Polyamine profiles and biosynthesis in somatic embryo development and comparison of germinating somatic and zygotic embryos of Norway spruce

LENKA GEMPERLOVÁ,1 LUCIE FISCHEROVÁ,1 MILENA CVIKROVÁ,1,2 JANA MALÁ,3 ZUZANA VONDRA´KOVA´,1 OLGA MARTINCOVÁ1 and MARTIN VÁGNERS1
1 Institute of Experimental Botany v.v.i., Academy of Sciences of the Czech Republic, Rozvojova´ 236, 16502 Prague 6, Czech Republic
2 Corresponding author (cvikrova@ueb.cas.cz)
3 Forestry and Game Management Research Institute, v.v.i., 156 04 Praha 5, Zbraslav-Strnady, Czech Republic

Received April 27, 2009; accepted July 24, 2009; published online 25 August, 2009

Summary The polyamine (PA) contents and activities of PA biosynthetic enzymes in Norway spruce somatic embryos [Picea abies L. (Karst.), genotype AFO 541] were studied in relation to anatomical changes during their development, from proliferation to germination, and changes in these variables associated with the germination of mature somatic and zygotic embryos were compared. Activities of PA biosynthetic enzymes steadily increased during the development of somatic embryos, from embryogenic suspensor mass until early cotyledonary stages. In these stages, the spermidine (Spd) level was significantly higher than the putrescine (Put) level, and the increases coincided with the sharp increases in S-adenosylmethionine decarboxylase activity in the embryos. The biosynthetic enzyme activity subsequently declined in mature cotyledonary embryos, accompanied by sharp reductions in PA contents, especially in cellular Put contents in embryos from 6 weeks old through the desiccation phase (although the spermine level significantly increased during the desiccation phase), resulting in a shift in the Spd/Put ratio from ca. 2 in early cotyledonary embryos to about 2, similar to the ratio in mature and germinating zygotic embryos. The accumulation of high levels of PAs in somatic embryos may be causally linked to their lower germinability than in zygotic embryos.

Keywords: ADC, ODC, Picea abies, SAMDC, somatic embryogenesis.

Introduction

Tissue culture approaches, in particular somatic embryogenesis, hold a considerable promise for accelerating breeding programs of woody plants. However, although the induction, proliferation and maturation of somatic embryos under suitable conditions can yield sufficient embryos of some coniferous species, in other species the germination frequency of somatic embryos is often too low for practical applications (Igasaki et al. 2003). Research into somatic embryogenesis of conifers has increased rapidly ever since the first reliable protocol for Picea abies L. (Karst.) was published (Chalupa 1985, Hakman et al. 1985). Coniferous somatic embryogenesis can be divided into a number of stages, most of which are comparable to zygotic embryogenesis (Attree and Fowke 1993). However, since somatic embryos develop without the protective environment of the surrounding maternal tissue, there is a need to supply both nutrients and regulatory compounds exogenously. Induction and continuous proliferation require auxin and cytokinins, whereas the further development and maturation of embryos depends on abscisic acid (Attree et al. 1991). By the end of the maturation phase, all of the anatomical structures of the embryo have been established (Find 1997), but
the embryo only becomes biochemically mature after a desiccation phase (Flinn et al. 1993).

Despite the availability of a successful protocol for generating Norway spruce plants using a somatic embryogenesis technique, there is a lack of data concerning the endogenous composition of biologically active compounds in both somatic and zygotic embryos. Generally, the development of embryos, as well as their conversion into complete plantlets, is closely associated with the changes in endogenous phytohormone levels. We have recently reported the changes in endogenous hormone levels (IAA, ABA and ethylene) during somatic embryo development and maturation of Norway spruce (Vagner et al. 1998). Another recent study on hybrid larch embryos has shown pronounced differences in the concentration of ABA between the mature embryos of somatic and zygotic origin (Von Aderkas et al. 2001).

Several developmental processes controlled by cytokinins also appear to involve polyamines (PAs). Polyamines, low molecular mass polycations, are ubiquitous cell components that are essential for normal growth of both eukaryotic and prokaryotic cells (Tabor and Tabor 1984). Most of the biological functions of PAs can be explained by their polycationic nature, which facilitates interactions with anionic macromolecules (such as DNA and RNA) and negatively charged groups of membranes. Biosynthesis of the three commonly occurring PAs – putrescine (Put), spermidine (Spd) and spermine (Spm) – is initiated in plants either by direct decarboxylation of ornithine by the enzyme ornithine decarboxylase (ODC; EC 4.1.1.17) or by decarboxylation of arginine by the enzyme arginine decarboxylase (ADC; EC 4.1.1.19) via agmatine and N-carbamoylputrescine intermediates. The exact role of these two pathways in Put biosynthesis is not clear, but ADC and ODC activities vary both between species and between plants of the same species at different stages (Santanen and Simola 1999). Another essential enzyme for PA synthesis is S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50), which is required for the production of the aminopropyl group used in the biosynthesis of Spd and Spm (for a review, see Wallace et al. 2003).

There have been a number of reports, some regarding conifers, indicating that PAs play a crucial role in somatic embryo development. The formation of somatic embryos of wild carrot in tissue culture appears to be associated with high Spd levels, and much more Spd than Put has been found in these embryos in the torpedo stage (Mengoli et al. 2007). The Spd, in particular, has been implicated in the somatic embryogenesis in tissue cultures of *Picea abies* (Minocha et al. 1999); and high levels of Put have been found in the pro-embryogenic tissue of *Picea rubens* Sarg., but Spd predominates during embry development in this species (Minocha et al. 2004).

This study was undertaken to establish changes in the metabolism of PAs during the development of somatic embryos of *P. abies*, (i) to relate PA concentrations to the development of the embryos; (ii) to compare PA levels in mature somatic embryos with those in mature zygotic embryos and megagametophytes; and (iii) to characterize changes in the PA levels during the germination of both types of embryos. The acquired data provide a detailed anatomical characterization of *P. abies* somatic embryo development, from proliferation to germination, and comparative information on the anatomy and germination of mature zygotic embryos, related to the changes in free PA contents and the activities of enzymes involved in their biosynthesis (ADC, ODC and SAMDC).

**Materials and methods**

**Plant material**

An embryogenic culture of *P. abies*, genotype AFO 541, was obtained from AFOCEL, France, and dry mature seeds of *P. abies* from cones were collected during the summer of 2007, before the end of July, in the grounds of the Forestry and Game Management Research Institute, Strnady, Czech Republic.

**Proliferation of embryogenic culture**

The embryogenic culture was grown on GD medium (Gupta and Durzan 1986) solidified by 0.75% agar (Sigma-Aldrich, Czech Republic), with pH adjusted to 5.8 before autoclaving, supplemented with 5 µM 2,4-D, 2 µM kinetin, 2 µM BAP (all Sigma-Aldrich, Czech Republic) and 30 g l⁻¹ sucrose (Lachema, Czech Republic). All organic components, except sucrose, were separately prepared and diluted, filter-sterilized and added to the cooled, autoclaved media. The embryogenic cultures were maintained by subculturing, weekly, in Magenta vessels (Sigma-Aldrich, Czech Republic) containing 40 ml of fresh medium and incubated in darkness at 24 ± 1 °C.

**Maturation of somatic embryos**

In the GD medium used for maturation, the cytokinins and auxin were substituted by 20 µM ABA (Sigma-Aldrich,
Czech Republic), which was used in combination with 3.75% polyethylene glycol 4000 (PEG). The PEG and ABA solutions were separately autoclaved and filter-sterilized, respectively, then added to the medium after autoclaving. During maturation, cultures were subcultured, weekly, in a fresh medium in Magenta vessels and incubated in the dark at 24 ± 1 °C for 5–6 weeks.

**Desiccation of somatic embryos**

Fully developed embryos after maturation were selected and desiccated, by transferring them onto dry paper in small Petri dishes (3 cm diameter), which were placed in large Petri dishes (18 cm diameter) with several layers of paper wetted by sterile water (to maintain 100% humidity). The large Petri dishes were covered by lids and sealed by parafilm, then incubated in a cultivation room with a 12-h photoperiod (PPFD about 15–25 μmol m⁻² s⁻¹, daylight fluorescent tubes; Osram AG, Winterthur, Switzerland) at 18 ± 1 °C for 3 weeks.

**Germination of somatic embryos**

The desiccated embryos were transferred into Magenta dishes filled with 40 ml of half strength GD medium, supplemented with sucrose (1%) and active charcoal (0.4%), but no phytohormones, with pH adjusted to 5.8 before autoclaving. The dishes containing embryos were placed in a cultivation room with a 12-h photoperiod (PPFD about 15–25 μmol m⁻² s⁻¹, daylight fluorescent tubes) at 24 ± 1 °C for 3 weeks.

**Imbibition and germination of seeds**

Cold-stored (4 °C) seeds of *P. abies* were imbibed in distilled water for 24 h at room temperature. During this time, hydration of the cotyledons, hypocotyl and finally the radicle was completed, and the zygotic embryos were ready to germinate (Tillmansutela and Kauppi 1995). This period of imbibition was necessary to compensate for the extra barriers surrounding zygotic embryos (the seed coat and mainly the lipophilic layers surrounding the endosperm) compared to their somatic counterparts. Subsequent germination took place in unsealed Petri dishes with wet filter paper under a 16-h photoperiod (PPFD about 60–80 μmol m⁻² s⁻¹, daylight fluorescent tubes) at 24 ± 1 °C for 2 weeks.

**Material for biochemical analyses**

The concentrations of PAs and the activity of biosynthetic enzymes (ADC, ODC and SAMDC) were measured at 1-week intervals, over the course of the development of somatic embryos and their desiccation. For the first 3 weeks of maturation, samples contained the whole embryogenic suspensor mass (ESM); thereafter (from the fourth week), the embryos were separated from the remaining mass. Samples of germinating somatic embryos were collected on days 1, 2 and 6. Biochemical analyses of zygotic embryos were performed on separated embryos and on the megagametophyte tissue isolated from dry mature seeds, imbibed seeds and seeds germinated for 1, 2 and 6 days. The samples (25–50 somatic or 50–100 zygotic embryos) were frozen in liquid nitrogen and stored at −80 °C until analysis.

**Anatomical study**

Paraffin sections (12-μm thick) of ESM and embryos of both origins were prepared according to Johansen (1940) and stained by the two-step procedure, using alcian blue and nuclear fast red, described by Svobodova et al. (1999). Alcian blue binds specifically to polysaccharides in the cell wall, and nuclear fast red counterstains chromatin structures. At least 10 samples (ESM or embryos) representing each of the developmental stages, described above, were used in the biochemical analyses (see below).

**ODC, ADC and SAMDC assays**

Ornithine decarboxylase (ODC; EC 4.1.1.17), arginine decarboxylase (ADC; EC 4.1.1.19) and S-adenosylmethionine decarboxylase (SAMDC; EC 4.1.1.50) activities were determined using a radiochemical method as described by Tassoni et al. (2000). Samples were extracted in three volumes of ice-cold 0.1 M Tris–HCl buffer, pH 8.5, containing 2 mM β-mercaptoethanol, 1 mM EDTA and 0.1 mM pyridoxal phosphate, and centrifuged at 20,000g for 30 min at 4 °C. Portions (0.1 ml) of both supernatant (soluble fraction) and resuspended pellet (particulate fraction) were used to determine ODC and ADC activities, by measuring the 14CO₂ evolution from 7.4 kBq L-[U-14C]arginine (2.15 GBq mmol⁻¹, Amersham Pharmacia Biotech UK) or 7.4 kBq L-[U-14C]arginine (11.5 GBq mmol⁻¹, Amersham Pharmacia Biotech UK), for ODC and ADC, respectively, in the presence of 2 mM unlabeled substrate during a 1.5-h incubation at 37 °C. The CO₂ was trapped in hyamine hydroxide, and the radioactivity was counted using a Tri-Carb 2900TR liquid scintillation analyzer (Packard Instrument Co., Meriden, CT).

To determine SAMDC activities, samples were homogenized in three volumes of 0.1 M phosphate buffer, pH 7.6, containing 2 mM β-mercaptoethanol and 1 mM EDTA, and centrifuged at 20,000g for 30 min at 4 °C. The supernatant and the resuspended pellet (0.1 ml portions) were incubated separately with 3.7 kBq [1-14C]S-adenosylmethionine (2.15 GBq mmol⁻¹, Amersham Pharmacia Biotech UK) in the presence of 2.8 mM unlabeled substrate and 3 mM Put. The amount of 14CO₂ released during a 1-h incubation at 37 °C was then measured using a Tri-Carb 2900TR liquid scintillation analyzer.

The protein contents of the samples were measured according to Bradford’s method, using bovine serum albumin as a standard (Bradford 1976).
**PA 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 mass tissue. 1,7-Diaminoheptane was added as an internal standard, and the extracts were centrifuged at 21,000g for 15 min. Standards (Sigma-Aldrich, Czech Republic) and PCA-soluble free PAs were benzyolated according to the method of Slocum et al. (1989), and the resulting benzyolamines were analyzed by HPLC using a Beckman-Video Liquid Chromatograph (Beckman Instruments, Inc., Fullerton, CA) equipped with a UV detector (monitoring the eluate at 254 nm) and a C18 Spherisorb 5 ODS2 column (particle size of 5 μm, column length of 250 × 4.6 mm) according to the method of Slocum et al. (1989).

**Statistical analyses**

Two independent experiments were carried out, in which similar results were obtained. Mean values ± SE obtained in one experiment (with three replicates) are shown in the figures. Data were analyzed using Student's t distribution criteria.

**Results**

**Anatomical study**

During proliferation, the embryogenic culture AFO 541 was characterized by the presence of highly bipolar structures, consisting of clusters of small meristematic cells with dense cytoplasm forming meristematic centers, attached to elongated, highly vacuolized suspensor cells (Figure 1A). Transition of the proliferating culture to the maturation medium containing ABA resulted in a rapid growth of meristematic centers, followed by disintegration of the suspensor cells. After 3 weeks of maturation, protoderm was formed as the first inner structure of embryos (Figure 1B).

![Figure 1. Anatomical characterization of somatic and zygotic embryos of P. abies in various developmental stages.](image-url)
The establishment of inner structures further continued until the sixth week of maturation, when somatic embryos were anatomically fully developed. The mature somatic embryos had a distinct apical meristem, procambial strands, well-developed cotyledons and root meristem with a root cap. During desiccation, virtually no further visible developmental changes were observed (Figure 1C). The morphology of mature zygotic embryos was comparable to that of somatic embryos, except that their shape differed slightly due to the surrounding megagametophyte (Figure 1G). In the embryos of both origins, the start of germination was characterized by frequent cell divisions throughout the embryos. As germination proceeded, the root of somatic embryo elongated, and in some cases a new root cap was formed (Figure 1F). The apical pole of germinating somatic embryos remained unchanged until the sixth day (Figure 1E). In contrast, the apical meristem of zygotic embryos had already formed leaf primordia within 6 days of germination (Figure 1H). At the same time, the root of zygotic embryo protruded from the megagametophyte (Figure 1I).

**Activities of ODC, ADC and SAMDC biosynthetic enzymes during development of somatic embryos**

The activities of biosynthetic enzymes were measured in both the soluble and particulate fractions. In ESM, the activity of ODC continually increased during the culture period and peaked 3–4 weeks after transfer to the maturation medium (Figure 2A). The level of ODC activity at this time was 2.5-fold higher than in ESM growing on the proliferation medium. Thereafter, the ODC activity declined until the end of the maturation phase and remained low throughout the whole desiccation phase. A further rise in
ODC activity was connected to the start of germination of somatic embryos. The ODC activity was equally distributed between the soluble and pellet fractions within all monitored phases. Although the activity of ADC was several-fold lower than that of ODC on any given day of analysis, two increases were observed (Figure 2B), both correlated with the observed maxima of ODC activity, in week 4 of the maturation phase and on day 6 of germination. During the desiccation and maturation phases, ADC activity was two times higher in the soluble fraction than in the particulate fraction but, conversely, after the start of germination the activity was two times higher in the particulate fraction than in the soluble fraction. SAMDC activity showed a time course similar to that of ODC (Figure 2C). However, the maximum observed in the embryos after 4 weeks on the maturation medium was much more pronounced. This peak of SAMDC activity corresponded with a marked increase in Spd content (Figure 4). The SAMDC activity was predominantly found in the pellet fraction in all developmental stages of somatic embryos.

Activities of ODC, ADC and SAMDC biosynthetic enzymes in zygotic embryos

The activities of the three biosynthetic enzymes ODC, ADC and SAMDC are shown in Figure 3. In zygotic embryos, ADC was mainly responsible for Put biosynthesis in both embryos and megagametophytes. However, in megagametophytes, the ADC activity, detected predominantly in the cytosolic fraction, was significantly lower and represented only 17–35% that found in embryos (Figure 3B). In contrast to ADC, ODC activity was detected at very low levels and was predominantly detected in the pellet fraction (Figure 3A). Most of the SAMDC activity was detected in the pellet of megagametophytes, except on day 6 of germination (Figure 3C), when marked increases in the activities of all three biosynthetic enzymes were observed in zygotic embryos, whereas in megagametophytes their levels decreased. This finding correlates with the high PA levels observed in the embryos during germination, but not megagametophytes (Figure 6). The imbibition of mature zygotic embryos resulted in only moderate increases in the activities of ODC, ADC and SAMDC. However, marked increases in their activities were observed on their first day of germination. In the samples of germinating embryos on the first day, ODC activity was detected mainly in the supernatant, but on days 2 and 6 higher activities were found in the pellet, in samples from both embryos and megagametophytes. The ADC activity was very high in germinating embryos and was detected mainly in the soluble fraction on days 1 and 2.

Comparison of ODC, ADC and SAMDC activities in somatic and zygotic embryos

In contrast to the predominance of ODC activity found in somatic embryos, in zygotic embryos ADC played the most important role in PA biosynthesis (Figures 2A and 3B). In mature zygotic embryos, the ADC activity was 18- and 34-fold higher than that of ODC on days 1, 2 and 6, respectively. In somatic embryos, the difference between the ODC and ADC activities detected at the start of the desiccation phase subsequently declined, and there was very little difference between them at the end of this phase.
PA contents during development of somatic embryos

At a very early stage of development, during proliferation of the embryogenic culture of Norway spruce, ESM contained approximately equal levels of Put and Spd, thus the Spd/Put ratio was about 1 (Figures 4 and 5). The content of Spm at this stage was rather low. After 3 weeks of culture on maturation medium, when the ESM contained globular and partly polarized embryos, increases in levels of all three amines were observed, which correlated well with the observed increases in ODC activity. However, pronounced changes in the PA levels and the Spd/Put ratio took place after 4 weeks of maturation in torpedo stage embryos (Figures 4 and 5). From this stage, the embryos were separated from the associated subtending tissue (embryogenic and suspensor cell masses), and a marked increase in PAs was observed in embryos at this stage of development. The Spd level was significantly higher than the Put level, and the increase coincided with a sharp increase in SAMDC activity in separated embryos (Figures 2C and 4). In the residual ESM, after separation of somatic embryos, similar PA contents were found to those detected in 3-week-old ESM, with approximately equal Put and Spd levels (data not shown). After 5 weeks of growth on maturation medium, the level of Put in embryos declined, and this stage of embryo development was characterized by very high Spd contents (Spd/Put ratio > 2; Figures 4 and 5). In mature 6-week-old cotyledonary embryos, the contents of Put and Spd decreased (Figure 4). During the desiccation period, the Put and Spd levels declined, whereas the Spm level significantly increased. After 3 weeks of desiccation, the Spm level was nearly as high as that of Spd (Figure 4). The decline in cellular Put contents in embryos after 3 weeks of desiccation resulted in an increase in the Spd/Put ratio to nearly 10 (Figure 5). In germinating somatic embryos, marked increases in Put and Spd levels, correlating with the increases in biosynthetic enzyme activities on the first and second day, were detected and the Spd/Put ratio decreased, but Spm contents remained high (Figures 4 and 5). A strong subsequent increase in the Put content on day 6 was reflected in a decline in the Spd/Put ratio to a value of about 2 (Figure 5).

PA contents in zygotic embryos

In mature Norway spruce zygotic embryos, the Spd level was twofold greater than the level of Put (thus the Spd/ Put ratio was slightly > 2, Figure 7), and PA concentrations were higher in embryos than in megagametophytes (Figure 6). After imbibition, the level of PAs slightly increased in the embryos and did not change in the imbibed megagametophytes. Increases in all three PAs were observed in embryos after 1 day of germination (but the Spd level was still higher than the Put level), while the content of all the three PAs in megagametophytes did not change or declined on day 1. On day 2, the PA contents in embryos increased slightly, and marked increases were observed in them after 6 days of germination (Figure 6).
The Spd/Put ratio was stable in both tissues (embryo and megagametophyte) during germination, with values ranging from 1 to 1.5 (Figure 7).

Comparison of PA content in somatic and zygotic embryos

In Figure 8, the Put, Spd and Spm contents in mature desiccated somatic embryos, mature zygotic embryos, and somatic and zygotic embryos 1, 2 and 6 days after germination are compared. Of note is the very high level of Spm found in somatic embryos. Pronounced differences between somatic and zygotic embryos were found not only in PA contents but also in Spd/Put ratios (Figures 5 and 7).

**Discussion**

The involvement of PAs in various plant growth and developmental processes, including a crucial role in somatic embryo development, has been reported in several coniferous species (Santanen and Simola 1994, Minocha et al. 1999, 2004, Silveira et al. 2004). The results presented here show that significant, characteristic changes in the endogenous levels of PAs and their biosynthetic enzymes coincide with the development of somatic embryos from early stages through maturation, desiccation and germination. On transfer from proliferation to maturation medium (time zero in the figures), Put biosynthesis was mediated largely by the activity of ODC, which continually increased for 4 weeks (Figure 2A). In addition, from 3 weeks ADC was found to be active in the ESM. Thus, from 3 weeks both enzymes contributed to PA biosynthesis, and their activities were highest in the early cotyledonary stage embryos separated from the associated subtending tissue at 4 weeks. Activity of the third biosynthetic enzyme, SAMDC, followed a time course similar to that of ODC (Figure 2). A transient decline in PA biosynthetic activity was observed during the late stages of embryo maturation (5 and 6 weeks) and during the desiccation phase, corresponding well with the observations of Vuosku et al. (2006) and Loukanina and Thorpe (2008).

Parallel rises in the contents of all three PAs were observed in the first 3 weeks in ESM cultured on a maturation medium, keeping the Spd/Put ratio close to 1 (Figures 4 and 5). In separated 4- and 5-week-old embryos, high Spd levels raised the Spd/Put ratio to about 2. A rapid subsequent decline in cellular Put in mature 6-week-old embryos and during the desiccation phase resulted in a shift in their Spd/Put ratios to as high as 10 at the end of desiccation. The decline in PA contents
in mature embryos was partly due to the decrease in biosynthetic enzyme activities (Figure 2) and partly due to the increased catabolism of Put and Spd during the later stages of embryo development, as previously described in *P. abies* (Santanen and Simola 1994). The pattern of changes in soluble free Put and Spd contents, and the Spd/Put ratio, during somatic embryo development are consistent with the previous studies on somatic embryogenesis in conifers (Minocha et al. 1999). Results indicating a direct role for Spd in somatic embryogenesis have been reported in *H. brasiliensis* (El Hadrami and D’Auzac 1992), *Medicago sativa* L. (Cvikrová et al. 1999) and *P. ginseng* (Monteiro et al. 2002). Significant increases in Spd levels are also reportedly associated with the formation of somatic embryos in *P. abies* (Santanen and Simola 1992) and *P. radiata* (Minocha et al. 1999).

Low levels of Spm were found in developing somatic embryos, corresponding to the levels that were previously determined in the embryogenic cultures of *P. abies* and *P. rubens* (Minocha et al. 1993). The Spm content slightly increased only in 4- and 5-week-old embryos, when Put and Spd reached their maximum levels. Although desiccated somatic embryos were anatomically comparable to the mature ones, a marked increase in the Spm content was observed in somatic embryos after two and, more markedly, 3 weeks of desiccation, when its level was comparable with that of Spd (Figure 4). Desiccation of zygotic embryos is, in most seeds, the terminal event in their development. In somatic embryos, desiccation for 3 weeks may represent a sort of osmotic stress, since accumulation of free Spd and Spm has been recognized as a biochemical indicator of drought-associated osmotic stress (Sanchez et al. 2005). The significant rise of Spm contents in embryos observed in the process of desiccation might therefore represent a response to this abiotic stress.

The morphology and the shape of mature somatic and zygotic embryos showed remarkable similarities, in spite of the difference in the environments in which they had developed (Figure 1C and G). However, the comparison of PA metabolism of mature somatic and zygotic embryos revealed some differences. In mature zygotic embryos of *P. abies* Put was synthesized more actively from arginine than from ornithine, and ADC activity (predominately cytosolic) was higher in the embryos than in the megagametophytes (Figure 3B). During somatic embryo development, ODC was mainly responsible for Put production, but in the mature somatic embryos, at the end of the desiccation phase, the biosynthetic activities of ODC and ADC were approximately equal. In mature somatic embryos, ODC activity was equally distributed between the cytosolic and pellet fractions, whereas the ADC activity was higher in the cytosolic fraction (Figure 2A and B). Previous analyses of the subcellular localization of ODC and ADC activities have indicated that both proteins are active in the cytosolic as well as in the particulate fractions (Tassoni et al. 2003). The difference in compartmentalization of biosynthetic enzymes may be related to distinct functions in different cell types (Bortolotti et al. 2004). In accordance with this hypothesis, assays performed on the leaves of *Arabidopsis thaliana* (L.) Heynh. have shown high particulate ODC activity to be associated with the nuclei and chloroplast-enriched fractions (Tassoni et al. 2003). However, the significance of this difference is not clear. Biosynthesis via ODC is generally connected with cell division, whereas the ADC activity is often linked to stress responses and cell elongation (Perez-Amador and Carbonell 1995, Acosta et al. 2005). Nevertheless, the exact role of these two pathways in Put biosynthesis is not clear, and both ADC and ODC activities vary in plants at different stages of development. It should also be noted that the differences in the relative magnitude of ADC and ODC activities in somatic and zygotic embryos of *P. abies* may not be important in the context of this study, since they both contribute to the production of Put, and thus to PA synthesis.

Mature zygotic embryos, as well as mature somatic embryos, contained higher levels of Spd than Put, and
PA contents were lower in megagametophytes than in embryos (Figure 6). Higher levels of Spd than Put have also been observed in zygotic embryos and megagametophytes of *P. radiata* (Minocha et al. 1999). However, the comparison of Put, Spd and Spm contents in somatic and zygotic embryos indicates that there are differences in PA metabolism between these two types of embryos (Figure 8). The Spd/Put ratio was as high as 10 in somatic embryos after desiccation, whereas the ratio was much lower (ca. 2) in zygotic embryos because they had higher Put and lower Spd contents (Figures 5 and 7). High concentrations of PAs are commonly observed in tissues undergoing somatic embryogenesis. However, a high level of free PAs is not the only important PA-related factor in somatic embryogenesis, and (for instance) it has been proposed that an inadequate Spd/Put ratio may be causally linked to abnormal growth and disorganized cell proliferation of grape somatic embryos (Faure et al. 1991).

We may hypothesize that the presence of relatively high concentrations of ABA in the maturation medium, and consequently high endogenous levels of ABA in developing somatic embryos of *P. abies* (Vágner et al. 1998), induces the biosynthesis and accumulation of PAs. Accordingly, Von Aderkas et al. (2001) found that ABA concentrations were about 100 times higher in hybrid larch somatic embryos than in embryos of zygotically origin, and in embryogenic cultures of *Araucaria angustifolia* (Bertol.) Kuntze the increases in endogenous ABA contents have been found to be positively correlated to the increases in Put and Spd levels, showing ABA and accumulation of PAs to be directly related (Steiner et al. 2007).

We were interested in the changes in PA composition that may occur during the germination of embryos, especially somatic embryos, and could potentially be linked to the developmental events. Morphological differences were observed between the two types of embryos as germination proceeded (compare Figure 1D–F and G–I). The start of germination was characterized by cell division in root meristems of both types of embryos, and in apical meristems of zygotic embryos, connected with the rise in PA biosynthetic activity (Figures 2 and 3). We analyzed zygotic embryos and megagametophytic tissue separately. Whereas ADC activity increased in zygotic embryos during seed imbibition and at the beginning of germination, SAMDC activity markedly increased only in the megagametophytes. In spite of a rise in Put levels in germinating zygotic embryos, Spd remained the major PA both in these embryos and megagametophytes (which serve as a storage tissue supporting the germinating embryos in coniferous seeds; Kong et al. 1997). The accumulation of Spd in the germinating zygotic embryos (without a corresponding increase in SAMDC activity in the embryos themselves) suggests that Spd is supplied from megagametophytes, where we found high SAMDC activity, but low Spd levels. However on day 6, the megagametophytes probably lost this function as their biosynthetic activity became much lower than that of the embryos. The Spd/Put ratio was stable in both the tissues (embryo and megagametophyte) during germination, with values ranging from 1 to 1.5 (Figure 7).

Rises in cellular concentrations of Spd, and stronger increases in Put contents, in somatic embryos during the first 6 days of germination decreased the amount of Spm as a percentage of total PA contents (to 41% and 21% in mature somatic embryos, respectively) and reduced the Spd/Put ratio to about 2, showing a pattern similar to that found for zygotic embryos (Figures 5 and 7). The accumulation of high levels of PAs in somatic embryos might contribute to their 'reserves', consisting predominately of proteins and triglycerides, which are used during their germination. Another important aspect that should be considered concerns the involvement of PAs in the synthesis of nitric oxide (NO), which is known to act as a signaling molecule in plant cells (Besson-Bard et al. 2008). The application of NO-releasing compounds has been shown to stimulate cell division and embryogenic cell formation in alfalfa cells (Besson-Bard et al. 2008) and in vitro development of *Arabidopsis* seedlings (Yamasaki and Cohen 2006). On the other hand, the significantly higher total content of PAs in somatic embryos (twofold and threefold higher levels of Spd and Spm, respectively) than in zygotic embryos might be responsible for their lower germinability.

**Acknowledgments**

We thank See-Editing Ltd. for linguistic editing. This work was supported by the Ministry of Agriculture of the Czech Republic (Project Nos. QHB2303 and 0002070202) and by the Institutional Grant AV0Z 50380511.

**References**


**Appendix**

**Abbreviations**

ADC arginine decarboxylase
BAP 6-benzylaminopurine
2,4-D 2,4-dichlorophenoxyacetic acid
ESM embryogenic suspensor mass
ODC ornithine decarboxylase
PAs polyamines
PCA perchloric acid
PEG polyethylene glycol
Put putrescine
SAMDC *S*-adenosylmethionine decarboxylase
Spd spermidine
Spm spermine