old *K. septemlobus*. MS and WPM medium in combination with either BA or kinetin were used to induce shoot proliferation. There was no significant difference in the rate of shoot multiplication between the two media. On average two to three shoots per explant were induced on WPM medium supplemented with 0.5mg/L kinetin. The *in vitro* rooting rate was low (less than 10%) but was significantly enhanced up to 60% in *ex vitro* cuttings. Rooted plantlets were successfully transferred to an artificial soil mixture and acclimatized. More than 90% of the plants survived after 4 weeks. These results suggest the possibility of using shoot proliferation via axillary bud culture of *K. septemlobus* as a means to propagate this species. However this technique is inferior to the somatic embryogenesis system that was recently developed by Moon *et al*. (2002).

Somatic embryogenesis (SE)

SE from immature zygotic embryos

Embryogenic callus was obtained from zygotic embryos on MS medium with 1.0 mg/l 2,4-D and the rates of embryogenic callus induction were different depending on the days of seed harvest. Embryogenic callus was transferred to MS medium without 2,4-D, and subsequently produced cotyledonary somatic embryos within 6 weeks. Plant conversion from somatic embryos was low (35%) on hormone free MS basal medium, while it increased to 61% on the MS medium supplemented with 0.05% charcoal. GA_3 (5 mg/l) treatment markedly enhanced the germination rate of embryos (up to 83%). Ninety-eight percent of the plantlets obtained through somatic embryogenesis survived after transfer to TKS2 artificial soil matrix. Approximately 30,000 plantlets have been micropropagated via somatic embryogenesis and have grown in the field for 3 years. These results indicate that propagation of medicinal *K. septemlobus* trees is practical through somatic embryogenesis (Moon *et al*. 2005).

Synthetic seed production

The effects of alginic acid concentration, somatic embryo size, additives in the capsules and nursery

seedbeds on the germination of artificial seeds that were produced by encapsulating somatic embryos of *K. septemlobus* were investigated. The optimal concentration of alginic acid was 3%,; higher than 3% alginic acid suppressed the germination of the synthetic seeds. Half-strength MS medium containing 0.02% of activated charcoal was the optimal basal medium. There was no significant difference in the germination rate among the different sizes of somatic embryos. Additives in the hydrated capsules were very important for germination of the artificial seeds and for post-germination growth. Germination of hydrated capsules was severely inhibited if they contained distilled water only. The addition of sucrose or MS medium to the hydrated capsule promoted proper germination of the artificial seeds. When artificial seeds were directly transferred to a soil-bed composed of perlite with 3% sucrose, the germination rate was higher than in perlite with water only. These results indicate that nursery additives in both the hydrated capsules and in the soil-bed were important for germination of artificial seeds of *K. septemlobus* (Kim *et al*. 2007).

SE from mature trees

An effective micropropagation technique, via somatic embryogenesis, has been developed using tissue from serially grafted shoots of mature *K. septemlobus* trees (~40 years old). Callus was induced from leaf segments of grafts cultured on MS medium supplemented with 2,4-D and sucrose in the dark. The effects of sucrose, coconut water and polyethylene glycol (PEG-3350) on the development of somatic embryos from embryogenic callus were evaluated.

More than 90% of the explants formed callus; however, only 20 segments of a total of 800 explants (0.4%) formed embryogenic callus after 8 weeks of culture. High concentrations (3% and 5%) of sucrose induced somatic embryogenesis. The treatment with 2-10% coconut water also had a positive effect on somatic embryo induction. A synergistic effect on somatic embryo induction was obtained by using 7% sucrose and 10% PEG. Generally PEG treatment resulted in smaller, more uniform somatic embryos.

Embryo germination and conversion to plantlets were significantly influenced by the gelling agents in the 1/2MS medium. In general, better results were achieved with gelrite rather than with agar. In gelrite-gelled medium, gibberillic acid (GA_3) enhanced embryo germination regardless of the GA_3 concentrations, while in agar-gelled medium the conversion rate decreased as the GA_3 levels were increased. Subsequently 91% of plantlets were successfully transferred to an artificial soil mixture

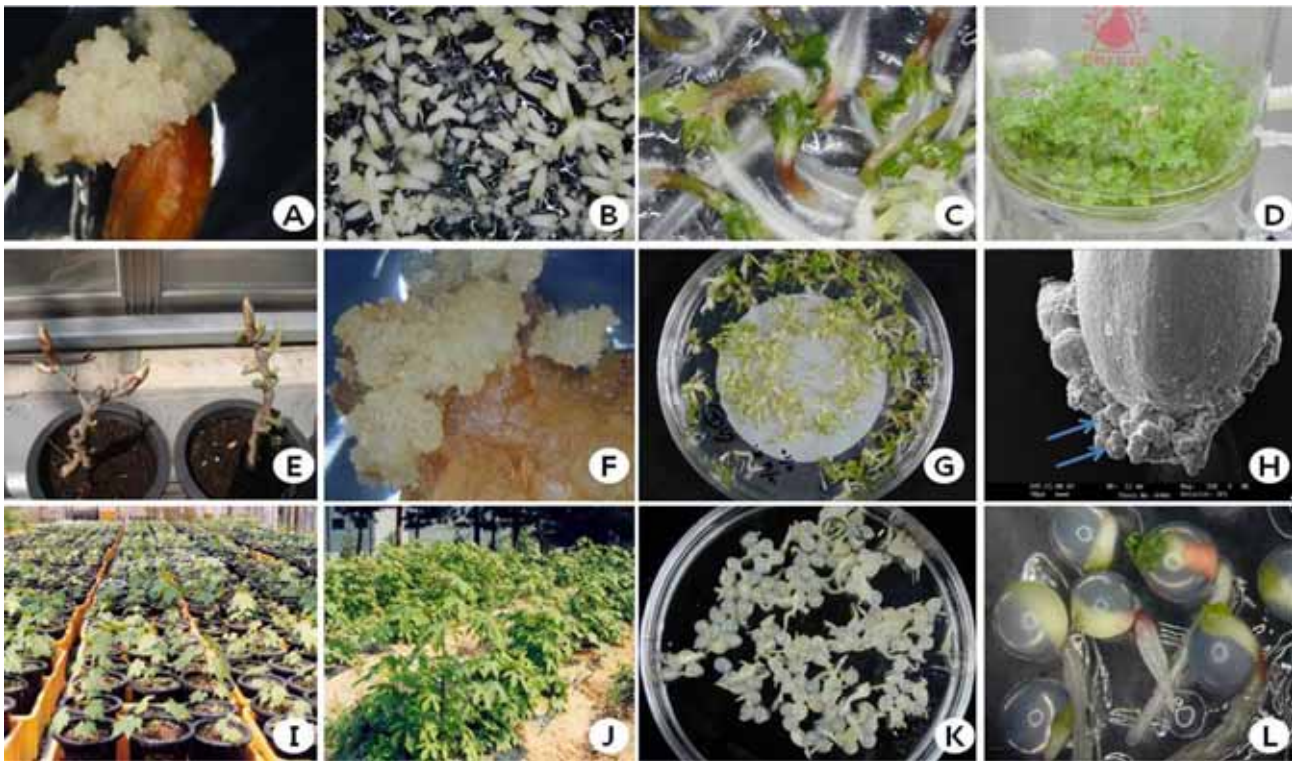

Figure 1. *Somatic embryogenesis and plant production in Kalopanax septemlobus tree. A~D: somatic embryogenesis and plant conversion from immature zygotic embryos (A- embryogenic callus from a zygotic embryo; B- somatic embryos at the early cotyledon stage; C- germinating somatic embryos; D- converted plants from SEs in a bioreactor culture system). E~J: SEs and plant propagation from a mature tree (E- mature tree scions grafted on juvenile rootstocks; F- embryogenic callus from leaf segment of the grafted scions; G- germinating somatic embryos synchronously; H- secondary somatic embryos formed at the radical part a of somatic embryo; I- acclimatized emblings in pots; J- 3-year-old plants grown at a nursery site; K– artificial seeds made by algenic acid; L- germinating artificial seeds.*

without distinct morphological alteration. This suggests that reliable somatic embryogenesis and plant propagation can be achieved by using tissues of mature *K. septemlobus* trees that were rejuvenated by serial grafting (Moon *et al*. 2008).

Establishment of a suspension culture system

Somatic embryogenesis can be improved by using a cell suspension culture system. Embryogenic cell suspensions were cultured in Erlenmeyer flasks, optimal cell density was monitored and the number of subcultures was recorded. The proliferation rate of embryogenic cells (ECs) was reduced as the inoculum density increased; the highest rate was obtained by adding cells at the rate of 0.1 g/100 ml to the medium. According to an analysis of the cell growth pattern and cell cycle (G1, S and G2/M), cell growth started within 5 days after the initiation of the cell culture, grew rapidly until day 15 and then decreased gradually. Distinctive changes in the cell growth cycle also depended on the culture periods; the cells at S-phase doubled from an initial 5.6% to 11.7% during 5 days of culture and then reached a plateau. Therefore, a 15-day-cycle was the optimal culture period for the propagation of ECs through suspension culture. Furthermore, the cell inoculum density was also important for the induction of SE; more than 65% of SEs developed to the torpedo stage when a low density of cell inoculum (0.5 g/L) was used. In contrast, higher inoculum densities rapidly reduced the proportion of SEs that reached the torpedo stage. Although the higher inoculum density delayed the development of SE, it did not affect the proportion of SEs at the globular and heart stage. In conclusion, this study showed that optimization of inoculum density in suspension cultures of *K. septemlobus* ECs was an efficient way for both the propagation of ECs and the induction of SEs. This suggests that further development of this system might reduce the culture period required for the production of somatic embryos (Kim and Moon 2009).

Genotype variation and aging effect

As shown for many other species, the induction rate of ECs of *K. septemlobus* was significantly dependent on genotype. To investigate the genotype-dependent embryogenic capacity, several parameters such as endogenous levels of various hormones, the content of DNA, the proliferation rate of embryogenic callus and somatic embryos were studied by using several genotypes of *K. septemlobus*. The effect of age of the trees on their embryogenic capacity was studied by using two embryogenic cell lines of each selected genotype. We observed that cytokinins/abscisic acid ratio in the explants played an important role in their embryogenic capacity. A decrease in both embryogenic capacity and DNA content was observed when frequently subcultured tissue was used as inoculum, suggesting that young embryogenic callus is better for the formation of somatic embryos than callus that was maintained long-term (Park *et al*. 2011. Plant Cell Tiss Org Cult. In press).

Conclusion

The demands for *K. septemlobus* are increasing in parallel with the continuing discovery of new pharmaceutical active substances in the species (Hyun and Kim, 2009). As an alternative to conventional propagation, *in vitro* propagation by SE has proven to be a powerful tool that can be applied for practical mass clonal propagation. Our results suggest that mature *K. septemlobus* trees can be propagated efficiently via SE in combination with rejuvenation by serial grafting (Figure 1). The SE system developed by KFRI can be applied to tree improvement programs of *K. septemlobus* and can also be helpful in studies aimed at obtaining a better understanding of tree phase change.

References

Ducos, J.P., Alenton, R., Reano, J.F., Kanchanomai, C., Deshayes, A., P´etiard, V. 2003. Agronomic performance of *Coffea canephora* P. trees derived from large-scale somatic embryo production in liquid medium. Euphytica. 131:215–223.

Kim, Y.W., Choi, Y.E., Yi, J.S., Moon, H.K. 2007. Germination of artificial seeds by encapsulation of somatic embryos of *Kalopanax septemlobus* with alginic acid. J Plant Biotechnology 34:229–235.

Kim, S.J., Moon, H.K. 2009. Establishment of suspension culture condition for embryogenic callus proliferation and somatic embryo development of *Kalopanax septemlobus*. J Plant Biotechnology 36:7-12.

Hyun, T.K., Kim, J.S. 2009. The pharmacology and clinical properties of *Kalopanax pictus*. J Medicinal Plants Research. 3:613–620.

Moon, H.K., Kim, S.H., Kim, B.K. 2002. Micropropagation of *Kalopanax pictus* Nakai via axillary bud cultures. Jour Korean For Soc. 91:75–780.

Moon, H.K., Kim, Y.W., Lee, J.S., Choi, Y.E. 2005. Micropropagation of *Kalopanax pictus* tree via somatic embryogenesis. In Vitro Cell Dev Biol-Plant. 41:303–306.

Moon, H.K., Park, S.Y., Kim, Y.W., Kim, S.H. 2008. Somatic embryogenesis and plantlet production using rejuvenated tissues from serial grafting of a mature *Kalopanax septemlobus* tree. In Vitro Cell Dev Biol-Plant. 44:119–127.

Park, S.Y., Cho, H.M., Moon, H.K., Kim, Y.W., Paek, K.Y. 2011. Genotype variation and aging effect in embryogenic capacity of *Kalopanax septemlobus*. Plant Cell Tiss Org Cult. In press.

Sutton, B. 2002. Commercial delivery of genetic improvement to conifer plantations using somatic embryogenesis. Ann For Sci. 59:657–661.

Yeoung, Y.R., Lee, M.H., Kim, B.S., Kim, H.K., Kim, J.H. 2001. Seed germination and softwood cutting technique of *Kalopanax pictus* Nakai. Korean J Plant Res 14:53–59.

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The Case for Hardwood Varietals: How Hardwood Somatic Embryogenesis Can Enhance Forest Productivity in the Southeastern United States

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Abstract

Forest industry in the southeastern U.S. has long focused on southern pines. By contrast, primarily due to problems with productivity, hardwoods have long played a secondary role in commercial forestry in the region. Impressive gains in pine productivity made by university/industry breeding programs over the past 50 years have recently been further enhanced by the rise of varietal forestry, based on somatic embryogenesis (SE) technology. The projected productivity gains with southern pine somatic seedling technology make a strong case for adopting parallel technologies with southern U.S. hardwoods. Given the rapidly-changing priorities in the region for new applications such as biomass energy, southern hardwood varietals are likely to become a much more important component of southern U.S. forestry. Systems for producing somatic seedlings of multiple southern U.S. hardwoods have been developed, and in some aspects are already more amenable to scale-up and other manipulations than conifer systems. In particular, the fact that hardwood SE cultures can not only be grown but manipulated as suspensions makes them powerful tools for genetic transformation and scaled-up somatic seedling production. The feasibility of scaling-up production of elite, varietal hardwoods using SE has already been demonstrated. By combining this propagation system with hybrid breeding, thousands of somatic seedlings of elite hybrid sweetgum (*Liquidambar*) and hybrid yellow-poplar (*Liriodendron*) have been generated, some of which have demonstrated impressive biomass productivity in field tests. In addition, the ability to initiate embryogenic cultures from mature tree tissues has made direct cloning of elite sweetgum genotypes possible. With regard to the Southern Appalachian Mountains, varietal blight-resistant American chestnuts (*Castanea dentata*) are now being developed as part of a new Forest Health Initiative for the purpose of species restoration. However, beyond restoration, elite chestnut varieties have enormous potential to be deployed in plantations for biomass energy and production of durable lumber for outdoor uses.

Introduction

With its long growing seasons, relatively inexpensive land and established forestry infrastructure, the southeastern United States appears to be a prime location for biomass energy plantations. Exactly what energy crop or crops would be the most suitable choice for the region is up for debate. Some varieties of switchgrass seem to offer promise of outstanding productivity in the southeast. However, switchgrass suffers from the disadvantage of not being amenable to long-term storage. It must be harvested on a schedule and used before it begins to deteriorate. By contrast, biomass produced by woody species can be stored "on the stump." Thus rapidly-growing forest trees might provide a more suitable source of bioenergy feedstocks for the region. It might seem that loblolly pine (*Pinus taeda*) would be the obvious choice for rapid production of woody biomass in the region. Not only is the fast-growing tree well-adapted to the soils and climate in the region, but it has benefited from decades of genetic improvement, established silvicultural protocols and forest industry infrastructure geared to get the most productivity from this type of tree. However, the pulp and paper industry of the region is highly reliant on loblolly pine as its primary feedstock and there is already some concern that an increase in demand for loblolly pine fiber for biomass may drive up pulpwood prices. The hardwood forest trees most commonly considered for biomass energy plantations are hybrid poplars (e.g. Tuskan 1998) and willows (e.g. Keoleian and Volk 2005). However, hybrid poplars have yet to be proven broadly suitable for plantation culture in the southern U.S., and the climatic extremes that characterize the region, in particular drought, may severely limit their productivity (Tschaplinski *et al.* 1998). Given recent progress with poplar genomics, it may be possible to engineer selected hybrid poplar clones with genes targeting drought tolerance. However, to date, no transgenic forest trees have been approved for deregulation in the U.S., making it likely that deployment of such drought-resistant poplars is several years off. Willows, which are mainly of Boreal origin, are characterized by very high evapo-transpiration rates, and therefore would probably require irrigation to survive and make rapid growth (Kozovkina and Quigley 2005). Some *Eucalyptus* species and hybrids characterized by outstanding biomass production rates of up to 25 dry tons/hectare/year have been proposed as potential biomass producers for the region, and *Eucalyptus grandis* x *Eucalyptus urophylla* hybrid clones engineered with a cold-tolerance gene that would enable their survival in the region have been produced (Hinchee *et al.* 2009). However, deployment of genetically engineered *Eucalyptus* trees in the U.S. may be delayed by several years, due to opposition from anti-GMO groups, which cite potential issues of

invasiveness, high water use and fire hazard associated with the genus, in addition to their general opposition to releasing a genetically engineered exotic tree. Thus there is a need for alternative short-rotation woody crops especially adapted for growth in southeastern soils and climatic conditions.

One intriguing alternative to the above-mentioned options would be to grow short-rotation plantations of fast-growing hardwood trees with strong coppicing ability that evolved in the southeastern U.S. and thus are already well-adapted to conditions in the region. Employing native short-rotation hardwoods for biomass production in the southeastern U.S. is certainly not a new idea. In fact, the idea of planting and harvesting such trees like an agricultural row crop was conceived and introduced as "silage sycamore" over 40 years ago (McAlpine *et al.* 1966). Although southern hardwoods are rarely mentioned in current technical reviews of potential feedstocks for cellulosic biomass energy or in popular press articles on the subject, a number of southern hardwoods, including American sycamore (*Platanus occidentalis*), sweetgum (*Liquidambar styraciflua*), yellow-poplar (*Liriodendron tulipifera*) and black locust (*Robinia pseudoacacia*), were extensively tested for biomass productivity (e.g. Steinbeck and Skinner 1985; Steinbeck 1999) in the 1970s and 1980s. The fact that substantial research has already been done on growing these trees on short rotations, including analysis of spacing, weed control and energy balance data, means there is no reason to "reinvent the wheel" in order to put these trees into production. In addition, a bioenergy product that can make ready use of biomass from these species as feedstocks is already being manufactured in the region. Factories producing biopellets, mainly for export to European countries, are already operating in Georgia, Alabama and Florida. The pellets are used for generating electricity via co-firing with coal or gasification. Although these factories currently use forest residues from harvesting and sawmill operations as feedstocks, they will soon require dedicated sources of biomass, a substantial proportion of which could be provided by short rotation southern hardwoods.

Many advances in silviculture and other aspects of plantation forestry have been made since the 1980s that have the potential to further enhance biomass production efficiency and feedstock uniformity. Not least among these is the rise of "varietal forestry." Elite varieties of loblolly pine (*Pinus taeda*), slash pine (*Pinus elliottii*) and other commercially important conifers are already being propagated by the millions using somatic embryogenesis (SE) by companies like CellFor and ArborGen, for forestry operations. Over the past few decades, our program at the University of Georgia has made advances developing SE technology for a number of important southern hardwood species, including elite varieties and

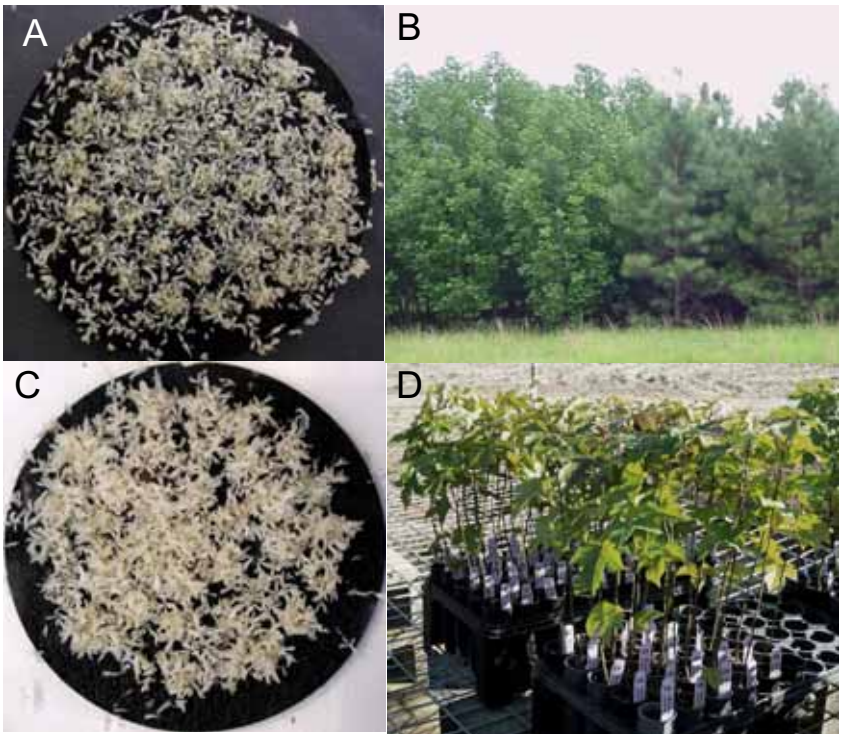

Figure 1. *Hybrid yellow-poplar and hybrid sweetgum somatic embryogenesis.* ***A.*** *Developing yellow-poplar somatic embryos following size-fractionation and plating of embryogenic suspension cultures.* ***B.*** *Some hybrid yellow-poplar clones in a test planting near Bainbridge, GA in their fifth growing season grew at least as fast as the adjacent loblolly pine trees.* ***C.*** *Hybrid sweetgum somatic embryos following size-fractionation and plating of embryogenic suspension culture.* ***D.*** *Hybrid sweetgum somatic seedlings prior to planting in field test.*

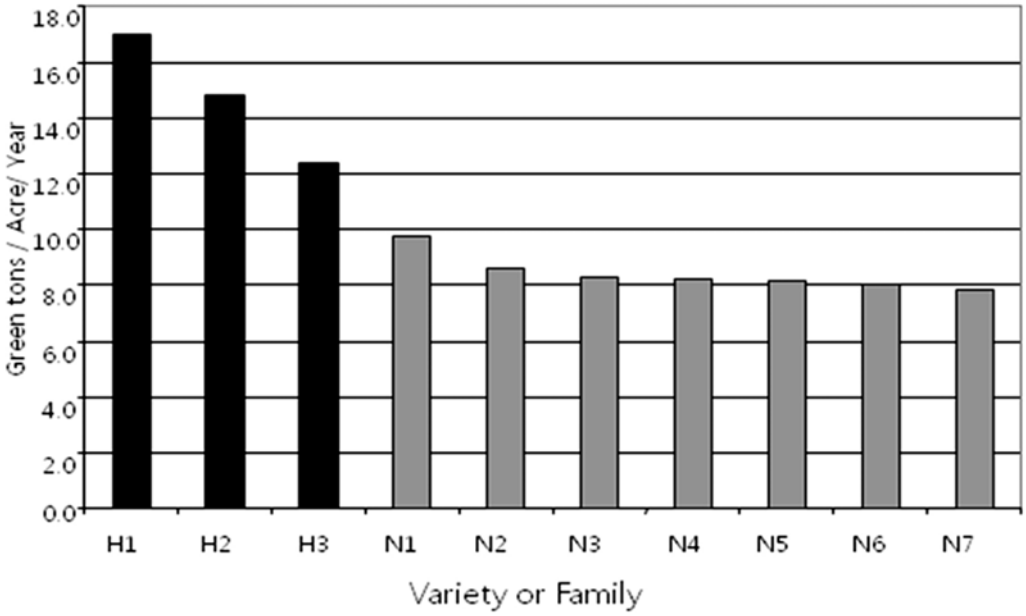

Figure 2. *Biomass yield at 8 years for American and hybrid sweetgums on an upper coastal plain site in South Carolina. H1 – H3 are somatic seedlings from the three top-performing hybrid varieties in the test. N1- N7 are American sweetgum seedlings from second generation elite open-pollinated families.*

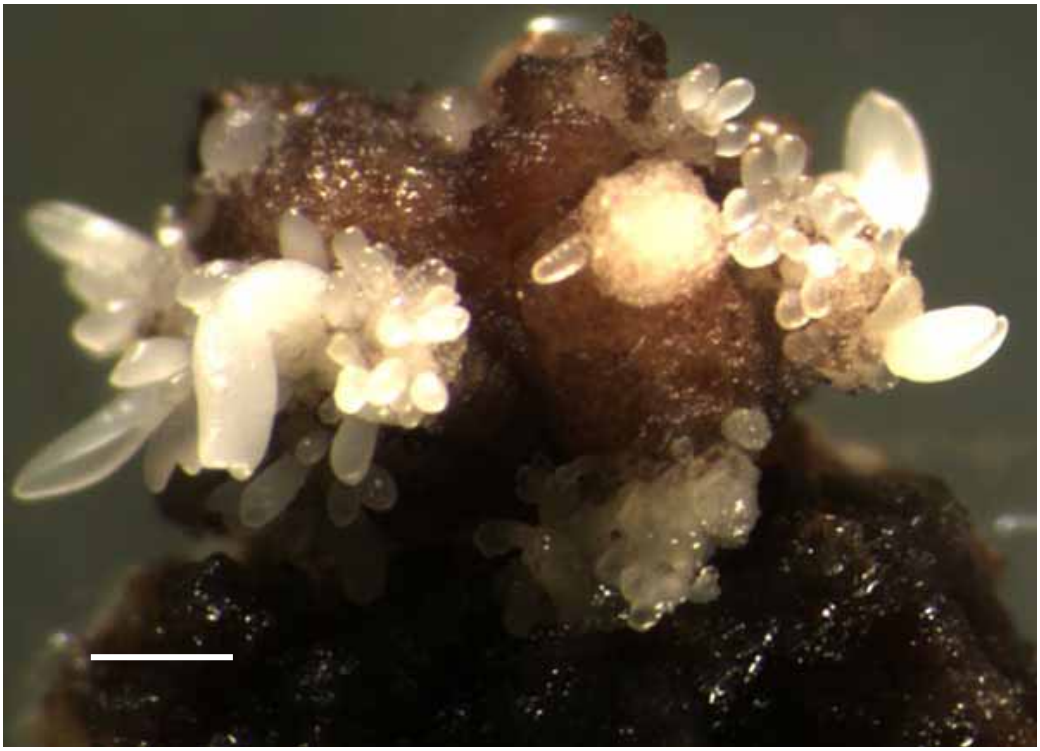

Figure 3. *Somatic embryos arising from a staminate inflorescence dissected from a dormant bud of an elite hybrid sweetgum tree and cultured on medium with NAA. Bar is 1 mm.*

hybrids. These advances parallel the advances with pine SE technology and in some cases may offer even greater potential for operational scale-up. Here, I will give some examples of the SE-based propagation systems we have developed for southern hardwoods and how some may make major contributions to cellulosic biomass production in the southeastern U.S.

Yellow-poplar model system

Yellow-poplar was the first species for which we developed an embryogenic propagation system (Merkle and Sommer 1985), and became the model for all the succeeding systems we developed for hardwood trees. Briefly, the cultures are initiated from immature seeds collected in July, when zygotic embryos are at the globular to early heart stage. Culturing the immature seed embryos on semisolid medium with 2 mg/l 2,4-D induces the embryos to form proembryogenic masses (PEMs). PEMs continue to rapidly proliferate with monthly transfer and the cultures can be maintained for several years this way with no loss in SE production potential. PEMs grow more rapidly as suspension cultures and populations of thousands of singularized, synchronous somatic embryos can be generated by size-fractionating the PEMs on stainless steel screens and collecting the fraction of PEMs between 38 and 400 µm on a filter paper in a Büchner funnel for culture on semisolid basal medium (Merkle *et al.* 1990). Yellow-poplar somatic embryos are picked from the filters and moved to germination medium, where they readily convert to somatic seedlings and these are transferred to potting mix, hardened off and moved to the greenhouse or shade house with very high survival rates.

Hybrid sweetgum and hybrid yellow-poplar varieties

While both yellow-poplar and sweetgum are generally characterized by fast growth, genetic improvement programs for these trees have lagged far behind that of the top commercial southern pines. One way to rapidly breed for elite, fast-growing trees is via hybrid breeding. Just as individual hybrid poplar clones have shown promise for biomass production, we hypothesized that crossing two *Liriodendron* species or two *Liquidambar* species would produce populations of hybrids from which individual heterotic clones could be selected for superior productivity in the southeastern U.S. Both yellow-poplar and sweetgum have counterparts native to eastern Asia, which have been separated from the North American species for at least 10 million years by continental drift (Parks and Wendel 1990). Chinese tuliptree (*Liriodendron chinense*), native to China and Vietnam, is interfertile with yellow-poplar (Santamour 1972a; Parks *et al.* 1983), and Formosan sweetgum (*Liquidambar formosana*), found in the temperate forests of eastern Asia, is interfertile with American sweetgum (Santamour 1972b). To produce hybrid *Liriodendron* clones, we worked with collaborators at the University of Tennessee and the University of North Carolina to pollinate selected yellow-poplar mother trees with pollen collected from Chinese tuliptree parents. Then, the immature hybrid seeds were explanted to initiate multiple hybrid embryogenic cultures, from which somatic seedlings were produced (Merkle *et al.* 1993). In later work, handling of embryogenic suspension cultures was optimized to provide up to 25,000 embryos per 0.5 g of starting material in a 125 ml flask (Fig. 1A; Dai *et al.* 2004). Yellow-poplar hybrid somatic seedlings have shown promising performance in a field test in south Georgia (Fig. 1B).

Banks of hybrid sweetgum clones were generated in a similar manner to the hybrid yellow-poplar clones. Selected American sweetgum mother trees were pollinated with pollen collected from unimproved Chinese sweetgum parents and the resulting seeds were used to initiate embryogenic cultures, from which hybrid somatic seedlings were produced (Vendrame *et al.* 2001). Suspension cultures of some of the hybrid lines were capable of producing over 6000 embryos from 0.5 g of starting material in a 125 ml flask (Fig. 1C; Dai *et al.* 2004). Thousands of hybrid sweetgum somatic seedlings were produced from multiple hybrid lines (Fig. 1D) and planted in field tests by industry cooperators. Based on volume and wood density measurements taken during the eighth growing season in the field, some of the hybrid varieties are out- performing seedlings from elite, open-pollinated second generation American sweetgum families with regard to biomass productivity, with one hybrid clone producing over 16 green tons/acre/year (Fig. 2). Embryogenic cultures of both the yellow-poplar and sweetgum hybrids have been successfully cryostored and recovered (Vendrame *et al.* 2001), so that the cultures can be held for years while somatic seedlings are field tested to identify elite varieties for scaled-up production.

Cloning elite sweetgum varieties using SE

While embryogenic cultures initiated from zygotic embryos are a powerful propagation tool, the full power of SE for propagation could be realized if embryogenic cultures could be initiated directly from tissues of mature trees that have already demonstrated elite performance in the field. We have developed such a system for sweetgum. Pieces of staminate inflorescences (clusters of male flowers) dissected from dormant buds and cultured on medium with thidiazuron (TDZ) or naphthaleneacetic acid (NAA) produce small clusters of somatic embryos after a few months, and some of these continue to produce new somatic embryos for years (Merkle *et al.* 1998; Merkle and Battle 2000; Merkle *et al.* 2003). We recently applied this approach to re-constitute embryogenic cultures of some of the elite hybrid sweetgum clones for which we did not have cryostored cultures (Fig. 3; Merkle *et al.* in press).

American chestnut varieties using SE

Prior to the early 20th century, American chestnut (*Castanea dentata*) was one of the most important forest trees in the United States, accounting for one in four

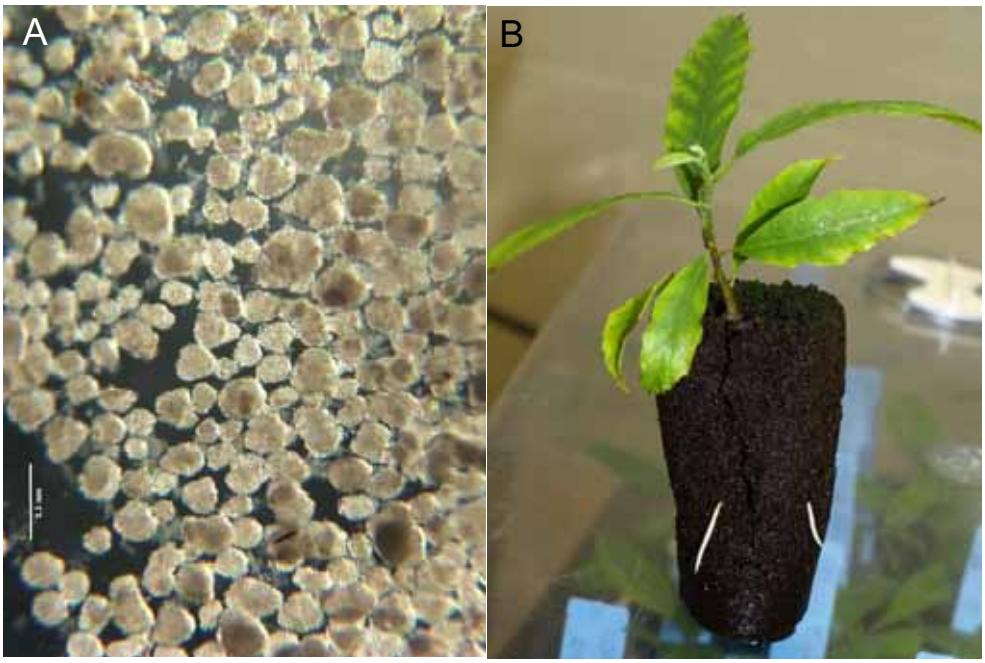

Figure 4. *American chestnut somatic embryogenesis.* ***A.*** *Suspension culture of American chestnut somatic embryos following size fractionation to synchronize development. Bar is 500 µm.* ***B.*** *American chestnut somatic seedling 8 weeks following germination on a polymerized peat plug.*

trees in the southern Appalachian forest. It had many uses, including construction lumber, furniture, tannins, fuel wood and nuts. Its timber was especially well-known for its durability and was widely used for outdoor purposes. The accidental introduction of the chestnut blight fungus, *Cryphonectria parasitica,* on Asian chestnut stock around the turn of the century resulted in the death of virtually every mature American chestnut tree within 40 years (Anagnostakis 1987). Before the blight arrived, American chestnut was among the fastest-growing species on upland sites in the Appalachian region. If the tree could be restored, it would have the potential not only to return to its prior status as a top timber provider, but its rapid growth and coppicing ability would make it an ideal candidate for short-rotation energy plantations on the currently underutilized slopes of the southern Appalachians. For example, a recent study indicated that American chestnut significantly outperformed northern red oak (*Quercus rubra*) and black walnut *(Juglans nigra*) when grown in a blight-free plantation setting in Wisconsin (Jacobs and Severeid 2004). Research on chestnut and chestnut blight should result in the availability of blight-resistant genotypes within the next 5 years. The American Chestnut Foundation's (TACF) breeding program has worked for the past 25 years using hybrid backcross breeding to introgress blight-resistance genes from the Chinese chestnut (*Castanea mollissima*) into American chestnut. TACF's first B3F3 seedlings, carrying (on average) 15/16 of the American chestnut genome and 1/16 of the Chinese chestnut genome, should express the blight resistance of Chinese chestnut and possess the canopy tree form of American chestnut (Hebard 2006). While TACF's goal is restoration of the species, the potential utility of varietal chestnut plantations for biomass and other applications is enormous, and SE technology developed in our lab and by collaborating labs is likely to be critical for mass propagation of the best chestnut varieties. In addition, the ability to regenerate American chestnut trees via SE also opens the potential to directly engineer blight-resistance genes into the tree.

An SE-based propagation system for the American chestnut has been under development for almost two decades (Merkle *et al.* 1991; Carraway *et al.* 1994; Xing *et al.* 1999, Robichaud *et al.* 2004), but only in the past five years has a scalable, suspension culture-based system become a reality (Andrade and Merkle 2005). Embryogenic American chestnut cultures are started from immature seeds. Green burs with nuts containing seeds at the correct stage of development are collected and the nuts are removed from the burs, surface-disinfested and the developing seeds dissected from them for culture. Only about 3% of the explanted seeds produce embryogenic cultures (Carraway and Merkle 1997). As with the yellow-poplar and sweetgum cultures, using suspension culture and our size-fractionation protocol allows the production of large populations of relatively synchronous, singularized chestnut somatic embryos (Fig. 4A). At least 12 weeks of cold treatment is needed for the somatic embryos to germinate and adding activated charcoal to the germination medium aids somatic seedling growth (Andrade and Merkle 2005). Temporary immersion bioreactors are being tested to see if they will improve somatic embryo maturation and germination. Direct

germination of somatic embryos in polymerized peat plugs in vitro is also being tested to see if it may enhance root system development compared to germination in gelled medium (Fig. 4B; Maner and Merkle 2010).

Embryogenic American chestnut suspension cultures have also been used to develop a gene transfer system for the tree. Andrade *et al.* (2009) co-cultivated embryogenic chestnut cultures with *Agrobacterium tumefaciens* carrying pCAMBIA2301, which includes the *nptII* selectable marker gene and *uidA* reporter gene, and subsequently used selection with geneticin in suspension culture to obtain transgenic material. Somatic seedlings were regenerated from the cultures, some of which have already produced staminate flowers (Andrade *et al.* 2009). Using this protocol, chestnut somatic seedlings expressing different candidate genes for blight resistance have been regenerated, although it will be some time before these are large enough to be screened for blight resistance. As part of the new Forest Health Initiative, multiple labs at different institutions in the U.S. are using genomics to identify Chinese chestnut genes that may be associated with blight resistance, and the plan is to clone these genes and engineer them directly into American chestnut. This approach may avoid the decades of hybrid breeding and backcrossing required by conventional breeding programs.

Conclusions

Combined with the substantial knowledge base already available on growing short rotation southern hardwoods in the southeastern U.S., hardwood SE technology can help the region realize its potential as a prime location for growing these trees for biomass energy. Some qualities of hardwood SE cultures, in particular the ability to manipulate them as suspensions for scaled-up propagation and gene transfer applications, make them especially useful tools for hardwood tree improvement. Hybrid breeding and SE technology are a powerful combination that could provide new short-rotation woody crops with outstanding biomass productivity for the region.

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References

Anagnostakis, S.L. 1987. Chestnut blight: the classical problem of an introduced pathogen. Mycologia. 79: 23-37.

Andrade, G.M., Merkle, S.A... 2005. Enhancement of American chestnut somatic seedling production. Plant Cell Rep. 24:326-334.

Carraway, D.T., Wilde, H.D., Merkle, S.A. 1994. Somatic embryogenesis and gene transfer in American chestnut. J. Amer. Chestnut Foundation. 8:29-33.

Carraway, D.T., Merkle, S.A. 1997. Plantlet regeneration from somatic embryos of American chestnut. Can. J. For. Res. 27:1805-1812.

Dai, J., Vendrame, W., Merkle, S.A. 2004. Enhancing the productivity of hybrid yellow-poplar and hybrid sweetgum embryogenic cultures. In Vitro Cell. Dev. Biol.- Plant40:376-383.

Hebard, F.V. 2006. The backcross breeding program of the American Chestnut Foundation. In: Steiner K.C., Carlson, J.E. (eds.), Restoration of American chestnut to forest lands—Proceedings of a Conference and Workshop, May 4-6, 2004. The North Carolina Arboretum, Natural Resources Report NPS/NCR/CUE/NRR — 2006/001, USDI National Park Service, pp. 61-77.

Hinchee, M., Rottmann, W., Mullinax, L., Zhang, C. S., Chang, S. J., Cunningham, M., Pearson,L., Nehra, N. 2009. Short-rotation woody crops for bioenergy and biofuels applications. In Vitro Cell. Dev. Biol – Plant 45: 619-629.

Jacobs, D. F., Severeid, L.R. 2004. Dominance of interplanted American chestnut (*Castanea dentata*) in southwestern Wisconsin, USA. For. Ecol. Man. 191: 111-120.

Keoleian, G.A., Volk, T.A. 2005. Renewable Energy from Willow Biomass Crops: Life Cycle Energy, Environmental and Economic Performance. Crit. Rev. Plant Sci. 24:385-406.

Kozovkina, Y.A., Quigley, M.F. 2005. Willows beyond wetlands: uses of *Salix* L. species for environmental projects. Water Air Soil Poll. 162:183-204.

Maner, L., Merkle, S. 2010. Polymerized peat plugs as an alternative matrix for germinating American chestnut somatic embryos in vitro. J. Amer. Chestnut Foundation. 24:16.

McAlpine, R.G., Brown, C.L., Herrick, A.M., Ruark, H.E. 1966. Silage sycamore. Forest Farmer 26: 6-7.

Merkle, S.A., Sommer, H.E. 1986. Somatic embryogenesis in tissue cultures of *Liriodendron tulipifera* L. Can. J. For. Res. 16:420-422.

Merkle, S.A., Hoey, M.T., Watson-Pauley, B.A., Schlarbaum, S.E. 1993. Propagation of *Liriodendron* hybrids via somatic embryogenesis. Plant Cell Tiss. Organ Cult. 34:191-198.

Merkle, S.A., Sotak, R.J., Wiecko, A.T., Sommer, H.E. 1990. Maturation and conversion of *Liriodendron tulipifera* somatic embryos. In Vitro Cell. Dev. Biol. 26:1086-1093.

Merkle, S.A., Wiecko, A.T., WatsonPauley, B.A. 1991. Somatic embryogenesis in American chestnut. Can. J. For. Res. 21:16981701.

Merkle, S.A., Neu, K.A., Battle, P.J., Bailey, R.L. 1998. Somatic embryogenesis and plantlet regeneration from immature and mature tissues of sweetgum (*Liquidambar styraciflua*). Plant Sci. 132:169-178.

Merkle, S.A., Battle, P.J. 2000. Enhancement of embryogenic culture initiation from tissues of mature sweetgum trees. Plant Cell Rep. 19:268-273.

Merkle, S.A., Battle, P.J., Ware, G.O. 2003. Factors influencing production of inflorescence-derived somatic seedlings of sweetgum. Plant Cell Tiss. Org. Cult. 73:95-99.

Merkle, S, Montello, P., Kormanik, T., Le, H. Propagation of novel hybrid sweetgum phenotypes for ornamental use via somatic embryogenesis. Propagation of Ornamental Plants (in press).

Parks, C.R., Wendell, J.F. 1990. Molecular divergence between Asian and North American species of *Liriodendron* (Magnoliaceae) with implications for interpretation of fossil floras. Amer. J. Bot. 77: 1243-1256.

Parks, C.R., Miller, N.G., Wendel, J.F., McDougall, K.M. 1983. Genetic divergence within the genus *Liriodendron* (Magnoliaceae). Ann. Missouri. Bot. Gard. 70:658-666.

Robichaud, R.L., Lessard, V.C., Merkle, S.A. 2004. Treatments affecting maturation and germination of American chestnut somatic embryos. J. Plant Physiol. 161:957-969.

Santamour, F. S. 1972a. Interspecific hybrids in *Liriodendron* and their chemical verification. For. Sci. 18:233-236.

Santamour, F.S. 1972b. Interspecific hybridization in *Liquidambar*. For. Sci. 18:23-26.

Steinbeck, K. 1999. Thirty years of short-rotation hardwoods research. In: Proceedings of the Tenth Southern Silvicultural Conference, February 16-18, 1999, Shreveport, LA, pp. 63-65.

Steinbeck, K., Skinner, T.M. 1985. Growing short-rotation forests in the southeastern U.S.A. In: Egneus, H., Ellegard, A. (eds.), Bioenergy 84, Vol. 2. Biomass Resources (Elsevier, London, pp. 63-69.

Tschaplinski, T.J., Tuskan, G.A., Gebre, G.M., Todd, D.E. 1998. Drought resistance of two hybrid *Populus* clones grown in large-scale plantation. Tree Physiol. 18:653-658.

Vendrame, W.A., Holliday, C.P., Montello, P.M., Smith, D.R., Merkle, S.A. 2001. Cryopreservation of yellow-poplar (*Liriodendron tulipifera*) and sweetgum (*Liquidambar* spp.) embryogenic cultures. New For. 21:283-292.

Vendrame, W.A., Holliday, C.P., Merkle, S.A. 2001. Clonal propagation of hybrid sweetgum (*L. styraciflu*a X *L. formosana*) via somatic embryogenesis. Plant Cell Rep. 20:691-695.

Xing, Z., Powell, W.A., Maynard, C.A. 1999. Development and germination of American chestnut somatic embryos. Plant Cell Tiss. Org. Cult. 57: 47-55.

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Propagation of Cork Oak Selected Trees by Somatic Embryogenesis: from Solid to Liquid Medium

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Abstract

The cork oak (*Quercus suber* L.) is one of the most important broadleaved species of the Mediterranean ecosystem. This evergreen tree produces cork, used for several industrial applications, and acorns to feed pigs of the Iberian race. We developed a protocol that allows the cloning of adult cork oak trees by somatic embryogenesis. More than forty selected trees have been cloned, and clonal tests established by the Spanish company TRAGSA are in progress to validate these genotypes as tested varieties. To implement multivarietal forestry with cork oak, a mass propagation system using liquid medium is required. Recurrent proliferation gives somatic embryogenesis its high production ability. In our protocol regeneration of cork oak takes place on gelled medium lacking plant growth regulators, with almost no callus production. Secondary embryos arise directly from primary somatic embryos, forming clusters of embryos that are quite different from the usual undifferentiated explants used for establishing suspension cultures. However, a method for the initiation of suspensions with embryogenic ability has been defined. Factors affecting multiplication and differentiation phases are being studied using Erlenmeyer flasks as culture vessels. The multiplication rate when culturing small embryogenic clumps was relatively high, ranging between 2 to 3 per week. The type of flask, the orbiting speed and their interaction affected both the number of newly formed embryogenic clumps and their size. At K_{La} values between 0.11 h^{-1} and 1.47 h^{-1}, which are obtained under standard working conditions, oxygen availability was not a limiting factor for growing. Genotype plays a substantial role. Some genotypes proliferate as proembryogenic masses while others do so as more organized embryogenic clumps. Adding different combinations of plant growth regulators to the culture medium did not modify this behaviour.Three different types of cork oak somatic embryo differentiation occur in suspension cultures. From embryogenic clumps, embryos usually develop resembling those obtained by the multicellular pathway of regeneration in semisolid cultures, forming embryo clusters. The second type of differentiation occurs in differentiated embryos or in organized structures with polar shape; it often shows globular embryos that separate from the surface of the culture and that develop into isolated cotyledonary embryos. The third type happens in small non-organized aggregates of a few cells; sometimes globular embryos appear that are attached to these aggregates; these embryos follow the developmental pattern observed in model species such as carrot. Conditions influencing these types of differentiation are under study.

Key words: Forest biotechnology, micropropagation, multivarietal forestry, *Quercus suber*, vegetative propagation

The cork oak

The cork oak is an evergreen hardwood tree that grows in the Mediterranean region. The Iberian Peninsula has the biggest area, with Portugal accounting for 33% of its distribution and Spain 23%, followed by Argelia, Morocco, Italy, Tunisia and France. In Spain and Portugal this species grows mainly in agroforest systems, called "dehesas" and "montados" respectively, which have great ecological and socioeconomic value (Martín-Vicente and Fernández-Alés 2006).

http://wwf.panda.org/what_we_do/where_we_work/mediterranean/about/forests/cork

From an economic point of view the cork oak can be considered a multipurpose species:
This forest tree produces acorns used to feed Iberian pigs, which are important in the high quality food industry. It also forms mycorrhiza with edible fungus, some of them gastronomically very appreciated, such as *Boletus edulis*, *Amanita caesarea* and *A. Rubescens* (Machado and Santos 2002). The main product is cork, used in wine bottling and other industrial applications (Silva *et al*. 2005), which is sustainably harvested from trees periodically each 9-12 years. In addition, recent studies have indicated that some cork processing by-products have several bioactive properties, such as insecticidal and phytotoxic qualities, selective cytotoxicity in insect and mammalian cells, and antiparasitic effects (Moiteiro *et al*. 2006). Therefore, the economic importance of this species justifies the implementation of genetic improvement programs and the development of different tools for implementing multi-varietal forestry, such as clonal regeneration by somatic embryogenesis.

Following the Council Directive 1999/105/EC of the European Union, on the marketing of forest reproductive material, nine main provenance regions of cork oak and thirteen restricted areas have been determined in Spain (Díaz *et al*. 1995). Studies on the genetic variation of the species have been carried out (Jiménez *et al*. 1999,

Jiménez *et al*. 2004, Soto *et al*. 2007), and genetic improvement programs of the species have started with different selections of plus trees (García-Valdecantos and Catalán 1993, Catalán *et al*. 1997, Bueno *et al*. 2002). Also, provenance and progeny tests were established (Castro *et al*. 2003) and technical guidelines for conservation of genetic resources of the species have been outlined recently (Gil and Varela 2008).

Somatic embryogenesis of cork oak on semisolid medium

Somatic embryogenesis is the enabling propagation biotechnology for implementing multi-varietal forestry (Park 2004). It is also the most suitable regeneration technique to obtain transgenic plants from transformed cells (Merkle and Nairn 2005). Our team is involved in defining protocols for regeneration by somatic embryogenesis in cork oak. We first observed the formation of embryo-like structures in fragments of mature acorns (Toribio 1986). Somatic embryo initiation and plantlet regeneration was described in immature zygotic embryos of the species (Bueno *et al*. 1992). As in other species , in zygotic embryos of cork oak there is a developmental window of response for inducing the embryogenic response. A genetic control on this window was observed: different half-sib families showed different developmental window profiles (Fernández-Guijarro 1997). The discovery that somatic embryogenesis can be induced in leaves from young seedlings, was a breakthrough. While 2,4-D was ineffective, a mixture of NAA and BAP, first at high concentration and then at low concentration, induced the embryogenic process in intact leaves (Fernández-Guijarro *et al*. 1994). All somatic embryos formed secondary embryos on medium lacking plant growth regulators, giving rise to embryogenic lines that could maintain the recurrent process for years (Fernández-Guijarro *et al*. 1995). The secondary embryos were mainly of multicellular origin, but embryos of unicellular origin were also observed (Puigderrajols *et al*. 1996).

The next important discovery was that the same protocol could be used to induce somatic embryogenesis in leaves from adult trees. These leaves were taken from epicormic shoots that had been obtained by forcing normally dormant buds in pieces of branches taken from the crown (Hernández *et al*. 2001). The protocol was optimised, showing that a critical factor to obtain somatic embryos was the size of the expanding leaves used as starting explants (Hernández *et al*. 2003a). This protocol was used to recover clonal plants from all the seven selected trees that were ramdomly chosen to study the effect of the time of collection on the induction of somatic embryogenesis (Hernández *et al*. 2003b).

Therefore, currently a very robust protocol to clone any adult cork oak tree is available (Toribio *et al*. 2005). It has been used to obtain clonal copies for conservation of an endangered and very valuable genotype of cork oak that grows in Minorca, one of the Balearic Islands in Spain (Hernández *et al*. 2008, Lorenzo *et al*. 2009). In order to compare plants of somatic and zygotic origin, and plants from somatic embryos induced in leaves from adult trees and in leaves from seedlings, a field trial was established (Celestino *et al.* 2009). As a practical application of these studies 44 cork oak trees selected for productivity and quality of cork in Extremadura (southwest Spain) have been cloned in collaboration with the Spanish company TRAGSA. Clonal tests to validate them as varieties are in progress (Hernández *et al*. 2011).

Culture of embryogenic tissues of cork oak in liquid medium

Having developed a robust protocol to clone selected cork oak trees, and having started trials to test selected varieties, now the challenge is to be able to mass produce plants at reasonable cost. To achieve this goal it is necessary to develop a system for culture in liquid medium.

The most common way to initiate embryogenic suspension cultures begins with proembryogenic masses (PEMs) growing on semisolid medium supplemented with auxins, usually 2,4-D, which are innoculated in the same medium lacking the gelling agent (Finer 1994). However in cork oak the embryogenic tissue under recurrent proliferation does not take the form of PEMs. Completely differentiated somatic embryos, up to their cotyledonary stage, are continuously produced on medium without plant growth regulators, forming clusters of embryos. Suspension cultures have to be initiated with these unusual explants.

We observed in previous studies that sometimes friable, proliferating masses detached from embryo clusters when these were transplanted onto semisolid medium. When these embryo clusters were shaken in liquid medium, and the remaining supernatant was collected and subcultured again onto semisolid medium, embryogenic cultures were restarted. Microscopical examination of the supernatant showed isolated cells and cell aggregates that developed globular and torpedo embryos (Puigderrajols *el al*. 1996). With this basis, we defined a protocol for establishing embryogenic suspensions. To initiate cultures in liquid medium we tried several procedures, but the best one was to place embryo clusters from semisolid cultures in liquid medium, apply a vigorous and brief shake, collect the fraction between 41 and 800 µm, and then inoculate it into fresh medium without regulators (Jiménez *et al*. 2007). The growth of this fraction showed initial differences among genotypes, mainly in proliferation and differentiation abilities.

The next step for establishing the suspension is the stabilization of embryogenic cultures. To do that we followed the “preferential subculture” and “low inoculum rule” methodologies defined by Finer (1994) transferring selected embryogenic clumps monthly into fresh medium. This way the embryogenic suspensions could be maintained. At the beginning cultures tended preferentially to differentiate rather than to proliferate. However, after three months of culture, proliferation was predominant. In consequence the features of cultures gradually change, but the type of production is still characteristic of each genotype. To date, we have initiated suspension cultures

with embryogenic material from twelve selected trees from several Spanish provenances. Although they showed different growth pattern, all of them produced the embryogenic structures needed to establish embryogenic suspensions.

Growth in liquid medium is usually very active, demanding frequent medium refreshment. This is labour intensive, and therefore, to define conditions that allow delayed subculture in the maintenance of cultures is desirable. By culturing four embryogenic clumps of 800-1200 µm in 70 ml of culture medium, at an initial density of inoculation of about 50 mg fresh weigth per liter, it was possible to maintain the cultures for four to six weeks without a deleterious effect, confirming one of the advantages of low inoculum culture (Finer 1994). After several cycles under these conditions, differences among genotypes were less marked than earlier, but differences among them were still evident. We observed two extreme models: genotype ALM-80 and genotype TRG-3. The most important difference was that proliferation of some genotypes resulted in a higher degree of breaking up (disintegration), while in others the structures tended to enlarge. In addition, some of them produced more differentiated structures, while others produced less differentiated nodular structures preferentially.

For establishing proper embryogenic suspensions, it is desirable to achieve cultures with the highest proliferation ability and composed of structures as much dispersed and undifferentiated as possible, such as PEMs. A number of treatments were carried out to accomplish that objective. The addition to the culture media of several plant growth regulators at various concentrations and combinations was ineffective to obtain PEMs. We also studied the effect of other factors such as the type of vessel and orbiting speed on the growth of embryogenic clumps. Both factors and their interaction affected both the number of newly formed embryogenic clumps and their size. The multiplication rate was relatively high, ranging between 2 to 3 per week. We also observed that the hydrodynamic stress influenced growth of cultures much more than oxygen availability. At K_{La} values between 0.11 h^{-1} and 1.47 h^{-1}, which were obtained under the working conditions, oxygen availability was not a limiting factor (Jiménez *et al*. 2011). We achieved much more homogeneous, less differentiated (mainly composed by small cell aggregates) cultures with fewer differences among genotypes, by adjusting the size of the initial inoculum, the density of inoculation and the orbiting speed.

Differentiation of cork oak somatic embryos

In our cultures in liquid medium we observed three different types of differentiation of somatic embryos. The first one, which occurred mostly in nodular clumps, showed cotyledons of somatic embryos emerging from the embryogenic clumps, and embryos growing from these and bearing a basal broad attachment to the mother tissue (Fig. 1). This pattern of development resembles the secondary embryogenesis through the multicellular pathway of regeneration observed in cultures on semisolid medium (Puigderrajols *et al*. 1996). The consequence of this regeneration pattern is that the somatic embryos

Fig. 1. *First type of differentiation. First signs of somatic embryo differentiation are cotyledons emerging from the surface of nodular embryogenic structures*

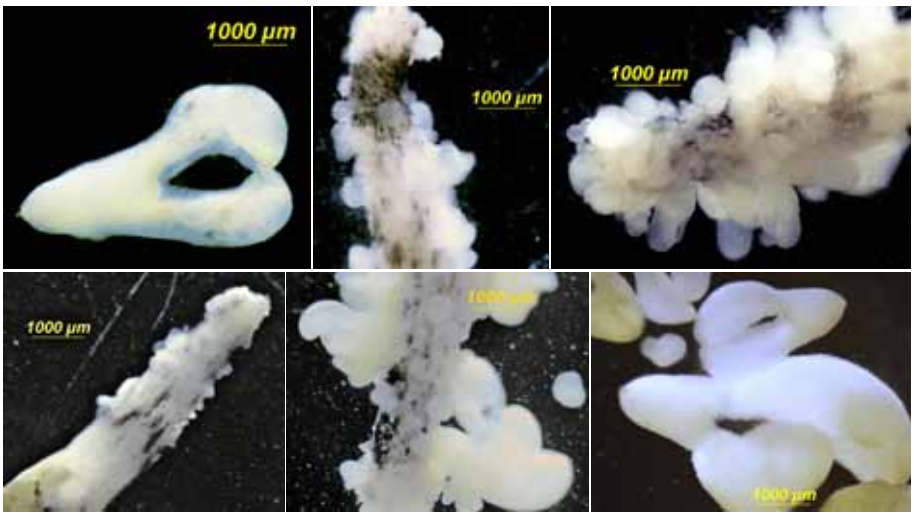

Fig. 2. *Second type of differentiation. Somatic embryo appeared as discrete globular structures on the surface of organized structures (usually cotyedonary embryos or polar structures), and they developed up to the cotyledonary stage free from the initial structure.*

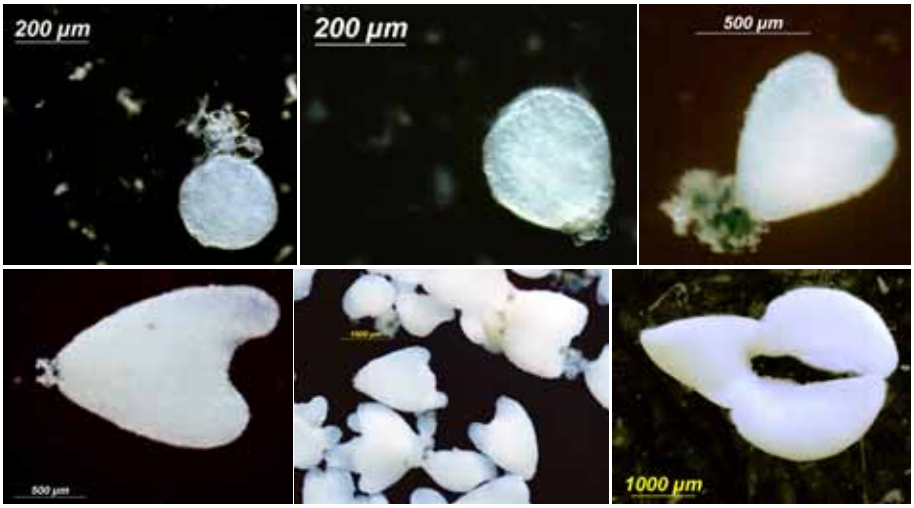

Fig. 3. *Third type of differentiation. Somatic embryos arise as discrete structures from small cell agregates not structured, showing the stages globular, heart, torpedo and cotyedonar.*

formed clusters of embryos that remained attached to each other by their root poles.

In the second type of differentiation, which happened mainly in more organized structures (such as polar structures or cotyledonary embryos), the embryos differentiated on the surface of the structure as discrete globular forms from the beginning. Later on, they differentiated cotyledons and became free easily (Fig. 2).

In the third type the embryos differentiated as in the previous one, but they developed not from structures more or less organized, but from small cell aggregates (Fig. 3). Somatic embryos arose as discrete structures, following the same pattern observed in zygotic embryogenesis and somatic embryogenesis of model species: stages globular, heart, torpedo, and cotyledonar. At present, ways to control these types of differentiation are under study.

In conclusion, we are well on the way to develop an embryogenic system for implementing multivarietal forestry in cork oak, for increased productivity and quality of cork (and other products) and for more uniformity of products that are required by industry.

Acknowledgements

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References

Bueno, M., Astorga, R., Manzanera, J. 1992. Plant regeneration through somatic embryogenesis in *Quercus suber*. Physiol. Plant. 85:30-34.

Bueno, M.A., Manzanera, J.A., González-Adrados, J.R., Pintos, B., Cuenca, B., Ocaña, L., Santos, I. 2002. Cork-oak phenotypic selection based on cork production and quality: biotechnological propagation and commercial application. Abstracts of the International Congress "Sustainable Foresty Wood Products & Biotechnology. Vitoria-Gazteiz. Novembrer, 11-14, p. 473-478.

Castro, L., Jiménez, P., Díaz-Fernández, P.M., Aranda, I., Iglesias, S., Gil, L. 2003. Review of the activities related to Mediterranean Oaks conservation in Spain. In "Mediterranean Oaks Network, Report of the second meeting, 2–4 May 2002—Gozo, Malta". Compiled by M. Bozzano and J. Turok, International Plant Genetic Resources Institute, Rome, Italy. p. 13-24.

Catalan, G., Ezquerra, F.J., Gil, l. 1997. *Quercus suber* genetic improvement programme in Spain: plus trees selection. In "*Quercus suber* Network. Report of the third and fourth meetings, 9-12 June 1996, Sassari, Sardinia, Italy, and 20-22 February 1997, Almoraima, Spain". Compiled by J. Turok, M.C. Varela and C. Hansen, International Plant Genetic Resources Institute, Rome, Italy. p. 54-59.

Celestino, C., Fernández-Guijarro, B., Hernández, I., López-Vela, D., Carneros, E., Jiménez, J., Cardo, L., Alegre, J., Toribio, M. 2009. Growth data from a field trial of *Quercus suber* plants regenerated from mature selected trees and from their half-sib progenies by somatic embryogenesis. Acta Hort. 812: 493-498.

Díaz, P., Jiménez, P., Catalán, G., Martín S., Gil, L. 1995. Regiones de procedencia de *Quercus suber* L. MAPA–ICONA/ETSIM, Madrid, Spain.

Fernández-Guijarro, B. 1997. Embriogénesis somática en alcornoque (*Quercus suber* L.). PhD thesis, Polytechnic University of Madrid, Spain. 195 pp.

Fernández-Guijarro, B., Celestino, C., Toribio, M. 1994. Somatic embryogenesis in *Quercus suber* L. In "Biotechnology of Trees". Edited by J.A. Pardos, M.R. Ahuja and R. Elena-Rossello, Investigación Agraria - Sistemas y Recursos Forestales, Fuera de Serie nº 4, INIA-MAPA, Madrid, Spain. pp. 105-110.

Fernández-Guijarro, B., Celestino, C., Toribio, M. 1995. Influence of external factors on secondary embryogenesis and germination in somatic embryos from leaves of *Quercus suber*. Plant Cell Tiss. Org. Cult. 41:99-106.

Finer, J.J. 1994. Plant regeneration via embryogenic suspension cultures. In "Plant Cell Culture: A Practical Approach". Edited by R.A. Dixon and R.E. Gonzales, Oxford University Press, Oxford. Pp. 99-125.

García-Valdecantos, J.L., Catalán, G. 1993. Possible criteria for selection of *Quercus suber* plus trees. Ann. For. Sci. 50: 448s-451s.

Gil, L., Varela, M.C. 2008. EUFORGEN Technical Guidelines for genetic conservation and use for cork oak (*Quercus suber*). Bioversity International. Rome, Italy. 6 pp.

Hernández, I., Celestino, C., Martínez, I., Manjón, J.L., Díez, J., Fernández-Guijarro, B., Toribio, M. 2001. Cloning mature cork oak (*Quercus suber* L.) trees by somatic embryogenesis. Melhoramento. 37:50-57.

Hernández, I., Celestino, C., Toribio, M. 2003a. Vegetative propagation of *Quercus suber* L. by somatic embryogenesis: I. Factors affecting the induction in leaves from mature cork oak trees. Plant Cell Rep. 21:759-764.

Hernández, I., Celestino, C., Alegre, J., Toribio, M. 2003 b. Vegetative propagation of *Quercus suber* L. by somatic embryogenesis: II. Plant regeneration from selected cork oak trees. Plant Cell Rep. 21:765-770.

Hernández, I., Celestino, C., López-Vela, D., Carneros, E., Jiménez, J., Alegre, J., Gil, L., Toribio, M. 2008. Plant regeneration from an endangered valuable cork oak tree by somatic embryogenesis. In "Suberwood: New Challenges for Integration of Cork Oak Forest and Products". Edited by J. Vázquez, H. Pereira and A. González-Pérez A, University of Huelva Publisher, Huelva, Spain. pp 205-212.

Hernández, I., Cuenca, B., Carneros, E., Alonso-Blázquez, N., Ruiz, M., Celestino, C., Ocaña, L., Alegre, J., Toribio, M. 2011. Application of plant regeneration of selected cork oak trees by somatic embryogenesis to implement multivarietal forestry for cork production. Tree For. Sci. Biotech. 5 (Special Issue 1):19-26.

Jiménez, J, López-Vela, D, Hernández, I, Carneros, E, Celestino, C, Toribio, M, Alegre, J. 2007. Iniciación del cultivo de embriones de alcornoque (*Quercus suber* L.) en medio líquido: caracterización de suspensiones obtenidas en función del tiempo en cultivo. Abstracts of the VII Reunión de la Sociedad Española de Cultivo in vitro de Tejidos Vegetales. Alcalá de Henares, España. 25-27 Junio. pp. 80-81.

Jiménez, J., Alonso-Blázquez, N., López-Vela, D., Celestino, C., Toribio, M., Alegre, J. 2011. Influence of culture vessel characteristics and agitation rate on gaseous exchange, hydrodynamic stress and growth of embryogenic cork oak cultures. In Vitro Cellular and Developmental Biology-Plant (under review).

Jiménez, M.P., Agúndez, D., Alía, R., Gil, L. 1999. Genetic variation in central and marginal populations of *Quercus suber* L. Silvae Genet. 48:278-284.

Jiménez, P., López de Heredia, U., Collada, C., Lorenzo, Z., Gil, L. 2004. High variability of chloroplast

DNA in three Mediterranean evergreen oaks indicates complex evolutionary history. *Heredity* **93**: 510-515.

Lorenzo, Z., Burgarella, C., López de Heredia, U., Lumaret, R., Petit, R.J., Soto, A., Gil, L. 2009. Relevance of genetics for conservation policies: the case of Minorcan cork oaks. Ann. Bot. 104: 1069-1076.

Martín-Vicente, A., Fernández-Alés, R. 2006. Long term persistence of dehesas. Evidences from history. *Agroforestry Syst***. 67**:19-28.

Machado, H., Santos, M.N. 2002. La symbiose ectomycorhizienne du chêne-liège dans les plantations portugaises: synthèse des connaissances et perspectives.Integrated Protection in Oak Forests. IOBC/wprs Bull. 25:61-66.

Merkle, S.A., Nairn, C.J. 2005. Hardwood tree biotechnology. In Vitro Cell. Dev. Biol. – Plant. 41: 602-619.

Moiteiro, C., Marcelo Curto, M.J., Mohamed, N., Bailén, M., Martínez-Díaz, R., González-Coloma, A. 2006. Biovalorization of Friedelane Triterpenes Derived from Cork Processing Industry Byproducts. *J. Agric. Food Chem. 54*:3566-3571.

Park, Y.S. 2004. Commercial implementation of multi-varietal forestry using conifer somatic embryogenesis. Proceedings of the IUFRO Joint Conference of Division 2, Forest Genetics and Tree Breeding in the Age of Genomics: Progress and Future, 1-5 November, 2004, Charleston, SC, USA, p. 139.

Puigderrajols, P., Fernández-Guijarro, B., Toribio, M., Molinas, M. 1996. Origin and early development of secondary embryos in *Quercus suber* L. Int. J. Plant Sci. 157:674-684.

Soto, A., Lorenzo, Z., Gil, L. 2007. Differences in fine-scale genetic structure and dispersal in *Quercus ilex* L. and *Q. suber* L.: consequences for regeneration of Mediterranean open woods. Heredity 99:601-607.

Silva, S.P., Sabino, M.A., Fernandes, E.M., Correlo, V.M., Boesel, L.F., Reis, R.L. 2005. Cork: properties, capabilities and applications. Int. Materials Rev. 50:345-365.

Toribio, M. 1986. Callus initiation and primary morphogenic responses from *Quercus suber* L. cultured *in vitro*. Abstracts 18th I.U.F.R.O. World Congress. Div.2, Ljubljana, Yugoslavia. p. 863.

Toribio, M., Celestino, C., Molinas, M. 2005. Cork oak, *Quercus suber* L. In "Protocol for Somatic Embryogenesis in Woody Plants". Forestry Sciences (Vol 77, Chapter 35). Edited by S.M. Jain and P.K. Gupta, Springer, The Netherlands. pp. 445-457.

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Proteomic and Genetic Transformation Studies of Somatic Embryogenesis in the Solanaceous Tree *Cyphomandra betacea*

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Abstract

Tamarillo (*Cyphomandra betacea*) is a woody plant of the Solanaceae family, known for its edible fruits. In recent years, different aspects of somatic embryogenesis induction and somatic embryo development of tamarillo have been studied in our lab in order to develop a suitable model for the cytological and molecular mechanisms involved in somatic embryogenesis, a morphogenic process with important applications both for plant cloning and genetic transformation.The main goal of this research is to achieve a better understanding of some of the molecular mechanisms underlying the somatic embryogenesis induction process of this species by using proteomic and genetic transformation techniques.

Key words: 2D-electrophoresis, *Agrobacterium*- mediated transformation, embryogenic proteins, non-embryogenic proteins, tamarillo.

Introduction

Cyphomandra betacea (Cav.) Sendt. is a solanaceous softwood tree (Fig. 1A) grown for its edible fruits (Fig. 1B) and usually known as tamarillo or tree tomato. The species can attain 2 – 4 meters in height. The fruits are tomato-like and can be red, orange or yellow according to the cultivar. The fruit of the red cultivar is the most popular due to its more striking appearance and flavor (Slack 1976). They are generally 5-10 cm long and 3-5 cm in diameter possessing many seeds (Hooker 1899). Flowers (Fig. 1C), which are rose-shaped and perfumed, usually develop in small groups at the top of the branches. They blossom mostly during the transition from summer to fall but can, however, appear at other times of the year. In Portugal the first pinkish flowers appear in spring, while the mature fruits are collected from October to April (Guimarães *et al*. 1996).

The species originated in South America, more specifically in the Andean region of Peru, Chile, Ecuador and Bolivia (Dawes and Pringle 1983), and has spread to Central America and the West Indies and, later on, to the Portuguese islands of Azores and Madeira and to southern Europe (Hooker 1899, Atkinson and Gardner 1993). By the end of the 19th century, it had reached Australia and New Zealand (Slack 1976). Nowadays tamarillo is grown in many regions of the globe such as India, China, USA, Kenya, Australia, Southern Europe and New Zealand (Guimarães *et al*. 1996). In this last country the plant is intensely cultured as a crop and the fruits are exported to several countries (http://www.crfg.org/pubs/ff/tamarillo.html).

This species is usually cultivated because of its fruits which, when ripe, can have several uses, from salads to industrial processed products like juices or jams. In terms of nutritional value, tamarillo fruits have a relatively high content of proteins, vitamin C, vitamin E, provitamin A and minerals like potassium and iron (McCane and Widdowson 1992). In recent years several studies aimed at an evaluation of the content and type of anthocyanins and carotenoids in fruits of tamarillo have been carried out (De Rosso and Mercadante 2007; Kou *et al.* 2008; Hurtado *et al.* 2009). The results of these studies have shown that some of these compounds may have important biological, therapeutic, and preventative antioxidant properties making them a valuable food resource that deserves to be explored both as a fruit and as a source of compounds that can eventually improve human health.

An increasing number of assays describing tamarillo micropropagation through axillary shoot proliferation (Barghchi 1986; Cohen and Elliot 1979; Obando *et al.* 1992), organogenesis (Guimarães *et al*. 1996; Obando and Jordan 2001) and somatic embryogenesis (Guimarães *et al.* 1988, 1996; Lopes *et al*. 2000; Canhoto *et al*. 2005; Correia *et al*. 2009) have been published. Genetically modified plants of tamarillo have also been obtained (Atkinson and Gardner 1993; Cohen *et al*. 2000; Cruz and Tomé 2007).

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Somatic embryogenesis induction of tamarillo

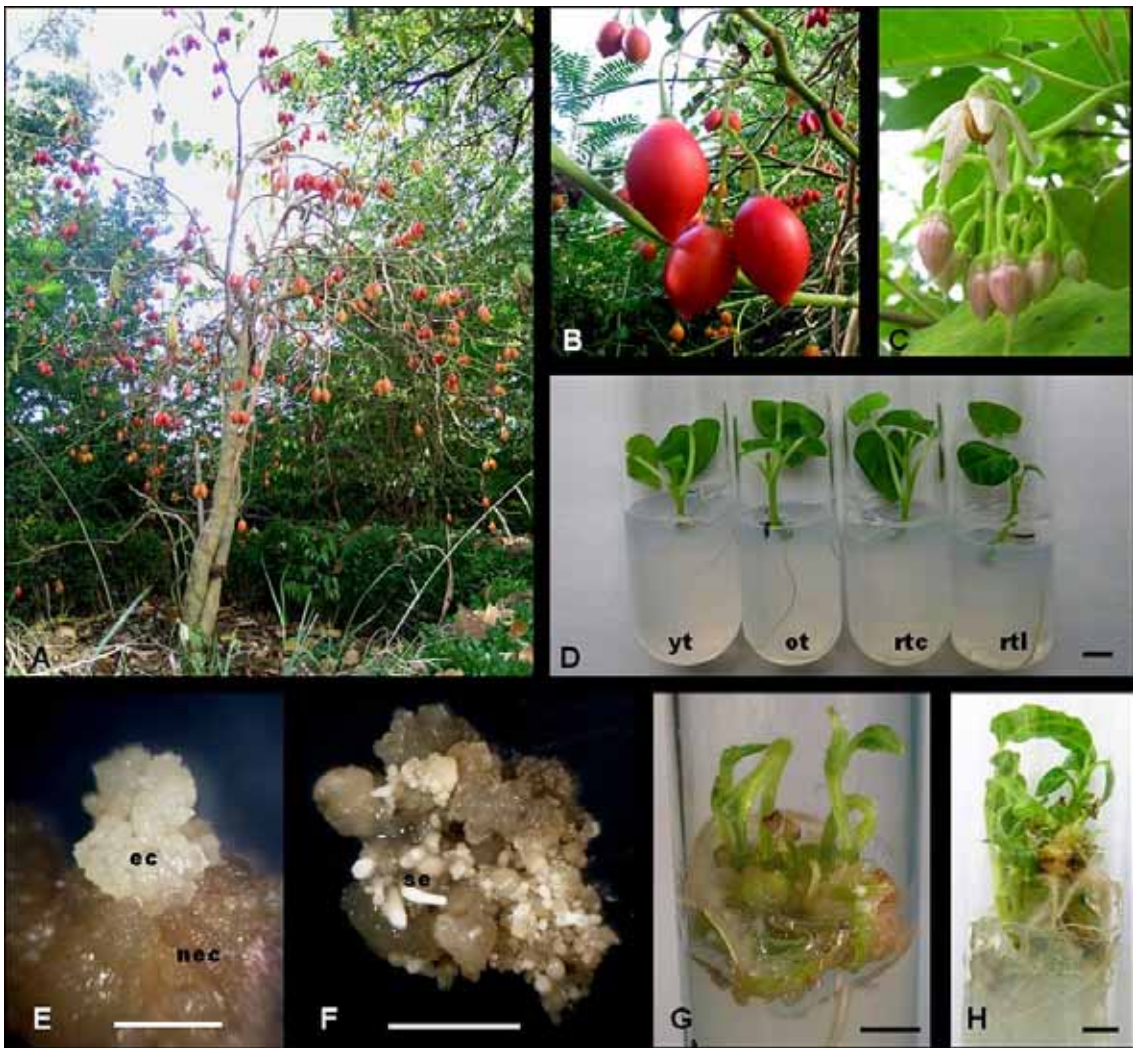

Figure 1. *A-C - Different aspects of Cyphomandra betacea: A - Tamarillo tree growing at Botanical Garden of the University of Coimbra; B – Flowers; C - Fruits. D - Propagation of 4 different tamarillo genotypes (yt – yellow tamarillo, ot – orange tamarillo and two red tamarillo clones – rtc and rtl) by shoot proliferation. E-H - Somatic embryogenesis in tamarillo: E – Leaf explant after 10-12 weeks on induction medium - MS supplemented with picloram. Note the embryogenic (ec), whitish, and non-embryogenic (nec), darker, zones formed. F – Somatic embryos at different developmental stages after 2 weeks on MS basal medium without auxin. Note the asynchronous development of the somatic embryos. G – Somatic embryo germination on MS medium. H - Plantlet development from somatic embryos, after 6 weeks on MS medium. (Bar = 0.5cm)*

The first successful induction of somatic embryogenesis of tamarillo was reported by our lab (Guimarães *et al*. 1988) and was obtained from cultures of mature zygotic embryos and hypocotyls of young seedlings. In a subsequent report (Guimarães *et al*. 1996) we described the regeneration of tamarillo plants by organogenesis and somatic embryogenesis from different explants (hypocotyls, cotyledons, roots and mature zygotic embryos) and from protoplasts.

The ability of different genotypes (Fig. 1D) to undergo somatic embryogenesis was also tested with preliminary results showing that some cultivars are more capable than others for somatic embryo formation. Attempts to induce somatic embryogenesis from adult plants were also made with the objective of cloning selected genotypes. To achieve this goal, assays with pith tissue obtained from stem segments and floral explants were carried out (Correia *et al.* 2009), revealing a recalcitrance of the adult material to somatic embryogenesis induction that was similar to that so often reported for other woody species. However, cloning of selected adult plants through somatic embryogenesis was achieved by first establishing offspring of these by axillary shoot prolifereation *in vitro* followed by induction of embryogenesis in leaf segments of these plants.

However, a more direct approach would be helpful to reduce costs and time.

Somatic embryogenesis induction in tamarillo is routinely achieved on an auxin-rich culture medium in which the inclusion of higher sucrose levels (9%) strongly increases somatic embryo formation raising the efficiency of somatic embryogenesis induction to values around 85% (Guimarães *et al*. 1996). Therefore, the methodology used for somatic embryogenesis induction in tamarillo was the one previously described (Guimarães *et al*. 1988, 1996). Briefly, when zygotic embryos or leaf sections from young seedlings or shoots are cultured on MS basal medium (Murashige and Skoog 1962) supplemented with an auxin (2,4-dichlorophenoxyacetic acid or picloram), a slow growing callus is induced after 4 - 6 weeks of culture. By the 8^{th} to 10^{th} week of culture whitish clusters of embryogenic cells are formed in some areas of the callus (Fig. 1E), which kept proliferating. Non-embryogenic cell masses that originated from the same explant also proliferate. Subculture of the embryogenic *calli* on media devoid of auxins allowed the differentiation of the embryogenic masses in somatic embryos that progressed through the stages characteristic of embryo development (Figs. 1F-H). Therefore, somatic embryos were obtained through a two-step process since two different media are necessary to achieve full somatic embryo differentiation.

Since the conditions for somatic embryogenesis induction and plant regeneration are well characterised in tamarillo, biochemical and molecular studies can be performed to better understand this morphogenic process. One advantage of using tamarillo for this purpose is the fact that embryogenic and non-embryogenic cell lines can be obtained from the same explant. This is particularly important since we can compare the two types of cell lines at the molecular level and try to find proteins or mRNA specific for each situation. This could lead to the identification of genes promoting or inhibiting the embryogenic process.

Proteomic and genetic transformation assays

Previous work, that aimed to identify protein markers associated with embryogenic and non-embryogenic calli, compared protein electrophoretic patterns of the two types of *calli* and found qualitative differences between them (Faro *et al*. 2003). Following the identification of a 26.5 kDa protein associated with non-embryogenic *calli* of tamarillo somatic embryogenesis induction is now under investigation in knocked out lines of *Arabidopsis thaliana* in which the orthologous non-embryogenic gene is inactivated. These knocked out lines do not display any particular discernible phenotype. However, preliminary results showed that somatic embryogenesis is induced at higher rates in the knocked out line than in the wild phenotype. These results indicate a post-transcriptional mechanism of control for somatic embryogenesis induction, in which the presence of the gene is inhibitory for somatic embryogenesis induction and that its down regulation apparently increases the embryogenic potential.

To study the possible function of this protein in the embryogenic process in tamarillo, a polyclonal antibody was generated and the specificity of it tested by Western blotting. The expression of the protein is being evaluated in different plant tissues and organs, including embryogenic and non-embryogenic *calli* of tamarillo. Imunocytochemical studies to establish the distribution of this protein within the cells are also being carried out.

In our lab a regeneration system for genetic transformation of tamarillo was established, based on our the work on tamarillo regeneration by organogenesis and somatic embryogenesis (Cruz and Tomé 2007). These genetic transformation studies and our efficient regeneration protocols contribute not only to the development of clones resistant to virus, pests and diseases but are also useful tools in functional genomics studies.

Genetically transformed plants of tamarillo with the putative non-embryogenic gene silenced have been obtained. GATEWAY cloning technology was used to prepare a construct, encoding a hairpin RNA targeting the non-embryogenic protein gene. This gene was incorporated into LBA4404 *Agrobacterium tumefaciens* competent cells. An improved *Agrobacterium*-mediated transformation protocol for tamarillo leaf explants was developed based on the regeneration of transformed plants by somatic embryogenesis. Leaves that had been cultured for one month on somatic embryogenesis induction medium were used as explants for a 2 days co-culture period with *A. tumefaciens*. In approximately 6 months more than 50 self-rooted transformed plants were obtained from a single embryogenic cell line, which demonstrates the efficiency of our protocols. Putative transgenic plants are now being analyzed by PCR and RT-PCR. The effect of non-embryogenic protein gene silencing is being estimated by proteomic analysis. Furthermore, silenced or down-regulated tamarillo plants are undergoing a somatic embryogenesis induction process to evaluate the role of the protein on somatic embryogenesis induction. Like in the knocked out lines of *Arabidopsis thaliana*, tamarillo transformed plants do not display any particular phenotype different from the wild type.

Trying to obtain more data on embryogenic-specific proteins, a proteomic analysis by 2D-electrophoresis was carried out to compare embryogenic and non-embryogenic *calli*. The results so far obtained have shown several differentially responsive proteins in embryogenic versus non-embryogenic callus. Despite these differences the analysis revealed strong similarities between expressed proteins in induced *calli* regardless of the auxin or explant used. Several potential proteins of interest are being analyzed by liquid chromatography tandem mass spectrometry (LCMS/MS).

References

Atkinson, R.G and Gardner, R.C. 1993. Regeneration of transgenic tamarillo plants. Plant Cell

Rep.12:347-351.

Barghchi, M. 1986. *In vitro* rejuvenation of *Cyphomandra betacea* (tamarillo). Plant Physiology Division Biennial Report, Department of Scientific and Industrial Research(DSIR), New Zealand. PP.52.

Canhoto, J.M., Lopes, M.L., Cruz, G.S. 2005. Protocol for Somatic Embryogenesis: Tamarillo (*Cyphomandra betacea* (Cav.) Sendt.). In " Protocol for Somatic Embryogenesis in Woody Plants". Edited by S.M. Jain, P.K. Gupta. Dordrecht. Springer. PP. 379-389.

Cohen, D. and Elliot, D. 1979. Micropropagation methods for blueberries and tamarillos. Comb Proc Int Plant Prop Soc. 29:177-179.

Cohen, D., van den Brink, R.C., MacDiarmid, R.M., Beck, D.L., Forster, R.L. 2000. Resistance to tamarillo mosaic virus in transgenic tamarillos and expression of the transgenes in F1 progeny. Acta Hort. 521:43-49.

Correia, S.I., Lopes, M.L., Canhoto, J.M. 2009. Somatic embryogenesis in tamarillo (*Cyphomandra betacea*): recent advances. Acta Hort. 839:157-164.

Cruz, F.P., Tomé, M.C. 2007. *Agrobacterium*-mediated transformation of tamarillo plants. Proc of Plant Transformation Technology. Vienna. Austria.

Dawes, S.N., Pringle, G.L. 1983. Subtropical Fruit from South and Central America. In "Plant Breeding in New Zealand". Edited by G.S. Wratt and H.C. Smith. Wellington, Butterworths. PP. 123-138.

De Rosso, V.V., Mercadante, A.Z. 2007. HPLC-PDA-MS/MS of anthocyanins and carotenoids from dovyalis and tamarillo fruits. J Agric Food Chem. 55:9135-9141.

Faro, M.R., Lopes, M.L., Canhoto, J.M., Cheung, A., Cruz, G.S. 2003. Identification and molecular characterization of a non-embryogenic *calli* protein in *Cyphomandra betacea*. 7th Int Bot Meeting – Plant Cell Biology. Lisbon.

Guimarães, M.L., Cruz, G.S., Montezuma-de-Carvalho, J.M. 1988. Somatic embryogenesis and plant regeneration in *Cyphomandra betacea* (Cav.) Sendt. Plant Cell Tiss. Org. Cult. 15:161-167.

Guimarães, M.L., Tomé, M.C., Cruz, G.S. 1996. *Cyphomandra betacea* (Cav.) Sendt. (Tamarillo). In "Biotechnology in Agriculture and Forestry". Vol. 35. Trees IV. Edited by Y.P.S. Bajaj. Springer-Verlag, Berlin. PP.120-137.

Hooker, J.D. 1899. *Cyphomandra betacea*. Curtis's Bot. Mag. 55:7682.

Hurtado, N.H., Morales, A.L, González-Miret, M.L., Escudero-Gilete, M.L., Heredia, F.J. 2009. Colour, pH stability and antioxidant activity of anthocyanin rutinosides isolated from tamarillo fruit (*Solanum betaceum* Cav.). Food Chem. 117:88-93.

Kou, M., Yen, J., Hong, J., Wang, C. 2008. *Cyphomandra betacea* Sendt. Phenolics protect LDL from oxidation and PC12 cells from oxidative stress. LWT – Food Sci Technol. 42:458-463.

Lopes, M.L., Ferreira, M.R., Carloto, J.M., Cruz, G.S., Canhoto, J.M. 2000. Somat Embryogenesis Induction in Tamarillo (*Cyphomandra betacea*). In "Somatic Embryogenesis in Woody Plants". Edited by S.M. Jain, P.K. Gupta and R.J. Newton. Vol. 6. Dordrecht, Kluwer Academic Publishers. PP. 433-455.

McCane, J. and Widdowson, D.A. 1992. In: Fruit and nut. Suppl. to the composition of foods. 5th ed. Holland, Unwin & Buss. London. PP. 74-77.

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

Obando, M. and Jordan, M. 2001. Regenerative responses of *Cyphomandra betacea* (Cav.) Sendt. (tamarillo) cultivated *in vitro*. Acta Hort. 560:429-432.

Obando, M., Goreux, A. and Jordan, M. 1992. Regeneration *in vitro* de *Cyphomandra betacea* (tamarillo), una especie frutal andina. Ciencia e Investigacion Agraria. 19:125-130.

Slack, J.M. 1976. Growing tamarillos. Agric. Gaz. 86:2-4.

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Detection of Seed Contamination of Seedlots of *Larix kaempferi* by Microsatellite Analysis

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Abstract

Seed contamination of seedlots was detected by microsatellites. This method was used to assess the availability of a molecular method in certification of forest seed. Three hundred sixty nine ramets of three seed orchards of *Larix kaempferi* and 864 seeds known to be harvested from the seed orchards were examined for maternity using five microsatellite markers. Genetic diversity of the overall samples was 0.700, that of plus trees and seeds was 0.779 and 0.620, respectively. Exclusion probability by five microsatellites used was 0.946 which was considered to reflect the necessity of additional markers for exact detection of seed contamination. The tendency of clustering of ramets and seedlots from the same seed orchards was not found by pairwise F_{ST} and principal component analysis. Further studies should be conducted with more accurate samples to verify the availability of pairwise F_{ST} and principal component analysis in detection of seed contamination as the result is not consistent with the other studies. Simulation was done to find the possibility of using maternity analysis in identification of contaminating seeds. The test was performed with the seed samples which were collected from each seed orchard at 95% confidence level by maternity analysis. Six contaminated seedlots were simulated by mixing the seeds from the correct seed orchard and the other seed orchard in the ratio about four to one. Eleven and 8 of 12 contaminating seeds, 14 and 14 of 16 contaminating seeds, 12 and 8 of 17 contaminating seeds were recognized in each set as they appeared to be assigned to a certain tree at a lower than 80% confidence level or unassigned. As the analysis could not sort out contaminating seeds exactly, more accurate study should be done to confirm the power of the method in detection of contaminated seedlots. However, it is considered that the method used in this preliminary study will be useful in detection of seed contamination which will support the identification of seed source under a OECD scheme for Certification of Forest Reproductive Material (CFRM) when genetic information of all individuals of the source populations can be assessed.

Introduction

The ontinuous supply of high-quality seeds is an important factors leading to successful reforestation (Tigabu *et al.* 2005). In particular, the genetic quality of forest seeds is important because of the long life span of forest species (Tigabu *et al.* 2005). Establishment of seed orchards and harvest of seeds from the plus trees of the seed orchards are the most common way to guarantee the high-quality of the forest seeds (Zobel and Talbert 1984; Varghese *et al*. 2000). Thus not only have several countries made provisions in their legislation regulating the marketing or use of reproductive materials (seeds and plants) (Lexer *et al.* 1999), but also the OECD has opened the Scheme for the Control of Forest Reproductive Material Moving in International Trade. Also Korean forest policy aims at constructing a system to certificate superior seeds based on OECD-CFRM.

Still, there could be a contamination of seedlots by seeds collected from outside the seed orchards in practice. A false declaration of the origin of the seed has been revealed by comparing samples taken at different stages of the production process using chloroplast DNA (Leinemann, unpublished). Methods to control the production of forest reproductive material and specifically to test the reliability of the declared origin of seeds are highly desirable (Finkeldey *et al*. 2010).

However, the method of detecting the seed contamination is hardly developed. There have been several studies about the detection of the mother trees of seeds using tissue of maternal origin (Grivet *et al*. 2005; Robledo-Arnuncio and García 2007) in natural forest stands, but it is difficult to isolate DNA from maternal tissues of most of the seeds.

The sustainable conservation of genetic diversity is an important issue of international concern (Lexer *et al.* 1999; Shanjani *et al.* 2008). As the number of mother trees predetermines the level of genetic diversity, seeds which are used in reforestation should be harvested from a sufficient number of mother trees. Studies aiming at the estimation of the number of mother trees have been conducted (Lexer *et al.* 1999; Shanjani *et al.* 2008). They used similar methodology which makes the estimation by pairwise F_{ST} and Principal Component Analysis (PCA) but derived somewhat contradictory results.

The objectives of this study are to confirm the previous analysis method estimating the number of mother trees and to assess the availability of a molecular method in detection of seed contamination.

Microsatellite markers are the ideal genetic markers for these purposes because they are highly polymorphic and codominantly inherited (Lexer *et al.* 1999). *Larix kaempferi* (Lamb.) Carrière which is one of the major

plantation species in Korea was used in this study.

Materials and Methods

L. kaempferi plus trees in three seed orchards (SS, WT, CC) and their offspring were used to detect the seed contamination and estimate the number of mother trees. SooHoe (SH) and WonTong (WT) seed orchards were established in 1985 and the ChoonChun (CC) seed orchard was established in 1977 (Table 1). In SH, leaves of all plus trees were sampled. Trees with cones which are supposed to be the mother trees of the seeds were sampled in WT. Plus trees which constitute CC seed orchard were sampled from the clone bank. As a result, 148, 117 and 104 trees were used as the candidate mothers. To analyze the offspring of the seed orchard, 864 seeds from each seedlot harvested in 2005 were sampled. Seedlings were germinated from the seeds.

DNA isolation and Microsatellite analysis

Genomic DNA was isolated from leaves of 373 adult trees and 864 seedlings using DNeasy Plant Kit (Qiagen). Five microsatellite primers were selected after screening 19 primers from Isoda and Watanabe (2007) (Table 2). Microsatellite loci were amplified in reaction volume of 11μL containing 9ng of DNA, 1x PCR buffer, 2mM

Table 1. Characteristics of the three seed orchard of *L. kaempferi*

Seed Orchard	Area(ha)	Total number of trees	Number of sampled trees	Number of seedlings
SH('85)	2	400	150	228
WT('85)	2	400	119	228
CC('77)	22	2657	104	228
Total		3357	373	864

Table 2. Information of the seven selected microsatellite primer of *L. kaempferi*

Locus	Prirmer sequence(5'-3')	Repeat	Tm(ºC)
bcLK033	F: GGAAATGTAGAGATGAGCAATAA R: AGGTGCGGTAGTACAAAGTGA	$[TC]_{14}$	53
bcLK056	F : ATGGGCTAAGGTATGTTTTACG R: TTGCCAACATCTATACCAAGTCT	$[AG]_{20}$	53
bcLK187	F : AGGACGGAGAGAGTCATTCTG R: AACCCTAGTGATTTTAAGGAGAGA	$[AG]_{13}$	53
bcLK225	F: CGTGGTTCCCATCCTCTAAA R: TGGCAGCTAAAGGATTAAAGAA	$[GA]_{20}$	52
bcLK228	F: CCCTAACCCTAGAATCCAATAA R: GAGGAAGGCGACAAGTCATT	$[AG]_{18}$	53
bcLK241	F: TGAGGTTAGGAGCATCTCGT R: GTCCTTCATCGCCTCTTCTT	$[GA]_{12}$	53

Table 3. Genetic statistics by 5 microsatellite loci of *L. kaempferi*

Locus	N_A	N_E	H_O	H_E	**PIC**	**P(exclusion)**
bcLK056	15	5.2	0.815	0.809	0.793	0.475
bcLK187	16	3.8	0.547	0.586	0.568	0.213
bcLK225	19	8.2	0.68	0.887	0.87	0.622
bcLK228	20	8.2	0.803	0.879	0.877	0.633
bcLK241	5	1.7	0.334	0.347	0.305	0.06
overall	15	5.4	0.636	0.7	0.683	0.946

Table 4. . Genetic statistics by seed orchards of *L. kaempferi*

	TOTAL	TREE				SEED			
		SH	WT	CC	Total	SH	WT	CC	Total
N_A	12.1	11.8	11.2	13.4	12.1	12.8	12.8	10.4	12.0
N_E	5.4	6.3	5.8	6.0	5.9	5.0	4.5	5.0	4.8
H_O	0.636	0.690	0.690	0.695	0.692	0.700	0.566	0.474	0.580
H_E	0.700	0.783	0.776	0.773	0.779	0.720	0.622	0.515	0.620

Table 5. Pairwise F_{ST} between trees and seedlots

	SH-P[1)]	SH-S[2)]	WT-P	WT-S	CC-P	CC-S
SH-S	0.043	-				
WT-P	0.006	0.031	-			
WT-S	0.088	0.042	0.065	-		
CC-P	0.015	0.029	0.010	0.048	-	
CC-S	0.160	0.074	0.130	0.022	0.113	-

[1)]P: tree group; [2)]S: seedlot.

$MgCl_2$, 0.2μM of forward and reverse primer with fluorescent dye, 1U of Taq DNA polymerase. PCR was performed in GeneAmp PCR System 9700 (Applied Biosystems) according to the following procedure: 10 cycles of 94ºC for 1 min, annealing temperature (Tm) presented in table 2 for 1 min, 72ºC for 2 min and 25 cycles of 94ºC for 30 sec, Tm for 30 sec, 72ºC for 1 min then a final extension at 72ºC for 10 min. Electrophoresis of PCR products were performed in Applied biosystems 3130*xl* Genetic Analyzer (Applied Biosystems) using GeneScan ROX as size standard and genotypes were confirmed using GeneMapper 4.0 (Applied Biosystems).

Data Analysis

Number of alleles (N_A), observed heterozygosity (H_O), expected heterozygosity (H_E), tests for Hardy-Weinberg Equilibrium (HWE) were computed by GenAlEx 6.2 (Peakall and Smouse, 2006). Principal Component Analysis (PCA) based on Pairwise F_{ST} was performed to estimate the number of mother trees.

he maternity analysis based on likelihood-based method was conducted using Cervus 3.0 (Kalinowski *et al*. 2007). Simulation of maternity analysis was performed 10000 cycles to find critical values of Δ, the difference in LOD scores between the most-likely mother and the second most-likely mother, for strict (95%) and relaxed (85%) confidence levels. The maternities were assigned for 85% of sampling of the candidate parents and the error rate was set of 1% as default value.

Results

The number of alleles ranged from 5 to 20 and exclusion probability by the 5 markers was 0.946 (Table 3). Genetic diversity of the overall samples was 0.700, that of plus trees and seeds was 0.779 and 0.620, respectively (Table 4).

Pairwise F_{ST} between groups and PCA plot

Pairwise F_{ST} value between trees and seedlots of each seed orchard are shown in table 5. Grouping tendency of trees with its seeds was not found by PCA (Fig 1). Axis 1 explained 95.45% and axis 2 explained 4.55% of variation.

Maternity analysis

Among 288 seeds, 51, 64 and 69 seeds from each seed orchard were assigned correctly at 80% confidence level. Subsequent tests were performed with these assigned data only. It was possible to find the mother tree in the seed orchards based on the analysis. Fifty one seeds were from 18 trees in SH, 64 seeds were from 17 trees in WT and 69 seeds were from 21 trees in CC (Fig 2). In the graphs, a few individuals of the mother trees which were revealed as mother trees of multiple seeds were mainly shown. Lastly, six contaminated seedlots were simulated by mixing the seeds from the

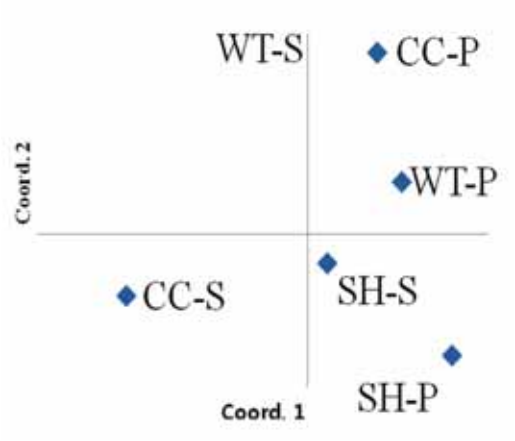

Figure 1. *PCA plot by pairwise* F_{ST}

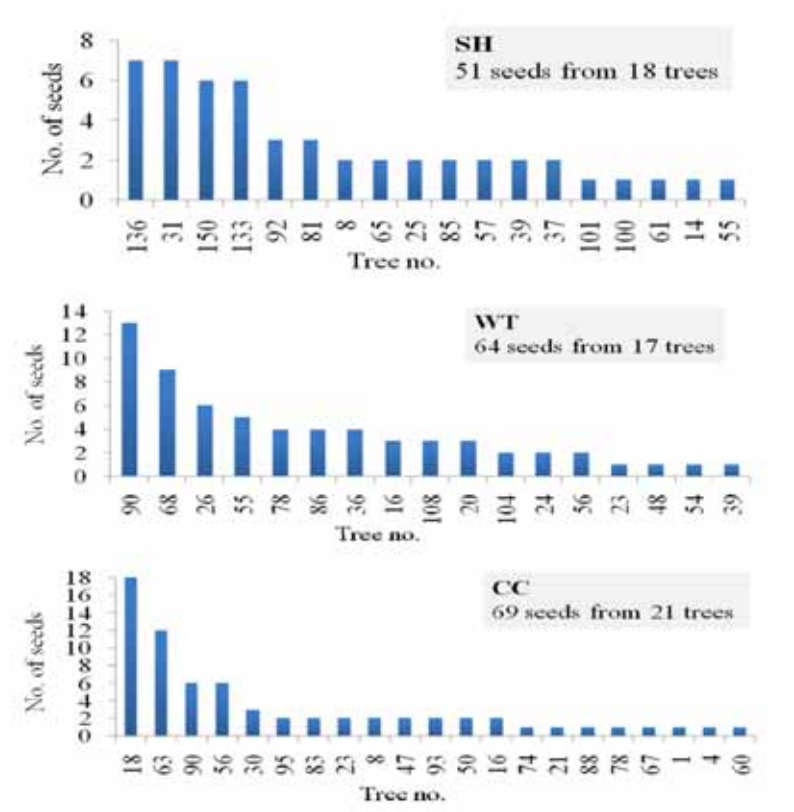

Figure 2. *Tree code (Tree no.) and the number of seeds assigned to the individuals*

correct seed orchard and the other seed orchard in the ratio about four to one. Eleven and 8 of 12, 14 and 14 of 16, 12 and 8 of 17 contaminating seeds were recognized in each set as they were assigned lower than 80% confidence level or unassigned (Table 6).

Discussion

Genotyping by microsatellite marker, estimation of pairwise F_{ST} to find differentiation between groups and maternity analysis to identify the mother tree of each seed were performed. We could not find genetic similarity between mother trees and seedlot samples.

Although the analysis could not sort out contaminating seeds exactly, it is considered that the method used in this preliminary study will be useful in detection of seed

Table 6. Maternity analysis results of six contaminated seedlots produced by simulation

	No. of assigned seed			
	Confidence Level			
Seedlot /mixed seeds	**95%**	**80%**	**<80%**	**unassigned**
SH (51)	16	48	3	-
/WT (12)	-	1	3	8
SH (51)	12	48	3	-
/CC (12)	2	4	6	2
WT (64)	16	60	4	-
/SH (16)	-	2	9	5
WT (64)	12	58	6	-
/CC (16)	0	2	7	7
CC (69)	11	66	3	-
/SH (17)	0	5	7	5
CC (69)	18	67	2	-
/WT (17)	1	9	6	2

contamination which will support the identification of seed source under OECD CFRM. A more accurate study should be done to confirm the power of the method in detection of contaminated seedlots considering the causes of low level of assignment in this study. First, we did not assess the detailed information of seed orchards, but there could be overlapping clones. Second, we did not collect seed samples directly so there is possibility of using mixed seedlots as samples. Lastly, assignment ratio would be increased if more microsatellite loci were genotyped.

Therefore, it is necessary to confirm the method with reliable samples in future. And the important thing in this kind of study is the choice of the method considering species, spatial patterns of genetic diversity and management intensity. First of all, research direction should be decided. According to the direction, the most appropriate marker and sampling strategy can be determined. If genetic differentiation is going to be used, various approach such as provenance characterization, reference sample approach can be applied.

References

Finkeldey, R., Leinemann, L., Gailing, O. 2010. Molecular genetic tools to infer the origin of forest plants and wood. Appl. Microbiol. Biotechonol. 85: 1251-1258.

Grivet, D., Smouse, P.E., Sork, V.L. 2005. A novel approach to an old problem: tracking dispersed seeds. Mol Ecol. 14:3585-3595.

Heinze, B., Lexer, C. 2000. Oak seedlots and forest seed trade regulations: an investigation using molecular markers. Glas. Šum. Pokuse. 36:347-360.

Isoda, K., Watanabe, A. 2006. Isolation and characterization of microsatellite loci from *Larix kaempferi*. Mol Ecol Notes. 6:664-666.

Kalinowski, S.T., Taper, M.L., Marshall, T.C. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol Ecol. 16:1099-1006.

Lexer, C., Heinze, B., Steinkellner, H., Kampfer, S., Ziegenhagen, B., Glössl, J. 1999. Microsatellite analysis of maternal half-sib families of *Quercus robur*, peduculate oak: detection of seed contaminations and inference of the seed parents from the offspring. Theor Appl Genet. 99:185-191.

Nason, A. 2001. The new OECD scheme for the certification of forest reproductive material. Silvae Genet. 50:177-280.

Peakall, R., Smouse, P.E. 2006. GENALEX 6: Genetic Analysis in Excel. Population genetic software for teaching and research. Mol Ecol Notes 6:288-295.

Robledo-Arnuncio, J.J., García, C. 2007. Estimation of the seed dispersal kernel from exact identification of source plants. Mol Ecol 16:5098-5109.

Shanjani, P.S., Vendramin, G.G., Calagari, M. 2008. Microsatellite analysis for differentiation and identification of the source tree of *Fagus orientalis* Lipsky. Iranian Journal of Biotechnology 6:85-91.

Tigabu, M., Oden, P.C., Lindgren, D. 2005. Identification of seed sources and parents of *Pinus sylvestris* L. using visible-near infrared reflectance spectra and multivariate analysis. Trees 19:468-476.

Varghese, M., Nicodemus, A., Nagarajan, B., Siddapa, K.R.S., Bennet, S.R.R., Subramanian, K. 2000. Seedling seed orchards for breeding tropical trees. Scroll Press, Coimbatore.

Zobel, Talbert 1984 Applied forest tree improvement. Wiley, New York.

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Current Applications of Coffee (*Coffea arabica*) Somatic Embryogenesis for Industrial Propagation of Elite Heterozygous Materials in Central America and Mexico

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Abstract

Of all the possible micropropagation techniques, it is widely accepted that vegetative propagation by somatic embryogenesis is by far the most promising for rapid, large-scale dissemination of elite individuals. Yet, to date, examples of somatic embryogenesis processes applied on an industrial scale are very few and far between. There are many complications. They usually involve a major genotypic effect, particularly for obtaining embryogenic tissues, or are related to the quality of regenerated somatic embryos, the incidence of somaclonal variation and, more generally, a lack of reproducibility and efficiency at certain stages of the process, leading to production costs that are prohibitive. Research on coffee somatic embryogenesis began at the end of the 1970s at various institutes, including CIRAD. Between 1995 and 2001, CIRAD moved the technique forward from a research laboratory scale to a technique enabling industrial dissemination of extremely promising *Coffea arabica* F1 hybrids. Over that period, two technological innovations made technology transfer economically feasible: mass production of somatic embryos in temporary immersion bioreactors and the possibility of sowing them directly in the nursery. At the same time, reassuring data were obtained on the genetic conformity of regenerated plants (somaclonal variation frequency < 3%). In 2002, in partnership with the ECOM group, CIRAD decided to transfer the somatic embryogenesis method on an industrial scale to Central America so that four Arabica hybrid clones, that were selected for agroforestry-based farming systems, could be disseminated throughout that part of the world. This article describes the different stages and the difficulties we had to overcome before successful technology transfer could occur in 2010. It describes one of the first examples of somatic embryogenesis technology applied on a commercial scale.
Keywords: Somatic embryogenesis, micropropagation, technological transfer, coffee tree, production costs, clonal conformity, somaclonal variations, *in vitro* plantlet, nursery

Introduction

Somatic embryogenesis, a long-awaited technology!

Somatic embryogenesis enables rapid and massive vegetative propagation of elite genotypes by doing away with lengthy and costly pedigree selection processes. Still, to date, examples of somatic embryogenesis processes applied on an industrial scale are few and far between. Nonetheless, a few examples can be mentioned, such as loblolly pine (*Pinus taeda*) [Gupta PM, pers. comm.], oil palm (Khaw *et al*. 1999) , *Coffea arabica* (Menéndez-Yuffá *et al*. 2010, present communication] and *C. canephora* (Sampote *et al*. 2006, Ducos *et al.* 2010), for which annual production now exceeds one to several million plants annually. Yet, this vegetative propagation technique is widely accepted as being by far the most promising for capturing genetic gain quickly through rapid and largescale dissemination of elite individuals. This is all the more true with woody species for which biological cycles are long. In the 1980s, there was great enthusiasm for developing this technology and expectations were running high, which explains why research was undertaken on a large number of species, without any immediate justification in some cases. There are many complications in developing this technology. They usually involve a major genotypic effect, particularly for obtaining embryogenic tissues, or they are related to the mediocre quality of regenerated somatic embryos, the incidence of somaclonal variations, and more generally a lack of reproducibility and efficiency at some stages, leading to production costs that prove prohibitive.

Quickest possible dissemination of genetic progress in the Arabica species

Research on coffee somatic embryogenesis began in the early 1980s at various institutes, including CIRAD, without any clear objective. At the beginning of the 1990s, CIRAD, in partnership with the Central American research network, PROMECAFE, set out to create *Coffea arabica* intraspecific F1 hybrids, by crossing the varieties traditionally grown in Latin America with wild individuals originating from Ethiopia and Kenya. The resulting hybrids proved to be extremely promising as they displayed a high level of heterosis, producing an average

40% more than the best cultivated varieties, with some of them producing coffee exhibiting better sensory qualities than those of the reference varieties (Bertrand *et al*. 2005). The co-breeders of these new varieties soon found the need for a somatic embryogenesis process capable of massively propagating Arabica F1 hybrid clones. However, moving from a technique developed in a research laboratory to an industrial process enabling the annual production of several million plants is a major leap forward. The cobreeders decided to go ahead and fund the research required to achieve this first change of scale. It took place under CIRAD management at CATIE, a regional research centre in Costa Rica.

Results

Situation prior to technological transfer (1995-1996): identification of points for improvement

Several limitations were identified that were an obstacle to technological transfer of the somatic embryogenesis process developed by CIRAD at that point in time. First of all, production costs. With the development of a software package capable of estimating a range of production costs under different culturing conditions, the verdict was announced: 1.52 USD/plant, whereas a conventional seedling cost 0.25-0.35 USD! There was a further handicap, because the planting densities practised with Arabica in Latin America were between 6 and 8,000 trees/hectare. This is a large number that is only possible because of the dwarfism of the varieties used, and that thus allows intensification of production. The additional cost of planting *in vitro* plantlets needed to be limited, even though significant added value was expected with hybrid material. The software also proved useful for precisely identifying the stages in the process responsible for the high cost of production; it involved some later stages, including germination and the development of weanable plantlets, i.e. possessing at least two pairs of leaves to withstand the shock of acclimatization to *ex vitro* conditions. This *in vitro* growth period was classically labourintensive because of the required subculturing and manufacturing of nutrient media and because it took up a great deal of space in the culture rooms. The second limitation was a risk that had so far been overlooked, namely that the somatic embryogenesis process could lead to a high frequency of somaclonal variation. These "photocopy errors" are undesirable since "variant" plants do not display all the agricultural qualities of the selected "mother-plant". Somaclonal variation is a recurring problem in *in vitro* cultures, particularly with somatic embryogenesis systems, which used relatively high concentrations of auxins, such as 2,4-D and IAA to induce the formation and multiplication of embryogenic cells. These growth regulators have often been shown to be implicated in the induction of somaclonal variation (Karp 1994).

Technological innovations and reassuring information on genetic conformity (1996-2001)

Over this period, two technical innovations made technological transfer economically feasible: i) mass production of pregerminated somatic embryos in temporary immersion bioreactors (Albarran 2005) and ii) the possibility of sowing them directly on horticultural substrate to achieve the regeneration of photoautotrophic plantlets in the nursery Barry-Etienne *et al.* 1999, Etienne *et al.* 2002a, Barry-Etienne *et al*. 2002b). These two technological leaps made it possible to transfer most of the late stages (germination, embryo conversion into plants) from the laboratory to the nursery and this considerably reduced production costs. The cost price per plantlet was thus estimated at 0.5 USD. It was wagered that costs could be reduced further by moving on to industrial production conditions.

At the same time, reassuring data were obtained on the genetic conformity of regenerated plants. Firstly, the frequency of somaclonal variation in the field proved relatively low (less than 3%). Secondly, the only variations observed over five years were qualitative, i.e. easily identifiable on a phenotypic level, and not quantitative. For example, the quantity of coffee produced or the amount of a given biochemical contained in the beans were not modified (Etienne and Bertrand 2001). Seven types of phenotypic variants were thus described: the Angustifolia (narrow leaves), Variegata (variegated leaf colouring) and Dwarf variants were the most frequent (Etienne and Bertrand 2003). In addition, multiplication conditions were specified for embryogenic material in cell suspensions, whereby the regeneration of somaclonal variants could be controlled. In 2001, the process (Fig. 1) was therefore considered transferrable to the industrial level, particularly as it had functioned on all nineteen of the F1 hybrids tested.

Establishing the partnership (2003)

In 1999-2000, CIRAD decided to go all the way in commercially developing this somatic embryogenesis process for large-scale multiplication of F1 hybrids, but also endeavoured to acquire useful experience for other tropical species for which application of this technology was being considered. It sought a partner interested in technological transfer for *C. arabica*. A contract was signed with the ECOM group in 2003. The Swiss group, which is a trader of quality coffees and well established in Latin America, notably in Mexico and Central America, proved to be greatly interested, as it was keen to secure its top-of-the-range coffee supplies in that zone. At the time, agronomy trials involving F1 hybrid clones were revealing their remarkable adaptation to agroforestry conditions and confirming the excellence of certain clones in sensory terms (Bertrand *et al*. 2010). The ECOM group was logically very interested, as the majority of coffee plantations in Latin America are managed as agroforests. The adoption of F1 hybrids might make it possible to increase the quantity and quality of coffee produced. The partners chose Nicaragua as the technological transfer site to disseminate Arabica hybrids throughout Central America.

However, both partners were aware of the difficulty of

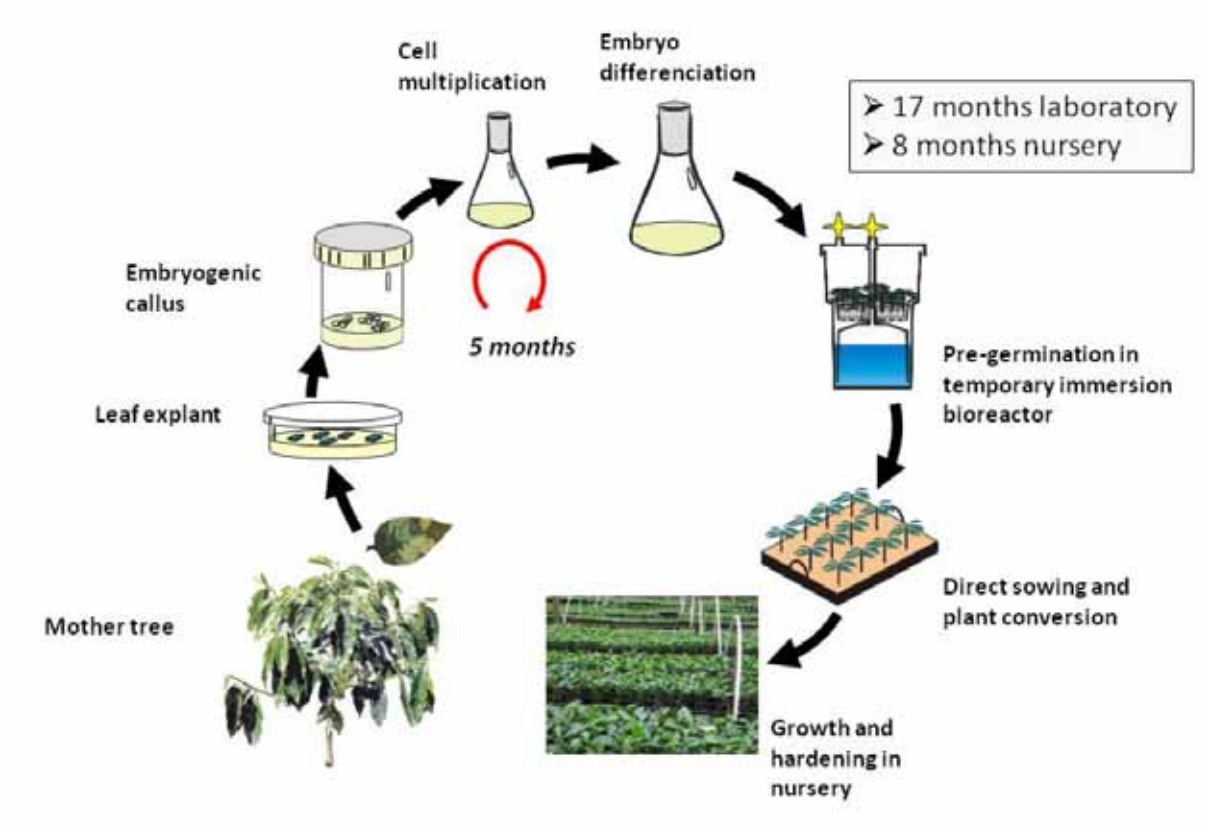

Figure 1: *Schematic representation of the industrial scale coffee somatic embryogenesis process that was transferred.*

such a technological transfer, though probably for different reasons. CIRAD focused on technical difficulties linked to the actual technological transfer itself, and the major change of scale to be achieved (increasing from an annual production of 50,000 plants to several million). For its part, ECOM's main concern was to be able to sell *in vitro* plantlets, as it was justifiably worried about the dual particularity of this new planting material: F1 hybrid and *in vitro* plantlet. Indeed, at that time, there were no known examples of coffee tree breeding programs leading to the commercial distribution of F1hybrids or *in vitro* plantlets. The market had to be created from scratch and there was likely to be a lot of hesitation on the part of coffee growers.

Construction of infrastructures and first adjustments (2004-2006)

The choice was made to construct a small operational 300 m^2 laboratory (Fig. 2), a facility small enough not to increase production costs with unavoidable expenses (fluids, work surfaces, etc.). It was also decided to locate it on the same site as a large coffee processing factory ('beneficio') at Sebaco, a small town 100 km away from the capital Managua, so that the many producers bringing their de-pulped coffee to be processed could also discover the hybrid material and familiarize themselves with this new *in vitro* propagation method. A collection of "mother-plants" (horticultural cuttings or graftings of selected hybrids) was set up near the laboratory. It was to provide the basic material required for *in vitro* propagation. Six to eight clonal copies of each selected tree were maintained at the site under stringent phytosanitary conditions to encourage plant reactivity once placed under *in vitro* conditions. Acclimatization structures were also installed near the laboratory because acclimatization, which is a very delicate stage of the process, calls for meticulous preparation and monitoring after completion of the *in vitro* phase. The acclimatization, hardening and development nurseries were installed at one farm of the ECOM group ('La Cumplida'), located near Matagalpa in the coffee growing zone 30 km from the laboratory.

Many problems were encountered over this period, preventing routine production. Firstly, on a technical level, the water used proved to be extremely hard and heavy limescale deposits covered the heating elements of the autoclaves, the stills, the bioreactors and the leaves of the mother-plants. There were numerous power-cuts which considerably hindered laboratory operations. High contamination levels were recorded during the wet season. Access to locally unavailable manufactured products proved to be a complication. The impossibility of finding staff trained in *in vitro* culture was a major difficulty and meant that the personnel had to be fully trained in the different tasks involved in production operations.

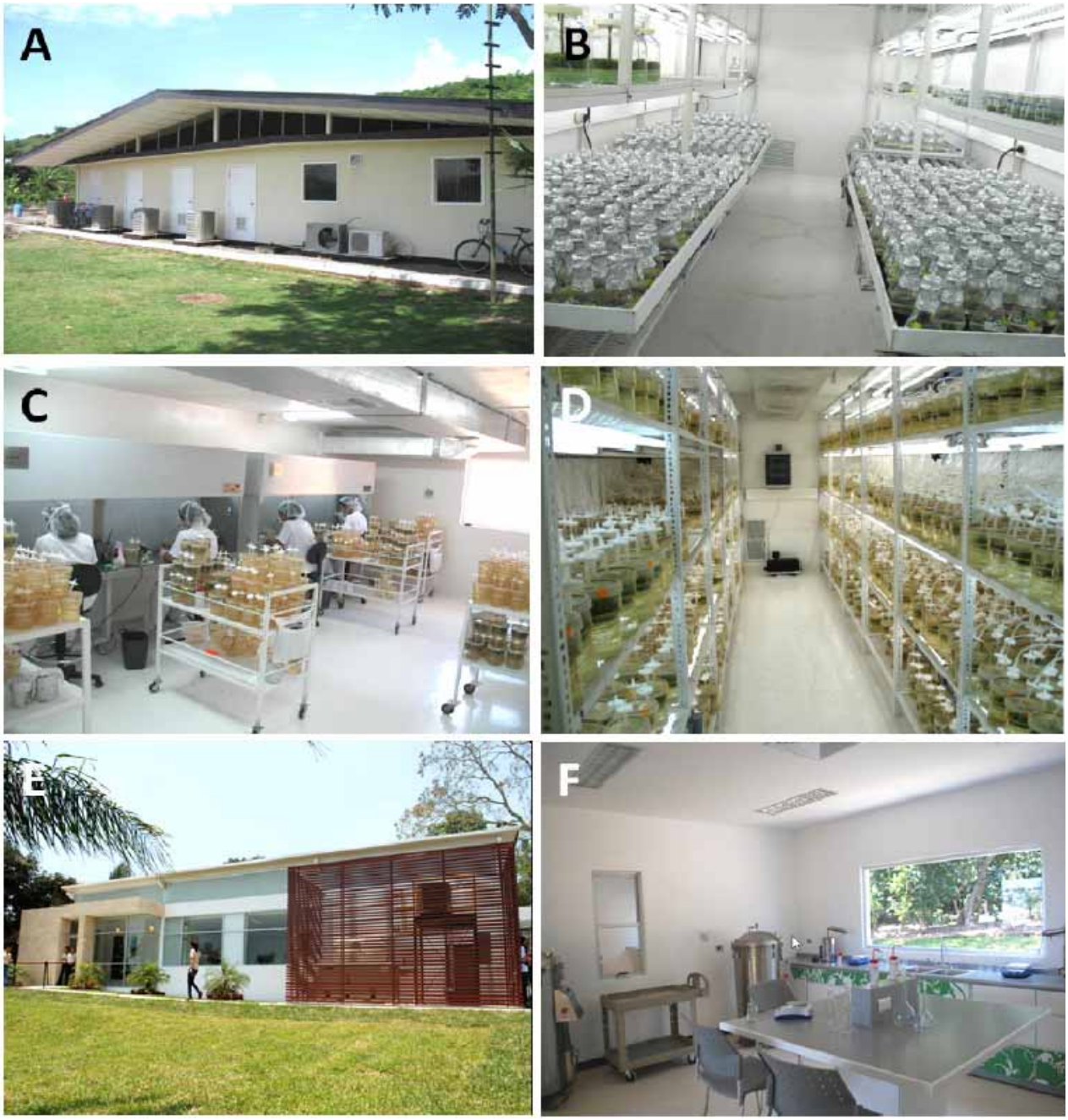

Figure 2: *Coffee in vitro propagation laboratories of Sebacco, Nicaragua (A, view of the building; B, culture room for cell suspension; C, sub-culturing room; D, culture room for bioreactors) and Xalapa, Mexico (E, view of the building; F, room for nutrient media preparation).*

Industrial production and change of scale (2007-2010)

By 2007, most of the technical problems mentioned above had been ironed out and a team of 25 people, eleven of whom were working in the laboratory, had been trained and organized. Several people were trained for each specialized job (medium preparation, autoclaving, preparing cell suspensions, data reporting, acclimatization, etc.), so that there was a replacement for anyone leaving their post. Production started at the beginning of 2007 and rose steadily over 3 years: 30,000 plants sold in 2007, 280,000 in 2008, 650,000 in 2009, and 1,300,000 so far in 2010 with a forecast of 2,500,000 plants by the end of 2011. Eventually, the production target for this laboratory is 5 million plants per year, without any modifications or additional facilities. As we shall see later, it will be possible to achieve this increase in

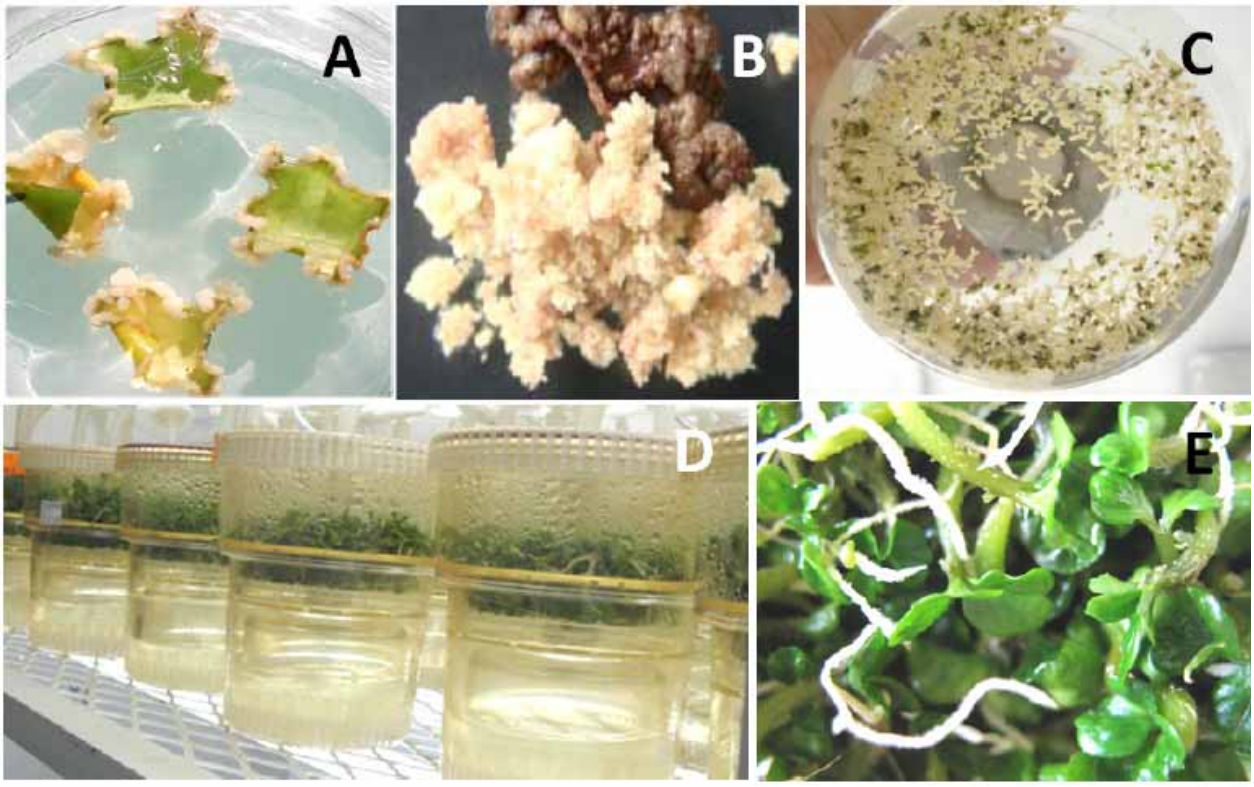

Figure 3: *Development stages of coffee tissues during the in vitro steps of the somatic embryogenesis process. A, leaf explants after one month in culture ; B, embryogenic callus regenerated 8 months after in vitro introduction; C, somatic embryo differentiation in Erlenmeyer flasks ; D, embryo pre-germination in 1 L-RITA® temporary-immersion bioreactors ; E, pre-germinated embryos ready for nursery transfer*

production by optimizing the process. Around ten F1 hybrids were cloned and used to establish a network of several hundred thousand plants grown under agroforestry conditions in Meso-America and Mexico. The first pre-industrial output provided an opportunity to test each stage of the somatic embryogenesis process, identify trouble spots and implement major optimizations. This experience is detailed below, stage by stage.

Industrial feasibility of the different stages of the process

The different stages of the somatic embryogenesis process are diagrammatically represented in Figure 1. Cloning, from the culturing of leaf fragments to the production of plants transferrable to the field, takes 2 years, of which 8 months are spent in the nursery.

Commercial production was launched once the genetic conformity of all the mother-plants had been checked by microsatellite molecular markers (SSR). Three mother trees did not conform to the expected genotype and were discarded.

- *Embryogenic tissue production*. This stage does not raise any problems in Arabica, apart from the fact that it is relatively long (8-10 months). All the explants react by producing a primary scar callus (Fig. 3A) and between 10 and 40% of them, depending on the genotype, produce a secondary embryogenic callus (Fig. 3B). These frequencies are enough for large-scale production, particularly as the embryogenic tissues are subsequently multiplied in the form of suspended cell aggregates. A genotype effect exists but it is easily taken into account by adapting the quantities of leaf explants used.
- *Multiplication of embryogenic tissues and embryo differentiation*. These two stages are carried out in a stirred liquid medium in Erlenmeyer flasks (Fig. 2B), which drastically reduces manpower and laboratory space requirements. For example, 5 million embryos are produced annually on 4 m^2 of stirring tables. This does not raise any problems industrially, but it requires major technical know-how in relation to the other stages, particularly for initially establishing the suspensions. These stages also enable important synchronization of plant material development, which will subsequently persist and help to reduce the work involved in sorting acclimatizable embryos. Consequently, each of these stages corresponds to a single development stage, i.e. embryogenic aggregates, then fully developed embryos at the torpedo stage at the end of the differentiation stage (Fig. 3C).
- *Pre-germination of somatic embryos in bioreactors*. The scaling-up at this stage has been successfully achieved over the last 3 years; 4 million pre-germinated embryos were produced in 2010 in

Figure 4 : *Nurseries of somatic embryo-derived coffee plants in Nicaragua. A and B, acclimatization tunnels ; C, plantlets obtained by direct sowing of pre-germinated embryos ; D and F, transfer of plantlets in 'tubetes' and hardening (E) ; G and H, growth nurseries (farm 'La Cumplida', Matagalpa, Nicaragua).*

one-litre RITA® temporary immersion bioreactors (Teisson and Alvard 1995) (Figs. 2C,D). The contents of these bioreactors had to be harvested 2 to 3 times to collect all the pre-germinated embryos (Fig. 3E) capable of continuing their development into nursery plantlets. However, we found that the volume of the bioreactors (Fig. 3D) was too small for industrial production and prevented any further change of scale by negatively affecting several production parameters. Tests with larger bioreactors (3 litres) revealed several advantages. The reduction in the total number of bioreactors led to greater efficiency, i.e. a larger number of acclimatizable embryos produced for the same amount of work involved. Fewer bioreactors also mean less investment and less cleaning work for the different constituent parts. In addition, the embryo stirring achieved in a larger volume is much more effective and makes for better synchronization during the initial germination stages. Thanks to this optimization all the embryos suitable for transfer to the nursery can be harvested in one go

- *Direct sowing of pre-germinated embryos on horticultural substrate and conversion into plantlets*. This is the trickiest stage of the process during which embryos have to be left to adapt to non-sterile *ex vitro* conditions, which are more subject to variations in temperature and relative humidity than under laboratory conditions. The transfer is made under conditions of saturated relative humidity in plastic tunnels (Fig. 4A). Somatic embryos are grown in the tunnels at high density in an inert, peat-based substrate (Fig. 4B). At the moment, this stage is an industrial bottleneck, as only 60% of embryos regenerate plantlets (Fig. 4C), on average, for all 11 genotypes propagated. Quite a strong genotype effect is found between the propagated hybrid clones. The average time taken for conversion into plants after sowing is relatively long (22 to 24 weeks) compared to seedlings (14 to 15 weeks after seed sowing). Conversion into plants is asynchronous and two to three successive harvests are needed. These observations illustrate that there is major room for improvement at this stage.
- *Growth in the nursery*. Plantlets with two to three pairs of leaves are transferred to more traditional nursery conditions where they are "hardened" to outside conditions by gradually reducing relative humidity and increasing light intensity (Figs 4D, E, G,

Figure 5. *A, 'variegata' somaclonal variant ; B, 'angustifolia' somaclonal variant ; C, Arabica F1 hybrid clone in the field ; D, transport of hybrid vitroplants to coffee growers ; E, Arabica F1 hybrid clones in agroforestry systems.*

H). This stage is well mastered and raises no problems. Although the initial growth of plantlets derived from the somatic embryos is slower and more heterogeneous than that of their seedling counterparts, they catch up by the end of the nursery phase (Fig. 4H) and prove to be even more vigorous than seedlings (Menéndez-Yuffá *et al*. 2010). Losses are minimal, amounting to around 9% of plants, which are discarded in quality checks at the end of the nursery stage. The rejects mostly consist of plants with horticultural defects (lack of vigour, curved stem, etc.); a few plants displaying early symptoms of somaclonal variations (Angustifolia (Fig. 5A) and Variegata (Fig. 5B) are also discarded, but they only amount to 0.3% of total production. The genotype effect is limited or nonexistent during the plant growth stage. The main difficulty for a change of scale at this stage was the choice of containers. If they were too big, very large volumes of horticultural substrate were needed and they took up a great deal of room in the nursery. Moreover, plant transportation to producers and planting out, were subsequently complicated, especially in the predominantly mountainous zones of Central America. A small 200 ml container, called a 'tubete', was chosen (Fig. 4F) because the laboratory has as objective to disseminate *in vitro* plantlets in an inert substrate, free of nematodes and diseases, throughout the Central American zone (Fig. 5D).

Genetic conformity of plants derived from somatic embryos

One of the expectations from this technological transfer was to obtain information about somaclonal variation. We have so far only identified morphological variations (qualitative) in *Coffea arabica* and we have demonstrated that phenotypically normal plants grow and produce normally (Etienne and Bertrand 2001,Etienne and Bertrand 2003). Some variants can be detected and discarded early in the nursery; such is the case with Angustifolia and Variegata variants. As we have seen, these variants only amount to 0.3% of total nursery production. The other phenotypic variants can only be detected at the mature stage, one or two years after planting. The first observations carried out on 100,000 plants established in 2008 in commercial plots in Nicaragua revealed a frequency of around 1% being abnormal, which is commercially perfectly acceptable. The most common abnormalities that were not detected in the nursery were dwarf variants (84%).

Apart from sorting in the nursery, various strategies have been introduced upstream to limit the occurrence of this problem. Firstly, the multiplication time for suspended embryogenic tissues has been shortened, as we

previously showed that the frequency of variation is affected by that parameter Etienne and Bertrand 2003). Likewise, multiplication is carried out in the presence of reduced levels of auxin (2,4-D). Another strategy has consisted in diluting the risk of encountering ahigh frequency of somaclonal variation by grouping together plants regenerated from different cell lines at the end of the nursery phase. To do this, it was necessary to introduce a system of traceability of production batches derived from independent cell lines.

Conclusions on technological transfer

We acquired important experience during this technology transfer. We found that it was unavoidable to move forward by trial and error, as it was impossible to foresee all the possible problems, particularly in a developing country. We learned from this experience that, in the event of problems, it is necessary to analyse these and intervene rapidly, so as to avoid production losses that can quickly become dramatic. In order to do that, it is necessary to establish faultless cohesion and communication beforehand within the team established with the partner. This technology transfer brought together partners from two different worlds, industry/private and research/public. To achieve an efficient partnership based on balanced complementarity, we had to train a joint, multidisciplinary team, which took a while. This partnership was exemplary as it demonstrated that public/private synergy can effectively work for a sustainable agricultural policy under certain conditions. Indeed, providing F1 hybrids to the farmers can serve as a catalyst for them to return to agroforestry (Fig. 5E), after they turned away from it in the 1980s for productivity reasons. At that time they favoured the less ecologically sound practices of the green revolution in vogue during that era.

The technology transfer of somatic embryogenesis is now complete for the Arabica species and demonstrates the feasibility of mass propagation by somatic embryogenesis. A substantial change of scale has been possible for each stage of the process, enabling a continuous flow of production from the laboratory to the nursery. This has given us a precise identification of the strengths and weaknesses of the technical process adopted. The commercial operation was launched in 2008 and the landmark million plants sold in a same year to Central American producers was reached in 2010. In addition, F1 hybrids have confirmed their superiority over traditional lines (Fig. 5C) and are generating such enthusiasm that demand now outstrips production capacity (2 million plants for Nicaragua alone). The aim of the partnership is therefore to respond to demand as quickly as possible through a major change of scale at the production unit in Nicaragua (5 to 6 million plants within 4 years), but also by setting up other units in the region. Indeed, a model is now available for the laboratory and nursery aspects that can be "copied" at other sites. Another production unit was established in May 2010 in the state of Veracruz, Mexico (Figs. 2E, F), to propagate other hybrids; in addition, several industrial nurseries have been established in Mexico, Guatemala and El Salvador.

Lastly, the demonstration that it is feasible to propagate *C. arabica* on a large scale by somatic embryogenesis renews the range of possibilities for this species in the field of genetic selection. Indeed, the success of this technological transfer now means we can consider introducing new varieties from hybrid or mutant materials and also the dissemination of GM varieties by somatic embryogenesis, since efficient genetic modification methods are available for this species (Ribas *et al*. 2010).

References

Bertrand, B., Alpizar, E., Llara, L., SantaCreo, R., Hidalgo, M., Quijano, J.M., Montagnon, C., Georget F & Etienne, H. 2010. Performance of Arabica F1 hybrids in agroforestry and full-sun cropping systems in comparison with pure lines varieties. *Euphytica,* in press.

Bertrand, B., Etienne, H., Cilas, C., Charrier, A. & Baradat, P. 2005. *Coffea arabica* hybrid performance for yield, fertility and bean weight. *Euphytica.* 141:255-262

Albarran, J., Bertrand, B., Lartaud, M. & Etienne, H. 2005 Cycle characteristics in a temporary immersion bioreactor affect regeneration, morphology, water and mineral status of coffee (*Coffea arabica* L.) somatic embryos. *Plant Cell, Tiss. Organ Cult.* 81:27-36.

Barry-Etienne, D., Bertrand, B., Vasquez, N. & Etienne, H. 1999. Direct sowing of *Coffea arabica* somatic embryos mass-produced in a bioreactor and regeneration of plants. *Plant Cell Reports*. 19:111-117.

Barry-Etienne, D., Bertrand, B., Schlongvoïgt, A. & Etienne, H. 2002a. The morphological variability within a population of coffee somatic embryos produced in a bioreactor affects the regeneration and the development of plants in the nursery. *Plant Cell Tiss. Org. Cult.* 68:153-162.

Barry-Etienne, D., Bertrand, B., Vásquez, N. & Etienne, H. 2002b. Comparison of somatic embryogenesis-derived coffee (*Coffea arabica* L.) plantlets regenerated *in vitro* or *ex vitro* conditions : morphological, mineral and water characteristics. *Annals of Botany*. 90:77-85

Ducos, J.P., Gibault, E. & Lambot, C. 2010. Coffee propagation by somatic embryogenesis at Nestle R&D Center-Tours. In: Proceedings of the Conference on Advances in Somatic Embryogenesis of Trees and its application for the future forests and plantations, IUFRO-KFRI 2010 conference, August19-21, 2010, Suwon, Korea.

Etienne, H. & Bertrand, B. 2001. The effect of the embryogenic cell suspension micropropagation technique on the trueness to type, field performance, bean biochemical content and cup quality of *Coffea arabica* trees. *Tree physiology*. 21:1031-1038.

Etienne, H. & Bertrand, B. 2003. Somaclonal variation in *Coffea arabica* : effects of genotype and embryogenic cell suspension age on frequency and phenotype of variants. *Tree Physiology*. 23:419-426.

Karp, A. 1994. Origin, causes and uses of variation in plant tissue culture. In: I. Vasil and T. Thorpe, Editors, *Plant Cell and Tissue Culture*, Kluwer Academic Publishers, Dordrecht 1994, pp. 139–151.

Khaw, C.H., Ng, S.K. & Thong, K.C. 1999. Commercial production of clonal palms by tissue culture – prerequisites, constraints and issues. In: Proceedings of the 1999 PORIM International Palm Oil Congress – Agriculture. Emerging Technologies and Opportunities in the Next Millenium. Palm Oil Research Institute of Malaysia (PORIM), Kuala Lumpur, Malaysia, 1-6 February 1999.

Menéndez-Yuffá, A., Barry-Etienne, D., Bertrand, B., Georget, F. & Etienne, H. 2010. A comparative analysis of the development and quality of nursery plants derived from somatic embryogenesis and from seedlings for large-scale propagation of coffee (*Coffea arabica* L.). *Plant Cell Tiss. Org. Cult.* 102:297-307.

Ribas, A., Dechamp, E., Bertrand, B., Champion, A., Verdeil, J.L., Lashermes, P. & Etienne, H. 2010. *Agrobacterium tumefaciens*-mediated genetic transformation of *Coffea arabica* (L.) is highly enhanced by using long-term maintained embryogenic callus. *BMC Plant Biology*, accepted.

Sampote, S., Ducos, J.P., Lambot, C., Kunasol, T., Kasinkasaempong, Y., Chantanumat, P. & Pétiard, V. 2006. First industrial massive propagation of *Coffea canephora* through somatic embryogenesis organized in Thailand. In: Proceedings of the 21st International Conference on Coffee Science, Montpellier (France), 11th - 15th September 2006.

Teisson, C. & Alvard, D. 1995. A new concept of plant *in vitro* cultivation liquid medium : temporary immersion. In : M Terzi *et al*. (eds) Current Issues in Plant Molecular and Cellular Biology (pp 105-110). Kluwer Academic Publishers, Netherlands.

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Coffee Propagation by Somatic Embryogenesis at Nestlé R&D Center-Tours

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Abstract

A pilot process was implemented at Nestlé R&D Center-Tours for mass propagation of selected Robusta clones. A batch takes 4 to 6 months to complete and consists of three phases. The development of torpedo embryos is achieved using Erlenmeyer flasks. The pregermination is conducted in a 10-L temporary immersion bioreactor made of glass or flexible disposable bags. The latter type, the so-called "Box-in-Bags", insures a higher light transmittance to the biomass due to its horizontal design. It allows a higher torpedo-to-cotyledonary stage conversion rate. Before their shipment, the pregerminated embryos are maintained under storage by spreading them out in layers onto coconut fibres in ex vitro conditions. These storage conditions preserve their ability to develop plantlets for at least 2 months and induce their hardening. Germination tests are performed in the NR&DC-T greenhouse. A closer confinement, obtained by placing a transparent plastic cover at 2 to 3 cm above the embryos, significantly improves the development of leaves and roots. This positive effect is due to the CO2 released from the horticultural substrate. The current production capacity of this pilot process is around 4.0 million pregerminated embryos per year, able to regenerate plantlets with a frequency of 70-76 %. Embryos produced during this implementation step were sent to different coffee producing countries, mainly to Thailand. In the local greenhouses, the embryos are directly transplanted into trays containing commercial peat or coconut fibres. The usage of the "micro-environment" method, combined with media releasing CO2, is well adapted for the large-scale acclimatization of very small vitro-plants in tropical greenhouses. By June 2010, 2.9 million somatic seedlings were produced, out of witch 1.8 million are already planted in the field in this country.

Introduction

Robusta (*Coffea canephora* var. Robusta) is traditionally propagated by seeds although the species is allogamous. The planting material derived from seeds is therefore usually highly heterogeneous with a majority of poor yielding trees. Nevertheless, high yielding clones exist of which some were selected from the Coffee Core Collection established by Nestlé. These are normally propagated by rooting of cuttings but there is a need for a vegetative propagation method faster than that.

Tissue cultures of coffee species exhibit a high embryogenic potential which offers a powerful alternative to other vegetative propagation techniques. During the 90s', three major breakthroughs led to a scaling up of this method: 1) in 1991, in our laboratory, the multiplication of embryogenic cells and the production of embryos (up to the torpedo stage) in liquid media (Zamarippa . 1991, Ducos . 1993) was achieved, 2) in1995, at the CIRAD (France) we obtained the pregermination of embryos (up to the cotyledonary stage) by temporary immersion in liquid media (Berthouly *et al.* 1995, Etienne and Berthouly 2002), 3) in 1999 we achieved the *ex vitro* germination by direct sowing of cotyledonary embryos in the greenhouse, thus avoiding individual manipulation of the embryos in the laboratory (Ducos *et al.* 1999, Barry-Etienne *et al.* 1999).

Starting from these progress steps, we started in 2003 to implement a pilot process for large scale propagation of selected Robusta clones by somatic embryogenesis. We are giving in this paper a summary of various publications about this R&D work.

Material and methods

Callus induction and multiplication

Embryogenic calli of selected clones are induced then multiplied in solid or liquid media as described in previous papers (Ducos *et al.* 2003, 2007a).Young and fully expanded leaves obtained from mature plants derived from cuttings and growing in a greenhouse are washed with tap water then dipped for 30 s. in a 70 % solution of ethanol. The leaves are surface sterilized 30 min. in a solution of calcium hypochlorite ($CaCl_2O_2$) at 40 g l^{-1} and rinsed three times in sterile water. The leaves are cut in small fragments avoiding mid-veins, margins, and apical and basal portions. The explants are placed on callus induction semi-solid medium containing 6-benzylaminopurine (BA) as the only growth hormone (Yasuda *et al.* 1985). It is composed of 1/4 strength macro salts and half strength micro salts of MS medium (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al.* 1968), and is supplemented with 1.0 mg l^{-1} BA, 30 g l^{-1} sucrose and 8.0 g l^{-1} agar. The explants are incubated in darkness at 25 °C and then subcultured for 3 to 4 months on the same medium and exposed to the same conditions.

Six to ten months after the disinfection, yellowish friable primary calli are selected and subcultured on semi solid medium of the same composition except that it has a higher agar concentration (15.0 g l^{-1}). They are subcultured every 6 to 8 weeks and kept under the

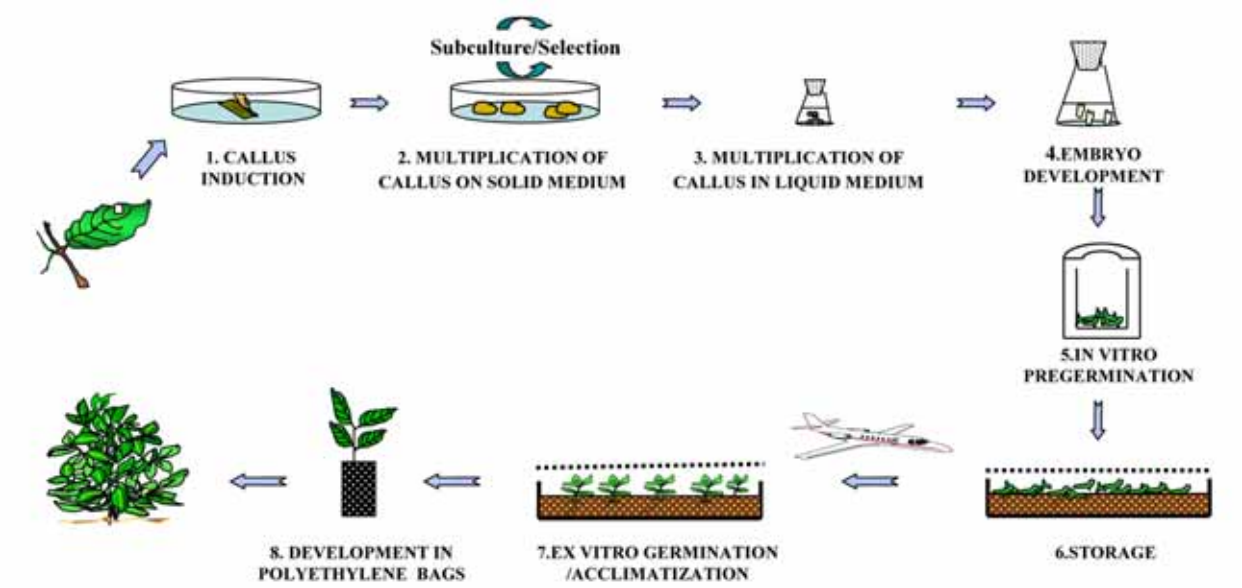

Figure 1. *Propagation of Coffee Robusta selected clones by somatic embryogenesis in liquid medium at Nestlé R&D-Tours Center*

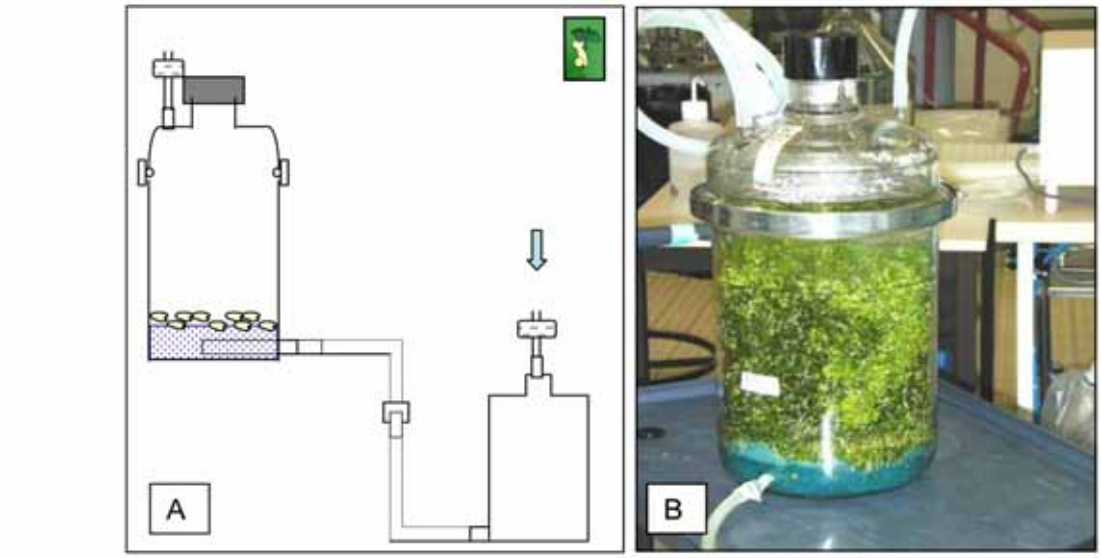

Figure 2. *Glass bottle Temporary Immersion Bioreactor: a) diagram, b) view of a culture of pregerminated Robusta somatic embryos after 2 months.*

earlier mentioned conditions. During each subculture, a binocular microscope is needed to carefully select the right kind of calli, using visual criteria (yellowish color, friable and granulous aspect) in order to maintain the embryogenic potential of the cultures.

To multiply embryogenic calli in liquid media, 0.10 g FW of friable calli are transferred into 10 ml of liquid medium contained in 25 ml Erlenmeyer flasks. Except for the gelling agent, the composition of this medium is identical to the callus induction medium. After 2 weeks, the medium is discarded and the calli transferred into 20 ml of fresh medium in 50 ml flasks. The transfers are repeated every 2 weeks to increase the culture volume: 50 ml in 100 ml flasks (T_{4W}), 100 ml in 250 ml flasks (T_{6W}). At T_{8W}, the cultures are subcultured by re-starting from 0.10 g FW in 10 ml, then by repeating the previous operations. The cultures were maintained on a gyratory shaker at 110 rpm and grown at 26° C with a light intensity of 5 µmol m^{-2} s^{-1} (16-h light day period).

Production and storage of pregerminated embryos

To start a batch production, 0.25-l flasks containing 100 ml of liquid medium are inoculated with 0.10 g FW of selected calli. This medium is composed of macro and micronutrients of MS medium (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al.* 1968) and 30 g l^{-1} sucrose. After 3 weeks, the suspensions are grouped by transferring the contents of two to six 0.25-l flasks into one 1.0-l flask containing 0.5 l of production medium. Then the medium is renewed every 2 weeks until the suspensions are mainly composed of torpedo embryos, generally within 8 to 10 weeks.

For their pregermination, i.e. their development from the torpedo to the cotyledonary stage, the embryos are transferred into 10-l temporary immersion bioreactors (TIB) (Ducos *et al.* 2007ab). The contents of two to six 1.0-l flasks, generally four, are grouped into 0.5 l of medium then transferred into a TIB that had been autoclaved 40 min. at 121 C° with 4.5 l of pre-germination medium. This medium consists of half strength macro and micronutrients MS medium (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al.* 1968), 0.1 to 1.0 mg l^{-1} BA and 20 g l^{-1} sucrose. When most of the torpedo stage embryos turn green, generally within 2 to 4 weeks, the medium is replenished by fresh media of same composition, but without BA. Within 2 to 3 months, the biomass is collected from the TIB and weighed. The temperature ranges between 24 to 27 C° and light intensity between 30 to 60 µmol m^{-2} s^{-1} (16-h light day period), depending on the place of the TIB in the culture room.

For the storage phase, the embryos are spread out in layers onto commercial coconut fibers (Falienor, France) containing Osmocote Mini Plus fertilizer (16+8+11) at 1.0 g l^{-1}. The embryos are kept in stock in the greenhouse inside 420 x 350 x 85 mm polystyrene boxes covered with transparent polycarbonate sheets (Makrolon®). Each storage box contains 2 l of substrate and about 5,000 pre-germinated embryos. The temperature ranges from 20 to 32 C° and the mid-day irradiance from 20 to 100 µmol m^{-2} s^{-1}. The pregerminated embryos are kept in stock in these conditions for 2 to 8 weeks before their shipment.

Ex vitro Germination tests

One germination test consists of 5 replicates, each one of 25 pregerminated embryos sown onto 0.5 l of coconut fibers (Falienor, France) supplemented with Osmocote Mini Plus (16+8+11) at 1.0 g l^{-1} and placed in a 100 x 100 x 100 mm plastic box (Sterivent, Duchefa, Netherlands). The boxes are kept under protective conditions inside a plastic tunnel (0.5 m height, 1.0 m width). Environmental conditions are identical to the ones of the storage phase. The germination rate (i.e. embryo-to-plantlet conversion rate) is calculated 4 months after sowing by counting the embryos which had developed at least one pair of true leaves.

Description of the pilot process

From the pool of selected calli, a production batch is started every month by inoculating 350 to 400 0.25-l flasks. Each batch lasts 4 to 6 months and consists of three phases: the production of torpedo embryos in flasks (phase 4), the pregermination in the bioreactor (phase 5) and the storage of the pregerminated embryos in the greenhouse (phase 6) (Figure1).

The development of torpedo embryos is achieved using Erlenmeyer flasks. Additionally to the rigorous selection of the calli, another very important parameter is the inoculation density which must be lower than 0.5 to 1.0 g FW l^{-1}. Contrary to other groups (Etienne-Barry *et al.* 2010, Menendez-Yuffa *et al.* 2009), we produce coffee torpedo embryos by continuous culture in liquid medium rather by temporary immersion in liquid medium because they are more homogeneous in size and more separated at the end of this phase.

For their conversion into green cotyledonary embryos, the torpedo embryos are transferred into 10-l glass jar temporary immersion bioreactor (TIB). It is similar to the twin flask system described by Escalona *et al.* 1999. It is composed of two glass jars (Figure 2): i) a 10-l jar containing the somatic embryos (200-mm diameter, 300-mm height), ii) a 5-l bottle containing the culture medium and placed below the 10-l jar. Overpressure (0.5 bar) is applied to the vent filter of the medium bottle for 5 min. every 12 h. When the overpressure stops, the medium goes down by gravitation.

Before their shipment, the embryos are maintained under storage by spreading them out in multi-layers onto coconut fibers in *ex vitro* conditions. These storage conditions preserve their ability to develop plantlets for at least 2 months and induce their hardening as the water content decreases by 5 to 10% (Ducos *et al.* 2009). When the embryos are sent to coffee producing countries, one test per clone and per batch of production is performed in our own greenhouse to estimate their ability to develop plantlets.

Results and Discussion

Evaluation of the pilot process

The yearly production of pregerminated embryos was increased from 0.7 million in 2003 to 4.2 million in 2009 (Table 1). During the last year, three operators were required in the laboratory, as well as 0.4 Kg of selected calli, 80 TIB and 8 shakers.

On the average, around 8,000 pre-germinated embryos were collected per TIB. The only embryos counted as "pregerminated" are those having a minimum hypocotyl size of 5 mm and a characteristic "Y" form due to small cotyledonary-like appendages. These criterions will be the main ones used to select good quality embryos at the sowing time in the greenhouse for their development into complete plantlets. Approximately, only half of the embryos collected at the end of the culture in TIB were be scored as "pregerminated".

In 2009, for a total of 8 clones, the conversion rate into complete plantlets was 76 %, on the average.

Table 1. Pilot process for the production of pregerminated somatic embryos of Robusta coffee

Year	Torpedo production (flasks)	Torpedo Pregermination (TIB)	*Ex vitro* Germination	
	Inoculum	Pregerminated Embryos	Germination tests	Theoric Plantlets
	Kg (FW)	*nbr (M)*	*%*	*nbr (M)*
2003	0,1	0,7	37	0,3
2004	0,3	1,3	37	0,5
2005	0,8	2,5	40	1,0
2006	0,7	2,9	67	1,9
2007	0,5	3,2	73	2,3
2008	0,5	2,9	70	2,0
2009	0,4	4,2	76	3,2
Total	3,3	17,6		*11,2*

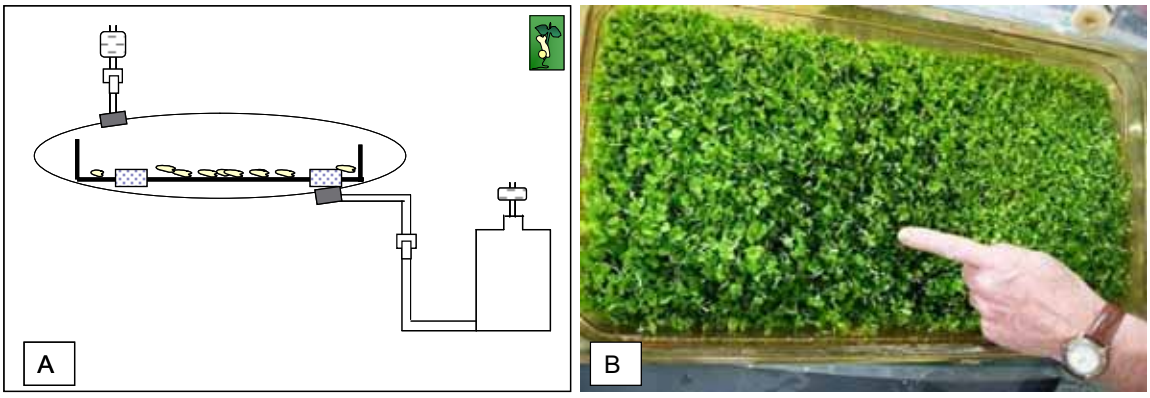

Figure 3. *Horizontal disposable Temporary Immersion Bioreactor ("Box-in-Bag"): a) diagram, b) view of a culture of pregerminated Robusta somatic embryos after 2 months.*

Recent major progress

Two main improvements were achieved.

The first one concerns the development of a new kind of TIB. During the culture in glass TIB light becomes a rate-limiting factor as it can only penetrate the first few centimetres into the embryo biomass. Moreover, the embryos with large cotyledons settle last at the end of each immersion period and thus cover the biomass underneath. A non-uniform light distribution inside the TIB may be responsible for differences in growth and quality among embryos.Our hypothesis was that a horizontal design will be more convenient than a vertical one by providing a higher light illumination to the embryos due to a greater surface-to-volume ratio. Different types of bioreactors were tried, but at the end the most simple and best solution was to place a rigid box inside a flexible plastic bag (Figure 3) (Ducos *et al.* 2007bc). This last one, called "Box-in-Bags", allows a higher torpedo-to- pregerminated cotyledonary stage conversion rate (Table 2).

The second improvement concerns the germination in *ex vitro* conditions. We had observed that a closer confinement, obtained by placing a transparent plastic cover at 2 to 3 cm above the embryos, significantly improved the development of leaves and roots. We could not explain the reason for that, until we noticed that coconut fibres as well as peat release significant quantities of CO2. As a result, the CO2 concentration ranged from 0.1 to 1.5 % in the headspace of the germination boxes. Different experiments demonstrated that the positive effect of the micro-environment was due to the CO2 released from these horticultural substrates (Ducos *et al*. 2009). As a result, the conversion rates into plantlets increased from less than 40% to more than 70%.

Shipment to producing countries

Embryos produced during this implementation step were sent to coffee producing countries, mainly to Thailand but also to Mexico and the Philippines. In the local greenhouse, the pregerminated embryos were directly transplanted into trays containing commercial peat or coconut fibers. The usage of the "micro-environment"

Table 2. Comparison of the pregermination in two Types of Bioreactor. Data are the average of 8 clones with 2 to 10 comparisons per clone. Each bioreactor are inoculated with 40 to 45 g of torpedo-stage embryos.

Bioreactor	Final FW	Pregerminated Embryons	Germination ex vitro
	g	*nbr X 1000*	*%*
Glass Jar	260	8.6	80
Box-In-Bag	437[s]	16.0[s]	69[ns]

[s] significantly different , t-Test , Pvalue < 0,01

method, combined with the media that released CO2, is well adapted for the large-scale acclimatization of very small vitro-plants in tropical greenhouses. In Thailand, it has been applied since the autumn of 2006 and led to a germination rate higher than 60 %. By June 2010, 2.9 million somatic seedlings were produced, out of witch 1.8 million were planted in the field in this country. In addition to our production activity, scientists of national institutes of different coffee producing countries were trained in our center to handle somatic embryogenesis technology.

Conclusions

Recent progress in plant micropropagation mainly consist on two majors trend: i) bulk-cultivation of small propagules in liquid medium under photomixotrophic conditions (with sugar), followed by their direct transfer to the greenhouse for their conversion to plants, ii) production of fully developed and individualized (singulated) plantlets on solid medium under photoautotrophic conditions (in sugar-free medium with CO_2-enrichment and a high light intensity), followed by their transfer to the greenhouse.

Up to now, our research was based on the first approach, but future progress may depend on a combination of both approaches, both in the laboratory for the pregermination and in the greenhouse for the germination.

Concerning the pregermination phase, Uno *et al*. (2003) reported that white coffee torpedo stage embryos can convert into green cotyledonary embryos on solid culture medium under photoautotrophic conditions. Obviously, the pregermination conducted in the temporary immersion bioreactor with this mode of culture will only be efficient if the coffee embryos are well illuminated, i.e. enough dispersed on the support. Advances can be expected by the development of horizontally designed bioreactors, especially if facilitated by plastic bags technology. During preliminary experiments, we confirmed that even white torpedo stage embryos can convert into green cotyledonary ones without sugar in the medium but with CO_2 enrichment in a Box-in-Bag bioreactor.

Concerning the germination in the greenhouse, the micro-environment method is empirical: its efficiency depends on the container, the cover and the nature of the substrate. However, we expect that further improvements are possible. Currently we are validating a tentative model in which the CO_2 concentration in the headspace will reach an equilibrium that is a function of the CO_2 diffusing rate from the substrate to the headspace ($Kla_{CO2media}$) and of the CO2 diffusing rate from the headspace to outside the container ($Kla_{CO2leackage}$):

$$CO_{2equilibrium} = CO_{2max} \cdot (Kla_{CO2\ media} / (Kla_{CO2\ media} + Kla_{CO2\ leackage})$$

Finally, we observed that, if the embryos are dispatched in layers on coconut fibres under micro-environment conditions, a significant part of them spontaneously developed into plantlets, even though they have not been individually sown. This suggests that at least a part of the sowing operations can probably be automated.

References

Berthouly, M., Dufour, M., Alvard, D., Carasco, C., Alemano, L., Teisson, C. 1995. Coffee micropropagation in a liquid medium using the temporary immersion technique. In: ASIC (ed) Proceeding of 16th Colloquium of International Coffee Science Association, ASIC, Vevey, Switzerland, pp 514-519.

Barry-Etienne, D., Bertrand, B., Vasquez, N., Etienne, H. 1999. Direct sowing of *Coffea arabica* somatic embryos mass-produced in a bioreactor and regeneration of plants. Plant Cell Rep. 19:111-117.

Ducos, J.P., Zamarripa, A., Eskès, A., Pétiard, V. 1993. Production of somatic embryos of coffee in a bioreactor. In: ASIC (Eds), 15th Int Sci Colloquium Coffee, Montpellier, pp. 89-96. ASIC, Paris.

Ducos, J.P., Gianforcaro, M., Florin, B., Pétiard V., Deshayes, A. 1999. A technically and economically attractive way to propagate elite *Coffea canephora* (Robusta) clones: in vitrosomaticembryogenesis. In: ASIC (Eds) 18th Int Sci Colloquium Coffee, Helsinki, pp. 295-301. ASIC, Paris.

Ducos, J.P., Alenton, R., Reano, J.F., Kanchanomai, C., Deshayes, A., Pétiard, V. 2003. Agronomic performance of *Coffea canephora* P. trees derived from large-scale somatic embryo production in liquid

medium. Euphytica. 131:215-223.
Ducos, J.P., Labbé, G., Lambot, C., Pétiard, V. 2007a. Pilot scale process for the production of pre-germinated somatic embryos of selected Robusta clones. In Vitro Cell. Dev. Biol.-Plant. 43:652-659.
Ducos, J.P., Chantanumat, P., Vuong, P., Lambot, C., Pétiard, V. 2007b. Mass Propagation of Robusta Clones: Disposable plastic bags for pre-germination of somatic embryos by temporary immersion. Acta Hort. (ISHS). 764:33-40.
Ducos, J.P., Lambot, C., Pétiard, V. 2007c. Bioreactors for Coffee Mass Propagation by Somatic Embryogenesis. International Journal of Plant Developmental Biology. 1:1-12.
Ducos, J.P., Prévot, A., Lambot, C., Pétiard, V. 2009. Positive effect of the CO2 released by commercial substrates on the ex vitro germination rates of coffee somatic embryos. Acta Hort. (ISHS) 812:329-336.
Etienne, H., Berthouly, M. 2002. Temporary immersion systems in plant micropropagation. Plant Cell Tiss. and Org. Cul. 69:215-231.
Escalona, M., Lorenzo, J.C., Gonzales, B., Daquinta, M., Gonzales, J.L., Desjardins, Y., Borotto C.G. 1999. Pineapple (*Ananas comosa L. Merr*) micropropagation in temporary immersion systems. Plant Cell Rep. 18:743-748.
Gamborg, O.L., Miller, R.A., Ojima, K., 1968. Nutrients requirements of suspension cultures of soybean root cells. Exp. Cell. Res. 50:151-158.
Menendez-Yuffa, A., Barry-Etienne, D., Bertrand, B., Georget, F., Etienne, H. 2010. A comparative analysis of the development and quality of nursery plants derived from somatic embryogenesis and from seedlings for large-scale propagation of coffee (*Coffea arabica* L.). Plant Cell Tiss. Organ. Cult. 102:297–307.
Murashige, T, Skoog, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissues cultures. Physiol Plant 15:473-497.
Uno, A., Ohyama, K., Kosai, T., Kubota, C. 2003. Photoautotrophic culture with CO2 enrichment for improving micropropagation of *Coffea arabusta* using somatic embryos. Acta Hort. (ISHS) 625:271-277.
Yasuda, T., Fujii, Y., Yamaguchi, T. 1985. Embryogenic callus induction from *Coffea arabica* leaf explants by benzyladenine. Plant and Cell Physiol. 26: 595-597.
Zamarripa, A., Ducos, J.P., Bollon, H., Dufour, M., Pétiard, V. 1991. Production of coffee somatic embryos in liquid medium: effects of inoculation density and renewal of the medium. Café Cacao Thé. 35:233-244.

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Somatic Embryogenesis in Whitebark pine (*Pinus albicaulis*) and Its Implication for Genetic Resource Conservation and Restoration

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Abstract

Whitebark pine (*Pinus albicaulis*) is an ecologically important keystone species that is an integral part of the ecosystem in western North America; however, the species is seriously threatened due to susceptibility to white pine blister rust (*Cronartium ribicola*), attack by mountain pine beetle (*Dentroctonus ponderosae*) and prolonged fire suppression, preventing natural regeneration. Projected climate change also casts further uncertainty on the species' future. Whitebark pine is in serious trouble and urgent effort is needed to ensure its protection, genetic resource conservation, and restoration. Somatic embryogenesis (SE) in whitebark pine has been obtained, embryogenic lines have been cryopreserved and thawed, and plants have been generated. In combination with other conservation measures, SE provides an additional tool for developing rust-resistant genotypes as well as long-term storage of valuable genotypes by cryopreservation. The latter does not change the genetic makeup or cause loss of juvenility. The application of SE for ecologically important and threatened species provides a new dimension for genetic resource conservation and species rescue.

Key Words: blister rust, cryopreservation

Introduction

Whitebark pine (*Pinus albicaulis*) is a soft pine growing in high-elevation montane forests of western North America (Arno and Hoff 1989), extending along mountain slopes from British Columbia (BC) to California and eastward to Montana and Wyoming (Critchfield and Little 1966). In Canada, it is distributed in the mountains of British Columbia and Alberta. It is a slow-growing, long-lived small tree of upper subalpine ecoregions of BC and Alberta, often found on steep mountain slopes from 1000 m to the tree line (Farrar 1995). Typically, it grows under drought conditions. The species is an ecologically important keystone species (Lanner 1982), because the seed of whitebark pine is an important food source for animals, including red squirrels, bears, and particularly, Clark's nutcracker, *Nucifraga columbiana*. Furthermore, Clark's nutcraker is the main means of seed dispersal for whitebark pine as the seed has no wing; forgotten caches provide a source of regeneration for the species. Whitebark pine forests also protect watersheds by regulating snow capture and melt (Lee 2003) and they stabilize soil at high elevations (Alberta SRD and ACA 2007). However, over much of its range, the species is seriously threatened due to susceptibility to white pine blister rust (*Cronartium ribicola*), attack by mountain pine beetle (*Dentroctonus ponderosae*) and prolonged fire suppression preventing natural regeneration. By far, the most serious threat is white pine blister rust; the vast majority of whitebark pine stands in Alberta are infected, resulting in rapid and significant mortality (Anon. 2007). A survey of the health of whitebark pine across its range in BC revealed that about 19% were dead and another 31% had active blister rust infection. The survey also concluded that the combination of high mortality, lack of a suitable substrate for regeneration, and incursion of climax species such as subalpine fir (*Abies lasiocarpa*) indicates further decline in whitebark pine (Zeglen 2002). Also, projected climate change casts further uncertainty on the species' future. Therefore, protection, genetic resource conservation, and restoration efforts are urgently needed for the species. Currently, the species is on the "watch list species" in Alberta and is "blue listed" as a species of concern in BC. Due to seed predation, tree decline associated with blister rust infection and difficulties in accessing trees, little to or seed is available for planting stock production, although BC made a significant seed collection in 2007 (D. Simpson, Canadian For. Serv., Pers. Comm.).

Somatic embryogenesis (SE) is a recently developed biotechnology technique whereby genetically identical trees are produced through the formation of embryos using tissue culture methods and is the primary enabling technology for all conifer biotechnology products. Plant propagation by SE involves four distinct stages, including initiation of embryogenic tissue, maturation of somatic embryos, germination of somatic embryos, and acclimation of somatic trees (Klimaszewska *et al*. 2007). However, the most important advantage of plant propagation by SE is that embryogenic tissue can be cryogenically stored in liquid nitrogen (Kartha 1985). This offers not only a means of storing germplasm but also provides opportunities to develop well-adapted and pest-resistant trees through lengthy screening and testing (Park and Bonga 2011).

Initiation of SE is influenced by several factors: In many pine species, the initiation is limited to the first few weeks of embryo development before the emergence of cotyledonary primodia (Becwar *et al*. 1990, Park *et al*. 1998, Percy *et al*. 2000). Thus, targeting the most responsive stage of zygotic embryo development is important. Furthermore, for pines (Garin *et al*. 1998, Lelu *et al*. 1999, MacKay *et al*. 2006) and other conifers (Cheliak and Klimaszewska 1991, Park *et al*. 1993), SE initiation is under strong genetic control. Therefore, it is important to include several seed families in the initiation experiments. Another important factor is the composition of tissue culture media, and various formulas have been used in pines, including DCR (Gupta and Durzan 1985), LV (Litvay *et al*. 1985) and MSG (Becwar *et al*. 1990).

In this paper, we report on successful plant regeneration of whitebark pine by SE and discuss its implication in protection, conservation, and restoration of this species.

Materials and methods

Plant material

For the initiation experiment, open-pollinated cones were collected at three different dates in 2008, i.e., 26 and 31 July and 11 August at Window Mtn. Lake (49.76467° N lat. 114.63980° W long. 2149 m ASL), Table Mtn. (49.36641° N lat. 114.25193° W long. 2204 m ASL) and Mt. DeVeber (53.70696° N lat. 119.60741° W long. 1909 m ASL), respectively. The Table Mtn. collection was chosen to represent the southern distribution and the Mt. De Veber collection the northern distribution of the range in Alberta. Although these stands were generally healthy, signs and symptoms of white pine blister rust were present at Window Mtn. Lake and Table Mtn. whereas the Mt. DeVeber stand within the Willmore Wilderness Park is in an area of mountain pine beetle activity and control. This stand had received anti-aggregation pheromone (Verbenone) treatment as a protective measure. Due to limited availability of cones, 10 cones from each of five OP families were collected at different locations and collection dates and shipped to the laboratories of Canadian Forest Service in Fredericton, New Brunswick and in Ste-Foy, Quebec. Seeds were extracted and subjected to surface sterilization. The sterilization procedure involved placing seed in a plastic basket stirred vigorously for 6 min in 200 ml of 3% (v/v) hydrogen peroxide with a drop of Tween 20. Seeds were then rinsed three times with sterile water. An alternative method was used in the laboratory in Quebec where intact cones were submerged in 98% ethanol for 10 min, air dried in a laminar flow unit after which the seeds were removed aseptically, hence the seed disinfection step was eliminated. The seed coat, nucellus, and membrane surrounding the megagametophyte were removed, and the excised whole megagametophyte was placed on initiation medium in 100 x 15 cm Petri dishes. Ten Petri dishes per family containing 10 explants each were placed, but the numbers varied slightly due to the availability of seeds. An experiment was also carried out to test the efficacy of "nicking" the micropylar end in order to expose the zygotic embryos to the medium more directly. At a later collection date when zygotic embryos were fully developed, i.e., 11 August, whole embryos were also excised and plated.

Tissue culture media

Two media formulations were tested for initiation, both based on modification of Litvay's medium (Litvay *et al*. 1985). The first consisted of half-strength macroelements and full-strength microelements plus Fe-EDTA and vitamins. The medium was supplemented with 1 g/L caseine hydrolysate, 0.5 g/L of L-glutamine, 20 g/L of sucrose and solidified with 4 g/L of gellan gum (Phytagel, Sigma) and contained 2.2 µM each of 2,4-D and BAP or 9.5 µM 2,4-D and 4.5 µM BAP. Media pH was adjusted to 5.8. These media are designated as mLV-L and mLV-S, respectively. The other medium, called 1/2LV, consisted of half strength macro- and microelements and vitamins with 9.5 µM of 2,4-D and 4.5 µM of BAP. Petri dishes were kept in the dark and, after 12 weeks, initiation of embryogenic tissue was assessed.

For maturation, varying numbers of embryogenic cultures (9 to 29 lines) were randomly selected and proliferated to obtain a sufficient quantity for the maturation experiment. The mLV maturation medium contained 60 g/L of sucrose with either 60 or 120µM of racemic abscisic acid (ABA). In addition to these ABA concentrations, medium with maltose instead of sucrose and coating the cells with particles of activated charcoal (AC) (Sigma) (Lelu-Walter *et al*. 2006) were also tested. A total of six media formulations were used in the maturation experiments. For each cell line approximately two hundred milligrams of fresh mass of embryogenic tissue were suspended in plant-growth-regulator-free (PGR-free) liquid medium (without or with 1 gl^{-1} CH), and then poured over a filter paper disk (Whatman no. 2) placed on approximately 25 ml of gelled maturation medium (Klimaszewska *et al*. 2000). Cultures were kept in the dark at 24 ºC for 12 weeks.

Of the mature somatic embryos, 22 randomly selected lines were subjected to germination and subsequent plant regeneration. The germination medium contained 20 g/L of sucrose, 6 g/L of gellan gum and no PGRs (Klimaszewska *et al*. 2000). After about 8 weeks, germinated somatic embryos were transferred to a soil mix (1:1 peat: vermiculate) and raised in a greenhouse.

Cryopreservation

Vigorously growing embryonal masses (EM) were cryopreserved using the protocol described in Klimaszewska *et al*. (2007). This protocol involves culturing 3 g of fresh mass in 7 ml of liquid initiation medium supplemented with 0.4 M sorbitol for 18–24 h. Subsequently, 3 ml of cooled dimethylsulphoxide (DMSO) was added to the suspended EM on ice. The EM suspensions remained on the ice and then were poured into cryo-vials, which were placed in alcohol-insulated containers (Cryo 1°C Freezing Containers, Nalgene™).

The containers were placed in a -80 ºC freezer for 2 h, and then the cryo vials were submerged in liquid nitrogen (- 196ºC).

To verify successful cryopreservation, one cryo-vial from each family was thawed after 24h in liquid nitrogen. For re-growth, the contents of cryo-vials were rapidly thawed in a water bath at 37ºC for 1-2 min. and the cell suspension was poured over a filter paper disk placed on thick pad of blotting paper. The storage solutions were allowed to drain for several minutes and the filter paper with cells was transferred onto initiation medium in Petri dishes. Growth of cultures typically occurs in 1-2 weeks after thawing.

Results

The process of somatic embryogenesis in whitebark pine is illustrated in Figure 1.

Initiation of somatic embryogenesis

Of the three collection dates, the first collection (27 July) produced the highest initiation percentage with a mean of 14.6%. The initiation percentage among the five OP families ranged from 9.9% to 24.2%, indicating variability among OP families (Table 1). The initiation percentage, while using the same mLV medium, decreased drastically to an average of 3.6% and 5.4% for the subsequent collection dates on 31 July and 14 August, respectively. This decrease in initiation percentage after 27 July was significantly ($p=0.05$) higher, but the difference between the dates 31 July and 14 August was not statistically significant. For the later two collection dates, it was possible to compare the effects of initiation media. The mean initiation rates on mLV and ½ LV were 4.5% and 2.6%, respectively. Although mLV produced a higher SE initiation rate, this difference was not significant. In another experiment we compared the initiation response on mLV-L versus mLV-S media and from uncut and cut megagametophytes (Table 2). On medium with reduced PGR, mLV-L, the response was almost double compared to the response on mLV-S (9.6 and 5.7%, respectively). It was also clear that the explants should not be cut before culture as this procedure decreased the response.

Maturation of somatic embryos

The production of mature somatic embryos was carried out using six maturation treatments. Due to the different availability of lines and quantity of embryogenic tissue, the number of lines and plates varied in each treatment combination, but several of the lines used in the experiments were common across the treatments. Although formal statistical analyses were not carried out, generally a higher level of ABA produced more mature somatic embryos per plate, i.e., 15.9 normal embryos per plate at 120 µM of ABA (control) *vs.* 2.5 for 60µM (Table 3). Maturation also produced abnormal embryos, such as those with fused cotyledons, and precociously germinating embryos as described in Park *et al*. (1998). Activated charcoal increased embryo production only slightly at the higher level of ABA, and substituting sucrose with maltose did not improve maturation. The use of maltose and AC seems to improve embryo production; however, this trend should be interpreted cautiously due to a lack of robust statistical analysis.

Cryogenic storage and thawing

Of about 100 embryogenic lines initiated, 20 were randomly selected for acryopreservation test using the protocol described previously. After a month in cryogenic storage, the lines were thawed, and all the lines re-grew.

Discussion

Somatic embryogenesis offers two important tools for conservation and restoration of whitebark pine. First, it provides an opportunity to cryogenically store a large number of genotypes for future restoration and secondly, genetically identical genotypes are produced in sufficient numbers to conduct clonally replicated genetic tests for disease resistance.

Somatic embryogenesis in whitebark pine has not been reported previously. In our laboratories, SE in whitebark pine was obtained on average at 14% when seed was collected in late July 2007, indicating the importance of collection timing and the collection timing X genotype interaction. As in many conifers (Park *et al*. 1993, MacKay *et al*. 2006), our data showed that genotype and stage of zygotic embryo (ZE) development were important for SE initiation. Improvement in SE initiation is important in order to capture a wider range of genetic variability. The data reported here were based on an exploratory experiment using our standard laboratory SE protocols. It is likely that SE initiation frequency may be improved by careful selection of the responsive stage of ZE development, manipulation of media components (Klimaszewska *et al*. 2000) and in some cases, crossing of responsive genotypes (MacKay *et al*. 2006).

As previously mentioned, the major threat to the existence of whitebark pine is blister rust and it is further threatened by the presence of mountain pine beetle and projected climate change. The whitebark pine genetic restoration program carried out for the Intermountain Region of the United States relies on plus-tree selection and rust screening (Maholovich and Dickerson 2004). Somatic embryogenesis can facilitate and complement these efforts. For example, in addition to collection of cones from healthy trees in high-risk areas, embryogenic lines can be produced and cryogenically stored. Due to animal feeding and difficulties of accessing stands, no seed is available for reforestation. Therefore, the SE system using immature seed can be used as a means of mass producing reforestation stock.

The most important factor for successful restoration of whitebark pine is to identify and quantify the amount of genetic variability in threat-resistance. In western white pine (*P. monticola* Dougl. ex D. Don), the current blister rust screening trials involved the use of offspring from

Figure 1. *Process of somatic embryogenesis in whitebark pine: (A) immature cones collected in late July; (B) seeds being excised to remove megagametophytes; (C) whole megagametophyte placed on initiation medium; (D) megagametophytes contain zygotic embryos (ze) at the poly-embryonic stage (upper right and lower left corner) at this time, and (E) embryo dominance among ze is subsequently established; (F) sometimes, translucent poly-embryonic ze are extruded from the megagametophyte (on the right); (G) embryogenic tissue is initiated and begins to grow rapidly; (H) on maturation medium, somatic embryos appear and (I,J) continue to develop; (K) fully mature somatic embryos; and (L) mature somatic embryos are germinated and ready for transplanting in a greenhouse.*

Table 1. Somatic embryogenesis initiation percentages by collection dates, media, and families[a].

Collection dates:	July 27	July 31		August 14	
Media:	mLV	mLV	½ LV	mLV	½ LV
Family 1	24.2	0.0	0.0	5.6	1.1
Family 2	15.1	1.8	2.0	3.0	0.0
Family 3	13.1	0.9	1.7	8.2	10.0
Family 4	10.7	5.0	1.0	6.0	1.0
Family 5	9.9	10.1	6.7	4.4	2.0
Family means	14.6	3.6	2.3	5.4	2.8

[a] OP families were randomly collected at different locations due to insufficient availability of cones at single sites, and thus, they are not the same families across the collection dates.

Table 2. Somatic embryogenesis initiation percentages averaged for all five seed families.

Medium	Explants not-cut	Explants cut
mLV-S	5.7	1.7
mLV-L	9.6	4.8

Table 3. Production of somatic embryos of normal and variant morphologies per Petri dish in 6 maturation treatments.

	60μM ABA		120μM ABA			
	No AC	AC[a]	No AC, No Maltose	AC	Maltose[b]	AC+Maltose
Normal	2.5	1.6	15.9	20.9	14.7	36.7
Variant	3.2	3.2	17.5	36.6	33.6	24.8
No. plates	51	23	90	65	37	22
No. lines	18	11	29	29	14	9

[a] Activated charcoal
[b] Substituted maltose in lieu of sucrose.

open- or control-pollinated families and they have shown varying degrees of resistance at the family level (King and Hunt 2004). A major gene for resistance has also been found in sugar pine (*P. lambertiana* Dougl.) (Kinlock *et al*. 1970); however, more complex resistance such as "partial resistance" or "tolerance" are more difficult to characterize (King and Hunt 2004). For a better understanding of the genetics of disease resistance, it is essential to use individuals clonally replicated by SE in challenge experiments to eliminate confounding by genetic effects of segregating family lots. Furthermore, once resistant genotypes are identified, they can be thawed from cryogenic storage and propagated for restoration plantings. King and Hunt (2004) suggest using a structured mating design in combination with the use of clonal replicates to gain a better understanding of the underlying genetics of rust resistance.

A simplified strategy for developing rust-free trees and deploying them in restoration efforts may involve using selections of putatively rust-free trees and conducting controlled crosses among them for clonal material production for testing and deployment. To study pathogen–host relationships, an elaborate mating design may be required. However, if the development of a rust-resistant line is the main purpose, a simpler pair-mating may be sufficient. The seeds obtained from these crosses are subjected to SE, and once SE is initiated, the embryogenic lines are cryogenically stored in liquid nitrogen. Then, a portion of frozen embryogenic lines are thawed, propagated to produce clonal replicates, and planted in rust screening trials. Hoff *et al*. (1994) suggest inoculating 2-year-old seedlings. It is also desirable to establish a longer-term, clonally replicated field test of embryogenic lines at sites with varying degrees of rust hazard. The short-term seedling test and longer-term field test results are used to identify

rust-resistant lines that can be used in restoration programs. The identification of resistant genotypes may be further enhanced by the development of molecular marker technology.

Although conventional breeding techniques may be used to develop rust-resistant genotypes, the use of SE and cryopreservation will likely expedite the developmental process with greater precision. In this strategy, SE and cryopreservation are used as tools for packaging natural variability in resistant genotypes. Furthermore, SE is the primary enabling technology for all conifer biotechnology products including genetic transformation. In most forest tree species, genetic transformation is usually not necessary as there is an abundance of natural genetic variability for commercial traits that can be packaged. However, in rare and endangered species with a complete lack of resistance to devastating diseases, genetic transformation can be one effective means of species rescue. Perhaps, major gene resistance found in other related species may be transferred to the ecologically important and endangered species using SE.

Conclusion

Somatic embryogenesis in whitebark pine has been obtained successfully and embryogenic lines were developed, cryopreserved, and thawed, and somatic plants were produced. Somatic embryogenesis, in combination with other conservation measures, provides an additional tool for developing rust-resistant genotypes as well as long-term storage of valuable genotypes. The application of SE in ecologically important and threatened species provides a new dimension for genetic resource conservation and species rescue.

References

Anon. 2007. Status of whitebark pine (*Pinus albicaulis*) in Alberta. Alberta Wildlife Status Rep. No. 63. Alberta Sustainable Resource Development (SRD) and Alberta Conservation Association (ACA). Edmonton, AB, Canada.

Arno, S.F., Hoff, R.K. 1989. Sivics of whitebark pine (*Pinus albicaulis*). GTR-INT-253, USDA Forest Service Intermountain Research Station, Ogden, UT.

Becwar M.R., Nagmani R., Wann S.R. 1990. Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Can. J. For. Res. 20:810–817.

Cheliak, W.M., Klimaszewska, K. 1993. Genetic variation in somatic embryogenesis response in open-pollinated families of black spruce. Theor. Appl. Genet. 82:185-190.

Critchfield, W.B., Little, E.L. 1966. Geographic distribution of the pines of the world. USDA Forest Service Misc. Pub. 991. Wash., D.C.

Farrar, J.L. 1995. Trees in Canada. Fitzehenery & Whiteside Ltd and Can. For. Serv., Nat. Res. Canada. Markham, ON, Canada.

Garin, E., Isabel, N., Plourde, A. 1998. Screening of large numbers of seed families of *Pinus strobus* L. for somatic embryogenesis from immature and mature zygotic embryos. Plant Cell Rep. 18:37-43.

Gupta, P.K., Durzan, D.J. 1985. Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Rep. 4:177-179.

Hoff, R.J., Hagle, S.K., and Krebill, R.G. 1994. Genetic consequences and research challenges of blister rust in whitebark pine forest. USDA For. Serv. Intermountain Res. Sta. Gen. Tech. Rep. INT-GTR-309, Ogden UT. pp. 110-135.

Kartha, K.K. 1985. Meristem culture and germplasm conservation. In: Kartha, KK (Ed) Cryopreserrvation of Plant Cell and Organs. CRC Press, Fl, USA. pp115-134.

King, J.N., Hunt, R.S. 2004. Five needle pines in British Columbia: Past, present and future. USDA For Serv. Proc. RMRS-P-32.

Kinlock, B.B. Jr., Parks, G.K., Fowler, C.W. 1970. White pine blister rust: Simply inherited resistance in sugar pine. Science 167:193-195.

Klimaszewska, K., Bernier-Cardou, M., Cyr, D.R., Sutton, B.C.S. 2000. Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. In Vitro Cell. Dev. Biol.-Plant 36:279-286.

Klimaszewska, K., Trontin, J.-F., Becwar, M.R., Devillard, C., Park, Y.S., Lelu-Walter, A.-M. 2007. Recent progress in somatic embryogenesis of four *Pinus* spp. Tree For. Sci Biotech. 1:11-25.

Lanner, R.M. 1982. Adaptation of whitebark pine for seed dispersal by Clark's nutcracker. Can. J. For. Res. 12:391-402.

Lee, I. 2003. Whitebark pine: keystone species in Peril. Ecoforestry Spring 2003, 28-32.

Lelu-Walter, M.-A., Bastien C., Drugeault A., Gouez M.L., Klimaszewska K. 1999. Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without growth regulators. Physiol. Plant. 105: 719-728.

Lelu-Walter, M.-A., Bernier-Cardou, M, Klimaszewska, K. 2006. Simplified and improved somatic embryogenesis for clonal propagation of *Pinus pinaster*. Plant Cell Rep. 25:767-776.

Litvay, J.D., Verma, D.C., Johnson, M.A. 1985. Influence of loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucus carota* L.). Plant Cell Rep. 4:325-328.

MacKay, J.J., Becwar, M.R., Park, Y.S., Corderro, J.P., Pullman, G.S. 2006. Genetic control of somatic embryogenesis initiation in loblolly pine and implication for breeding. Tree Genet. Genom. 2:1-9.

Mahalovich, M.F., Dickerson, G.A. 2004. Whitebark pine genetic resource program for Intermountain West

(United States).USDA For Serv. Proc. RMRS-P-32.
Park, Y.S., Bonga, J.M. 2011. Application of Somatic Embryogenesis in Forest Management and Research. *In* Proc of the IUFRO 2.09.02 conference on Somatic Embryogenesis of Trees. Aug. 19-22, 2010. Suwon, Korea.
Park, Y.S., Pond, S.E., Bonga, J.M. 1993. Initiation of somatic embryogenesis in white spruce (*Picea glauca*): genetic control, culture treatment effects, and implications for tree breeding. Theor. Appl. Genet. 86:427-436.
Park, Y.S., Barrett, J.D., Bonga, J.M. 1998. Application of somatic embryogenesis in high-value clonal forestry: Deployment, genetic control, and stability of cryopreserved clones. In Vitro Cellular and Develop. Biol.- Plant. 34:231-239.
Park, Y.S., Bonga, J.M., Cameron, S.I., Barrett, J.D., Forbes, K., De Verno, L.L., Klimaszewska, K. 1998. Somatic embryogenesis in jack pine (*Pinus banksiana* Lamb.). *In*: Jain S.M., Gupta P.K., Newton R.J. (eds.) Somatic embryogenesis in woody plants, vol. 4. Kluwer Academic Pub, The Netherlands, pp 491-504.
Percy, R.E., Klimaszewska, K., Cyr, D.R. 2000. Evaluation of somatic embryogenesis for clonal propagation of western white pine. Can. J. For. Res. 30:1867-1876.
Zeglen, S. 2002. Whitebark pine and white pine blister rust in British Columbia Canada. Can. J. For. Res 32:1265-1274.

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"Somatic Embryogenesis of Trees" conference on "Advances in Somatic Embryogenesis of Trees and Its Application for the Future Forests and Plantations" August 19-21, 2010, Suwon, Republic of Korea

Maturation of Somatic Embryos of *Nothofagus alpina*, Induced from Mature Seeds

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Abstract

We carried out several tests to evaluate the effect of carbon type, abscisic acid and duration of cold treatment on the maturation and germination of somatic embryos of *Nothofagus alpina* induced from cotyledons of embryos excised from mature seed. Different treatments were applied to pro-embryogenic masses containing cotyledonary somatic embryos that were harvested and cultured in basal Broadleaved Tree Medium without plant growth regulators. The three factors studied significantly influenced both maturation and germination. The highest level of germination (53,3%) and plant conversion (22,2%) were obtained by increasing the concentration of sucrose in the culture medium to 60 gL^{-1}. This resulted in microplantlets that were morphologically normal. The cold treatment also significantly influenced the germination of the SEs. Thus, after a period of 6 weeks at 4°C, germination of SEs reached 43,4%, although the conversion rate did not exceed 8%. The application of ABA brought premature germination under control and resulted in continued growth of the somatic embryos. It also reduced the incidence of recurrent embryogenesis to 13,3%. These results show that we have managed to optimize the conversion of raulí SEs to viable plants.
Key words: micropropagation, plant growth regulators, raulí.

Introduction

Nothofagus alpina ((Poepp. *et* Endl.) Oerst.), known in South America as raulí, is a species that is distributed west of the Andes. It attracts attention, particularly because of its forestry potential and ecological importance as a endemic species in the sub-antarctic forests. Because of the quality of its timber, its distribution and relative abundance in southern Chile, it has become, together with two other species of the same genus, namely roble (*N. obliqua*) and coigüe (*N. dombeyi*), of economic interest, mainly in the sawmilling industry (Donoso *et al*. 1998, Grosse and Quiroz 1998).

Traditionally, the regeneration of this species is naturally by seed, but due to its intolerant characteristics regeneration is successful only in places where mineral soil is exposed and in the presence of light (Donoso *et al*. 1998). Direct seeding is difficult due to special germination requirements, low viability, cyclical production of seeds and insects consuming the endosperm and embryo (Loewe *et al.* 1997).

Micropropagation by organogenesis has been attempted with *Nothofagus*, mainly with juvenile material of roble (Martínez-Pastur and Arena 1995, Muñoz 1999), raulí (Jordan *et al*. 1996, Martínez-Pastur and Arena 1996, Sanchez-Olate *et al*. 2004), *N. antartica* (ñirre) (Martínez-Pastur *et al*. 1997) and *N. pumilio* (Lenga) (Martínez-Pastur and Arena 1999). This has resulted in adventitious shoot and root development *in vitro*, but with only modest proliferation and rooting rates, varying considerably between species and sources. Somatic embryogenesis, on the other hand, could be a valuable tool for propagation of all species within the genus because it regenerates bipolar structures with a high regenerative capacity (Margara 1988, Pierik 1990).

There are only a few references dealing with plant regeneration via somatic embryogenesis, or induction of pro-embryogenic masses (PEMs), for *Nothofagus* species (Castellanos *et al*. 2005). However, investigations have been performed with other species of the Fageceae family, such as those conducted with the genus *Quercus* (Manzanera 1992, Fernández Galiano *et al*. 1997, Cuenca *et al*. 1999) and *Castanea* (Piagnani and Eccher 1990, Xing *et al*. 1999, Corredoira *et al*. 2003). With these, somatic embryos at different stages of development, maturation, germination and plantlet conversion were obtained (Piagnani and Eccher 1990, Manzanera 1992, Fernández-Galiano *et al*. 1997, Xing *et al*. 1999, Mauri and Manzanera 2003).

Thus, along with the induction of pre-embryogenic masses from differentiated tissue, maturation of somatic embryos is a key stage in the propagation of plants through somatic embryogenesis. Maturation occurs after differentiation of the polar embryo and its different organs and meristematic regions (West and Harada 1993). During maturation the morphogenic process is interrupted by a shift to the synthesis and accumulation, mainly in the cotyledons, of reserve substances such as proteins, lipids and, in some cases, many carbohydrates (Thomas 1993). Therefore, dynamic events occur during the maturation phase of seed development.

Although the development of somatic embryos passes through the same successive developmental stages as

zygotic embryos, they do not undergo dormancy (Zimmerman 1993). Additionally, the integuments and the endosperm, which are required for preservation and germination of the zygotic embryo, are not formed (Dodeman *et al*. 1997). Reserve products accumulated by somatic embryos have similar characteristics to those that are stored by zygotic embryos. Similarly the sub-cellular compartments to which these substances are directed are similar in both cases (Von Arnold *et al.* 2002). However, the amount of a particular substance stored, and the time at which this occurs, may vary between somatic and zygotic embryos (Merkle *et al*. 1995).

Consequently, the maturation process of somatic embryos needs to be studied for each species and/or the *in vitro* culture conditions have to be managed in such a way that natural conditions that promote embryo maturation are approximated as much as possible to achieve rates of germination and plant conversion at operationally acceptable levels. Clearly, the level of knowledge about the phenology and reproductive cycle of the species in question plays a crucial role.

In angiosperms and particularly in the Fagaceae, the study of somatic embryo maturation has focused, as was done with conifers, on analyzing the effect of plant growth regulators (PGRs), osmotic components of the culture medium and low temperature treatments on the advanced states of embryo development (Cuenca *et al*. 1999, Corredoira *et al*. 2003, Robichaud *et al*. 2004).

Thus, ABA has been used in a wide range of concentrations to improve maturation, showing a positive effect on the prevention of precocious germination and accumulation of reserve substances (Gupta and Grob, 1995). Where ABA is ineffective, an increase in osmotic potential in the culture media has often been successful. Generally, an increase in the concentration of sucrose or other osmotic agent such as polyethylene glycol has been used to confer dehydration tolerance to the embryoid, as a pre-condition for germination (Sunderlíková and Wilhelm, 2002).

The objective of our study was to analyze the influence of maturation factors, such as abscisic acid (ABA), low temperatures and the choice and concentration of carbohydrate, on the *in vitro* germination of *Nothofagus alpina* somatic embryos derived from the cotyledons of mature zygotic embryos. In addition, their conversion to microplants is an additional objective.

Materials and methods

Plant material

We used an embryogenic line, induced from isolated cotyledons of mature seeds of raulí that were obtained through controlled pollination in the clonal seed orchard at Huillilemu in the Tenth Region of Chile. After the induction of embryogenic callus and initial embryo development, the culture was placed on maintenance medium consisting of a mineral and vitamin solution BTM (Chalupa 1983), plus benzyladenine (BA) and naphthaleneacetic acid (NAA) at concentrations of 0,1 mgL^{-1} each, plus 30 gL^{-1} sucrose and 6,0 gL^{-1} agar. Subcultures were established on fresh medium every 28 days, alternating two subcultures on medium with growth regulators with one subculture on BTM basal medium without hormones. The explants remained on the latter at the start of the trials.

Initial explants

The starting explant for each of the tests consisted of embryogenic masses (EMs) showing cotyledonary somatic embryos (Figure 5A). EMs were selected, whose fresh weight at the start of the maturation treatments was between 50 to 80 mg. The initial fresh weight of each explant was recorded.

For germination cotyledonary somatic embryos were isolated from the PEMs after the maturation treatments were finished. Pre-sterilized surgical equipment was used under a stereomicroscope, to select embryos with a normal morphology and axis length of 3 mm or higher as the explants for this stage (Figure 5B).

Maturation treatments

We performed maturation assays with ABA, cold treatment and different carbon sources. Each of these trials was followed by germination and plant conversion while keeping track of the origin of each maturation treatment. In all cases, the BTM mineral solution was used as base medium plus 30 gL^{-1} sucrose and 7,0 gL^{-1} agar, plus plant growth regulators BA and NAA at a concentration of 0,05 mgL^{-1}, unless otherwise indicated.

The chilling effect on the maturation and germination of somatic embryos was evaluated by testing six treatments, a normal temperature of cultivation (25± 1ºC) for 2 (T1), 4 (T2) and 6 (T3) weeks and the same periods of time under a constant temperature of 4ºC (T4, T5 and T6, respectively). To evaluate the abscisic acid effect, four treatments were applied, including a control without PGRs (T1) and 3 levels of this hormone, 0,2; 1,0 and 2,0 mgL^{-1} (T2, T3 and T4, respectively and, without BA and NAA) for 4 weeks. The influence of concentration and type of carbon was evaluated after application of five treatments, a control without addition of carbohydrates (T1), 30 gL^{-1} and 60 gL^{-1} sucrose (T2 and T4) and 30 gL^{-1} and 60 gL^{-1} of maltose (T3 and T5), for 4 weeks. For subsequent germination, which lasted a total of 21 days, the BTM mineral solution and vitamins was applied, without PGR and the macronutrient level was reduced to 25%.

Culture conditions
Results

Effect of cold treatments on maturation and germination of somatic embryos

During the maturation phase, there was a significant cold duration effect on the fresh weight increase of the EMs (v-p = 0.0000) both at normal and at 4°C temperature. However, there was no significant difference in response between the treatments at 4°C and normal temperature (Figure 1). The trend was the same for both, reaching the highest fresh weight after 6 weeks (20,9 mg

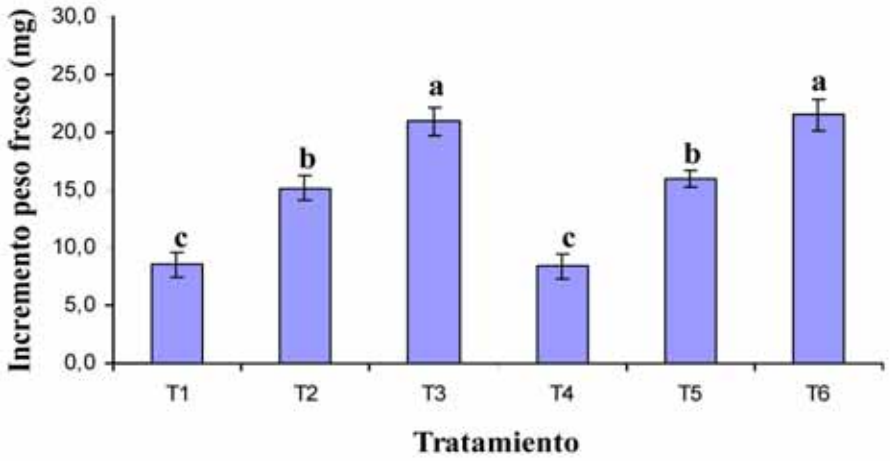

Figure 1. *Effect of temperature and duration of exposure on fresh weight increase of raulí EMs.*

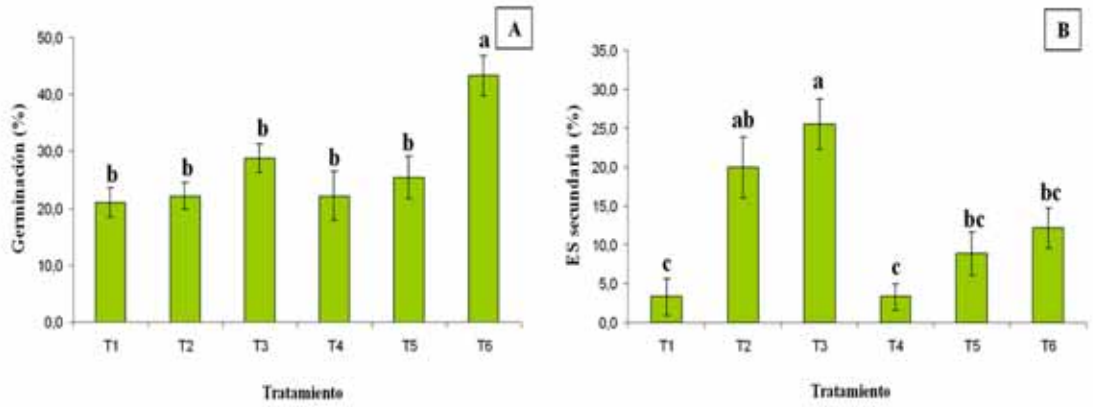

Figure 2. *Effect of cold treatments on germination (A) and secondary somatic embryogenesis (B) in somatic embryos of N. alpina.*

for T3 (25°C) and 21,5 mg for T6 (4ºC)).

The treatments did not significantly influence the organogenic response of the EMs (v-p = 0.4514), although at normal temperature organogenesis occurred in 6,7% of the cultures (in the T2 and T3 treatments) and in 2,2% of the cultures at 4°C (in the T5 and T6 treatments) . The survival rate after the normal temperature treatment was 100% and 97,8% after the 4°C treatment, a difference that was not statistically significant.

However, the cold treatment significantly affected germination and plant conversion. After 6 weeks of incubation at 4ºC (T6), the germination rate reached 43,3%, which was significantly (v-p = 0.0000) different from the response obtained with the other treatments (Figure 2A). After 10 days on germination medium the embryos showed normal morphology with a pair of well-developed cotyledons, although these were generally smaller than those observed in their zygotic counterparts (Figure 5C). There was no statistically significant effect of the duration of the cold treatment on the frequency of secondary embryogenesis. At 25°C, however, duration had a significant effect on that parameter (v-p = 0.0000). At that temperature, secondary embryogenesis started to appear after 5 days, mainly in the hypocotyl region. The highest incidence of SSE was obtained at 23°C after 6 weeks of culture (T3). At 25,5% this was not significantly different from the rate at T2, but higher than at the other treatments (Figure 2B). At low temperature, SSE occurred at equal or significantly (v-p = 0.0077) lower rates than at 25°C in an overall analysis between the two temperatures.

The maturation treatments did not significantly affect the conversion rate of germinating embryos to plants (v-p = 0.2243), reaching the highest value (7.8%) with the T6 treatment.

Effect of ABA on the maturation and germination of SEs

There were no significant (v-p = 0.4346) differences

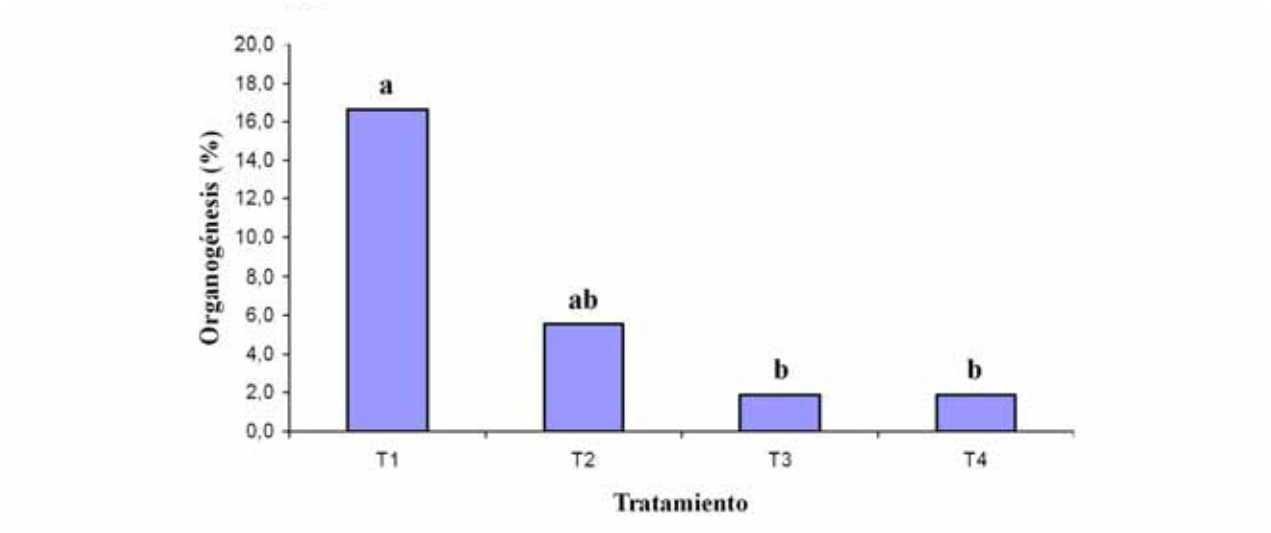

Figure 3. *Effect of ABA treatments on the organogenic response of raulí EMs.*

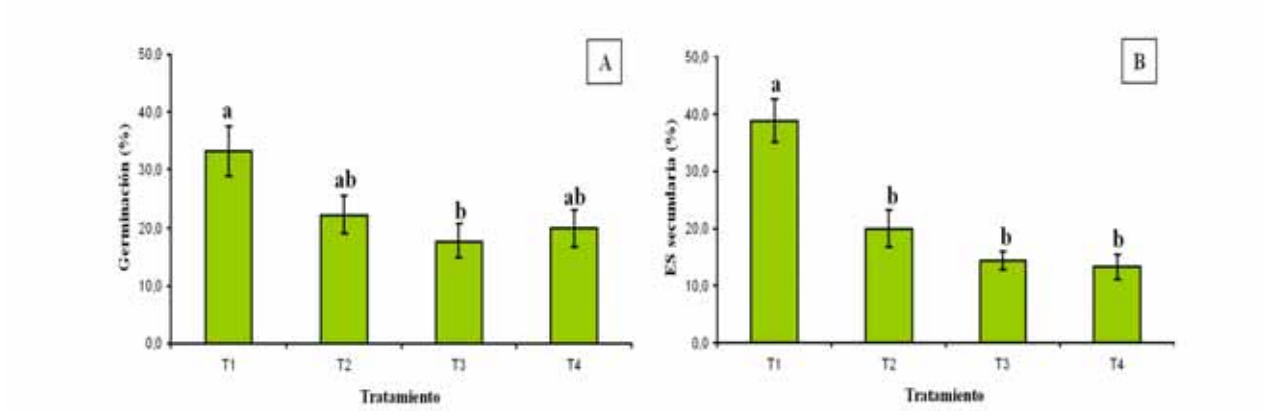

Figure 4. *Effect of ABA treatments on germination (A) and secondary somatic embryogenesis (B) in somatic embryos of N. alpina.*

in fresh weight increase (FWI) of the embryogenic aggregates between the ABA treatments. The FWI values ranged from 11,2 mg with T3 (1,0 mL^{-1} of ABA) and 13,6 mg in the control without ABA.

The organogenic response of the PEMs was significantly influenced by ABA (v-p = 0.0146). The highest percentage (16,7%) of organogenesis occurred in the control , but this was not significantly different from the value obtained with T2 (0,2 mgL^{-1} of ABA). With the other treatments the response fell to 1,9% (Figure 3). Although the cultures were maintained in continuous darkness, elongating buds appeared that could potentially be used for further multiplication by placement on organogenic stem proliferation medium (Figure 5D). All cultures survived the ABA treatments, except that with the highest concentration of ABA (T4) the survival rate dropped slightly to 98,2% after 4 weeks of culture. However, this difference was not significant (v-p = 0.4132).

ABA significantly (v-p = 0.0170) affected germination of the somatic embryos. The highest rate of germination occurred in the control (33,3%). This was significantly different from the result obtained with T3 (1,0 mgL^{-1} of ABA) but not significantly different from that obtained with T2 and T4 (Figure 4A) . For its part, the application of ABA had a significant effect on the control of secondary embryogenesis (v-p = 0.0000), decreasing its incidence with respect to the control treatment (Figure 4B, 5E).

The treatments had no significant (v-p = 0.9726) effect on the rate of conversion to plants. The highest percentage of somatic embryos that became plants was 5,6%, which was achieved both in the control treatment and after application of 1,0 mgL^{-1} of ABA (T3).

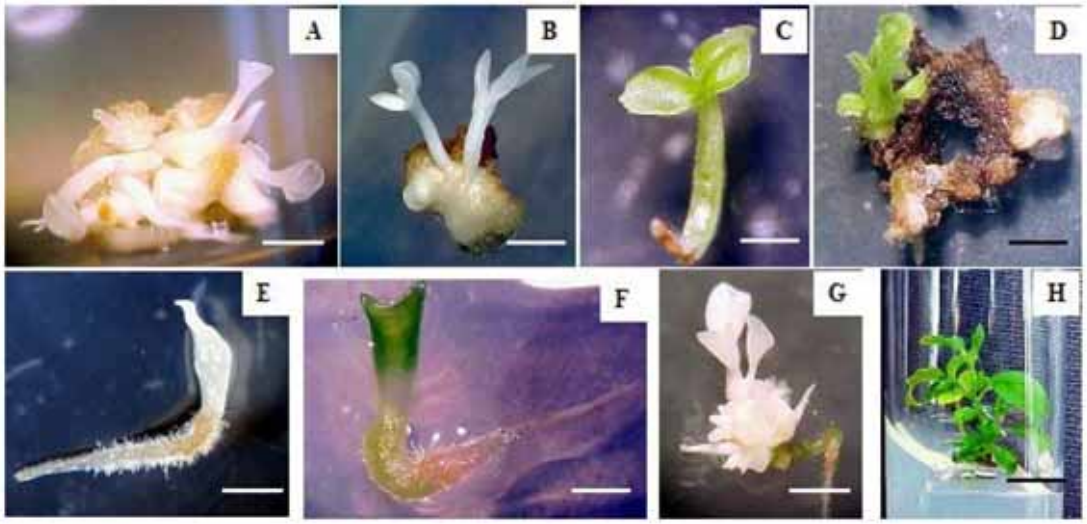

Figure 5. *Different aspects of the maturation of Nothofagus alpina somatic embryos.* ***A.*** *Embryogenic masses (EMs) used in the maturation treatments (bar = 2 mm).* ***B.*** *Cotyledonary somatic embryo stage being used in the germination treatments (bar = 1,5 mm).* ***C.*** *SE after 10 days on germination medium, after treatment at 4º C for 6 weeks (bar = 2 mm).* ***D.*** *Organogenic response of EM in medium without PGR (bar = 4mm).* ***E.*** *Control of SSE during the germination of SE, by application of 0.2 mgL^{-1} of ABA (bar = 2 mm).* ***F.*** *Germination of SE after 15 days of culture, after maturation in medium containing 60 gL^{-1} sucrose (bar = 3 mm).* ***G.*** *Expression of SSE during germination, after maturing in medium with 30 gL^{-1} sucrose (bar = 3 mm).* ***H.*** *A somatic embryo converted into a raulí plant (bar = 7 mm).*

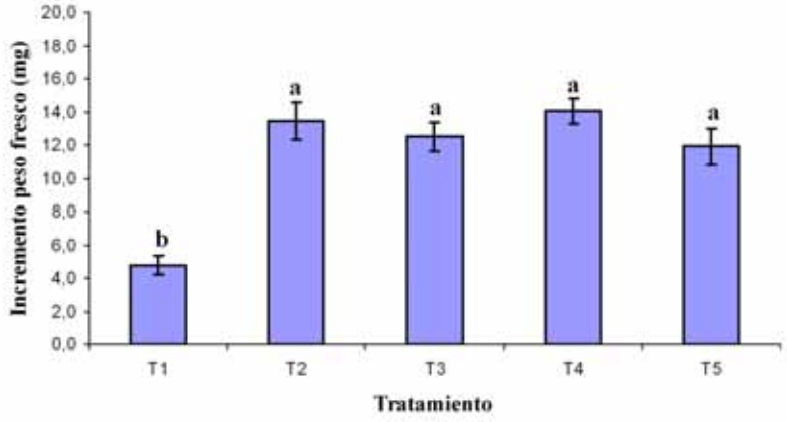

Figure 6. *Effect of treatment with 30 gL^{-1} and 60 gL^{-1} sucrose and maltose, on fresh weight increase of raulí PEMs.*

Effect of carbon type on the maturation and germination of SEs

No significant differences in FWI were found after exposure to the two different carbon types. Sucrose and maltose at 30 gL^{-1} and 60 gL^{-1} produced a significantly (v-p = 0.0000) higher FWI than the control. The control FWI was 4,8 mg after 4 weeks in culture and 14,0 after 4 weeks on 60 gL^{-1} sucrose (T4) (Figure 6). The percentages of EMs presenting organogenesis were not significantly influenced by the treatments (v-p = 0.1110). Only 4,4% of the explants showed an organogenic response in the control. This was not significantly different from the responses obtained with the other treatments where it peaked at 20,0% after treatment with maltose (30 gL^{-1} and 60 gL^{-1}).

The survival rates of the EMs were 100% except that in the T1 (no carbon control) it was slightly lower (95,6%). In the latter there was a slight mortality of cellular aggregates after two weeks.

The treatments had a significant effect on the germination rates of somatic embryos (v-p = 0.000), reaching 53,3% with T4 (60 gL^{-1} sucrose), which was significantly higher than obtained with T3 and T1 but not than the values obtained with T2 (30 gL^{-1} sucrose) and T5 (60 gL^{-1} of maltose), where the average rate of germination was 44,4% and 43,3% respectively. With 60 gL^{-1} sucrose (T4), germination is triggered on the fifth day of culture, resulting in simultaneous elongation of the

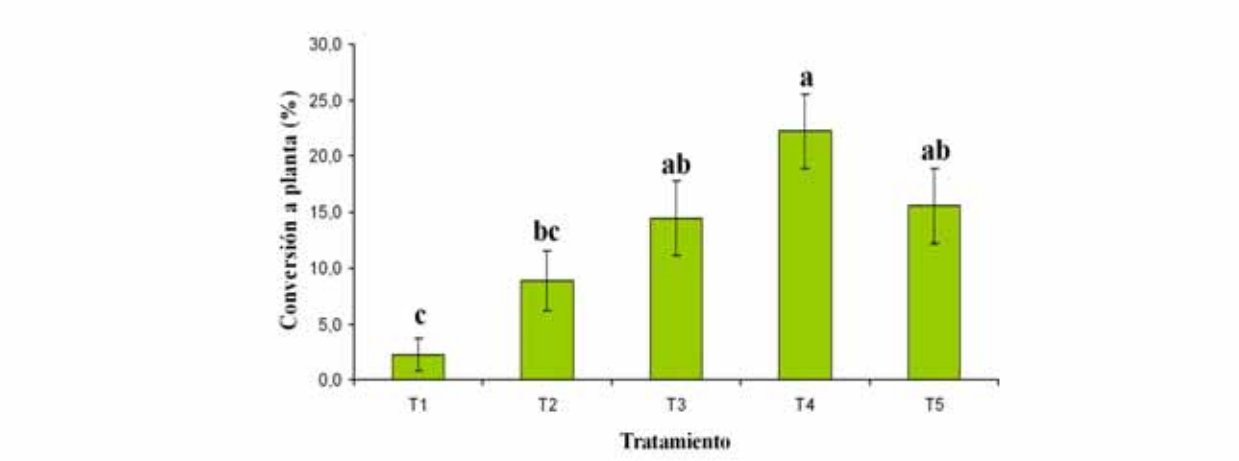

Figure 7. *Effect of carbon type and concentration on the plant conversion of N. alpina somatic embryos.*

hypocotyl and root axis. The aerial parts became photosynthetically active when transferred to light and developed morphologically normal cotyledons (Figure 5F). This had a clear impact on the subsequent plant conversion rate after this treatment. The SSE incidence rates were also significantly influenced by the treatments applied (v-p = 0.0002). The manifestation of secondary somatic embryogenesis was highest in T2 (30 gL^{-1} sucrose) with 47,8% of the explants showing this process (Figure 5G). This response did not differ significantly from that obtained with T3 and T5 (maltose treatments), but differed significantly from the control response and that obtained with the application of 60 gL^{-1} sucrose (T4), where only 34,4% produced secondary embryos.

Carbohydrate type and concentration significantly influenced the conversion rate of somatic embryos into normal plants (v-p = 0.0003). Of the SEs that matured in medium with 60 gL^{-1} sucrose (T4) 22,2% underwent synchronous development of both poles of growth. After 35 days of culture they developed true leaves, an epicotyl and a main root with laterals (Figure 5H). The response to 60 gL^{-1} sucrose, although higher, was not significantly different from that obtained with 30 gL^{-1} and 60 gL^{-1} of maltose, where the conversion rates were 14,4% and 15,5%, respectively (Figure 7).

Considering together the three variables analyzed, germination percentage, SSE and conversion to plants, the best results were achieved by increasing the concentration of sucrose in the medium to 60 gL^{-1}. Those plantlets that did not develop proper roots could be multiplied via organogenesis.

Discussion

There are morphological and biochemical similarities between somatic and zygotic embryogenesis. However, in many species, especially in woody ones, we must use special culture sequences that promote the maturation of somatic embryos prior to germination (Palada-Nicolau and Hausman 2001). In zygotic embryos the maturation process usually involves some dehydration, which reduces the metabolism to an inactive or quiescent state. However, in somatic embryos this slowdown to dormancy does not occur so that its tolerance to dehydration is very limited (Von Arnold *et al.* 2002). Precocious germination, and consequently, low rates of survival and conversion into plants, is common in woody plants, as was observed in *Quercus robur*, in which epicotyl development was rare when a culture protocol was used that did not include a proper maturation treatment (Cuenca *et al*. 1999).

The increase in osmotic potential in the culture medium imitates the water stress conditions that occur in the seed and thus it increases the tolerance of SEs to dehydration. We observed this in our study and this was also noted in cultures of *Quercus ilex*, where an increase in the concentration of sucrose to 90gL^{-1} had a positive effect on fresh and dry weight and on the germination of somatic embryos (Mauri and Manzanera 2003).

An increase in the concentration of sucrose to 60 gL^{-1} , combined with BA and NAA in the medium, controlled precocious germination of somatic embryos of *Castanea dentata* and achieved a plant conversion rate of 3,3% (Xing *et al*. 1999). In this same study, ABA application increased shoot regeneration, but decreased the rate of rooting and plant conversion. In a study of *Quercus robur* somatic embryogenesis in explants obtained from adult trees, it was necessary to apply such osmotic agents as sorbitol (60 gL^{-1}) and sucrose (30 gL^{-1}) to achieve plant conversion rates of 60% and 17% for 2 genotypes (Toribio *et al.* 2004).

Carbon type and concentration also had a marked effect on the maturation and subsequent germination of somatic embryos of *Castanea sativa* (Corredoira *et al*. 2003). However, unlike in raulí where 60 gL^{-1} sucrose was the best, in chestnut the best results were obtained with 30 gL^{-1} of maltose, yielding a conversion rate of 6% but with 33% of the embryos showing only stem development. In addition, it was necessary to expose the

cultures to 2 months at 4°C to achieve these levels of conversion, showing that partial dehydration alone was not sufficient.

A cold period of 3 months was also necessary to improve the conversion rates of somatic embryos of *Castanea dentata* to seedlings (Andrade and Merkle 2005). The conversion frequency rose from 7% without cold treatment, to 47% after cold treatment. Although some SEs germinated without cold treatment, none of them completed the conversion process. This corresponds with what was observed in sexual reproduction where the American chestnut seeds require a cold period to break dormancy. This period of stratification is also necessary for *Nothofagus* (Donoso *et al*. 1998) where the seeds need a prolonged period of cold in the ground before they will germinate. With regard to raulí, additional studies are needed to determine the optimum cooling time for improved germination and conversion.

The addition of 2 mgL^{-1} of ABA significantly increased the dry /fresh weight ratio of somatic embryos of *Castanea dentata* (Robichaud *et al*. 2004). Starch accumulation increased and the embryos assumed an opaque white appearance while in the control the embryos remained translucent. In our work with raulí the control of secondary embryogenesis and precocious germination was possible through the application of ABA. However, the levels of germination and plant conversion were low and, as observed in *C. dentata*, it is probably necessary to use only short pulses of ABA during the early stages of development prior to the addition of any osmotic agent such as polyethylene glycol. However, these studies should be complemented with those of low temperature application.

In *Quercus suber* cultures the addition of 0.25 mgL^{-1} of ABA to the culture medium promoted the maturation of somatic embryos in a pattern that was similar to that obtained with endogenous ABA (Garcia-Martin *et al*. 2005). It was determined that the content of endogenous ABA during secondary embryogenesis is similar to that observed in immature embryos, demonstrating that secondary embryos are produced from primary embryoids with low levels of ABA.

References

Andrade, G.M., Merkle, S.A. 2005. Enhancement of American chestnut somatic seedling production. Plant Cell Reports. 24:326-334.

Castellanos, H., Sánchez-Olate, M., Ríos, D. 2005. La embriogénesis somática como alternativa para la regeneración *in vitro* de raulí y roble. In "Clonación de raulí: estado actualy perspectivas". Edited by B. Gutiérrez, O. Ortiz, M.P. Molina. CEFOR-INFOR-UACH. Concepción, Chile. PP. 59-74.

Corredoira, E., Ballester, A., Vieitez, A.M. 2003. Proliferation, maturation and germination of *Castanea sativa* Mill. somatic embryos originated from leaf explants. Annals of Botany. 92:1-8.

Cuenca, B., San-José, M., Martínez, M., Ballester, A., Vieitez, A. 1999. Somatic embryogenesis from stem and leaf explants of *Quercus robur* L. Plant Cell Reports. 18:538-543.

Chalupa, V. 1983. Micropropagation of conifer and broadleaved forest trees. Communicationes Instituti Forestalis Cechosloveniae. 13:7-39.

Dodeman, V., Ducreux, G., Kreis, M. 1997. Zygotic embryogenesis *versus* somatic embryogenesis. Journal of Experimental Botany. 48:1493-1509.

Donoso, P., González, M., Escobar, B., Basso, I., Otero, L. 1998. Viverización y plantación de Raulí, Roble y Coigüe en Chile. In "Silvicultura de los Bosques nativos de Chile". Edited by C. Donoso, A. Lara, Editorial Universitaria, Santiago, Chile. PP. 177-244.

Fernandez-Galiano, E., Mauri, P.V., García, G. 1997. Somatic embryogenesis induction on *Quercus faginea* LAMK. Acta Horticulturae. 447:143-145.

García-Martín, G., Manzanera, J.A., González-Benito, M.E. 2005. Effect of exogenous ABA on embryo maturation and quantification of endogenous levels of ABA and IAA in *Quercus suber* somatic embryos. Plant Cell, Tissue and Organ Culture. 80:171-177.

Grosse, H., Quiroz, I. 1998. Silvicultura de los bosques de segundo crecimiento de Roble, Raulí y Coigüe en la región centro sur de Chile. In "Silvicultura de los Bosques nativos de Chile". Edited by C. Donoso, A. Lara, Editorial Universitaria, Santiago, Chile. PP. 95-128.

Gupta, P,K., Grob, J.A. 1995. Somatic embryogenesis in conifers. In "Somatic embryogenesis in woody plants". Vol. I. Edited by S. Jain, P. Gupta, R. Newton, Kluwer Academic Publishers, Netherlands. PP. 81-98.

Jordan, M., Velozo, J., Sabja, A.M. 1996. Organogenesis *in vitro* of *Nothofagus alpina* (P. *et* E.) Oerst., Fagaceae. Plant Cell Reports. 15:795-798.

Loewe, V., Toral, M., Mery, M., Camelio, M., López, C., Urquieta, E. 1997. Monografía deRoble. *Nothofagus obliqua*. CONAF, INFOR, FIA. Santiago, Chile. 90 pp.

Manzanera, J.A. 1992. Inducción de embriogénesis somática en Roble (*Quercus robur* L.). Investigación Agraria, Sistema recursos forestales. 1:73-81.

Margara, J. 1988. Multiplicación vegetativa y cultivo *in vitro*. Ediciones Mundi-Prensa. Madrid, España. 232 pp.

Martínez-Pastur, G., Arena, M. 1995. *In vitro* propagation of *Nothofagus obliqua* (Fagaceae). Austral Journal Botany. 43:601-607.

Martínez-Pastur, G., Arena, M. 1996. *In vitro* propagation of *Nothofagus nervosa* (Phil.) Dim. *et* Mil. Phyton. 58(1/2):1-7.

Martínez-Pastur, G., Arena, M., Caso, O.H. 1997. *In vitro* propagation of *Nothofagus antartica* from sphaeroblasts and saplingns. Biocell. 21:231-236.

Martínez-Pastur, G., Arena, M. 1999. Micropropagation of *Nothofagus pumilio* (Poepp. *et* Endl.) Krasser. Bosque. 18:43-50.

Mauri, P.V., Manzanera, J.A. 2003. Induction, maturation and germination of holm oak (*Quercus ilex* L.)

somatic embryos. Plant Cell Tissue and Organ Culture. 74:229-235.

Merkle, S.A., Parrott, W.A., Flinn, B.S. 1995. Morphogenic aspects of somatic embryogenesis. In "*In vitro* embryogenesis in plants". Edited by T.A. Thorpe, Kluwer Academic Publishers, Dordrecht, Boston, London. PP. 155-203.

Muñoz, C. 1999. Alternativas biotecnológicas para la propagación de fagáceas chilenas. El caso del roble. Universidad de Concepción, Concepción, Chile. 40 pp.

Palada-Nicolau, M., Hausman, J.F. 2001. Comparison between somatic and zygotic embryo development in *Quercus robur* L. Plant Biosystems. 135:47-55.

Piagnani, C., Eccher, T. 1990. Somatic embryogenesis in chestnut. Acta Horticulturae. 280:159-161.

Pierik, R. 1990. Cultivo *in vitro* de las plantas superiores. Ediciones Mundi-Prensa, Madrid, España. 326 pp.

Robichaud, R., Lessard, V., Merkle, S. 2004. Treatments affecting maturation and germination of American chestnut somatic embryos. Journal of Plant Physiology. 161:957-969.

Sánchez-Olate, M., Ríos, D., Pedraza, M., Pereira, G., Castellanos, H., Escobar, R. 2004. Propagación *in vitro* de *Nothofagus procera* ((Poepp. *et* Endl.) Oerst.) a partir de embriones aislados. Bosque. 25: 123-128.

Sunderlíková, V., Wilhelm, E. 2002. High accumulation of legumin and *Lea*-like mRNAs during maturation is associated with increased conversion frequency of somatic embryos from pedunculate oak (*Quercus robur* L.). Protoplasma. 220:97-103.

Thomas, T. 1993. Gene expression during plant embryogenesis and germination: An overview. The Plant Cell. 5:1401-1410.

Toribio, M., Fernández, C., Celestino, C., Martínez, T.A., San-José, M.C., Vieitez, A.M. 2004. Somatic embryogenesis in mature *Quercus robur* trees. Plant Cell, Tissue and Organ Culture. 76:283-287.

Von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., Filonova, L. 2002. Developmental pathways of somatic embryogenesis. Plant Cell Tissue and Organ Culture. 69:233-249.

West, M., Harada, J. 1993. Embryogenesis in higher plants: an overview. The Plant Cell. 5:1361-1369.

Xing, Z., Powell, W., Maynard, Ch. 1999. Development and germination of American chestnut somatic embryos. Plant Cell Tissue and Organ Culture. 57: 47-55.

Zimmerman, J.L. 1993. Somatic Embryogenesis: A model for early development in higher plants. The Plant Cell. 5:1411-1423.

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Somatic Embryogenesis in Norway spruce: Improving Embryo Maturation Efficiency

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Abstract

The efficiency of somatic embryo maturation can be influenced by many factors. In this work we have investigated the effects of gelling agent type, cold treatment and the presence of reduced glutathione in the culture medium. Gellan gum, agarose and different agar brands were used as the gelling agents in our experiments. The largest number of embryos was obtained on agarose medium. To study the influence of low temperature on maturation of somatic embryos we used two treatment variants: maintenance at 10°C from the second week and from the third week of maturation. When cold treatment was used from the second week of maturation a decrease in culture necrosis was observed and the largest number of embryos was formed. In order to investigate the influence of reduced glutathione in cultures during the proliferation stage, the cultures were exposed to the following glutathione concentrations: 0.2, 0.5, 1 mM. For the lines 110/7 and R84/2 a 0.5 mM concentration was optimal, while 1 mM GSH was optimal for line 110/9. In summary, the presence of reduced glutathione in the medium resulted in an enhanced number of embryos and increased maturation efficiency.
Kew words: *Picea abies,* gelling agents, cold treatment, reduced glutathione

Introduction

Somatic embryogenesis is the preferred method for propagation of valuable conifers. Several studies have been published regarding the optimization of different stages of somatic embryogenesis and on an understanding of their development mechanisms. In spite of our current ability obtain somatic embryos, the influence of many development factors is still unclear. Norway spruce (*Picea abies*), white spruce (*Picea glauca*), and black spruce (*Picea mariana*) can be model objects for experiments that aim to obtain optimization of somatic embryogenesis.

In our work we have investigated the influence of such factors as gelling agent type, cultivation temperature and the presence of reduced glutathione in the culture medium.

The water availability in the medium for callus cultures is directly dependent on type and concentration of the gelling agent. This parameter is also influenced by the presence of polyethylene glycol (PEG) and high sucrose concentration in the medium. Furthermore, the availability of mineral substances also depends on the gelling agent type. In our study we used such commonly used gelling agents as gellan gum, agar and agarose.

Metabolic pathways leading to desiccation and cold tolerance are similar. Cold treatment can partially substitute for desiccation, since both are a mild application of stress and are simple to use (Pond, 2005). Such treatment can result in improved quality of somatic embryos. In our work we studied the influence of cold treatment on different stages of somatic embryos maturation.

It is known, that besides their antioxidant function, ascorbic acid and glutathione play a significant role in plant growth and development. The balance of reduced and oxidized glutathione (GSH/GSSG) is crucial during somatic embryo maturation, has a positive effect on the organization of shoot apical meristems and can serve as a regulator of gene expression. Belmonte *et al*. (2004, 2005) investigated the effect of glutathione on somatic embryogenesis of white spruce (*Picea glauca*). In our work we determined the optimal GSH concentration for Norway spruce (*Picea abies*).

Materials and Methods

Plant material and culture medium

To initiate somatic embryogenesis we used mature zygotic embryos of Norway spruce (*Picea abies*) seeds. The seeds were put in water at +37°C overnight, then sterilized in hydrogen peroxide 10% for 5-7 minutes and washed three times in sterile distilled water. Mature zygotic embryos were isolated from seeds and placed horizontally on the initiation medium NSIII, containing 1 g l^{-1} casein hydrolysate, 0.45 g l^{-1} l-glutamine, 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or Picloram, 1 μM 6-benzylaminopurine (BAP), 1 mg l^{-1} vitamins LP/AE (Jain *et al*. 1988), 1% (w/v) sucrose and 0,3% (w/v) gellan gum. Several lines with a different somatic embryo maturation capacity were obtained (110/1, 110/7, 110/9, 75/6 and others) (Popova *et al*. 2010). Embryogenic cultures were transferred to fresh medium every 11-14 days. The NSIII medium with the BAP concentration increased to 5 μM cytokinin (BAP) was used for proliferation.

Maturation of somatic embryos

Maturation of somatic embryos occurred on NSIII medium without plant growth regulators (PGR) over a 6 day period. During the next 6 weeks the embryogenic tissues were cultivated on ½ NSIII medium (1/2 macroelements and Ca^{2+}) supplemented with 0.5 g l^{-1} casein hydrolysate, 0.2 g l^{-1} l-glutamine, 40 µM abscisic acid (ABA), 1 mg l^{-1} vitamins LP/AE, 6% (w/v) sucrose. Gelling agents (see below) were added to the medium.

The media used for initiation, proliferation and maturation, were autoclaved at 121°C under 1 atm pressure for 25 minutes. The pH for all media was 5,8 before autoclaving. Filter-sterilized vitamins LP/AE, ABA, l-glutamine, 2.4-D, Picloram, BAP were added to the cooled liquid medium.

Embryogenic cultures at all stages of somatic embryogenesis were incubated in the dark at 23°C.

Experiment 1. The influence of the gelling agents

To investigate the influence of gelling agents on maturation of somatic embryos the lines 110/7, 110/9, 110/1, 111/1 were used. We used 5 gelling agents: gellan gum, european type agar (Panreac), agarose (Lachema), agar (Fisher BioReagents), bacteriological agar (Helicon). Each agent was added to medium without PGR at the following concentrations: 0.3% (w/v) for gellan gum and 0.8% (w/v) for the other types. Embryogenic tissue was cultured on the medium for 6 days and then was transferred to the media with a higher gelling agent concentration: 0.8% gellan gum, 1.5% european type agar, 1% agarose, 1.5% agar, 1.5% bacteriological agar (w/v). The cultures were observed during the 5-6 weeks of cultivation.

Experiment 2. The influence of temperature and gelling agents

For this experiment lines 75/6, 110/8, 110/3 were used. Maturation of *Picea abies* somatic embryos during the first six days was performed on NSIII medium without PGR. Gellan gum 0.3% and european type agar 0.8% (w/v) were chosen as the gelling agents. After 6 weeks the embryo suspensor mass (ESM) was incubated on ½ NSIII medium supplemented with 4% PEG and solidified with 0.8% gellan gum or 1.5% european type agar (w/v). In the first variant of the experiment the temperature was 10°C after the second week of maturation and in the second variant after the third week. In the control the ESM was not exposed to low temperature. Observations of development were made after 6 weeks.

Necrosis determination

To evaluate the degree of necrosis of the embryogenic tissue we used the L parameter of Lab colour space. Embryogenic cultures were photographed with a Canon PowerShot SX20 IS camera under standard conditions. Then, using Adobe Photoshop CS2 Version 9.0, the color of all cultures of all analyzed lines was averaged and the chromatic components of the resulting color were calculated.

Experiment 3. The influence of reduced glutathione

To investigate the influence of reduced glutathione, lines 110/7, 110/9, 75/6, R84/2 were used. Embryogenic cultures were put on proliferation medium NSIII solidified with 0.8% european type agar. Three levels of reduced glutathione (GSH) were used: 0.2, 0.5, 1 mM. The control was GSH free. After 14 days cultures were placed on medium without PGR solidified with 0.8% european type agar. After 6 days of cultivation embryogenic cultures were transferred to the maturation medium, ½ NSIII solidified with 1.5% european type agar. The results were evaluated after 5-6 week of somatic embryo maturation.

Statistical analysis

ANOVA 1 followed by Fisher LSD test (STATISTICA) was used on data from experiment 3 and the necrosis determination. ANOVA 2 followed by Fisher LSD test (STATISTICA) was used for data of experiment 1 and 2. The confidence level was $p = 0.05$ in all cases.

Results and Discussion

Experiment 1. The influence of the gelling agents

Gelling agent concentration and type have a strong effect on somatic embryo formation. The water availability for embryogenic cultures is reduced with increasing of the gelling agent concentration. The quality of embryos improves when high concentrations of gelling agent are used. This results in good germination rates and a normal phenotype. Most embryos grown on medium with a low gelling agent content either do not germinate or the germinated embryos are vitrified and possess an abnormal phenotype (disorders in the shoot and root apical meristem formation are observed). Gelling agent type also plays an important role. At the same concentrations gelling agents differ in their capacity to capture water in the medium and in their ability to make nutrients available for tissue cultivation (Klimaszewska *et al*. 2000). Phytagel is often used as gelling agent (Iraqi *et al*. 2001; Jain *et al*. 1988) while in other experiments agar is chosen (Stasolla 1999, 2001).

In our study we have used gellan gum, agarose and different agar brands. All lines grew best on 1% agarose and line 110/9 gave the best overall performance. All lines performed much poorer on medium with the other gelling agents. The worst performance was 1.5% Fisher agar (Table 1).

We have also calculated the numbers of cotyledonary somatic embryos. Lines 110/1 and 110/9 didn't form cotyledonary somatic embryos on any medium. Line 111/1 formed cotyledonary somatic embryos only on the medium containing 1.5% Panreac agar. Line 110/7 formed cotyledonary embryos on all media types, but the best result was obtained on medium with 1% agarose. Using this medium type we got an average of 26 cotyledonary embryos per callus (data not shown).

We have also evaluated changes, such as browning and secondary growth, in the embryogenic tissues. For

Table 1. *Average number of somatic embryos per callus (data are shown for six calli for each variant)*

Line	1% agarose	0.8% Phytogel	1.5% european type agar (Panreac)	1.5% bacteriological agar (Helicon)	1.5% agar (Fisher BioReagents)
110/7	62	34	40	39	21
111/1	16	15	12	31	8
110/9	143	55	27	10	29
110/1	92	62	38	39	55

Table 2. *Average embryo number per callus on media supplied with gellan gum. i – cold treatment from the second week of maturation, ii – cold treatment from the third week of maturation*

Line	Treatment		
	control	i	ii
110/3	1.3±0.8	35.7±5.2	19.3±3.5
110/8	0	0.9±0.5	1.3±0.5
75/6	0	0	0

ANOVA 2 followed by Fisher LSD test was used for data analysis. The confidence level was p = 0.05.

Table 3. *Average embryo number per callus on media supplied with Panreac agar. i – cold treatment from the second week of maturation, ii – cold treatment from the third week of maturation*

Line	Treatment		
	control	i	ii
110/3	2.7±1.6	43.7±6.7	11.6±4.7
110/8	4.4±1.3	26.3±7.5	5.5±1.9
75/6	0	0	0

ANOVA 2 followed by Fisher LSD test was used for data analysis. The confidence level was p = 0.05.

line 110/7 no browning and secondary growth were observed. The same results were obtained for the 110/9 line on agarose-supplied medium. All other media gave secondary growth in the cultures. Lines 110/1 and 111/1 displayed secondary growth on all media. On gellan gum and Panreac agar lines 111/1 and 110/9 turned brown; line 110/9 turned brown on medium with Helicon bacto agar and line 110/1 did so on medium supplied with Panreac agar.

Thus, cultivation on the medium with 1% agarose led to a positive effect for three out of the four lines. On that medium browning and secondary growth were minimal and the number of somatic embryos was high. Furthermore, line 110/7 produced significantly more cotyledonary somatic embryos on the medium with 1% agarose than on the other media.

Experiment 2. The influence of temperature and gelling agents

It is known, that temperature influences the efficiency of regeneration and development processes. Pond (2005) found that quality and quantity of white spruce embryos depended on temperature and the length of the treatment. According to her results, exposing callus to 10^{o}C was most effective, resulting in a decrease of the number of vitrified embryos. Lower temperatures (1-5^{o}C) only slightly affected embryo quality, so that they can be used for storage of embryos and seedlings. While exposing embryos to low temperatures we must take into account their development stage and length of treatment. The quality of embryos significantly improves if exposed to cold treatment during the last stages of their development. Excessively long treatment with low temperatures results in a decrease in the number of normal embryos and an increase in that of vitrified embryos.

In our work we studied the effect of cold treatment and two different gelling agents in the media on the number of somatic embryos produced. Line 75/6 produced no somatic embryos on any of the media. Line 110/3 grown on gellan gum as well as on Panreac agar

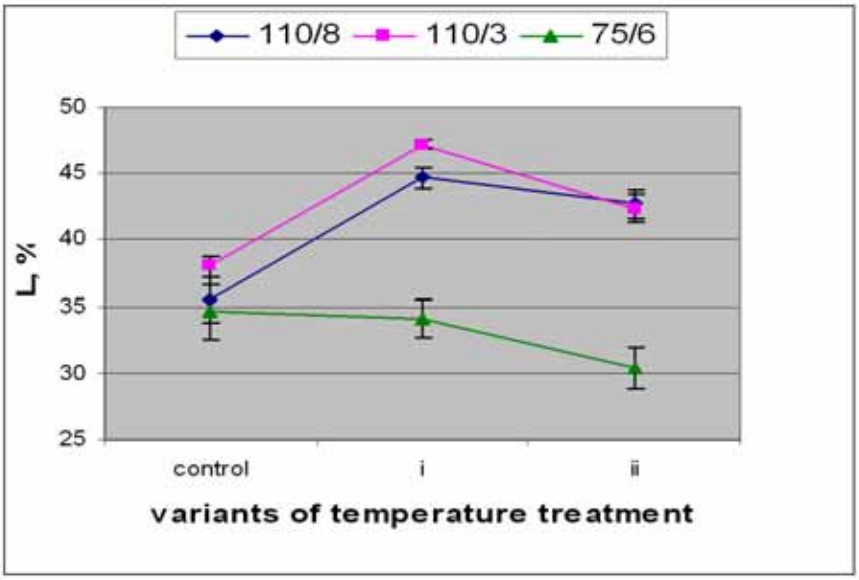

Figure 1. Changes in the L parameter of Lab color space depending on the temperature treatment. i – embryogenic cultures treatment with 10°C from the second week of maturation; ii – embryogenic cultures treatment with 10°C from the third week of maturation

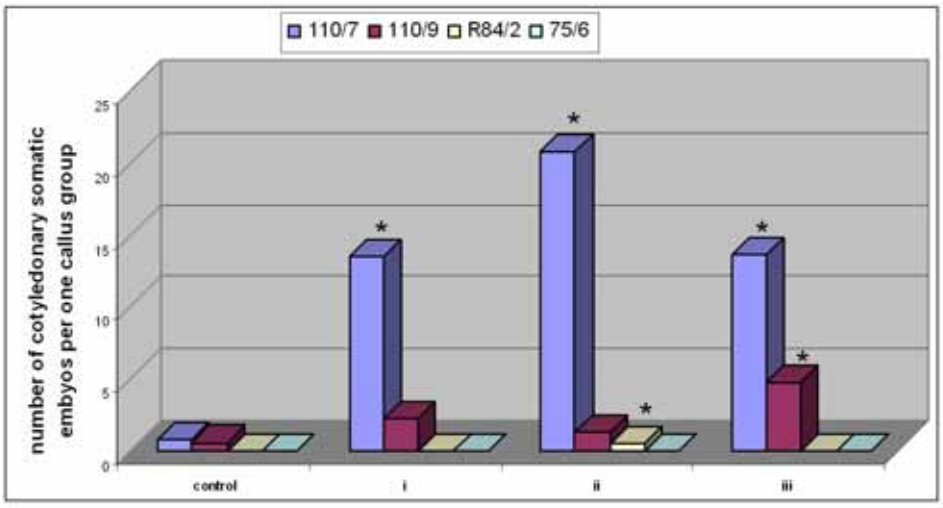

Figure 2. Average number of cotyledonary somatic embryos per callus. i – medium with addition of 0.2 mM GSH, ii - medium with addition of 0.5 mM GSH, iii - medium with addition of 1 mM GSH. Asterisk symbol (*) indicates values that are significantly different from control ($p<0,05$)

medium formed significantly more embryos after cold treatment that started two weeks after maturation. For line 110/8 on gellan gum medium there were no statistically significant differences between the treatments. However, when grown on Panreac agar the low temperature treatment that started after two weeks of maturation was best (Tables 2, 3).

We also determined the number of torpedo and cotyledonary embryos. Line 110/3 formed such embryos whenever grown on media with Panreac agar. On medium with gellan gum torpedo-like or cotyledonary embryos were formed only when given a cold treatment starting after the third week of maturation. Line 110/8 formed torpedo-like or cotyledonary embryos only on the medium with Panreac agar when given no cold treatment and after cold treatment starting the third week of maturation (data not shown).

Chromatic analysis can be used to determine color patterns in plants (Yadav *et al*. 2010). We chose the L parameter of Lab color space to show % lightness from the darkest (0%) to the lightest (100%). This correlates with the necrosis level. For lines 110/8 and 110/3

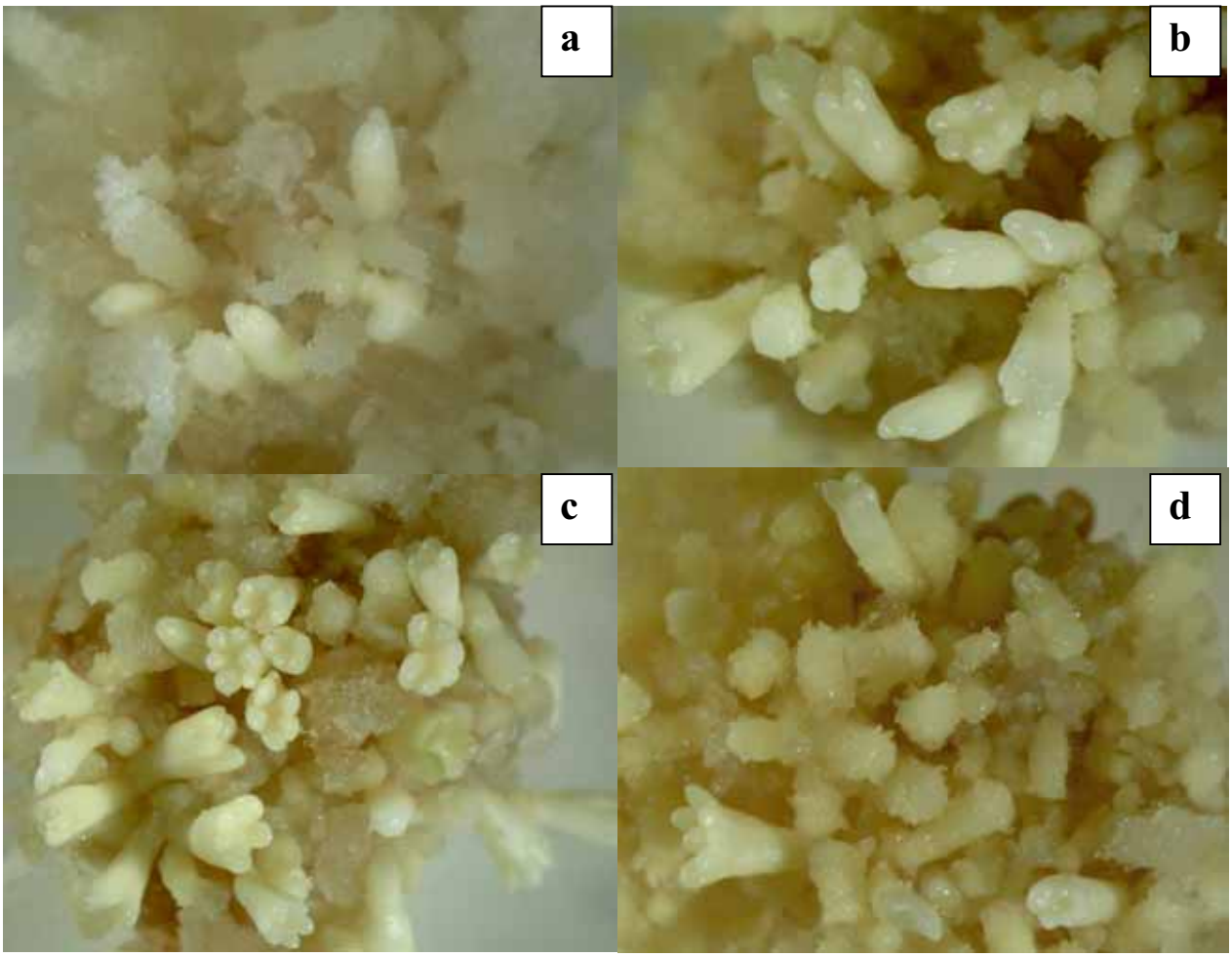

Figure 3. *Embryogenic tissue with embryos for line 110/7 obtained with GSH at different concentrations: a) control; b) 0.2 mM; c) 0.5 mM; d) 1 mM*

incubation of ESM at 10°C always had a positive effect (Figure 1) with the necrosis level being lower than in the control. No significant differences between treatment effects were noted for line 75/6.

Experiment 3. The influence of reduced glutathione

In the studies of Belmonte and co-workers (2004, 2005) it was shown that the presence of reduced glutathione created favorable conditions for cell proliferation and embryo formation. Subsequent addition of oxidized glutathione to the medium improved their further development. GSH treatment has a positive effect on cell organization in the shoot apical meristem, thus enhancing the quality of somatic embryos.

We evaluated the influence of GSH at different concentrations on the number and morphology of somatic embryos (Fig. 2). Glutathione did not improve the low embryogenic potential of line 75/6 but improved the performance of line R84/2 when supplied 0.5 mM GSH in the medium.

Line 110/7 cultured with 0.5M GSH produced the highest number of embryos (20/callus). Line 110/9 was most productive (5 embryos/callus) with 1 mM GSH. Besides increasing the number of cotyledonary embryos, treatment with GSH during the proliferation stage also improved embryo quality (Figure 3). Line 110/7 became vitrious after 4 weeks of control treatment while GSH at 0.2 mM and 0.5 mM improved embryo quality and GSH at 1 mM had a negative effect. The optimal GSH concentration varied with genotype.

Conclusions

Embryogenic cultures have been established for many conifer species. Improved knowledge in biochemistry and physiology has helped to optimize cultivation of ESM and maturation of somatic embryos. To select optimal conditions for somatic embryo production, we must take into account gelling agent type, temperature treatment and the presence and concentration of reduced glutathione in the culture medium. Gelling agent type and concentration play a key role in water availability and thus affect vitrification of somatic embryos. A decrease in the number of vitrified embryos can be obtained by cold

treatment during the maturation stage. The stage of embryo development and the duration of treatment are also important. Reduced glutathione in the medium optimizes the redox status of the cells and thus contributes to normal development.

Future optimization of these and other factors that affect embryo development is expected further to enhance the efficiency of somatic embryogenesis, both qualitatively and quantitatively and to increase the rate of embryo conversion to seedlings.

References

Belmonte, M.F., Donald, G., Reid, D.M., Yeung, E.C., Stasolla, C. 2005. Alterations of the glutathione redox state improve apical meristem structure and somatic embrio quality in white spruce. Journal of Experimental Botany. Vol. 56, № 419. P. 2355-2364.

Belmonte, M.F., Stasolla, C., Katahira, R., Loukanina, N., Yeung, E., Thorpe, T.A. 2004. Glutathione-induced growth of embryogenic tissue of white spruce correlates with changes in pyrimidine nucleotide metabolism. Plant Science. 168:803-812.

Belmonte, M.F., Yeung, E.C. 2004. The effects of reduced and oxidized glutathione on white spruce somatic embryogenesis. In Vitro Cell. Dev. Biol. – Plant. Vol. 40. P. 61-66.

Iraqi, D., Tremblay, F.M. 2001. The role of sucrose during maturation of black spruce (*Picea mariana*) and white spruce (*Picea glauca*) somatic embryos. Physiologia plantarum. Vol. 111. P. 381-388.

Jain, S.M., Newton, R.J., Soltes, E.J. 1988. Enhacement of somatic embryogenesis in Norway spruce (*Picea abies* L.). Theor. Appl. Genet. Vol. 76. P. 501-506.

Klimaszewska, K., Bernier-Cardou, M., Cyr, D.R., Sutton, B.C.S. 2000. Influence of gelling agents on culture medium gel strength, water availability, tissue water potential, and maturation response in embryogenic cultures of *Pinus strobus* L. In Vitro Cell. Biol. – Plant. 36: 279-286.

Pond, S.E., 2005. The effect of temperature on conversion of white spruce somatic embryos. Propagation of Ornamental Plants. Vol. 5, №1. P. 35-44.

Popova, O.G., Churochkina, O.A., Schestibratov, K.A. 2010. Influence of reduced glutathione and types of gelling agents on maturation of *Picea abies* somatic embryos. Materials of the X International Conference of Young Scientists. Eurasian Forests. P. 215-216.

Stasolla, C., Loukanina, N., Ashihara, H., Yeung, E.C., Thorpe, T.A. 2001. Ascorbic acid changes the pattern of purine metabolism during germination of white spruce somatic embryos. Tree Physiology. Vol. 21. P. 359-367.

Stasolla, C., Yeung, E.C. 1999. Ascorbic acid improves conversion of white spruce somatic embryos. In Vitro Cell. Dev. Biol. – Plant. Vol. 35. P. 316-319.

Yadav, S.P., Ibaraki, Y., Gupta, S.D. 2010. Estimation of the chlorophyll content of micropropagated potato plants using RGB based image analysis. Plant Cell Tiss Organ Cult. 100: 183-188.

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Somatic Embryogenesis of Forest Trees in Europe: What's Going on? An Overview

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Abstract

Somatic embryogenesis (SE) is a powerful tool for both the basic understanding of the development of zygotic embryos as well as of the rapid multiplication of material produced in tree improvement programs (Klimaszewska *et al.* 2007). However, it is very difficult to fully appreciate the overall level of activity of the research and application of somatic embryogenesis of forest trees in Europe. Publications and reports only highlight a small fraction of the ongoing work. Towards this end, as part of the EU funded Research Infrastructure Concerted Action "Treebreedex" (http://treebreedex.eu, contract Number 026076) a questionnaire was developed to document the species involved, the stage of development and the application of SE in tree improvement programs in Europe.

A total of 43 organisations covering 25 European countries responded to the questionnaire (including Belarus). The majority of these organisations were university academic departments (43%) and national forest research institutes (37%) followed by other state organisations (19%) and tree improvement programs,

Table 1. *Countries involved in somatic embryogenesis in Europe*

Country	Species	
	Angiosperms	Gymnosperms
Austria	*Quercus*	
Belarus	*Quercus*	
Belgium		*Abies*
Bulgaria	*Quercus*	
Croatia		*Picea*
Czech Rep.	*Quercus*	*Abies, Picea*
Denmark	*Fagus,Quercus*	*Abies, Picea*
Finland		*Pinus*
France		*Larix,Picea,Pinus*
Germany	*Acer,Fagus*	*Abies, Larix, Picea, Pseudostuga*
Hungary	*Aesculus,Populus*	
Ireland		*Picea*
Italy	*Fraxinus*	
Latvia		*Picea*
Lithuania	*Populus*	
Norway		*Abies,Picea*
Poland		*Abies,Larix,Picea*
Portugal	*Eucalyptus,Quercus*	*Pinus*
Romania	*Fraxinus,Prunus,Quercus,Sorbus*	*Picea,Pinus*
Russia		*Larix,Pinus*
Serbia	*Aesculus*	
Slovakia		*Abies,Pinus*
Spain	*Castanea,Quercus*	*Pinus*
Sweden		*Picea,Pinus*
UK		*Picea*

public and private companies. In total, 45 forest tree species are being studied (27 gymnosperm and 18 angiosperm species). The most studied gymnosperm species were *Abies* (8 laboratories) *Picea* (18 laboratories) and *Pinus* (14 laboratories) while *Quercus* (10 laboratories) was the most studied angiosperm species (Table 1).

Most programs considered that they were doing "basic research" (50%) while about 30% said they were doing both "basic and applied" research. Only about 20% of the programs were part of a tree improvement program (about 12% for angiosperm and 21% for gymnosperm species). Respondents of about 46% of the programs believed that the SE process requires further improvement, although in several species (*Quercus*, *Abies, Picea* and *Pinus*) it was deemed to work well enough for industrial application. The major technical bottleneck for most species was maturation (20%) and germination/conversion (20%). The majority (37%) of respondents said that the reason for using SE was to study the basic process of SE, although several programs (Denmark, Ireland and France) are producing impressive numbers of emblings at present (*Abies*, *Picea* and *Pinus*). Other than propagation, the major applications of SE technology was for cryogenic storage of germplasm (29%), physiological and biochemical studies (23%) and comparison of zygotic and somatic embryos (22%). Improved production protocols (55%) and economic analysis of the process (28%) were seen as the major technical research needs.

Most of the material that is being produced as part of a tree improvement program are use in clonal testing programs (47%) with 23% being used in variety deployment and 16% for clonal for GCA estimation. The majority of this material is used in bulk propagation (51%), although some clonal propagation is used. Among the angiosperms propagation use was equally divided between bulk and clonal. Most SE plants (emblings) are planted directly in the field (51%), whereas a few programs (18%) use them as stock plants to produce rooted cuttings which then go to the field. The main use of SE in *Abies* species is in the production of selected clones for Christmas tree production.

Most programs (81%) said that they were at the "experimental" stage while a few (27%) believed that they had advanced to the "pilot-stage" and 6% to the commercial-scale. Most programs reported that they produced less than 100 emblings per year, whereas several programs working with *Picea*, *Abies* and *Pinus* ranged between 10 to 30,000 emblings per year. Many programs were working with between 10 to 20 clones, but the larger programs worked with 10 to 200 clones.

Most programs deployed clones either in mosaics of monoclonal mixtures or in large monoclonal blocks. Multi-clonal mixtures were the least common deployment option. About 35% of the programs had material in field trials. The age of these trials ranged from 2 years for *Abies*, 4 years for *Pinus,* 10 years for *Quercus* and 15 years for *Picea*.

The main non-technical bottlenecks of the process were seen as the cost to produce emblings (60%) and a lack of appreciation of the value of the improved material (35%). Public acceptance and lack of an end use (both about 15%) were also seen as non-technical bottlenecks. Only about 9% of the respondents believed that national regulations presented a serious problem.

In other parts of the world including Canada, the United States and New Zealand, large commercial-scale SE propagation programs are underway producing up to several million emblings per year (actual production figures are impossible to obtain). The question is then why is not the same thing happening in Europe? The presence of many small private forest land owners and the resulting lack of large forest land owning companies may be part of the difference. In contrast, in Canada where SE technology is practised on a large scale most of the land is owned by the state. In this case it is the need to reforest land within a specified time after harvesting that has encouraged the use of SE planting stock. The fact that about 35% of the respondents cited the "Lack of interest in improved material" may be part of the problem. Without an understanding of the value and benefits of improved material from tree breeding programs it is hard to sell planting stock produced by SE that will always cost more than unimproved seedling material.

In conclusion, Europe appears to have the necessary scientific base (knowledge, skills and improved material) to use SE on a larger scale. After all somatic embryogenesis was first identified in gymnosperm species in Europe which demonstrates the point that we have the R&D capability. The problem seems to be the lack of a well developed market for this improved material. Certainly the production costs of emblings is important in this, but a program to "sell" this technology is needed. In order to do this we need to have both facts and figures as well as well designed demonstration trials to convince production nurseries as well as forest land owners of the value of planting improved material as well as the use of advanced propagation techniques that can make it available in the shortest time required.

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References

Klimaszewska K., Trontin J.-F., Becwar M., Devillard C., Park Y.S., Lelu-Walter M.A. 2007. Recent progress on somatic embryogenesis in four *Pinus* spp. Tree and Forestry Science and Biotechnology, 1:11-25.

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An Overview of Current Achievements and Shortcomings in Developing Maritime Pine Somatic Embryogenesis and Enabling Technologies in France

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Introduction

Pinus pinaster Ait. is a major conifer in France covering over 1 million ha in intensively managed plantation forests. Together with high productivity (11.8 m^3/ha/year), mechanization of marketed harvests (70%) resulted in a significant contribution of this species to the national pulpwood (27.1%, 3.3 million m^3) and softwood timber production (24.7%, 5.6 million m^3). In France 34 000 workers are employed in this industry (16.5% of the forest sector) with an annual turnover of around 2.5 billion Euros (36% as exports). Breeding programs launched in the early sixties by FCBA and INRA were combined in 1995 into a joined initiative called "Maritime Pine for the Future" that involved all other major forest actors (CPFA, CRPF, ONF). Up to 15% genetic gains were achieved for volume and straightness in first and second generation varieties. Lower genetic gains (10%) are however expected by the third round of selection owing to genetic redundancy within breeding populations. Moreover, maritime pine has a low ability for conventional clonal propagation that would facilitate efficient capture and deployment of the best genetic stocks. The need for an efficient mass propagation system has also dramatically increased because of two recent heavy storms that resulted in a complete clearing of 300 000 ha of forest. To face this challenging task, FCBA and INRA are jointly developing somatic embryogenesis (SE) as a critical enabling technology for efficient elite tree selection, clonal propagation of improved varieties and cryopreservation of both natural and newly obtained genetic resources (Klimaszewska *et al.* 2007). SE is also providing the plant regeneration system for genetic engineering (Trontin *et al.* 2007) and serves as a research tool to access the reverse genetics towards marker-aided selection of valuable traits. We will briefly review our major achievements pointing out the current limitations for practical use.

Somatic embryogenesis initiation

SE is initiated from immature zygotic embryos excised from the megagametophyte. Following an international collaborative project for pines (Park *et al.* 2006) we discovered that culture on Litvay basal formulations (mLV, Litvay *et al.* 1985) in use at INRA (Lelu-Walter *et al.* 2006) provided a significant within family shift in genotype capture compared to culture on DCR-based media (mDCR, Gupta and Durzan 1985). From 2003 to 2009 we obtained a 63.0 ± 11.2% (confidence limits, α = 0.05) mean initiation rate with mLV over a large panel of controlled (27) and open families (17). This is a 3-fold increase compared to the one obtained on the mDCR medium in use at FCBA from 2000 to 2005 (21.7 ± 7.6%). Supplementation of mLV with 1-4 µM CPPU [N-(2-chloro-4-pyridyl)-N'-phenylurea], a potent cytokinin, as sole plant growth regulator instead of 2,4-D (2,4- dichlorophenoxyacetic acid) and BA (benzyladenine) further increased the mean initiation rate to scores high enough (76.8 ± 6.4%, Park *et al.* 2006, Trontin *et al.* 2009b) to be of practical value. However, as in most conifers, the capture of the full genetic potential of field-tested trees is delayed by recalcitrance of adult trees to SE. Embryogenic cultures and emblings could be obtained at FCBA from *Picea abies* somatic plants up to 3 years old (Harvengt *et al.* 2001). Similar attempts with maritime pine were unsuccessful for both somatic and zygotic plants. Ongoing developments include participation in international initiatives to gain knowledge for different pine species as to how to improve SE initiation by using common induction protocols and rejuvenation methods for pre-conditioning mature plant material.

Embryogenic tissue maintenance, cryopreservation and genetic transformation

The embryogenic tissue initiated from immature zygotic embryos is efficiently propagated on filter paper discs using mLV (Lelu-Walter *et al.* 2006, Klimaszewska *et al.* 2007) or mDCR supplemented with 2,4-D and BA. A growth rate survey of 6 lines from 3 families (biweekly subculture, 100 mg per filter) concluded that mean relative increase in fresh mass is significantly higher (t test, p = 0.0013) on mLV (1582 ± 152%) than on mDCR (1268 ± 113%). Cryopreservation of embyogenic tissue is very effective (near 100% regrowth) using a simplified, slow cooling method (Lelu-Walter *et al.* 2006). A cryo-collection of more than 1800

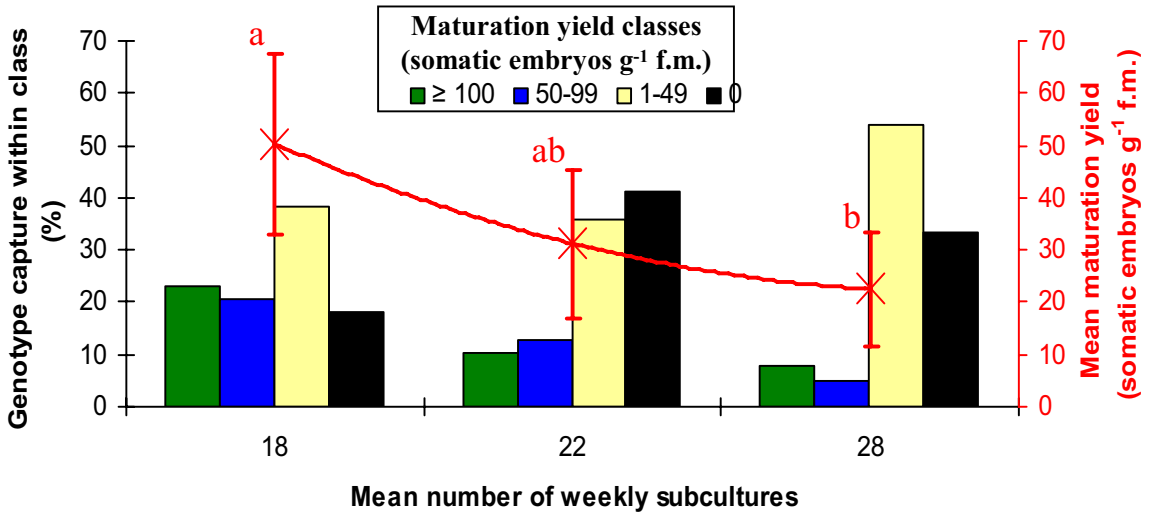

Figure 1. Maturation ability of 39 embryogenic lines cultivated on mLV-based media as a function of subculture number post-initiation (weekly subculture). All lines were initiated in 2007 from 4 seed families (9-10 lines per family).
Red curve: mean number of cotyledonary somatic embryos g^{-1} f.m. Bar = 5% confidence limits. Significant variations between means are indicated by different letters (t test, $p < 0.01$).
Histogram: genotype capture (% embryogenic lines) within 4 maturation yield classes, i.e. 0, 1-49, 50-99 or over 100 cotyledonary somatic embryos g^{-1} f.m.

embryogenic lines from elite families has been established at FCBA for implementation in the breeding program. Genetic transformation of embryogenic tissue and regeneration of transgenic plants was achieved through *Agrobacterium*-mediated gene transfer (Trontin *et al.* 2007) and currently offers around 30% genotype capture. In contrast to the poor SE obtained on mDCR, this medium yielded a better transformation rate than mLV (Trontin *et al.* 2009a). In both media formulations, phosphinothricin (1 mg l^{-1}) was a more effective selective agent than hygromycin B (10 mg l^{-1}) while an additional problem with the latter was that it lowered maturation yields (Trontin *et al.* 2007). Genetic transformation has also been used in ongoing reverse genetics studies in our laboratories but the method is still restricted to only a few genotypes with high and consistent transformation rates of up to 89 ± 18 events g^{-1} fresh mass tissue (f.m.).

Somatic embryo development and conversion to plantlets

The use of mLV instead of mDCR basal salts provided a clear improvement in both genotype capture and yield in cotyledonary somatic embryos at the maturation step. Mean yield computed from 547 lines matured on mLV from 2005 to 2009 was 64.2 ± 3.6 embryos g^{-1} f.m. This is a 20-fold higher yield than obtained on mDCR (3.1 ± 0.3 embryos g^{-1} f.m.) in a sample of 1830 lines studied from 2000 to 2004. Despite this major achievement, maturation ability of embryogenic lines is invariably lost within a few months as previously noticed (Breton *et al.* 2006). In a survey of 39 lines (**Figure 1**), mean maturation yield significantly decreased from 50 ± 17 to 22 ± 10 embryos g^{-1} f.m. within 10 weeks and quite shortly after initiation (18 weeks). At the same time, genotype capture calculated as the frequency of lines producing at least 50 embryos g^{-1} f.m. was reduced from 43.6% to only 12.8%. Germination ability of mature embryos from 6 elite genotypes is high (69.4 ± 3.7%) on mLV but the conversion rate into standard plantlets remains low (34.5 ± 3.7%). Moreover initial growth of cotyledonary somatic embryos is much lower than that of their zygotic counterpart, i.e., of mature zygotic embryos excised from the megagametophyte and similarly germinated on mLV as a control. The physiological quality of cotyledonary somatic embryos has thus to be improved in maritime pine. Ongoing work at INRA and FCBA includes transcriptomics and proteomics of embryo development as well as genetic and epigenetic studies.

Field performances: preliminary results

We have established 7 field tests since 1999 representing a total of about 2000 somatic plants from 217 clones growing in the field. Emblings are producing normal trees but usually at a lower initial growth rate than that of control seedlings. However, since the mean relative increase in height is similar or even higher in specific lines, the disadvantage of an initial low growth rate can probably be overcome by selecting the top elite clones within each family.

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References

Breton, D., Harvengt, L., Trontin, J.-F., Bouvet, A., Favre, J.-M. 2006. Long-term subculture randomly affects morphology and subsequent maturation of early somatic embryos in maritime pine. Plant Cell Tiss. Organ Cult. 87: 95-108.

Gupta, P.K., Durzan, D.J. 1985. Shoot multiplication from mature trees of Douglas fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). Plant Cell Rep. 4: 177-179.

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

Klimaszewska, K., Trontin, J.-F., Becwar, M., Devillard, C., Park, Y.-S., Lelu-Walter, M.-A. 2007. Recent progress on somatic embryogenesis of four *Pinus* spp. Tree For. Sci. Biotechnol. 1: 11-25.

Lelu-Walter, M.-A., Bernier-Cardou, M., Klimaszewska, K. 2006. Simplified and improved somatic embryogenesis for clonal propagation of *Pinus pinaster* (Ait.). Plant Cell Rep. 25: 767-776.

Litvay, J.D., Verma, D.C., Johnson, M.A. 1985. Influence of loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of the wild carrot (*Daucus carota* L.). Plant Cell Rep. 4: 325-328.

Park, Y.-S., Lelu-Walter, M.-A., Harvengt, L., Trontin, J.-F., MacEacheron, I., Klimaszewska, K., Bonga, J.-M. 2006. Initiation of somatic embryogenesis in *P. banksiana*, *P. strobus*, *P. pinaster*, and *P. sylvestris* at three laboratories in Canada and France. Plant Cell Tiss. Organ Cult. 86: 87-101.

Trontin, J.-F., Debille, S., Vallance, M., Quoniou, S., Lesage-Descauses, M.-C., Label, P., Harvengt, L., Lelu-Walter, M.-A. 2009a. Basal medium formulation strongly affects transformation efficiency in maritime pine. Tree IUFRO Biotechnology 2009 (Whistler, BC, Canada), p. 62 (P-122).

Trontin, J.-F., Reymond, I., Quoniou, S., Hoebecke, J., Saint-Denis, L., Laffray, A., Alazard, P., Debille, S., Besson, F., Rousseau, J.-P., Canlet, F., Predhomme, J.-P., Bruneau, G., Bouvet, A., Lelu-Walter, M.-A., Park, Y.-S., Harvengt, L. 2009b. Some insights into initiation rate of somatic embryogenesis in maritime pine using Litvay basal formulations containing CPPU. Tree IUFRO Biotechnology 2009 (Whistler, BC, Canada), p. 61 (P-121).

Trontin, J.-F., Walter, C., Klimaszewska, K., Park, Y.-S., Lelu-Walter, M.-A. 2007. Recent progress on genetic transformation of four *Pinus* spp. Transgenic Plant J. 1: 314-329.

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Somatic Embryogenesis and Genetic Transformation of Korean Fir, A Korean Native Conifer

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Abstract

Korean fir (*Abies koreana*) is a native conifer grown on the top of the mountains such as Mts. Halla and Jiri of Korea and is found to be naturally growing at altitudes over 1,000-1,900m. In recent years its number has been depleted at a fast rate due to the climatic changes in Korea and thus there is an urgent need for the conservation of this rare conifer. For this purpose we studied the possibility of establishing a high efficiency somatic embryogenesis protocol for conservation of the species.

In conifers, somatic embryogenesis represents the best system for plant regeneration and gene transfer technology (Klimaszewska *et al*. 2001, Park *et al*. 1998). Propagation of several *Abies* species has been reported by somatic embryogenesis using immature zygotic embryos (Vooková & Kormuťák, 2004) and also in hybrids of *Abies* species using mature zygotic embryos (Salaj & Salaj, 2003). We intended to establish somatic embryos from both mature and immature zygotic embryos by optimizing culture conditions. For mature zygotic embryo culture we used a total of twenty seed families collected from both Mts. Halla and Jiri, whereas for immature zygotic embryo culture two genotypes from the Korea National Arboretum have been selected. Embryogenic cell lines were initiated from both immature and mature ZEs and maintained as proembryonal masses (PEMs) in the presence of cytokinin only. Induction rates of EM were significantly different between seed sources, and it also negatively correlated with polyembryony tendency. Compared to mature ZEs, efficiency of EM initiation was 4-10 folds high in immature ZEs culture that could be easily maintained.

Since loss of maturation capacity has been reported in long term embryogenic cultures of *Abies* (Roth *et al*. 1997), for the maturation of long term maintained cultures in the present study 3-year-old embryonal mass was treated with the various treatments e.g., ABA, PEG and TIBA. We incorporated TIBA in the maturation medium as we hope to achieve proper embryo development by controlling endogenous auxin concentration. Three of eight EM lines induced from mature ZEs were developmentally arrested. To help better understand the developmental aberrations at the late stage of embryogenesis, the gene expression patterns were analyzed from these cell lines, together with normal lines as a control. A transformation procedure was also developed for Korean fir EM using *Agrobacterium tumefaciens*. Stable integration of the transgene was confirmed by PCR and absolute real time qRT-PCR on

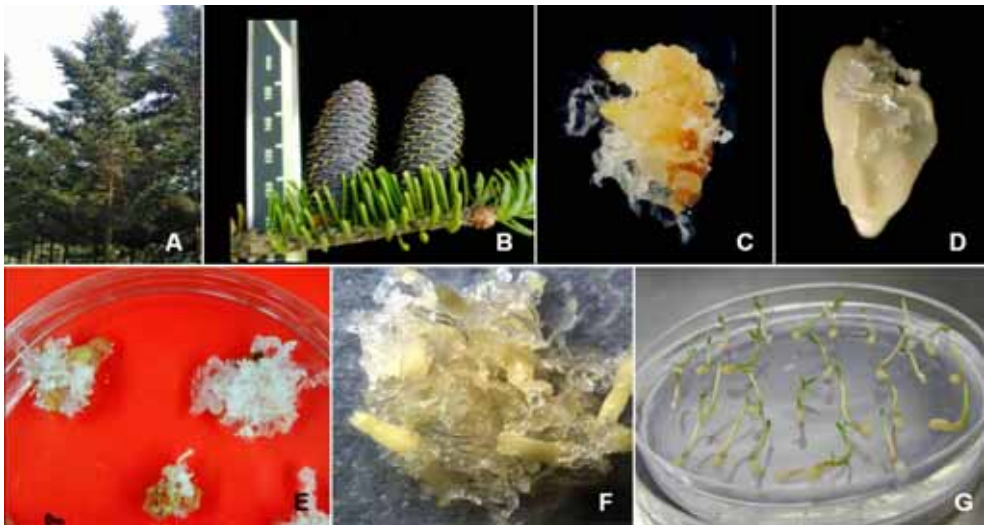

***Fig. 1**. Process of somatic embryogenesis (SE) in Korean fir. A. Forty-year-old Korean fir, B. Immature cones, C-D. EM initiation from mature (C) and immature zygotic embryos (D), E. Embryonal masses proliferation, F-G. SEs maturation and plantlet conversion (G).*

transformed tissues.

In conclusion, we found that seed sources and genotypes had an important influence on embryogenic culture initiation in Korean fir, and mature ZE could be a good material to produce EM, which promises to produce normal SEs. However the efficiency might be affected by polyembryony. Proper embryo development could be achieved by control of endogenous auxin to block the auxin transport. *Agrobacterium*-mediated gene transfer using embryonal masses is a useful technique for large-scale generation of transgenic Korean fir and may prove useful for other native conifer species.

With the above proposed work we achieved a high efficiency somatic embryogenesis protocol for Korean fir that would be helpful for breeding and conservation of this rare conifer and a genetic transformation protocol that would help in introducing desirable traits in the species.

References

Aitken-Christie, J., Connett, M., 1992. Micropropagation of forest trees. In Transplant Production Systems, eds Kurata, K., Kozai, T. pp. 163-194.

Klimaszewska, K., Lachance, D., Pelletier, G., Lelu, M.A., Segiun, A. 2001. Regeneration of transgenic *Picea glauca*, *P. mariana* and *P. abies* after cocultivation of embryogenic tissue with *Agrobacterium tumefaciens*. In Vitro Cell Dev Biol-Plant. 37:748-755.

Park, Y.S., Barrett, J.D., Bonga, J.M. 1998. Application of somatic embryogenesis in high-value clonal forestry: Deployment, genetic control, and stability of cryopreserved clones. In Vitro Cellular and Develop. Biol.- Plant. 34:231-239.

Roth, R., Ebert, I., Schmidt, J. 1997. Trisomy associated with loss of maturation capacity in a long term embryogenic culture of *Abies alba*. Theor. Appl. Genet. 95:353-358.

Salaj, T., Salaj, J. 2003. Somatic embryo formation on mature *Abies alba* x *Abies cephalonica* zygotic embryo explants. Biologia plantarum. 47:7-11.

Schrader, S., Kaldenhoff, R., Richter, G. 1997. Expression of novel genes during somatic embryogenesis of suspension-cultured carrot cells (*Daucus carota*). Journal of Plant Physiology 150:63-68.

Vooková, B., Kormuťák, A. 2004. Propagation of some Abies species by somatic embryogenesis. Acta Universitatis Latviensis, Biology. 676: 257–260.

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Regulation of Early Stages During Somatic Embryogenesis in Norway Spruc and Scots pine

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Abstract

Somatic embryogenesis is an attractive method to propagate conifers vegetatively both as a tool in the breeding program and for large scale clonal propagation. The whole procedure of conifer plant regeneration through somatic embryogenesis is comprised of a sequence of steps including initiation of embryogenic tissue, proliferation of embryogenic cultures, differentiation of early embryos and development of late and cotyledonary embryos (von Arnold and Clapham 2008). To execute this pathway efficiently, a number of critical physical and chemical treatments should be applied with proper timing. Many conifers belonging to the genus *Picea* can be propagated on a large scale by somatic embryos. However, for several species belonging to *Pinus* the initiation frequency of embryogenic cell lines is low and regeneration of high quality cotyledonary embryos is insufficient. In order to control the formation of plants via somatic embryos, it is important to understand how the somatic embryos develop. The earliest events in embryogenesis are the most critical for plant body formation, since it is then that the primary meristems and the embryo polarity are established. Therefore, we are focusing on gaining a better understanding of the regulation of the earliest stages during somatic embryogenesis in Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvetris*).

Somatic embryogenesis in Norway spruce has become a model system for studying embryology in conifers. It provides a well-characterized sequence of developmental stages, resembling zygotic embryogeny, that can be synchronized by specific treatments, making it possible to collect a large number of somatic embryos at specific developmental stages. We have used this model to analyze global changes in gene expression during early stages of embryo development by generating an expression profile of 12,536 complementary DNA clones (Vestman *et al*. 2010). In total 720 clones were differentially expressed. In order to gain a general picture of which processes take place during early stages of embryogenesis, we related each conifer gene to the Arabidopsis genes with which it shared most sequence similarity. By this approach we identified putative processes associated with early embryogenesis that have not been identified on the molecular level in conifers before. We recognized notable changes in the expression of genes involved in regulating auxin biosynthesis and auxin response, gibberellin-mediated signaling, signaling between the embryo and the female gametophyte, the switch from embryonic to vegetative development, and tissue specification including the formation of boundary regions. In addition our results confirmed the involvement of previously described processes, including differentiation of a protoderm, programmed cell death (PCD), and stress related processes. Our analyses of genes and putative processes that take place during differentiation of early embryos and development of late embryos in Norway spruce can now serve as a basis for further studies of the processes in conifers.

Polarization during differentiation of early embryos in Norway spruce proceeds through the establishment of three major cell types: meristematic cells of the embryonal mass, the embryonal tube cells and the terminally differentiated suspensor cells. The organization of the apical-basal polarity in the early embryos is dependent on a gradient of PCD from the embryonal tube cells committed to death to the cell corpses at the basal end of the suspensor (Bozhkov *et al*. 2005). We have investigated the role of intracellular free zinc in the maintenance of a balance between cell survival and PCD during early embryo development (Helmersson *et al*. 2008). The results showed that accumulation of zinc in the embryonal mass but not in the suspensor is required for correct embryonic patterning and embryo survival. Zinc deficiency is lethal to embryos, whereas supplementation of extra zinc suppresses terminal differentiation and death of the suspensor, causing a delay of suspensor elimination and embryo maturation. The dual role of zinc in cell death/survival is related to its inhibitory effect on metacaspase activity.

In order to investigate the importance of polar auxin transport for appropriate apical-basal polarization during early embryo differentiation in Norway spruce, somatic embryos at different developmental stages were treated with the polar auxin transport inhibitor 1-N-naphtylphthalamic acid (NPA) (Larsson *et al*. 2008). NPA-treatment led to increased indole-3-acetic acid content, abnormal cell divisions and decreased PCD, resulting in aberrant development of the embryonal tube cells and suspensors. Mature embryos that had been treated with NPA showed abnormal cotyledon formation and irregular cell divisions in the area of the root meristem. The aberrant morphologies are comparable with several auxin response and transport mutants in Arabidopsis. In accordance, the expression of a Norway spruce *CUP-SHAPED COTYLEDON* orthologue (*PaNAC01*) is regulated by polar auxin transport and is

associated with differentiation of the shoot apical meristem and formation of separated cotyledons (Larsson *et al*. in preparation).

A number of genes that are required in the female gametophyte for its development into the embryo sac, and post fertilization for the progression of embryogenesis, have been described in Arabidopsis, including *MATERNAL EFFECT EMBRYO ARREST* (*MEE*) genes. Putative homologues of *MEE* genes are up-or down-regulated during differentiation of early embryos in Norway spruce (Vestman *et al*. 2010). It is tempting to assume that ”nurse cells” expressing these genes have megagametophytic signaling functions required for differentiation of early somatic embryos.

In the microarray analyses (Vestman *et al*. 2010) we also found that the expression of a homologue of *LEAFY COTYLEDON 1* (*LEC1*) decreases during development of late embryos in Norway spruce simultaneously as the expression of a homologue of an *ABA INSENSITIVE 3* (*ABI3*) increases. More detailed analyses of the expression of these genes during development of zygotic and somatic embryos have confirmed the array results (Uddenberg *et al*. in preparation). In Arabidopsis it has been shown that the activity of the *LEC* genes must be repressed post-embryonically to allow vegetative development to proceed. Based on these results we assume that the switch from embryonic to vegetative growth in conifers takes place in the beginning of late embryogeny.

The mature seeds of most gymnosperms contain only one embryo, but the presence of more than one embryo in a young seed is common in pines but not in spruces. During polyembryony the early embryo goes through several rounds of cleavage resulting in many equal-sized embryos. The embryos start to compete, resulting in that one embryo becomes dominant, while the subordinate embryos are aborted by PCD. In most pine species, initiation of embryogenic cultures is limited to the first weeks of zygotic embryo development during the cleavage stage. It has been suggested that embryogenic cultures initiated from immature zygotic embryos during the cleavage stage are a result of a continuation of the cleavage process. In contrast, embryogenic cultures of spruces are usually initiated from differentiated cells in mature zygotic embryos, which first are simulated to dedifferentiate. These differences in initiation of embryogenic cultures between pines and spruces might be one of the reasons why it is more difficult to regenerate high quality cotyledonary embryos in pine species.

Embryogenic cell lines of Scots pine vary significantly in their ability to differentiate cotyledonary embryos (Burg *et al*. 2007). Furthermore, the morphology of the cotyledonary embryos differs among cell lines. In order to elucidate the development of somatic embryos and to identify deviations from the normal plan leading to aberrant embryos, we have compared the developmental pathway of somatic embryos in representative cell lines yielding cotyledonary embryos with normal and abnormal morphologies. We found no fundamental differences in the early patterning between cell lines. However, a characteristic of cell lines that give rise to a high proportion of abnormal cotyledonary embryos was that they first produced both early and late embryos that carried supernumerary suspensor cells (Abrahamsson *et al*. in preparation). We are now studying if aberrant polar auxin transport and PCD during early differentiation of embryos in Scots pine causes the unbalanced ratio between the embryonal mass and the suspensor. Before somatic embryogenesis can be used for clonal propagation of Scots pine, further studies are required in order to control the differentiation of early somatic embryos.

References

Bozhkov, P., Filonova, L., Suarez, M., Helmersson, A., Smertenko, P., Zhivotovsky, B., von Arnold, S. 2004. VEIDase is a principal caspase-like activity involved in plant programmed cell death and essential for embryonic pattern formation. Cell Death Diff. 11:175-182.

Burg, K., Helmersson, A., Bozhkov, P., von Arnold S. 2007. Developmental and genetic variation in nuclear satellite stability during somatic embryogenesis in pine. J. Exp. Bot. 53:687-698.

Larsson, E., Sitborn, F., Ljung, K., von Arnold, S. 2008. Inhibited polar auxin transport results in aberrant embryo development in Norway spruce. New Phytol. 177:356-366.

Helmersson, A., von Arnold, S., Bozhkov, P. 2008. The level of free intracellular zink mediates programmed cell death/cell survival decisions in plant embryos. Plant Physiology 147:1158-1167.

Vestman, D., Larsson, E., Uddenberg, D., Cairney, J., Clapham, D., von Arnold, S. 2010. Important processes during differentiation and early development of somatic embryos of Norway spruce as revealed by changes in global gene expression. Tree Genetic and Genomes DOI 10.1007/s11295-010-0336-4.

von Arnold, S. and Clapham, D. 2008. Somatic embryogenesis. Methods in Molecular Biology 427:31-47.

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Climatic Adaptation in Norway Spruce: Molecular Dissection of Growth and Dormancy

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Abstract

The work described here deals with the issue of climatic adaptation in the ecologically and economically important gymnosperm trees. The anticipated change in global climate may present a significant challenge to the rapid adaptation of the growth-dormancy cycle in trees (Kvaalen and Johnsen 2008). Therefore, an understanding of climatic adaptation of trees is crucial, especially in a scenario of rapid climatic change. In woody species cycling between growth and dormancy must be precisely synchronized with seasonal climatic variation. The length of photoperiod is an important factor to control shoot elongation, growth cessation and bud dormancy in woody species (Olsen *et al*. 2004 and 2010). Photoperiod and light qualities are sensed by the phytochrome light receptors (Olsen *et al*. 1997). Phytochrome is a photoreceptor that is sensitive to light and of crucial importance throughout the life cycle of higher plants.

The critical photoperiod increases with increasing northern latitude of origin of the trees. And northern ecotypes, like herbaceous long day (LD) plants, show a limited response to night break. This, as well as their requirement for prolonged far red (FR) exposure to sustain growth suggests a domination of a phyA-like-based system (Mølmann *et al*. 2006). Like herbaceous short day (SD) plants, southern ecotypes of woody species respond to night breaks in a red-far red (R-FR) reversible manner (Clapham *et al*. 1998 and 2002) and we assume that a domination of a phyB-like-based system prevails in southern ecotypes as well. This implies that the relative significance of photoperiod and light quality differs in different latitudinal ecotypes. To test the hypothesis that this might be linked to dominance of different phytochrome types in different latitudinal ecotypes would be of fundamental importance for understanding the mechanisms underlying climatic adaptation in latitudinal ecotypes of trees. We propose to test this hypothesis by studying bud dormancy and shoot elongation in our recently obtained transgenic Norway spruce (*Picea abies*) with increased or decreased levels of phyA-like and phyB-like phytochromes (denoted PhyN and PhyP, respectively).

In the dark the phytochromes are located in the cytoplasm and they translocate to the nucleus upon exposure to light to initiate signal transduction. We also use material with GFP-tagged phytochromes to test the hypothesis that light and temperature modulation of the phytochrome effect on the control of shoot elongation and bud formation might be associated with an effect of light on the translocation of active phytochromes (in the Pfr-form) from the cytoplasm to the nucleus. Thereafter we want to elucidate how important this light-regulated event is in bud formation and shoot elongation. We hypothesize that increasing the content of a phyA-like phytochrome will make the plants behave like a more northern ecotype having a longer critical day length and higher demand for FR light compared to control plants. On the other hand, reducing the phyA-contents should then result in a shorter critical day length and lower demand for FR light, similar to in more southern ecotypes. Also, increasing the content of the phyB-like phytochrome should result in plants behaving like a more southern ecotype, whereas reducing the content of this phytochrome should result in a more northern behaviour. To test these hypotheses, growth experiments under controlled conditions will be performed and relevant plant traits characterized.

Temperature is also known to modulate photoperiodic responses both with respect to induction of dormancy and depth of dormancy in trees (Mølmann *et al*. 2005). Such effects of temperature may explain some of the annual variation in the dormancy and chilling requirement that has to be fulfilled before dormancy release in nature. Our studies of wild type and phytochrome A over expression in *Populus* indicates that phytochrome action is modulated by temperature (Olsen *et al*. 1997; Mølmann *et al*. 2005). Studies of flowering in *Arabidopsis* also suggest that the functional relationships between different phytochromes are dependent on temperature (Halliday *et al*. 2003; Halliday and Whitelam 2003). Thus, it might be hypothesized that phytochromes act as temperature sensors. However, the mechanism behind the temperature modulation of photoperiodic responses in plants is unknown. It is well known that after conversion of phytochromes from their inactive (Pr) to their active form (Pfr), they are translocated into the cell nucleus where they interact with different transcription factors (Rockwell *et al*. 2006). An early hypothesis suggested that the phytochrome photoequilibrium (Pr to Pfr conversion) is sensitive to temperature (Borthwick *et al*. 1952). Accordingly, it can also be hypothesized that the translocation of active phytochrome to the nucleus is affected by temperature. To test this hypothesis, we have also made plants in which the phytochromes are tagged with a green fluorescent protein (GFP) label. By using plants with such GFP-tags on the different types of phytochromes in microscopy studies, we will be able to

test the hypothesis that the phytochrome photoequilibrium and translocation of active phytochrome into the nucleus is affected by temperature. Such knowledge will have wide implications for our ability to predict the responses of economically important trees such as Norway spruce to rapid climatic change.

References

Borthwick, H.A., Hendricks, S.B., Parker, M.W., Toole, E.H., Toole, V.K. 1952. A reversible photoreaction controlling seed germination. Proc. Natl. Acad. Sci. USA. 38:662-666.

Clapham, D., Dormling, I., Ekberg, I., Eriksson, G., Qamaruddin, M., Vince-Prue, D. 1998. Latitudinal cline of requirement for far-red light for the photoperiodic control of bud set and extension growth in *Picea abies* (Norway spruce). Physiol. Plant. 102:71-78.

Clapham, D., Ekberg, I., Eriksson, G., Norell, L., Vince-Prue, D. 2002. Requirement for far-red light to maintain secondary needle extension growth in northern but not southern populations of *Pinus sylvestris* (Scots pine). Physiol. Plant. 114:207-212.

Halliday, K.L., Salter, M.G., Thingnæs, E., Whitelam, G.C. 2003. Phytochrome control of flowering is temperature sensitive and correlates with expression of the floral integrator *FT*. Plant J. 33:1-11.

Halliday, K.J., Whitelam, G.C. 2003. Changes in photoperiod or temperature alters the functional relationships between phytochromes and reveal roles for phyD and phyE. Plant Physiol. 131:1913-1920.

Kvaalen, H. and Johnsen, Ø. 2008. Timing of bud set in *Picea abies* is regulated by a memory of temperature during zygotic and somatic embryogenesis. New Phytol. 177:49–59.

Mølmann, J.A., Asante, D.K., Jensen, J.B., Krane, M.N., Ernstsen, A., Junttila, O., Olsen, J.E. 2005. Low night temperature and inhibition of gibberellin biosynthesis overrides phytochrome action and induce budset and cold acclimation, but not dormancy in *PHYA* overexpressors and wild-type of hybrid aspen. Plant Cell Environ. 28:1579-1588.

Mølmann, J.A., Junttila, O., Johnsen, Ø., Olsen, J.E. 2006. Effects of red, far-red and blue light in maintaining growth in latitudinal populations of Norway spruce (*Picea abies*). Plant Cell Environ. 29:166-172.

Olsen, J.E., Junttila, O., Nilsen, J., Eriksson, M., Martinussen, I., Olsson, O., Sandberg, G., Moritz, T. 1997. Ectopic expression of phytochrome A in hybrid aspen changes critical daylength for growth and prevents cold acclimation. Plant J. 12:1339-1350.

Olsen, J.E., Jensen, J.B., Mølmann, J.A., Ernstsen, A., Junttila, O. 2004. Photoperiodic regulation of apical growth cessation in northern tree species. The role of phytochrome and gibberellin. J. Crop Improvement. 10:77-112.

Olsen, J.E. 2010. Light and temperature sensing and signaling in induction of bud dormancy in woody plants. Plant Mol. Biol. 73:37–47.

Rockwell, N.C., Shu, Y.S., Lagarias, J.C. 2006. Phytochrome structure and signalling mechanisms. Annu. Rev.Plant Biol. 57:837-858.

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Increased Gelling Agent Concentration Promotes Somatic Embryo Maturation of Hybrid Larch (*Larix* x *eurolepis*): a 2-DE Proteomic Analysis

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Abstract

Advances in biotechnology offer attractive new opportunities for propagating conifers. The speed with which new material can be produced and the high potential for clonal multiplication make somatic embryogenesis a powerful and flexible tool for the release of improved varieties. One of the critical steps in somatic embryogenesis is the production of high quality somatic embryos and the conversion of embryos to plants. Somatic embryo maturation is a complex process that is influenced by many factors. Among them the water availability of the culture medium is important since desiccation treatments applied to hybrid larch somatic embryos promote plantlet development (Lelu *et al*. 1995). In plants, most seeds become desiccated during the later stages of zygotic embryo development; a process that plays an important role in the transition between embryo maturation and germination (Kermode 1990). Furthermore, somatic embryos subjected to desiccation for a week reportedly showed transient increases in their endogenous ABA content, within 6 to 24 hours (Dronne *et al*. 1997). Thus, somatic embryos appear to be capable of adapting and responding very rapidly to environmental changes. Instead of attempting to desiccate mature somatic embryos we tried to reduce hydration of the somatic embryos earlier, i.e., during the maturation process. Water availability for the culture can be modulated *via* the gelling agents of the culture medium. The maturation of somatic embryos of various pine species is routinely promoted by using media with gellan gum concentrations exceeding the standard 4gL^{-1} (up to 12 gL^{-1}, as reviewed by Klimaszewska *et al*. 2007). This effect was also recently observed in hybrid larch, for which an increase in gel strength from 4 gL^{-1} to 8 gL^{-1} greatly enhanced recovery of well-developed cotyledonary somatic embryos that were able to germinate and develop into plantlets at a high rate (Lelu-Walter and Pâques 2009). Our objective was to better understand this effect.

We, therefore, compared the effect of high gellan gum concentration (8 gL^{-1}) to the normal concentration (4 gL^{-1}) in the medium on different physiological parameters during somatic embryo maturation of hydrid larch. Therefore, we also initiated a proteomic study to identify proteins that are differentially regulated in the presence of a high or normal concentration of gellan gum, after one week of maturation. Studies at the proteomic level may also help to identify protein markers of optimal or stressed culture conditions.

Dry weight and water content

To characterize the water status of the somatic embryos during maturation, the water content and dry weight of the embryos on both types of media were followed throughout their development (Table 1).

Under reduced water availability such as on 8 gL^{-1} (results not shown), water content decreased whereas dry weight increased.

Osmotic potential

The osmotic water potential ($\Psi\pi$) of the somatic embryos, and the respective media, was measured at various maturation stages (Table 2).

Somatic embryos developed on 8 gL^{-1} of gellan gum, had lower osmotic potential than on 4 gL^{-1}, the difference increased with maturation duration. Hence, the results suggest that since water is less available in the medium with 8 gL^{-1} gum (result not shown), somatic embryos might possess mechanisms that adjust their $\Psi\pi$ sufficiently to ensure the transfer of the water from the medium. This hypothetical adjustment of the somatic embryos $\Psi\pi$ to water availability could explain the greater dehydration observed in the somatic embryos cultured on 8 gL^{-1} with the water content measure.

Proteomic analysis

At one week of maturation, 62 spots were significantly different between embryos on both gellan gum concentrations. Two-third were more abundant on 4 gL^{-1}. The identified proteins were classified according to their functional categories (Table 3).

Most of the proteins fell into the "Metabolism" category (38%). Forty one percent of these proteins are involved in carbohydrate metabolism, mainly in gluconeogenesis. All proteins involved in environmental information processing were over-expressed only on the 8 gL^{-1} gellan gum medium.

Water availability and $\Psi\pi$ were lower in the medium with 8 gL^{-1} gellan gum, suggesting that the latter may have induced drought stress. However, this hypothesis was not supported by the profiles of various proteins that are reportedly induced by drought, including 6-phosphogluconate dehydrogenase (decarboxylating), actin, enolase, fructose phosphate aldolase, phosphoglucomutase

Table 1 *: Percentage of dry weight and water content in embryos during maturation according to gellan gum concentration. Error represents standard error (n=9). Significantly different groups, obtained by multiple comparison analysis, are indicated by different letters (p = 0.05).*

Percentage of dry weight[a]		
	4 gL^{-1}	**8 gL^{-1}**
1 week	10.13 ± 0.4 (a)	12.44 ± 0.2 (a)
3 weeks	12.59 ± 0.4 (b)	17.76 ± 0.5 (b)
6 weeks	21.72 ± 2.6 (c)	25.10 ± 1.2 (d)
8 weeks	23.63 ± 1.4 (cd)	29.21 ± 2.1 (e)
Water content (g $H_2O.g^{-1}$ dry weight)		
	4 gL^{-1}	**8 gL^{-1}**
1 week	8.88 ± 0.4 (a)	7.04 ± 0.1 (b)
3 weeks	6.95 ± 0.2 (b)	4.63 ± 0.1 (c)
6 weeks	3.66 ± 0.5 (d)	2.99 ± 0.2 (de)
8 weeks	3.25 ± 0.3 (d)	2.44 ± 0.2 (e)

[a]: The percentage dry weight was calculated by multiplying the dry weight to fresh weight ratio by 100. The dry weight (DW) was determined after oven-drying at 70°C for 6 h.

Table 2 *: Osmotic potential (mosmole/kg H_2O) measured for the embryogenic cultures according to gellan gum concentration. Different letters indicate significant differences among treatments by multiple comparison analysis (p< 0.001, n=10).*

	4 gL^{-1}	**8 gL^{-1}**
1 week	-1.21 ± 0.05 (a)	-1.25 ± 0.05 (ac)
8 weeks	-1.15 ± 0.11 (ab)	-1.35 ± 0.10 (c)

Table 3 *: Biological interpretation of the putatively identified proteins.*

markers of favourable conditions on 4 $g.L^{-1}$	**non-informative markers**	**markers of favourable conditions on 8 $g.L^{-1}$**
Metabolism		
adenosine kinase [EC:2.7.1.20] argininosuccinate synthase [EC:6.3.4.5] aspartate aminotransferase [EC:2.6.1.1] alcohol dehydrogenase [EC:1.1.1.1] chalcone synthase [EC:2.3.1.74] 2 * enolase [EC:4.2.1.11] flavanone 3-hydroxylase [EC:1.14.11.9] glucose 6-phosphate isomerase cytosolic A phosphoglucomutase [EC:5.4.2.2] pyruvate decarboxylase [EC:4.1.1.1]	3-isopropylmalate dehydrogenase [EC:1.1.1.85] acetyl-CoA C-acetyltransferase [EC:2.3.1.9] dihydrolipoyl dehydrogenase 1 mitochondrial fructose-bisphosphate aldolase [EC:4.1.2.13] isopentenyl diphosphate isomerase [EC:5.3.3.2] NAD-dependent sorbitol dehydrogenase	1-aminocyclopropane-1-carboxylic acid oxidase 6-phosphogluconate dehydrogenase chalcone synthase [EC:2.3.1.74] inositol-3-phosphate synthase [EC:5.5.1.4] UDPglucose 6-dehydrogenase [EC:1.1.1.22]
Cell development		
26S proteasome subunit P45 type IIIa membrane protein cp-wap13		actin 3 * Ras type protein 2 * 20S Proteasome subunit beta type-3
Stress		
heat shock 70 kDa protein		8 * heat shock type proteins superoxide dismutase [EC:1.15.1.1]
Others		
chromosome chr8 scaffold_29	thiosulfate sulfurtransferase [EC 2.8.1.1]	

and superoxide dismutase. In contrast, the putatively identified differentially abundant proteins suggest that the embryos' physiological status was better on the medium with the higher gellan gum concentration, as indicated mainly by the reductions in abundance of enzymes involved in the glycolysis pathway and HSPs.

Conclusion

The data presented here describe the physiological effects of an increased gellan gum concentration in the medium used to culture somatic embryos of hybrid larch. We observed a decrease in osmotic water potential and water content, and an increase in DW. The putatively identified differentially abundant proteins suggest that the somatic embryos physiological status was better on the medium with the higher gellan gum concentration, as indicated by the reduction in abundance of enzymes involved in the glycolysis pathway and HSPs. These results justified our choice to mature hybrid larch embryogenic lines with 8 gL^{-1} gellan gum. We are now investigating this medium modification for other conifer species.

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References

Dronne, S., Label, P., Lelu, M.A. 1997. Desiccation decreases abscisic acid content in hybrid larch (*Larix* x *leptoeuropaea*) somatic embryos. Physiologia Plantarum. 99: 433-438.

Kermode, A.R. 1990. Regulatory mechanisms involved in the transition from seed development to germination. Crit. Rev. Plant Sci. 9: 155-195.

Klimaszewska, K., Trontin, J.F., Becwar, M., Devillard, C., Park, Y.S., Lelu-Walter, M.A. 2007. Recent progress in somatic embryogenesis of four *Pinus spp*. Tree For. Sci. Biotech. 1:11-25.

Lelu, M.A., Klimaszewska, K., Pflaum, G., Bastien, C. 1995. Effect of maturation duration on desiccation tolerance in hybrid larch (*Larix* x *Leptoeuropaea dengler*) somatic embryos. In vitro Cell Dev. Biol. 31:15-20.

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

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Somatic Embryogenesis and Plant Regeneration in 10-year-old Somatic *Picea glauca*

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Abstract

Domestication of trees is limited due to several constraints hampering breeding efforts. Some of the constraints include delayed flowering, intolerance to inbreeding that prevents fixation of desirable alleles, and long time frames required to assess tree health and productivity. Vegetative propagation of select trees is the most direct means of capturing a large proportion of the genetic diversity in a single selection cycle. However, most trees lose the ability for vegetative regeneration with increasing ontogenic age. Rejuvenation attempts of adult vegetative and/or reproductive age conifers in tissue culture through organogenesis or micrografting of shoot buds onto juvenile root stock have not produced entirely satisfactory results and indicated that reversal of maturation in conifers is difficult and indeed it has not been proven yet. Despite many studies on maturation and phase change of conifers, there are still many aspects of the process that need elucidation.

Recently, we initiated SE from primordial shoot (PS) explants of 10-year-old somatic white spruce clonal trees and regenerated seedlings (truly juvenile propagules), which are under field evaluation. The stages of SE and embryo development did not differ from those initiated from seed embryos and were amenable to cryopreservation and re-growth after thawing. The SE initiation was dependent, among others, on the genotype and culture medium. Two nutrient media were tested initially: MSG (Becwar *et al*. 1990) and MLV (modified Litvay 1985, Klimaszewska *et al*. 2001), and a total of 14 medium variants. For the medium survey experiments, 11 genotypes of 2 to 3 year-old somatic white spruce were tested. PS explants were excised from lateral shoot buds (after surface disinfection) and subdivided into 2 to 4 longitudinal segments which were placed with the cut surface in contact with the medium. The media contained PGRs some of which have never been tested for induction of SE in conifers. There was no difference in the explant response between MSG and MLV medium, and the SE consistently occurred in the presence of 2,4-D and BA. Interestingly, the positive response was also demonstrated on MLV medium where the auxin was BSAA (1.0 uM) in combination with BA (4.4 uM). The other auxins such as DICAMBA (13.5 uM) and Picolinic acid (24 uM), tested in combination with a cytokinin TDZ (0.23 uM) or BA (4.4 uM), did not promote SE. Of the 11 genotypes SE was observed in the explants of three genotypes on both MSG and MLV media with 2,4-D (at 9.0 or 13.5 μM) and BA (at 4.4 μM) and with 2,4-D and BA plus 0.4 μM Ancymidol. Based on these results all subsequent experiments with older trees (from 4 to 10 years) were carried out with clonal trees of four genotypes, established in a plantation, on MLV medium with 9.0 μM 2,4-D and 4.4 μM BA (MLV-S). Of these genotypes, 893-2 and 893-12 never responded, 893-1 responded only up to year 4 and 893-6 consistently responded every year. Three 10 years-old clonal trees of 893-6 had produced male strobili as well as SE from PS explants challenging the existing hypothesis that explants taken from reproductive phase conifers loose the ability for SE. The SE was associated with formation of a nodule on the surface of an elongated needle primordium or in callus. PS explants responded when collected in the spring (after dormancy) as well as in late summer and fall (during development and before dormancy). Early somatic embryos were detectable after about 3 weeks of culture. Upon subculture onto maturation medium, mature somatic embryos were recovered in great numbers. Each year, the somatic embryos converted to somatic seedlings and young trees with juvenile characteristics.

Unequivocally, this result provides evidence that certain populations of cells within PS, when collected at the "right" time, express embryogenic potential. Although the responding trees are of tissue culture origin, at present they are the only well-characterized material that may be used for addressing questions concerning the molecular basis for phase change and the causes of the loss of ability to vegetative propagation. Identification of key genes that may have different expression patterns in responding and non responding genotypes early in culture could be used in the future as markers of embryogenic potential.

References

Becwar, M.R., Nagmani, R., Wann, S.R. 1990. Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). Can J For Res. 20:819-817.

Klimaszewska, K., Lachance, D., Pelletier, G., Lelu, M.A., Segiun, A. 2001. Regeneration of transgenic *Picea glauca*, *P. mariana* and *P. abies* after cocultivation of embryogenic tissue with *Agrobacterium tumefaciens*. In Vitro Cell Dev Biol-Plant. 37:748-755.

Klimaszewska, K., Overton, C., Stewart, D., Rutledge,

R.G. 2010. Initiation of somatic embryos and regeneration of plants from primordial shoots of 10-year-old somatic white spruce and expression profiles of eleven genes followed during the tissue culture process. Planta. DOI 10.1007/s00425-010-1325-4

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Recent Research Activity on Conifer Somatic Embryos at FFPRI

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Abstract

Somatic embryos of conifers (*Chamaecyparis obtuse* (Maruyama *et al*. 2005), *Chamaecyparis pisifera* (Maruyama *et al*. 2003), *Cryptomeria japonica* (Maruyama *et al*. 2000), *Pinus armandii* var. *amamiana* (Ishii *et al*. 2008) and others) were induced and studied for micropropagation and genetic engineering. However, practical application of somatic embryo technology in the forestry industry has not been achieved because of the high production cost, low percentage of induction, uncertainty of regeneration and the occurrence of aberrations. The growth of plantlets obtained by organ culture was better than by somatic embryo formation in the case of *Pinus armandii* var. *amamiana* (Fig. 1)..

Most reports that deal with somatic embryogenesis in conifers describe the use of immature zygotic embryos as explant which, unfortunately, cannot yet display eventual adult characteristics. We tried to produce male sterile Cryptomeria (Sugi cedar; the most important plantation conifer tree in Japan) by introducing an anther specific male sterile gene into somatic embryos. Regenerated germinants were obtained through somatic embryogenesis from transgenic callus grown from embryogenic cells. This will solve the problem of unwanted pollination by transgenic trees in the spring time and will provide us with environmentally friendly propagules, sustainable biodiversity and prevention of gene flow from transgenic trees. In parallel, a study of micropropagation by tissue culture, including somatic embryogenesis, from true-to-type male sterile Cryptomeria trees that were discovered recently in the wild has started. Callus similar in appearance to embryogenic callus was obtained from shoot segments of male sterile *Cryptomeria japonica* (Fig. 2). However, plant regeneration from such callus is still under development.

References

Ishii, K., Hosoi, Y., Maruyama, E., Kanetani, S. 2008. Micropropagation of an endangered species *Pinus armandii* var. *amamiana*. Annals For. Res. 51:5-10.

Maruyama, E., Hosoi, Y., Ishii, K. 2003. Somatic embryo culture for propagation, artificial seed production, and conservation of sawara cypress (*Chamaecyoaris pisifera* Sieb. et Zucc.). J. For. Res. 8:1-8.

Maruyama, E., Ishii, K., Hosoi, Y. 2005. Efficient plant regeneration of Hinoki cypress (Chamaecyparis obtusa) via somatic embryogenesis. J. For. Res. 10:73-77.

Maruyama, E., Tanaka, T., Hosoi, Y., Ishii, K., Morohoshi, N. 2000. Embryogenic cell culture, protoplast regeneration, cryopreservation, biolistic gene transfer and plant regeneration in Japanese cedar (*Cryptomeria japonica* D. Don). Plant Biotechnology 17:281-296.

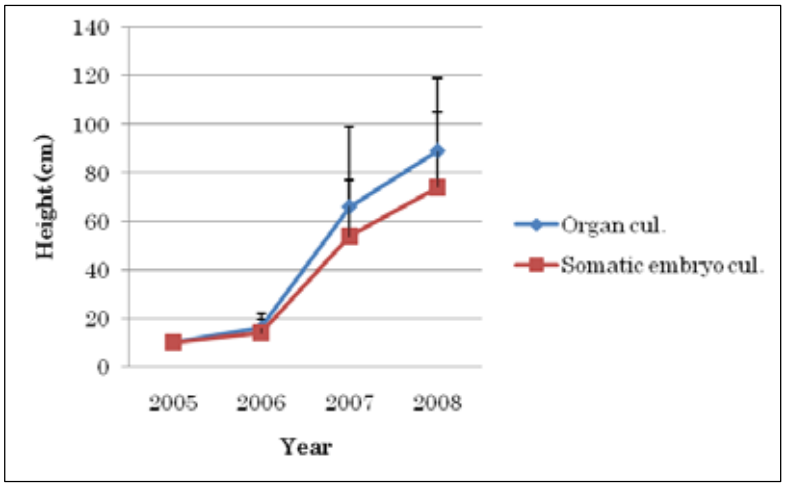

Figure 1. *Growth of plantlets of Pinus armandii var. amamiana. Nine plants from organ culture and 41 plants from somatic embryo culture were examined.*

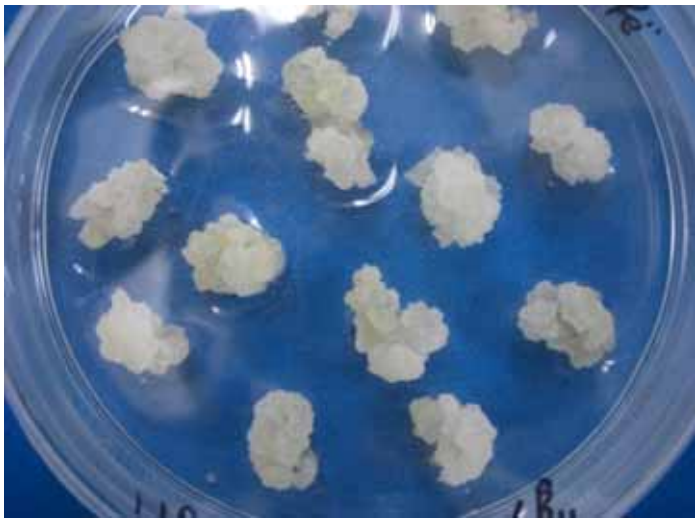

Figure 2. *Callus similar in appearance to embryogenic callus obtainded from male sterile Cryptomeria japonica.*

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SHORT ABSTRACT

Significant Advances in Genotype Capture in Somatic Embryogenesis of *Pinus radiata*

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Here we highlight some of the research conducted over the last three years aimed at improving the early stages of somatic embryogenesis in *Pinus radiata* D. Don. Previous work had shown recalcitrance in many *Pinus* species to *de novo* embryogenesis, from the culture-initiation step through to maturation and germination of somatic embryos. Our research has improved initiation rates from 13%, typical of former protocols, to an average of 50%, through improved explant preparation and media formulations, in combination with optimising the state of zygotic-embryo maturation at time of plating. The operator effect in relation to the excised-embryo technique was also analysed, with significant differences among operators, for some crosses. Early cell-line proliferation was improved from an average of 49% over all lines to 94%, using a combination of improved media formulations and tissue-handling techniques. Genetically diverse populations of open- and control-pollinated *P. radiata* families were tested over the three years. The higher numbers of cell lines initiated in every family resulting from the techniques described here offer the potential to increase selection opportunities for a wide range of plantation tree characteristics. These developments should lead to reductions in cost and an increase in the utility of somatic embryogenesis to New Zealand forestry.

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Maritime Pine Somatic Embryogenesis at IBET/ITQB - Portugal

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Maritime pine (*Pinus pinaster*) is the most widely planted conifer species in South-western Europe and pine plantations represent a major economic activity in the EU. Maritime pine economical importance is mainly due to the industries of sawing, cellulose and agglomerates, resin and resin products. In Portugal, it can be found mainly north of Tagus river spreading from the Atlantic coast to an interior with marked Atlantic influence. *P. pinaster* accounts for approximately 25% (711.000 ha) of the total forest, just after cork oak (with 26%). Somatic embryogenesis in maritime pine has been previously reported by several research groups but some constraints to the efficient production of somatic embryos from a wide range of genotypes still remain. Although the primary goal of somatic embryogenesis is to allow the large scale vegetative propagation of selected genotypes, we have been interested in initiating several lines of research related to somatic embryogenesis aiming to understand the biological mechanisms underlying some of the observed difficulties during the propagation process. One of these lines of research has focused on the analysis of somaclonal variation in somatic embryogenesis tissues including embryonal cell masses and regenerated embryos and plants. By using flow cytometry and SSR analysis for detecting variation at the ploidy and DNA sequence levels, we concluded that although variation occurred in somatic embryogenic tissues and derived plants, the variation events can be minimized by cryopreserving embryogenic cell lines as soon as they are initiated. We have also been studying global gene expression at different stages of zygotic embryo development aiming to identify important molecular players that regulate embryo development, particularly at early stages. In such studies we have already identified different gene clusters according to their expression profile and a few of these genes have been further characterized. More recently, somatic embryogenesis is being used as a tool in projects focused on the characterization of gene function, namely genes associated with different traits including resistance to biotic stresses and several growth traits. An overview of the ongoing activities will be presented.

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The Role of Spermidine and Spermine in Cell Function and Pattern Formation in Scots Pine Embryogenesis

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Polyamines (PAs) are important regulators of cellular

functioning, promoting e.g. cell division, growth, differentiation or programmed cell death (PCD) depending on the cell type and environmental signals. In the PA biosynthesis pathway, putrescine can be metabolized further to spermidine (Spd), spermine (Spm) and thermospermine by spermidine synthase (SPDS), spermine synthase (SPMS) and thermospermine synthase. In our studies, we have focused on the role of Spd and Spm in cell function and pattern formation in Scots pine embryogenesis. We have sequenced 1038 bp and 1125 bp long cDNA fragments covering the coding sequences of the putative Scots pine SPDS and SPMS genes, respectively. The phylogenetic analyses of the SPDS and SPMS sequences supported the conception that all eukaryotic SPDS genes are derived from a common ancestor, whereas two groups of SPMS genes (SPMS and ACL5) exist in plants. The sequenced Scots pine gene belongs to the ACL5 group and it was named as PsACL5. In Scots pine zygotic embryos, Spd is the most abundant PA and both free and soluble conjugated forms of Spd and Spm fluctuate in a consistent way during the embryogenesis. In zygotic embryos, the SPDS expression is localized close to cytoskeletal structures in the cells. The PsACL5 gene expression is found in the apical rib meristem and in the megagametophyte cells surrounding the corrosion cavity at the early embryogeny and in the vascular region of developing cotyledons at the late embryogeny. Currently we are using somatic embryogenesis and embryogenic cultures of Scots pine as an experimental platform (SENBIT® bioreactors) to study the roles of Spd and Spm in oxidative stress, cell division and PCD during embryogenesis. To conclude, our results suggest that Spd may act in the basic metabolism of Scots pine embryogenic cells being essential for cell viability, whereas the role of Spm may be mostly involved in tissue differentiation and growth. Furthermore, Spm may protect embryogenic cells against developmentally programmed as well as environmental stresses. The better understanding of cellular mechanisms of polyamine metabolism during zygotic embryogenesis may help the development of improved protocols for somatic embryogenesis.

Cellular Levels of ATP, Glucose-6-Phosphate, and NADP(H) during Somatic Embryogenesis of *Abies cephalonica* Loud. and *Abies alba* Mill

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All living cells need sufficient energy to keep metabolic processes running and to maintain important functions such as growth, polymer biosynthesis and defense from biotic and abiotic stresses. In zygotic embryos the nutrients and energy are partly provided by the megagametophyte tissue which is lacking in the case of somatic embryos. Therefore, the main objective was to monitor the major bioenergetic metabolites (cellular levels of ATP, NAD(P)H and glucose-6-phosphate (glu-6P)) during several experiments related to the proliferation (*A. cephalonica* and *A. alba*) and maturation periods (*A. alba)*, and to correlate these biochemical parameters with morphological characteristics of developing somatic embryos. Proliferation experiments were focused on the effects of different embryogenic cell lines (2 of *A. cephalonica* and 4 of *A. alba*), sampling days and the number of embryogenic cell masses (ECMs) per Petri plate on the proliferation rate, ATP, NAD(P)H and glu-6P cellular levels. The proliferation rate, the cellular levels of ATP and glu-6P were significantly affected by tested cell lines, sampling days as well as number of ECMs per Petri plate (exception was only the non-significant effect of number of ECMs on glu-6P level). The highest ATP and NAD(P)H levels ware found at approx. 9 and 12 day lasting proliferation, respectively, but the temporal patterns were not consistent among tested cell lines and species. During the maturation period, the ATP cellular levels were affected only by the duration maturation period. The tested concentrations of polyethylene glycol (PEG)-4000 (5 and 10%) and abscisic acid (ABA: 32 and 64 μM) did not affect the ATP and NAD(P)H levels. However, all tested factors had a significant influence on the levels of glu-6P. ATP levels, detected during proliferation period at the time when the early embryogeny was finished, were similar to those detected in the first 6 weeks of maturation. Thereafter, formation of precotyledonary and cotyledonary embryos, a new morphogenic event, was accompanied by increased levels of ATP and glu-6P. Our results suggest that cellular level, of especially ATP might be used as a potential biochemical marker for monitoring the morphogenic events occurring during the somatic embryo development.

Transcriptomic Analysis of Somatic Embryogenesis in Sweet Orange

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Somatic embryogenesis in citrus is critical for germplasm in vitro conservation and genetic improvement via biotechnological approaches such as protoplast fusion, genetic transformation and somaclonal variation selection. Herein, suppressive subtractive hybridization (SSH) and microarray analysis were carried out using embryogenic

callus line of *Citrus sinensis* cv. Valencia' sweet orange which has been preserved invitro forover 26 years and still has strong embryogenic competence. Two SSH libraries were constructed. Library I was with embryogenic callus as teste randnon-embryogenesis callus as driver ; library II was constructed with induced and non-induced embryogenic callus as tester or driver separately. After screening by microarray and sequencing, a total of 988 differential expressed uni genes was obtained, among which the functions of 726 genes (73%) could be assigned using existing data bases. LEC1, ABI3, and FUS 3 genes which are major regulators of embryo development were found for the first time in fruit tree crop. Some genes, such as members of ABA and GA path ways, glycolysis / gluconeogenesis metabolism were also discovered. Molecular function analysis showed that the differentially expressed genes mainly contained oxidoreductase (12.6%), transcription factor (3.8%), translation regulator (1.3%), transporter (4%), signaltransducer (0.6%) ; while biological process analysis showed that these genes mainly involved in stimulus (18.1%), post-embryonic development (1%), embryonic development (6.2%) and establishment of localization (6.5%). Time-dependent genes of differential expression showed 9 distinct profiles by using SOM method. The information generated in this study provides new clues to understand the molecular mechanism of somatic embryogenesis in citrus.

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Somatic Embryogenesis of Coniferous Species in Russia

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Despite the active studies of somatic embryogenesis in gymnosperms this technique still remains problematic for coniferous species growing in Siberia. The aim of this study is the development of efficient biotechnological protocols of *Pinus sibirica, Pinus pumila, Larix sibirica, Larix dauruca, Picea obovata, Picea ajanensis* involving somatic embryogenesis and optimization using cytoembryological control. Experiments of culturing the immature isolated embryos of Siberian coniferous species were carried out on modified media MS, MSG, LV, DCR and MA with different hormone concentrations and proportions. For induction of embryogenic callus every species needs an optimized medium supplemented with L-glutamine, casein hydrolysate, ascorbic acid and hormones. The active proliferation of embryonal mass (EM) is observed on the same medium with reduced concentration of cytokinins. The proliferation of EM was significantly improved when they were subcultured after dispersing in liquid medium. The somatic embryos from embryonal masses mature on basal medium with ABA(mM60-120mM)andPEG.Inspiteofspeciesspecificity the morphogenesis of embryogenic structures follow the same scheme: elongation of somatic cells, formation of initial cells and embryonal tubes, development of globular, torpedo and bipolar somatic embryos. The first step of formation of an embryogenic callus is elongating somatic cells and their asymmetric division. The second step of formation of embryogenic callus is an active formation of embryogenic mass: the elongated cells divide and produce globular embryos and embryonal tubes surrounding them. The third step of somatic embryogenesis is formation of bipolar embryos and their maturing on basal medium with ABAandPEG.Processesofsomaticembryogenesisin*Larix* and *Picea* proceeded 4-6 months, in *Pinus* − 7-10 months. However, not all donor-plants of coniferous species can form morphogenic callus and somatic embryos. As a rule, heterotic genotypes and hybrids formed somatic embryos intensively. The active development of embryonic callus and formation of somatic embryos is observed in hybrid seeds of *Pinus sibirica* and *Larix sibirica*. Emryogenic callus was produced from the unique genotypes of *P. sibirica* with annual development cycle of female cones and their hybrids. We have found 5 selective cell lines in *Larix sibirica* and one line in *Picea ajanensis.* The success of the somatic embryogenesis is due to the stage of explant development, medium components, hormonal regulation and tree genotypes. Using the effective biotechnology of somatic embryogenesis in combination with selective programs (hybridization works - controlled pollination with selection of parents, early selection, testing of improved genotypes, mass propagation) is one perspective task of forest genetics and selection.

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Somatic Embryogenesis and Plant Regeneration in Elite Genotypes of *Picea koraiensis*

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Picea koraiensis, called Korean spruce, is an evergreen tree and found mostly in northeast Asia. In this study, plant regeneration via somatic embryogenesis from open-pollinated immature zygotic embryos of 9 genotypes of elite trees was established. Immature zygotic embryos were cultured onto RJW medium modified from 505 medium (Pullman *et al*. 2003) with 21.48 μM NAA, 2.22

μM BA and2.32μM KT. The frequency of total 9 genotypes was 74.2%. Embryogenic callus of 9 genotypes of elite trees were subcultured on RJW basal medium containing 8.06 μM NAA, 1.11 μM BA and 1.16 μM kinetin. The calluses of three lines, $3^{\#}$, $9^{\#}$ and $2^{\#}$ were actively proliferated but others are not. Somatic embryogenesis was induced from embryogenic callus in genotypes of $3^{\#}$, $9^{\#}$ and $2^{\#}$ on RJW medium with ABA and 60 gl^{-1} sucrose. Cotyledonary somatic embryos were subjected to drying process. The drying of embryos by uncap the culture bottle for 5 days on clean bench resulted in high frequency germination of somatic embryos (87% in RJW medium). However, plantlet conversion from germinated embryos was greatly reduced and optimal medium for plant conversion was 1/2 WPM (Lloyd and McCown 1980) or 1/2 BMI medium. Conclusively, we firstly established the plant regeneration system via somatic embryogenesis in Korean spruce, which can be applied for rapid micropropagation of elite trees of *P. koraiensis*.

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Developmental Plasticity of Glandular Trichomes into Somatic Embryogenesis in *Tilia amurensis*

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In *Tilia amurensis*, two types of trichomes (hairy and glandular) develop from epidermal surfaces of cotyledons and hypocotyls of zygotic embryos soon after germination. Here, it is demonstrated that glandular trichome initials develop directly into somatic embryos when treated in vitro with 2,4-dichlorophenoxyacetic acid (2,4-D). Zygotic embryos of *Tilia amurensis* were cultured on Murashige and Skoog medium with 3 % sucrose and various concentrations (0, 2.2, 4.4 and 8.8 mM) of 2,4-D. Morphological development of trichomes and somatic embryos was analysed by scanning electron microscope and light microscope after histological sectioning. In zygotic embryos cultured on edium with 4.4 mM 2,4-D, formation of hairy trichomes was completely suppressed but formation of andular trichome initials increased. That some filamentous trichome initials developed directly into somatic mbryos was confirmed by histological and scanning electron microscope observation. When explants with different stages of trichome initials (two-, four- and eight-celled filamentous and fully mature ichomes) were temporally pre-treated with 4.4 mM 2,4-D for 24 h and transferred into hormone free medium, two-celled and four-celled filamentous trichome initials were the effective stage of trichomes for somatic embryo induction. In conclusions it is suggested that early developing filamentous richome initials have developmental plasticity and that with 2,4-D treatment these trichome initials develop directly into somatic embryos.

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Scale-up Somatic Embryogenesis of Conifers for Reforestation

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Clonal forestry offers very significant advantages for forest productivity due to the genetic volume gain and quality improvements which can be obtained through selection and mass propagation of elite clones. Somatic embryogenesis is a highly efficient method for cloning genetically improved trees and offers the potential for storage and testing of clones as well as production of unlimited numbers of plants. Significant progress has been made during the past 25 years in somatic embryogenesis (SE) of conifer species. SE technology is becoming commercial for loblolly pine with the few genotypes that currently work well in the process. Several companies are producing somatic seedlings for internal clonal testing or are selling somatic seedlings from a few high-value selections. However, full commercialization is still limited due to inability to produce zygotic like somatic embryos. We have produced good quality embryos by adding glucose with maltose in development media (USpatent#7,598,073B2) and adding sucrose in late maturation media (USpatent#7,521,237B2). We have scaled−up embryonal suspensor mass multiplication in liquid in biobags by continuously supplying media (US patent # 7,725,754 B2). Cultures can be directly plumped onto development media from the biobag. Large-scale embryo production and manufactured seed delivery system will be discussed in this presentation.

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Improved Maturation and Conversion Protocols in *Pinus pinea* L. Somatic Embryogenesis

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Stone pine (*Pinus pinea* L.) is a characteristic conifer species of the Mediterranean ecosystem that has economic interest because of its edible pine nuts. Spain's breeding program is mainly focused on improving seed yield.

Development of somatic embryogenesis for cloning selected genotypes is required to put multivarietal forestry into practice. We reported plant regeneration by somatic embryogenesis from stone pine zygotic embryos, although the number of mature somatic embryos obtained was very low. Now several procedures have been tested for improving embryo maturation and plant development using the responsive embryogenic lines 1F11and 2F47. A preconditioning treatment, based on reduced nutrient and PGR levels, was applied before maturation to lower the growth rate of embryo-suspensor masses. Suspensions of embryogenic tissue with activated charcoal were poured onto filter paper disks and cultured under maturation conditions. The standard maturation medium was made up of a modified Litvay' medium (mLV) with 121µM ABA, 60 g l^{-1} sucrose and 10g l^{-1} Gelrite. Cultures we rekeptin darkness at 23±1ºC for 16weeks. Effects of ABA level (80,121,161,181µM) and subculture regime (monthly*vs* continuous) were tested. Partial desiccation of samples previor to undergoing the maturation treatment was also assayed. Filter paper disks with attached tissue were partially desiccated under laminar hood flux for 2, 4, 6 and 24 h. Then, samples were transferred to the standard maturation medium and monthly subcultured. To assess the effect of an auxin antagonist on maturation, 5.4 mg l^{-1} PCIB was added to the standard maturation medium at different subcultures or along the whole 16-week period of maturation. A pre-germination treatment (cold storage for 30days) was applied to mature embryos for conversion. Medium with 121 or 161 µM ABA produced the highest number of mature embryos in both line 1F11 and line 2F47. Repeated subculturing resulted in 13-fold increase in mature embryos in both lines. Tissue desiccation for 2-4 h to a 7,5-17% relative water content enhanced maturation up to 1,7-fold in the line 1F11 and 5-fold in the line 2F47. The inclusion of PCIB did not improve and even reduced the number of mature embryos as regards the control. Conversion was enhanced (36 %) when germinated embryos were transferred for further growth to vent culture boxes filled with SH medium.

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Cryopreservation of Norway spruce Embryogenic Cultures: Levels of Polyamines

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Two embryogenic cultures of Norway spruce (*Picea abies* (L.) Karst.) of various cryotolerances were subjected to cryopreservation in liquid nitrogen. The procedure comprises preculture in a liquid medium with a rising concentration of sorbitol, DMSO treatment and driven temperature reduction before storage in liquid nitrogen. After thawing, we monitored the re-growth of these cultures. The morphology and viability of these embryogenic cultures were studied using simple trypane blue staining and green-fluorescent fluoroscein diacetate/red-fluorescent propidium iodide double staining. I addition, endogenous free polyamines (putrescine, spermine and spermidine) and their bonded and conjugated forms were analyzed during the process.
The endogenous free polyamines were maintained at high levels with spermidine being the predominant polyamine in the embryogenic suspensor mass (ESM) of cryotolerant cell line AFO 541. In the cryosensitive line C110 the contents of putrescine and spermidine were almost identical and rather low, the content of spermidine being approximately one third of that in the ESM of AFO 541. Osmotic pretreatment resulted in the continual disintegration of polyembryogenic centers and suspensors in both cell lines. Perchloric acid-soluble conjugates of polyamines increased significantly in the ESMs treated with cryoprotectants. The cells that retained viability and re-growth ability after cryopreservation were the meristematic cells inside the embryonal heads, and the cells in the intermediate area between suspensor and meristems. The restoration of AFO 541 growth after cryopreservation was almost immediate; however, the C110 ESM re-grew with difficulty, often exhibiting callogenesis. High levels of soluble conjugates of polyamines and a marked increase in the amount of polyamines bound to the high molecular mass substances, were observed in the cells of AFO 541 on day 6 after thawing and also to some extent on day 11. On days 15 and 21 after thawing, the amount of free putrescine and spermidine in the AFO 541 cells reached the levels observed in the ESM before the cryotreatment. The extremely low levels of polyamines determined in the ESM of C110 cell line 3 w after thawing agreed with the cell viability and rate of re-growth observed in this culture.

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Thirty Years of Forest Biotechnology Programs at Korea Forest Research Institute

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The roots of the current forest biotechnology program at KFRI dated back to the late 70's when Dr. YG Park dreamed of the future years of forest biotechnology and established a poplar tissue culture lab in Suwon. During 80's, by numerous trials and errors, we were able to develop micropropagation and regeneration systems for more than a dozen tree species including conifers and hardwoods *via* shoot tip, embryo and callus cultures. Recognizing the importance of these works, the government provided a large sum of research money to

support biotechnology program at KFRI. In 1995, the tissue culture lab was expanded and upgraded to a research division accommodating 3 research labs. These included a cell culture lab, a tissue culture lab and a genetic engineering lab. During the years, cell culture lab focused on the production of taxol from cell suspension cultures of yew trees and later on the production of adventitious roots of wild ginseng in bioreactors. Tissue culture lab developed somatic embryogenesis protocol for larch, pines and some tree crop plants like *Aralia*. In the genetic engineering lab, a number of genes were isolated from poplars and other organisms and introduced to poplar *via Agrobacterium* vectors. In the new millennium, we are still doing basically the same work but in more focused and sophisticated manners. Cell culture labs are working on symbiotic relationship of *Gastrodia* with *Armillaria* to improve tuber production. Somatic embryogenesis technology is being pursued toward realization of clonal forestry using yellow poplars. Emblings are being planted for their on-site performance. Genetic engineering lab employs DNA microarray to profile genes involved in stress response, growth, and development. Some candidate transcription factors are being tested in transgenic poplar plants. However, developing new techniques is a never-ending process. Now it may be the time to put all of the knowledge, techniques and resources into a single project to demonstrate what forest biotechnology can do to realize some of perspectives we originally dreamed of.

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The Effects of SCB Fertilizer on the Growth of *Quercus acutissima* Planted at Abandoned Mine Area

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This study was conducted to analyze the effect of SCB(Slurry Composing and Biofiltration) liquid fertilizer on the growth of *Quercus acutissima* seedlings (at age two) which were planted at abandoned mine area. Significant increment of growth (height and root collar diameter) and chlorophyll contents in leaves were found when using SCB fertilizer and heavy metal contents in soil were reduced after SCB fertilization. Height and root collar diameter were increased about 20% and 23%, respectively, and chlorophyll (a) content was increased about 21% in leaves compared to control treatment. Lead(Pb), Manganese(Mn), and Iron(Fe) concentration were reduced more than 20% in the soil after SCB fertilization. The effect of SCB liquid fertilizer was similar to chemical fertilizer, so it can be applied to be a useful fertilizer in abandoned mine area.

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Growth Performance by Provenances of Norway Spruce (*Picea abies*) in Korea

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Norway spruce (*Picea abies* (L.) H. Karst.) with twenty-four provenances from Germany and Rumania were investigated to analyze the growth performance among provenances and ages, and select the superior provenances at five sites in Korea (planted in 1980). Significant differences were found in growth (height, DBH, volume) among both provenances and sites. While mean individual volume growth of *P. abies* was best in Chuncheon site(0.214 m^3), inferior in Cheongwon site (0.127 m^3). Best provenance was different in each site, but G1-64-057(Dorna Cindreni, Rumania) provenance was proved to be superior in average (0.133 m^3). G1-64-057(from Rumania), 840-23, and 840-19(from Germany) provenances were superior to other ones in volu me growth, and G1-64-047, G1-64-48, G1-64-55 (from Rumania) provenances proved to be inferior ones. Growth ranking of Norway spruce has been changed with ages. Regression analysis explained significant interaction between sites and provenances in both height and diameter growth. The interaction terms explained 37.4% in height and 48.8% in diameter growth. Norway spruce was bigger than *Abies holyphylla* (as a reference species) by 1.7 ratios in individual volume.

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Propagation of Plants Via(by) Cutting from 4-month-old Somatic Embling in *Liriodendron tulipifera* L.

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In an attempt to develop efficient propagation methods of somatic emblings in *L. tulipifera*, softwood stem cutting was conducted. Four-month-old somatic emblings' stems were cut at 5~10cm length and planted in cutting square box containing a mixed soil with peat moss, perlite, and vermiculite on March 15, 2008. The cuttings were assigned to each of three auxin treatments (IBA 100ppm+talc, 1000ppm+talc, Rootone) and one control group under two environment (an acclimation room adjusted at 25℃ and a vinyl greenhouse, temperature ranging from 0 to 15℃). The root development, investigated in every five days after taking cutting, started at 30 days after the experiment begins. At that time, the rooting rate, the mean number of roots per cuttings, and the mean length of root per cuttings were 26.7%, 5.5, and 2.1cm, respectively, and at 50 days after taking cutting, those values were increased to 73.3%, 6.2, and

12.4cm, respectively. The secondary roots were developed at 35 days, and root hardening started at 45 days after taking cutting. Under the vinyl greenhouse conditions, no roots were developed in all auxin treatments and control, and all cuttings were dead at 40 days after taking cutting. However, there were significant differences among auxin treatments in the rooting rate and the number of roots per cuttings in the acclimation room, but no difference was found in the length of roots. The rooting rate was highest in the treatment of Rootone and followed by IBA 100ppm, control, and IBA 1000ppm, 62.5, 52.5, 40, and 22.5%, respectively. The mean number of roots per cuttings was the highest in IBA 1000ppm and followed by IBA 100ppm, Rootone, and control, 11.0, 7.7, 7.3 and 5.6, respectively. We suggest that the root development of cuttings from somatic emblings in *L. tulipifera* is completed at 45 days after taking cutting, and after then, five days of acclimation would be enough to transfer them to a nursery bed. It is likely that mass propagation of *L. tulipifera* by the softwood stem cutting of somatic emblings is possible if the process is conducted under a temperature of 25℃ and a humidity of 80%, which increases the rooting rate.

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Effect of Cutting's Maturity, Genotype, and Growth Regulators on Vegetative Propagation of *Salix caprea*

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Salix caprea is one of promising species for phytoremediation of metal-contaminated soils. However, conventional cutting propagation does not produce sufficient roots for the establishment of clones that lead to low survival rates. Hence, this study was performed to explore efficient vegetative propagation techniques by cuttings through the establishment of optimum maturity of cuttings, root regulators among different *S. caprea* genotype. To establish the optimum cuttings' maturity, cuttings were obtained from the same mother plants of each three different clones (CB1, BH5, and BH10) at 3 weeks intervals on 6 March, 28 March, 17 April, and 8 May. Effect of growth regulators were evaluated by using indole-3-butyric acid (IBA) at 100, 500 and 2000 mg L^{-1} concentrations and rootone for cuttings collected on 17 April. Two months later the rooting success was evaluated. There were significant high interaction effect in rooting rate between cuttings' maturity and clones at p=0.0006. The best rooting rates (49%) and number of root per cutting (7.7) were obtained with CB1 cuttings collected in 28th March. In contrast, rooting rates and number of root per cutting of BH10 cuttings collected on 8th May were only 3 % and 2.7, respectively. Regardless of the clone type, stem cuttings collected on 28 March have rooted best followed by those collected on 6 March, while those taken on 8th May have the lowest rooting rate and average number of root per cutting. On the other hand, regardless of cuttings maturity, the rooting performance of the different clones was in the order CB1 (31%) > BH10 (27%) > BH5 (10%). Pretreatment of growth regulator at 2000 mg L^{-1} of IBA in quickdip method showed the best rooting traits (rooting percentage was 40 %, root number was 6 and root length per rooted cuttings was 11.3 cm) in CB1 clone. Similarly, shoot length and shoot dry weight were also affected significantly by age maturity of cuttings. Overall, younger cuttings and pretreatment with 2000 mg L^{-1} IBA could improve rooting percentage, root number, and root length of *Salix caprea* especially from the CBI clones.

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Effect of Different Irrigation Period on Photosynthesis and Growth Performances of Containerized Seedling of *Eucalyptus pellita* and *Acacia mangium*

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The objective of this study was to find optimal water condition of containerized seedling production of two tropical species for high seedling quality. This study was conducted to investigate photosynthesis, water use efficiency(WUE), Stomatal conductance(g_s) and growth performances of containerized seedlings of *Eucalyptus pellita* and *Acacia mangium* growing under three different irrigation periods(1time/1day, 1time/2days and 1time/3days). *E. pellita* showed outstanding photosynthetic capacity at 1time/1day irrigation and *A. mangium* showed good photosynthetic capacity at all treatments. As irrigation period were shortened, g_s of two species increased, while WUE of two species decreased. Root collar diameter and height of two species were the highest at 1time/1day irrigation, while the lowest at 1time/3days irrigation. As irrigation period were shortened, H/D ratio, biomass and seedling quality index(DQI) of two species increased but T/R ratio of two species showed the opposite tendency. These results showed that 1time/1day irrigation is optimal water condition of containerized seedling production of two tropical species and irrigation controlling is very important for growth and seedling quality of containerized seedling.

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Selective Breeding of Hawthorn (*Crataegus pinnatifida* Bunge) from Korea

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In order to develop new cultivar of hawthorn with high quality fruit, we have selected from Korea. In current study, we evaluate the characteristics of fruit and measured the content of phenolic compounds in the fruit of *C. pinnatifida*. Hawthorn has been used as herbal medicine in Korea for the treatment of various cardiovascular disease, arteriosclerosis and hypertension. Because phenolic compounds are known as active constituents of hawthorn, it is important to measure these compounds for selection superior tree and using as breeding materials. To assess the morphological variation of the hawthorn fruit 5 quantitative characteristics (FL : Fruit Length, FW : Fruit Weight, WF : Weight of Fruit, IFY : Individual Fruit Yield) were made on 5 individuals. Fruit length of hawthorn showed a range of 15.2 to 18.8 mm, and the weight of hawthorn fruit showed a range of 2.17 to 3.84 g. The finding determined that the clone of Chuncheon 8 had highest EC (834 mg/100 g) and ChA (331 mg/100 g) content and Chuncheon 15 had highest HP (876 mg/100 g) and IQ (427 mg/100 g) content. With their high functional components, the clone of Chuncheon 8 and Chuncheon 15 can be evaluated to be selected breeding material for cultivar development.

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Growth Performance of European Alder (*Alnus glutinosa*) in Korea

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The provenance test of European alder (*Alnus glutinosa* (L.) Gaertn.) was conducted to select adaptable provenances in Korea. Seeds of fifty-provenances from over 20 European countries were introduced in 1987 and planted at three sites. Only one site (Chungwon) has been existed up to present and twenty-four provenances were analyzed. Height and diameter growth were different among provenances significantly. Average height was 12.2 m(10.6~14.6 m) and diameter was 17.0 cm(12.4~22.0 cm) at age of twenty-two. YUG-341, BUL-037, and TUR-240 provenances were proved to be appropriate ones while GER-079, POL-103, and ITA-229 provenances were inferior in the volume growth. The difference of individual volume growth among provenances were very large (0.066~0.277 m^3, 46.1~192.5%, compared to mean). The growth (height, diameter, and volume) was negatively correlated with latitude and positively correlated with longitude, and no correlation with altitude.

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Disease Development and Gas Exchange Rate in the Seedlings of *Pinus densiflora* Artificially Inoculated with *Bursaphelenchus xylophilus* and *B. mucronatus*

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Four-year-old seedlings of *Pinus densiflora* were inoculated with a suspension of *B. xylophilus*, or *B. mucronatus* adjusted to 3,000 nematodes per 50μL sterilized distilled water in a greenhouse on July 21, 2008 to evaluate initial symptom development and the changes of gas exchange rate. *B. xylophilus* and *B. mucronatus* were distinguished by four restriction enzymes except Rsa I, and the result of ITS-RFLP of *B. xylophilus* used in this experiment was completely matched to that of pine wood nematode reported previously. Needle dehydration and subsequent yellowing were observed in all seedlings inoculated with *B. xylophilus* isolate while the appearance of the seedlings was normal in all seedlings inoculated with *B. mucronatus* and control. Needle dehydration was observed in most seedlings inoculated with *B. xylophilus* between 2 and 3 weeks after inoculation. In seedlings inoculated with *B. xylophilus*, continuous decrease in photosynthetic rate was observed after 6 days of inoculation. Photosynthetic rate decreased more markedly after 12 days of inoculation when external symptoms appeared in most seedlings, and ceased almost completely 19 days after inoculation. There was no remarkable decrease in gas exchange rate in seedlings inoculated with *B. mucronatus* until 12 days after inoculation. Photosynthetic decline was only observed at 19th day after inoculation but recovered above control level after 25 days of inoculation. Disease development and gas exchange rate changes in the seedlings of *Pinus densiflora* inoculated with *B. xylophilus* were not observed on those inoculated with *B. mucronatus*. Further studies are needed to clarify the factor which restricts symptom development.

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Genetic Variation of the Endangered Endemic Plant Species, *Lycoris uydoensis* (Amaryllidaceae) in Korea

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Lycoris uydoensis M. Kim (Amaryllidaceae), a herbaceous perennial grows in just uydo island of Buan-gun, Chollabuk-do in Korea. This plant species is endemic and have a few small natural populations. The natural populations have been greatly disturbed by development and vandalism. Some individuals of them have been transplanted for the ornamentals of whitish flower and cultivation. And it has been suggested based on karyotype (3M+1SM+4T+11A) analysis that *L. uydoensis* is a natural hybrid. Also, *L. uydoensis* is sterile (2n=19), propagates via strong vegetative reproduction by producing bulbels and found in streamsides favorable to moving bulbels. To estimate level of the genetic diversity for conservation strategies, ISSR analysis was conducted in forty five individuals of a population of *L. uydoensis*. Forty eight ISSR loci produced in five primer (#843, #849, #854, #855, #873) revealed no variation among forty five individuals of a population. The results indicated that one or a few individuals of *L. uydoensis* were maintained populations via asexual reproduction by producing bulbels.

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Genetic Diversity among the Natural Populations of Rare Endemic Plant Species, *Lycoris chinensis* var. *sinuolata* (Amaryllidaceae) in Korea

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The threatened and endemic plant species, *Lycoris chinensis* var. *sinuolata* K. Tae et S. Ko (Amaryllidaceae) is restrictedly distributed in just South western region of Korea peninsula. This plant species is herbaceous perennial and fertile (2n=16). And *L. chinensis* var. *sinuolata* propagates via both sexual by seed and asexual reproduction by bulbels. Three small natural populations of this species usually are located in gradual slope of valley of mountain region. All of three populations have been generally preserved by the national park and temple forest.

The genetic diversity and structure of *L. chinensis* var. *sinuolata* populations were assessed using ISSR marker. Seventy individuals in three natural populations were analyzed with five primer. Low levels of genetic diversity were revealed by ISSR marker. At the species level, the expected heterozygosity and Shannon' index were 0.135and 0.202 on average, respectively. The result of AMOVA analysis showed that forty five% of the total genetic diversity was shared among populations. Results from this study provide a scientific basis for estimating genetic diversity and for strategic conservation of this species.

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Study of Genetic Diversity and Taxonomy of Genus *Sorbus* in Korea Using Random Amplified Polymorphic DNA

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Genus *Sorbus* is a long lived woody species and plants of this genus is primarily distributed patchy throughout Asia and Europe. *Sorbus commixta* is primarily distributed throughout Europe. Eastern Asian *Sorbus* species are regarded as very important herbal medicines in Korea and China. Random amplified polymorphic DNA (RAPD) was used to investigate the genetic variation and phylogenetic analysis of four species of this genus in Korea. Although some Korean populations of these species were isolated and patchily distributed, they exhibited a high level of genetic diversity. Twenty-six primers revealed 205 loci, of which 128 were polymorphic (62.4%). *S. commixta* had the highest expected diversity (0.165), *S. aucuparia* the lowest (0.109). The estimated gene flow (*Nm*) was low high among intra-species (mean *Nm* = 0.755). A similarity matrix based on the proportion of shared fragments (GS) was used to evaluate relatedness among species. The estimate of GS ranged from 0.786 to 0.963. The molecular data allowed us to resolve well-supported clades in Korean taxa and European species. An addition, especially, species-specific markers for genus *Sorbus* by RAPD analysis may be useful in germ-plasm classification and agricultural process of several taxa of this genus.

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Isolation and Characterization of Salt-induced Genes in Poplar Cells by Suppression Subtractive Hibridization and cDNA Microarray Analysis

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Salt stress inhibits plant growth and productivity. The effects of salt stress on poplar physiology, biochemical and molecular changes have previously been studied in poplar leaves and roots. Here, we report microarray expression profiles of 542 genes from cDNA library of 150 mM NaCl treated poplar (*Populus alba* × *P.tremular* var.*glandulosa*) cells using suppression subtractive hybridization method. Among 542 genes on the microarray, merely 3.9% of genes displayed significantly up-and down-expression level in response to salt stressed poplar cells. The majority of the genes with altered transcript levels belong to metabolic process, cellular process, response to stress, and biological process unknown. Especially, these genes encoding LEA protein, myo-inositol-1-phosphate synthase, NAD-dependent dehydrogenase, and malate synthase were previously reported as salt tolerance gene in leaves and roots of *Populus euphratica*, which has physiological ability to tolerate extreme drought and salinity. These results suggest that the cell suspension may offer an opportunity for understanding function of salt-induced gene between organs and cells.

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Improved Tolerance to Salt in Transgenic Poplar Producing Mannitol

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The *mtlD* gene encoding mannitol-1-phosphate dehydrogenase, catalyzes the biosynthesis of mannitol from fructose. We cloned the gene from *Escherichia coli* and modified to be expressed in plants. The chimeric *p35S-mtlD* gene was stably incorporated into poplar (*Populus alba* × *P. glandulosa*). Transgenic poplar plants accumulated mannitol at concentrations ranging from 0.025 to 0.033 mg/g fresh weight, whereas nontransgenic poplar plants did not accumulated it at all. Seven transgenic poplar clones were subjected to salt stress with NaCl using leaf disk and shoot tip culture systems. Although the transgenic poplar plants did not show much difference from nontransgenic poplar plants in callus formation and growth in the presence of 100 or 125 mM NaCl, they exhibited improved rooting performance (both rooting rate and the number of roots per plant) in shoot tip culture. Overall, the transgenic poplar plants watered with 100 mM NaCl in the pots exhibited less injury than nontransgenic poplar plants measured by photosynthetic rate. Taken together, the transgenic poplar plants appeared to have attained a limited salt tolerance through the expression of *mtlD* gene.

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Cross-species Amplification of Microsatellite Loci in Some *Hibiscus* spp.

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Accurate identification of individuals, clones and cultivars for target species and information on their genetic relationship is essential for effective performance of breeding and genetic resources management programs. However, the isolation and characterization of microsatellite on a species from which markers have not yet been developed entails a significant input of time and cost. Therefore, if we could find available markers in existing microsatellite primer sets developed from closely related species, it would be very efficient in terms of eliminating the time-consuming and costly process. In this study, to validate as available markers for identification of clones and cultivars of *Hibiscus syriacus,* total 27 microsatellite primers derived from *H. rosa-sinensis, H. tiliaceus* and *H. glaber* were tested. After PCR optimization, 15(56%) tested primers successfully amplified *H.syriacus* DNA, of which 10(67 %) were polymorphic. These polymorphic microsatellite loci were expected to provide useful codominant markers for inexpensive and highly informative multi-locus data on identification of the number of *H. syriacus* cultivars. They seem to be available for assessing intraspecific variation, mating system, and paternity studies in the species.

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Somatic Embryogenesis of Siberian Larch

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Somatic embryogenesis was initiated from immature zygotic embryos of Siberian larch (*Larix sibirica* L.) which is resistant to larch bud midges. Seeds obtained in the result of controlled pollination were introduced into *in vitro* culture. Different mixtures of nutrient medium: ½ of MS, MSG and MA were used for induction of embryogenic callus formation. The formation of embryogenic callus proceeded on all the media during a month in 18-20% of cases. However, the proliferation and maturation of somatic embryos proceeded only on the MA medium. So, in the result of inducing the Siberian larch hybrid seeds into the culture the five actively proliferating cell lines were obtained. Doubling of

biomass of a proliferating culture was observed even on the 11-th day of cultivating. Somewhat about 250-380 somatic embryos per 100 g of embryonic mass were counted in the proliferating culture. To stop proliferation and go to the maturation stage the embryonic mass was cultivated during four days in the liquid with no hormone containing nutrient MA medium at steady stirring (60 revolutions per minute). Twelve media which contained different variations of concentration of ABK, sucrose, PEG and Gelrite were applied to reach maturation of somatic embryos. The maximum result was obtained at distributing the embryonic mass on filter paper on the surface of nutrient medium containing 40 g/l sucrose, 60 M ABK and 10% PEG. Development to mature somatic embryos made from 15 (the cell line 3) to 60 (the cell line 5) embryos on 500 mg of embryonic mass. Fully mature somatic embryos were brought to the medium for germination which was free from plant growth regulators and with decreased two times content of macro- and microelements as well as iron and which didn't contain organic nitrogen and vitamins. On this medium in four days already the lengthening hypocotyl and developed root were observed. As soon as a hypocotyl of germinated somatic embryo appeared (the third week on medium for germinating) it was considered as a germ. Germs (after five weeks of germination) were brought to the ecosoil and passed the period o f adaptation to environment.

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High Frequency Plant Regeneration from Abnormal Shoot Organogenesis in Medicinal Plant, *Hovenia dulcis*

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An efficient plant regeneration protocol for shoot organogenesis from *Hovenia dulcis* callus cultures was established. Induction of organogenic callus was achieved on Murashige and Skoog (MS) medium supplemented with 1.0 mg l^{-1} kinetine and 1.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). Further differentiation of organogenic callus into primordium, shoot like structures and plantlet was achieved on MS medium supplemented with 0.1 mg l^{-1} Gibberellic acid (GA3) and 0.1 mg l^{-1} kinetin. Numerous abnormal shoots developed on transfer of callus to MS medium with cytokinins, which however did not grow further into entire plantlets. The re-cultivation of abnormal shoots in MS medium without cytokinins resulted in the growth of normal shoots. The elongated shoots subsequently were rooted in basal MS medium and established in artificial soil. The RAPD analysis of the regenerated plants confirmed their genetic stability even after in vitro procedures.

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The Embryogenic Competency and Morphological Changes during Somatic Embryogenesis in *Iris Pseudacorus*

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In the present study, firstly we report the establishment of a high frequency plant regeneration system via somatic embryogenesis in *Iris pseudacorus*. Secondly, the morphological characteristics of somatic embryos in this species are also described. Embryogenic callus was obtained from bulb segments of *Iris pseudacorus* on Murashige and Skoog (MS) medium with 2,4-dichlorophenoxyacetic acid (2,4-D) alone or in combination with kinetin. When early globular somatic embryos were subcultured onto MS medium with 4.52 1M 2,4-D, high frequency of somatic embryogenesis was obtained. Deprivation of 2,4-D was required for maturation. Mature somatic embryos had an elongated scutellum with a notch on the base of scutellum. Separation of embryos from embryo clusters was necessary to enhance the frequency of germination. Germination was stimulated by separation of embryos from embryo clusters and transfer onto fresh half-strength MS medium with 3% sucrose. After acclimation in artificial soil in greenhouse for 2 months, 96.4% of plantlets survived.

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Long-term Cryopreservation of Embryogenetic Scots Pine Cultures

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Somatic embryogenesis (SE) is considered the most potential vegetative propagation method for Scots pine (*Pinus sylvestris* L.). The existing SE protocols for Scots pine are based on the use of immature zygotic embryos as explants for culture initiation. Using juvenile explants, the final performance of the SE clones can be evaluated only by field tests of regenerated plants taking 5-10 years at the minimum. The Scots pine SE cultures have, however, been found to gradually lose their embryo

production capacity within 1-2 years of continuous *in vitro* culture. Cryopreservation of SE cultures is thus a prerequisite for successful clone delivery, being able to maintain regeneration ability during field testing.

Cryopreservation techniques have been developed for Scots pine SE cultures, but there are no reports on long-term cryostorage. The aim of the present work was to study potential effects of the long-term cryopreservation and different cryoprotectants on the viability, proliferation ability or embryo maturation capacity of Scots pine SE lines.

Altogether 108 different SE lines from 4 donor trees were used material. The samples were cryopreserved using either PDG mixture 1:1 or 1:2½, or DMSO solely as cryoprotectant, and stored in liquid nitrogen for 2 to 14 years (from 1995-2007 to 2009).

At least 80% of the SE lines cryopreserved with PDG mixture 1:1 in 1998/1999 (93%) and in 2006/2007 (83%), and the lines cryopreserved with PDG mixture 1:2½ in 2000/2001 (80%) remained viable and started proliferating following thawing. The recovery was significantly lower for the lines cryopreserved with PGD in 1996/1997 (44%) and for the lines preserved in 1994/1995 using DMSO (0%). The PDG mixture used, cryostorage time, or donor tree did not impact significantly the growth ratio of the SE lines, W_1/W_0 in 6-week proliferation varying 19-24x. At the maturation phase, the length of cryopreservation affected significantly ($p < 0,01$) the production of cotyledonary embryos, the quantity of embryos being lower in the lines cryopreserved for 8-13 years (varying from 60± 21 to 68 ±29/gFW) than in the ones cryostored for two years (292±72/gFW). The PDG mixture used and donor tree had no significant effect on embryo production capacity.

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Regeneration of *Eleutherococcus seoulensis* using Immature Seeds Via Somatic Embryogenesis

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High-frequency somatic embryogenesis was achieved from the zygote seeds of *Eleutherococcus seoulensis.* Callus were induced from the immature zygote embryo on the callus induction medium. Among the callus induction medium, Mixtures of 2,4-D and TDZ hormones on the medium are the most effective way to induce the callus. Opaque and friable embryogenic calli were formed on medium without any hormones during about 2-weeks. Initiation of somatic embryogenesis and development up to the globular stage from embryogenic cell clumps occurred in callus materials. To find finest maturation condition on somatic embryos, several experiments were experimented. Concentrations of MS salt, Sucrose, Active Charcoal, PEG and density of embryogenic callus were manipulated. From that, the medium included 1/2MS 3% sucrose, ABA 0.1mg/L, A.C 0.02%, 0.5% gelrite was the best medium to maturate the embryogenic callus. The highest rate of germination was the medium with 0.2mg/L GA_3 hormone. Plentlets were transferred to 1/2 SH solid medium with 1.0 mg/L GA_3 and 0.2% activated charcoal for shoot and root elongation and them elongated plantlets further developed on 1/2 SH medium for 4 weeks. Plantlets produced somatic embryos were acclimatized in a greenhouse.

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Regeneration of Wild *Panax ginseng* Via Somatic Embryos

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This study was conducted to establish the optimal condition for plant regeneration and acclimatization from somatic embryos of wild *Panax ginseng*. Cotyledon segments of wild *Panax ginseng* produced primary and secondary somatic embryos when cultured on MS and WPM media with supplement of 7% sucrose. To induce plantlet conversion, cotyledonary somatic embryos were cultured on WPM solid medium with GA_3 at various concentrations (1-30 mg/L) for 4 weeks. The highest rate of plantlet conversion was the medium with 3.0 mg/L GA_3. Plantlets were transferred to 1/2 WPM solid medium with GA_3 at various concentrations (0-5 mg/L) and 0.5% activated charcoal for shoot and root elongations. Elongated plantlets further developed into well-developed leaf and root system on 1/3 SH medium with 0.5% activated charcoal under ventilation condition for 5 months. The highest survival rate to soil was 75% when plantlets were regenerated on 1/3 SH medium without sucrose under ventilation condition. And *in vitro* flowering occurred when 2~4 cm plantlets were cultured on 0.7, 0.9 and 1.1% Bacto-agar gelling 1/2 SH medium. Frequency of *in vitro* flowering of plantlets is 12, 8, 8%, respectively.

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In vitro Symbiotic Germination of *Gastrodia elata* Seeds with *Mycena* Species

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Gastrodia elata must establish the symbiotic relationships with beneficial fungal species, depending on its developmental stages such as seed germination and

vegetative growth. Although *Mycena osmundicola* has known to be only fungal species germinating *G. elata* seeds, we found that two other *Mycena* species (KFRI1212 and KFRI 1223) were also able to promote the germination of *G. elata* seeds at different rates. Final germination rates were not significantly different between *M. osmundicola* (90.4%) and KFRI1212 (94.4%), while KFRI1212 promoted the protocorm development more rapidly than did *M. osmundicola*. Molecular analysis based on sequencing internal transcribed regions revealed that KFRI1212 matched most closely to *M. chlorophos* (AB512312.1), indicating that symbiotic germination of *G. elata* seeds could be also achieved by another *Mycena* species rather than *M. osmundicola*.

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Effect of Growth Regulators on In Vitro Regeneration of *Kadsura japonica* (L.) Dunal

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Scarlet Kadsura (*Kadsura japonica* (L.) Dunal, 남오미자 in Korean, 南五味子 in Japanese and Chinese) is ever green broadleaved woody liana plants growing in the subtropical zones in Korea, China, Japan and Taiwan. It belongs to Schizandraceae(or Magnoliaceae) and grows 30m long . Its fruit ripens in red color. In this thesis in vitro culture of this species is reported. Material used was green twigs collected from one plant growing in a greenhouse. The original plant, a cutting by us, was from Jeju island in the southern part of Korea. Twigs collected were sterilized and cultured on WPM agar media with four concentrations(0.2, 0.5, 1.0, 5.0mg/1 and control) of BAP and Kinetin. Effect of cytokinins on the shoot development from axillary bud of *Katsura japonica* Duna were investigated after 5 weeks culture. The percentage of the shoot development was slightly inhibited by both kinds and concentrations over control(88%). The highest mean number of new shoots were obtained on WPM containing 0.5 mg/l BAP(1.8 shoots). In the test of rooting of shoots with one axillary bud on WPM containing IBA and NAA with variable concentrations(0.2, 0.5, 1.0, 5.0mg/1), the highest percentage of rooting, 95%, was obtained at 5% IBA. Thirty percent survival was obtained after 4 weeks when the plantlets were transferred to pots with peatmoss and perlite(1:1) and maintained in high humid conditions. Through this experiment we could expect the possibility of micropropagation of this species although some more research is necessary for commercialization.

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Commercialization of Conifer Somatic Embryogenesis for Multi-varietal Forestry through Partnerships in Canada

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In the past 30 years, tree improvement programs around the world have contributed to the significant increase in forest productivity by providing genetically improved seeds. These programs typically produce 3-5% gain in height when evaluated at about 10 years of age. Our experience also indicated that the efficient capture of variability among the individuals of elite crosses could yield 4-6 times the gain available from the use of seed orchard seeds. Multi-varietal forestry (MVF), defined as the use of genetically tested tree varieties in plantation forestry, is the means of utilizing additional genetic variances contained in the breeding population. The implementation of MVF requires efficient vegetative propagation techniques, the development of high-value tree varieties, and the management of plantation diversity. Owing to recent achievements in somatic embryogenesis (SE), the implementation of MVF is in progress in Canada. To help promote R&D and application of SE, the Canadian Wood Fibre Centre of the Canadian Forestry Service launched the National Network of Somatic Embryogenesis Laboratories in 2007 with two main objectives: (1) to build competency in SE of all commercially and ecologically important Canadian tree species through R&D and (2) to establish regional applied SE laboratories to implement industrial MVF. Furthermore, this SE network can function as a conduit for implementing other advances in tree biotechnology such as molecular marker technology.

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Application of Somatic Embryogenesis in Forestry for Improved Productivity, Genetic Conservation and Restoration

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Somatic embryogenesis (SE) and cryopreservation are the first biotechnologies that have many unexploited possibilities in forestry, including mass propagation of high-value trees with desirable attributes, species/germplasm conservation and restoration, and the development of pest resistant tree varieties. Recognizing these potentials, the Canadian Forestry Service, in partnership with the forestry sector, embarked on SE projects on both economically and ecologically important Canadian conifers. Immature seeds of whitebark pine (*Pinus albicaulis*), limber pine (*P. flexilis*), Douglas-fir (*Pseudotsuga menziesii*), western red cedar (*Thuja plicata*) and yellow cedar (*Chamaecyparis nootkatensis*) were used in 2007 and 2008. Whitebark and limber pines are ecologically important keystone species that are an integral part of ecosystems; however, over much of their range including British Columbia and Alberta, the species is seriously threatened due to susceptibility to white pine blister rust (*Cronartium ribicola*) and mountain pine beetle (*Dentroctonus ponderosae*). SE in whitebark and limber pines has been obtained for the first time, embryogenic lines were successfully cryopreserved and thawed, and plants were generated, providing a new dimension for genetic resource conservation and species rescue. In addition to commercial application in spruce species in eastern Canada, somatic seedlings in Douglas-fir was obtained at high frequencies offering a possibility of industrial implementation. Also, somatic seedlings of western red cedar have been obtained for the first time, but plant regeneration in yellow cedar was unsuccessful requiring further research.

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Masspropagation and Cryopreservation of *in vitro*-cultured Axillary Bud Meristems of *Melia azedarach*

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Melia azedarach is of great important tree because it has therapeutic as well as insecticidal properties. This tree has been used for wood resistant to termites, fodder, green manure, as well as oil from seeds, It was a originated from north-western India, and extended in many subtropical countries. Cryopreservation of plant meristems or cells, somatic and zygotic embryos, is an important technique for long-term preservation without genetic alteration. Simple and reliable cryopreservation protocols with vitrification have been developed and applied to many plant species. Vitrification methods have been applied to tropical plants such as banana that is not resistant to cold conditions. In this study, an optimal procedure for masspropagation of *M. azedarach* was achieved when using nodal segments with two axillary buds as explants. In each treatment, five nodal segments were cultured. On the MS (Murashige & Skoog) medium supplemented with BA(6-Benzyladenine) 1 mg/L, multiple shoots were developed within 4-5 weeks in 95% of the total cultures. As the concentration of BA decreased, shoot length was reduced and leaf development occurred. Generally, nodal explants more efficient the length than shoot tip explants. Formation of axillary shoots was observed on the media with cytokinins (BA). Shoot proliferation was influenced by cytikinin type and concentration. Plantlets of *M. azedarach* were cold-hardened at 10 ℃ for 16 hr photo-periods for 6 weeks. Excised axillary shoot-tips from hardened plantlets were precultured on a solidified MS medium supplemented with 0.7 M sucrose for 1 day at 25 ℃. Axillary shoot-tip meristems were dehydrated using a highly concentrated vitrification solution (PVS2) for 60 min at 0℃ prior to a direct plunge into liquid nitrogen (LN). The PVS2 vitrification solution consisted of 30% glycerol (w/v), 15% ethylene glycol (w/v), 15% DMSO (w/v) in MS medium containing 0.4M sucrose. After short-term warming in a water bath at 40℃, the meristems were transferred into 2 ml of MS medium containing 1.2 M sucrose for 15 min and then planted on solidified MS culture medium. Successfully vitrified and warmed meristems resumed growth within 2 weeks and directly developed shoots without intermediary callus formation. The survival rate of cold-hardened plantlets for 3 and 4 weeks was 90%. We did not find any difference in PCR-band patterns between control and cryopreserved plants.

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Somatic Embryogenesis of Flowering Cherry, *Prunus incise* 'February Pink'

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Factors affecting somatic embryogenesis from root explants of *Prunus incisa* Thunb. cv. February Pink were investigated. Using a medium containing Murashige and Skoog salts and vitamins supplemented with 10μ*M* 2,4-dichlorophenoxyacetic (2,4-D), we evaluated the effects of light, growth regulators, amino acids, carbohydrate source, and root induction medium. Explants cultured under light or dark conditions both resulted in the formation of embryos. Embryogenesis was inhibited by

the addition of 6-benzyladenine, thidiazuron, or gibberellic acid to the medium. Amino acids were not effective in promoting embryogenesis, with high levels of amino acids actually inhibiting it. Sucrose and glucose effectively induced embryogenesis, while sorbitol and mannitol completely inhibited it. Sucrose and glucose also promoted secondary embryogenesis. Embryos that formed in medium containing 4% or 5% sucrose were abnormally shaped and did not fully develop, while those that formed in medium with sucrose concentrations of 2% or 3% were much more vigorous. Root explants that were induced on medium containing 1.0 u*M* indole-3-butyric acid (IBA) produced more somatic embryos than explants induced on medium without IBA. Approximately 50% of the roots induced on medium containing 1.0 u*M* IBA produced somatic embryos on medium containing 10 u*M* 2,4-D and 3% sucrose.

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Incorporating Pedigree Reconstruction in Clonal Forestry Development and Production Systems

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The use of informative microsatellites molecular markers in combination with partial or full pedigree reconstruction created opportunities for the assembly of structured matings from natural pollination without the need to elaborate mating designs. The developmental phase of somatic embryogenesis focuses on the production of multiple cell lines for clonal testing. These cell lines are produced from immature or mature embryos from specific matings among selected parents, thus causing time delay associated with parents' selection, artificial controlled pollination, and the biological requirements for embryo and seed development. An alternative scenario for cell line production is proposed where the authenticity of the desired male parents is determined from wind-pollinated natural crosses from seed collected from desired female parents. This approach is made possible by the recently achieved high induction rate of cell lines permitting matings assembly at the tissue culture stage and thus the saving of critical time. Similarly, following the completion of clonal testing, the production phase of somatic embryogenesis focuses on the bulk-up of selected elite genotypes. The benefit from DNA fingerprinting and pedigree assessment as a quality control/quality assurance safeguard is also demonstrated.

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Quantitative-genetic evaluation of the induction success rate

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A deployment by somatic embryogenesis has been often considered by forest geneticists in order to capitalize on the entire genotypic superiority of their best breeding stock. Unfortunately, variability in propensity to produce somatic embryos (induction success rate) has been encountered in different species. We will discuss the impact of this differential success rate on selection response and diversity in the clone mixtures supplied to operational forestry. Results of our quantitative-genetic evaluation support the advantage of somatic embryogenesis even in the presence of relatively high induction success rate.

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Comparison of The Amenability of *Pinus patula* and Its Hybrid Crosses to Somatic Embryogenesis

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Somatic embryogenesis (SE) offers a means to overcome the constraints of vegetative propagation for maximizing the deployment of genetically improved *Pinus patula* cuttings. These constraints are caused by the onset of ontogenetic maturation in the hedge plants, resulting in low rooting and poor field performance of cuttings. The amenability of embryogenic tissue to cryopreservation offers a means of overcoming hedge maturation effects. As hedges age and the performance of the resulting cuttings declines, SE-derived clones can be drawn from cryostorage for substitution of the aging hedges with more juvenile material. Thus, a further benefit of this process is the ability to deploy this species clonally, a process that is currently not feasible. A serious threat to the South African softwood industry is the pathogen, *Fusarium circinatum*, which causes serious losses in *P. patula* both in the nursery and during early establishment in the field, but could potentially also result in an outbreak in mature trees. As part of the strategy to find a solution to this growing problem, which is restricting the continued deployment of this species, controlled pollinated (CP) crosses of *P. patula* with species that show resistance to *F.circinatum* have been developed and tested. The CP material was put through the same SE protocol used for *P. patula*, with positive results. These results were obtained in almost every step of the process, with improvements in maturation responses and germination of embryos through to the conversion to hardened hedges, without major protocol changes and

with an increased number of genotypes amenable to the process. Preliminary results obtained from several *in vitro* trials comparing the amenability of the embryogenic tissue from various *P. patula* and *P. patula* hybrid families to the SE process, showed a 44% increase in germination of well-developed embryos and a 25% increase in somatic plantlets ready to be planted out for hardening in responsive genotypes. The cutting production, rooting, *F. circinatum*-resistance and field performance of the SE-derived *P. patula* hybrid material is further tested to assess the feasibility of this propagation strategy.

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Development of Mass Reproduction Technology of selected Oil Palm Clones through Somatic Embryogenesis

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Oil palm tree (*Elaeis guineensis* Jacq.) was known as the most important oil producing crops in the world due to the highest productivity of the species compared with other crops. Nevertheless, two barriers made it difficult to commercialization of the tissue cultured plants. One obstacle is the absence of reliable Mass Reproduction Technology (MRT) and the other is somaclonal variation of the *in vitro* clones. Somatic embryogenesis in oil palm include multi-step process *in vitro* and it needs more than eighteen months of producing period from meri-stemic tissues of selected clone in the field. Moreover, the limitation of the explant source of the selected genotypes of oil palm is strongly related to just mentioned above obstacles. Using the 6 clones from the ASD, we could develop unique technologies for the MRT. Mass of somatic embryos was obtained from *in vitro* grown seedlings using meri-stem cultures and induced mass could be used as further explant sources for the second round process for the commercial-scale production of the somatic embryos. *In vitro* selection of the embryogenic cell lines from the mass were routinely conducted with 2 weeks of intervals. Detection of the phenotypic variants *in vitro* seemed to be powerful tools for early elimination of the somaclonal variants. Development of the germination process from the somatic embryos was focused on the rate of the abnormalities *in vitro.* Association of the mixer to separate cells from embryogenic mass was allowed us to establish cell suspension cultures. Pilot-scale cell suspension culture of the cell lines through bioreactor was revealed as quite successful system. From the selected lines of cell cultures to plantlets, it required just 6 months of culture period. Now, field test is undergoing to verify the relationship between *in vitro* abnormalities and *ex vitro* growth. Our results will suggest reliable MRT process for the oil palm clones from cell to seedlings *in vitro*.

PHOTO GALLERY

IUFRO Working Party 2.09.02
Somatic Embryogenesis of Forest Trees Conference

Suwon, Republic of Korea
August 19-21, 2010

Opening Ceremony (Aug 19, 2011)

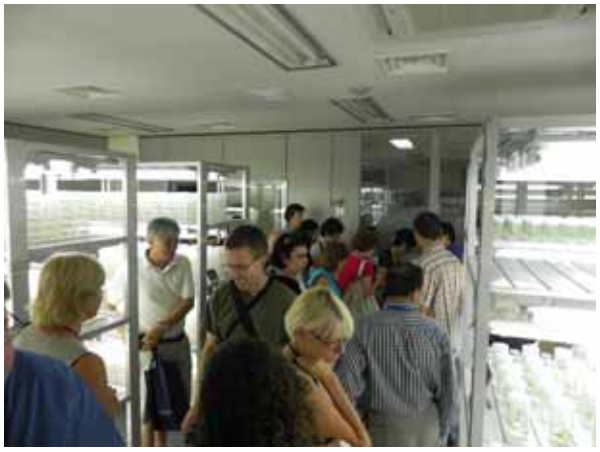
Opening by KFRI General Director (Dr. WY Choi) and IUFRO working party chair (Dr. YS Park)

Congratulatory speechs by President of the Korean Society for Plant Biotechnology (Dr. JR Liu) and the Korean Forest Society (Dr. KJ Lee)

Oral Presentation Sessions (Aug 19-20, 2011)

Reception Dinner at Ibis hotel (Aug 19, 2011)

Field Trip (Aug 21, 2011) KFRI and Eocheon Experimental Forest

Lab tour at Foret Biotechnology Div., KFRI


Field Trip to Eocheon Experimental Forest, Hwaseung

Field Trip (Aug 21, 2011) Korean Folk Village

Tasting Korean Cuisine before going to Folk Village

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FFPRI - Forestry and Forest Products Research Institute
INRA - Franch National Institute for Agricultural Research
KFRI - Korea Forest Research Institute
KFSVC - Korea Forest Seed & Variety Center
KRIBB - Korea Research Institute of Bioscience & Biotechnology
SOIBC - Branch of Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry

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