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Conifer somatic embryogenesis: I. Development

Abstract: The discovery of conifer somatic embryogenesis (SE) and the subsequent development of SE protocols for a range of genera and species have opened new research opportunities to forest biotechnologists and a means towards mass clonal propagation for the forest industry. This paper provides a general description of the conifer SE process, followed by a review of protocols developed specifically for several conifer species in the *Pinaceae* family for which production of somatic trees has been demonstrated. Additionally, future research needs, including approaches for developing markers to optimize SE process and the production of high quality embryos, are discussed.

Additional key words: cryopreservation, initiation, maturation, *Pinaceae*, somatic seedlings

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Introduction

Somatic embryogenesis (SE) depicts an asexual process that leads to the formation of an embryo from somatic cell/s. Somatic embryos are capable of germinating and producing plants that perform similarly to their zygotic counterparts. This particular attribute of SE, in combination with its potential to provide a large number of plants in a short period of time, has made it an attractive method for clonal propagation.

The phenomenon of asexual SE exists *in vivo* in many angiosperm species as a result of apomixis. In conifers, however, SE can only be induced *in vitro* from seed explants (immature and mature zygotic embryos) and, in some species, from seedling explants (cotyledons, shoot apices, young needles, Lelu et al. 1994a; Attree et al. 1990; Mo and von Arnold 1991; Salajova and Salaj 2001). Thus far, however, only the seed explants provide practical efficiencies of SE initiation and ease of handling. Nevertheless, there are continued research initiatives directed at developing protocols to induce SE from vegetative explants that are derived from juvenile and mature

trees (Bonga 1997; Ruaud 1993; Harvenget et al. 2001; and patent by Pâques and Bercetche 1998). Cloning of mature trees would be the most effective way to capture genetic gain (Park and Bonga 1992) but thus far it has remained a challenge.

At present, propagation of conifers through SE requires regeneration of somatic seedlings from a number of embryogenic lines, representing various seed families, for clonal trials. During the clonal testing period, the embryogenic cultures are stored in liquid nitrogen (–196°C, cryopreserved) under the premise that the juvenility of the clones and their genetic fidelity are retained. These embryogenic lines can be thawed at will, re-grown to the desired amount and then used for the mass production of clonal plants.

Apart from the potential applications, SE is of fundamental biological interest and has often been compared to zygotic embryogeny, particularly with reference to the “normal” development and phenotype of somatic embryos. Over the last decade, much information has been accumulated about the requirements to achieve this “normalcy” via the manipulation of conifer SE *in vitro*. This includes, among

others, knowledge of empirical selections of the explant, medium and culture environment; therefore well-established principles are currently in place.

This overview focuses only on the recent and most successful SE protocols (either published or patented) for certain conifer species belonging to the *Pinaceae* family, which resulted in the production of a number of somatic trees. This is not meant to undermine research results published by various scientists whose valuable work has either become the basis for protocol improvements or has not been pursued further due to specific circumstances.

History of conifer somatic embryogenesis

The first reports on conifer SE from seed explants were published for *Picea abies* (Chalupa 1985; Hakman et al. 1985). Interestingly, these publications were preceded by a patent issued to El-Nil (1980) (Weyerhaeuser Company) on a discovery of SE in Douglas-fir, illustrating that a disparity exists between industrial and academic research. To date, in the family *Pinaceae*, close to 41 species and hybrids belonging to five genera (*Abies*, *Larix*, *Picea*, *Pinus*, and *Pseudotsuga*) have been reported to undergo SE. By far, the largest body of work has been directed towards SE in *Picea* spp., particularly *P. abies*, *P. glauca*, *P. glauca* × *engelmannii*, and *P. mariana*. This has been due, in part, to the relative ease of SE initiation and regeneration of somatic seedlings from somatic embryos as compared to the other genera in the *Pinaceae* family, as well as to the economic importance of these species to the forest industry.

Current status in SE protocols and cryopreservation

SE in conifers is a multistage process. Each stage represents different challenges, and these are often dependent on the outcome of the previous stage. It is important to note that at each stage of SE, there is an attrition of embryogenic culture lines that will proceed from one stage to the next and produce plants; this has to be incorporated in the planning strategy when the application of this technology for clonal selection is considered. In general, the SE process is divided into several stages:

Initiation – onset of embryogenic cultures, typically from immature (all conifers) or mature zygotic embryos (primarily spruce), on semi-solid nutrient media with cytokinin (firs) or with a combination of auxin and cytokinin (all conifers). The end of this stage is marked by the explants showing a visible growth of embryogenic tissue that is composed of early somatic embryos, single cells and cell aggre-

gates. The frequency of SE initiation from immature embryos, in addition to other factors (i.e., genetic), strongly depends on the developmental stage. This stage of SE does not require light and the cultures are typically placed in darkness at approximately 22 to 25°C.

Proliferation (maintenance) – establishment of embryogenic cultures (embryonal masses) and continuous growth (increase in fresh mass) upon periodical subcultures onto a fresh semi-solid (all conifers) or liquid medium (spruces, some pines, Douglas-fir), usually of the same composition as the one used for initiation. For fast increase of the fresh mass of embryogenic tissue, recent protocols recommend dispersing the embryogenic tissue in a liquid medium and then culturing the embryogenic tissue that has been thinly spread on a filter paper disc placed on the surface of a semi-solid medium.

At this stage, the vigorously growing embryogenic culture may be cryopreserved. If not cryopreserved, the embryogenic tissue must be subcultured onto a fresh medium every 12 to 21 days. This stage of SE does not require light.

Maturation of somatic embryos – development of immature (early) somatic embryos through histodifferentiation into cotyledonary embryos on a nutrient medium that typically contains abscisic acid (racemic ABA) and provides reduced water availability to the developing cultures. The modulation of water availability is performed either by decreasing osmotic potential of the medium (addition of osmotically active solutes) or by increasing the medium's gel strength (physical means), or by a combination of both. A developed somatic embryo that morphologically and physiologically resembles a zygotic embryo marks the end of this stage, which lasts 6 to 12 weeks. This stage of SE most frequently proceeds under low light intensity (5 to 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 16h photoperiod).

Some protocols apply a pre-maturation step, which involves a brief (3 to 7 d) culture of embryogenic tissue on a medium devoid of plant growth regulators (PGR) and containing activated charcoal prior to the transfer onto a maturation medium. The medium can be either liquid or semi-solid.

Post-maturation treatment of somatic embryos – the mature somatic embryos can be either partially desiccated at a high relative humidity 98% (RH) prior to germination or dried to a low water content at a low relative humidity for short or long-term storage, respectively. The aim of partial desiccation of somatic embryos prior to germination is to reduce the water content and/or to complete the maturation process. However, partial desiccation is not necessary if the water content of mature somatic embryos is sufficiently low at the end of the maturation period. This may occur on media that contain higher than standard concentrations of gelling agents, in which case

the resulting mature somatic embryos are characterized by a high conversion rate to phenotypically normal plants.

Germination and conversion to plants – somatic embryos are usually germinated *in vitro* on a semi-solid nutrient medium that contains sucrose and may or may not contain a source of organic nitrogen and activated charcoal. This stage is completed after the elongation of an epicotyl and the development of needles occur, most frequently after 12 to 16 weeks, depending on the species. If the somatic embryo maturation medium contains a gelling agent concentration that is higher than the one routinely used, then the germination medium should also have an elevated level of this compound (but lower than in the maturation medium) to prevent hyperhydricity in the developing plants (Klimaszewska, personal observation). The light intensity is low for the first two weeks of germination ($5 \text{ mol m}^{-2} \text{ s}^{-1}$, 16 h photoperiod) and then it is gradually augmented during the growth of plantlets (up to $40 \text{ mol m}^{-2} \text{ s}^{-1}$, 16 h photoperiod).

Early growth ex vitro – establishment of *in vitro*-grown somatic seedlings in a substrate under greenhouse conditions. Typically, during the first 2 to 3 weeks of growth, a high relative humidity is provided to facilitate the plants' acclimatization to ambient conditions.

Cryopreservation – storage of conifer embryogenic tissue in liquid nitrogen was first reported for *Picea glauca* by Kartha et al. (1988). This protocol, with minor modifications, has been extended to numerous genera and species (Cyr 1999). The general approach is to facilitate the gradual removal of free water from the embryogenic cells and to minimize the formation of intracellular ice by using slow cooling. Most current protocols entail the incubation of a tissue suspension in a proliferation medium of decreased osmotic potential for a period of 24 to 48 hours. In general, 0.4 M sorbitol is used as the osmotic agent; this treatment is followed, just prior to freezing, by the addition of 5 to 10% (v/v) dimethylsulphoxide (DMSO). Then, vials are cooled at 0.1 to 0.5°C per minute in a programmable chamber or via incubation at -80°C for 1 to 2 hours using alcohol-insulated containers (Cyr et al. 1994; Percy et al. 2000). Upon reaching a terminal temperature of -35 to -45°C , cultures are plunged in liquid nitrogen and stored in the vapor (-140°C) or liquid (-196°C) phase. For regeneration, vials are rapidly thawed for 1 to 2 minutes at 37°C , the storage solution is removed via draining and the cultures are transferred onto a fresh semi-solid medium. Growth of cultures typically occurs within 1 to 2 weeks after thawing.

Picea spp.

Compared to other conifers, SE of spruce species has been the most successful and most advanced

commercially. Out of the 11 species reported to undergo SE process to date, five species are being evaluated in clonal trials and/or in large-scale propagation programs. These species are: *P. glauca* \times *engelmannii* (Webster et al. 1990; Sutton et al. 1993), *P. sitchensis* (Krogstrup 1990; Cyr et al. 2001), *P. mariana* (Adams et al. 1994), *P. glauca* (Park et al. 1998; Lamhamedi et al. 2000), and *P. abies* (Högberg et al. 1998, 2001).

The most frequently used nutrient media for spruce SE include the one described by von Arnold and Eriksson (1981) at full or half-strength and by Litvay et al. (1985) at half-strength or half-strength macroelements and full strength microelements. Media are supplemented with organic nitrogen (L-glutamine and casein hydrolyzate). Germination is carried out either on the same media or on media that do not contain organic nitrogen (e.g., Mohammed et al. 1986).

The initiation of SE is on average, always higher from immature zygotic embryos than from mature ones. This response also varies depending on the species, the developmental stage of the embryos and the seed family. Media typically contain 2,4-dichlorophenoxyacetic acid (2,4-D) at 9.0–10 μM , benzyl adenine (BA) at 4.5–5.0 μM , sucrose at 1 or 2 % and are solidified with agar (0.8%) or gellan gum (0.4%). Media of the same composition are usually used for the proliferation of embryogenic cultures.

The maturation of somatic embryos and generation of plants in spruce species occur by following protocols published by several research groups. These protocols may be summarized as follows:

1. Pretreatment (7 d) on a PGR-free medium, either liquid or semi-solid (the latter usually with 0.5 g/l activated charcoal), followed by culture on a semi-solid medium (0.8 % agar) containing ABA at 40 to 60 μM , IBA at 1 μM and 3.4% sucrose for 5 weeks. The cotyledonary somatic embryos must be partially desiccated prior to germination (5 to 15% water loss).
2. Pretreatment (3–6 d) on a PGR-free medium, either liquid or semi-solid (0.4–0.45% gellan gum or 0.8% agar) and usually with 0.5 g/l activated charcoal, followed by culture on a semi-solid medium containing ABA at 30 to 60 μM , 3% sucrose and 5 to 7.5% polyethylene glycol (PEG 4000) for 6 to 8 weeks. The cotyledonary somatic embryos must be partially desiccated prior to germination.
3. No pretreatment. Embryogenic tissue clumps are placed on a semi-solid medium (0.4 to 0.6% gellan gum) with 22 to 40 μM ABA and 6% sucrose. A post-maturation treatment is not required for high frequency germination.
4. No pretreatment. Embryogenic tissue is dispersed in a PGR-free liquid medium. A suitable volume of suspension is then poured over a filter paper circle in a Büchner funnel, the liquid medium is completely drained, and the filter paper with cells is

placed on the surface of a maturation medium for 8 to 9 weeks. This medium contains 60 μ M ABA, 3 to 6% sucrose and 0.6 to 0.75% gellan gum. No post-maturation treatment is necessary for high frequency germination.

Germination of cotyledonary somatic embryos occurs on semi-solid media containing 2 or 3% sucrose, 0.4 to 0.6% gellan gum or 0.54 to 0.9% agar.

The fact that spruce species respond to these various protocols is indicative of the amenability of this material to undergo somatic embryogenesis. It is important to note that many protocol modifications that contribute to the improvement of any SE stage are being introduced on an ongoing basis, particularly when the purpose is to produce plants for field-testing. Implementation of one or another protocol will ultimately depend on the production cost and efficiency in large-scale propagation programs, as well as evaluation of the long-term effects of various tissue culture treatments on the plant performance under field conditions.

***Pinus* species**

Sixteen species of *Pinus* have rendered positive results when tested for SE response but only a few species were more extensively studied. In these cases, a range of seed families was evaluated during multiyear programs that resulted in the production of plants. These species include *P. taeda* (Becwar et al. 1990; Li et al. 1998a, b and patents by Becwar et al. 1996; Handley 1997, 1998; Rutter et al. 1998), *P. radiata* (Smith 1997 and patents by Aitken-Christie and Gough 1996; Aitken-Christie and Parkes 1996; Smith 1996), *P. pinaster* (Lelu et al. 1999; Ramarosandratana et al. 2001a, b), *P. sylvestris* (Keinonen-Mettälä et al. 1996; Lelu et al. 1999), *P. nigra* (Salajova et al. 1999), *P. strobus* (Garin et al. 1998; Klimaszewska et al. 2001 and patent by Klimaszewska et al. 2001) and *P. monticola* (Percy et al. 2000).

Initially, it was assumed that the early protocols developed for spruce species would be applicable to pines; with time, however, it became apparent that pines were less responsive to these approaches. First of all, pine embryos dissected from mature seeds did not initiate SE or if they did, it was at a very low frequency. Second, the somatic embryo maturation was of poor quality when media with low ABA concentration and low osmolality were applied. Media used for pine SE include those described by Gupta and Durzan (1985), Becwar et al. (1990), Litvay et al. (1985) (modified to contain half-strength macroelements), Smith (1996), and Teasdale et al. (1986) (modified). Media are supplemented with an organic nitrogen source (L-glutamine and/or casein hydrolyzate). Germination is carried out either on the same media or on media that do not contain organic nitrogen.

Presently, there are two methods that can be used to produce somatic plants in pine species; one method involves high molecular weight PEG (M_r 4000) for somatic embryo maturation, followed by a cold treatment and partial desiccation of mature somatic embryos. The other method involves a reduction in water availability by physical means (i.e., by increasing the medium gel strength) to produce mature somatic embryos with low water content.

For *P. taeda*, SE initiation from immature embryos was found to improve on a medium with lower than standard gelling agent concentration, from 0.12 to 0.2% gellan gum, and in the presence of 14 μ M 2,4-D, 2.2 μ M ABA and 3% sucrose. An inclusion of ABA (40 μ M) in the medium was found to increase the initiation efficiency for some seed families. For maintenance, the medium is the same as above except that ABA is removed and the gellan gum concentration is 0.2%. At any time, the established embryogenic tissue clumps can be transferred from a semi-solid medium to liquid medium of the same nutrient and PGR composition but containing 0.5 g/l activated charcoal, for the establishment of cell suspension. For maturation, a suitable volume of cell suspension is poured over nylon membrane circles and the liquid medium is drained in a Büchner funnel. The membranes with cells are then cultured on a development/maturation medium containing 7% PEG, 476 μ M ABA, 1.25% activated charcoal and 6% maltose. For proper germination and plant production, the cotyledonary somatic embryos require low temperature treatment (4°C, in the dark for 4 weeks) on a medium without PEG and activated charcoal but containing 80 μ M ABA and 6% maltose. This treatment presumably circumvents the negative effect of PEG on germination. Somatic embryos are then partially desiccated at a high relative humidity (98%) for 3 weeks prior to germination on a medium containing 3% sucrose, 0.2% gellan gum and 0.5% activated charcoal. Approximately 40% of the somatic embryos have been germinated and produced plants that were planted in the field.

For *P. radiata*, SE is initiated from immature embryos that are dissected out from megagametophytes and cultured on a proprietary medium (with or without nurse tissue) containing 3% sucrose, 60 μ M ABA and 0.45% gellan gum. No auxins or cytokinins are necessary at this stage, but the medium may contain one or more amino acids (L-glutamine, arginine, asparagine, citrulline, ornithine, lysine, alanine, proline). The overall initiation frequency over eight seed families can reach approximately 10%. Interestingly, a medium of the same composition may be used for the maturation of somatic embryos, presumably due to the presence of ABA (the gellan gum concentration can be augmented at least transiently to 0.6%). The cotyledonary somatic embryos are then subjected to a starvation treatment at high RH (analo-

gous to partial drying) for maximum germination. Germination and plant conversion is carried out on a medium containing 3% sucrose and 0.45% gellan gum.

For *P. strobus*, *P. monticola*, *P. pinaster* and *P. sylvestris*, SE is initiated from immature embryos enclosed in the megagametophytes on semi-solid media with 2% sucrose and 0.3 to 0.4% gellan gum. For *P. strobus* and *P. monticola*, it was shown that media with low concentration of 2,4-D (2.2 M) and BA (2.2 M) promoted higher initiation frequencies than media with standard concentrations (10 M 2,4-D and 5 M BA). Moreover, the embryogenic tissue of *P. strobus* maintained on a low PGR medium produced on average more mature somatic embryos than the ones maintained on a standard PGR medium. This PGR adjustment resulted in over 50% initiation averaged over 14 seed families of *P. strobus*. However, in spite of the improvements made to the medium composition, the initiation in *P. monticola* remained below a practical level when averaged over a number of tested seed families. *P. pinaster* SE cultures may also be initiated on a medium with higher than standard concentration of 2,4-D (13.5 M 2,4-D in presence of 2.2 M BA) and sucrose (6%). Media of the same composition are usually used during the proliferation stage.

Maturation of early somatic embryos is best achieved by dispersing the embryogenic tissue in a liquid PGR-free medium, dispensing a suitable volume of suspension onto filter paper circles, draining the liquid and placing the filter paper with cells onto the maturation medium. Recent protocols emphasize the importance of using media with ABA at 60 to 120 M ABA, high gellan gum concentration (0.9 to 1.0%) and sucrose or maltose at 3 to 6%. The maturation period varies from 9 to 12 weeks. Developed somatic embryos are characterized by high plant conversion frequency without the need for prior desiccation. Germination and plantlet production is carried out on media with 2% sucrose and solidified with 0.45 to 0.6% gellan gum. A large number of *P. strobus* and *P. pinaster* somatic plants have been produced for field-testing studies.

Pseudotsuga menziesii

There is only one journal publication on SE process of Douglas-fir (Durzan and Gupta 1987); all subsequent advancements in this technology are included in various patents (Gupta and Pullman 1991, 1996; Gupta 1996; Carpenter et al. 2000). Media used for SE include those described by Murashige and Skoog (1962) and Gupta and Durzan (1985), each supplemented with casein hydrolyzate and L-glutamine.

The initiation of SE proceeds from immature embryos that have been placed on the medium but remain attached to the megagametophytes via the suspensor, producing a frequency of 1 to 10%. This is in

contrast to the type of explants used in other conifer species where immature embryos are typically left enclosed within the megagametophytes or dissected out. The patented protocols for plant production through SE involve a sequence of six different media; the first is used for initiation and is followed by two maintenance media, a medium for singulation (of early somatic embryos clumped together), one development/maturation medium, and one germination medium. The second maintenance medium and the singulation medium are liquid. The maturation medium may also be liquid, in which case the cells from the second maintenance medium are poured over a polyester pad soaked with 10 ml of maturation medium in a Petri dish. It is clear from the issued patents that the conditions for each SE stage are being constantly revised to ensure a high quality product resulting from a process that is also economically viable. The main proprietary claims relate to the manipulation of the osmolality of each medium that is suitably adjusted by supplementing the media with various amounts of carbohydrates. Another claim pertains to the use of adsorbent materials, such as activated charcoal, in the maturation medium.

The initiation medium contains high concentrations of auxin (2,4-D 500 M) and cytokinins (kinetin 200 M and BA 200 M), sucrose (1.5%) and charcoal (0.25%), and is solidified with 0.5% agar. For proliferation, the cultures are transferred onto a medium with reduced concentrations of auxin (5 M) and cytokinins (each at 10 M) and containing sucrose at 3% or maltose. The singulation medium may contain ABA at 19 M to 57 M gibberellic acid (GA₃) at 0 to 15 mg/l and 2% sucrose or maltose. Somatic embryo development proceeds on a semi-solid medium (0.3% gellan gum) or liquid medium that may contain ABA (up to 190 M), GA₃ (0.5 to 25 mg/l), activated charcoal (0.25%) and a high concentration of sucrose (6%). Cotyledonary somatic embryos are then germinated on a semi-solid medium (0.8% agar) devoid of PGR and containing 2% sucrose. In some genotypes, improvements in the cotyledonary embryo germinability can be achieved by prior partial drying at 98% RH for 4 days. Relatively high numbers of somatic plants can be produced following these protocols.

Larix spp.

Six *Larix* species and hybrids have been evaluated for SE, but efficient protocols using immature seeds have been developed only for *Larix × leptoeuropaea* (*L. leptolepis* (kaempferi) × *L. decidua*) (Lelu et al. 1994b, c). SE initiation frequencies varied depending on the developmental stage of the embryos, with 39% on BM3 medium (Gupta and Durzan 1985) and 55% on MSG medium (Becwar et al. 1990) achieved for several control-pollinated seed families. Media were sup-

plemented with 9 M 2,4-D, 4.5 M BA, 3% sucrose and 0.7% agar. For proliferation, the cultures were placed on MSG medium containing 9 M 2,4-D, 2.2 M BA, 2% sucrose and 0.4% gellan gum.

Large numbers of mature somatic embryos were produced after a one-week pretreatment on MSG with 1% activated charcoal, 3% sucrose and 0.4% gellan gum followed by culture on a MSG medium with ABA (40 to 60 M), IBA (1 M), 6.4% sucrose and 0.4% gellan gum.

The most recent protocols for the proliferation and maturation of the somatic embryos entail spreading the tissue on filter paper circles for maximum efficiency. For *L. × leptoeuropaea* embryogenic tissue, this culture technique led to a four-fold increase in the number of mature somatic embryos compared to the culture of comparable fresh tissue mass in clumps (Lelu, personal communication). Somatic plants were regenerated on a MSG PGR-free medium that contained 3% sucrose. Approximately 1000 plants were established in a field plantation at INRA (Orleans, France) in 1993.

To our knowledge there is currently no major program that focuses on the optimization and utilization of this technology in any larch species for commercial use.

***Abies* spp.**

Four fir species and two hybrids have been reported to undergo SE; however, plants were regenerated in a consistent manner only in *Abies nordmanniana* (Nørgaard 1997).

A variety of medium formulations were used in SE initiation of firs, including modified versions of Murashige and Skoog (1962), Schenk and Hildebrandt (1972) and Gupta and Durzan (1985) media. These media, also used for culture proliferation, typically contain cytokinin (BA) as a sole PGR at 5 M, 1 to 2% sucrose and 0.18 to 0.3% gellan gum. Somatic seedlings have been obtained by maturing somatic embryos on media containing ABA (10 to 80 M), PEG 4000 (7.5 to 10%), maltose (3%) or sucrose (2%) and solidified with gellan gum (1.8 to 3%), followed by mild desiccation of somatic embryos and germination on a medium containing 2% sucrose and 1% activated charcoal. Recently, an improvement in the quantity and quality of somatic embryo maturation of *A. nordmanniana* has been achieved by including p-chlorophenoxyisobutyric acid (PCIB), an auxin antagonist, in the maturation medium to prevent or minimize the undesirable proliferation of cultures (Find, personal communication).

To date, there is no published comprehensive report that would evaluate the potential of SE for clonal propagation of any fir species. The challenges identified so far are low SE initiation frequency, mortality of the initiated embryogenic lines in culture and no or

low maturation frequency of somatic embryos. It is clear that more research and development effort is necessary to bring fir SE technology to the application stage.

Indicators/markers of somatic embryo maturity/quality

Current maturation protocols lead to a development of mature somatic embryos that morphologically resemble zygotic embryos. These somatic embryos are harvested after arbitrarily chosen periods of time, usually 6 to 12 weeks, and are germinated and further grown in a greenhouse. Determination of the harvest time is decided on the basis of visual observation and past experience. This procedure only gives an indication that the maturation was successful if produced somatic embryos were able to convert to plants. However, such an empirical approach does not give any information of the quality of somatic embryos with respect to storage reserves accumulation or water content, nor does it give information on the optimal time for harvesting to achieve maximal plant conversion rates. Therefore, there is a need to develop marker/s that could be used for quality control of different batches of somatic embryos that are matured, or when different maturation protocols are applied.

One approach is to follow the evolution of storage reserves accumulation (such as proteins, lipids, carbohydrates, and dehydrin type proteins) in zygotic embryos and compare it to the accumulation pattern in maturing and mature somatic embryos. The anticipated outcome would be a biochemical marker of maturity/quality for somatic embryos. Research in this area has thus far been done with *Picea abies* (Hakman et al. 1990; Gösslová et al. 2001) on storage proteins and carbohydrates, *P. glauca* (Attree et al. 1992) on lipids, and with *Pinus* spp. (patent by Carpenter et al. 2000) on carbohydrates deposition and the presence of dehydrins. The latter claims that somatic embryos having elevated levels of sucrose series oligosaccharides and dehydrin group proteins exhibited improved germination frequency.

Future research directions

Research issues can be segregated into SE process steps and genotype response. Process steps that will require significant advances include the frequency of SE initiation and the development of synthetic seed technology, which is directly linked to embryo quality. SE initiation protocols in most species (except *Picea*) require the use of immature zygotic embryo explants thus limiting efforts to short seasonal windows. More importantly, initiation response is low (i.e., *Pinus* spp.) and results in the inability to capture

certain families. This has caused great concerns among breeders and foresters of potential adverse selection pressure. Ultimately, the area of greatest interest is developing the ability to obtain SE from tissues of mature trees. With respect to artificial seed, development and operational demonstration will be needed for somatic embryos in image analysis, machine separation, encapsulation or pelletization, storage packaging and automated seeding.

The improvement of genotype response to SE will have a significant impact on the development of a predictable and cost-effective operational process. However, the current reality is that the quality and yield of SE within and among seed families are highly variable. Emphasis on biochemical and physiological studies will be important for understanding this variability, with the ultimate aim to develop SE culture protocols that normalize response among genotypes at each step of the process. This will also aid the development of biochemical markers that can be used as selection or rejection criteria in a commercial process. Similarly, molecular tools can be developed for the detection of somaclonal variation to ensure genetic fidelity. These may help determine when such a change occurs, and may thus eventually lead to the optimization of tissue culture protocols that would eliminate this potential problem.

Conclusion

It is evident from the accumulated empirical knowledge that there are critical factors controlling the various stages of SE in conifers. However, to make use of this knowledge for such goals as mass propagation, the researchers must understand how these factors, active in one stage, influence behavior during subsequent stages. With informed manipulation of these factors, embryogenic cultures will not only exert their maximum potential for propagule production, but the somatic embryos produced will have the same vigor and germination that characterizes their zygotic counterparts.

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