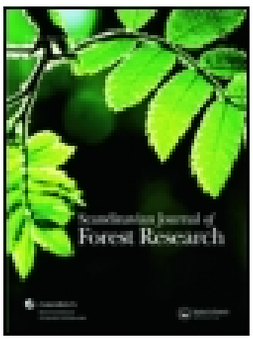

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Plant physiological and genetical aspects of the somatic embryogenesis process in conifers

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ABSTRACT

The processes for producing conifer planting stock by somatic embryogenesis (SE) in conifers are described. Implementation of SE presents opportunities and limitations at various stages of the *in vitro* process. The topic of genetic stability, or somaclonal variation, is a particular concern and reviewed. Following the *in vitro* processes, several factors affect the successful acclimation, early growth and field performance of SE planting stock. Experiences with other conifer species in the context of commercial production are reviewed. While SE production has historically been a very labor-intensive process, recent advances have been made to automate the various steps. Developments to enable SE for Norway spruce in Sweden are described.

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Somatic embryogenesis; Norway spruce; conifers; SE technology

Practical processes for somatic embryogenesis in conifers

Somatic embryogenesis (SE) is a process where (somatic) embryos develop from somatic cells. In conifer species, induction of the process in the laboratory works effectively only for somatic cells within the zygotic seed embryo. Once the first somatic embryos have been formed, they are capable of going through the same developmental process as their zygotic counterparts, progressing through early embryo development, maturation, germination and plant formation.

When the SE process is utilized for commercial production of plants for forest regeneration, the practical steps are to isolate zygotic embryos from the seeds under sterile conditions, and place them on a culture medium containing plant growth regulators (PGRs, typically cytokinin and auxin) that induce the formation of early-stage somatic embryos. These early-stage somatic embryos then continue to multiply, stimulated by the same PGRs, and will form masses of early-stage embryos, so-called pro-embryogenic masses, or PEMs. When the PEMs are removed from the medium containing the initial PGRs and subjected to abscisic acid, the multiplication process stops and the early-stage embryos of the PEMs continue development into mature somatic embryos that subsequently, given the right culture conditions, will germinate and form plants.

The complete SE process starting with initiation of an initial early-stage somatic embryos from a seed embryo until the somatic embryo has germinated is carried out under sterile conditions (*in vitro*) (Figure 1). Furthermore, PEMs cultures can be pre-treated for long-term storage at very low temperature (cryostorage). This is useful for field testing of different cell lines (e.g. from elite seeds in a breeding program) that can be cryostored as PEMs while somatic seedlings from these lines develop in the tests. Once results from the field trials are available and the best clones can be selected, cryopreserved PEMs cultures can be thawed and embryos multiplied from the stored cultures.

An additional and perhaps even more attractive use for SE plant production technologies is to directly generate SE plants from seeds of control-pollinated families without first testing the cell lines, i.e. family forestry with vegetative multiplication. With this approach, it is possible to generate unlimited numbers of plants from the same family, compared to the otherwise typically limited numbers of plants that can be derived from a control-pollinated seed lot. Such an approach can be beneficial both to the forestry industry by enabling production of a larger numbers of plants from elite crosses and to capture the values from the breeding programs at an earlier stage without waiting for often lengthy clone testing periods. SE also offers the possibility to produce plants from valuable interspecific hybrid crosses that generate few seeds (e.g. *Pinus elliottii* var. *elliottii* × *P. caribaea* var. *hondurensis*; Nunes et al. 2018, *Abies alba x A. cephalonica and Abies alba x A. numidica;* Salajova et al. 1996*)*. The SE technology can also be used in conservation programs to multiply scarce seeds from endangered hard-to-multiply species (e.g. *Chamaecyparis thyoides*; Ahn et al. 2017).

Application of SE: possibilities and limitations during the *in vitro* process

After a PEM culture has been initiated from a seed embryo, somatic embryos within the PEM culture are multiplied to sufficient numbers to obtain the required yield of plants at the

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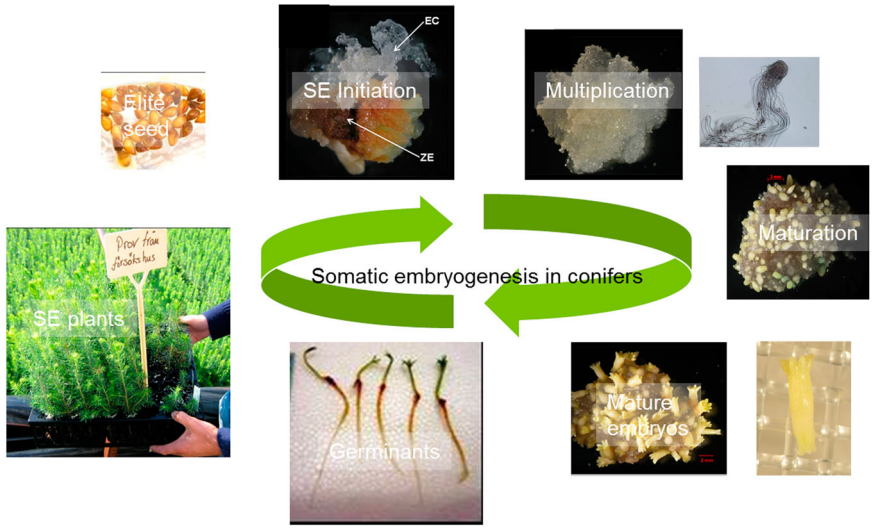

Figure 1. SE process in conifers. The SE process starts with initiation of early-stage somatic embryos from a zygotic seed embryo. Early-stage somatic embryos multiply indefinitely when kept on culture medium with the right composition and form cultures of PEMs. Embryo maturation starts after transfer of PEMs to a maturation medium, where mature embryos form from the early-stage somatic embryos of the PEMs. Germinated embryos with root and shoot development can be planted in compost and transferred to *ex vitro* conditions for acclimatization and plant growth. Shown photos are published with permission from SweTree Technologies AB.

end of the SE process. The target mass of PEMs depends on yields in the subsequent steps of the SE process, up until an SE plant is delivered for planting in the field. PEM cultures, or mature somatic embryos, can be stored under cryogenic conditions for prolonged time allowing for clonal field testings whilst SE material for propagation is under safe long-term storage. Yields for different steps vary among cell lines, as discussed in subsequent sections. To date, there are large differences in how well currently available SE laboratory methods work with different conifer species and different cell lines (genotypes, clones). Spruce species (*Picea* ssp.) generally respond well to initiation treatments and most responsive seeds give sufficient yields in all SE process steps up to the plant stage, whereas pine species (*Pinus* ssp.) overall give low yields in all of the SE process steps. This "recalcitrance" (inability to respond to the applied culture method) in pines presents a challenge for production of SE plants for large-scale plantings. For pines amenable to rooted cutting propagation like radiata pine (*Pinus radiata*), large-scale commercial plant production can be obtained through consecutive cutting cycles from limited numbers of initial SE donor plants. The SE process has also been demonstrated in many other conifer species with different rates of success, notably SE plants can be readily generated from species of larch (*Larix* ssp.) and less well from firs (*Abies* ssp.).

Initiation of SE cultures

When the SE process is applied for commercial plant production, the first step is to obtain improved seeds from a breeding program. Seeds collected before full maturity generally give higher yields in terms of SE initiations, but can often not be obtained for practical reasons of identifying and harvesting the responsive stages of immature cones from the field. For example, it was noted in white spruce (*Picea glauca*) that early cotyledonary-stage embryos could generate more than three times more SE initiations than fully mature embryos (Park et al. 1993). Genetically improved and valuable seed lots from controlled crosses commonly yield only a few hundred seeds from each cross. Amplification by SE of seeds from controlled crosses allows for theoretically unlimited numbers of plants to be produced from each improved seed through the multiplication step when early-stage embryos in the PEMs divide.

The standard process for SE initiation is not equally effective in all genotypes. A study of 30 full-sib families derived from six-parent complete diallel crosses of white spruce showed that the genetic variation in SE initiations is under strong additive genetic control. Selection and breeding for improved SE initiation might be a possibility, although there are some complications from large variances due to genotype–treatment interactions (Park et al. 1993). A similar study in loblolly pine (*Pinus taeda*) also found that SE initiation is highly influenced by additive effects, as 42% of the total variance was explained by general combining ability effects. The parental contribution to SE initiation potential in loblolly pine was confirmed by two independent experiments using a 3×3 factorial mating design and a three-parent diallel mating model, respectively (MacKay et al. 2006). Relatively large variances due to specific combining ability and maternal effects were found for SE initiation in white spruce when immature zygotic embryos were used as explants for SE initiation (Park et al. 1993, 1994) and in loblolly pine (MacKay et al. 2006), where to-date only immature zygotic embryos can initiate SE. The same pattern of large genetic variation for SE initiation among families was observed previously in 170 seeds collected from a total of twenty mother trees in a natural population of black spruce (*Picea mariana*) (Cheliak and Klimaszewska 1991). The annual variation in genetic potential for SE initiation was tested from seeds from the same open-pollinated mother trees of *Pinus radiata* over two consecutive years, showing that the same top three mother trees out of

seven tested also maintained their SE initiation potential over the two years tested (Montalbán et al. 2012). Although based on small experiments and imprecise estimates of genetic variance components, these experimental results suggest that selection of mother trees with good genetic potential for SE initiation can help expand the number of cell lines available for SE plant production.

SE initiation in Norway spruce (*Picea abies*) does not appear to be under strong genetic control as demonstrated in a study where all of 618 immature zygotic embryos derived from 18 control-pollinated families from 20 parental clones could be induced to form somatic embryos (Högberg et al. 1998). However, it has been noted in several recent SE initiation efforts in Norway spruce using mature seeds from controlled crosses provided by the Swedish Forest Research Institute and stored from 5 to 10 years that, on average, 1–3% of the families do not generate any SE initiations at all (U. Egertsdotter, unpublished data). The average initiation rates from mature and stored seeds are around 15% across families, which is in agreement with previous findings in white spruce where mature embryos yielded an initiation rate of 15.8% (Park et al. 1993).

We can conclude that the differences in capacity to initiate somatic embryos among families of Norway spruce will not pose any major problems for having a balanced representative sample of a population. However, to estimate genetic parameters for a character like initiation capacity of a population, much larger samples are needed than those reported above, which only represent the specific, often selected material, used in each study.

Multiplication of early-stage somatic embryos

Multiplication of PEMs occurs at different rates in different cell lines. It is not unusual for cell lines in spruce species that the multiplication process proceeds continuously for years without any notable change in multiplication rate or capacity for maturation. Multiplication in pine species is quite different and the limit for effective multiplication typically extends to a maximum of nine months. Lack of synchronization in the development of early somatic embryos of PEMs is a limiting factor for the transition from multiplication of PEMs to maturation of somatic embryos. Only the most developed early-stage embryos have the capability to respond to the maturation treatment whereas more immature embryos that fail to mature die.

PEMs cycle through phases of development such that the PEMs culture is composed of different developmental stages of early-stage embryos at any point in time (Filonova et al. 2000). This results in embryos appearing at different stages of maturation at the end of the maturation phase, where some will not be sufficiently developed to continue development into germination and plant formation. To some extent, the degree of synchronization depends on culture conditions, such that liquid culture medium provides better access to the medium components and stimulates a higher degree of synchronization, but synchronization also depends on the inherent capacity of the cell line to generate more or less developed early-stage somatic embryos.

For cost-effective production of large numbers of SE plants, it is an advantage for the *in vitro* SE culture process that the first two steps of the process can be supported by liquid media. In addition to the positive effect on synchronization, liquid media allow for automated handling and more effective growth of the cultures (Mamun et al. 2015). There are also other methods that can be applied to liquid-based cultures that will improve the degree of synchronization (Egertsdotter and Clapham 2015).

Maturation and germination of somatic embryos

In the next step of SE subsequent to multiplication, the early-stage embryos stop multiplying and start to accumulate storage reserves in the maturation process. Also in this step, there are variations among different genotypes in terms of how many mature embryos will develop, and how well the embryos develop toward a fully mature embryo that can germinate. Most genotypes that can establish an SE culture can however give at least some mature embryos, allowing the genotype to be represented in a clonal mix.

In the laboratory, mature somatic embryos typically are subjected to a period of desiccation to mimic the process that occurs naturally in seeds, before germination is started. Germination can also occur without desiccation in some species and cell lines, but desiccation is overall believed to help improve germination rates and subsequent plant formation. In pines, low rate of germination is currently the largest technical bottleneck of the SE process affecting its application on a commercial scale, whereas germination does not pose a serious problem in spruces.

Once germinants have formed, the next step is to establish germinants growth *ex vitro* by gradual transition to growth conditions outside the enclosed culture container used for *in vitro* culture. This step requires careful monitoring of the growth conditions to avoid too rapid changes, but is generally not considered a problem and causes few losses. Once the SE plants are fully acclimatized and have started to grow outside the *in vitro* conditions, they perform like seedlings at similar developmental stages.

Unintentional genetic selection

Concerns have been raised about the risk of direct and indirect genetic effects caused by selection at different SE plant production steps, from initiation of embryos to acclimated nursery crop. For vegetative propagation of forest trees, it is important that genetic diversity can be maintained by the following measures: most families are represented, a sample is representative by having a predictable mean value, and that unknown correlated genetic effects do not appear later.

In white spruce, the genetic variance due to cell lines was found to be highest for SE initiation, lower for maturation and still lower for germination. Interestingly, no correlation was found between the percentages of SE initiation, and maturation or germination rates (Park et al. 1994). In Norway spruce, 23 parents were used in a mating design to generate 25 families. Immature seeds from 15 of the families gave rise to SE cultures, of which 12 could produce mature embryos. No

clear correlation could be found between parent breeding values and the ability to form SE plants, or between embryogenic characters and field or nursery traits (Högberg et al. 1998). This agrees with a previous study showing no association between the capacity of families for SE and the phenological traits of the resulting SE plants after six growing seasons (Ekberg et al. 1993).

Variation among cell lines will be apparent in all stages of the SE process: initiation, multiplication, maturation, desiccation, germination, acclimation and plant formation, each step affecting the overall effective yield of SE plants. To obtain a representative sample of selected families and their parents for SE propagation, the variability is handled by compensating for specific cell line characteristics in different steps of the process. For example, large samples of seeds are initially used for SE initiation, the size of the PEM culture is adapted to cell line multiplication rate, mature embryos are harvested at optimal times for each cell line and conditions for desiccation and germination adjusted to obtain the highest number of viable germinants. Additional adaptation of the culture conditions is possible for the subsequent developmental steps at the nursery.

In conclusion, selection taking place under multiplication of nursery stock is common for all kinds of propagation techniques. For Norway spruce using SE propagation, the general conclusion is that selection effects due to genotypical differences in ability to perform through the SE process will not significantly affect the genetic gain and diversity of a clone mixture. Nevertheless, it is essential to ensure appropriate genetic representation by adjustments for cell line variability. Further basic research on regulation of embryo development will help design future SE procedures that allow more genotypes to respond at a higher level.

Genetic stability during the SE process

The concern has many times been raised about the genetic fidelity of plants produced by lengthy *in vitro* culture, so-called somaclonal variation. For conifer species, where rotation times in the field are often very long, it becomes of particular interest to ensure that there are no harmful genetic aberrations introduced during the *in vitro* part of the plant propagation process. Consequently, several studies have in depth addressed this potential issue. Indications of any genetic defects can, in principle, be identified by analyzing the target tissue with molecular markers and comparing the patterns with control tissue that is selected as normal based on appearance and performance. Chromosome counts and morphological characters of SE plants have also been used as tools to screen for any genetic discrepancies.

The results from the sample of studies presented below show that genetic aberrations may occur if the SE culture is maintained for an extended time at the proliferation stage (PEMs multiplication). However, none of the studies were able to demonstrate a correlation between genetic aberrations related to prolonged culture time, and the phenotype and growth of the resulting SE plants.

It is broadly accepted that SE cultures used for plant production should only be maintained in culture for multiplication for a maximum of nine months. To avoid extended time of multiplication, SE cultures can be cryopreserved and only thawed as required for plant production over a limited time period. It is anticipated that fewer genetic aberrations occur during storage under liquid nitrogen, although traditional cryoprotectants such as dimethylsylfoxide was previously shown to induce genetic aberrations detectable by RAPD analysis. However, the aberrations were not detectable after cryostorage suggesting that the aberrant cells did not survive in cryo-storage (Aronen et al. 1999). Recently, alternative one-step cryopreservation methods that do not include the use of cryoprotectants (Kong and von Aderkas 2011) have been shown to be at least as effective (Aery et al. 2014). Furthermore, *Picea abies* somatic embryos regrown from cryostorage after pretreatment by such one-step freezing method showed no change at any of five microsatellite loci tested (Hazubska-Przybyl et al. 2013).

Genetic stability during the SE process from multiplication to SE plant was evaluated in six white spruce SE cell lines by RAPD analyses using ten primers (DeVerno et al. 1999). Although variant fragments were detected in three of the clones after sub-culturing for twelve months (two clones) or two months, SE plants displaying normal development had RAPD patterns identical to those of the initial control SE culture at the beginning of the SE process, whereas SE plants showing aberrant development had RAPD patterns that deviated from the controls. In contrast, no intra-clonal variation could be detected from a study on 2154 RAPD profiles using three, ten or 29 primers from SE cultures and plants, including aberrant phenotypes with genomic chimeras (Fourré et al. 1997).

The genetic stability of a total of 11,042 SE plants from 65 black spruce cell lines originating from 18 controlled crosses and one half-sib family and 22 white spruce cell lines originating from seven controlled crosses were evaluated by morphological characteristics and chromosome counts (Tremblay et al. 1999). The SE plants were grown and evaluated for a five-year period. The phenotypic variation detected in the study resembled that reported for agricultural crops and other tree species: dwarfism, fasciation, variegated patterns, height alterations, modified branch angle and bushy shape (reviewed by Bajaj 1990; Hammerschlag 1992). Nevertheless, the frequency of variants among the total number of plants was found to be low both for black spruce (1.6%) and white spruce (1.0%). Two factors were identified as important for the appearance of variants: cell line and time in culture, where the former had a stronger effect. Family effects were not detected. Of the six variant phenotypes analyzed for chromosome counts, three showed aberrant counts (Tremblay et al. 1999). The variegated pattern in white spruce was previously shown to occur in only two out of seven clones derived from three controlled crosses. This variant was analyzed with 250 RAPD markers of which one was found to be specific for the variegated trait; it was not associated with any instability in chromosome numbers (Isabel et al. 1996). Chromosomal changes due to the SE process could also not be detected by flow cytometry in silver firs (Gajdošová et al. 1995) and Norway spruce (Mo et al. 1989) although trisomy associated with severe decline in maturation capacity was

detected in one cell line of *Abies alba* cultured for six years (Roth et al. 1997).

To summarize early efforts to detect any genetic variations induced by the SE process, studies using molecular markers such as RAPDs or isozymes did not reveal somaclonal variation in conifers (Eastman et al. 1991; Isabel et al. 1993; Heinze and Schmidt 1995), and only limited mtDNA changes (DeVerno et al. 1994). No chromosomal changes have been reported to occur as a result of the SE process.

Recently, more comprehensive analyses of the SE process have been conducted using microsatellite markers. No allelic differences were detected at three microsatellite loci during early stages of SE in two genotypes of Norway spruce (Helmersson et al. 2004). Further studies focused on the stability of four nuclear microsatellite loci in 314 somatic plants (38 clones) and 208 zygotic seedlings derived from six full-sib families and four half-sib families, respectively. Although no differences in growth and development of the somatic plants and seedlings could be detected, the mutation rate was higher in somatic plants than in seedlings of Norway spruce. Mutated microsatellites were observed in somatic plants derived from six out of 38 clones analyzed, with significant differences among families, and in three of 208 analyzed seedlings representing two of four half-sib families (Helmersson et al. 2008).

In general, the methods for the SE process in pines are less-well developed than for spruce species. This is likely due to a higher degree of polyembryony in most pine species, which confounds the early initiation of the SE process from the zygotic embryo(s). It has been suggested that the SE process in pines occurs by different mechanisms associated with the polyembryogenic process rather than a differentiation process. Indeed, it has been shown that SE cultures established from the same seed of loblolly pine (*Pinus taeda*) can differ genetically, indicating that more than one embryo was initiated having different fathers (Becwar et al. 1991). The yields of the subsequent steps of the pine SE process are considerably lower than in most spruce species, and the complete SE process resulting in a plant is so far only successful for a limited number of clones.

The genetic stability was analyzed in cultures of early-stage somatic embryos and zygotic embryos for four variable nuclear microsatellite loci in ten families of Scots pine. The results showed significant variation among families in microsatellite stability, where the families showing the highest variability also had the highest rate of somatic embryo initiation and proliferation rate. However, embryo maturation was more successful from families with lower variability. For six of the ten families analyzed, the genetic stability of early-stage somatic embryos and zygotic embryos was similar (Burg et al. 2007).

Abnormalities in formation of mature somatic embryos and germinants were observed in some clones of pine species, but no correlation with the applied SSR molecular markers was noted for Scots pine (*Pinus sylvestris*) (Burg et al. 2007). The effect of time in culture for multiplication was studied in samples from multiplying SE cultures and SE plants of 17 cell lines from six families of maritime pine (*Pinus pinaster*). Although analysis of seven SSR loci showed genotype-dependent variations after six, 14 and 22 months in culture and five out of 52 SE germinants showed abnormal phenotype, the results still showed no correlation between genetic stability and abnormal germinants (Marum et al. 2009).

It is a broadly accepted view that by utilizing optimized culture protocols and limiting the time in culture, all potentially negative effects from the SE process on plant development can be avoided (Bozhkov and Von Arnold 1998; Högberg et al. 2001; Högberg et al. 2003). Based on results available today from analyses of potential somaclonal variation due to SE processes in spruces and pines, the risk from somaclonal variation on the SE plant fidelity also appears limited. However, although the genetic variability reported in SE material is minor, it is still important to pursue research and analyses of the cause and results of such variability.

Recently, it was demonstrated for both SE plants and zygotic seedlings that photoperiodic control of growth cessation and bud set is at least partly under epigenetic control and that temperature during embryo development affect timing of bud set. This suggests that there may be an opportunity to "engineer" cell lines for different climate zones by adjusting the temperature during SE processes (Kvaalen and Johnsen 2008). It is currently not known if and how epigenetic mechanisms controlling other phenotypic characters are affected by the growth conditions during the SE processes. However, extensive field trials comparing SE trees with control non-SE trees have not shown any phenotypic traits related to the SE process (see overview in Table 1) although recent results show different epigenetic patterns in SE cultures and shoots from adult trees (Ausina et al. 2016). However, comparisons of epigenetic patterns and phenotypes of SE cultures and SE trees of the same genotypes, and zygotic embryos and non-SE trees, are still missing making it unclear to what extent these epigenetic differences are stably maintained and if they lead to any phenotypic alterations.

SE offers an attractive system for studying the effects from abiotic factors, e g related to climatic changes, on epigenetic control mechanisms by allowing different test scenarios to be applied to large numbers of embryos of the same genotypes, followed by production of sufficient numbers of clonal trees for field planting and phenotyping. Effects of climatic changes can be explored and documented. Possibilities to "engineer" trees at the embryo stages for increased adaptability are intriguing.

Characteristics of the SE plant after the *in vitro* SE process

Acclimatization from in vitro *growth conditions*

The *in vitro* part of the SE process ends with planting of a germinated embryo in a substrate suitable for continued growth in a greenhouse or growth chamber. During the first period of time outside *in vitro* culture, cultural conditions are critical, requiring uniformly high humidity, temperature and light conditions and a specialized greenhouse or culture chamber. The changes that occur during acclimatization of the SE plant have been studied at the microscopic level in white spruce. Several structural changes of the epidermal layers were observed that are probably necessary to restrict water loss and control stomatal aperture function (Lamhamedi et al. 2003).

Early growth of the SE plant in nursery growth environment

Depending on the cell line, large variations in growth pattern of SE plants have been observed, particularly during the first growing season, including both the acclimatization phase and subsequent growth at the nursery site. Some cell lines will perform even better in terms of growth and development than the control seedlings, whereas others may grow and develop less than controls, at least during the first few years in the ground. Superior characteristics of SE trees in comparison to related zygotic seedling have also been observed in field trials as discussed in the next section.

Variation among cell lines of white spruce in morphology, growth, physiology, anatomy and ultrastructure was studied in plants grown for six months in the greenhouse (Lamhamedi et al. 2000). SE plants from three cell lines from each of four families of white spruce were compared to zygotic seedlings from the same families. Significant differences between seedlings and SE plants were noted for several growth- and physiological traits, with greater variability in height, dry mass of new roots, needle dry mass and branch density among SE plants than zygotic seedlings.

Field performance of SE plants

Efforts toward commercial production of SE plants started in the early 1990s, and involved testing of SE plant performance in the field. This was first addressed on a larger scale by the Forest Biotechnology Center, BC Research Inc. (reviewed by Grossnickle 2011). Evaluation of SE plants in a seedling quality testing program for interior spruce (*Picea glauca* – *Picea engelmanni* hybrid complex) showed that SE plants have comparable performance to zygotic seedlings during the first two years in the field (Grossnickle and Major 1994a, 1994b). Further trials found no major differences in physiological or morphological attributes between SE plants and seed derived plants (Nsangou and Greenwood 1998; Lamhamedi et al. 2000; Benowicz et al. 2002). An SE seedling quality program was later developed to determine field performance on four different sites for SE plants from 34 cell lines arising from twelve full-sib families, with one to six cell lines per family, from interior spruce and white spruce crosses (Grossnickle and Folk 2007). The SE plants were originally deployed in 1999 as viable stocklots outplanted in British Columbia (Grossnickle and Folk 2005). The field test results showed that the tested genotypes displayed a wide range of performances for the measured morphological and physiological parameters, under four environmental regimes, suggesting that SE plants can be produced from a sufficiently wide genetic base to cope with variations in environmental stresses.

In another study aimed at evaluating genetic parameters and performance stability in SE clones, 52 white spruce somatic clones were tested on two sites in Quebec for survival, bud dormancy, stem form, growth and branching characteristics four years after outplanting. The results show that SE clones had high survival rates (98–99%) at the different sites, and a weak genotype–site interaction coupled to a relative ranking of clones for height being the same for both sites. SE clones also demonstrated superior performance in adaptive, stem form, growth and branching characteristics (Wahid et al. 2012).

Similar results for high survival rates (92–99%) were obtained for 70 SE clones from two full-sib families of Douglas fir outplaned on five sites in the Pacific Northwest USA. Height, diameter at breast-height and stem volume were measured after seven and a half years; stem sinuosity, stress wave velocity and pilodyn after six and a half years, the results demonstrating high between-test clonal correlations and low levels of variance due to clone × test interactions (Dean 2008).

Additional efforts to evaluate SE plants for field performance are summarized in Table 1.

It was recently shown that SE plants in black spruce can generate fully functional male and female strobili. Good-quality pollen suitable for pollination and generation of seeds was obtained from SE plants. Seedlings were generated from SE parent trees, showing the potential of SE plants for establishment of seed orchards (Colas and Lamhamedi 2014).

Overall, from the analyses of SE plant performance at the nursery or in the field, there are no apparent negative effects from the SE process on growth and/or development of SE plants compared with seed plants. Based on these results, there are no reasons to believe that abnormalities will be introduced due to the SE process.

Commercial production and use of conifer SE plants

The SE process has been applied for industrial production of conifer plants of different species, mostly for use in plantation forestry. Despite a less favorable SE process available for pine species, to date most large-scale SE plant production efforts are made with different pine species (Table 2).

The explanation for this may be that plantation forestry is more common in areas where pine species are dominating forestry production, particularly fast-growing subtropical pine species that are more suitable for plantation production through cuttings. Nevertheless, recent focus on the SE process for plant production in different regions includes white spruce, black spruce, Sitka spruce and Norway spruce. Globally, there are a handful of entities involved with commercial scale-up of conifer SE plant production, as summarized in Table 2.

Enabling SE plant production by automation

Implementation of the SE process for production of plants in commercial forestry operations has long been hampered by labor-intensive steps during the *in vitro* part of the process. Efforts have been made to reduce manual labor by applying image analysis and robotics for selection of good germinants of Nordmann fir (*Abies nordmanniana*) (Find and Krogstrup 2008) and to apply synthetic seed technology to simplify the transfer of germinants of Douglas fir to the field during the acclimatization process (Gupta and Kreitinger 1993). To date, these methods have not been shown to operate well on a large scale. This could be explained by difficulties

Table 1. Examples of studies to evaluate quality of SE plants at different stages of development and of field performance with emphasis on *Picea*.

Species	Test parameters	Results	Entity	Reference
Abies nordmanniana	Containerized SE plants grown for two years in nursery before field planting	2007: 9 clones, total 400 plants 2014: 150 clones, total 2000 plants 2015: 250 clones, total 8000 plants	Univ. of Copenhagen	Pers. comm. J. Find
Picea abies	Effects of maturation medium and light on early SE seedling growth	Improved plant survival and growth	SLU, Skogforsk	Högberg et al. (2001)
P. abies	Field trials with 50 half-sib families represented by both seedlings and SE plants on one site in Sweden	Started in 2015. No significant difference in shoot growth between SE plants and seed plants after one year of field growth	Skogforsk	Högberg (2015b)
P. abies	Containerized SE plants using standard conifer SE research methods for plant production	1994 (Nordic Forest Research SNS cryo project): 21 clones from 4 families, 120 trees in the field today are 10-13 m in height 2007: epigenetic project for "after effects": 800-900 trees from a single full-sib family	Norwegian Forest Research Institute	Pers. comm. H. Kvaalen
P. glauca	Test of SE germinant response to *ex vitro* acclimatization treatments	Important to induce higher net photosynthesis, lower epidermal transpiration, and more starch for successful acclimatization	Forestry Dept. MRNF, Quebec Laval, Quebec	Lamhamedi et al. (2003)
P. glauca	Analysis four years after planting for survival, bud dormancy, stem form, growth, branching: 52 clones replicated in 10 blocks, and 520 and 446 ramets at each of two locations	Superior performance in adaptation, stem form, growth, branching, but low clone heritability. Height showed relatively higher genetic gain	Forestry Dept. MRNF, Quebec Laval, Quebec	Wahid et al. (2012)
P. glauca	Testing of over 2000 varietal lines (SE clones) in 45 field sites. Oldest 15 years in the field	Five best of 233 lines in test show 85% more volume gain than seed orchard gain at age 10	JD Irving Ltd	Pers. comm. Y-.S. Park
P. mariana	Seed production from controlled crosses of SE plants	Production of viable seeds from crossings of SE trees. Maternal effect on cone size and seed mass.	Forestry Dept. MRNF, Quebec	Colas and Lamhamedi (2014)
Pseudotsuga menziesii	Field assessments of gas exchange, water relations and frost hardiness during two growth seasons: 192 plants from SE and 192 plants from seeds on one test site	Similar adaptive attributes of SE plants and zygotic plants. Only marginal differences in water relation parameters	Dept. of Forest Sciences, UBC and CellFor	Benowicz et al. (2002)
P. menziesii	Field testing in five locations of 37 (2008) and 70 (2009) SE clones from two full-sib families measured at 6.5 and 7.5 years	Demonstrated that clone heritability and genetic parameters are maintained in SE plants	Weyerhaeuser	Dean (2008); Dean et al. (2009)

maintaining sterility during handling and the complexity of the procedures, making them too complex and expensive for scale-up. Recently, another approach based on liquid handling of somatic embryos of Norway spruce for multiplication, maturation and harvest has shown promise to provide a cost-effective technology for large-scale production of SE plants (Aidun and Egertsdotter 2012). The fluidics-based technology is currently being pursued by Swedish forestry companies for pilot-scale testing and cost evaluation (Figure 2). The principle of the technology is described below and the possibilities and risks discussed.

SE plant production by the fluidics-based technology: the SE fluidics system

For Swedish forestry operations, the benefits from vegetative propagation will be realized primarily through family forestry. In addition to enabling capture of high values from the breeding program through multiplication of elite seeds, SE also offers advantages to seed orchard operations, as discussed previously (shortened turnaround times, avoidance of pollen contamination). The fluidics-based technology will enable large-scale production of SE plants for deployment or breeding.

The SE fluidics system: initiation of SE cultures

SE cultures are initiated using elite seeds from the Swedish breeding program (managed by Skogforsk), generally using standard procedures for isolating the zygotic embryo and inducing formation of early-stage somatic embryos on solid culture medium. The composition of the induction medium has been improved and recent results show rates of initiation above 20% from fully mature, dried seeds. Initiation rates from

Table 2. Summary of known currently active global entities with interests in commercial production of conifer SE plants

Location/producer	Method	Species	Status
Canada/JD Irving Ltd	Containerized SE plants	*Picea abies*, P. ssp	Aim to scale-up production of SE plants from clones tested since 1990s. Manual planting of SE germinants
Coillte/Ireland	Production of SE plants to be used as stock plants for cuttings	*P. sitchensis*	Annual production of 2.5–3 million cuttings from SE donor plant
Chile/Arauco	Production of SE plants to be used as stock plants for cuttings	*Pinus radiata*	120,000 SE stock plants annually for production of 10 million SE cuttings
NZ/Scion	Production of SE plants to be used as stock plants for cuttings	*P. radiata*	Long-term successful program for SE cutting plant production. Support to other entities in South America and elsewhere. Millions produced annually
US/Arborgen	Production of SE plants to be used as stock plants for cuttings	*P. taeda*	Annual goal 1 million
US/Weyerhaeuser	Synthetic seeds	*P. taeda*, *Pseudotsuga menziesii*	Annual goal 10 million

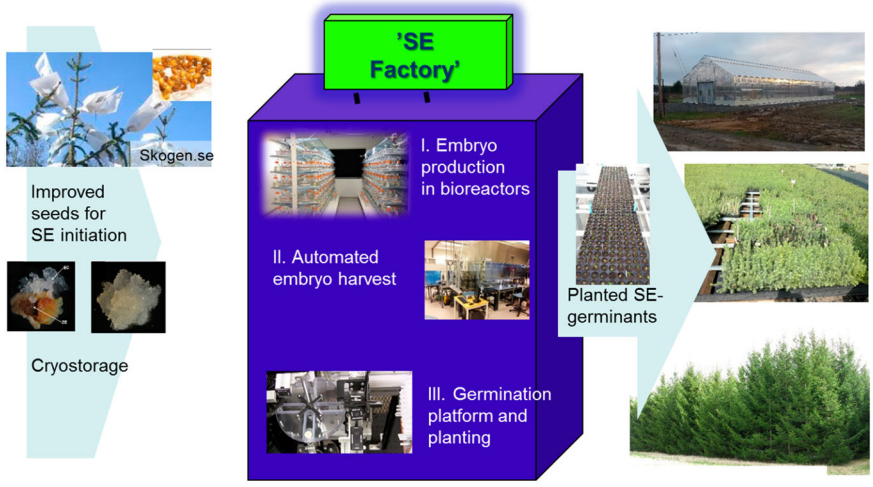

Figure 2. The fluidics-based technology for the automated harvest of mature somatic embryos (SE Fluidics system) is the core technology for SE plant production in the 'SE Factory'. Improved seeds from breeding programs are used for SE initiation to generate cultures of PEMs. The cultures can be kept for extended periods in cryostorage, or used directly for plant production. Mature somatic embryos are produced from multiplied PEMs in bioreactors (I); mature embryos are harvested from the bioreactors in the fluidics-based harvesting system, then germinated in the germination platform (III); germinants ready for *ex vitro* growth are planted in nursery planting trays. Germinants are acclimatized to greenhouse conditions and plants generated for field planting. Shown photos from SweTree Technologies AB SE-factory pilot installations 2015 are published with permission from SweTree Technologies AB.

immature seeds are considerably higher, on average 60%. With this rate of induction, even small seed lots can be captured in the SE process and represented in clone mixes.

The SE fluidics system: multiplication, maturation and harvest

Once a PEMs culture has been established from the first initiated somatic embryos and multiplication continues on solid culture medium, transfer to a temporary-immersion bioreactor will increase the rate of multiplication and reduce the need for manual labor for subculture. The solid-grown PEMs culture is dispersed into smaller clusters of PEMs at the start of the bioreactor culture period. This procedure is carried out with a specific tool designed and manufactured for the purpose, and has been shown to increase the yield of mature embryos and the degree of synchronization of the embryos. Multiplication rates depend on cell lines and can be as high as for PEMs cultures in liquid medium in suspension culture. The temporary-immersion bioreactor model is specifically designed and manufactured for Norway spruce PEMs cultures and maturation to reduce handling time, increase yields and dock with the automated harvest system. When most of the embryos in the bioreactor have reached full maturity, the mature embryos are harvested from the bioreactor by direct transfer to the automated harvest system. Harvest of mature somatic embryos from an SE culture requires individual selection of mature somatic embryos from the mixed mass of immature, non-responding embryos in the original PEMs and mature embryos, and is the most cumbersome manual step of the SE process. Mature embryos can be rapidly harvested with the SE fluidics system, with each embryo documented and sorted by an image analysis system. In the SE fluidics system, SE cultures composed of PEMs and mature embryos are dispersed into smaller units, mature embryos separated from the PEMs, and individual mature embryos analyzed and selected based on previously set selection criteria.

The SE fluidics system: the germination platform and planting

Mature embryos harvested from bioreactors are placed directly in the germination platform box in which they remain for the subsequent developmental steps leading up to the germinated embryo ready for planting. Planting is an automated process where a planting tray is filled with germinants in each of the processing lines of an automated planting system. A production system has many planting lines working in parallel. Newly planted germinants are transferred to a year-round greenhouse (or culture chamber) that can hold uniformly high humidity, temperature and light conditions for acclimatization. The SE plants are gradually transferred to regular nursery conditions though a series of greenhouse chambers also allowing for 'in-wintering' of plants for storage and later transplanting to larger containers and continued growth before planting in the field.

Concluding remarks concerning Swedish conditions and possible scenarios

The use of clonal propagation for families in Sweden's many climate zones implies using many clones and many clone mixtures, and developing new commercial clones over time. At present, to produce a well-functioning nursery clone, on average, one has to start with 200 mature seeds resulting in twenty cell lines and ten plant-producing cell lines, of which one is of highest quality. Using fresh seeds to stimulate initiation of cell lines and the number of seeds needed is reduced to about 35 instead of 200. In total, to generate a clone mix of 25 high-quality nursery clones, from 900 to 5000 seeds are needed.

Assuming an SE planting program within 25 million plants per year and limiting the number of ramets per clone to 1 million, 25 new clones must be introduced each year. If planting takes place in many climatic zones, different clone mixes are needed and many more clones will be in use each year, even if the annual introduction of new clones will still be 25. Every clone will be used to a lesser extent each year, but over more years.

Research priorities

The fundamental laboratory method for multiplying a zygotic seed embryo to generate many plants by SE was established in the 1980s. Today, plant generation by SE has been successfully demonstrated for many conifer species (Salaj et al. 2015; Klimaszewska et al. 2016). New improved methods, and recently automation for enabling cost-effective large-scale production, have now made SE plant production feasible as a tool for operational forestry to capture genetic gains from breeding programs. Furthermore, it has been shown that SE can be initiated from ten year old primordial shoot explants from one genotype of SE-derived plants of white spruce (Klimaszewska et al. 2011) suggesting possibilities to develop effective methods for initiation of SE from mature conifer tissue across species and genotypes.

There is however still a need to further our understanding of the SE process. The fact that not all seeds respond to the initiation treatment to form an SE culture, and the variability in yields from subsequent steps of the SE process, indicate that methods and culture conditions for somatic embryos at different developmental stages can be improved. In most research labs, a few well-performing cell lines are used for research. It is only when larger scale (commercial nursery) efforts attempt to initiate high-yielding cell lines from many seeds that variability becomes apparent. In particular, the later stages of germination and early plant growth have been largely neglected in research efforts up until now. Specifically, if SE is to be used for clonal propagation of families, increasing the yields and limiting variability become essential for full flexibility to capture sufficient numbers of cell lines to meet goals.

Furthermore, recent research results suggest that epigenetic modifications may occur during the early development of somatic embryos. Although so far reports on field performance of SE trees and their phenotypic characteristics show no correlation to the SE process, it will be a key research priority to further investigate the occurrence and characteristics of such modifications to understand their role for developmental processes and climate adaptations.

Disclosure statement

No potential conflict of interest was reported by the authors.

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