Chapter 7

Advances in Conifer Somatic Embryogenesis Since Year 2000

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Abstract

This review compiles research results published over the last 14 years on conifer somatic embryogenesis (SE). Emphasis is placed on the newest findings that affect the response of seed embryos (typical explants) and shoot primordia (rare explants) to the induction of SE and long-term culture of early somatic embryos. Much research in recent years has focused on maturation of somatic embryos, with respect to both yield and quality, as an important stage for the production of a large number of vigorous somatic seedlings. Attempts to scale up somatic embryo production numbers and handling have resulted in a few bioreactor designs, the utility of which may prove beneficial for an industrial application. A few simplified cryopreservation methods for embryonal masses (EM) were developed as a means to ensure cost-efficient long-term storage of genotypes during clonal field testing. Finally, recent long-term studies on the growth of somatic trees in the field, including seed production yield and comparison of seed parameters produced by somatic versus seed-derived trees, are described.

Key words Cryopreservation, Field tests, Somatic embryos, Somatic trees, Tree improvement

1 Discovery of Somatic Embryogenesis in Conifers and the General Pattern of Somatic Embryo Development

Since its discovery in *Picea abies* and *Larix decidua* [1, 2], somatic embryogenesis (SE) in conifers has been reported in many other species, with a large majority of them belonging to Pinaceae, and only a few to Cupressaceae, Taxaceae, Cephalotaxaceae, and Araucariaceae families. The general differentiation and developmental pattern of conifer somatic embryos is highly similar among most species tested to date and starts with an immature seed embryo (enclosed in a megagametophyte or excised) or with an excised mature embryo that is cultured on a nutrient medium containing plant growth regulators (PGRs) from either both the auxin and cytokinin groups or from cytokinin only. Subsequently, the

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cells of the embryo deviate from their previous pattern of division and differentiation into a mature embryo; instead, they start dividing profusely and differentiate into multiple early somatic embryos also known as embryonal masses (EM). For example, 50 mg fresh mass of white spruce proliferating cell culture may contain, at any given time, approximately 300 single early somatic embryos, 39–75 cleaving early somatic embryos, 60–90 multiple cleaving somatic embryos, small and large cell aggregates, and single small and large cells, the latter with large vacuole(s) (Klimaszewska, unpublished). Typically, the composition of the culture is highly heterogeneous and may change over culture time, a period that may last from several months to several years, during which EM has to be subcultured every 10–21 days (depending on the species) onto fresh medium of the same or slightly modified composition. EM of some species can be cultured on a semisolid medium or in liquid medium. The cultures are amenable to long-term storage through cryopreservation without losing their viability and growth characteristics. In most species, early somatic embryos will not develop further unless the culture conditions are changed. High frequency development and maturation of early somatic embryos in most conifer species of the Pinaceae family take place under remarkably similar conditions, namely in the presence of abscisic acid (ABA), sucrose and/or other sugars, and in a medium that imposes restriction on water availability either by physical means (high concentration of solidifying agents) or by a high molecular weight solute such as polyethylene glycol (PEG, MW 4000–8000) that mimics drought. Often the sugar concentration is also increased to lower the osmotic water potential of the medium. Once the somatic embryos resemble mature seed embryos (usually after 6–12 weeks, depending on the species), they are harvested and desiccated if matured on a medium with PEG for normal germination, or they can be germinated directly if developed on a medium with high gel strength. On a germination medium, the somatic embryos display a rapid radicle and hypocotyl elongation (usually within 1 or 2 days) followed by the growth of a shoot and a root. Once the plantlets reach the desired size, they are transferred into containers with a suitable substrate and acclimatized in a greenhouse or a nursery. Subsequently, they are planted in the field for research purposes, clonal selection or, eventually, for commercial production.

Several reviews describing various aspects of SE research in conifers have been published in both international journals and in books [3–9]. The main focus of the present review is to summarize the progress in SE research in conifers made over the last 14 years and to determine its impact on understanding the basic mechanisms governing the process and on the development of new, more efficient protocols for the production of somatic trees. We also include research results on conifer species for which somatic embryogenesis has been achieved only recently and the results obtained with adult trees.

2 Genetic Control in Somatic Embryogenesis

Perhaps the most basic factor that determines whether SE is initiated from the seed embryos is the genetics of parental trees, providing that suitable culture medium and conditions have been established. Experiments designed to establish the extent of genetic control in SE initiation have been conducted with a few pine species. A large study was undertaken with *Pinus sylvestris* using 49 seed families from diallel crosses among seven elite trees including reciprocals and selfing [10]. Four of the experimental trees were preselected for their propensity for SE based on an earlier study that tested 138 trees. Analysis of the data suggested a stronger maternal than paternal effect on culture initiation; however, specific combining ability (SCA) had no detectable effect. The maternal effect at the initiation stage could be explained by both the genotype and the developmental or physiological stage of the mother tree and the inherited maternal alleles of the zygotic embryo. Similarly, MacKay et al. [11] quantified the genetic control of SE initiation in *P. taeda* using seeds from diallel crosses and factorial matings. Thirty seed families were used in the experiments that tested two different culture treatments and resulted in large differences between treatments in SE initiation frequency among families. The variance due to treatments accounted for 41 % of total phenotypic variance, whereas that due to families accounted for 22 %. Significant variance due to interactions between families and treatments was also found, accounting for 13 % of the phenotypic variance. The latter indicated that different culture media might be better suited for different genotypes. In another study with 20 control-pollinated seed families of *P. radiata*, Hargreaves et al. [12], also challenged the notion that poor results should be attributed to genetic effects only and showed that it was possible to create laboratory conditions that increased the number of responding explants across all families. However, previous work with *P. taeda* showed that many mother trees produce seeds that do not respond to SE initiation, leading to the hypothesis that such trees possess unfavorable alleles at loci expressed in the mother tree, whereas favorable alleles at other loci may be inherited by zygotic embryos [11]. The estimates of large general combining ability (GCA) variance component and narrow sense heritability suggested that targeted breeding could influence SE initiation in *P. taeda*. To test this hypothesis, an experiment was carried out that involved a small number of control reciprocal crosses among trees that ranged from low to high SE initiation capacity when tested with seed from open-pollinated mother trees. By selecting a favorable mother tree for cross-pollination for each pair of parental trees, it was possible to increase SE initiation frequency from 1.5- to 9-fold. Also, some trees had strong additive effects as male parents, but had negative maternal effects; hence, using them in control

crosses as pollen donors might be yet another solution to increase SE initiation. The authors concluded that this knowledge of genetic control in SE initiation can now be easily applied to breeding schemes to capture valued genotypes. Smaller studies with seeds from control crosses of *P. pinaster* [13] and *P. sylvestris* [14] supported the above conclusions.

3 Improvements of Previously Established Protocols for Somatic Embryogenesis

SE biotechnology of conifers has constantly evolved since its discovery in 1985, and incremental improvements are being made according to the time and effort committed to a given species. The literature search revealed that since the year 2000, about 46 journal articles reporting improvements of SE protocols were published for *Pinus taeda*, *Pinus strobus*, *Pinus sylvestris*, *Pinus pinaster*, *Pinus radiata*, *Pinus patula*, *Pseudotsuga menziesii*, *Abies nordmanniana*, *Picea abies*, *Picea glauca*, *Picea mariana*, and *Larix* hybrids. In addition, the publication of approximately 40 articles on species for which SE is described for the first time is a clear indicator of the importance of this technology for conifer clonal propagation. For species of economic importance that are grown in forest plantations, a lot of research has been carried out by companies and patented, for example for *Pinus taeda* (Arbogen, Weyerhauser, WA, USA), *P. radiata* (Forest Genetics Ltd and Arborgen, NZ), *P. abies*, *P. pinaster*, and *P. radiata* (FCBA, France), *Pseudotsuga menziesii* (Weyerhauser, WA, USA), *Picea glauca* and *P. abies* (Natural Resources Quebec, QC, Canada and JD Irving Inc., NB, Canada). Among these economically valued plantation species, the largest body of literature exists for *Pinus taeda* (loblolly pine), which reports on the stepwise optimization approach to overcome low efficiencies at each stage of SE. In their recent review, Pullmann and Bucalo [9] have attributed these improvements to medium supplements including specific sugars, vitamins, organic acids, and redox potential modifiers. Other controlled factors, including medium water potential, pH, adsorption of medium components by activated carbon and use of liquid versus semisolid medium, also positively influenced SE. These modifications resulted from the analytical studies of *P. taeda* seed tissue, the seed environment, and gene expression in megagametophytes, zygotic embryos, and somatic embryos. The premise of the study was that duplication of the seed environment in vitro would lead to the design of efficient protocols for SE.

3.1 Initiation of SE and Growth of Early Somatic Embryos

Major improvements were made in the frequency of SE initiation in either open-pollinated or control-pollinated seed sources of several European and North American *Pinus* species, and *Pseudotsuga menziesii*. In *P. strobus*, the number of responding immature seed

embryos increased from the average of 20 % to the average of 53 % across five open-pollinated seed families by reducing the 2,4-dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA) concentrations from 9.5 to 2.2 μM and from 4.5 to 2.2 μM, respectively. Both concentrations were tested in modified [15, 16] Litvay's medium (MLV). The most striking difference in initiations occurred when the embryos were at the pre-cleavage and early post-cleavage stages, which were also linked to the morphological appearance of the megagametophytes becoming opaque as opposed to being translucent [15]. The same medium modifications were tested with a few control-pollinated seed families of *P. sylvestris* and the result was better on a medium with reduced PGRs, i.e., 24 % initiation versus 9 % [14]. However, contrary to the response of *P. strobus* and *P. sylvestris*, when eight control-pollinated seed families of *P. pinaster* were tested, MLV with reduced concentrations of PGRs decreased the initiation frequency from 93 to 80 %; nevertheless both culture medium variants were very productive [13]. The high response of the latter was also attributed to the selection of embryos that were at the uniform pre-cotyledonary stage of development by excising the embryos from the surrounding megagametophytes. When the embryos were cultured within megagametophytes, the SE response was only slightly reduced, suggesting that in the tested cones the development of embryos was relatively synchronized. This was in contrast to *P. radiata* for which the zygotic embryos had to be excised for the best response [17].

Another medium that is considered suitable for SE initiation in *P. pinaster* and *P. sylvestris* is Gupta and Durzan's medium (DCR [18]). Like other commonly used media [7, 19, 20], it also includes glutamine and casein hydrolysate as well as 2,4-D and BA. Recent research aimed at improving SE initiation in *P. radiata* showed that by making another modification to Litvay's medium (designated as GLITZ) it was possible to achieve considerably higher responses from both 19 open-pollinated and 20 control-pollinated seed families compared with those obtained on Embryo Development Medium 6 (EDM6) [12, 17, 21]. Average initiations were 70 % for both types of seed families when embryos were excised from the megagametophytes at an early stage of development. GLITZ medium contained glutamine (0.5 g/L), casein hydrolysate (1.0 g/L), 2,4-D (4.5 μM), and BA (2.25 μM). Likewise, a 2-year study on SE initiation in *P. nigra* demonstrated higher potential for two out of four tested medium formulations [22]. DCR and MLV media consistently supported approximately 10 % explants producing EM as opposed to Litvay medium (LV [16]) and Quoirin and Lepoivre (QP [23]) media, on which the response was negligible.

Among pine species, SE in *P. taeda* has been the most researched owing to its high commercial value in the USA and

elsewhere, but relatively low responses obtained in earlier work. Not surprisingly, a considerable effort was undertaken to improve the efficiencies of SE and understand the bottlenecks at each stage (*see* ref. 9 and references therein). Various supplements were tested in a unique loblolly pine (LP) medium formulation and found beneficial for SE initiation, such as $AgNO_3$,maltose instead of sucrose, high level of myo-inositol (up to 20 g/L), glutamine, casamino acids, 2-(*N*-morpholino) ethanesulfonic acid (MES, as pH stabilizer), biotin, folic acid, vitamins B_{12} and E, α-ketoglutaric acid, kinetin together with BA, activated charcoal, abscisic acid (ABA), brassinolide as well as D-xylose and D-*chiro*-inositol [24]. The number of initiated SE cultures increased further by adding liquid overlays 14 days after placement of explants on gelled medium. This technique allows replenishment or addition of nutrients and PGRs, or adjustment of pH without disturbing the tissue.

All the medium supplements that were beneficial to *P. taeda* were also tested with immature seed embryos of *P. menziesii*, which differ from those of pine species by the lack of cleavage polyembryony and the need for an embryo to be cultured while partially excised and still attached to the megagametophyte by a suspensor [25]. These tests resulted in the development of an effective medium formulation for initiation of SE in *P. menziesii* that included activated charcoal, ABA, biotin, brassinolide, folic acid, MES, pyruvic acid, D-xylose, and D-*chiro*-inositol in addition to 1 g/L myoinositol, 0.5 g/L casamino acids, 0.45 g/L glutamine and 2,4-D, BA, and kinetin [24, 25]. Tests with seeds from high-value crosses conducted over 2 years gave initiation frequencies of 40 and 57 %, respectively. Based on the above results, a new medium was designed for the culture of mature embryos of *P. abies* that resulted in doubling SE initiation from around 14–30 % when the medium contained 100 mg/L D-xylose. Some other medium additives were asparagine and brassinolide, and the PGRs were α-naphthaleneacetic acid (NAA) and BA [24].

Research on initiation of SE in somatic embryos of *Larix* x *leptoeuropaea* showed that 98 % of cotyledonary somatic embryos matured for 3 weeks produced SE; those matured for 6 weeks produced SE at a frequency of only 2 % [26]. The authors suggested that the loss of ability of somatic embryos to respond to the induction treatment might be caused by the synthesis/accumulation of ethylene, because enrichment of the vessel headspace with ethylene reduced the induction of SE from 3-week-old somatic embryos from 98 to 4 %. Ethylene was also found to influence the development of early somatic embryos as described below.

3.2 Growth of Initiated EM

Once SE is initiated and EM can be identified (after several days to several weeks), the next challenge is to ensure a rapid proliferation of EM upon subculture onto fresh medium to generate the amounts that are needed for various steps, such as cryopreservation

and/or production of mature somatic embryos. The majority of conifer species are usually subcultured onto media of the same composition, but in a few cases, such as *P. pinaster*, it has been shown that medium modifications were required to obtain better growth and/or to maintain the embryogenic potential of the cultures [27, 28]. These modifications included weekly subcultures, substitution of sucrose with maltose and withdrawal of PGRs (2,4-D and BA). To maintain satisfactory growth of *P. radiata* EM, it was necessary to increase the amino acid content in LV medium after initiation [12]. In some species and genotypes, the application of a culture technique that is based on dispersing the cells in a liquid medium, and then collecting the cells on a filter paper, draining the liquid, and placing the filter paper with the cells onto a fresh medium has been the most important for the survival and growth of *P. monticola* and *P. sylvestris* [14, 29].

Embryogenic cultures of *Cryptomeria japonica* (Cupressaceae) were composed of a mixture of EM and callus cells, and when an attempt was made to culture the EM separately; their embryogenic capacity was lost [30]. The culture medium was that of Campbell and Durzan [31], containing 1 μM 2,4-D and 0.6 g/L glutamine; however, when the medium was supplied with 2.46 g/L glutamine, the culture remained embryogenic and simultaneously its dry mass and endogenous level of glutamine increased. The high glutamine treatment might have increased the synthesis of certain macromolecules or metabolites that were essential for SE. The ability of EM to grow in the presence of callus cells was attributed to the high content of endogenous glutamine in the latter that might have supported the growth of EM in mixed culture. Based on the research results of others, the authors concluded that without an adequate supply of glutamine/glutamate, the embryogenic culture of *C. japonica* would lose its embryogenic characteristics. Phytosulfokine, which is a small sulfated peptide, was also found beneficial for *C. japonica* culture growth when included in the medium at 32 nM [32]. This peptide acts as an extracellular ligand at the onset of cell dedifferentiation, proliferation, and redifferentiation and plays a stimulatory role in SE. In particular, phytosulfokine promoted suspensor regeneration from basal cells of somatic embryos of *Larix leptolepis* [33]. The presence of suspensor in somatic embryo development and in the maintenance of the culture embryogenic characteristics was established as critical by Umehara et al. [34] and later by Larsson et al. [35] and Abrahamsson et al. [36] for *Picea abies* and *Pinus sylvestris*, respectively.

3.3 Development and Maturation of Early Somatic Embryos

To promote further development and maturation of early somatic embryos in a majority of conifer species, the proliferating cultures of early somatic embryos are transferred (after a pretreatment or without it) onto a medium with ABA that replaces auxin and/or cytokinin. Most often, the medium water potential is lowered at the

same time by increasing the concentration of sugar(s) and creating permeating osmotic stress, or by including PEG (MW 4000–8000), thus creating a non-permeating osmotic stress, the latter is due to the larger than cellular pores molecule size [37]. An alternative method of affecting somatic embryo development is to increase the gelling agent concentration in the ABA medium, which increases gel strength and reduces water availability to the cells [38].

3.3.1 Abscisic Acid

A study that unequivocally confirmed that ABA was crucial for the normal somatic embryo development and maturation in conifers was carried out with *Larix* x *leptoeuropaea* [39]. This larch hybrid is somewhat unique because it can produce cotyledonary somatic embryos and plantlets on a medium with ABA or without it, hence providing an ideal material for this study. However, the somatic embryos that developed on both media differed in structure, cell types, intracellular secondary metabolites and storage product accumulation, endogenous ABA concentrations, and extracellular mucilage build-up. Clearly, those from ABA medium displayed a coordinated growth and better-shaped somatic embryos with the concomitant accumulation of lipids and storage proteins that were lacking in embryos developed in the absence of ABA. Hence, somatic embryos developed without ABA did not go through maturation. Still, in all conifer species studied to date, a certain number of genotypes in a given species fails to produce mature somatic embryos even in the presence of optimized concentrations of ABA. It has been shown that the ability of embryogenic tissue to utilize ABA from the medium may reflect the capability of embryo maturation in different genotypes of *Picea glauca* x *engelmanni* [40]. The genotypes that produced mature somatic embryos on gelled medium with racemic ABA (equal amounts of (+)-*cis*, *trans-ABA* and (−)-*cis*, *trans*-ABA) were characterized by a greater utilization of exogenous ABA, when grown as cell suspensions, compared with a non-productive genotype. Furthermore, different forms of ABA were metabolized to various levels. For example, only half of racemic ABA was metabolized by the 22nd day of culture; the remainder was exclusively (−)-ABA. The natural ABA ((+)-cis, trans-ABA) was still available at the end of the test, but its amount may be influenced by species, cell density of the initial inoculation, tissue growth rates, and initial ABA concentrations. The natural ABA exerted by far the best bio-effect compared with racemic ABA and the mixture of ABA isomers.

3.3.2 Activated Charcoal

Improvement of somatic embryoquality and yield was achieved by combining ABA with activated charcoal (AC). In *P. abies*, AC introduced into a medium at 0.125 % with 189 μM ABA promoted a zygotic-like appearance of somatic embryos with more elongation and taper in the hypocotyl region as well as formation of a prominent shoot apical region compared with those developed

without AC [41]. These embryos grew faster and were produced at a reduced material and labor cost because the cultures did not require subculturing onto fresh medium. However, the authors cautioned against the types of AC to be utilized as these vary with respect to particle sizes and hence the adsorption properties causing potential deficiencies in the medium components. Alternatively, AC was used with *P. pinaster* by coating the cells with AC and culture on a filter paper placed on the maturation medium [13]. This method of culture resulted in a greater number of mature somatic embryos produced in a shorter time compared with cultures without AC coating. Similarly, *P. sylvestris* aged cultures (24 weeks old) responded favorably when coated with AC, whereas there was no effect on young cultures (8 weeks old) [14].

3.3.3 Carbohydrates, PEG (Medium Water Potential), and Gel Strength (Water Availability)

Changes in water status that occur during conifer zygotic embryo development and maturation are also critical for the progression of the development of early somatic embryos, but the type of compounds used to alter the medium water status must be the "right" type for a given species. For example, when mannitol (a plasmolyzing agent) was tested against PEG (a non-plasmolyzing agent) in cultures of *P. glauca*, the better quality of somatic embryos from the latter was accompanied by the accumulation of higher levels of reduced ascorbate, resulting in a physiological state similar to that of zygotic embryos [42]. Moreover, in the presence of PEG, there was a constant decline in the GSH (reduced)/GSSG (oxidized) ratio of glutathione, suggesting seed-like fluctuations of the ascorbate-glutathione metabolism in somatic embryos. In another study with somatic embryos of *P. glauca* and *P. mariana* it was found that sucrose at 6 % (in the absence of PEG) was highly beneficial when added to the maturation medium for both the number of matured somatic embryos and the accumulation of soluble and insoluble storage proteins [43]. The maturation response could not be matched by osmotic equivalents of glucose and fructose (products of sucrose hydrolysis) in the medium. Moreover, the embryo carbohydrate content was independent from the carbohydrate used in the maturation medium. The same conclusion was later reached for somatic embryos of *P. abies*, where endogenous carbohydrate patterns were stable irrespective of culture conditions, which indicated the carbohydrate status to be a robust feature of normal somatic embryo development [44]. Experiments aimed at the separation of the sucrose osmotic influence from its role as carbon and energy source suggested that sucrose might have an additional regulatory role in the maturation process. In *P. abies*, a medium with 7.5 % PEG and 3 % maltose promoted the development of a large number of somatic embryos, but with low germination frequency in spite of the post-maturation partial desiccation [45]. Conversely, somatic embryos developed on a medium with 3 % sucrose (without additional osmotic agent),

although low in numbers, were able to germinate. A combination of sugar assays, metabolic and proteomic analyses revealed that somatic embryos grown on sucrose medium contained high levels of sucrose, raffinose, and late embryogenesis abundant proteins, all involved in the acquisition of desiccation tolerance (reviewed by Trontin et al., Chapter 8). These embryos also accumulated starch whereas those from PEG and maltose medium had high levels of storage proteins. Therefore the poor germination of *P. abies* somatic embryos grown on PEG and maltose medium was most likely caused by the reduced desiccation tolerance.

Manipulation of water availability to the cells of EM was also achieved by physical means, without affecting water potential of the medium, by increasing the amount of gelling agent that increased medium gel strength and consequently reduced the amount of water available to the cells [16, 46]. By applying this method of water control to *P. strobus* early embryo cultures, high quality mature somatic embryos were produced on medium with 1 % gellan gum that were characterized by a lower water content compared with those from 0.4 % gellan gum medium. Combination of 0.8 or 1 % gellan gum with 6 % sucrose (instead of 3 %) in the maturation medium was even more beneficial because the somatic embryos accumulated higher quantities of storage proteins [47]. An intuitive interpretation of these results is that the developing embryos must have been exposed to the water stress (drought type of conditions) on the media with high gelling agent concentrations, a condition similar to that of a developing zygotic embryo, when the maturation of the embryo is accompanied by desiccation (loss of water). However, in a later study involving cultures of *Larix* x *eurolepis*, an opposite conclusion was reached [48]. The more numerous and higher quality (lower water content) somatic embryos that developed on medium with 0.8 % gellan gum were in fact less stressed than those developed on 0.4 % gellan gum. This conclusion was based on the measurements of physiological parameters and on the two-dimensional (2-D) protein gels that identified 62 proteins that differed between the two somatic embryo groups from the two treatments. Fifty six proteins were subsequently identified, and among them 6-phosphogluconate dehydrogenase (decarboxylating), actin, enolase, fructose phosphate aldolase, phosphoglucomutase, and superoxide dismutase, which are known to be associated with water stress, were expressed at a higher level in somatic embryos developing on medium with 0.4 % gellan gum. In addition to the increased abundance of heat shock proteins in somatic embryos cultured on the 0.4 % gellan gum medium, the observed increases in expression of pyruvate decarboxylase (which directs carbon metabolism toward glycolysis) and apparent detoxification capacity (indicated by the increased expression of superoxide dismutase) suggested that maturation medium containing 0.4 % gellan gum induced a water stress response in the developing somatic embryos. Contrary to this, somatic embryos developed on

0.8 % gellan gum medium accumulated stress proteins at a much lower level. Further evidence supporting utilization of high gellan gum medium for the maturation of somatic embryos came from a large study of *P. pinaster* where multi-scale integrated analysis was used to follow early molecular and physiological events involved in somatic embryo development [49]. Similarly to *P. strobus*, early somatic embryos of *P. pinaster* do not develop on medium with 0.4 % gellan gum; instead, abundant proliferation of EM occurs, which is not conducive to subsequent embryo development. According to the transcriptomic and proteomic analysis results, these cultures had enhanced glycolysis whereas those from medium with 0.9 % gellan gum had adaptive, ABA-mediated molecular and physiological responses marked by active protein synthesis and overexpression of proteins involved in cell division, embryogenesis and starch synthesis. Concomitantly, synthesis of protective secondary metabolites and regulation of oxidative stress were activated, most likely to adapt to the culture conditions. Furthermore, two genotypes of cotyledonary *P. pinaster* somatic embryos, after 10–14 weeks of culture on maturation medium, were compared with zygotic embryos excised from developing fresh and desiccated seeds with respect to dry mass, water content, sucrose and raffinose contents, raffinose/sucrose ratio, and total proteins ([50]; reviewed by Trontin et al., Chapter 8). The study demonstrated that somatic embryos were the most similar to zygotic embryos in seeds collected from late July to early August, and with respect to total protein content up to October (Northern Hemisphere). The somatic embryos, which typically are harvested after 12 weeks, are not at the same maturity level as their zygotic counterparts at the mature, desiccated stage.

3.3.4 Ethylene

There is evidence suggesting that ethylene, a gaseous plant growth hormone produced by cultured plant cells and tissues, may also affect the development of somatic embryos in *P. glauca* [51]. Due to the volatile nature of ethylene, it is difficult to control it in culture. However, the reduction in endogenous ethylene synthesis was achieved by incorporating α-aminooxyamino acid (AOA), a potent inhibitor of ethylene biosynthesis, into the medium, which proved to be beneficial to somatic embryo development. The mechanism of this stimulation was not elucidated, only suggesting that AOA may have interfered with the metabolism of other compounds, most likely through the availability of S-adenosylmethionine, a common precursor for both ethylene and polyamine biosynthesis. In *P. mariana*, it has been shown that limiting ethylene biosynthesis or its physiological action was beneficial to somatic embryo development in a poor line, but not beneficial in a line that was a good embryo producer [52]. These opposite reactions could stem from different initial ethylene levels in the two cultures, one having a super-optimal and the other an optimal level. Later study with cultures of *P. sylvestris* confirmed that ethylene production

varied among five embryogenic lines, both when cultured on proliferation medium with 2,4-D and on maturation medium, indicating a lack of definite trend [53]. Future experiments should include many more genotypes to realize the full impact of ethylene on SE in *P. sylvestris*. Also, any generalization to other conifer species should be avoided.

3.3.5 Antiauxin and Auxin Transport Inhibitor

Among *Abies* species, *A. nordmanniana* somatic embryos were stimulated to develop on maturation medium (with ABA) after a 4- to 8-week treatment with PCIB, 2-(*p*-chlorophenoxy)2-methylpropionic acid, an auxin antagonist that is believed to reduce the activity of endogenous indole-3-acetic acid (IAA) by competitive binding to auxin receptors [54]. *Abies* species do not require an auxin for initiation or proliferation of early somatic embryos and the subsequent problems pertaining to somatic embryo development have been attributed to the high activity of endogenous auxin, at least in *A. nordmanniana*. Treatment of *P. sylvestris* early somatic embryos with the auxin transport inhibitor 1-*N*-naphtylphthalamic acid (NPA) caused the embryos to form supernumerary suspensor cells at high frequency, which led to abnormal development [36]. Although treatment with PCIB increased the yield of somatic embryos, their morphology was not affected, suggesting that the supernumerary suspensor cells in early somatic embryos were stimulated by disturbed polar auxin transport.

3.3.6 Redox Compounds

P. glauca somatic embryo development and maturation were greatly improved through the manipulation of glutathione redox status in EM cultures. By employing a two-step protocol that first included reduced glutathione (GSH) and then its oxidized form (GSSG) in culture medium, which caused a shift in the total glutathione pool towards its oxidized state, proper somatic embryo development was achieved [42]. However, due to the high cost and labor associated with this protocol, a simpler alternative was developed involving dl-buthionine-[*S* , *R*]-sulfoximine (BSO) [55]. BSO is effective in reducing endogenous GSH levels through the inhibition of its de novo synthesis without affecting glutathione reductase, the GSH-recycling enzyme. These changes are similar to those observed when GSH and GSSG are applied sequentially to impose an oxidized environment. To maximize somatic embryo development, BSO concentration had to be at 0.01 mM, while higher concentrations were inhibitory. Therefore, it appears that certain threshold of cellular GSH must be maintained for embryo development to continue. In *Araucaria angustifolia* cultures of EM, manipulation of the GSH/GSSG ratio of the culture medium proved to be beneficial to somatic embryo development up to the pre-cotyledonary stage, but to achieve a complete development would require further modification of the redox potential of the cultures [56].

3.3.7 Inhibitors of SE

When *Larix laricina* early somatic embryos were cultured in high density suspension in a liquid medium the differentiation of suspensors was inhibited, thus negatively impacting the development of new somatic embryos [57]. It was confirmed that an inhibitory compound was present in the conditioned culture medium, which was subsequently purified and the compound was identified as vanillyl benzyl ether (VBE) [34]. Tests with synthetic VBE in the medium produced similar results. Interestingly, the low density suspension cultures also contained VBE, but at much lower concentrations, which did not prevent differentiation of the suspensors. This finding emphasizes the importance of the presence of suspensors in somatic embryo development of a conifer and increases awareness of the influence of cell density on the embryogenic characteristics of a culture. Another modifier of normal somatic embryo development is an inhibitor of polar auxin transport, 1-*N*-naphtylphthalamic acid (NPA), which was tested in *P. abies* [35]. Polar auxin transport is essential to proper embryo patterning and establishment of root/shoot polarity. During early somatic embryo development, treatment with NPA caused an increase in IAA content, abnormal cell division, and decreased programmed cell death resulting in the aberrant development of embryonal tube cells and suspensors. These embryos had abnormal morphology marked by malformed and fused cotyledons and irregular cell divisions at the site of root meristem.

4 Extending Somatic Embryogenesis Protocols to Numerous Conifer Species

Previously published protocols have been utilized, often with slight modifications and with various degrees of success, to test/achieve SE in numerous other conifer species.

4.1 Pinaceae

Among *Picea* species, results that showed regenerated somatic seedlings were published for *P. morrisonicola* [58], *P. koraiensis* [59], and *P. likiangensis* [60]; however, it is not clear whether any of the somatic trees were established in the field.

In the *Pinus* genus, at least 16 new species were reported to display SE; however, for many of them, initiation and/or maturation efficiencies and plant regeneration were low and needed further research and improvements. Notoriously low initiation and survival of EM has been reported for *P. contorta* [61], *P. monticola* [29], *P. roxburghii* [62, 63], *P. pinaea* [64], *P. banksiana* [65], *P. densiflora* [66, 67], *P. rigida x taeda* [68], *P. kesiya* [69], *P. thunbergii* [70], *P. armandii* [71], and *P. luchuensis* [72]. On the other hand, species such as *P. patula* [73], *P. nigra* [22], *P. bungeana* [74], *P. brutia* [75], *P. oocarpa* [76], and *P. halepensis* [77] responded at frequencies ranging from 9 to 30 %.

Significant progress in SE response and plant regeneration has been achieved for *Larix leptolepis* [78, 79], *L.* x *eurolepis* and *L.* x *marschlinsii* [80]. The reciprocal hybrids between *L. decidua* and *L. leptolepis* are important species in Europe, and the first hybrid variety ('REVE-VERT') was registered in France in 2005. SE modified protocols were subsequently tested as a means for rapid cloning of limited numbers of hybrid seeds and resulted in up to 48 % of initial zygotic embryos producing high numbers of vigorous somatic plants (Fig. 1). It is anticipated that SE will influence breeding strategies for these hybrids by offering an additional tool for the production of large quantities of plants for clonal field tests.

SE in *Abies* species has been very challenging, but recent progress made with some species is encouraging. A study with *A. alba* by Krajňáková et al. [81] tested several variables for maturation of somatic embryos, which improved the maturation yield by utilizing a method of spreading the cultures in a layer on Whatman #2 filter paper placed on the surface of a semisolid medium [82] supplemented with 32 μM ABA, maltose, organic N additives, and devoid of PEG. The medium formulation was Murashige and Skoog [83] modified by 50 % reduction in inorganic salt strength. The somatic embryos required desiccation for proper germination

Fig. 1 *Larix* x *eurolepis* "Reve-Vert" somatic seedlings (INRA, France, improved variety) acclimatized at the XYLOBIOTECH nursery (XYLOFOREST platform, www.xyloforest.org) located at the FCBA, Pierroton, France (0.8×)

but still the conversion to plants remained inefficient. In *A. lasiocarpa*, a novel medium was designed based on the elemental analysis of megagametophytes and designated AL, which was free of inorganic nitrogen and in which L-glutamine was supplied at 2 g/L as the sole nitrogen source [84]. AL medium was compared with Schenk and Hildebrand [85], and although initiation of SE (up to 37 %) did not differ significantly between the two media, EM growth after subculture and its survival was better on AL. However, the conversion to plants was very low (approximately 8 % of germinated somatic embryos), which seems to be a norm in this genus. Similarly, in *A. cilicica* and *A. cilicica* x *A. nordmaniana*, relatively high initiations of EM were obtained (63 and 28 %, respectively) but only a third of EM lines developed cotyledonary stage somatic embryos on both maltose or lactose media [86]. In *A. numidica*, both the maturation and germination of somatic embryos were studied. PEG and 6 % maltose in maturation medium were very effective followed by partial desiccation and germination of somatic embryos on medium with activated charcoal and indolbutyric acid (IBA) [87, 88]. In *A. cephalonica*, up to 25 % of the seeds produced EM lines, and somatic embryo development was achieved on medium with PEG and sucrose followed by medium without PEG for up to 12 weeks [89, 90]. However, germination was poor, most likely due to the omission of partial desiccation of somatic embryos before germination, which appears to be a requisite in this genus.

A complete protocol for SE and plant production, including cryopreservation, was reported recently for *Tsuga caroliniana* and *T. canadensis* [91]. Induction frequencies were from 17 to 52 % for immature embryos, respectively. The results confirmed the interplay among the collection date of the cones, medium composition and source tree on the frequency of SE induction, which has been reported in all previous publications. Maturation of somatic embryos was completed by slow drying under permeable plastic film. However, conversion of somatic embryos to plants was very low and requires further research.

4.2 Cupressaceae

Cryptomeria japonica SE was successful with up to 17 % of immature seed explants tested over 3 years, and the presence of PGRs was not required in the initiation medium [92]. The embryogenic characteristics of the cultures could be improved by increased glutamine concentration in the medium [30]. Somatic embryos developed better on a medium that in addition to ABA, PEG, charcoal and maltose, also contained 32 nM phytosulfokine [32].

Two species of *Chamaecyparis* produced plants through SE, namely *C. pisifera* [93] and *C. obtusa* [94, 95]. Initiation of SE occurred on both a medium with PGRs (2,4-D and BA) and on PGR-free medium at the frequency up to 33 and 48 %, respectively. Development of somatic embryos was promoted by PEG,

AC, ABA, and maltose in a medium gelled with 0.3 or 0.5 % gellan gum. Sixty-eight percent of *C. obtusa* EM lines produced cotyledonary somatic embryos, 91 % of which germinated and the plants were subsequently transferred to a greenhouse [95]. In both species, somatic embryo germination was very high and conversion to plants was not problematic. Field tests of *C. pisifera* somatic trees are underway [93].

Another species that was recently studied for its propensity to undergo SE in the same family was *Juniperus communis* [96]. This work confirmed that similarly to *Cryptomeria* and *Chamaecyparis*, the presence of an auxin and/or a cytokinin was not necessary for SE to be initiated and was even inhibitory in *J. communis*, with a reduction from 50 to 25 %. Proliferation of EM was rapid on PGR-free medium as well, but the maturation of somatic embryos was stimulated by 60 μM ABA after brief culture on ABA-free medium with a lower concentration of N and Ca. The development of somatic embryos was highly asynchronous and the culture produced a continuous supply of early and mature somatic embryos. The latter germinated after partial desiccation and converted to plants at a low frequency. A major impediment to plant production was the growth of new EM at the basal part of the somatic plant, which could not be controlled by the application of the gibberellin ($GA_{4/7}$) in the medium.

4.3 Taxaceae

In *Taxus wallichiana*, SE was achieved indirectly from calli that grew on zygotic embryos in culture [97]. Initially, the explants were cultured in the presence of BA and NAA, and after 8 weeks they produced compact yellow calli. Subsequently, when transferred onto a medium with 2,4-D, NAA, and BA, the calli changed in morphology, and two out of four displayed embryogenic characteristics. Somatic embryo development was achieved on medium with ABA and charcoal after 12 weeks; however, the conversion rate to plants was only 10 %.

4.4 Cephalotaxaceae

A complete SE protocol was developed for *Torreya taxifolia* [98]. High initiation of SE (60–100 %) from six seed families was accomplished on medium with various additives, including PGRs (2,4-D, BA, kinetin, and ABA) and maltose. The EMs were cryopreserved using the standard protocol. Somatic embryos developed on a medium with ABA, activated charcoal, maltose, biotin, brassinolide, MES, folic acid, and pyruvic acid. Two genotypes of clonal mature somatic embryos germinated at 64 and 95 %, respectively, and after 2 months the somatic seedlings were planted in a substrate. The species is under threat of extinction and SE will assist in the present and future conservation of this ancient plant.

4.5 Araucariaceae

Despite extensive research to develop SE protocols for *Araucaria angustifolia*, only pre-cotyledonary somatic embryos were obtained in culture [99, 100].

5 Genetic Stability of Cultured Embryonal Mass and Somatic Seedlings

Embryogenic cell lines from ten half-sib seed families of *P. sylvestris* were analyzed using four nuclear single sequence repeat (SSR) markers, also known as microsatellites or short tandem repeats (STR) [101]. The aim was to determine whether the genetic stability of the lines changes during in vitro culture. The results indicated that mutations occurred in the cell lines and that their frequency was dependent on the seed family. Interestingly, mutations were detected in cell lines cultured on both a medium with PGRs and on a medium without PGRs, undermining the present notion that 2,4-D is the main culprit causing genetic instability. The authors also found a considerable mutation rate in zygotic embryos, albeit at a much lower rate compared with cultured EM, which led them to conclude that the in vitro culture stress triggered the mutations in the microsatellite regions in *P. sylvestris*. Whether the instability in the studied microsatellite loci reflect alteration in functional genes remains to be investigated. In a similar study with *P. pinaster*, 17 EM lines from six seed families were analyzed using seven nuclear SSR markers after 6, 14 and 22 months of culture as well as regenerated somatic seedlings [102]. The SSR pattern at the time of line establishment was used as a reference for the cultures of increasing age. Genetic variation was detected in cultures of all ages and in 5 out of 52 somatic seedlings. Some somatic seedlings displayed plagiotropism and loss of apical shoot dominance, but no correlation was found between genetic instability at the analyzed loci and the abnormal phenotype. Nevertheless, there is a risk of genetic mutations during the cell proliferation stage in vitro, which may lead to the regeneration of mutant plants with different mutations occurring among somatic plants regenerated even from the same EM line. The latter could be caused by the presence of a mixture of cells that accumulated different mutations in a given cell line and, hence, the regenerated plants were not clonal. There is some speculation that the loss of embryo development capacity in aged EM lines might be attributed to the accumulation of mutations, perhaps together with epigenetic changes, during prolonged in vitro culture, as described below.

6 Aging of Embryogenic Cultures and its Influence on the Ability to Produce Mature Somatic Embryos

The age of embryogenic cultures maintained on semisolid medium can negatively influence their ability to produce mature somatic embryos in some conifer species. However, aging was not counterproductive in cultures of *Picea* and *Larix* hybrids. In *L.* x *eurolepis*, a line was still productive after 9 years of subculturing [80]. On the contrary, the somatic embryo regeneration ability of cultures of

A. lasiocarpa [84] and *C. japonica* [92] decreased overtime whereas in *Larix leptolepis*, embryogenic cultures became non-embryogenic [103]. The aging and associated changes appear critical in *Pinus* sp. In *P. pinaster*, maturation yield decreased rapidly within 6 months of culture [28, 104]. Reduction in the quantity of somatic embryos or cessation of somatic embryo development was accompanied by substantial modifications to the cellular organization/composition of the culture during proliferation [27]. Total culture time also affected the quality of cotyledonary somatic embryos, with progressive reduction of size and germination rate [28]. However, the embryogenic culture's ability to regenerate cotyledonary somatic embryos could be prolonged by modifying the culture medium composition and subculture frequency [27].

In *Larix* sp., the effect of aging on embryogenic ability has been studied at the molecular level (reviewed by Trontin et al., Chapter 8). Differential expression of various microRNAs (four major miRNA families: miR171, miR159, miR169, miR172) has been detected in embryogenic and in non-embryogenic cultures of *Larix kaempferi* [105]. In particular, miR171 and miR159 were found downregulated and upregulated in non-embryogenic cultures, respectively. Subsequently, the authors identified a MYB transcription factor (*LaMYB33* from *L. kaempferi*) as a target gene for miR159 and *Larix SCARECROW-LIKE 6* homolog (*LaSCL6*) was targeted by miR171 [106]. Post-transcriptional regulation of *LaMYB33* and *LaSCL6* by miRNAs may participate in the maintenance of embryogenic potential as part of the epigenetic complex of regulation of gene expression [103, 106].

To circumvent the recurrent problem of aging, the embryogenic cultures of most conifer species are routinely cryopreserved shortly after initiation (*see* Ozudogru and Lambardi, Chapter 32). Full embryogenic competence of old embryogenic lines could be restored in *P. pinaster* by inducing new cultures from cotyledonary somatic embryos (secondary SE; [104]).

7 Desiccation and Cryopreservation

The ability to cryopreserve EM has been a critical success feature of the development of SE to the level we utilize this technology in the afforestation programs today. The maturation competences of SE cultures under conditions of continuous subculture are highly variable both within and between species. Some species of *Picea* and *Larix* are seemingly unaffected by long periods of minimal or erratic subculture, but many *Pinus* species show a sharp decline in both quantitative and qualitative mature embryo production [28, 80, 107]. These differences, to some degree, influence the urgency to cryopreserve cultures. However, the cost savings associated with not subculturing and the importance of retention of genetic fidelity

and maturation competence make cryopreservation as critical as the other stages of the SE process.

Considering the progress made in cryopreservation over the past decade, key developments include a better understanding of the morphology of cell lines, the interaction of cryoprotectant treatments with these and the development of protocols that facilitate storage of immature and mature somatic embryos [108, 109]. It is also clear that relatively simple pretreatments and freezing protocols are proving as effective as earlier more complex methodologies. Embryogenic cell lines on proliferation media are highly heterogeneous, consisting of a range of cell types in a state of constant differentiation and dedifferentiation. This variability in cell types has been thought to differentially influence the responsiveness of cellular components to osmotic treatment, colligate cryoprotection and controlled cooling [107]. Recent results with a number of species indicate that many of the factors that contribute to successful cryopreservation still remain elusive [108]. The increasing body of work using more differentiated tissues may lead to this being the preferential material for long-term storage of conifers.

7.1 Cryotolerance of Embryonal Masses

Cryotolerance of *P. abies* was studied in association with growth rate, anatomical features and polyamines (putrescine, spermidine and spermine) in five embryogenic cultures [108]. The authors found that the ability to produce normal mature embryos was the only characteristic shown to have a positive correlation with cryotolerance. Of the two lines showing a high percentage of cryotolerance, one was a highly productive line, in terms of maturation ability, the other one had a negligible ability to produce mature embryos. Anatomically, the contrast between the embryo initials for these two lines prior to cryopreservation was striking, with the poor-embryo-producing line showing highly dedifferentiated initials. The same contrast was seen with total polyamine contents, with the two cell lines with the highest contents giving opposite results with regard to cryotolerance at 94 and 0 %. These observations indicate that the factors that confer cryotolerance in EM are yet to be fully elucidated.

New species to show successful recovery from liquid nitrogen storage include *P. nigra* and *P. omorika* [110–112], respectively. More unusually for conifer embryogenic tissues, the pretreatment stages for *P. omorika* were done on semisolid medium with increasing sucrose concentrations followed by air drying of the EM to 20 % of original fresh weight and subsequent immersion directly into liquid nitrogen. No other cryoprotectant agents were used. After cryostorage, *P. nigra* demonstrated growth rates and ability to produce mature embryos similar to the control material maintained in long-term culture [110]. Another less common pretreatment (maltose) and cryoprotectant formulation was applied to *P. pinaster*

that included PEG 4000 with dimethylsulfoxide (DMSO), resulting in 97 % recovery of the cell lines tested [113].

Vitrification using a modified plant vitrification solution (PVS2), developed primarily for nonembryogenic tissues and shoot apices, has been tested with the aim of developing a simplified cryopreservation procedure for conifer embryogenic tissues. Successful vitrification of tissues would facilitate immediate immersion into liquid nitrogen storage without intervening steps including transient storage at –40 to –80 °C in freezers or programmable cooling incubators. This was successfully achieved with some cell lines of *P. mariana* [114]. An encapsulation/dehydration method was tested with immature somatic embryos of *P. sitchensis* and resulted in the regeneration of EM following immersion in liquid nitrogen [115]. No –40 to –80 °C or programmable freezer are required, but the tissue treatment is labor intensive prior to storage.

A novel method for tissue regrowth was tested with *P. radiata* and resulted in significantly improved post-thaw growth with 60 cell lines stored from 6 months to 4 years prior to thawing [116]. The authors used a vigorous culture (nurse culture) of *P. radiata* to nurse the thawed cells; the nurse culture and thawed cells were separated from each other by a nylon screen. Further simplification of methods was achieved with *P. glaucax engelmannii* and *P. menziesii*. The method eliminated both the use of toxic cryoprotectants and freezing environments. Following culture on ABA medium at 4 °C, the tissue was immersed directly into liquid nitrogen [109]. The method relied on preconditioning of early somatic embryos and these retained the ability to regenerate EM following storage in liquid nitrogen.

Contamination of EM lines can still plague this step of the SE process, with a number of authors reporting significant losses of cell lines upon thawing from liquid nitrogen storage [108, 112]. *Picea omorika* cryopreserved as clumps of tissues rather than as cell suspensions had a decreased frequency of contamination if liquid nitrogen was prevented from entering the vials during freezing [112]. In embryogenic cultures of *P. radiata* cell lines stored for 6 months, none of the 37 genotypes (222 vials) were contaminated and only 5 % of the vials from a further 23 genotypes (138 vials) stored for 4 years were contaminated, despite the fact that all vials had been immersed in liquid nitrogen upon freezing [116]. Interestingly, antibiotic cephotaxime (100 mg/L) was used in the proliferation medium to reduce the risk of bacterial contamination of cultures during the frequent treatments before cryostorage of *P. abies* [108].

7.2 Mature Somatic Embryo Storage

Mature somatic embryo storage could potentially confer a range of advantages over the cryopreservation of embryogenic masses and would be especially useful in the application of this propagation technology. Effective storage would facilitate both the synchrony

of seed orchard and laboratory production with seasonal nursery and planting programs. Added advantages are that with careful pretreatment, no cryoprotectant chemicals or programmable freezing equipment is required. Successful desiccation without cryopreservation may also be an important aspect of improving quality and synchronizing germination in somatic embryos in many species. Continued development of direct sowing and artificial seed technologies may also benefit from more effective desiccation protocols.

The desiccation environment seems to be one of the key elements for successful storage. *Picea mariana* and *P. glauca* somatic embryos were slowly dried at 97 or 88 % relative humidity (RH) to reach a water content of 0.23 H_2O g/L dry weight before achieving a high post liquid nitrogen germination frequency of 93.8 % [117]. Desiccation at a lower RH of 63 % had a significantly negative effect on subsequent germination following cryopreservation. Interestingly, the somatic embryos that managed to survive this treatment showed a 100 % conversion to plantlets whereas the conversion of the somatic embryos from the 97 or 88 % RH treatments ranged from 26.7 to 46.7 %. These authors also tested the stored embryo potential for embryogenic tissue reinduction following thawing. Embryos that had been desiccated at high RH (97 %) and were rehydrated for 12 h at 100 % RH had reinduction rates that were similar to those of the controls [117]. Further work from this team has elucidated some of the mechanisms linked to fast desiccation tolerance in *P. mariana* [118]. Their studies showed that an initial short period of slow desiccation of the embryos increased their subsequent tolerance to a fast desiccation treatment. The mechanisms behind this indicated an increase in sucrose accumulation, occurrence of raffinose, and depletion of starch reserves within the somatic embryos. The occurrence of dehydrins was also investigated in reference to their suspected role in the development of desiccation tolerance. The authors noted a doubling of the dehydrin signal intensity after 48 h of slow desiccation (24–48 kDA), which coincided with the best treatment for subsequent germination of rapidly desiccated embryos.

Picea glauca and *P. glauca*×*engelmannii* complex somatic embryos were gradually dried over salt solutions to the level of dry seed embryos and retained their viability upon rehydration [119]. Desiccated somatic embryos also survived subsequent freezing in liquid nitrogen, without the addition of cryoprotectant or preculture steps. Highest survival (>80 %) after freezing in liquid nitrogen was in embryos pre-dried to Ψ of –15 to –20 MPa, which yielded relative water content (RWC) close to predicted bound (apoplastic) water values. In another study, somatic embryos of *P. glauca* survived a rapid desiccation treatment (2 h of air drying on a laminar flow bench at ambient temperature and humidity) if they were carefully preconditioned on maturation medium [120].

The optimum treatment was leaving embryogenic tissue on maturation medium for 51 days, making it possible for embryos to become cotyledonary before placing the Petri dishes into 5 °C for 8 more weeks of incubation. It should be noted that in *P. glauca*, cotyledonary embryos are fully developed after 51 days and precocious germination was observed in some embryos prior to incubation at 5 °C. In contrast to the results presented for *P. mariana* (and *P. glauca*) [118], shorter periods of incubation were detrimental to the quality of the germinant following rapid desiccation [120]. Elucidation of the mechanisms behind the cold tolerance of *P. glauca* somatic embryos has subsequently shown, with freezing damage tests based on electrolyte leakage, that somatic embryos matured at lower temperatures possessed significantly higher freezing tolerances than somatic embryos matured at 20 °C [121].

8 SE from Vegetative Tissues of Adult Conifers

Vegetative (also known as clonal) propagation of adult trees has a major advantage over propagation through seed because large genetic gains are achieved by capturing a large proportion of tree genetic diversity in a single selection cycle [122]. Hence, vegetative propagation of select superior forest conifers through SE is highly desirable, particularly because it has the potential to deliver a stable supply of superior seedlings for forest plantations. However, in spite of decades of research efforts, efficient propagation of adult conifers by any means is still beyond reach [61, 123, 124]. The first work that raised expectations in this area of research was induction of SE in buds (primordial shoots/needles) of 2- to 3-year-old *P. abies* grown from a somatic embryo [125] and *Ceratozamia* spp. [126], but no results on somatic plant growth have been published. Using four genotypes of somatic trees of *P. glauca*, it was subsequently demonstrated that one genotype produced SE from primordial shoot explants (Fig. 2a–f) consistently from age 2 (in 2002; [127]) to 15 years (in 2015; Klimaszewska, personal communication). The media for each stage of SE were the same as those used for seed embryo SE and a large number of juvenile propagules (somatic seedlings) have been grown in a greenhouse and subsequently planted in the field. These somatic trees derived from donor trees of increasing chronological and ontogenic ages

Fig. 2 (continued) (17.5×). (**c**) Primordial shoot cut longitudinally and showing slightly elongated needle primordia (24×).Fig. 2 (continued) (**d**) Embryonal masses growing from the explant after 33 days (magnification 25×). (**e**) Mature somatic embryos produced from embryonal masses induced from the same donor trees of different ages; 2, 7, and 8 years old (0.5×). (**f**) Somatic seedlings cultured on germination medium for 7 weeks (1.1×). (**g**) Clonal, juvenile G6 trees produced from primordial shoots collected from 7-year-old (on the *left*) and 8-year-old (on the *right*) donor trees (1×) growing in the nursery of NRCan-CFS, Valcartier, QC, Canada

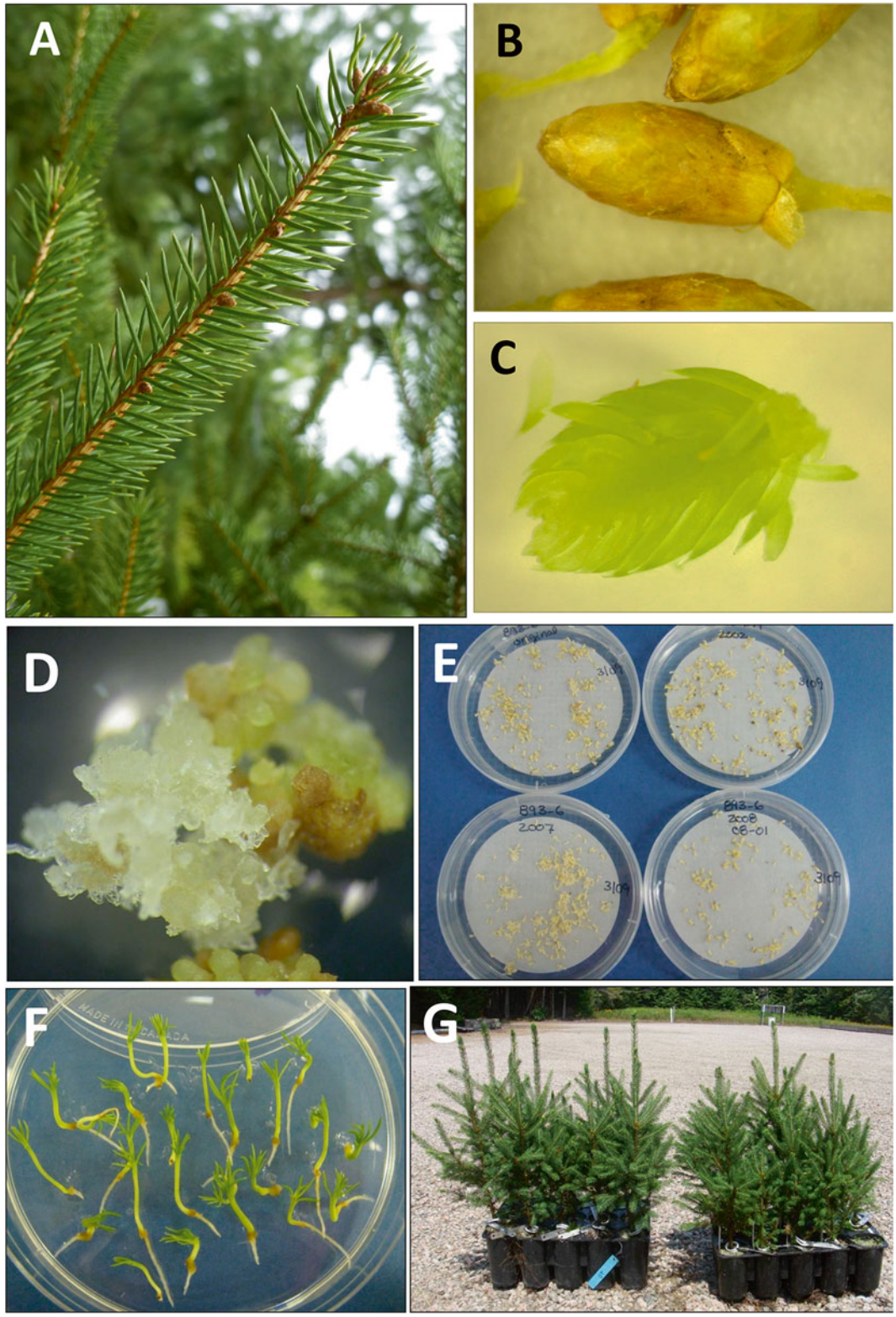

Fig. 2 Induction of somatic embryogenesis (SE) within primordial shoots of somatic *Picea glauca* 13-year-old trees, genotype G6. Vegetative buds were collected on May 6, 2013 from a plantation established in 2003 by NRCan-CFS in Valcartier, QC, Canada. (**a**) A branch with pre-flush buds (2.5×). (**b**) Cleaned and disinfected buds

are being evaluated for their growth rate and morphology, and are expected to provide evidence of true rejuvenation. Simultaneously, the donor trees of responsive and nonresponsive genotypes provided a unique opportunity to examine the molecular aspects underpinning SE within shoot tissues of adult *P. glauca* trees (reviewed by Trontin et al., Chapter 8). A 32,000 oligo-probe microarray was used for transcriptome-wide expression profiling of explants at day 0 and day 7 of culture, which led to the identification of four of the most differentially expressed genes in each of the two genotypes [128]. The absolute quantitative PCR (qPCR) of these genes was expanded to 21 days of SE induction and showed that the expression of all eight genes was maintained throughout the induction period. In contrast to the responsive genotype, explants of the nonresponsive genotype expressed high levels of stress-related genes, such as two extracellular serine protease inhibitors, a cell wall invertase, and a class III apoplastic peroxidase, whereas the former showed temperate expression of these genes. Instead, high expression of dehydrins and the QT-repeat and proline rich proteins that are conifer-specific were identified in the responsive genotype and suggested an adaptive stress response. These results further suggested that the possible causes of the lack of SE induction in an explant may not be necessarily due to an innate lack of SE promoting activity, but that biotic defense activation could potentially be a dominant antagonist. Therefore, future work should focus on determining how and if suppressing biotic defense activation could be used to promote SE induction in nonresponsive explants.

9 Field Growth of SE Trees

Clonal forestry offers significant advantages for forest productivity due to the genetic gain (volume and quality improvements) that can be realized through selection and mass propagation of elite individuals (clones) [129]. Somatic embryogenesis, with its capacity for long-term germplasm cryopreservation and scale-up technologies, is the preferred avenue to accelerate the selection and operational deployment of value-added genotypes, especially through multivarietal forestry [4, 7]. Over the past decade, more information has become available from field performance trials of planting stock derived from SE. As described in an earlier review, the majority of reports was for *Picea* spp. due to their responsiveness to SE relative to other genera [4]. A number of early trials with *Picea* spp. and *P. menziesii* have been established for several decades and while the *Pinus* spp. have been more recalcitrant to SE, some information is available [7].

Further studies looking at the possible long-term effects on field growth of somatic seedlings caused by in vitro conditions

was undertaken with *P. abies* [130]. The somatic plants were assessed for survival and early growth after 4 months in the field. The authors confirmed that prolonged exposure to ABA during the maturation period of somatic embryo formation inhibited early growth. Another treatment of continuous light routinely given to *P. abies* seedlings to improve early growth in the greenhouse had a negative effect on the growth of somatic plants. The authors concluded that direct inwintering of somatic plants after transfer to ex vitro conditions should be avoided. Early greenhouse work studying clonal variation in morphology, growth, physiology, anatomy, and ultrastructure of 6-month-old container-grown *P. glauca* somatic plants found a number of differences when compared with zygotic seedlings of the same families [131]. Height ranges of clones were greater (14.4–31.8 cm) than that of seedlings (15.8–24.3 cm), and root collar diameters were generally greater in clones. Variation within families was larger among somatic clones than among zygotic seedlings for height, needle dry mass and branch density. Light microscopy showed that tannins were more abundant in somatic plants than seedlings; otherwise all needle samples displayed a similar morphology. Of more concern was the incidence of root deformation in somatic plants which had to be transplanted from culture vessels to styroblock containers. Only 52 % of somatic plants had a normal root form, a rate that is comparable with that observed in zygotic seedlings that were not transplanted. Another interesting observation was that plants from specific clones suffered from copper deficiency symptoms in all replications despite fertilizer application. What was clear and encouraging from this study was the early screening potential for selection of superior clones based on both physiological and morphological characteristics [131]. Subsequent work presenting pooled data that compared zygotic seedlings and somatic plants of *P. menziesii* for gas exchange rates, water relations and frost hardiness after 2 years in the field concluded that there were no significant differences between the two stock types [132]. However, no data was presented for individual clone performance. There were only three clones in this trial derived from control crosses versus the seedling controls, which were from bulked open pollinated seed collected from the same orchard. When considering frost hardiness and bud break, no significant differences were found between the two stock types in the latter study. More recent work raised the possibility that there may be some interaction between temperature at the time of somatic embryo maturation and subsequent frost hardiness and bud break especially in the first few years of plant establishment [117]. Based on field performance studies, it appears that clones produced from SE, at least those of *P. menziesii* and *P. glauca*, can be highly acclimated to different climatic conditions [133, 134].

Evaluation of genetic parameters and examination of genotype x environment interactions to characterize the genetic stability of somatic seedlings of *P. glauca* have been done 4 years after establishment of the field tests [134]. In these tests, 52 clones (from 14 control-crossed families) were compared and they are the first of a series of trials established under different ecological site conditions comparing over 1000 somatic clones. Encouragingly, the percentage of somatic seedlings (52 clones) exhibiting normal adaptive characteristics for survival (98–99 %) and bud frost damage and stem form (90–99 %) characteristics were high, and therefore, genetic parameters were not calculated for these characteristics. Strong positive genotypic correlations were found between height, diameter, annual shoot length and volume. The authors felt that the stability of the clonal performance at the two sites reflected the efficiency of clonal selection and was therefore a good reason to promote the selection of generalist clones for future applications in multiclonal forestry [134]. Older somatic plantings (5.5 years) of *P. menziesii* var. *menziesii* have been assessed for survival and performance, clonal genetic parameters such as variances, heritability, and correlations, and for stability of clonal performance across five sites in Washington and Oregon, in the Pacific Northwest, USA [133]. There were 70 clones in the test and the somatic seedlings were grown in the same greenhouse for 1 year prior to planting. All exhibited growth rates and morphology within the normal range exhibited by zygotic seedlings in nurseries. The survival of the somatic seedling clones at 5.5 years ranged from 92 to 99 % and the general conclusion from this study was that the stability of these clones was encouraging for future clonal forestry applications in coastal Douglas fir.

A set of *P. radiata* trials was established in New Zealand and Australia (three in each country) to investigate clonal stability focusing on growth and form traits [135] (Fig. 3). The planting stock was derived from cuttings taken from hedges established from somatic embryos rather than using germinated somatic embryos directly. One reason for this approach was to improve plant quality within clones (height, root mass, and stem diameter, all of which were positively affected). There were 245–280 clones tested at the three New Zealand trials and 44–69 clones at the three Australian sites. In general, clonal stability was good across the New Zealand sites, and although there was only a small number of clones that were common between Australia and New Zealand, clones stable for growth could be identified across both countries. The authors did note that age 5 may still be too young to draw firm conclusions with regard to genotype rankings. Forest Genetics Ltd. planted their first trials of *P. radiata* derived from somatic seedlings in 1999. They have been able to clearly identify outstanding clones, which now form the basis of field-proven material being sold to commercial clients (www.forest-genetics.com).

Fig. 3 Somatic *Pinus radiata* in a field test of the Forest Genetics Ltd., New Zealand

These plants command a premium price relative to seedlings of control pollinated seed lots. Evaluation of somatic seedlings has been also ongoing in France since 1999 with *P. pinaster* for which ca. 3200 clonal trees from more than 200 genotypes were established in eight field tests [7]. Data analysis at age 6, from 24 clones planted in 2004, indicated that somatic seedlings are producing normal trees but usually with a lower initial growth rate than those from seedlings (Trontin, personal communication). However, it has been shown that mean relative increase in height was similar or even higher in specific somatic lines after 6 years, suggesting that normal growth can be recovered later.

Seed production from somatic clonal trees has been studied in *P. mariana* [136]. The authors found that the somatic trees produced both viable pollen and female cones that were able to be crossed to produce equally viable seeds. The authors noted that male strobili were produced about 6 years after planting and 2 years after the early onset of female flower production, which is earlier than what is generally observed in zygotic trees. The authors also noted the incidence of albino germinants (up to 14 % in one particular inbred cross). No direct non-somatic clone controls were used in this research to determine if earlier male and female cone production had an adverse effect on vegetative growth. Results were generally compared with other data available for *P. mariana* and there were no outstanding anomalies (pollen germination, seed mass, and morphophysiological standards for planting stock). The authors concluded that the stock produced

through SE and selected for exceptional performance could be used for subsequent seed production and would enhance gains from multivarietal forestry.

10 Bioreactor/Scale-Up Studies

For commercial application of SE, laboratory-scale protocols must be scaled up and fulfill several criteria such as production of high quantities of uniform somatic embryos at a given time and at a reasonable cost per unit. This can be achieved by utilizing bioreactors, which are amenable to automation and allow continuous monitoring and control of growth conditions (agitation, pH, oxygen, and carbon dioxide), large volumes, and maintenance of a homogeneous culture. In a study with *P. sitchensis*, two EM lines were grown in bioreactors of different configurations (air-lift, bubble, stirred tank, and hanging stirrer bar) and compared with shake flask cultures [137]. The bioreactors were 5, 2, or 1 L in volume. Both lines exhibited larger increases in biomass when grown in bioreactors, but one line proliferated as single early somatic embryos while the other one formed large aggregates of somatic embryos. Samples taken from all cultures were transferred onto maturation medium with 40 μM ABA, 1 μM IBA, 3 % sucrose, and 0.1 % activated charcoal. There were two medium variants in Petri dishes: one semisolid (0.6 % agar) and another semisolid covered with 7 mL of liquid medium for submerged culture. One line produced cotyledonary somatic embryos at the highest number when proliferated in bubble bioreactor on both variants of maturation medium. For the second line, the submerged way of culture was unsuitable. The results suggested that the bioreactor configuration, design, and operating conditions must be adequately chosen to suit the physiological, metabolic, and morphological characteristics of a line. For *P. menziesii*, a large-scale somatic embryo production system was developed by Weyerhauser Co. (WA, USA) [138]. It involved growing the EM in 1 L flasks in liquid medium on a rotary shaker in darkness, followed by culture in perfusion bioreactors on liquid medium soaked pads containing PEG, ABA, $GA_{4/7}$, and charcoal for development and maturation. The development medium was pumped from the reservoir into the bioreactor until it made contact with the lower surface of the pads. The medium was absorbed in the pads by capillary action and, after a few hours, it was pumped out to the reservoir. This was repeated at regular intervals until mature cotyledonary embryos developed. Twenty to fifty cotyledonary somatic embryos were produced from the initial 1 mL of settled EM, but different genotypes showed variations in both the number and quality of mature somatic embryos. The somatic embryos were cold-treated before germination. The authors concluded

that the solution for low-cost mass production of conifers was the combination of liquid culture with bioreactors, automation technology, and manufactured seed delivery system.

11 Conclusions and Future Research

The multidiscipline approach that is taking place to find solutions to some of the problems still facing large-scale production of conifer trees through SE should prove fruitful in the future. SE research has generated knowledge and protocols that can be immediately applied from one species to another and have their utility verified. For example, in *Pinus* species and other conifer genera where initiation of SE is proving problematic, the viability of crosses prior to sampling needs to be considered. Following sampling for embryogenesis, a further collection of cones should be made when the seed is mature to verify that the seed is viable. It has been shown with a range of conifers that embryos often form early in development only to abort prior to becoming cotyledonary, especially in situations where self-fertilization or hybridization may be occurring. In some cases, SE may be a way of rescuing these embryos that could be advantageous, especially with the establishment of novel first generation hybrids. However, for general protocol development, it is better to have known high viability crosses to work with. Another confounding factor with protocol development is the presence of the megagametophyte, which is likely to have a confounding effect with regard to development of an optimal induction medium. The megagametophyte tissue dies as soon as it is dissected away from the seed coat and may start to produce toxic leachates. Examination of the literature does show mixed responses and it is possible that the megagametophyte acts as a buffer against suboptimal media interactions, and that it even provides some nutritive benefits in the short term. It is recommended that both methods of dissection be tested as part of the matrix including media modifications when protocols are being developed or improved for conifer species. Optimization of culture medium should benefit from using software tools for experimental design, computation, and graphic visualization of multifactor interactions that together influence the culture productivity from its onset in vitro to the plants in a greenhouse/nursery as demonstrated for herbaceous species by Halloran et al. [139] and Adelberg et al. [140].

We expect to see increased integration of somatic embryogenesis within current nursery practices. In the laboratory, methods to reduce plant production costs will continue to develop; these include liquid culture and sorting mechanisms for mature somatic embryos. Somatic embryos of a number of conifer species are amenable to organogenesis, and this step will be used to provide

plants for stool bed and subsequent cutting regeneration. Where physiological age constraints limit the life of stool beds, liquid nitrogen storage will ensure a continuous supply of juvenile stock plants. Soft tissue manipulation robotics for medical procedures is improving as well as visual multidimensional graphics, and this may create new opportunities for automation of the SE process.

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