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ORIGINAL PAPER

Exogenous putrescine affects endogenous polyamine levels and the development of *Picea abies* somatic embryos

Zuzana Vondráková · Kateřina Eliášová · Martin Vágner · Olga Martincová · Milena Cvikrová

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Abstract Embryogenic cultures of Norway spruce (*Picea abies*) were treated with exogenous putrescine (Put) applied via either proliferation or maturation media to determine how such treatment affected endogenous polyamine levels, the histological structures of the embryogenic suspensor mass (ESM), and the yield of mature embryos. Treatment with exogenous Put at 10, 100, or 500 μM significantly increased the endogenous free and conjugated Put contents of the treated ESM in a concentration-dependent manner. All of the Put treatments also reduced endogenous spermidine (Spd) levels. In conjunction with the increased abundance of endogenous Put, this caused a pronounced decrease in the Spd/Put ratios of treated ESMs relative to untreated controls. Exogenous Put stimulated meristem cell division and enlargement. However, single embryos were not readily released from polyembryonic centers and the frequency of development of malformed embryos was high.

Keywords Exogenous putrescine · Somatic embryogenesis · *Picea abies* · Polyamines

Abbreviations

ESM	Embryogenic suspensor mass
FM	Fresh mass
MPC	Malformed polyembryogenic center
PC	Polyembryogenic center
Put	Putrescine
SE	Somatic embryo
Spd	Spermidine
Spm	Spermine

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Z. Vondráková · K. Eliášová · M. Vágner · O. Martincová · M. Cvikrová (✉)
Institute of Experimental Botany AS CR, Rozvojová 263, 165 02, Prague 6, Czech Republic
e-mail: cvikrova@ueb.cas.cz

Introduction

Somatic embryogenesis is considered to be an advantageous method for plant micropropagation in vitro, and the number of published studies on somatic embryogenesis in conifers has increased rapidly since the 1980s (Hakman et al. 1985). Some promising results have been achieved, especially with Norway spruce (*Picea abies*) for which complete plants have successfully been regenerated in this way (Bornman 1983; Attree and Fowke 1993). Spruce somatic embryos are established via a sequence of developmental stages that resembles zygotic embryogenesis and is regulated by phytohormones (Vestman et al. 2011). The first stage, which is usually facilitated by cytokinins and auxins, involves the induction of the embryogenic suspensor mass (ESM) from the primary explants (zygotic embryos). The embryogenic culture consisting of early embryogenic structures can be reproduced during proliferation; the embryos continue to mature if treated with abscisic acid (ABA). In total, the maturation of spruce somatic embryos takes 5–7 weeks (Bozhkov and von Arnold 1998). The mature embryos are then desiccated under controlled conditions at a high relative humidity prior to germination. However, although the induction, proliferation and maturation of somatic embryos under suitable conditions can yield sufficient embryos to enable

propagation in some coniferous species, there are many species for which the germination frequency of somatic embryos is generally too low for practical applications (Igasaki et al. 2003). Furthermore, both the quality of the somatic embryos obtained in culture and their conversion rate (i.e. the embryos' ability to generate functional root and shoot systems) are strictly dependent on the genotype of the original explants.

In general, the development of embryos and their conversion into complete plantlets are both closely linked to changes in endogenous hormone levels. Auxins, cytokinins and ABA all play vital roles in these processes, as do polyamines (PAs). The roles of PAs during in vivo and in vitro development have been reviewed (Baron and Stasolla 2008), and there have been multiple reports (some based on studies of conifers) indicating that PAs play a crucial role in somatic embryo development (Santanen and Simola 1994; Minocha et al. 1999, 2004; Silveira et al. 2004). We have previously shown that there are significant and characteristic changes in the endogenous levels of PAs and the associated biosynthetic enzymes that coincide with the progression of Norway spruce somatic embryos through the different phases of their development, from the early stages through to maturation, desiccation and germination. At a very early stage in the development of a proliferating embryogenic Norway spruce culture, the ESM contained approximately equal quantities of putrescine (Put) and spermidine (Spd). However, in embryos that had undergone 4 weeks of maturation, the Spd level was significantly higher than the Put level (Gemperlova et al. 2009). High levels of Put have been observed in the proembryogenic tissues of *Picea rubens*, but Spd became more abundant during embryo development in this species (Minocha et al. 2004). In addition, developing somatic and zygotic embryos of *Pinus radiata* were observed to have high Spd levels that increased over time (Minocha et al. 1999).

It is widely known that efficient somatic embryogenesis requires treatment with exogenous phytohormones. Several studies have shown that exogenous PAs can both induce cell division and promote regeneration in plant cell cultures (Kakkar et al. 2000; Takeda et al. 2002). The study presented herein was undertaken to investigate the effects of Put treatment during the maturation and/or proliferation of spruce embryogenic culture and to determine how (if at all) elevated Put levels affect the various processes involved in somatic embryo development. The specific aims were: (1) to determine the endogenous PA levels in the embryogenic cultures after treatment with exogenous Put; (2) to relate the observed changes in endogenous PA levels to changes in the histological structure of the ESM if any such changes occurred; and (3) to compare the yields of mature embryos under the different tested treatment regimes.

Materials and methods

Plant material

An embryogenic culture of *P abies*, genotype AFO 541, was obtained from AFOCEL, France. Cultures were kept in darkness at 23 °C during proliferation and maturation. Cultivation proceeds on GD medium (Gupta and Durzan 1986) supplemented with sucrose. During proliferation 2,4-D, kin and BAP (all Duchefa, Haarlem, The Netherlands) were added to the medium. Maturation was initiated by replacing these phytohormones by ABA and polyethylene glycol 4000 (all Sigma-Aldrich). Cultivation of embryogenic culture in detail is described by Gemperlova et al. 2009.

The yield of embryos was estimated at the end of maturation (after 5 weeks of maturation). Images of embryo clusters were recorded using a Nikon SMZ 1500 stereomicroscope and a Nikon DS-5M digital camera. The images were processed using the Nis-Elements analysis system AR 3.0 (Laboratory Imaging, Prague, Czech Republic); the total number of embryos (including those that were malformed or only partially developed) in each image was counted along with the number of mature cotyledonary embryos. The embryo yields after 5 weeks of maturation were expressed on a per-gram basis relative to the fresh mass of the embryogenic culture at the start of the experiment.

The addition of putrescine to the culture medium

Filter-sterilized Put solution was added to the liquid GD medium after autoclaving. In the first set of experiments, the embryogenic cultures were cultivated on medium supplemented with 10, 100, or 500 μM Put during the first 2 weeks of maturation and then further subcultured on Put-free medium for the next 3 weeks. In the second set of experiments, Put was applied for 2 weeks during proliferation just before the start of maturation, after which the cultures were cultivated on Put-free maturation medium. Embryogenic cultures cultivated on Put-free GD medium throughout were used as controls.

Material for biochemical analyses

The PA contents of the control and Put-treated embryogenic cultures were measured weekly over the course of the somatic embryos' development. During proliferation and the first 3 weeks of maturation, samples contained the whole ESM; thereafter (from the fourth week), the embryos were separated from the remaining mass. The samples were frozen in liquid nitrogen and stored at −80 °C until analysis.

Microscopy and histological study

The structure and appearance of the ESM was evaluated during the proliferation stage and the first 2 weeks of maturation. The morphology of the early somatic embryos' ESMs was investigated by placing them on a microscopic slide and treating them with 1 drop of 0.04 % trypan blue (Sigma-Aldrich, Germany). A cover glass was then placed onto the ESM after 2 min of exposure, and the dye was rinsed out with distilled water. Paraffin sections of the ESM were prepared according to Svobodova et al. (1999) for histological observation. Briefly, samples were fixed with 50 % FAA (formaldehyde/acetic acid/ethanol/water 1/1/9/9, v/v/v/v) for at least 24 h, gradually dehydrated in an ethanol/butanol series, and infiltrated with paraffin wax. Longitudinal sections (12 μm) were cut on a Finesse rotary microtome (Thermo Shandon, UK). Sections were stained with 0.1 % alcian blue in 3 % acetic acid and 0.1 % nuclear fast red in 5 % $Al_2(SO_4)_3$ according to Benes and Kaminek (1973). The preparations were examined using a Zeiss Jenaval transmission light microscope. All images were recorded using a Nikon DS-5M digital camera and processed using the Nis-Elements AR 3.0 computer image analysis system.

Polyamine analysis

Extraction and HPLC analysis of benzoylated polyamines was performed on HPLC Beckmann System Gold (125S Solvent Module Pump, 168 Detector), with C 18 Spherisorb 5 ODS2 column (250 × 4 mm) in methanol gradient according to Slocum et al. (1989).

Statistical analyses

Two independent experiments (three replicates each) were conducted, each of which yielded similar results. The mean values (± the standard error, SE) obtained in one experiment with three replicates are shown in the figures. Data were analyzed using Student's *t* distribution criteria. Asterisks above the bars in the figures indicate significant differences ($P < 0.05$) between the values observed in treated cultures and those in the corresponding controls.

Results

Treatment with putrescine during maturation

Histological observations

In the control experiments with no added Put, somatic embryo development was initiated during the first 2 weeks of ESM cultivation on maturation medium. The meristematic centres enlarged, individual embryos were released from polyembryogenic complexes, and their embryonal heads were elongated (Fig. 1a). Cells in the basal region that were connected with suspensors (future root caps) and aligned in vertical rows perpendicular to the embryo axis were readily apparent in the longitudinal sections (Fig. 1c). The suspensors subsequently disintegrated and the embryos became polarised in the 3rd week. Fully developed embryos with apical meristems, cotyledons, procambial strands and root meristems with root caps were first seen after around 4 weeks of maturation, and complete maturation was observed after 5 weeks.

Exogenous Put (10, 100, or 500 μM) was added to the maturation medium for the first 2 weeks, after which the cultures were transferred to Put-free media. The polyembryogenic meristematic centres (PCs) formed under these conditions were larger than those observed in the control experiments (supplementary Fig. 1a, b, c, d). However, the frequency of malformed embryos was high at all tested Put concentrations, and single embryos were rarely released from the large and often malformed polyembryogenic centers (MPCs). Cell divisions were not properly directed, and the cells did not arrange themselves in vertical rows on the basal region of the meristematic centers (Fig. 1b, d). The complexes then disintegrated extensively over the following weeks of maturation. At the end of the maturation period, many of the embryos remained non-developed and morphologically abnormal. The fully developed mature embryos were slight and long, consisting of apical and root meristems, root caps, hypocotyls and a ring of cotyledons around the apical meristem.

Polyamine contents

At the start of maturation, in untreated control ESMs, the content of free Spd was higher than that of Put and the content of Spm was the lowest one (Fig 2a, c, d; results for the controls are indicated with the label 0 μM). The Spd/Put ratio was approximately 1.50 (Fig. 3a). After 3 weeks of cultivation on maturation medium, when the globular and partly polarized embryos had developed, the levels of all three amines increased (Fig. 2a, c, d). There was a further pronounced increase in the PA contents of the embryos after 4 weeks of maturation when they were separated from the rest of ESM. From this stage of development onwards, the Spd level was significantly higher than that of Put (Fig. 2c). The cellular Put content declined after 5 weeks of maturation; together with the pronounced increase in Spd levels, this increased the Spd/Put ratio to around 5 (Fig. 3a).

Significant increases in endogenous free Put levels were observed on days 7 and 14 after treatment with exogenous

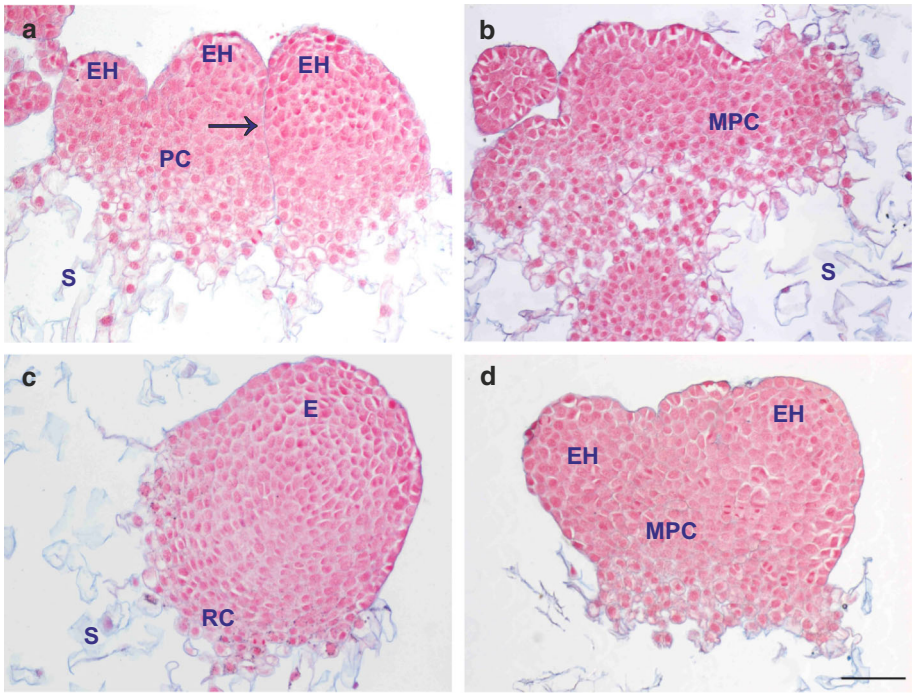

Fig. 1 Changes in the histological structures of Norway spruce embryogenic cultures treated for 1 week with 10 μM putrescine during maturation. **a** Non-treated control showing the cleavage of embryonal heads in the polyembryogenic meristematic centre; the *arrow points* to the separation of two embryonal heads from the polyembryogenic meristematic centre. **b** A huge malformed meristematic centre formed after Put treatment; no organisation into individual embryonal heads is apparent. **c** Non-treated control; a single early somatic embryo with a well-organized basal region corresponding to the future root cap; **d** poorly developed embryonal heads that cannot be released from the malformed polyembryogenic meristematic centre formed after Put treatment; *PC* polyembryogenic meristematic centre, *EH* embryonal head, *S* remaining suspensor cells, *E* single somatic embryo, *RC* root cap region, *MPC* malformed polyembryogenic meristematic centre. Sections were stained with *alcian blue* and nuclear fast red. The *scale bar* represents a length of 100 μm

Put at either 100 or 500 μM (Fig. 2a). However, while the levels of Put conjugates (predominately amide conjugates of hydroxycinnamic acids) declined continuously over the course of the maturation period in the control experiments, their abundance in the ESMs treated with 10 or 100 μM Put increased continuously, peaking on day 14 (i.e. after 1 week of cultivation on Put-free media). However, after a week of further cultivation on Put-free media (i.e. on day 21), the levels of Put conjugates in these ESMs were comparable to those observed in the controls (Fig. 2b). The time course of Put conjugate formation on media containing 500 μM exogenous Put differed due to a 1 week lag-phase. However, as in the 10 and 100 μM cases, the levels of Put conjugates in the 500 μM treated cultures peaked on day 14 (Fig. 2b). After 4 and 5 weeks of maturation, there were no significant differences between the control embryos and those treated with either 10 or 100 μM Put with respect to their contents of free Put or Put conjugates. However, significantly higher levels of both forms of Put were observed in the embryos treated with exogenous Put at 500 μM (Fig. 2a, b). All of the tested exogenous Put concentrations reduced the Spd levels (Fig. 2c) in both the ESM and SE. However, there were pronounced increases in the Spm contents in embryos treated with 100 μM of exogenous Put, and very pronounced increases in those treated with 500 μM (Fig. 2d). In addition, the Spd/Put ratios for ESMs treated with exogenous Put after 21 days of cultivation were very different to those observed for the control ESMs (Fig. 3a). There were no great differences between the control cultures and those treated with either 10 or 100 μM Put in terms of the Spd/Put ratios in 4-week old embryos separated from the associated subtending tissue. However, cultures treated with 500 μM exogenous Put exhibited significantly lower Spd/Put ratios. The Spd/Put ratio for the somatic embryos of the control culture increased significantly during the 5th week of cultivation whereas those in the SEs of the treated cultures remained approximately further unchanged.

Yield of somatic embryos

There were pronounced differences between the control and Put-treated cultures with respect to the yield of somatic embryos at the end of maturation (Fig. 4a). Exogenous Put

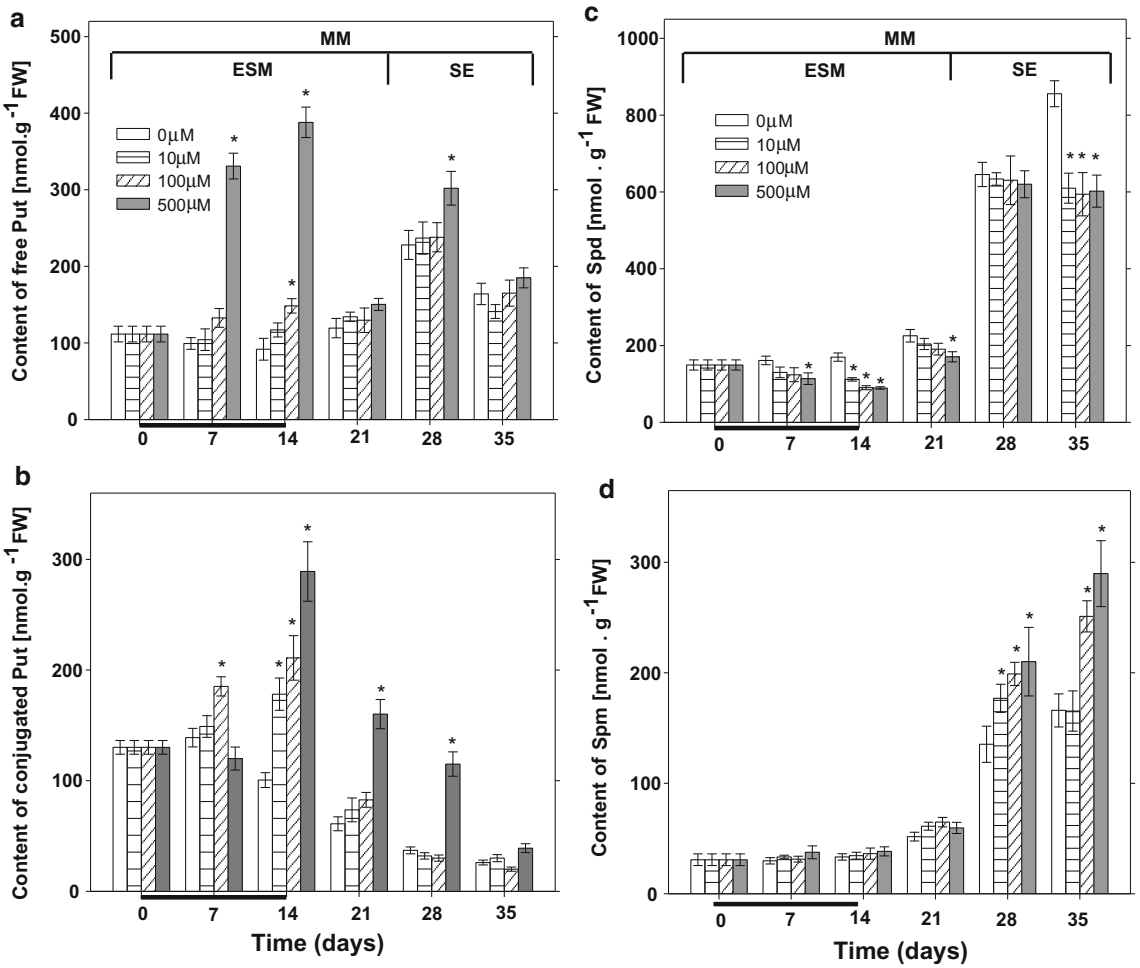

Fig. 2 Endogenous putrescine (Put), spermidine (Spd) and spermine (Spm) contents in the ESM and separated 4 and 5 week old Norway spruce somatic embryos (SE) after treatment with 10, 100 or 500 µM Put for 2 weeks during growth on maturation media (MM), which was followed by growth on Put-free MM. **a** Free Put contents; **b** conjugated Put contents; **c** Spd contents; **d** Spm contents. *ESM* embryogenic suspensor mass. Control culture—0 µM. The Put treatment is indicated by the *black box* on the abscissa

decreased the numbers of all embryos and fully developed mature embryos in a concentration-dependent manner. Treatment with 10, 100, or 500 µM Put reduced the number of all embryos relative to the control by 25, 57, or and 60 %, respectively. The number of fully mature somatic embryos was also lower in Put-treated cultures. However, the number of fully developed embryos as a proportion of all formed embryos was around 40 % for all cultures.

Treatment with putrescine during proliferation

The induction of meristematic activity due to treatment with exogenous Put during maturation prompted us to investigate the effects of Put treatment during proliferation, i.e. the stage when new embryogenic structures emerge. Due to the observed formation of extended meristematic centers following Put treatment during maturation, we expected the application of exogenous Put to have positive effects on meristem growth in proliferating ESMs and to thereby enhance the yield of embryos at the end of maturation.

Histological observations

During proliferation, the ESM consisted of early embryos and/or polyembryogenic complexes (supplementary Fig. 2a). During proliferation, new embryogenic structures emerge and old ones are degraded. The application of Put (at concentrations of 10, 100 or 500 µM) to the ESM during the 2 weeks immediately prior to maturation stimulated meristem growth. The meristematic centers of the treated ESMs were large, long and often malformed (supplementary Fig. 2b, c). The effect was much more pronounced in the ESMs treated with higher Put concentrations (supplementary Fig. 2d). The treated ESMs

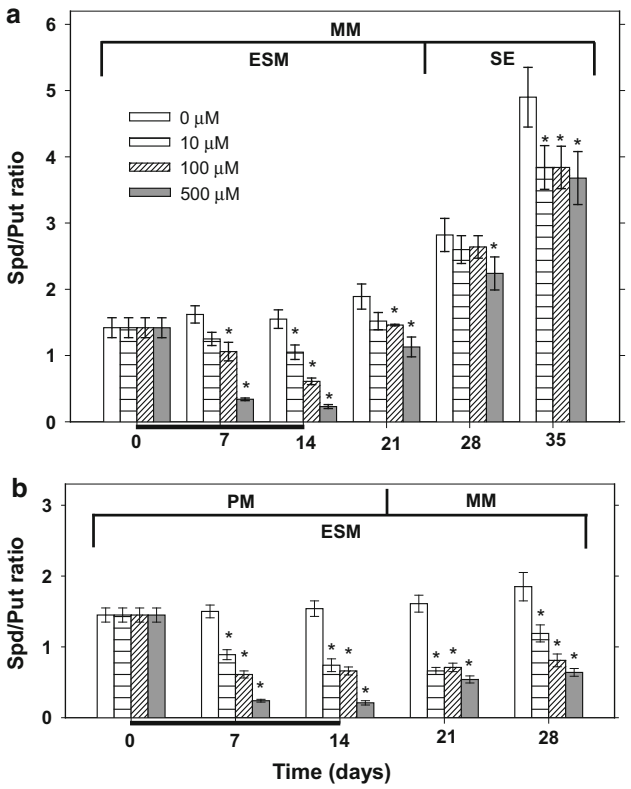

Fig. 3 Changes in the Spd/Put ratios in ESM and somatic embryos (SE) of Norway spruce. **a** Treatment with 10, 100 or 500 µM putrescine (Put) during the growth on maturation media; **b** treatment with 10, 100 and 500 µM putrescine (Put) during the growth on proliferation media. Control culture—0 µM. The Put treatment is indicated by the *black box* on the abscissa

were allowed to mature on Put-free media. However, single embryos were only rarely released from these polyembryonic centers and malformed embryos were common.

Polyamine contents

During the 14 days of proliferation growth, the Spd content of the non-treated control ESM (results for the controls are indicated with the label 0 µM in the figures) was greater than that of Put, and that of Spm was the lowest one (Fig. 5a, c, d). The PA levels did not change significantly during proliferation, so the Spd/Put ratio was stable at around 1.50 (Fig. 3b). At all tested concentrations, exogenous Put promoted increases in the ESM's levels of endogenous free Put on days 7 and 14. During the subsequent cultivation on Put-free maturation media (from day 14 onwards) the endogenous levels of free Put declined even in samples that had been treated with the highest tested Put concentration (Fig. 5a). Treatment with exogenous Put at concentrations of 10 or 100 µM during proliferation caused significant increases in the levels of Put conjugates measured on days 7 and 14 (Fig. 5b). During the subsequent cultivation on Put-free maturation media, the levels of Put conjugates declined. However, their levels in cultures that had been treated with 100 µM Put were still significantly greater than in control cultures. Treatment with the highest tested Put concentration (500 µM) caused the conversion of free Put into conjugated forms after a 7 day lag-phase. The concentration of conjugated Put derivatives therefore peaked later, i.e. on day 21, 1 week after the cultures had been transferred to Put-free maturation media. The abundance of the conjugates then decreased on day 28, 2 weeks after the cessation of Put treatment, but remained many times higher than in the control cultures (Fig. 5b). While treatment with exogenous Put caused an increase in endogenous Put levels within the ESM in proportion to the applied concentration, all Put treatments caused reductions in Spd levels (Fig. 5c). A

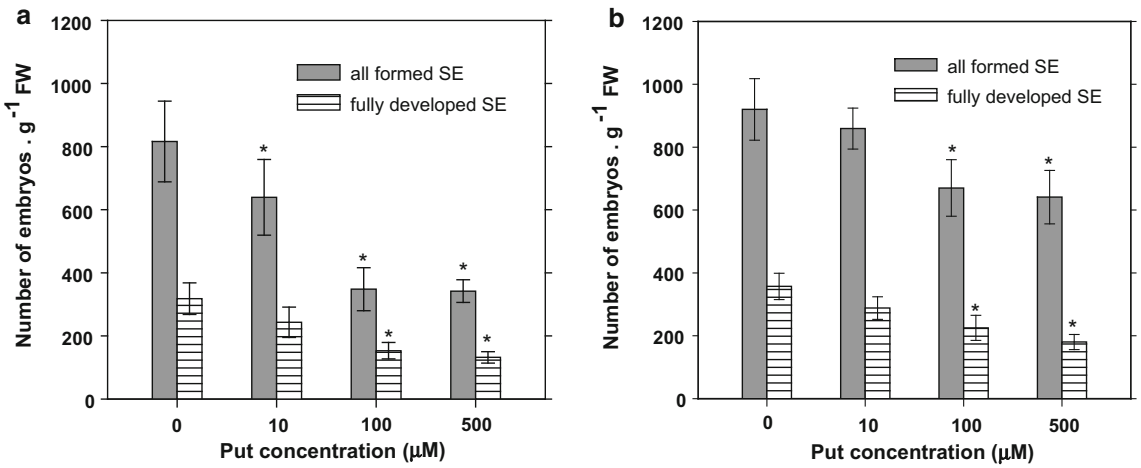

Fig. 4 Yield of Norway spruce somatic embryos determined at the end of maturation. **a** Treatment with 10, 100 or 500 µM putrescine (Put) during the growth on maturation media; **b** treatment with 10, 100 and 500 µM putrescine (Put) during the growth on proliferation media. The embryo yields after 5 weeks of maturation were expressed on a per-gram basis relative to the fresh mass of the embryogenic culture at the start of the experiment

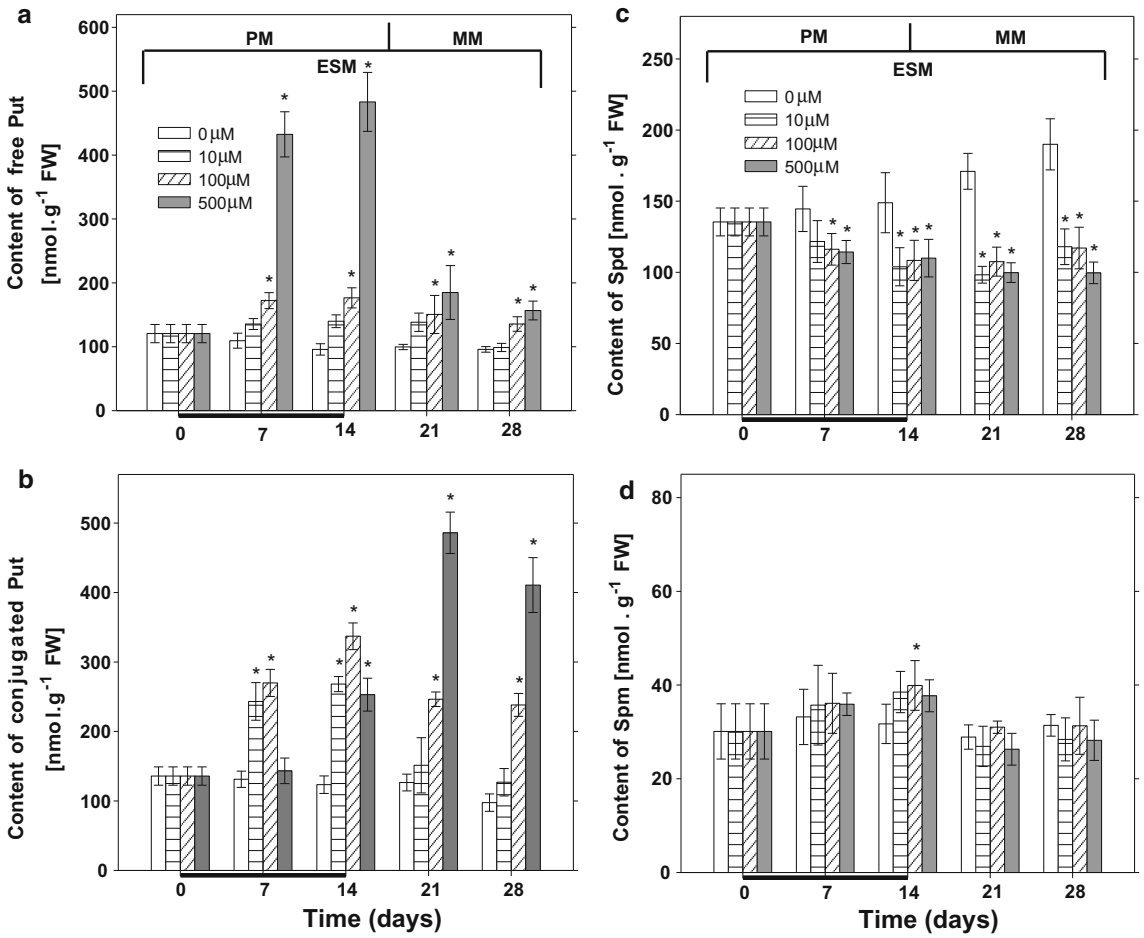

Fig. 5 Endogenous putrescine (Put), spermidine (Spd) and spermine (Spm) contents in the ESM of Norway spruce after treatment with 10, 100 or 500 µM Put during 2 weeks' growth on proliferation medium (PM) followed by 2 weeks' growth on Put-free maturation media (MM). **a** Free Put contents; **b** conjugated Put contents; **c** Spd contents; **d** Spm contents. Control culture—0 µM. The Put treatment is indicated by the *black box* on the abscissa

slight increase in Spm levels was observed in the treated cultures on day 14 (Fig. 5d). The increase in endogenous Put levels in the ESM during cultivation on Put-treated proliferation media and the associated decline in Spd levels caused a pronounced decrease in the Spd/Put ratio compared to the control (Fig. 3b). Due to the slight increase in Spd levels in ESMs treated with 10 µM Put on day 28 (after 2 weeks' cultivation on Put-free maturation media), the Spd/Put ratio increased relative to its value on day 21 in this particular case (Fig. 3b).

Yield of somatic embryos

As was observed for Put treatment during maturation, the application of Put during proliferation reduced embryo yields (Fig. 4b). There were no significant differences between the control cultures and those treated with 10 µM Put with respect to the numbers of all embryos or fully developed embryos. However, higher exogenous Put concentrations reduced the number of all embryos by around 25 % relative to the control. The number of fully developed embryos as a percentage of the total number of formed embryos decreased from about 40 % in the control to approximately 30 % in the cultures treated with 100 or 500 µM Put.

Discussion

The effects of exogenous PAs on the development of embryonal-suspensor masses and the formation of somatic embryos have previously been investigated in an effort to increase the frequency of somatic embryogenesis in *Picea glehnii* cultures. However, the addition of Put at 10 or 100 µM had only slight positive effects on the development of *P. glehnii* ESMs while treatment with Put at 500 µM inhibited ESM proliferation and development (Nakagawa et al. 2011). In this work, the ESM of Norway

spruce was cultivated in maturation and/or proliferation media containing exogenous Put at concentrations of 10, 100, or 500 μM. In contrast to the results obtained with *P. glehnii*, Put treatment promoted meristem growth in Norway spruce ESM whether applied in the maturation or the proliferation (Fig. 1, supplementary 1, supplementary 2). However, the polyembryogenic complexes generally did not successfully release embryos during their subsequent development and reduced yield of mature somatic embryos was observed at the end of the maturation period (Fig. 4a, b). The role of Put in cellular growth has yet to be fully elucidated. In *Pinus taeda* suspension cultures, high levels of endogenous Put were associated with reductions in cellular growth (Silveira et al. 2004) whereas Put stimulated cellular division in *Araucaria angustifolia* embryogenic cultures (Steiner et al. 2007).

The cellular contents of endogenous free and conjugated Put in the ESMs increased linearly with the concentration of exogenous Put in the medium (Figs. 2a, b, 5a, b). However, after 5 weeks of ESM maturation, there were no significant differences between the separated embryos from the control ESMs or those treated with 10 and 100 μM Put with respect to their contents of free or conjugated Put. Higher contents of both forms of Put were only observed in embryos that had been treated with the highest exogenous Put concentration (Fig. 2a, b). In some plant species, the conjugation of free PAs with hydroxycinnamic acids is an alternative to oxidative deamination as a way of regulating endogenous PA levels (Bouchereau et al. 1999; Bagni and Tassoni 2001; Biondi et al. 2001; Cvikrova et al. 2008). The levels of soluble Put conjugates detected in the ESMs were high and proportional to the concentration of exogenous Put in the media, suggesting that hydroxycinammic amides are formed in the cells of the ESM to maintain PA homeostasis (Figs. 2b, 5b). The difference between the Put levels determined in ESMs treated with exogenous Put during maturation and proliferation could be due to the diversity of PA metabolism or to the different physiological processes occurring in the two stages: the proliferation stage is primarily characterized by cell multiplication whereas maturation also involves histodifferentiation. In addition, it may be that the two stages differed in PA metabolism because of the different phytohormone contents of the proliferation and maturation media. In *A. angustifolia*, the addition of Put increased the levels of endogenous IAA and caused ABA accumulation relative to untreated controls. This suggests that Put has specific effects on the balance of IAA and ABA during cell growth (Steiner et al. 2007) and is important because ABA metabolism plays a vital role in the development of conifer somatic embryos, as reviewed by Stasolla and Yeung (2003). Significant increases in Spd levels are reportedly associated with the formation of somatic embryos in *P. abies* (Santanen and Simola 1992) and *P. radiata (*Minocha et al. 1999). The changes in the levels of soluble free Put and Spd, and the Spd/Put ratio, that were observed during somatic embryo development in this work are consistent with the results of our previous studies on somatic embryogenesis in *P. abies* (Gemperlova et al. 2009). Treatment with exogenous Put during both maturation and proliferation increased the levels of endogenous Put (Figs. 2a, 5a), whereas all applied concentrations of Put decreased the Spd content of the ESM (Figs. 2c, 5c). Similar results were observed in embryogenic cultures of *A. angustifolia*, where treatment with exogenous Put increased levels of endogenous Put without affecting either Spd or Spm levels in one case (Silveira et al. 2006) but reduced Spd levels in another (Steiner et al. 2007). Interestingly, treatment with exogenous Put also decreased the endogenous Spd concentrations in Scots pine calli (Sarjala et al. 1997). These results suggested that a lack of available aminopropyl groups may explain the poor conversion of exogenous Put to free endogenous Spd and Spm (Silveira et al. 2006). The increase in endogenous Put levels in ESMs cultivated on Put-containing media together with the concomitant decrease in Spd levels caused a pronounced reduction in the Spd/Put ratios of treated ESMs compared to untreated controls (Fig. 3a, b). In 5 week old embryos separated from the associated subtending tissue, the reduced Put levels (Fig. 2a) and the pronounced increase in Spd concentrations (Fig. 2c) increased the Spd/Put ratio (Fig. 3a). Nevertheless, the differences in the Spd/Put ratios for the 5 week old SEs from the Put-treated and control cultures were reflected in their different levels of embryo development and embryo yields (Fig. 4a). Increased Spd/Put ratios have previously been observed in ESMs ESMs (Santanen and Simola 1992; Kong et al. 1997) and developing somatic embryos (Minocha et al. 1999; Gemperlova et al. 2009), and were suggested to be essential for SE formation. In vitro, Put is generally associated with the stimulation of cellular division and Spd with morphogenic potential. Increases in Spd levels are known to be indicative of cellular competence for somatic embryogenesis in several species, such as in tissue cultures of *Hevea brasiliensis* (El Hadrami et al. 1992), in the initiation phase of somatic embryogenesis in *Panax ginseng* (Monteiro et al. 2002) and in the development of globular pro-embryos in alfalfa (Cvikrova et al. 1999). The reduced SE yields observed at the end of maturation in cultures treated with exogenous Put during maturation or proliferation may be linked to the lower levels of Spd in these cultures (relative to untreated controls). The differences between the effects of Put application in the different treatment regimes on the yield of embryos (estimated after 5 weeks of maturation) probably result from the different time after Put application (3 and 5 weeks after application

in maturation and proliferation, respectively). Put-treated cultures in proliferation might cope better with increased level of Put due to prolonged time for further free Put metabolic channelling (PA conjugation and/or catabolism). All of the tested Put concentrations reduced Spd levels in both the ESM and SE. However, there was a pronounced increase in the Spm content of Put-treated embryos (Fig. 2d). Spm is more biologically dynamic than the other PAs. It is involved in stabilizing the cellular membrane and shows certain antioxidant effects under stress conditions (Bouchereau et al. 1999). Spm accumulation was observed in *P. abies* ESM that had been treated with a cryoprotectant, and significantly increased Spm levels were reported in Norway spruce SE during desiccation. Both of these findings are consistent with the suggested role of Spm under abiotic stress conditions (Vondrakova et al. 2010; Gemperlova et al. 2009). In keeping with our previous results, one could reasonably suggest that the significant increase in Spm levels observed in embryos formed from ESMs treated with Put at 100 μM and especially 500 μM (Fig. 2d) represents a stress response induced by the high levels of exogenous Put.

In conclusion, the exogenous applications of Put during proliferation and/or maturation had no discernible positive effects on subsequent embryo development.

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