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Recent Progress in Somatic Embryogenesis of Four *Pinus* spp.

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ABSTRACT

Advances in conifer biotechnology offer new opportunities in the field of vegetative propagation and genetic engineering. Development of clonal propagation methods, especially somatic embryogenesis (SE), has numerous potential applications. Owing to its efficiency in plant regeneration, coupled with the ease of long-term storage in liquid nitrogen, SE became an indispensable tool for accelerating the development of tree varieties and deployment. Early SE protocols were developed for *Picea* species, however, when applied to *Pinus* species, especially at the somatic embryo maturation stage, they were unsuccessful. It became apparent that pines required more research and development to bring the SE biotechnology to its potential. This review emphasizes the most recent progress made in SE and cryopreservation in the genus *Pinus*, focusing on major plantation and forest species in Europe (*Pinus pinaster*), New Zealand, Australia, and South America (*P. radiata*), as well as North America (*P. taeda* and *P. strobus*). Much consideration is also given to applying SE in pine improvement and deployment strategies in multivarietal forestry.

Keywords: clonal trees, conifers, cryopreservation, deployment strategies, embryonal mass, immature seed, mass propagation, multivarietal forestry, pines, plantations, somatic embryos, zygotic embryos
Abbreviations: **ABA**, abscisic acid; **AC**, activated charcoal; **BA**, benzyladenine; **CPPU**, N-(2-chloro-4-pyridyl)-*N'*-phenylurea; **2,4-D**, 2,4-dichlorophenoxyacetic acid; **DMSO**, dimethylsulphoxide; **EM**, embryonal mass; **fm**, fresh mass; **MVF**, multivarietal forestry; **PEG**, polyethylene glycol; **PGR**, plant growth regulator; **SE**, somatic embryogenesis

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INTRODUCTION

Softwood species, typically represented by conifers, are an important source of timber and fiber for the forest and pulp-and-paper industries, respectively. Species belonging to the genus *Pinus* cover vast forest areas of Europe, North America, South America, and Asia. A few, such as loblolly pine (*Pinus taeda* (L.)), maritime pine (*P. pinaster* (Ait.)), radi-

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ata (or Monterey) pine (*P. radiata* (D. Don)), and to certain extent eastern white pine (*P. strobus* (L.)), have been extensively used as plantation species. Maritime pine is predominantly planted in the Aquitaine region of France (1.5×10^6 ha) and has been given high priority in breeding programs. In Portugal, maritime pine occupies about 33% of the forest area (1×10^6 ha) and breeding began there in the 1980s. Loblolly pine is the most widely planted tree by the US forest products industry, with 1.4×10^9 trees planted each year. Radiata pine has been used in a few Southern Hemisphere countries – mainly Chile, New Zealand, and Australia – where, overall, the plantations occupy 4×10^6 ha. Eastern white pine is planted mainly in the eastern parts of Canada and the USA, but plantings have been temporarily suspended because the exotic fungus *Cronartium ribicola* is causing large losses. For example, in Quebec, approximately 1×10^6 seedlings have been planted each year for the last 3 years; this number will likely increase in the next few years.

In conifer breeding programs, genetic gains are mainly achieved through progeny tests, followed by selection. Superior genotypes within the best families are then used to establish seed orchards to produce genetically improved seeds. A tissue culture process for mass propagation of conifers by somatic embryogenesis (SE), which was developed over the last two decades, has become the first biotechnology showing great potential for application in forestry. The SE process can produce any number of zygotic-like somatic embryos and plants from one seed; thus, it is a means of mass clonal propagation. The use of clonally replicated trees can help establish more informative progeny testing to allow optimal selection and more efficient assessment of parental breeding values. Clonal tests can also improve the process for selecting elite genotypes compared with seedling progeny tests. Any selected clones can be easily and massively deployed from the cryopreserved, juvenile stock of embryogenic tissues by direct propagation of somatic embryos, establishment of clonal seed orchards, or rooting of cuttings from somatic donor-plants in the case of responsive pine species. Combined with cryopreservation, SE would also prevent the selected plant material from depreciation as is often observed in cuttings, which show decreased rooting ability with aging.

After the first report of plant regeneration through SE in *P. taeda* (Gupta and Durzan 1987), more results were published on other pines, indicating that this tissue culture process may be practiced with many species (**Table 1**). Because most of the public and private research effort was focused on those pine species that are vital to forest industry, progress in optimizing SE protocols, in cryopreservation, and in field tests of somatic trees was the greatest with *P. taeda*, *P. radiata*, *P. pinaster*, and *P. strobus*.

This review describes recent progress in protocol development for SE and cryopreservation of the above-mentioned four pine species. It presents strategies for integrating these biotechnologies in tree improvement programs and for deploying somatic trees in multivarietal forestry (MVF).

SE OF PINES: GENERAL CONSIDERATION

Gupta and Durzan (1987) first reported SE in *P. taeda* and described "somatic polyembryogenesis" for the proliferating embryonal mass (EM) that originates from the embryonal-suspensor cells of developing zygotic embryos. Smith *et al*. (1985) first introduced the method of initiating SE by culturing the intact or whole megagametophyte of *P. radiata* containing the developing zygotic embryos. This method of initiation by extrusion of the immature zygotic embryos through the micropyle of an immature megagametophyte soon became the preferred method for initiating embryogenic cultures in *P. taeda* (Becwar and Pullman 1995) and other *Pinus* species. The method is relatively easy and rapid, compared with extracting and culturing tiny immature zygotic embryos at the optimum precotyledonary stages of development, unless cotyledonary–stage embryos are used (see *P. pinaster*, *P. radiata*).

Explants for SE initiation

Research carried out over the last two decades has proven that SE in pines is initiated most efficiently from immature zygotic embryos. There has been limited progress toward initiating SE from mature seed or from selection-age trees of *P. taeda* (Gupta and Durzan 1987; Tang *et al*. 2001) and from mature seed of *P. strobus* (Garin *et al*. 1998). This approach, further described by Tang and Newton (2005), has the potential to eliminate the narrow initiation window imposed when using immature seed explants. So far, the initiation frequencies reported for mature seed explants are too low for practical application. Recently, initiation of SE from mature trees has been achieved with other *Pinus* species (Malabadi *et al*. 2004; Malabadi and van Staden 2005), and these advancements may have direct application to the species of interest.

In some instances, initiation of SE may not result in establishment of an embryogenic line because the ensuing EM ceases to proliferate. Thus, it is important to distinguish between the initial outgrowth/extrusion from an explant and continuous growth, when assessing the success rate.

A potential problem in pine SE is caused by polyembryony. Multiple immature embryos in a developing seed are a feature common to all *Pinus* species (Singh 1978). Simple polyembryony is the fertilization of more than one egg (archegonium) per seed. The number of archegonia per seed ranges from one to seven in several *Pinus* species (Lill 1976; Owens *et al*. 1982; Skinner 1992). Fertilization of multiple archegonia by different pollen results in multiple,

Table 1 First reports of SE in *Pinus* spp.

Species	Response[a]	Established in soil mix	Reference
Pinus banksiana Lamb.	SE, PL	yes	Park *et al.* 1998b
Pinus caribaea Morelet	SE, PL	no	Lainé and David 1990
Pinus elliottii Engelm	SE		Jain *et al.* 1989
Pinus koraiensis Sieb et Zucc.	SE		Bozhkov *et al.* 1997
Pinus lambertiana Lamb.	SE, PL	no	Gupta and Durzan 1986
Pinus monticola Dougl.	SE, PL	yes	Percy *et al.* 2000
Pinus nigra Arn	SE		Salajova and Salaj 1992
Pinus palustris	SE		Nagmani *et al.* 1993
Pinus patula Schiede et Deppe	SE, PL	no	Jones and van Staden 1995
Pinus pinaster Ait.	SE		Bercetche and Pâques 1995
Pinus radiata D. Don	SE		Smith *et al.* 1985
Pinus roxburghii Sarg.	SE		Arya *et al.* 2000
Pinus strobus L.	SE		Finer *et al.* 1989
Pinus sylvestris L.	SE		Keinonen-Mettälä *et al.* 1996
Pinus taeda L.	SE, PL	yes	Gupta and Durzan 1987

[a] SE: somatic embryos; PL: plants

genetically different zygotes. Cleavage polyembryony also occurs in *Pinus* species, where the embryos from each fertilization event cleave into four genetically identical embryos. For example, the observed total number of zygotic embryos per immature *P. taeda* seed ranged from 5 to 12 (average 7.4) among five different control-crossed families (MacKay *et al*. 2001). Therefore, immature *P. taeda* seeds, similar to other *Pinus* species, frequently have two or more genotypes of developing zygotic embryos. As the seed matures, one zygotic embryo develops more rapidly, becoming the dominant embryo, and most subordinate zygotic embryos degenerate. There is evidence in one pine species that some subordinate zygotic embryos survive to seed maturity (Bozhkov *et al*. 1997).

Polyembryony is important because the explant most frequently used to initiate SE in *Pinus* species is the immature whole megagametophyte at the precise stage of seed development when multiple zygotic embryos are viable, and it is from the zygotic embryos that SE originates (Becwar *et al*. 1990; Percy *et al*. 2000; Pullman and Johnson 2002). This early stage of seed development corresponds to 3-6 weeks post-fertilization, when the dominant zygotic embryo is at a precotyledonary stage of development (Becwar *et al*. 1990). Genetic markers have been used in *P. taeda* to show that SE can be initiated from more than one genotype of zygotic embryos in an individual immature megagametophyte explant (Becwar *et al*. 1991). Although this study clearly showed that embryogenic cultures can be initiated from subordinate zygotic embryos that are genetically different than the dominant zygotic embryo, it is not clear how frequently this occurs, or what impact it has on using SE for clonal propagation in *Pinus* species.

There are two possible implications of initiating from early stage polyembryonic explants in *Pinus* species. First, the resulting embryogenic cultures could be genetically heterogeneous – originating from two or more genotypes. Genetic fidelity within lines is such an important issue for the application of SE to clonal forestry that additional work is warranted to verify the ramifications, if any, of initiating SE from polyembryonic explants. One approach is to screen for genetic fidelity using markers only in selected cell lines that, based on results of field tests, show high potential for superior genetic gain. This would greatly reduce the breadth and cost of such genotyping. If evidence for genetic heterogeneity within a selected cell line is found, then isogenic lines could be initiated from individual somatic embryos through secondary SE (see *Secondary SE*) to ensure genetic fidelity within lines (Park 2002).

It has been hypothesized that polyembryony evolved in conifers is an adaptive mechanism to eliminate the effects of self-pollinated embryos, by having multiple, viable, outcrossed embryos in each seed (Sorensen 1982). If the one genotype that becomes dominant (and eventually matures in the seed) is a random event, then polyembryony should have no impact on selecting superior genotypes through SE from polyembryonic explants. Conversely, it has also been hypothesized that polyembryony provides a post-fertilization selection mechanism among heterogenic out-crossed embryos having different vigor (Buchholz 1926). If this latter hypothesis is correct, then initiation from subordinate zygotic embryos may have an impact on the effectiveness and efficiency of selecting desirable traits using SE in *Pinus*. As it is now possible, with genetic markers, to determine from which genotype (dominant or non-dominant) the *Pinus* SE culture originates, the above two hypotheses can be tested.

MacKay *et al*. (2001) also studied the relationship between the number of immature zygotic embryos per seed and initiation of SE. The results indicated that the number of zygotic embryos per seed may be a driver of initiation and thus could be a useful indicator of initiation potential. Clearly, additional work is needed to better understand and determine the effect that initiating from polyembryonic immature explants has on the SE process in *Pinus* species.

Although most *Pinus* subordinate zygotic embryos fail to develop as the seed matures, at least some subordinate zygotic embryos in mature *P. koraiensis* seeds were viable and were identified as the main prerequisite for initiation of SE from the mature seed explants (Bozhkov *et al*. 1997). Therefore, it is possible that SE initiation from mature *Pinus* seeds (Gupta and Durzan 1986; Tang *et al*. 2001) may originate from the few subordinate zygotic embryos that survive to seed maturity in some *Pinus* species. That is, the low frequencies of initiation from mature seed of *Pinus* species may be causally related to the low frequency of survival to seed maturity among subordinate zygotic embryos.

Culture medium formulations for SE initiation and embryonal mass (EM) proliferation

Several formulations of media are used in pine SE: DCR (Gupta and Durzan 1985), or modified DCR with MS (Murashige and Skoog 1962) microelements; EM, EDM, EMM (Smith 1996); MSG (Becwar *et al*. 1990); modifications of P6-based medium from Teasdale *et al*. (1986) as described by Pullman and Johnson (2002); WV5 (Coke 1996); mLV (Litvay *et al*. 1985) modified to contain half-strength macroelements (except iron and EDTA); and PR medium (Quoirin and Lepoivre 1977) modified to contain half-strength macroelements. The media are typically enriched with sources of organic nitrogen (L-glutamine and casein hydrolysate or with a mixture of several amino acids), plant growth regulators (PGRs), and many other additives (see *P. taeda*). Carbon is provided in a form of sucrose (1-3%) or maltose, and/or other carbohydrates. Agars or most often gellan gum (Phytagel™ or Gelrite™) are used to solidify the media, although some researchers also reported success with liquid medium (van Winkle and Pullman 2005; Pullman and Skryabina 2007) and establishment of cell suspensions (see *P. taeda*). Most frequently, the PGRs include 2,4-D, BA, and occasionally also ABA. In one study, a potent cytokinin CPPU was used as the sole PGR. Media may also contain AC. In most cases, Petri dishes are used as culture vessels because they facilitate culture observation and frequent subcultures. The explants may be left on initiation medium with or without subculture for up to 16 weeks. Cultures are kept in darkness at 24-25°C. Initiation of SE is not perfectly synchronized and may occur between 2 and 16 weeks, however, most explants produce macroscopically identifiable EMs between 6-10 weeks. During this time, the proliferating EM is subcultured onto fresh medium (either semi-solid or liquid) of the same or slightly modified composition for further growth. In order to amass large quantities of EM in a relatively short time on a semi-solid medium, a technique may be applied that involves transient suspension of the tissue in liquid medium (approximately 200-300 mg in 4 or 5 mL) in a centrifuge tube and dispersal of the aggregates into a fine cell suspension by vigorous shaking (Percy *et al*. 2000; Lelu-Walter *et al*. 2006). The suspension (4 or 5 mL) is then poured over a filter-paper disk (Whatman #2, 7.5 cm diameter) placed in a Büchner funnel. A low pressure pulse is then applied for a few seconds to drain the liquid, and the filter paper with attached cells is placed on the surface of a medium. This procedure yields 2-4 g fresh mass (fm) EM after 2 weeks of culture. Initiation of SE and proliferation of EM is carried out in darkness at 23 to 25°C.

Genetic control of SE initiation

It has been shown for several pine species (and other conifers) that SE initiation is genetically controlled perhaps independently of other traits. Effect of seed families was prevalent in studies with *P. pinaster*, *P. strobus*, and *P. taeda* (Klimaszewska *et al*. 2001; MacKay *et al*. 2001; Miguel *et al*. 2004; Lelu-Walter *et al*. 2006; Park *et al*. 2006). In the latter species, genetic control of SE initiation and its implications for breeding were studied (MacKay *et al*. 2006). Initiation of SE was highly variable among 30 full-sib seed families ranging from 4% to 23% using one culture medium

and procedure, and from 23% to 73% using a different culture medium and procedure. The highest initiation frequencies were obtained using a WV5-based medium. The family ranking of initiation values between the two media and procedure treatments were significantly correlated (r = 0.83), indicating that parental contributions to initiation were consistent despite the differences caused by medium and procedure. This study provided evidence for strong additive genetic effects on initiation of SE in *P. taeda*, supporting the hypothesis that initiation rates can be improved by a selective breeding approach. There was evidence for a negative maternal effect on initiation of SE in some trees, and positive maternal effect in other mother trees. Certain female parents can be used to effectively circumvent putative negative maternal effects in other trees. This approach improved initiation from 1.5- to 9.2-fold simply by switching the mother and pollen parent in controlled crosses.

Maturation of somatic embryos

An inorganic and organic medium composition used for this stage of SE is usually the same as for previous stages and both the auxin and the cytokinin are replaced by ABA. Early work on maturation of pine somatic embryos proved to be unsuccessful despite using protocols developed for spruce (*Picea*) and larch (*Larix*) species. This is clearly reflected in **Table 1**, where the first reports on SE did not include regeneration of plants in most species. A critical factor that was discovered to promote development of large numbers of somatic embryos was restriction of water availability either by physical means (Klimaszewska and Smith 1997) or osmotic (Li *et al*. 1998) or combination of both (Percy *et al*. 2000; Klimaszewska *et al*. 2001) (see *P. strobus* and *P. pinaster*). To maximize mature somatic embryo production, the EM is first suspended in PGR-free liquid medium (100-200 mg f.m. per 4 or 5 mL) and cultured on a filter-paper disk as described above (*P. strobus*, *P. pinaster*) or on a nylon mesh (*P. radiata*), or other membrane supports (*P. taeda*). Accordingly, there are two methods that are being used for somatic embryo maturation in pine species. One method involves decreasing the medium water potential by high molecular weight PEG (M_r 4000) (*P. taeda*). The other method involves reduction in water availability to the cultured cells by physical means (i.e., by increasing the medium gel strength up to 1000 g cm^{-2}) to produce mature somatic embryos with low water content (see *P. strobus*, *P. pinaster*). In both methods, the maturation media contain a relatively high ABA concentration (60-480 µM). Activated charcoal is also used for some species, either included in the medium (*P. taeda*) or by coating cultured cells (see *P. pinaster*). Somatic embryos that develop on a medium with PEG require 3-4 weeks of cold treatment and/or partial drying before germination. No post-maturation treatment is required when somatic embryos develop on a medium with a high gelling agent concentration. In the latter method, the EM does not require transfer to a fresh medium throughout entire maturation period, which lasts up to 10 weeks. Storage of Petri dishes with mature somatic embryos at 4°C for several weeks is also effective in some species and has practical implications for simultaneous germination of different maturation batches.

During maturation of somatic embryos the cultures are kept most frequently at 16 h photoperiod under low light intensity (approximately 5 µmol m^{-2} s^{-1}).

Markers of maturity and quality of somatic embryos

The need to improve the quality of somatic embryos remains a limiting factor to broader application of SE in clonal propagation and genetic engineering. High quality somatic embryos germinate at high frequency and the resulting germinants convert to vigorous planting stock. Although sufficient quantities of pine somatic embryos can be produced from some cell lines, other lines are recalcitrant to large-scale embryo production, or the embryos produced are not of high quality. Maturation success is based on the fact that somatic embryos are subsequently able to convert to plants, and for all the practical purposes, this is the ultimate goal. However, such an empirical approach does not give any information about the optimal time for harvesting to achieve maximal conversion rates or on the quality of somatic embryos with respect to storage reserve accumulation and other compounds that define a mature stage. Therefore, there is a need to develop markers that can be used to verify or control the quality of somatic embryos. One approach is to follow the accumulation of storage reserves (such as storage proteins, lipids, starch) or sugar alcohols and organic acids, and other proteins (such as the late embryogenesis abundant proteins) in zygotic embryos and compare these with the accumulation pattern in maturing and mature somatic embryos (see *P. strobus*, *P. pinaster*, and *P. taeda*). Another approach is to study temporal and spatial gene expression patterns in zygotic and somatic embryogenesis to gain insights into the molecular events taking place during development, which may explain existing differences (see *P. pinaster* and *P. taeda*).

Secondary SE

There are no published results on initiation of secondary SE from mature somatic embryos of pines. However, it has been achieved for at least two species, *P. strobus* and *P. pinaster*, on the same medium as used for primary SE initiation (Klimaszewska; Lelu-Walter *et al*., unpublished). Pine somatic embryos, even at the mature stage, may still have some loose cells attached to the radices and these may proliferate on medium with PGR. Therefore, it is important and prudent to thoroughly examine the cultures daily (before the secondary SE initiation occurs) under the microscope and remove whatever proliferating EM that has been carried over from previous culture on the maturation medium. The initiation of secondary SE, which lasts up to 6-8 weeks, is preceded by callus formation over the whole somatic embryo, followed by the appearance of groups or single early somatic embryos protruding from the callus surface. Once the EM is separated from callus and subcultured, it is indistinguishable from the original young culture.

Conversion to plants

Germination of zygotic-like mature somatic embryos and development of aerial parts is carried out most frequently on a semi-solid medium without PGR. It has been found beneficial for *P. strobus* and *P. pinaster* to culture the somatic embryos in darkness or dim light for the first 7-10 days before exposure to light. This ensures elongation of hypocotyls, reduces anthocyanin synthesis – which is predominant in these species – and facilitates later handling. It is also beneficial to place somatic embryos horizontally on a medium and then tilt the Petri dishes vertically to 45 or 60º so the roots do not penetrate the medium but instead develop on the surface (Klimaszewska *et al*. 2001; Lelu-Walter *et al*. 2006). Once the somatic embryos convert to plantlets, they are transferred to the same substrate as used for seedlings and grown either under controlled greenhouse conditions or in the nursery. Usually during the first 7 to 10 days, high relative humidity is maintained, which is gradually reduced over a period of a few days to the ambient conditions. Fertilization and pesticide treatments are the same as used for seedlings except that somatic seedlings are fertilized immediately after transplanting.

Cryopreservation

Long-term storage of pine EM at ultra-low temperatures (-140 to -196ºC) is a routine and integral element of any SE program. Since the first publication on cryopreservation of white spruce EM by Kartha *et al*. (1988), the protocol has

been modified for use with *P. monticola* and *P. radiata* (Percy *et al*. 2000; Hargreaves *et al*. 2002) and has since been applied to *P. strobus* and *P. pinaster* (Klimaszewska, unpublished, Lelu-Walter *et al*. 2006). This current protocol entails incubating EM (1 or 2 g fm.) in 3 or 7 ml of liquid culture medium (of the same composition as for maintaining the growth of EM), respectively supplemented with 0.4 M sorbitol for a period of 18-24 h. All subsequent steps are carried out on ice. Just before freezing, cold DMSO solution (in a culture medium) is added to the cell suspensions to a final concentration of 5-10% (v/v). The cell suspension is then left on ice for 1 or 2 h, and then dispensed into cryovials, which are placed in alcohol-insulated containers (Nalgene Cryo 1°C Freezing Container) that are pre-cooled for 2 h at -80ºC. The containers with vials are then placed at -80ºC for 1-2 h, during which a slow cooling of the cell suspension (approximately -1ºC/min) takes place. The vials are subsequently plunged in liquid nitrogen and stored in a cryofreezer. Thus far, no published report is available on the recovery percentage of cryopreserved EM over time but our results are showing that storage of five *P. strobus* genotypes for up to 7 years has not resulted in any deleterious effect on the recovery of cultures (K. Klimaszewska, unpublished). For regeneration, the contents of the vials are rapidly thawed in water bath at 37ºC for 1-2 min and the cell suspension is poured over a filter-paper disk placed on a thick pad of sterile blotting paper. The storage solution is allowed to drain for several minutes and the filter paper with cells is transferred onto semi-solid medium. Growth of cultures typically occurs within 1-2 weeks after thawing. Some species, such as *P. taeda*, require ABA in the recovery medium, which improves both recovery frequency and growth rate of cultures retrieved from cryostorage (Becwar and Krueger 2004). The recovery rate of cryopreserved genotypes has been as high as 95% (Pullman *et al*. 2003a). *P. radiata*, however, required a nurse culture to grow cells efficiently after cryostorage (Hargreaves *et al*. 2002). The latter involved culturing the thawed cells on nylon mesh placed on top of five pieces (3-4 mm in diameter) of nurse EM arranged in the center of a Petri dish. The nurse EM was genetically distinct from all of the thawed lines and was maintained by routine subcultures for 2 years. Sixty cell lines were tested and the majority showed improved post-thaw growth, i.e., vigor and health compared with those grown without the nurse culture; some lines could be recovered only when cultured on nurse EM. The growth enhancement could be attributed to a better aeration of the cells when placed on a "physical platform" and possibly to more rapid removal of the cryoprotectants away from the recovering EM. Compounds released by damaged cells could also be absorbed by nurse tissue, a factor that might adversely affect the growth. The nurse culture technique has been used for over a decade at Scion (trade name for New Zealand Forest Research Institute Ltd, New Zealand) to recover cryostored EM of radiata pine.

PINUS PINASTER

Somatic embryogenesis was first reported in maritime pine by Jarlet-Hugues (1989). Extensive research has been undertaken since then in France (Bercetche and Pâques 1995; Lelu *et al*. 1999) and Portugal (Miguel *et al*. 2004). Particular attention has been placed on initiation and proliferation of EMs, which were thoroughly characterized according to their morphology and cytology. Two different basal media are commonly used for maritime pine SE, depending on the laboratory: DCR-based and mLV.

The highest initiation frequencies are obtained with zygotic embryos during the early stages of late embryogeny (from established dominance to precotyledonary stages, Bercetche and Pâques 1995; Miguel *et al*. 2004). Interestingly, SE has also been initiated in maritime pine at advanced stages, e.g., cotyledonary embryos, but with reduced yields (Lelu *et al*. 1999). When possible, culture of more advanced zygotic embryos excised from the surrounding megagametophyte is generally preferred, compared with embryo culture within the intact megagametophyte (Miguel *et al*. 2004). Using zygotic embryos enables better control of the developmental stage, ensuring that all explants are developmentally uniform, and also limiting the risk of having mixed genotypes in a given embryogenic line.

Considering the culture medium, the results vary according to the laboratory and the material (its origin and culture). Open-pollinated seed families from Portugal had the highest initiation frequencies on DCR medium, ranging from 5% to 49% (Miguel *et al*. 2004). However, full-sib families from France had the highest mean initiation, 75%, on mLV medium and, much lower, 37%, on DCR-based medium (Park *et al*. 2006). The use of isolated zygotic embryos from full-sib seed families could explain the high initiation frequencies obtained on mLV medium, which ranged from 87% to 100% (Lelu-Walter *et al*. 2006).

Initiation media are usually supplemented with 2,4-D and BA. High PGR levels have been routinely used, e.g., 2,4-D at 10 µM and BA at 5 µM (Bercetche and Pâques 1995) or 2,4-D at 13.6 µM and BA at 4.4 µM (Miguel *et al*. 2004). Reduced concentrations of PGRs (2,4-D at 2.2 µM and BA at 2.3 µM) did not improve the initiation rate (Lelu-Walter *et al*. 2006). Interestingly, with early zygotic embryo stages, EM could be initiated without PGRs, but the frequency was drastically reduced (1% to 13%, Lelu *et al*. 1999). Maintenance of EM on mLV medium lacking PGRs resulted in proliferation and simultaneous somatic embryo development up to the cotyledonary stage. Therefore, the absence of PGRs led to asynchronous cultures, making control of regeneration difficult. Recently, 2,4-D and BA were replaced by CPPU, a potent cytokinin, as the sole PGR. This was effective with mLV medium, but had no beneficial effect when used with DCR-based medium (Park *et al*. 2006).

Embryonal masses can be cultured in clumps on proliferation medium. However, in order to rapidly multiply the regenerative tissue, the plating method previously described by Klimaszewska and Smith (1997) for *P. strobus* (see *Somatic Embryogenesis of Pines: General Consideration*), has been adapted to maritime pine (Lelu *et al*. 1999). Comprehensive work has been carried out to describe EM morphology and cytology precisely, in relation to its proliferation and maturation capacity (Ramarosandratana *et al*. 2001a; Breton *et al*. 2005). Two different morphotypes of EM were defined as smooth and spiky, the spiky one being characterized by the presence of somatic embryos at late stages of early embryogeny protruding from the EM periphery (Ramarosandratana *et al*. 2001a). Embryogenic tissue with a spiky aspect provided the highest biomass production on DCR-based medium (Breton *et al*. 2005). However, EM growth was also found to be strongly affected by genotype (variation among cell lines) and subculture frequency (7 days promoted increased biomass compared with 14 days). During proliferation, microscopic changes of EM occurred, leading to a low occurrence of developed early stage somatic embryos concomitant with a gradual increase in growth rate. Growth of EM on proliferation medium and early somatic embryo development thus appeared disconnected. In order to preserve a spiky morphotype, i.e., to retain early embryogenic ability during proliferation, it was recommended that the pieces of EM be subcultured weekly for a short period (less than 6 months) on a maltose-containing medium without PGRs (Breton *et al*. 2005).

Somatic embryo maturation remains a critical step triggered by many factors, the first being the cultures themselves. The morphology of the EM is an indicator of the subsequent maturation capacity of the tissue. The spiky morphotype produced the best maturation yields, in terms of cotyledonary somatic embryos, with peripheral parts of EM pieces being more productive than inner parts (Ramarosandratana *et al*. 2001a). There was no relationship between biomass production, EM spatial (dish) and temporal (subline) subdivision during proliferation, and maturation ability (Breton *et al*. 2006). Similarly, no relationship could be

established between biomass production of EM lines on maturation medium and maturation performance (Ramarosandratana *et al*. 2001a). In close relation with progressive loss of early embryogenic ability of EM lines during proliferation on DCR-based medium, maturation yield was found to decrease as a function of subculture number, and was lost in less than 10 months (Breton *et al*. 2006). Therefore, the EM should be cryopreserved as soon as a sufficient amount is produced (see cryopreservation). An additional consequence of the aging effect (number of subcultures) was a decrease in the length of cotyledonary somatic embryos. Aging of EM affected not only the quantity but also the quality of somatic embryos regenerated (Breton *et al*. 2006).

Cotyledonary somatic embryo development varied among the lines (Ramarosandratana *et al*. 2001b). Eighteen of 20 open-pollinated seed families produced cotyledonary somatic embryos (representing 11% of 896 lines tested, Miguel *et al*. 2004). From controlled crosses, it is suggested that the embryogenic capacity of a line depended on both parent trees, and not solely on either parent tree alone. Embryogenic potential depended on the cross, as well as on the specific line (Lelu-Walter *et al*. 2006).

Cotyledonary somatic embryo development and maturation in maritime pine require at least 12 weeks (**Fig. 1A**). Cultures on DCR-based medium were transferred to fresh medium at 3- to 4-week intervals (Ramarosandratana *et al*. 2001b; Miguel *et al*. 2004). However, when mLV medium was used, recent work shows that there was no need to transfer cultures to fresh medium during the entire maturation period (Lelu-Walter *et al*. 2006). In general, maturation has been achieved using the EM plating method, as previously used for tissue proliferation (Lelu *et al*. 1999; Ramarosandratana *et al*. 2001b). There was no indication that the initial amount of EM (50-200 mg fm) plated on a filter-paper disk influenced somatic embryo production. However, because of the abundant proliferation of some lines on mLV maturation medium, it is recommended that 50-100 mg fm of EM be plated per filter-paper disk. Recently, the plating method has been modified by suspending EM in liquid culture medium with AC at 10 g l^{-1} and collecting the cell aggregates coated by AC particles on the filter paper, which then is placed on the maturation medium. Coating the cells with AC particles reduced EM proliferation and significantly enhanced maturation of maritime pine somatic embryos. The average number of cotyledonary somatic embryos produced was five times higher when EM was coated with AC than without (Lelu-Walter *et al*. 2006). Somatic embryos also developed faster in the presence of AC. The beneficial effect of AC may be attributed to its ability to adsorb residual PGRs (von Aderkas *et al*. 2002), but its mechanism of action remains complex.

Gellan gum has been used as the gelling agent in the embryo development and maturation medium at concentrations of 9-10 g l^{-1} (Lelu *et al*. 1999; Ramarosandratana *et al*. 2001b), as well as high ABA concentrations ranging from 80 µM (Ramarosandratana *et al*. 2001b; Lelu-Walter *et al*. 2006) to 120 µM (Miguel *et al*. 2004). Reducing the medium water potential by adding PEG to the culture medium at a 10% concentration (Miguel *et al*. 2004) also promoted somatic embryo development, however, its effect was in most cases not significant and appeared to vary among lines (Ramarosandratana *et al*. 2001b). The medium's water potential is more often reduced by increasing sucrose concentration from 3% to 6% (Ramarosandratana *et al*. 2001b; Lelu-Walter *et al*. 2006), with 6% being better than either 3% or 9%. Attempts to replace sucrose with maltose did not improve the embryo maturation yield.

In conclusion, based on the best results, in mature young embryogenic lines of maritime pine, the EM should be coated with AC particles, spread over filter paper placed on mLV medium supplemented with 0.2 M sucrose (6.8%), 80 µM ABA and gelled with 9 or 10 g l^{-1} of gellan gum for 12 weeks without any subculture. Among 18 tested lines representing eight full-sib seed families, the mean number of cotyledonary somatic embryos per g fm varied from 124 to 440, depending on the line (Lelu-Walter *et al*. 2006). However, in maritime pine, maturation remains a challenge mainly because of two major difficulties. First, despite the high ABA and gellan gum concentrations used, excessive proliferation of EM can interfere with embryo maturation. Second, the somatic embryo development remains asyn-

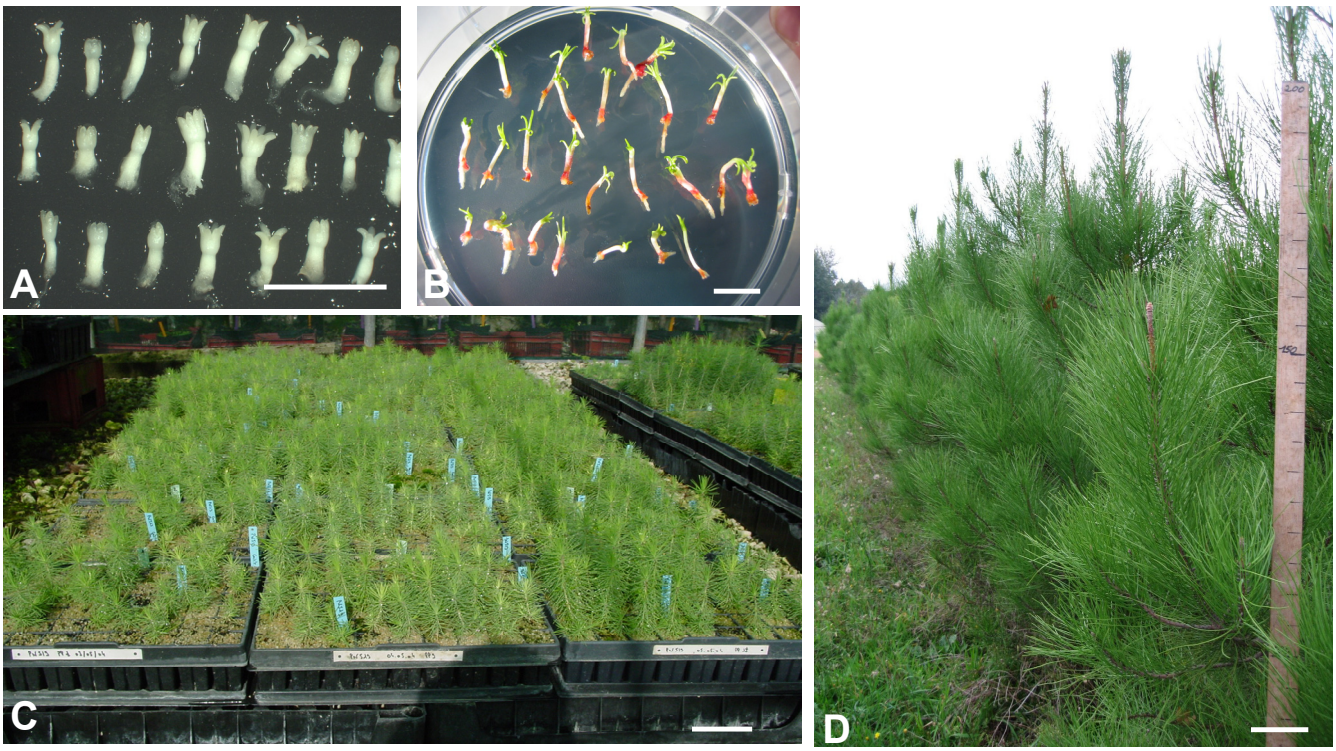

Fig. 1 ***Pinus pinaster*** **somatic embryo maturation, somatic plant regeneration, and field testing.** Cotyledonary somatic embryos after 12 weeks of maturation. Bar = 5 mm (**A**), early germination of cotyledonary somatic embryos. Bar = 1 cm (**B**), one clone acclimatized and grown in the greenhouse for 6 months. Bar = 10 cm (**C**), somatic plants in field test 2 years after planting. Bar = 10 cm. The ruler is 2 m in height (**D**).

chronous. After 12 weeks, the cultures are composed of cotyledonary somatic embryos as well as immature embryos. More research is needed to optimize the differentiation capacity of the cultures. It would be interesting to thoroughly investigate somatic vs. zygotic embryo development in physiological and proteomics studies in order to improve understanding of embryogenesis.

In a comparative study of storage reserves in somatic and zygotic embryos, it has been found that starch was predominant in mature somatic embryos, whereas zygotic embryos preferentially accumulated proteins and lipids (Jordy and Favre 2003). Recently, Perez-Rodriguez *et al*. (2006) studied the expression patterns of two glutamine synthetase genes (*GS1a*, *GS1b*) in zygotic and somatic embryos based on the importance of glutamine biosynthesis during the early stages of somatic embryogenesis, in particular during proliferation and maturation of somatic embryos (Bozhkov *et al.* 1993; Khlifi and Tremblay 1995; Joy *et al.* 1997). The expression of the cytosolic *GS1a* isoform was confined to green tissue and partly induced by light; it was barely detectable in EM cultured in dark. In contrast, the constitutively expressed *GS1b* isoform was highly expressed in EM. In the cotyledonary stage somatic embryos, the expression of *GS1b* was present in the vascular system similarly to zygotic embryos. However, *GS1b* transcripts were much more difficult to detect in cotyledonary and developing somatic embryos from EM with low regeneration ability compared with better performing lines. These results indicated deficient vascular development, particularly at the embryo root pole, even at the cotyledonary stage. The vascular pattern formation shown by *GS1b* analysis was thus correlated with the morphological characteristics of somatic embryos. Mature somatic embryos were also found to accumulate *GS1a* transcripts in the cotyledons, whereas *GS1a* expression remained undetectable in zygotic embryos. Accumulation of *GS1a* transcripts was found to correlate with nuclear genes (*rbcs* and *psbO*) encoding photosynthetic proteins and an arginase gene induced in the shoot pole of the seedling during germination. Thus, *GS1a* analysis suggested that germination was already initiated in maturing somatic embryos. In conclusion, both *GS1a* and *GS1b* could be useful molecular markers for screening somatic embryo conformity at both the photosynthetic activity and vascular formation levels.

Less work has been done on *P. pinaster* somatic embryo germination and conversion to plants. Perhaps this is because it is difficult to regenerate large quantities of high quality somatic embryos (i.e., that morphologically resemble zygotic embryos). Currently, somatic embryos are harvested from the maturation medium starting at 12 weeks, and are selected based on their morphology. Germination is carried out as described earlier (see *Somatic embryogenesis of pines: General Consideration*) and resulted in a high mean germination frequency (**Fig. 1B**), 78% from over 2000 cotyledonary somatic embryos (**Table 2**). However, there was considerable variation in germination among the lines (ranging from 61% to 96%). Aerial parts (i.e., epicotyl growth) developed after 6-7 weeks on germination medium, but conversion to plants remained more difficult to achieve than germination, with frequencies varying from 46% to 89% depending on the line (**Table 2**). The success rate of somatic embryos producing plantlets varied among crosses and among lines within a given cross. Because of this variability and the plant production requirements for clonal tests, the number of somatic embryos required to produce 1000 plants was estimated from 12 tested lines. The relative yield of embryos required varied from 1140 to 2330, depending on the line (Lelu-Walter *et al*. 2006).

Once the plants developed in Petri dishes, attempts were made to shorten *in vitro* culture duration to minimize plant deterioration. Plants grown on germination medium for 14-16 weeks in central France, in the Orléans region, have been transferred from Petri dishes directly to *ex vitro* conditions (Lelu-Walter *et al*. 2006). Trays containing potting mix have been placed in a shade house in mid-May and covered with a plastic sheet during the first 2-3 weeks to maintain high humidity. The average survival of acclimatized plants after 4 months was 65%, varying from 36% to 100% among lines (**Fig. 1C, Table 2**).

In conclusion, extensive work during the last 10 years has resulted in a much more efficient SE regeneration system in *P. pinaster*. Significant progress was made on initiation and proliferation of EM, and this is now considered to be optimized. Although advances have been obtained in embryo maturation, further studies are still required to synchronize and better understand somatic embryo development. The results obtained so far are promising, and SE will be progressively implemented in maritime pine breeding programs as data from field tests accumulate. Since 1993, a significant collection of embryogenic lines (over 1700) from a few elite families has already been cryopreserved by FCBA. Field tests of somatic trees have been established since 1999. About 100 cryopreserved clones originating from 25 elite families are currently under evaluation. After two growing seasons in the field, some clones are more than 2 m high (**Fig. 1D**), and display similar vigor and morphology as the seedlings. SE can reasonably be considered as an alternative tool in breeding programs to establish more informative clonal tests for improved individual selection and, ultimately, accelerate the deployment of new maritime pine varieties in MVF.

PINUS RADIATA

Somatic embryogenesis of *P. radiata* embryogenic tissue can be initiated from whole megagametophytes with enclosed immature embryos placed either on EM medium with AC without PGR or on EDM medium containing 4.5 µM 2,4-D and 2.7 µM BA (EDM6). The frequency of explants initiating SE from full-sib seed families varied from 0.3% to 16% over the entire 5-6 weeks of the cone collection period. Each seed family responded at different fre-

Table 2 *Pinus pinaster* somatic embryo germination and plant development (percentages in parentheses).

Parent trees		Line	No of somatic embryos			No of plants	
Mother	Father		Isolated	Germinated	Converted to a plant	Acclimatized	Survived[a]
A	D	PM2	183	151 *(82)*	98 *(53)*	50	31 *(62)*
A	E	PM16	123	113 *(92)*	97 *(79)*	56	44 *(78)*
B	D	PM3	337	230 *(68)*	165 *(49)*	60	28 *(47)*
B	D	PM4	114	95 *(83)*	78 *(68)*	30	11 *(37)*
B	E	PM10	65	60 *(92)*	49 *(75)*	27	14 *(52)*
B	E	PM18	90	59 *(65)*	41 *(46)*		
B	F	PM5	375	362 *(96)*	336 *(89)*	252	177 *(70)*
B	F	PM6	120	109 *(91)*	101 *(84)*	70	41 *(58)*
C	D	PM12	332	202 *(61)*	179 *(54)*	84	73 *(87)*
C	D	PM13	197	152 *(77)*	128 *(65)*	61	22 (*36)*
C	E	PM15	83	60 *(72)*	47 *(57)*	22	14 (*64)*
C	F	PM9	112	80 *(71)*	59 *(53)*	26	26 (*100)*
Total			2131	1673 *(78)*	1378 *(65)*	738	481 *(65)*

[a] Survival after 4 months in a shade house.

quencies, the best at 65% to 87%, and the worst at 0.3% to 14% (Smith *et al*. 1994; Walter and Grace 2000). However, Smith (1997) reported initiation of SE from all tested full-sib seed families at an average frequency of 30% to 35%. Radiata pine SE can also be initiated from excised zygotic embryos cultured on EDM6 medium (Walter *et al*. 2005). Alternatively, modified LV medium containing either sucrose or maltose can be used to initiate EM from cotyledonary zygotic embryos (Grace *et al*., unpublished). Cerda *et al*. (2002) used PR medium supplemented with 14 µM 2,4-D, 5 µM BA, 0.8% agar (Sigma), 500 mg l^{-1} L-glutamine, 400 mg l^{-1} L-arginine, and 400 mg l^{-1} L-asparagine, on which the immature zygotic embryos produced embryogenic tissue within 2-4 weeks.

Radiata pine EM can proliferate on various media: BLG1, EM, and EDM6 (Jones 1990; Smith 1996; Walter and Grace 2000; Walter *et al*. 2005), and PRPM (Cerda *et al*. 2002). The PRPM medium contains the following mixture of amino acids: 1000 mg l^{-1} L-glutamine, 522 mg l^{-1} L-arginine, 8 mg l^{-1} L-alanine, and 7 mg l^{-1} proline. Walter *et al*. (2005) reported that EM could be also proliferated in liquid BLG1 medium. In general, cultures maintained on BLG1 remain in a less differentiated state and maintain their somatic embryo maturation capacity for longer than those on EDM6. However, EDM6 is the medium of choice, as EM lines tend to remain healthier and tissue is easier to bulk up for maturation and cryopreservation purposes (Walter and Grace 2000). Recently, and for the first time, modified LV medium containing either sucrose or maltose has also been successfully used to proliferate *P. radiata* EM (Devillard *et al*., unpublished).

Maturation protocols applied to *P. radiata* include both culture of EM pieces directly on a medium or dispersal of EM in liquid medium followed by culture on filter paper. A sequence of two media is used during the maturation period: EMM1 for the first 10-14 days, and then the tissue pieces are divided and placed on EMM2 medium. The tissue pieces or filter papers with EM are subcultured onto the latter medium every 2-3 weeks until the mature somatic embryos develop. Usually after 4-6 weeks on EMM2, the first white cotyledonary somatic embryos are harvested, followed by multiple harvests during the next 4-6 weeks (Walter *et al*. 2005). Both EMM1 and EMM2 media contain 57 µM ABA and 3% sucrose, and vary only in the concentration of gellan gum (Gelrite™), which is added at 0.6% and 0.45%, respectively. Recently, a novel maturation technique, combining somatic embryogenesis and organogenesis to regenerate plants from somatic embryos, was tested (Devillard *et al*., unpublished). Isolated precotyledonary somatic embryos were placed on a sequence of organogenic media for plant regeneration. The development of somatic embryo meristem was induced on PGR-free medium, leading later to axillary budding. Alternatively, adventitious buds regenerated from meristematic tissue formed on precotyledonary somatic embryos maintained on organogenic medium containing 5 mg l^{-1} BA. These preliminary results showed that it was possible to regenerate buds using both axillary and adventitious shoots. However, further experiments to elucidate the incidence of hyperhydricity and poor shoot elongation need to be undertaken. This approach may enable the regeneration of plants from EM that are recalcitrant to maturation, or that are poor embryo producers.

To synchronize and accelerate root emergence, mature somatic embryos are partially desiccated on a nylon membrane, which is placed in each of the three wells of a six-well Falcon Multiwell® dish (the remaining wells are filled with water) and stored sealed at 5°C for at least 7 days. Subsequently, germination involves two subcultures of the somatic embryos onto germination medium (Walter *et al*. 2005). More recently, a less time-consuming and more cost-effective germination method has been developed that avoids the use of nylon membrane and requires only one transfer to the germination medium instead of two (Devillard, unpublished). When the somatic embryos have germinated and developed epicotyls, they can be transferred to potting mix according to the procedure described by Walter *et al*. (2005).

Similarly to other pine species, radiata pine EM capable of plant production produce both normal and abnormal cotyledonary-stage somatic embryos on maturation medium. Minocha *et al*. (1999) reported that mature cotyledonary-stage somatic embryos capable of germination and conversion to plants could be distinguished by their higher spermidine:putrescine ratios compared with the abnormal cotyledonary-stage somatic embryos, which were incapable of regenerating plants.

Several thousand somatic seedlings have been produced for small field trials, on a number of sites, since 1992. Field trials set up since 1995 were done with somatic seedlings of higher quality because of improved germination techniques and handling of plants in the nursery, and some clones have been performing as well as seedlings and rooted cuttings (Walter and Grace 2000).

PINUS STROBUS

Initiation of SE in *P. strobus* was first described by Finer *et al*. (1989, **Table 1**) but no plants were regenerated from those cultures. Later studies were devoted to assessing and improving SE initiation frequencies in a large number of seed families, and subsequently, to the development of protocols for somatic embryo maturation and production of plants (Klimaszewska and Smith 1997; Garin *et al*. 1998; Klimaszewska *et al*. 2000, 2001, 2004; Park *et al*. 2006) (**Fig. 2**).

Medium formulations that have been used for all stages of SE were MSG and mLV. However, most protocol optimization was done with mLV medium containing 0.5 g l^{-1} L-glutamine and 1.0 g l^{-1} casein hydrolysate. Sucrose is added to the medium at 2% and gellan gum at 0.4% for both initiation of SE and proliferation of EM. It has been determined that 2,4-D and BA each at 2.2 µM (designated L-PGR) are more beneficial for SE than the standard 9.5 µM 2,4-D and 4.5 µM BA (S-PGR) or 1.1 µM of each (UL-PGR). On the L-PGR medium, the mean over five open-pollinated seed families of established lines was 49% and over 14 open-pollinated seed families was 60% when the zygotic embryos were cultured between the pre- to just post-cleavage stages compared with 21% on S-PGR. In the earlier study screening 13 open-pollinated seed families on a medium with S-PGR, approximately 12% of explants initiated SE (Garin *et al*. 1998). Embryonal mass lines were also established from embryos at the later stages of development, but at lower frequencies. For example, for embryos cultured at the cotyledonary stages, the mean over five seed families was 17% and over 14 families was 8% (Klimaszewska *et al*. 2001). Later studies, testing a number of medium modifications (including CPPU), confirmed that culture of explants on mLV with L-PGR resulted in the highest SE occurrence, an average of 44% over 20 full-sib seed families (Park *et al*. 2006).

The first zygotic-like development of somatic embryos was achieved by Klimaszewska and Smith (1997) by employing a high gel strength maturation medium, in which the concentration of either agar or gellan gum exceeded the one used for other SE stages and with a relatively high concentration of ABA (80 to 120 µM). The most productive culture technique is when the EM is spread over a filter-paper disk (**Fig. 2B**). Medium (mLV) that supported the development of large numbers of somatic embryos and reduced undesirable proliferation of EM had a gel strength between 800 and 1050 g cm^{-2}, which was achieved by using 0.8% and 1.0% gellan gum (Phytagel™) or approximately 2.0% and 2.5% agar (Difco-Bacto), respectively (Klimaszewska *et al*. 2000). Gellan gum at concentrations from 0.4% to 1.0% did not change the medium water potential (Ψ), which remained between -0.43 and -0.44 MPa (in the presence of 3% sucrose). However, gelling agent concentration had a pronounced effect on the amount of water or

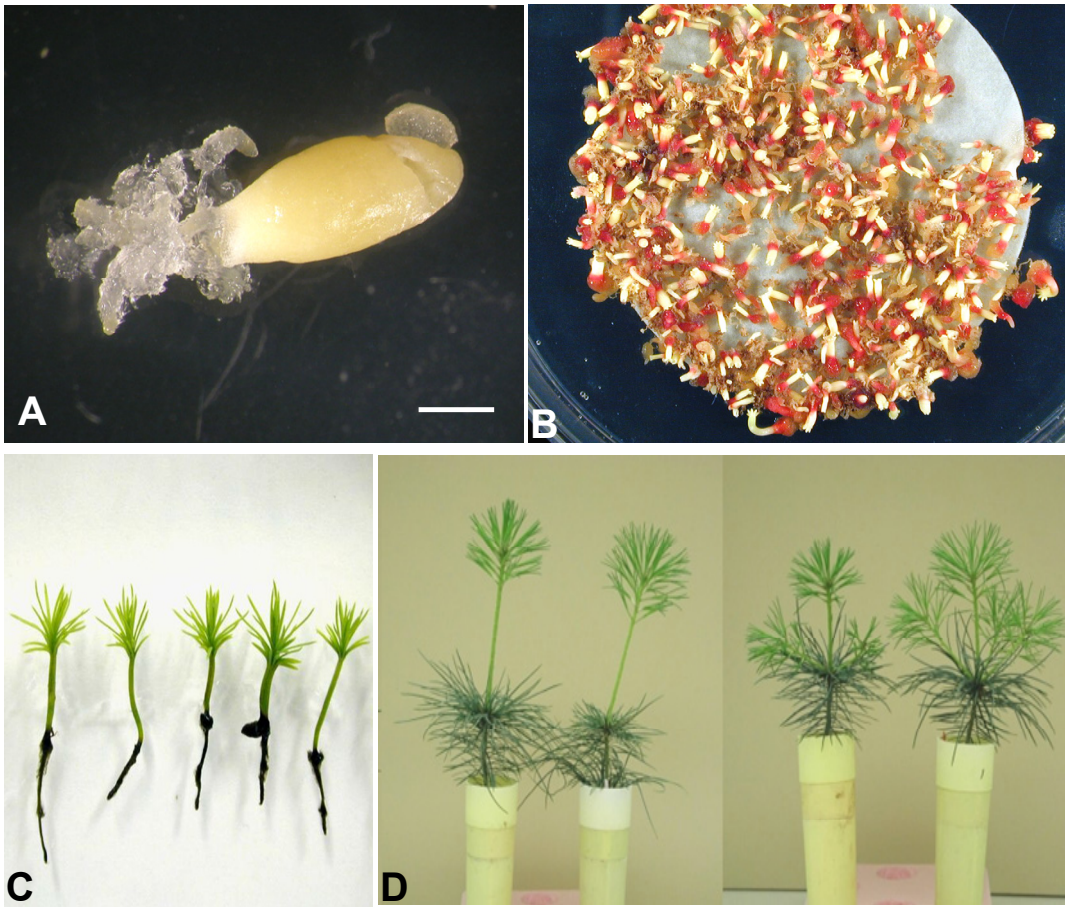

Fig. 2 Clonal plant production in *Pinus strobus* via SE. SE initiation from an immature megagametophyte explant. Bar = 1.3 mm (**A**), mature somatic embryos after 10 weeks of culture. Bar = 1 cm (**B**), three month-old somatic plants *in vitro*. Bar = 1.6 cm (**C**), clonal fidelity of regenerated plants from two different lines in greenhouse containers. Note the difference between clones and identical branching pattern within a clone. Bar = 4.7 cm (**D**).

liquid absorbed by filter papers, and therefore, on the availability of water or liquid to the EM cultured in a layer spread over its surface. Measurement of the hydration state of filter papers equilibrated on the medium surface revealed a linear decrease in the amount of water with increasing concentrations of gelling agent. As expected, and based on the gel strength data at any fixed concentration, filter papers set on gellan gum medium absorbed less water than those on agar. An obvious conclusion from this study was that water availability plays an important role in the maturation of somatic embryos, which is not surprising as the same applies to *in vivo* seed development and maturation (reviewed by Bradford 1994). Furthermore, mature somatic embryos developed on either 0.8% or 1.0% gellan gum had lower Ψ than those from lower concentrations. These embryos developed over 8-10 weeks and were characterized by high conversion to plants without prior post-maturation treatments. In a subsequent study aimed at improving somatic embryo quality, it was found that sucrose added to the medium at 6% (instead of 3%) increased storage protein accumulation in mature somatic embryos, perhaps because the Ψ of the maturation medium was lowered from -0.44 to -0.61 MPa (Klimaszewska *et al*. 2004). Similarly, reduction in Ψ of somatic embryos was also detected without any deleterious effect on plant regeneration.

Accumulation of the most abundant storage proteins in mature somatic embryos – the buffer-insoluble 11S-globulins MW 59.6 kDa and buffer soluble 7S vicilin-like proteins MW 46.0-49.0 kDa – depended on the maturation medium and the time in culture (Klimaszewska *et al*. 2004). The highest accumulation was detected in mature somatic embryos that were cultured for 9 weeks on a medium with 6% sucrose, solidified with 1.0% gellan gum. Extension of the maturation to 12 weeks on media with both 3% and 6% sucrose resulted in reduced amounts of proteins. Nonetheless, the somatic embryos matured under the best of tested conditions and compared with the mature zygotic embryos had slightly higher water content, and overall, accumulated approximately 50% less storage proteins. The identity of both types of proteins in somatic embryos matched those in zygotic embryos, which were confirmed by amino acid sequencing and tandem mass spectrometry (MS/MS).

Culture of EM on L-PGR medium was also beneficial for subsequent mature somatic embryo yield, which was nearly double compared with the yield on medium with standard PGR concentration (123 vs. 76 somatic embryos g fm^{-1} EM, respectively) in the five seed families. Of 152 lines cultured, 145 produced somatic embryos, and these converted to plants at frequencies from 70% to 82% after approximately 10 weeks. The medium for somatic embryo germination and conversion to plants is mLV with 2% sucrose and 0.6% gellan gum. Approximately 5000 plants were transferred to the potting mix once they showed shoot growth and were placed in a greenhouse (**Fig. 2D**). A subset of these plants (2500) representing 210 genotypes was planted in the field (Park, unpublished).

In conclusion, SE in *P. strobus* is sufficiently effective to be used in clonal propagation of elite germplasm. Presently, a study has been launched to determine if somatic seedlings derived from backcrossed seeds of a hybrid between *P. wallichiana* × *P. strobus* and *P. strobus* could be used for white pine blister rust (*Cronartium ribicola*) challenging tests. The hybrids were created with the expectation of transferring white pine blister rust tolerance from *P. wallichiana* to *P. strobus* (Lu *et al*. 2005).

PINUS TAEDA

The most frequently used culture medium formulations for *P. taeda* SE include DCR, MSG, modified P6, and WV5. Initiation of SE has been improved by supplementing these media with several addenda, including ABA (Handley 1997), brassinolide (Pullman *et al*. 2003c), ABA, silver nitrate, and cytokinin adjustments (Pullman *et al*. 2003b), modifying culture pH and addition of biotin and folic acid (Pullman *et al*. 2005a), gibberellin inhibitors (Pullman *et al*. 2005b), adjusting 2,4-D and AC concentrations (Toering and Pullman 2005), and addition of organic acids and vitamins B12 and E (Pullman *et al*. 2006). van Winkle and Pullman (2005) achieved desired PGR levels in liquid culture media for *P. taeda* SE by including AC. Pullman and Skryabina (2007) used liquid medium and liquid overlays to improve SE initiation in *P. taeda*.

Uddin (1993) first reported that embryo development medium containing maltose enhanced somatic embryo matu-ration in *P. taeda*. Subsequent reports have verified the importance of maltose and the following factors for enhanced somatic embryo production in *P. taeda*: (1) maltose as a carbohydrate (sugar) either alone or in combination with glucose in the tissue proliferation medium before embryo development (Gupta 1996), (2) PEG as an osmotic agent in combination with ABA (Gupta and Pullman 1991; Li *et al*. 1998; Rutter *et al*. 1998; Pullman *et al*. 2003b), and (3) using ABA at very high concentrations compared with levels used with other conifers, in combination with AC (Handley 1998). Maltose and PEG together significantly enhanced embryo maturation frequency and germination capacity of *P. taeda* somatic embryos compared with a medium with only maltose, or with sucrose combined with PEG (Li *et al*. 1998; Pullman *et al*. 2003c). Desiccation-tolerant somatic embryos of *P. taeda* were produced on an embryo development medium containing ABA and PEG (Tang 2000a, 2000b).

Several approaches, including the following, have been taken to improve *P. taeda* somatic embryo quality. Somatic embryos having elevated levels of certain oligosaccharides, sucrose, raffinose, and stachyose have been shown to have improved germination (Carpenter *et al*. 2000). Sugar alcohols also show potential as markers of embryo quality (Carpenter and Koester 2000). Pullman and Buchanan (2006) studied the accumulation of organic acids in developing seeds of *P. taeda*. The major organic acids contributing to osmotic potential were malic acid early in seed development and oxalic acid late in seed development. This finding may be useful for modifying the embryo development medium and may yield higher quality somatic embryos. Recent publication on patterns of storage protein and triacylglycerol (TAG) accumulation in developing somatic embryos showed that, compared with zygotic embryos, the former had lower levels of TAG but higher levels of storage proteins (Brownfield *et al*. 2007). Mature zygotic embryos had about a 3:2 ratio of soluble protein whereas somatic embryos had five times the soluble protein compared with the amount of insoluble protein. This indicates possible differences in metabolic activity at the time of seed desiccation.

Cairney *et al*. (2006) generated Expressed Sequence Tags (ESTs) from zygotic and somatic embryos of *P. taeda* to better understand genes that control embryo development. Vales *et al*. (2007) studied ABA-responsive gene expression in somatic and zygotic embryos to improve somatic embryo development, maturation, and embryo quality.

Different light quality treatments were applied to *P. taeda* somatic embryos during the pre-germination and germination steps, using cool white fluorescent bulbs or light-emitting diodes (LEDs), or both (Merkle *et al*. 2005). In general, red wavelengths provided by LEDs during these steps resulted in higher frequencies of somatic embryo germination (up to 64%) and conversion (up to 50%), longer tap roots, and more first-order lateral roots than the standard cool white fluorescent treatments or treatment with blue wavelengths from LEDs. In addition, exposure to red light allowed germination of somatic embryos of some clones that failed to produce germinants under fluorescent light.

In summary, SE in *P. taeda* has advanced significantly since its first report in 1987. Coupled with cryogenic storage, SE overcomes many of the difficulties associated with other clonal propagation methods, and provides a robust clonal propagation system. Improvements in embryo quality and obtaining initiation from mature explants are technology advances that are on the horizon and will make SE in *P. taeda* and other *Pinus* species an even more powerful technology.

SOMATIC EMBRYOGENESIS APPLICATIONS IN PINE IMPROVEMENT AND DEPLOYMENT STRATEGIES IN MULTIVARIETAL FORESTRY

Multivarietal forestry

The most important application of SE is in implementing MVF, which is defined as the deployment of tested tree varieties in plantation forestry. It is also known as clonal forestry but, with advances in conifer SE, MVF is considered to be a more descriptive term when applied to commercial plantation forestry (Park 2004). In general, a clone refers to a genotype with its genetic copies or ramets, whereas a variety refers to a clone that has been selectively bred for certain attributes as in an agricultural variety. The use of MVF offers many advantages (Libby and Rauter 1984; Carson 1986; Libby 1990; Park *et al*. 1998c), including: (1) Obtaining much greater genetic gain than conventional tree breeding based on seed orchards; (2) Flexibility to rapidly deploy suitable varieties with changing breeding goals and environments; and (3) Ability to design and balance genetic gain and diversity in plantations. However, MVF has rarely been practiced with conifers, particularly pines, because of the general lack of an efficient vegetative propagation system that can produce tested genotypes consistently over time. Owing to recent achievements, the commercial potential for SE has been explored (Adams *et al*. 1994) and the deployment of trees produced by SE has begun (Parke *et al*. 2002).

The first requirement for MVF is the availability of an efficient vegetative propagation system such as SE and a method for long-term storage (cryopreservation). This provides an opportunity to produce genetically tested identical genotypes consistently over time, which is analogous to the production of agricultural varieties. The second requirement for MVF is the development of high-value tree varieties. Development of SE varieties is usually closely coordinated with multi-generation tree breeding programs, which generally adapt a form of recurrent selection and manage breeding populations for the next generation. In many well-established programs, the "nucleus" breeding strategy is a hierarchical structuring of the breeding population, where the top tier contains the elite (nucleus) parents. This strategy is adapted to obtain fast delivery of genetic gain from the elite crosses (Coterill *et al*. 1989). Therefore, it is best to start varietal development with the nucleus population using a system of controlled crosses. However, owing to the recent development of highly informative molecular marker technology, such as micro-satellites, pedigrees of progenies resulting from unstructured matings (e.g., open-pollination, polycross, and supplementary mass pollination) can be reconstructed (El-Kassaby *et al*. 2006). This technique will simplify execution of the breeding strategy for MVF and be more efficient by removing the need for laborious controlled pollination.

Park and El-Kassaby (2006) proposed a strategy for MVF using SE and molecular markers. The strategy is illustrated in **Fig. 3**. Following the numbered boxes in the figure, the MVF strategy begins with selection of parents from a long-term breeding program, e.g., a nucleus breeding population or elite subline, where 10-15 parents are usually included. If the program is new, it can be started with phenotypic selection from wild stands (**Fig. 3-1**). In typical tree

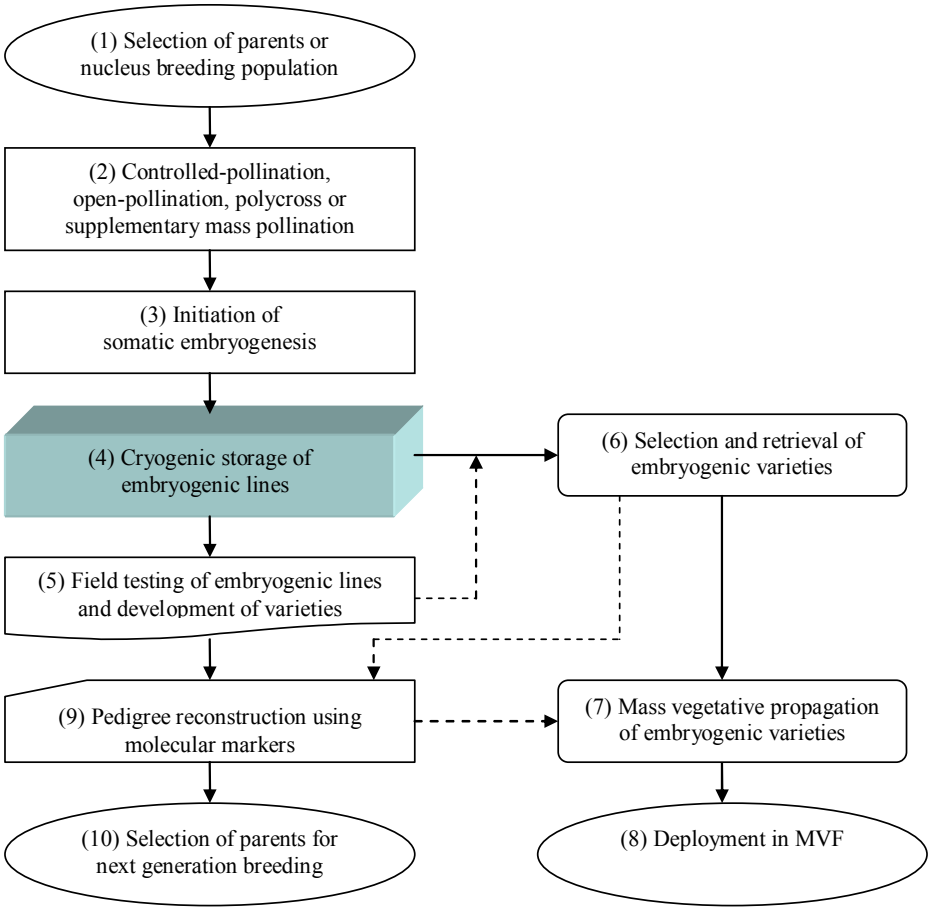

Fig. 3 MVF strategy using SE and molecular markers. Solid lines indicate material flow and dotted lines indicate information flow.

breeding programs, controlled crosses among the selected elite parents will be performed to produce offspring; however, if a breeder wants to use pedigree reconstruction using molecular markers, open-pollination, supplementary mass pollination, or other mating schemes can be used to circumvent controlled pollination (**Fig. 3-2**). The resulting seeds from the selected parents are then subjected to SE initiation to develop SE lines (**Fig. 3-3**). Once embryogenic lines are developed and proliferated, they are stored in liquid nitrogen (**Fig. 3-4**). Field testing is the most important part of MVF and is essentially the process for identifying tree varieties for deployment. Field tests are established using the embryogenic lines retrieved from cryogenic storage, and the tests are evaluated periodically to identify suitable tree varieties (**Fig. 3-5**). Once field testing has identified the suitable varieties, they are retrieved from cryogenic storage (**Fig. 3-6**), mass propagated (**Fig. 3-7**), and deployed in MVF (**Fig. 3-8**). The embryogenic lines growing in field tests, including the selected varieties, can be genotyped using molecular markers, e.g., informative micro-satellite markers, to reconstruct the pedigree or mating matrix (El-Kassaby *et al*. 2006). This genotyping data will also be used to control coancestry for the multi-varietal mixes for deployment (**Fig. 3-9**). Using the field test data combined with the reconstructed pedigree, parents for the next generation breeding population will be recruited. At this time, infusion of elite parents from long-term breeding program can also be incorporated (**Fig. 3-10**).

Technical requirements of somatic embryogenesis

SE and cryopreservation are the two key technologies that make the implementation of MVF possible. However, there are two basic requirements for the technology. First, initiation of SE and plant conversion rates must be sufficiently high. This is important to obtain the high levels of genetic gain for various attributes when incorporating them into variety development and to maintain genetic diversity in plantations. The second requirement of SE for MVF is that the cryopreserved embryogenic varieties must be genetically stable. Data on the stability of cryopreserved pines are scarce, but Park *et al*. (1998a) demonstrated that embryogenic lines of *Picea glauca* were generally stable when examined for *in vitro* and *ex vitro* characteristics. Trees regenerated from normally maturing or germinating somatic embryos after cryopreservation appeared to maintain genetic integrity; however, somatic embryos identified as variant phenotypes at either the maturation or germination stage exhibited an altered RAPD fragment pattern (De Verno *et al*. 1999). In operational situations, such variant embryos could be selected against and discarded before deployment.

Phenotypic and genotypic alterations could occur during prolonged *in vitro* culture of conifer somatic embryogenic tissue (De Verno 1995; Isabel *et al*. 1996; Fourré *et al*. 1997). In *P. sylvestris* the comparison of stability of four variable nuclear microsatellite loci in embryogenic cultures and zygotic embryos showed significant difference among families (Burg *et al*. 2007). In six out of 10 families analysed, the level of genetic stability was similar between somatic and zygotic embryos. The four remaining families showed higher mutation rate during SE. However, it has not been assessed if the studied microsatellite loci reflected alteration in functional genes.

Cryopreservation can maintain juvenility and minimize undesirable genetic change caused by prolonged subculture because ultra-low temperatures stop cellular metabolic functions (Kartha 1985). Therefore, it is prudent to use cryogenic storage as a means of minimizing any potential genetic change. As noted in the flow chart (**Fig. 3-4**), embryogenic lines will be cryopreserved first and then subjected to field testing on the thawed embryogenic lines. This is because, if an embryogenic line cannot be cryopreserved, it will not be useful for MVF. Also, any change that might occur during the initial cryogenic storage will be irrelevant to MVF as the MVF program will use post- cryopreserved material only.

As discussed previously, an embryogenic line of a pine species may contain more than one genotype. Various

molecular marker technologies can be used to examine and monitor genetic stability and integrity of embryogenic varieties.

Field testing

Field testing in tree breeding is a well-established discipline and is equally relevant to varietal testing. An additional aspect of field testing SE-derived trees is to determine genetic fidelity of trees relative to seedlings. To date, no indication of any abnormality has been reported. The genotypes to be tested are usually derived from some form of mating scheme on the selected parents, and the field testing, in general, has two main objectives: (1) to identify suitable varieties for deployment, namely the production population, and (2) to select parents for generation advancement, thus forming next generation breeding population. Normally, depending on the objectives pursued, a breeder has to make a compromise over which mating design to employ because no single mating design can meet both objectives effectively.

The first goal is essentially the process of identifying tree varieties and is the key element of MVF. For this purpose, a number of candidate embryogenic lines are evaluated over a range of environments with respect to traits of interest such as growth, wood quality, and pest resistance. The tests are evaluated at regular intervals throughout the rotation age and beyond. The tests will provide the most current information for varietal deployment for MVF, thus offering the flexibility of adjusting the composition of varietal mixtures.

Testing a large number of embryogenic lines will result in larger genetic gain, but field testing is often constrained by limitations in resources and logistics. Therefore, it is necessary to accept some trade-offs so testing will be manageable in size. In general, the production population (embryogenic lines) will be generated from a nucleus population (NP) or a group of elite parents ranging from 10 to 15 individuals through controlled pollination independent of genetic testing. For example, 10 elite trees in a NP may generate up to 45 full-sib crosses using a half-diallel mating scheme. In eastern Canada, a test area of about one hectare per site is considered manageable, and is suitable for testing about 2500 trees at 2 × 2 m spacing. Using this guideline, about 250 to 300 embryogenic lines can be tested at each site using 10 clonal replicates. However, a breeder has considerable latitude to plan a test design depending on the particular situation.

Differential SE success rate has an impact on capturing potential genetic gain through MVF; however, a simulation study demonstrated the embryogenic propensity among families has no significant impact on gain (Lstibůrek *et al*. 2006). They also indicated that, even though there is reduced variation among families because of differential success in SE, the variation within family is unchanged. Thus, it is reasonable to expect much of the genetic gain will be derived from variation within families using fewer families that produce a high rate of SE. Thus, in a practical tree breeding situation, one would generate more embryogenic lines within a family than using more families with a smaller number of individuals within a family. However, balancing is necessary to obtain optimal gains from both among- and within-family selection.

The use of pedigree reconstruction techniques based on molecular markers offers a new dimension to streamline the MVF strategy, primarily by circumventing the need to perform laborious controlled mating (El-Kassaby *et al*. 2006; Park *et al*. 2006). For example, open-pollinated or mass-pollinated seeds collected from each elite parent in the NP are normally considered half-sibs, but they contain full-sibs. These full-sib relationships within maternal parents will be identified by genotyping using molecular markers. This would result in the reconstructed pedigree or mating matrix without performing controlled crossing. The reconstructed pedigree will allow for estimation of genetic parameters necessary for selecting parents for the next generation breeding population and predicting genetic gain. This information will also be used in controlling co-ancestry in both breeding and deployment populations.

Deployment considerations

The diversity of multi-varietal plantations is a concern because of the perception that a narrow genetic base may result in MVF plantations being more vulnerable to diseases and insects than seedling plantations, and may result in plantation failure. For known diseases and insects, MVF has an advantage because resistant varieties may be developed in combination with improved economic traits. But, for unknown or introduced diseases and insects, protection is limited despite the high degree of genetic variability existing among forest trees. It is difficult, if not impossible, to design a protection scheme against unknown pests. However, it is generally assumed that, the more varieties deployed in a plantation, the lower the risk. The use of an increased number of varieties in a plantation will reduce genetic gain, so it will be necessary to balance genetic gain and diversity, leading to the question of what is an appropriate number of varieties in a MVF plantation (Libby 1982). This is a difficult question; however, using various approaches, scientists generally agree that 10-30 varieties mixed in a plantation should be sufficient for protection and still yield the benefits of MVF (Hühn 1987; Libby 1982; Zobel 1993; Roberds and Bisher 1997). Lindgren (1993) suggested some basic considerations for determining an appropriate number of varieties in a plantation: (1) if the species being deployed is short lived or of short rotation, a lower number of varieties may be used because the exposure to potential risk is short; (2) a lower number of varieties may be acceptable if plantation management is intensive and includes pest management; and (3) the more well-known a variety, the more acceptable is its extensive use. In addition, once an appropriate number of varieties is decided, the MVF strategy must consider the configuration of the deployed varieties, either as a random mixture or in varietal blocks (Libby 1982). In general, a random mixture is appropriate when varieties are not well known or future pest situations are uncertain (Lindgren 1993).

An approach called "desired gain and diversity" is used in eastern Canada for the multivarietal deployment of spruce species. In this approach, the number of varietal lines is decided based on the desirable or predetermined level of genetic gain and diversity using the best available field test data. For example, it is likely that a large number of varieties may be included in the multivarietal mixture at an early stage of the field test; however, when the test is mature or varietal characterization is sufficient, a smaller number may be used. Therefore, determining an appropriate number of varieties is a dynamic process based on best available field test data, co-ancestry, juvenile and mature correlations, etc. This strategy can also be combined with other proposed strategies such as a mixture of varieties and seedlings, which takes commercial thinning into consideration (Park *et al*. 1998a). Therefore, diversity of plantations is dynamically managed spatially and over time, where selection of varieties will be continuously revised based on field test data throughout the rotation age and as new varieties are introduced at each breeding cycle, resulting in different compositions of varietal mixtures.

Industrial production

Industrial production and commercial deployment of embryogenic varieties in conifers is at an early stage. CellFor Inc. (Canada) is a leader for commercial production of SE-derived conifers. The company sold six million SE-derived loblolly pine seedlings to the customers in southern USA in 2006, and expects to produce 27 million seedlings in 2007 (www.cellfor.com). Arborgen (USA) is also known to produce a significant number of SE-derived loblolly pine seed-

lings for its affiliated companies in the southeastern USA, but its rate of commercial production is not known. Other private sector companies and organizations active in this area include FCBA (France), GenFor (Chile), Horizon2 (New Zealand), and J.D. Irving, Ltd. (Canada); however, at this time, the annual production is estimated at about one million trees, whereas several companies are at a field test establishment stage.

Most conifer SE application has been developed using Petri dish-based systems and *in vitro* germination. Although these approaches are suitable for establishing varietal field tests and small-scale production, they are viewed as laborious for commercial application, raising the cost of production. Improving the efficiency of SE as a mass propagation system is an important aspect of commercialization. Promising developments in the automation of the SE process include liquid maintenance culture, bioreactor maturation, and embryo purification and desiccation. Liquid culture methods, which facilitate rapid bulk-up, uniformity, and improved embryo yield, have been developed for several conifers (Timmis 1998; Cyr *et al*. 2001). However, further development in automating the SE process for commercial production is needed. Despite the lack of an automated process, the current cost of SE-derived trees is equivalent to that of rooting-of-cuttings production in eastern Canada.

The SE systems for pine species reviewed here are sufficiently refined to implement MVF. However, at this time, there is a shortage of sufficiently field-tested SE varieties to deploy. Therefore, well-designed field tests must be established by a collaboration of tissue culturists, and tree improvement and nursery specialists. As indicated previously, field testing is a key element in developing value-added varieties and deployment strategies. The current productivity and quality improvements through MVF have been obtained by careful exploitation of existing natural genetic variability. However, MVF is likely to be the delivery mechanism for tree biotechnology products in the future.

CONCLUDING REMARKS

Conifer SE is the first biotechnology to be applied in tree improvement (preferably within MVF), and has opened an array of new commercial opportunities for the forest industry. These opportunities are being captured by various companies worldwide, both by forming partnerships with public or other private research organizations and by starting in-house programs. There is no doubt that research investments in this area were needed and were very productive, not only for the development of "workable" SE protocols for deployment of new pine varieties maximizing genetic gain but also for the advancement of our knowledge in conifer embryology. Conifer SE is a highly controllable process with distinct developmental stages that enables many fundamental research questions to be addressed more easily. It can deliver ample amounts of experimental material, thus providing a valuable research tool. Developing somatic embryos have been used to study temporal and spatial molecular events taking place from the early stages to maturity. Furthermore, SE offers an opportunity to elucidate certain physiological and molecular processes involved in wood formation and disease resistance through genetic transformation, which has been optimized in the last decade (see review Trontin *et al*. 2007). Genetic transformation of EM and regeneration of large numbers of transgenic plants are also greatly benefiting the ongoing functional genomics research in pine species.

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