Cryotolerance in Norway spruce and its association with growth rates, anatomical features and polyamines of embryogenic cultures

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Summary Our study focused on the possible association between the cryotolerance of Norway spruce (Picea abies (L.) Karst.) embryogenic cultures and the anatomical structures of their embryogenic suspensor mass (ESM), their growth rate and their content of endogenous polyamines (PAs). The anatomical characteristics and PA content during cryopreservation and regrowth were studied in the ESMs of AFO 541 and C110 cultures, which have comparable ESM anatomy but diverse growth rates, PA content and regeneration abilities after cryopreservation. Different levels of tolerance to exogenous treatment were already apparent after transfer of the ESMs to liquid media. The endogenous free PAs were maintained at high levels, with spermidine being the predominant PA in the ESM of AFO 541, while in the ESM of C110 the content of putrescine and spermidine was almost identical and rather low, the content of spermidine being approximately one-third that in the ESM of AFO 541. Osmotic pretreatment, using a double application of sorbitol followed by an application of dimethyl sulfoxide (DMSO) resulted in the continual disintegration of polymorphic centers and suspensors in both cell lines. A continual decrease in the level of PAs was observed during the cell osmotic pretreatment. The cells that retained their viability and regrowth ability after cryopreservation were the meristematic cells inside the embryonal heads and the cells in the intermediate area between suspensor and meristems. Restoration of AFO 541 growth after cryopreservation was almost immediate; however, the C110 ESM culture regrew with difficulty, often exhibiting callogenesis. The possible role of PAs in the process of cryopreservation of Norway spruce cultures is discussed.

Keywords: cryopreservation, Norway spruce (Picea abies (L.) Karst.), somatic embryogenesis.

Introduction

Cryopreservation offers the potential for the economical and reliable long-term storage of genetic resources by creating a stable physico-chemical state for germplasm where conventional methods such as seed-banking are inappropriate (Cyr 1999).

The main difficulty of cryopreservation is the lethal damage caused by the formation of intracellular ice crystals. To prevent such damage, cells must undergo several preparative steps before storage at −196 °C. A double application of sorbitol followed by the application of dimethyl sulfoxide (DMSO) (Kartha et al. 1988) has been used in the cryopreservation of Picea sitchensis (Bong.) Carr. (Find et al. 1993), Pinus patula Scheide ex Schltd. & Cham. (Ford et al. 2000), Pinus roxburghii Sarg. (Mathur et al. 2003, Malabadi and Nataraja 2006) and other species. The success of these processes is strongly influenced by the physiological status of the plant tissue used for cryopreservation and its ability to regenerate (Find et al. 1998).

The embryogenic suspensor mass (ESM) is well suited for cryopreservation because of its fast growth rate and its large population of meristematic cells. Moreover, partially differentiated cultures, such as those produced in conifer somatic embryogenesis, exhibit less variation than callus-type cultures (Wang et al. 1993). The ESM of some coniferous species, such as Pseudotsuga menziesii (Mirbel) Franco, Picea glauca (Moench) Voss and Pinus pinaster Solaud., non Ait. are quite tolerant to cryopreservation, while other species show marked differences in their cryotolerance (Norgaard...
et al. 1993a, 1993b, Cyr et al. 1994, Park et al. 1994). Large genotype variations in cryotolerance have been observed among 70 different embryogenic cultures of Norway spruce (Norgaard et al. 1993a) and among angiosperms (McLellan et al. 1990).

The plasma membranes of cells are the primary site of freezing injury during the process of cryopreservation. Oxidative damage of membrane phospholipids and proteins, severe dehydration associated with freezing and the mechanical injury of cells can all influence cell survival rates and recovery rates following cryopreservation (Harding 2004). Polyamines (PAs) stabilize the molecular composition of membranes and suppress lipid peroxidation, thereby preventing membrane injury, retaining plasma membrane permeability and reducing leakage under stress conditions. In plant cells, PAs occur not only as free molecular bases but may also be covalently linked to phenolic acids, usually hydroxycinnamic acids (soluble conjugated PAs), as well as to high-molecular-mass substances such as hemicelluloses and lignins and in small amounts to proteins (insoluble conjugated PAs). The diamine putrescine (Put), the triamine spermidine (Spd) and the tetraamine spermine (Spm) participate in the control of such important processes as cell division, growth, morphogenesis and differentiation (Paschalidis et al. 2001, Theiss et al. 2002). The high flexibility of PA metabolism in response to environmental stress, the metabolic link between PA and ethylene synthesis and their inevitable role in cell division and proliferation strongly suggest that PAs may play a significant role in cell survival after cryopreservation. To our knowledge, only one report (on work with sucrose pre-cultured banana meristems) has shown a correlation between survival rate after cryopreservation and increased levels of Put (Ramon et al. 2002).

In the present paper, we focus on the cryopreservation of Norway spruce ESM. The ESM consists of meristematic and suspensor cells: meristems are arranged as the meristematic embryonal heads of immature somatic embryos and linked in the organization of these immature embryos, exemplified by the thickness of meristems and the length and size of suspensors. The anatomical structures of different embryogenic lines of spruce differ in the organization of these immature embryos, exemplified by the thickness of meristems and the length and size of suspensors. The present study was undertaken in order to examine the cryotolerance of five different cell lines of Norway spruce embryogenic cultures. The specific aims of the present study are: (i) to relate the anatomical structure of ESM to cryotolerance; (ii) to compare PA content in the ESM of different spruce genotypes and relate them to cryotolerance; and (iii) to relate changes in the anatomical structure of ESM to changes in the pool of PAs in the course of cryoprotectant treatments and during regeneration after cryostorage. The data acquired provide a detailed anatomical characterization of Norway spruce ESM during a double application of sorbitol, followed by an application of DMSO, and during regrowth after thawing in relation to changes in the pool of PAs.

Materials and Methods

Plant material

The embryogenic cultures of Picea abies (L.) Karst. were obtained either as a gift (cell line AFO 541 from AFOCEL, Nangis, France) or induced in our lab from zygotic embryos of mature seeds on GD medium (Gupta and Durzan 1986) (cell lines C106, C110, C112 and C203; for the details, see Vágner et al. 2005).

Proliferation of embryogenic cultures

The embryogenic cultures were grown on GD medium (Gupta and Durzan 1986) solidified by 0.75% agar (Sigma-Aldrich, Prague, Czech Republic), with pH adjusted to 5.8 prior to autoclaving, and supplemented with 100 mg l⁻¹ cephotaxime (Sefotak, Valeant Pharma, Prague, Czech Republic), 5 μM 2,4-D, 2 μM kinetin, 2 μM BAP and 30 g l⁻¹ sucrose (all Duchefa, The Netherlands). The addition of the antibiotic cephotaxime decreases the risk of bacterial contamination of cultures during the frequent treatments before cryostorage. All phytohormones and organic components, except sucrose, were prepared separately and diluted, filter-sterilized and added to the cooled, autoclaved medium. The embryogenic cultures were maintained by weekly sub-culturing in Magenta vessels (Sigma-Aldrich, Prague, Czech Republic) containing 40 ml of fresh medium and incubated in darkness at 24 ± 1 °C.

Maturation of somatic embryos

In the liquid GD maturation medium, the cytokinins and auxin were substituted with 20 μM abscisic acid (ABA) and 3.75% (w/v) polyethylene glycol 4000 (PEG) (both Sigma-Aldrich, Prague, Czech Republic). The PEG and ABA solutions were autoclaved and filter-sterilized, respectively, and then added to the cooled, autoclaved medium. During maturation, cultures were sub-cultured weekly into fresh liquid medium on membrane rafts (Osmotek, Israel) in Magenta vessels and incubated in the dark at 24 ± 1 °C for 5–6 weeks.

Transfer to liquid medium

Cultivation of ESM in liquid media was necessary for the treatment of ESM with gradually increasing concentrations of cryoprotectants. To establish suspension cultures, approximately 1–1.3 g fresh weight of ESM grown on solid media were inoculated into 30 ml of liquid GD medium in 250 ml flasks and grown at 24 °C in the dark. The cultures were gently agitated in a vertical gyratory roller (20 rpm, CSAV Prague, Czech Republic). After 1 week of cultivation, sieved clusters of cells were transferred to the fresh medium and used for the experiments described below after a further 4 days.
Treatments with cryoprotectants

On the 11th day of cultivation, the cultures were treated with sorbitol (Duchefa, The Netherlands). A stock 4 M solution of sorbitol was gradually added to the flasks over a period of 30 min until a 0.2 M concentration of sorbitol was achieved in the medium. The same procedure was repeated the following day, resulting in a 0.4 M sorbitol concentration. On the following day (i.e., the 13th day of cultivation in liquid medium), the flasks with cultures were cooled on ice, and DMSO was gradually added over a period of 30 min to reach the final 2% concentration. The cooled ESM was then filtered and transferred into 2 ml cryovial tubes (Simport, Canada). Freezing was performed in a commercial freezing container (Mr. Frosty, Nalgene, USA). The container was cooled to 4 °C, cryovials containing ESM were placed inside and the container was put in deep freezer to reach the temperature −45 °C over a period of 3 h. Thereafter, the cryovials were stored in liquid nitrogen in an Arpege 70 cryoscontainer (Nalgene, USA) (for details of cryoprotocols, see Vágner et al. 2005).

Regrowth after thawing

The cryovials were briefly immersed in water at a temperature of between 40 and 45 °C. As soon as the contents were seen to begin to thaw, the cryovials were transferred onto a mixture of water and ice. After the surface of the cryovials had been sterilized with ethanol (70% v/v), the ESM was spread on a filter paper and placed onto the solid proliferation medium in a Petri dish. To decrease DMSO concentration, the filter papers with the ESM were transferred to fresh medium after 1 h, again after 24 h and again after 7 days. The ESM was cultivated in darkness at 24 ± 1 °C. The media were changed after 24 h and again after 7 days. The ESM were stained with 150 μl of 2.25 μM PI solution (stock solution 1 mg ml⁻¹ H₂O). A stock solution of FDA (2 mg ml⁻¹ acetone) was freshly diluted with water to 0.02% (w/v) concentration, and 20 μl of this solution was mixed on a microscope slide with the ESM already stained with PI. Slides were examined under a fluorescence microscope, Eclipse E600 (Nikon, Tokyo, Japan) equipped with a color digital camera DVC 1310 C (Austin, TX, USA). To detect FDA staining, the filter set for FITC was used (excitation at 450–490 nm, barrier filter 520 nm); PI was detected with the filter set for TRITC (excitation at 510–560 nm, barrier filter 590 nm). Images were digitally stored with the computer image analysis system Lucia G/F, version 4.6 (Laboratory Imaging, Prague, Czech Republic). Confocal microscopy was performed using a LSM 5 Duo confocal laser scanning microscope (Zeiss, Jena, Germany) equipped with an Argon/2 laser (FDA excitation at 488 nm, emission filter-set BP 505–550) and a DPSS laser (PI excitation at 561 nm, emission filter-set LP 650). In the viable cells, FDA is hydrolyzed by intracellular esterases to fluorescein. Fluorescein accumulates inside the cell and exhibits a bright green fluorescence. PI only penetrates through damaged membranes of non-viable or dead cells. It intercalates with nucleic acids to form a bright red fluorescent complex seen in non-viable cells, particularly in the nuclei.

Material for biochemical analyses

The concentrations of PAs were measured in the ESM of five different cell lines of Norway spruce cultured on solid media for 1 week (material source); PA concentrations were then measured every day during ESM cultivation in liquid media; during the course of the cryoprotectant treatment (namely, 1 and 24 h after the first application of sorbitol (S₁ and S₂); 1 and 24 h after the second application of sorbitol (S₁ and S₄); 1 h after the application of DMSO (D); and on the 6th, 11th, 15th and 21st days during the regrowth phase after thawing). The samples were frozen in liquid nitrogen and stored at −80 °C until required for analysis.

Polyamine analysis

The cells were ground in liquid nitrogen and extracted overnight at 4 °C with 1 ml of 5% perchloric acid (PCA) per 100 mg fresh weight tissue. 1,7-diaminoheptane was added as an internal standard, and the extracts were centrifuged at 21,000×g for 15 min. PCA-soluble free PAs were determined in one-half volume of the supernatant. The remaining supernatant and pellet were acid hydrolyzed in 6 M HCl for 18 h at 110 °C to obtain PCA-soluble and PCA-insoluble conjugates of PAs as described by Slocum et al. (1989). The standards (Sigma-Aldrich, Prague, Czech Republic), the PCA-soluble free PAs and the acid-hydrolyzed PA conjugates were benzoylated according to the method of Slocum et al. (1989), and the resulting benzoyl-amines were analyzed by HPLC using a Beckman chromatographic system equipped with a 125S Gradient Solvent Delivery Module, 507 Variable mode.
Injection Autosampler and 168 Diode Array Detector (Beckman Instruments, Inc., Fullerton, CA, USA). A Gold Nouveau software data system was used to collect, integrate and analyze the chromatographic data. A C18 column (Phenomenex Aqua, 5 μm, 125A, 250 × 4.6 mm, Phenomenex, Utrecht, the Netherlands) was used for the separation of polyamines. Elution was carried out at a flow rate of 0.4 ml min⁻¹ at 45 °C. Standard samples (5 or 10 μl) were injected for each single run. The mobile phase consisted of solvent A (10% v/v methanol) and solvent B (80% v/v methanol). The gradient program (expressed as percentages of solvent A) was as follows: 0–10 min, 45–0%; 10–30 min, isocratic 0%; 30–40 min, 0–45%. The column was washed with 45% solvent A for 30 min between samples. Eluted polyamines were detected with a UV detector at 254 nm by comparing their retention times with those of the standards (Sigma-Aldrich, Prague, Czech Republic).

Statistical analyses
Two independent experiments were carried out, from which similar results were obtained. In the figures below, the means ± SE obtained from one of the experiments (comprising three replicates) are shown. Data were analyzed using Student’s t distribution criteria.

Results
Embryogenic capacity and cryotolerance of spruce embryogenic cultures
The embryogenic capacity (i.e., the ability of the ESM to produce mature somatic embryos capable of germination) and cryotolerance were determined in a number of cell lines of Norway spruce ESM. Five of them, with diverse features, were further selected for the determination of PA content. The anatomical characterization of the embryogenic cultures grown on proliferation and maturation media is presented in Figure 1; their ability to regenerate after cryopreservation is presented in Table 1.

The ESM of AFO 541 represented plant material with the most stable rapid growth during proliferation. The culture was composed of large polyembryogenic complexes linked with large suspensors (Figure 1A). A huge yield of somatic embryos was obtained at the end of maturation (Figure 1B). The embryogenic culture AFO 541 had very high cryotolerance, and its restoration of growth after cryostorage was almost immediate (Table 1).

The anatomical structure of the ESM of C110 was very similar to that of AFO 541 (Figure 1C). The culture grew rapidly during proliferation; however, the yield of mature somatic embryos was lower than in AFO 541 (Figure 1D), and this embryogenic line was sensitive to cryoprotectant treatments. The regeneration of C110 ESM after cryopreservation was very slow; the culture regrew with difficulty, often with callogenesis (Table 1).

The polyembryogenic complexes in C112 ESM were not as compact as those of C110 and AFO 541: the pure meristematic layers of C112 ESM were connected by shorter suspensors, and many free long suspensor cells were present in the ESM (Figure 1E). Only a few somatic embryos were able to complete their development (Figure 1F). Only a low percentage of cryostored ESM was able to regrow, and even then, only individual scattered cells survived. Regrowth of C112 was markedly slower than AFO 541 (Table 1).

The growth of the ESM of C106 was extremely slow. The C106 ESM was characterized by pure non-compact meristems, chaotically structured suspensors and the presence of many free suspensor cells in the ESM (Figure 1G). No matured somatic embryos were obtained from C106 (Figure 1H), and the ESM did not regenerate after thawing (Table 1).

The ESM of C203 was characterized by extremely rapid growth. The meristems usually developed without any junction with suspensors, which were often vacuolized (Figure 1I). Polyembryogenic complexes were not created in this culture, and somatic embryos did not finish maturation (Figure 1J). However, the cryotolerance of this embryogenic culture was high, and the ability to regenerate was similar to that of AFO 541 (Table 1).

PA content of ESM of Norway spruce
The endogenous contents of PCA-soluble free Put, Spd and Spm, determined in the ESM of five different cell lines of Norway spruce grown on solid proliferation medium are presented in Figure 2. The predominant PA in the ESM of AFO 541 was Spd followed by Put, while in genotype C106, where no matured embryos were formed, the PA distribution pattern was reversed. The ESM of the remaining studied cell lines contained approximately equal levels of Put and Spd. The content of Spm at this developmental stage of embryos was rather low in all cultures studied.

Anatomical characteristics and PA content of the ESMs of AFO 541 and C110 after transfer to liquid medium
The growth characteristics and alterations in PA content during the process of cryopreservation were studied in more detail in AFO 541 and C110 since these cultures have comparable ESM anatomy. However, these two cultures differ in their rate of growth, their yield of matured somatic embryos and particularly in their regeneration ability after cryopreservation.

Figure 1. Anatomical characterization of embryogenic cultures of five cell lines of Norway spruce (A and B, AFO 541; C and D, C110; E and F, C112; G and H, C106; I and J, C203). Left column (A, C, E, G, I): proliferation of ESM (light microscopy, trypan blue staining). Scale bar 1 mm. Right column (B, D, F, H, J): yield of somatic embryos after 5 weeks of maturation. Scale bar 5 mm. S, suspensor; SC, free suspensor cells; PC, polyembryogenic complexes; EH, embryonal heads.
The ESM of Norway spruce does not usually tolerate long-term cultivation in a liquid medium. However, cultivation in liquid medium is necessary for the efficient cryotreatment of ESM before cryostorage. For this reason, we focused on the changes associated with the transfer of the ESM from solid to liquid media. The changes induced by the transfer of ESM to the liquid phase and/or by the cryoprotectant treatment can thus be evaluated separately.

The ESMs of both cell lines retained their growth rates. The polyembryogenic character of AFO 541 remained conserved, and most suspensor cells were still attached to polyembryogenic complexes. However, the length of the suspensor cells was shortened, and a part of the suspensor cells was loosened from these structures. In C110, a distinct disintegration of polyembryogenic complexes was visible: the ESM was composed of free suspensor cells, free small somatic embryos and small complexes with fragile embryonal heads. The accumulation of starch grains in the cytoplasm and around the nuclei of suspensor cells was a typical feature of C110, although this could occur in both cell lines (Figure 6I and J).

The PA content of the ESM cultivated in liquid medium prior to cryopreservation are shown in Figure 3. In both cultures, the highest PA concentration was of Spd; its concentration rose and fell periodically during the 2 weeks in culture, with the maximum always occurring on the second and/or third day after changing the medium. However, by the end of the 14 days of cultivation, the embryogenic cultures were in a different physiological state, characterized not only by different growth rates and anatomical characteristics but also by distinct PA levels. The endogenous level of free PAs was markedly higher in the AFO 541 culture, with Spd being the predominant PA in the course of the whole cultivation. In the C110 ESM, the concentrations of Put and Spd were almost identical and rather low after the 12th day, the Spd concentration being almost 2.5 times lower than in the ESM of AFO 541.

The PA content of the ESMs of AFO 541 and C110 under treatment with cryoprotectants

The cryoprotectant treatment influenced the level of PCA-soluble free PAs (Figure 5). Marked decreases in free Put

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Growth rate (%)</th>
<th>Cryotolerance (%)</th>
<th>Total PAs (μmol g⁻¹ DW)</th>
<th>Embryogenic capacity (embryos g⁻¹)</th>
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<tbody>
<tr>
<td>AFO 541</td>
<td>300 ± 8</td>
<td>94</td>
<td>6.78 ± 0.83</td>
<td>52 ± 6</td>
</tr>
<tr>
<td>C110</td>
<td>215 ± 6</td>
<td>26</td>
<td>4.50 ± 0.52</td>
<td>37 ± 8</td>
</tr>
<tr>
<td>C112</td>
<td>154 ± 4</td>
<td>14</td>
<td>4.15 ± 0.46</td>
<td>9 ± 3</td>
</tr>
<tr>
<td>C106</td>
<td>161 ± 3</td>
<td>0</td>
<td>5.24 ± 0.73</td>
<td>0</td>
</tr>
<tr>
<td>C203</td>
<td>355 ± 8</td>
<td>95</td>
<td>2.97 ± 0.33</td>
<td>0</td>
</tr>
</tbody>
</table>

Growth rate: fresh weight increase of ESM (%) after 1 week of cultivation on proliferation medium. Cryotolerance: percentage of successful regrowth after cryostorage. Embryogenic capacity: number of mature somatic embryos per gram fresh weight of ESM after 5 weeks of maturation. Developed cotyledons and embryo length 2.2 mm as minimal were set as the criteria of embryo maturity, cultures were evaluated by computer image analysis.

**Treatment with sorbitol and DMSO**

The treatment of the ESMs with sorbitol on the 11th and 12th days of cultivation and with DMSO on the 13th day, just before freezing, caused the continual disintegration of polyembryogenic centers and suspensors in both C110 and AFO 541. The long vacuolized cells of suspensors are much more sensitive to osmotic changes elicited by sorbitol treatment than are the small cells of embryonal meristems. The majority of suspensor cells were therefore injured or damaged, embryonal meristems were partly disintegrated and polyembryogenic complexes were rarely present (Figure 4). Vital staining showed regions of dead cells in viable meristems of AFO 541 (Figure 4D), whereas the loose meristems of C110 became a mosaic of viable and dead cells (Figure 4H). Injury to the long vacuolized suspensor cells of both cell lines was obvious after the viability test. Generally, the ESM of AFO was markedly less damaged than that of C110 ESM. However, the stress reaction of treated cells was not only manifested in structural changes: starch accumulation further intensified during the cryoprotectant treatments; nuclei of suspensor cells in both cell lines were enclosed with starch grains; and free starch grains were observed to be present in their cytoplasm (Figure 4B, D, F, H–J).
and Spd after sorbitol applications were observed in the ESM of AFO 541 \((P < 0.05\) compared with the values prior to cryoprotectant treatment; Figure 5A). The level of free Spm did not change, and its content in DMSO-treated cells, before freezing, was comparable with that in cells before receiving the cryoprotectant treatments. Alterations in the total content of free PAs and their soluble and insoluble conjugates are presented in Figure 5C. A continual decrease in the level of polyamines observed during the cell osmotic pretreatment was caused predominately by a significant decrease in PCA-soluble free PAs \((P < 0.05\) compared with the values prior to cryoprotectant treatment). Thus, while the content of total PCA-soluble free PAs was double that of PCA-soluble conjugates in cells before the cryoprotectant treatments, in DMSO-treated ESM, the levels of both PA forms were approximately equal (Figure 5C). A gradual decrease in free Put, Spd and Spm, as well as changes in the fractional composition of the PA pool in C110 ESM, was similar to that observed in the AFO 541 embryogenic culture (Figure 5B and D).
Anatomical characteristics of the ESMs of AFO 541 and C110 after thawing

Freezing the ESM before cryostorage resulted in massive selective damage to cells. All suspensor cells, long and vacuolated, were destroyed. The character of the ESM changed, as only the scattered small cells of embryonal meristems survived. No integral viable somatic embryos were detected in either culture 1 day after thawing, when using the double-staining method for viable and dead cells. At the start of regeneration, only a few sporadic viable meristematic cells were found inside the embryonal heads and in the intermediate area between suspensor and meristems.

Significant differences were observed in the initial lag phase of regeneration between AFO 541 and C110. In the ESM of AFO 541, the first newly developed early somatic embryos were observed on Days 4–5 after thawing (Figure 6A). Enlarged embryos, with more robust embryonal heads connected with large suspensors, occurred in the AFO 541 culture on the 11th day after thawing (Figure 6B) when the development of polyembryogenic centers started. The large polyembryogenic centers were clearly visible from the 15th day after thawing. After 3 weeks of regrowth, regrown ESM did not differ structurally from the ESM that had never been cryostored (Figure 6C). One month after cryostorage, the growth rate of AFO 541 ESM did not differ significantly from the non-cryostored control. Similarly, the embryogenic capacity (i.e., ability to produce mature embryos capable of germination) remained constant after cryostorage.

The initial lag phase of regeneration of C110 ESM was markedly longer than in AFO 541. In a similar manner to that observed in AFO 541, the scattered viable cells appeared in the ESM just after thawing and persisted in the culture for 2 weeks (Figure 6D and E). Only 3 weeks later, the early stages of the developing embryos were observed in a small part of the C110 culture (Figure 6F). Furthermore, occasional callus development was observed during the process of regeneration, indicating an origin in non-embryogenic culture rather than in ESM. A part of the ESM was not able to regenerate at all.

PA content of the ESMs of AFO 541 and C110 after thawing

We were aware of the fact that analyses of PAs in ESM cultures 1 week after thawing were conducted on samples that contained a large number of dead cells. The separation of living cells in amounts necessary for PA determination would have been impossible; the values presented here might therefore have been influenced by the presence of dead cells in the sample.

The levels of free Put and Spd determined in the ESM of AFO 541 on Day 6 were low due to there still being a high proportion of dead cells present. However, a marked increase in Put and particularly Spd 2 weeks after thawing correlated well with the observed anatomical changes of the culture (P < 0.05 compared with the values determined in ESM 1 h after DMSO treatment, i.e., prior to freezing; Figure 7A). The level of free Put and especially the high amount of Spd 2 and 3 weeks after cryostorage corresponded well with the PA values in the ESM cultivated in liquid medium before freezing (Figure 3). The fractional composition of the PA pool in the ESM of AFO 541 showed significant changes after thawing (Figure 7C). A level of PCA-insoluble PA conjugates observed on Days 6 and 11, more than twice times higher than was observed in the tissue before freezing, was probably due to the decompartmentalization of dead cells still present in tissue samples (Figure 6A and B). A relatively high proportion of soluble PA conjugates occurred in the ESM, especially on Day 6 after thawing, which reflects the unsatisfactory state of embryogenic tissue after freezing. The successful regrowth of AFO 541 ESM on Days 15 and 21 was characterized by an increase in the total content of free PAs (P < 0.05 compared with the values determined in ESM prior to freezing; Figure 7C).

The results of the PA analyses of C110 ESM corresponded well with the markedly longer lag phase of regeneration as revealed in the microscopic analysis (Figure 6D–F). The levels of free Put and Spd in the ESM of C110 were very low during the 21-day cultivation period after thawing (Figure 7B). The very low level of PAs in this culture (compared with the values in the ESM of C110 prior to freezing) on Day 15 was most probably due to the leakage of the intracellular contents of dead cells into the medium. A slight increase in free Put and Spd characterized the ESM, in which a slight regrowth was observed 21 days after thawing (21R). The level of PAs remained practically unchanged and very low in the ESM where no marks of proliferation were observed (21N). No marked changes were found in the fractional composition of the PA pool in the ESM of C110 after thawing (Figure 7D).

Discussion

The relationship between embryogenic capacity and the total content of free PAs confirmed the role of PAs (with Spd pre-
dominating, Figure 2) during somatic embryo development in spruce as crucial and in agreement with that previously described by Minocha et al. (2004) and Gemperlová et al. (2009).

Growth rate is the only characteristic shown to have some association with cryotolerance (Table 1). Stable and rapid growth rates of cultures AFO 541 and C203 correlated with the cells’ ability to survive cryoprotectant pretreatments, freezing and thawing. However, because the embryogenic potential of C203 was negligible, this cell line was excluded from further studies. To test for any possible relationships between the morphology of ESMs, the components of PAs and cryotolerance, we compared the behavior of the AFO 541 and C110 cultures, which had similar ESM structures but exhibited different levels of cryotolerance. Although the cultivation of the ESM in liquid medium before cryopreservation enables a good contact between plant cells and the cryoprotectants, it also causes a certain level of stress. Surprisingly, different levels of tolerance to exogenous treatments by these two cell lines was already apparent after their transfer to the liquid medium with structural changes of the ESM being much more visible in the C110 culture (Figure 4A, C, E and G). The first symptom of the stress effect, which was most pronounced in the C110 culture, was the accumulation of starch grains around the nucleus of suspensor cells (Figure 4D, and G–J). The ESM of AFO 541 was in a very good physiological state at the beginning of cell treatment with cryoprotectants, i.e., on the 11th day of cultivation in the liquid medium. The endogenous free PAs were maintained at a steady high level, with Spd being the predominant PA: a fact that might be important for the maintenance of high embryogenic potential after cryostorage (Figure 3). By contrast, most of the polyembryogenic complexes in C110 ESM were disintegrated before the treatment with cryoprotectants, and the concentrations of Put and Spd were almost identical and rather low, with the concentration of Spd being almost 2.5 times lower than its concentration in the ESM of AFO 541. It is generally accepted that a critical step in achieving the post-thaw survival of plant material lies in the dehydration step and not in cooling per se. The induction of tolerance to dehydration is induced by sugar, osmotic or ABA treatments depending on the plant species and tissue type (Ramon et al. 2002). Our protocol was of osmotic pretreatment, using a double application of sorbitol followed by the application of DMSO as the cryoprotective agents. These compounds contribute to cellular dehydration, prevent cell

Figure 5. Changes in the content of PCA-soluble free Put, Spd and Spm in the ESMs (A and B) and the total content of PCA-soluble free Put, Spd and Spm and their PCA-soluble and insoluble conjugates (C and D) in the process of cryoprotectant treatment. A and C, AFO 541; B and D, C110. C on x axis represents PA content at the 11th day of ESM culture in liquid medium; S1, 1 h after the first sorbitol application; S2, 24 h after the first sorbitol application; S3, 1 h after the second sorbitol application; S4, 24 h after the second sorbitol application; D, 1 h after DMSO application. Bars represent SE of three replicates.
damage and restore regrowth of cultured cells after cryostorage (Winkelmann et al. 2004). The process of treating the ESM culture with cryoprotectants enhanced the anatomical differences between the AFO 541 and C110 cell lines. The destruction of suspensors, which was observed after the first sorbitol application, was even more intense after the second application, while the disintegration of polyembryogenic centers and suspensors occurred continually in both cell lines. The DMSO treatment eroded the embryogenic structure by inducing damage to the embryonal heads. A key aspect of DMSO’s action is pore formation; consequently, the significant enhancement in permeability of membranes to hydrophilic molecules explains DMSO’s cryoprotectant activity (Gurtovenko and Anwar 2007). The stress reaction of treated cells did not only result in structural changes. Because the accumulation of starch grains intensified in cells treated with cryoprotective agents, the quantity observed might indicate the degree of stress that has occurred in ESM cells (Figure 6). The accumulation of calcium oxalate crystals and starch grains, which are adaptive responses to water loss, have been observed in the parenchyma cells of *Stenocereus beneckei* (Ehrenberg) Buxbaum seedlings in response to water deficit (Ayala-Cordero et al. 2006).

The stress response of cells towards the osmotic shock induced by dehydration during the treatment with cryoprotectants included an altered PA metabolism. Parallel decreases

Figure 6. Regeneration of the ESMs of AFO 541 (A–C) and C110 (D–F) after thawing: confocal laser scanning microscopy, double vital staining FDA/PI; red colored dead cells of former embryonal heads and suspensors, green fluorescence in viable cells of newly developed embryos. A and D, 5th day; B and E, 11th day; C and F, 21st day. Scale bar 100 μm.
were observed in the free Put and Spd content in both cell lines during osmotic pretreatment. The relatively high content of Spm (compared with the levels of Put and Spd) as a consequence of cryoprotectant treatment of ESM is in accordance with previous results obtained from plants under hyperosmotic salt stress (Figure 5A and B) (Sanchez et al. 2005). Being biologically more dynamic than the other PAs, Spm is involved in stabilizing the cellular membrane and shows certain antioxidant effects (Bouchereau et al. 1999). The increased level of Spm observed prior to the cryostorage of AFO 541 ESM corresponds well with the suggested role of Spm in cellular membrane stabilization and as an antioxidant (Silveira et al. 2004). To our knowledge, only one report—this one on mouse embryos—describes the use of Spm as a cell membrane protector during cryopreservation (Berger et al. 1987).

Microscopic observations indicated that the cells that retained their viability and ability to regrow after cryopreservation were the meristematic cells inside the embryonal heads as well as the cells in the intermediate area between suspensor and meristems. A similar observation has been made after thawing an embryogenic culture of *P. sitchensis* (Bong.) Carr. where only cells located in the periphery of the embryogenic region of the embryos survived cryostorage (Kristensen et al. 1994). As mentioned above, the determination of PAs in AFO 541 on Day 6 after thawing (and to a lesser extent, also on Day 11) included the contents of a certain amount of dead cells. Decompartmentation, which accompanies cell death, might have led to the marked increase in the amount of PAs bound to high-molecular-mass substances like proteins and nucleic acids that was observed in cells on these days (Figure 7C). We suppose that stress conditions and cell death during cryostorage and thawing caused the disturbance of PA homeostasis, which was characterized by the high level of PA-soluble conjugates determined in AFO 541 on Day 6 (Figure 7C). On Day 21 after thawing, the amount of PAs in the AFO 541 cells reached approximately the same level as was observed in the suspension culture before the cryotreatment (Figure 7A).

Survival after cryopreservation is influenced by several factors such as genotype, physiological state of donor plant, volume of embryogenic load, retained cellular water level and the method used for freezing and thawing (Kim et al. 2006). The involvement of PAs in the stress responses of plants indicates their importance for plant survival (Kusano et al. 2008). Nevertheless, despite the intense interest in PA metabolism in plants exposed to abiotic stresses, there is rela-

![Figure 7. Changes in the contents of PCA-soluble free Put, Spd and Spm in the ESMs (A, B), and the total contents of PCA-soluble free Put, Spd and Spm and their PCA-soluble and insoluble conjugates (C, D) during the regrowth phase after thawing. A and C, AFO 541; B and D, C110. 0 on x axis represents PA amounts determined in ESM 1 h after DMSO treatment, i.e., prior to freezing. R, regenerating; N, non-regenerating. Bars represent SE of three replicates. Bars representing SE for PAs in B and C are partially hidden by the columns.](https://academic.oup.com/treephys/article-abstract/30/10/1335/1661979)
tively little information on the roles of PA metabolism in the process of cryopreservation. Ramon et al. (2002) were the first to report alleviated stress conditions related to endogenous levels of putrescine, which they observed during the cryopreservation of a Musa meristem culture.

The results presented here have shown that: (i) the size and structure of polyembryogenic complexes determines the quality of somatic embryos but does not correlate with their cryotolerance; (ii) growth rate is the only characteristic shown to have some association with cryotolerance; and (iii) a clear connection between the total content of PAs and cryotolerance was not determined.

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References


