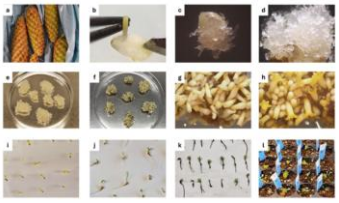

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Clonal propagation of conifers by somatic embryogenesis (SE) – an introduction to methodology and examples of applications for research and plant production

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Abstract

This article is aimed as a basic introduction to conifer somatic embryogenesis for someone with a general interest in applications of this technique and/or who is interested in starting some practical work in the field. We also present data from an example study on individual trees' ability to initiate somatic embryogenesis in Norway spruce (*Picea abies*). A total of 158 trees were each tested for ability and relative competence to initiate somatic embryogenesis from the zygotic embryos by observing the initiation frequency for ten zygotic embryos from each individual tree. Furthermore, we conducted a case study by following the process for SE plant formation for a selection of 48 cell lines that were monitored through the SE developmental pathway, with data collected on the success rates at different steps. We then evaluated the relative importance of different steps for the outcome of plant formation and yield.

Keywords

Somatic embryogenesis; Conifer; Methodology; Applications; Initiation

Contents

1 Introduction

The purpose of this article is to provide a popular scientific overview and introduction to researchers who are not working in the somatic embryogenesis (SE) field by presenting an overview of various SE applications in conifers and highlight possibilities and limitations by discussing the results from an SE initiation study in Norway spruce (NS) that covered a relatively large number of genotypes (mother trees). It is not intended as a full review of the literature on NS SE methods nor as a full research article. We specifically discuss many of the practical challenges for obtaining SE plants for forestry applications or research objectives by analysing the results for the SE process starting from cone collection, through initiation of SE cultures, proliferation, maturation and to germination of the somatic embryos. We also analyse the data by an integrated analysis of yields across different developmental steps.

Future forests need to be composed of trees with resilience both to climate-imposed direct abiotic stresses, such as droughts and temperature changes, and to increased pressure from insect and pest attacks, in order to survive and sustain their important roles for wood and fibre supply, and as essential parts of the global ecosystem. It has been well documented that climate change has a negative impact on forests through the growing intensity and frequency of natural disturbances, including insect attacks, droughts, wildfires, and global stressors such as air pollution (Peterson et al. 2018). The Fifth National Climate Assessment reports that climate change will increase tree mortality and decrease the capacity to store carbon (Domke et al. 2023). Models predict that tree death related to climate stress will roughly double by 2099 (Anderegg et al. 2022). To mitigate climate effects on our future forests and sustain productivity to meet human needs, with regard to wood and fibres, carbon capture and ecosystems, the genetic design and production of future trees need to be moving at a faster pace than natural evolutionary processes can accomplish.

Tree breeding strategies typically target both traditional adaptation and increased production (Pâques 2013; Haapanen et al. 2015) and encompass strategies to mitigate climate impact (Lstibůrek, García-Gil and Steffenrem 2023; Chakraborty et al. 2024). For many forestry species, seed orchards are the main link between breeding efforts and silviculture needed for providing the improved trees for forestry operations (Funda and El-Kassaby 2012). Seed orchards are, however, frequently challenged in different ways, including attacks by insects and other pests, that affects the availability of seeds. The long timespans from selection of elite trees to commercial seed availability also puts limits on the timely availability of improved trees for planting. SE is a clonal propagation technique that fundamentally relies on the clonal multiplication of seeds. SE offers several advantages for conifer plant production both timewise, by the direct route from improved seeds to a plant without the need to generate a seed orchard for plant production, and by the potential for scale-up and automation of plant production under climate- and weather-independent conditions. SE as a clonal propagation technique also has several technical advantages over cutting propagation as discussed below.

1.1 Steps for clonal propagation of conifers by SE

SE is a method for clonal propagation where somatic cells of the initial explant are induced to form early-stage somatic embryos. In conifers, the initial explant to start a culture of somatic embryos is typically a zygotic embryo, although a few reports have shown that SE can also be induced from shoots, including shoots from NS plants derived from SE (Varis, Klimaszewska and Aronen 2018). Conifer somatic embryos develop in a similar way to zygotic (seed) embryos, from the earliest stages (proembryogenic masses; PEMs) to plants, by a process analogous to embryo development in a seed (Figure 1). In brief, the *in vitro* methods for plant formation by SE include several steps, from the initial initiation and further multiplication of the PEMs on a culture medium containing auxins and/or cytokinins, followed by a maturation phase where the somatic embryos stop multiplying and start the maturation process stimulated by abscisic acid (ABA). Mature somatic embryos are then sometimes partially desiccated before transfer to a culture medium without any plant growth regulators (PGRs) that stimulates germination. Once germination has occurred with visible growth of roots and shoots, the small SE seedling can be transferred to *ex vitro* conditions.

The SE cloning process allows in theory for unlimited numbers of copies of the original seed embryo genotype to be generated. It is almost trivial to mention the experimental benefits from working with clones for studying various physiological mechanisms, and for the high accuracy phenotyping in association with genetic studies. SE as a cloning method can be utilized for producing and testing many plants of each sibling from a crossing whilst the genotype is maintained as an SE culture under cryogenic storage for future plant production once the most suitable genotype for a certain growing situation has been identified, or for providing test data from the field for evaluation of that particular crossing. Furthermore, in an experimental set up, variations between siblings in their responses to the experimental conditions can be monitored and can inform about the genetic background for certain traits.

1.2 SE protocols and methods

With the few exceptions of studies covering larger number of genotypes conducted by the forestry industry, studies on SE protocol optimizations have been limited to a few model cell lines. Although this approach limits the applicability of the results to be used across genotypes, it is also a prerequisite for demonstrating any significant effects owing to the large variability between different genotypes (and cell lines). This situation is at least partially the cause for why the SE method still lacks the efficacy of the seed embryo developmental process despite over 40 years of efforts to improve yields.

There are differences between the protocols for SE plant production in different conifer species (Jain and Gupta 2018) but also differences in the protocols within species like NS. It is not well understood why different protocols should be needed for the same species when applied in different laboratory locations. One hypothesis is that cell lines get preferentially initiated on a certain initiation medium composition and that this affects the downstream culture requirements for further development to take place. Here we used a protocol based on one of the first protocol established for NS in 1985 with some key modifications (Arnold and Clapham 2008). As for most conifer SE protocols, it goes through initiation of PEMs, 'capture' of multiplying PEMs to establish a culture, maturation of PEMs into somatic embryos and germination followed by plant formation (Figure 1).

The actual laboratory handling processes during the steps for NS SE are still more or less similar between laboratories with some differences, notably which zygotic embryo stage that is used as the initial explant for the initiation process , the application of an optional pre-maturation step without PGRs before start of maturation and whether a partial desiccation of the mature embryo is applied before germination. The main differences for NS initiation, proliferation and maturation are found in the medium composition, where the types and concentrations of the nitrogen sources, levels of ABA, carbohydrate source(s) and addition of osmotic agents vary among different laboratories. In recent years, as SE is increasingly explored for large scale production of conifer plants including NS (Egertsdotter, Ahmad and Clapham 2019), protocol optimizations of the SE process have mostly focused on the downstream germination and plant formation processes, including acclimatization to *ex vitro* conditions, and how the methods for these steps vary with respect to light conditions, timing and substrates (Klimaszewska et al. 2016).

The SE protocols for manyconifers have been well worked out at a sufficiently effective level to support limited research studies that only require a few plants and typically involve only one or a few well-known cell lines. Scale-up of SE plant production across many cell lines including the new, latest improved seeds from breeding programs, poses different challenges to protocol efficiency. It is therefore essential to understand the bottlenecks of the SE protocols to improve the overall success rate of SE propagation across genotypes.

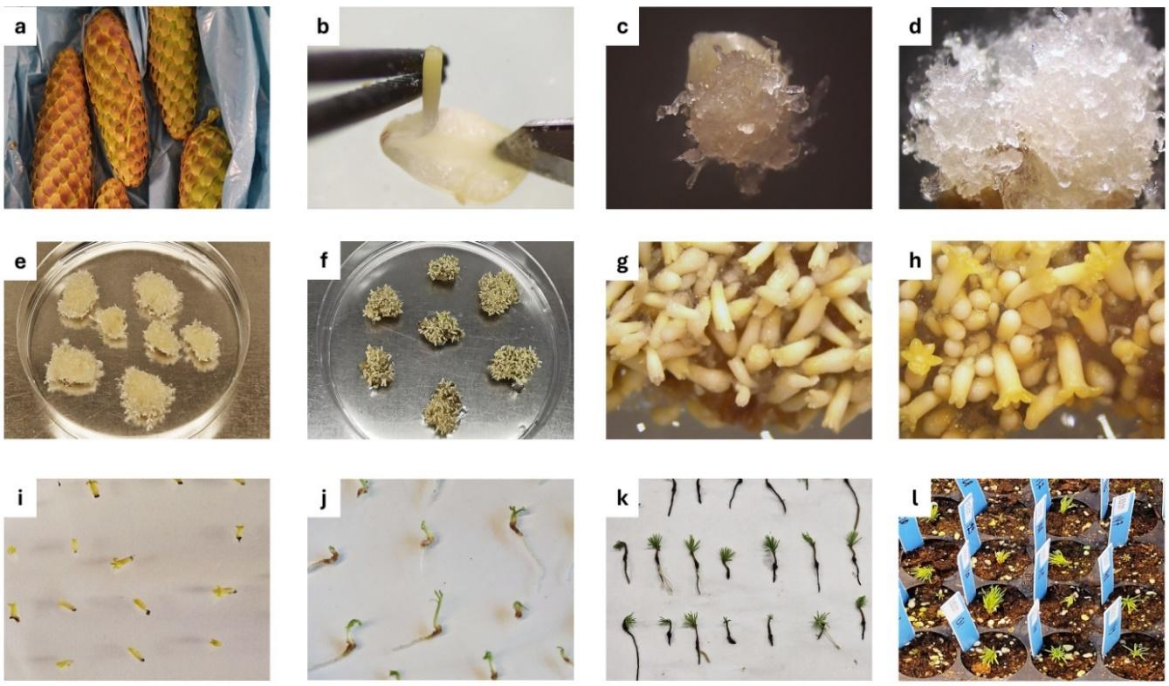

Figure 1. Somatic embryogenesis (SE) process. (a) Cones from selected trees. (b) Extraction of zygotic embryos. (c) SE culture initiation with PEMs protruding from the zygotic embryo. (d) Developing PEM ready for isolation from the zygotic embryo. (e) Proliferating PEMs are multiplied by biweekly subcultures onto fresh medium of the same composition. (f) PEMs with maturing embryos on medium containing ABA. (g) Maturing embryos. (h) Mature somatic embryos ready for harvest. (i) Mature somatic embryos isolated for partial desiccation. (j) Germination *in vitro*. (k and l) Germinants with a shoot apical meristem and root system planted *ex vitro*.

1.3 SE in forestry applications: use for testing in breeding programs and production of elite plants with improved traits

SE is much less hampered, but still challenged, by biological and technical-economical limitations than plant production by rooted cuttings; the method has a great potential to be scaled up and yields plants unaffected by plagiotropic growth. Conifer SE is also a powerful research tool to study gene functions and has huge propagation potential for reforestation and production forestry (Zhao et al. 2024). The SE process for plant production offers a long-term safeguard of germplasm by cryogenic storage. It is highly amenable to field testing and to automation for cost-effective scale-up, and the output plants match the quality of those obtained from the seeds. Gene function in conifers can be studied both by a transgenic approach (Klimaszewska et al. 2016)) and by genomic modification as recently demonstrated to work in both spruce (Cui et al. 2021) and pine (Poovaiah et al. 2021). The transgene is introduced early in embryo development; gene functions can therefore be studied across developmental stages, from meristematic centers *in vitro* to whole plants under controlled conditions or in the field. The high propagation potential of SE means that large numbers of similarly genetically or genomically altered plants can be produced for testing across different growth conditions and stress situations.

SE is particularly suitable for production of clonal plants of conifers, as they are valuable trees for the global economy. One of the most economically valuable forestry

species in the Nordic countries, Norway spruce, shows an irregular flowering pattern that can cause shortage of high-quality seeds for forest regeneration (Tikkinen, Varis and Aronen 2018). By using SE techniques to produce NS seedlings, the problem with lack of seeds will be reduced as the cultures can be cryopreserved for long-term use and then multiplied as needed independent of external factors (Zhu et al. 2022). Traditional clonal propagation of conifers by cuttings is limited in scale owing to ageing of the donor plant, plagiotropism and high labour costs. SE offers a method that can be scaled up and automated for cost-effective large scale plant production. The use of SE in forestry operations is increasing; SweTree Technologies AB in Sweden annually produces about 100 000 SE seedlings for forest regeneration (UBI 2024) and in Finland approximately 180 000 SE seedlings were distributed for planting in 2023 (Mikko Tikkinen, personal comm. 2023-12-22). At J.D Irving Limited, Sussex, New Brunswick, Canada, SE plant production from tested SE cell lines of NS and White spruce (*Picea glauca*) was started already in 2010 with an initial 300 000 SE seedlings produced annually (Park et al. 2016). That number has gradually increased and since 2010, over 16 million SE seedlings have been produced (Pers. Comm. Pamela Nicks, Maritime Innovation Limited, Irving Woodlands, Sussex, New Brunswick, Canada). Since 2015, the Quebec provincial nursery at St Modeste has produced about 200 000 SE plants from White spruce annually. Commercial SE plant production also occurs in the Southern hemisphere mostly from pines (mainly Radiata pine (*Pinus radiata*)) but typically through a process involving both SE and cuttings since the yields from the SE process in pines are relatively low and don't meet the production demands.

Furthermore, the SE propagation method opens the door to very early testing of trees from the breeding program by molecular markers, already at the embryo stage. There is increasing interest in applying genome-wide association studies (GWAS)as an approach where genomic variants are statistically associated with a particular trait identified my molecular markers, for early selection at the embryo-stage of superior trees. The potential for utilizing GWAS for early selection has already been demonstrated based on the existing heterogeneous phenotypic data from breeding programs (Chen et al. 2021) but more studies still need to be performed and verified on early-stage SE cultures.

1.4 An example of practical use fore SE to conifer plant production – SE for Christmas tree production

The SE technology is particularly well suited for production of conifer plants intended as Christmas trees, where *Abies* species are the most common conifer on the European market. The annual turnover for the Christmas tree industry is approximately 1,5 billion EUR. With a total market of 140 million trees used each year, artificial trees add up to 50 million, or 35% of the market, with large country-to-country variation depending on the Christmas tree tradition (Christensen 2024).

Christmas tree production compared to traditional forestry is characterized by its short rotation (9 – 12 years) and by every grown tree being the final product intended for the consumer. Furthermore, the production cycle involves extensive cultivation that includes weed and pest control, fertilization, leader-length control and side trimming. Central characteristics of importance are generally a symmetric appearance and good post-harvest quality, and, varying with the market, also tree density, narrowness and choice of species. A superior tree therefore holds several characteristics that make

cloning an interesting way to conserve these traits in the product to be sold. Today, *Abies nordmanniana* is the most used Christmas tree in Europe, with an annual production of 50 million trees.

SE research in *Abies nordmanniana* was initiated in the 1990's (Norgaard and Krogstrup 1991; Nørgaard 1997), further developed for initiation based on fresh as well as dried seeds (Nørgaard and Krogstrup 1995; Find et al. 2002; Kristensen et al. 2005) and supported by cryo-preservation techniques (Norgaard, Baldursson and Krogstrup 1993). The first demonstration plot holding SE clones of *A. nordmanniana* was established in 2007, and by using standard protocol for initiation, proliferation, cryopreservation of embryogenic cultures, maturation of somatic embryos, and plant regeneration, about 400 clones were established and ready for field testing in 2014 – 15 (Find 2016). The use of a standard well-working protocol is a practical necessity when dealing with such large number of genotypes.

The approximately 400 clones were established in 5 field trials. After 5 – 6 growing seasons the first clonal Christmas tree evaluation on the surviving sites was carried out (Xu and Nielsen 2023). In general, survival was excellent despite one sandy site. When comparing somatic seedlings with standard commercial zygotic seedlings after 5 years, overall, the somatic seedling height was approximately 20%lower than seedlings, whereas hardly any difference was seen for Christmas tree quality. The clonal uniformity in appearance is striking, but presently, growth seems more variable and comparable to the variation among seedlings. Genotype by environment interaction was present for several traits, however, some of the within clone variation could be due to protocol issues and lack of culling in the used testing material (Xu and Nielsen 2023). Nielsen et al. (2022) documented strong accumulated effects on seedling height from the preceding steps in the protocol. This first study based on unselected material from natural stands demonstrates the importance of the seed harvest area and also a huge variation among families – highlighting the importance of superior starting material for cloning by SE techniques (Xu et al. op.cit.).

Lately, close to 275 new clones (in total 14.000 somatic seedlings) was established in field trials 2019 – 2021 based on superior seeds (crossings or selections) originating from the Danish breeding program (Xu, Nielsen and Hansen 2018; Nielsen, Xu and Hansen 2020) and these are presently under testing in field trials (Nielsen, Xu and Hansen 2020). Use of somatic clones for disease testing were proven successful across treatments based on the genetic uniformity of the test plants(Xu et al. 2018).

1.5 Impact from the SE method on SE tree plant traits

Interestingly, it has been found that the actual somatic embryogenesis process itself can positively influence tree traits (Kvaalen and Johnsen 2008; Puentes et al. 2018). Resistance in NS seedlings when fed to the pine weevil (*Hylobius abietis*) was 30% higher in plants produced through SE (somatic seedlings here) compared to seedlings produced from seeds from the same mother tree (Puentes et al. 2018). In a subsequent study, somatic seedlings were found once more to be less damaged by pine weevils than nursery seedlings, indicating that there may be a plant protection advantage associated with SE propagation (Berggren et al. 2023). Furthermore, somatic seedlings have been found to heal and close stem wounds (such as those made by insect bark-feeding) much faster and to a greater extent than nursery seedlings (Berggren et al. 2025). The underlying mechanisms behind these effects have not been fully understood yet.

However, the transcriptome of somatic and zygotic embryos has been found to differ strongly in the expression of a high number of stress-associated genes (Jin et al. 2014; Winkelmann 2016). Thus, exposure early in life to SE and the stress it entails (Fehér 2006) may affect plants' defense responses later in life. In addition to direct benefits for plant resistance, SE also provides a way to have a large number of genetically identical plants. This is particularly useful in studies examining resistance to various forest pests (i.e., clones can be exposed separately to each pest) and examining the possibility of tree breeding to improve resistance traits.

The SE methodology has also been an essential tool to study epigenetic effects, where it has been possible to demonstrate that the temperature during embryogenesis has an impact on seasonal behavior in NS(Kvaalen and Johnsen 2008), *Abies nordmanninana* (Lobo et al. 2022) and *Pinus pinaster* (Trontin et al. 2025). Such studies can enlighten understanding of climate change and of the mechanisms behind adaptability and potentially result in treatments that could be applied during plant production for enhanced tree response.

Somaclonal variation, the change of growth pattern and traits of certain individuals within a clone of an *in vitro* culture, is another symptom of epigenetic effects that have been mostly observed in long-term tissue cultures of other plants than conifers. This variation is for the most part considered problematic as it may result in different characteristics of the clonally propagated plants but can also be utilized as a tool to increase genetic diversity (Krishna et al. 2016). In conifers, the presence of somaclonal variation detectable with various molecular markers showed minor or no somaclonal variation (Sarmast et al. 2024).

1.6 A case study on SE initiation in Norway spruce

Although SE is increasingly used for conifer plant production on a commercial scale, the different steps of the SE-cloning process pose different challenges. The first step, initiation of the SE culture, does not work equally well across all genotypes for the methods available to date. This is perhaps the most limiting step of the SE process for forestry applications as it is essential to be able to multiply trees from all seed sources. Several factors affect the initiation success rates, where the timing of the cone collection plays an important role (Varis et al. 2023). The use of molecular markers to identify trees receptive to SE induction would greatly facilitate breeding efforts (Sandberg 2023 and in progress). At present, each of the following steps, through embryo maturation and germination, to acclimatization of the planted germinant *ex vitro* and plant growth, all add additional limitations that lead to losses and a reduced number of resulting plants.

In this study, we used the SE protocols that are routinely used by us (Arnold and Clapham 2008) to demonstrate that the results from 158 genotypes are in line with previously published results, showing that all steps of the SE process affect plant formation success. We specifically observed that the highest initiating trees all produced mature embryos whereas those with lower initiation rates did not.

2 Materials and methods

2.1 Selection of trees

The trees were selected from the base populations of plus trees in the southernmost Skogforsk archives that contain approximately in total 3500 genotypes. Out of these we specifically selected 512 that were previously genotyped in a project called "Genomic breeding of Norway spruce for new bio-products also called Bio4Energy" (Baison et al. 2019). From two archives located in either Ekebo (Svalöv) or Maltesholm (Tollarp), 158 trees were selected from over 200 trees based on availability of cones in the trees and accessibility from the aerial lift.

2.2 Collection and handling of cones

Cones were collected from 158 different mother trees at Skogforsk's clone archives in Ekebo and Maltesholm at the time point around 800 degree days in July 2022 by Skogforsk and SLU's School for Forest Management as described earlier in Björs and Sjögren (2023). The cones were transported from Ekebo and Maltesholm in coolers to the laboratory in Skinnskatteberg. Upon arrival the cones were transferred to paper bags and kept in cold storage with a steady temperature at +3˚C (+/- 2˚C).

The cones were sorted by size and by estimated degree of maturation; the cones with closed scales were deemed unripe and placed in cold storage at +3˚C to allow for further ripening. The more mature cones with open scales were selected for immediate cleaning and seed extraction. Two cones from each tree were cleaned with detergent and a brush, then rinsed with tap water for five minutes. Next step was to rinse the cones with 70% alcohol, then the seeds were collected from the cones using tweezers. About 25 seeds were collected from each cone, resulting in 50 seeds from each tree. The seeds were placed in 50 ml Falcon tubes marked with the ID-number of the tree and put into cold storage at +3 ˚C. Before sterilizing the seeds, the degree of maturation of five embryos was determined for each tree following the classification of embryo stages established previously in Pullman and Buchanan (2003). Only trees with embryos at stage 3 or above were included in the study. The seeds were sterilized according to a standard protocol (Björs and Sjögren 2023). Briefly, the seeds were sterilized by immersion for one minute in 96% ethanol followed by 20 minutes in 30% hydrogen peroxide with a drop of Tween and then washed with sterile water. The sterilized seeds were stored in 50 ml Falcon tubes at +3˚C until used for SE initiation.

2.3 Cone processing

In the present study, the cones were initially inspected for coloration and morphology to determine a correlation between detectable cone maturity level and embryo developmental stages present within the cone. Each cone collection contained cones with different appearances designated immature or mature, based mainly on scale appearance rather than coloration after examination of the embryo stages detected within each type of cone (Figure 2a). The developmental stages of embryos within each cone type were to some degree correlated such that immature cones contained more immature embryos (Figure 2b), whereas the targeted stages of early cotyledonary stage embryos were preferentially found in more mature cones (Figure 2c and d).

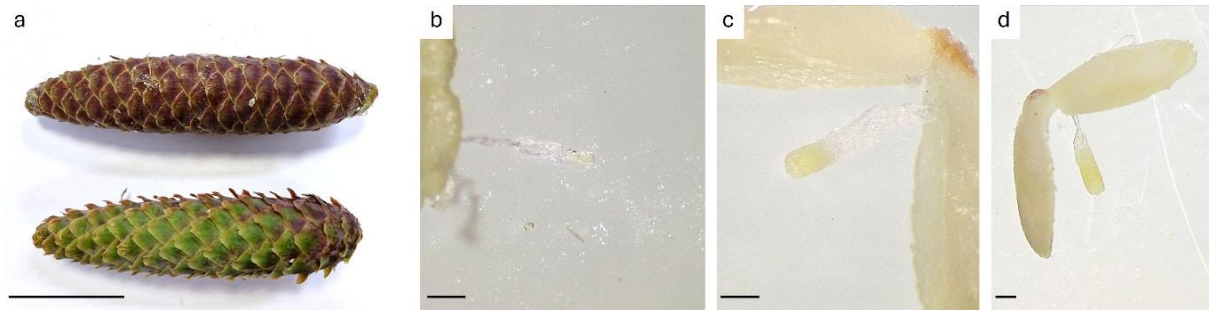

Figure 2. Cones used for SE initiation. (a) The main types of cones in the collection were distinguished by scale appearance where cones with less open scales predominantly contained immature zygotic embryos and cones with more open scales contained early cotyledonary embryos. Scalebar 5 cm. (b) Immature zygotic embryos. (c and d) Early cotyledonary embryos. Scalebars for b, c, and d are 1 mm.

2.4 Initiation of SE cultures

Zygotic embryos were aseptically extracted from the previously sterilized seeds. 10 embryos per tree were placed in 9 cm Petri dishes containing modified ½ LP medium (Arnold and Clapham 2008) with 3.5% Gelrite whilst still attached to their megagametophytes. The cultures were placed in the dark at 20˚C. Zygotic embryos that showed PEM development within six weeks since the start of the initiation process were noted as a successful SE initiation.

2.5 Proliferation of SE cultures by multiplication of PEMs ("capture")

Proliferating PEM masses from the successful SE initiations were subcultured biweekly onto fresh medium of the same composition whilst still attached to the initial explants until about 5 mm of PEM mass had developed, when the PEM mass could be separated from the initial explant. The PEM masses that continued to proliferate after six weeks of biweekly subcultures without the initial explants were noted as 'captured'. The proliferating PEM cultures were maintained at 20˚C in the dark.

2.6 Pre-maturation treatment and maturation of somatic embryos

Pre-maturation, maturation and germination were performed as described in Dobrowolska et al. (2017). Briefly, after around 20 weeks of maintaining proliferating PEMs, approximately 1 g of tissue, each divided into eight clumps, was transferred to 9 cm petri plates (Sarstedt, Germany) containing pre-maturation medium composed of DKM maturation medium devoid of PGRs. Two weeks later, the tissue clumps were transferred onto DKM maturation medium supplemented with ABA (16 mg l−1) and kept on this medium with subcultures every second week for up to six weeks or until mature embryos had appeared. Mature somatic embryos separated from the non-responsive PEM tissue and transferred manually with forceps to 5 cm Petri dishes (Sarstedt, Germany) were subjected to partial desiccation for three weeks. Pre-maturation, maturation and desiccation were carried out at 20˚C in the dark. To enable an equal comparison of maturation capability between SE cultures from specific trees, all the captured PEM tissue from the initiations were transferred for maturation, followed by harvest of all mature embryos for subsequent germination.

2.7 Germination of somatic embryos

Desiccated embryos were placed on solid germination medium containing casein hydrolysate (0.5 g l−1) and sucrose (30 g l−1), with a maximum of 30 embryos per plate. To initiate germination, the embryos were subjected to different light types and intensities each for two-week periods following a modified growth plan from Dobrowolska et al. (2017). Germination was started with two weeks in the dark followed by red light (wavelength: 660 nm; TL-D 18W/15, Philips, Stockholm, Sweden), then white light (Fluora L 18W/77, Osram, Johanneshov, Sweden) at different light intensities, until the embryos had germinated sufficiently for planting.

3 Results and discussion

3.1 Effects from the initial explant

The developmental stage of the seed embryo affects the success rate of the SE initiation. To accurately determine the stages of the developing embryo, cones need to be collected at regular intervals, and the embryo stage checked under the microscope. The optimal developmental stage for SE initiation and cone collection is more or less important for various conifers where; broadly, spruce species are less sensitive, whereas pines typically require careful examination of the embryo developmental stage to respond to the initiation treatment (Pullman and Webb 1994). In this study, the initial examination of embryos collected at the targeted time of optimal 800 day degrees showed that the cone collection contained embryos both at the targeted developmental stage (early cotyledonary) as well as embryos at a lower degree of maturation (pre-cotyledonary) known from previous studies to be less productive or non-responsive. The earlier stage embryos were discarded, and some trees that only contained empty seeds, or too immature embryos, were also omitted from the study. Cold storage for up to eleven weeks of the less mature cones did not noticeably affect the degree of embryo maturation.

3.2 SE initiation frequency

In this study, the average initiation frequency across 158 trees was close to 70%. In a previous study of NS, 274 of 354 embryos (77%) from ten families showed positive SE initiation (Högberg et al. 1998). More recently, a large study of NS based on results from SE initiation over several years (2011 to 2015) from 126 families showed a 59.8% mean rate of initiation from 12,907 initiation events (Varis et al. 2023).

Interestingly, the largest cohort of initiation frequencies at 20% initiation rate also included the highest proportion of failures to capture a proliferating SE culture beyond the first occurrence of PEMs (capture) and thereby failure to establish a proliferating SE culture (Figure 3). The cohorts of higher initiation frequencies also gradually showed higher proportions of captures and at 50% initiation and above, the capture rates were 100%.

Previous studies have suggested that SE initiation success is under genetic control and specifically correlated with the mother tree (reviewed in Egertsdotter 2019). In the present study, there was no statistically significant correlation between the origin of the mother tree and SE initiation rates. This may be explained by the

random selection of trees of various genetic backgrounds and the relatively low numbers of trees included in this study not providing sufficient data for such analysis.

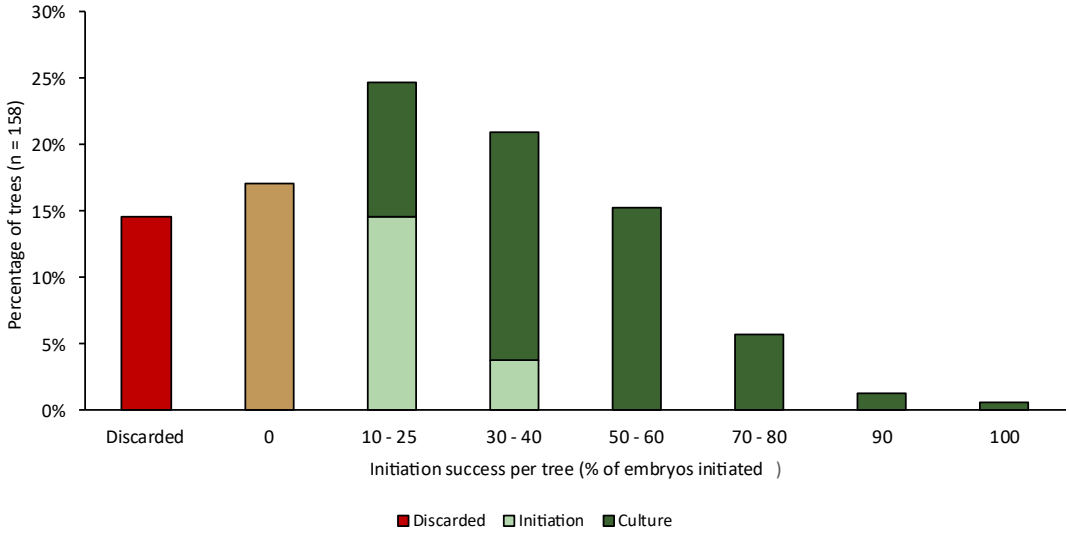

Figure 3. Distribution of trees with successful initiation results (n = 158). Each bar represents the percentage of trees falling into a specific range of initiation success, calculated as the number of embryos with successful initiation divided by the total number of tested embryos per tree. Each bar (light and dark green together) represents successful initiation results, dark green represents the subset of those trees that also produced at least one successful culture (i.e., first capture). Bars are grouped by initiation success rate (shown on the x-axis as percentage intervals), allowing comparison of initiation and ensuing culture development across trees.

3.3 External factors that can affect SE initiation

The proportion of trees with SE initiations increased for each laboratory working week during the first four weeks, with a peak at 90% successful initiations in week 32 (Figure 4). After this week, the percentage of initiated trees dropped significantly ($p < 0.05$). We believe the reason for this difference is that our working methods over time improved over the first four weeks, with less embryo damage during the extraction process. In week five, the training of a new person in the lab started and presumably contributed to the lower output. Furthermore, the cold storage of cones can also have contributed to the increasingly successful initiation attempts, since the seeds that were handled in the laboratory later after collection were also stored longer in the cold room. Cold-treatment has been shown to positively affect germination in Nordmann fir (*Abies nordmanniana*) (Nielsen et al. 2022). From week 36 the trend showed a decrease in initiation frequencies, possibly reflecting a negative effect from the storage time of cones.

3.4 Capture of proliferating culture

After the initial steps of inducing the formation of PEMs from the zygotic embryo, embryogenic tissue composed of multiplying PEMs was isolated from the initial explant (the zygotic embryo) when the PEM-clumps had reached 5 mm in diameter. If the tissue continues to proliferate after isolation for the first two subcultures, it was scored as a first capture of an embryogenic culture previously described for *Pinus taeda* (Pullman and Bucalo 2011). Trees from different origins showed differences in their

responses in both initiation and first capture with performances not correlated (Table 1). The reason for such differences in response is not known but may be related to the developmental stage of the zygotic embryo used as initial explant, as this varies between cones and within cones (Björs and Sjögren 2023). In addition to the initial capture, here we also accounted for a second capture which was scored if the embryogenic culture continued to proliferate beyond two months (Table 2). The loss of initially well-growing PEM cultures during the first few months after initiation is well known and has been recently reported (Varis et al. 2023).

Table 1. Percentage of trees (n = 158) with successful initiation results from each population cluster (Milesi et al. 2019). First capture is considered successful if at least one initiated embryo per tree developed into a proliferating culture.

Cluster	Trees tested	Initiation (%)	1st capture (of initiated trees %)
ALP	26	42.31%	72.73%
CEU	9	22.22%	100.00%
CSE	80	75.00%	83.33%
NPL	12	75.00%	66.67%
RUS	6	83.33%	100.00%
No cluster	25	84.00%	71.43%

Figure 4. Percentage of trees with successful initiations per test week in 2022 (n = 158). The green line represents the proportion of trees each week that produced at least one successful embryo initiation.

3.5 Proliferation of PEM tissue (multiplication phase)

At the stage of proliferation of PEMs, the early-stage somatic embryos (constituting the PEMs) keep multiplying as long as they are kept in contact with regularly refreshed culture medium containing 2,4-D and BA. In this study, proliferation success for each tree was measured after four months by the accumulated number of plates with proliferating PEM tissue. Each plate is prepared at the start of the culture interval by transferring approximately 1 g of PEM tissue divided into 6 – 7 clumps to fresh medium in a petri plate. At the next subculture, the PEM tissue was divided into the same amount of tissue per plate generating several plates corresponding to the proliferation rate of the cell line.

From this stage of development and onwards, 48 trees were selected for evaluation of success rates at different steps and ultimately the relative importance of different steps for the overall SE cloning process. These trees displayed different performances through the steps leading up to proliferation of PEMs where the relatively best performing clusters changed between the three different time-points scored (1st and 2nd capture, and proliferation after four months) demonstrating that an initial good initiation rate does not necessarily give the best multiplying cell line (Table 2).

Table 2. Average number of cell lines and plates per initiated trees (n = 48) of different tested population clusters (Milesi et al. 2019). A = Average number of 1st capture cell lines per tree in each cluster, B = Average number of 2nd capture cell lines per tree in each cluster, and C = Average number of proliferation plates produced by each cell line after the 2nd capture, calculated per cell line and cluster.

Cluster	Number of initiated trees	Number of cell lines 1st cap.	Number of cell lines 2nd cap.	Number of plates 2nd cap.	A	B	C
ALP	3	7	6	28	2.33	2.00	4.67
CSE	29	78	64	190	2.69	2.21	2.97
NPL	2	5	4	28	2.50	2.00	7.00
RUS	3	4	3	10	1.33	1.00	3.33
No cluster	11	29	26	80	2.64	2.36	3.08

The established cell lines from the 48 different mother trees that originated from different population clusters (Milesi et al. 2019) showed different success rates of proliferation (Figure 5).

From these results, it appears that even when the trees are open-pollinated, most of the seeds from those mother tree genotypes that are amenable to SE initiations, will respond to the SE initiation treatment leading to established SE cultures. This agrees with previous findings that demonstrated the relatively higher importance of the mother over the father tree in determining the potential for SE initiation (reviewed in Egertsdotter 2019).

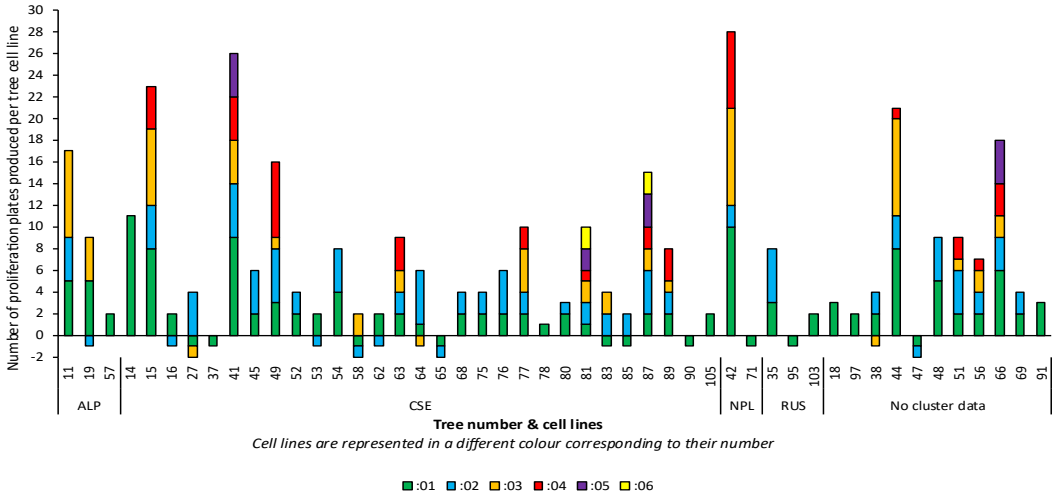

Figure 5. Proliferation success of captured PEMs from SE initiations from different trees and cell lines from individual seeds from each tree one month after the second capture. Amount of PEM tissue accumulation counted as number of plates from the trees that had successful initiation. The relative contributions from different cell lines from the same tree indicated by different colors. Cell lines that did not continue to grow beyond the second capture stage are labelled -1.

3.6 Maturation success from different mother trees and specific cell lines

To get an estimate of embryo-developmental capabilities of the initiated cell lines from the 48 selected trees, all captured PEM tissue from each tree was transferred to the maturation treatment starting with two weeks on pre-maturation medium. The PEM tissue will continue to multiply on pre-maturation medium even though there are no PGRs in the medium. Proliferation on pre-maturation medium differs among cell lines resulting in a change in starting weight of PEM tissue by the time for the onset of maturation when PEM tissue has been transferred to maturation medium containing ABA. If the PEM tissue is then not subdivided and weighed before transfer to maturation medium, calculation of yield based on the starting weight of PEM tissue before pre-maturation will be skewed towards a higher yield per fresh weight. The underlying reason for the continued growth on medium free of PGRs is likely an effect from accumulated 2,4-D in the tissue. The continued effect from auxins on PEM proliferation after transfer to auxin free medium has been demonstrated in *Abies nordmanniana* (Find, Grace and Krogstrup 2002). The biochemical mechanisms for retention of 2,4-D in plant tissue were demonstrated for soybean and carrot and suggested to explain the different responses to SE in these two plants (Montague et al. 1981).

In the present study, we both recorded the number of plates at the start of maturation (Table S1) and estimated the maturation capacity of different cell lines and mother trees by counting the total number of mature embryos generated from all plates of a specific cell line (Figure 6).

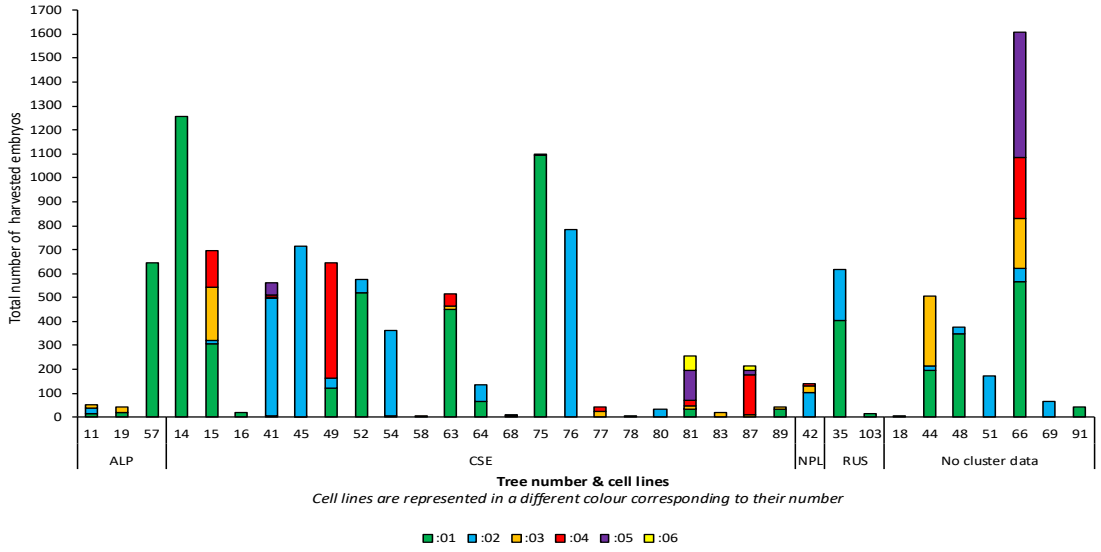

Figure 6. Maturation success from different trees and cell lines from individual seeds from each tree 6 – 7 weeks after start of maturation. The total number of mature embryos for each cell line is shown. The relative contributions from different cell lines from the same tree are indicated by different colors.

3.7 Germination test of mature embryos

The germination capability of mature embryos from different mother trees and cell lines was evaluated by transferring the mature embryos to germination medium. Germination was scored after two to three months as germinated embryos ('germinants') were deemed plantable when they showed approximately 5 mm root

development and some epicotyl development. The number of plantable germinants for each cell line was counted (Figure 7).

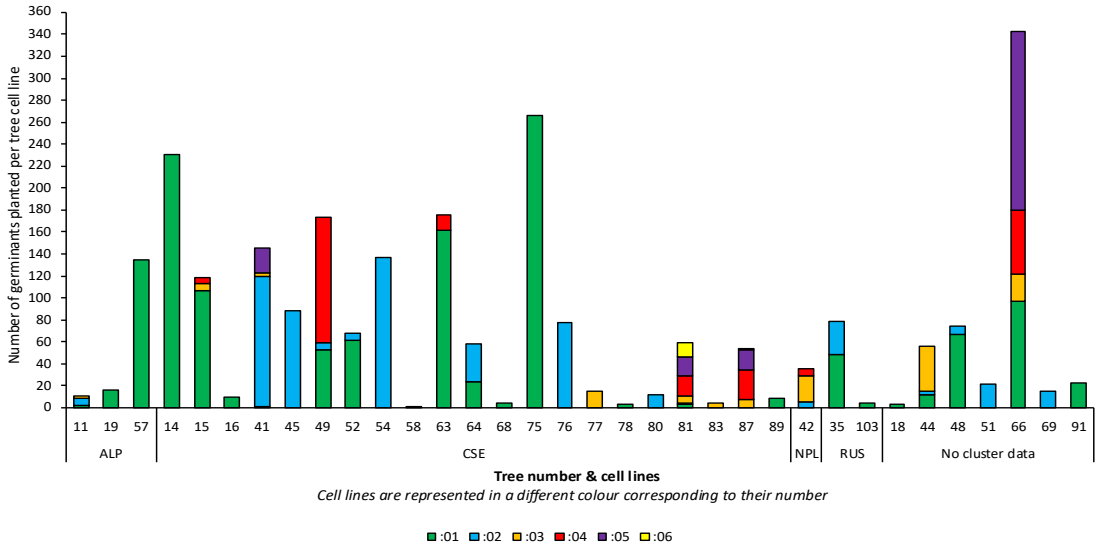

Figure 7. Total number of planted germinants from each of the selected cell lines from different trees.

The ten best cell lines with respect to production of good germinants were selected for further evaluation and imaging of quality. The selected cell lines had all produced over 400 mature embryos each but the results from the germination showed large variations in the capacity to develop further (Figure 8). Cell line 22:63:01 produced the most uniform and more fully developed germinants when compared to the other cell lines. 22:75:01 produced over 1000 embryos, but the majority lacked epicotyl development and had poorly developed roots resulting in poor plant formation, whereas germinants from 22:42:02 showed promising root length but no cotyledon growth.

Figure 8. Overview photo with ruler as scalebar of 30 representative germinated embryos after two to three months on germination medium from each of the 'top ten' performing cell lines with respect to germination success.

Representative germinants from six cell lines showing active epicotyl growth (with one exception) and more than 5 mm root were selected and development after planting *ex vitro* in compost was followed over a seven-week period (Figure 9). In this selection, there appears to be a clear correlation between the presence of an active epicotyl and success of plant formation where the cell lines with weak epicotyl development at the time for planting (22:41:02, 22:87:04) showed poor performance after planting and the cell line with almost no epicotyl development did not continue growth after planting (22:42:02). The importance of epicotyl development has previously been noted for SE germinants (Dobrowolska et al. 2017).

Figure 9. Evaluation of growth of planted germinants from six selected cell lines until six weeks after planting. Three germinants were from the same cell line (22:63:01). Scalebar is 1, cm.

3.8 Evaluation of factors affecting the overall results from the SE process

The success of the *in vitro* SE process is noted in terms of number of established and growing plants *ex vitro*. Each step of the SE process is challenged by losses assumed to be due to suboptimal culture conditions based on the deviation in visible traits of the propagule relative to its zygotic counterpart. The final step of acclimatization from *in vitro* to *ex vitro* is then the ultimate test for the SE-derived propagule and also where significant losses occur, particularly during larger scale production. It has been previously demonstrated that each of the sequential steps of the SE process can affect the outcome from the SE process. A comprehensive analysis of different factors affecting the SE plant production process in Nordman fir showed that the nursery stage of SE plant development was impacted by the germination temperature ($p<0.001$), initial embryo score ($p=0.007$), clone ($p<0.001$) and to a lesser extent, week of germination ($p=0.017$) (Nielsen et al. 2022). Here we have attempted to make a similar but more limited analysis for NS, evaluating the relative effect of initiation rate, yield of mature embryos and germination success (Figure 10). From the present study, the data sets were too small to make any statistical analysis. The results presented show the relative success rates from subsequent steps where the highest yields of germinants come from the medium initiation success rates whereas initiating trees that did not form embryos showed various success rates. The trees with initiation rates above 90, however, all generated embryos.

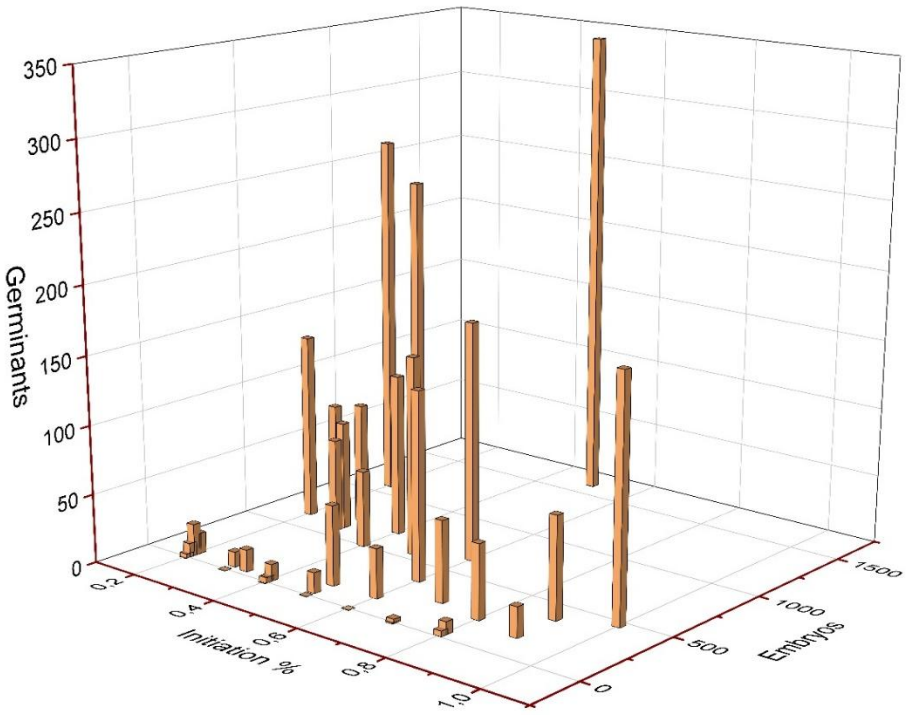

Figure 10. 3D bar chart representing three data metrics for 48 trees: (1) successful initiation rates; (2) total number of somatic embryos; and (3) number of planted germinants. Each bar represents data from an individual tree, enabling visualization of key component stages in the somatic embryogenesis process across different samples.

3.9 Consideration for SE in forestry applications

The starting step of SE initiation in conifers is collection of the cones from selected trees. Zygotic embryos are used for successful SE initiation despite some reports on successful SE initiation from shoots and buds (Klimaszewska et al. 2011; Varis, Klimaszewska and Aronen 2018) which, however, have proved hard to repeat. Cloning conifers using vegetative starting material (like buds or shoots) would be preferred for many applications such as traditional clonal forestry and testing of selected potentially elite mature trees. Micropropagation techniques based on shoot regeneration from buds or callus have been demonstrated for various conifers but so far no protocols have been established for efficient multiplication and regeneration (Sarmast 2018). However, for clonal propagation of families, which is an alternative way of preserving genetic diversity, somatic embryogenesis representing cloning of the seed embryo is preferred as the seeds represent the latest gains from the breeding program (Rosvall, Lindgren and Mullin 1998).

Trade-offs between gain and genetic diversity have been part of the strategic considerations in tree breeding for decades, for breeding base populations and deployment populations (Wei and Lindgren 1995; Lindgren, Gea and Jefferson 1996). By definition, clonal propagation conserves the genotype and thereby challenges the aims for genetic diversity unless accounted for by e.g. applying numerous clones, and/or keeping a family structure in combination with clonal propagation. Despite the uneven rate of germinants noted in this study (Figure 10) and that has also been previously observed in seed orchards (El-Kassaby and Thomson 1996), as long as the relationship and records of numbers of germinants of each clone are kept, for the individual clone representation, it is possible to calculate or even predict the actual diversity of the commercial somatic seedling population. Skewness and uneven contribution due to variable flowering (Eriksson 1973) and nursery practice (El-Kassaby and Thomson 1996) is well known also in zygotic seed and seedling production.

However, climate resilience can be considered for populations as well for individuals combining traits that secure superior adaptation.

4 Final remarks

With this summary of results from a limited range of experiments to clonally propagate seeds by somatic embryogenesis (SE), we offer a basic explanation of the SE method for producing plants and point to some practical hurdles that typically will occur during the process, from starting the cultures of immature somatic embryos through to the planting of germinated somatic embryos. A consideration when using SE is that although the responses to the culture treatments to go through the steps of somatic embryogenesis differ between trees of different genotype and here most tested trees (70%) *did* respond and produced a culture of immature somatic embryos; however, not all continued to develop to form plantable germinated embryos. This limitation on development through all the steps to the planting stage is important to consider when applying SE in breeding programs, or for research projects, as it affects the output in terms of plants needed for replanting, or for evaluating experimental data, respectively. For reforestation purposes, the failure of some trees to produce plants from the SE process may challenge the genetic diversity and should be accounted for when selecting trees for propagation by SE by including extra numbers of trees. With the increasing availability and affordability of molecular analysis techniques for large data sets and

improved genomics resources for conifers, it is feasible to assume that new results will support development of improved SE methods that can provide higher yields at each step of the SE process, and ultimately from all trees, irrespective of genotype.

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