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Polyamines during somatic embryo development in Norway spruce (*Picea abies* [L.])

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ABSTRACT: Contents of free polyamines (putrescine, spermidine and spermine) were determined in different developmental stages of Norway spruce (*Picea abies* [L.] Karst.) somatic embryos by means of HPLC. Determinations were performed embryogenic tissue after 4 weeks of the growth on proliferation medium, after 2 and 5 weeks of the culturing on maturation medium, and 2 weeks after desiccation. Maturation of somatic embryos (after 5 weeks) was accompanied by increase of concentrations of putrescine (2.3 times) and spermidine (3.2 times). In comparison with above mentioned polyamines, spermine concentrations were significantly lower (4.3 times). Two weeks after desiccation, the concentrations of putrescine decreased 5.4 times and spermidine 2.2 times in comparison with mature embryos. To improve the efficiency of somatic embryogenesis of less responsive genotypes, the supplementation of growth media by polyamines is discussed.

Keywords: Norway spruce; somatic embryogenesis; putrescine; spermidine; spermine

Abbreviations: abscisic acid – ABA; 6-benzylaminopurine – BAP; cytokinins – CKs; 2,4 dichlorophenoxyacetic acid – 2,4-D; indolylbutyric acid – IBA; putrescine – Put; spermidine – Spm; spermine – Spd

Somatic embryogenesis is considered as an advantageous technique for *in vitro* propagation of conifers. Moreover, it could be usefully utilized for a detailed study of developmental processes accompanying differentiation of embryo and its conversion during polyembryogenesis.

Regeneration of complete plants by means of somatic embryogenesis in the Norway spruce (*Picea abies* [L.] Karst.) was described by many authors (Boenman 1985; Hakman et al. 1985; Attree, Fowke 1993; Chalupa 1985; Chalupa et al. 1990; Malá et al. 1995). The process of somatic embryogenesis is divided into three stages according to the grade of embryo differentiation: induction of embryogenesis, proliferation and maturation of somatic embryos, and conversion of mature embryos into complete plants. However, embryo maturation and low germination frequencies are main limitations for a broader use of this technique (Attree, Fowke 1993).

The induction of embryogenic tissues can be achieved by applying phytohormone treatments on mature or immature zygotic embryos. Induction and continuous proliferation require auxin and cytokinins (CKs), whereas the further development and maturation of embryos depend on abscisic acid (ABA) (Attree et al. 1991). The initiation rate is higher when immature zygotic embryos are used;

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however, it is difficult to determine an optimal cone harvest time (Chalupa 1985; Vágner et al. 2005a). The transfer of the embryogenic tissue from proliferation onto maturation medium leads to the embryo development. Despite of the successful protocol for the establishment of Norway spruce somatic embryogenesis technique, there is a lack of data concerning the endogenous composition of biologically active compounds both in somatic and zygotic embryos. Generally, the development of embryos as well as their conversion into plantlets is closely associated with changes in endogenous phytohormone levels. Changes in endogenous hormone levels (IAA, ABA and ethylene) during Norway spruce somatic embryo development and maturation have been reported recently (Vágner et al. 2005b).

Besides the key roles of auxin and cytokinins, polyamines (PAs) have a very important function in differentiation processes. PAs are ubiquitous cell components essential for normal growth and are considered to be a new class of plant hormones implicated also in the regulation of somatic and zygotic embryogenesis (Kong et al. 1998; Silveira et al. 2004). PAs have a wide spectrum of action with some similarities both with auxins and CKs and in cooperation with plant phytohormones they modulate morphogenic processes (Altamura et al. 1993). Most of the biological functions of PAs can be explained by their polycationic nature, which allows interactions with anionic macromolecules such as DNA, RNA and with negative groups of membranes. Three commonly occurring PAs in plants are diamine putrescine (Put), triamine spermidine (Spd) and tetramine spermine (Spm).

There are a number of reports indicating that PAs play a crucial role in somatic embryo development including few conifers (Minocha et al. 1993). Putrescine was the most abundant of PAs in the embryogenic suspension culture of *Pinus taeda* (Silveira et al. 2004), whereas the development of both somatic and zygotic embryos of *Pinus radiata* was characterized by a high level of spermidine (Minocha et al. 1999). A high level of putrescine was determined in the pro-embryogenic tissue of *Picea rubens*, while spermidine was predominant during the stages of embryo development (Minocha, Long 2004).

The present study was undertaken to establish changes in the levels of PAs during development of somatic embryos of highly responsive Norway spruce. This knowledge will enable us to improve the method of somatic embryogenesis in a less responsive hurst ecotype of Norway spruce.

Contents of free polyamines (putrescine, spermidine, and spermine) were determined in different developmental stages of Norway spruce somatic embryos.

MATERIALS AND METHODS

Induction of embryogenic tissue growth

Immature cones of 140 years old elite open pollinated Norway spruce (*Picea abies* [L.] Karst.) growing in the Labské Pískovce habitat conservation area in Northern Bohemia were collected in late July 2006 and stored at 4°C. After seed sterilization in 1% NaClO (Savo, Biochemie, CR), the extirpated immature embryos were cultivated (dark, 24°C) onto the solid E medium (Gupta, Durzan 1986) modified with 0.2 mg/l gelerit (Sigma – Aldrich, Germany) and phytohormones (0.5 mg/l of BAP, 1.0 mg/l of 2, 4-D and 0.5 mg/l of Kin; all Sigma – Aldrich, Germany) for the induction of embryogenic tissue differentiation (Malá 1991; Chalupa 1997).

Proliferation stage

After 4 weeks, the cultures were transferred onto a fresh medium of the same composition. The samples were taken for analyses after 4 weeks of culturing under the same conditions as mentioned above.

Maturation stage

For maturation, the cultures were transferred onto the solid E medium without phytohormones, supplemented with 8 mg/l of ABA and 20 mg/l of PEG of m. w. 3350 (both Sigma, Chemical Co., USA). ABA solution was filter-sterilized and added after autoclaving. The cultures were transferred onto the fresh medium every week. Cultures were kept in the same conditions as described above. After 2 weeks, the somatic embryo cultures were transferred onto the solid E medium containing 0.1 mg/l of IBA (Sigma Chemical Co., USA) and 20 mg/l of PEG and cultured under white fluorescent light (30 μmol/m^2/s) and 16 h photoperiod (Malá 1991). The samples were taken for analyses after 2 weeks and 5 weeks of the culturing.

Desiccation

The fully developed embryos only were desiccated. The embryos were carefully transferred on dry paper in small Petri dishes (3 cm in diameter). These open dishes were placed into large Petri dishes (18 cm in diameter) with several paper layers wetted by sterile water (100% humidity). Large Petri dishes were

covered by lids and sealed by Parafilm® (Chicago, Il, USA). They were kept under the light regime of 12 hours of light and 12 hours of darkness, at 20 ± 1°C for 2 weeks (Vágner et al. 2005a,b). The samples were taken for analyses after 1 and 2 weeks during desiccation.

Preparation of samples for analyses

For PAs determination, the 200 mg samples (fresh weight) were taken in the course of above-mentioned intervals of somatic embryo development. The samples were immediately frozen in liquid nitrogen and than stored at –80°C until determinations.

Polyamine analysis

Two analyses were carried out independently. Briefly, 200 mg of frozen sample was extracted with 2 ml of 5% (v/v) perchloric acid overnight at 4°C and 1,7-diaminoheptane was added as an internal standard. The extracts were centrifuged at 21,000 *g* for 15 min. Standards (Sigma-Aldrich, St Louis, MO, USA) and perchloric acid soluble free PAs were benzoylated according to the method of Slocum et al. (1989). HPLC analysis of benzoyl-amines was performed on Beckman-Video Liquid Chromatograph equipped with UV detector (detection at 254 nm) and C_{18} Spherisorb 5 ODS2 column (particle size 5 μm, column length 250 × 4.6 mm) according to the method of Slocum et al. (1989).

Statistical evaluations

Data obtained from two independent experiments with two parallel analyses were evaluated by Student's *t* distribution criteria. Means ± S.E. are shown in Table 1.

RESULTS AND DISCUSSION

The contents of free PAs (putrescine, spermidine and spermine) at different developmental stages of Norway spruce embryos were determined by the means of HPLC. During the growth of the embryogenic culture on proliferation medium, the embryogenic tissue contained approximately equal Put and Spd concentrations. The content of Spm at this stage was rather low. After 2 and 5 weeks of cultivation on maturing medium, when the culture contained globular and partly polarized embryos, a significant increase in the concentration of all three amines was observed. However, pronounced changes in PA levels and the changed proportion Spd/Put occurred after 5 weeks culture. At this stage the embryos could be separated from the remaining tissue. Ivory-coloured torpedo stage embryos with not yet well-developed cotyledons formed the major part of embryos (Fig. 1). A significant increase in PAs was observed in embryos in this stage of development. The concentration of Spd was significantly higher than that of Put. The embryos were characterized by 230, 324 and 275% increase in Put, Spd and Spm contents, respectively (compared with the contents in the embryogenic tissue grown on proliferation medium) (Table 1).

High Spd contents and higher concentrations of Spd than Put were also found in the torpedo stage of *Daucus carota* somatic embryos (Mengoli et al. 1989). Similarly, Spd was verified during somatic embryogenesis of *Vigna aconitifolia* (Kaur-Sawhney et al. 1985), *Hevea brasiliensis* (El Hadrami,

Table 1. Contents of free putrescine, spermidine and spermine in the Norway spruce embryogenic culture growing on proliferation (4 weeks) and maturation (2 weeks) media, in mature somatic embryos (5 weeks on maturation medium) and in embryos in the course of desiccation (1 and 2 weeks). In columns, the values are significantly different according to *t*-test at the 0.05 level (nmol/g DM)

	Putrescine	Spermidine	Spermine
Embryogenic culture	1,234.4 ± 112.2	1,361.7 ± 139.5	302.3 ± 26.5
Proliferation medium 4 weeks			
Embryogenic culture	1,850.5 ± 185.2	2,096.4 ± 199.3	392.4 ± 40.1
Maturation medium 2 weeks			
Mature embryos 5 weeks	2,831.7 ± 254.4	4,406.9 ± 450.2	833.7 ± 79.5
Mature embryos	780.1 ± 70.8	2,473.1 ± 248.0	631.6 ± 57.5
Desiccation 1 week			
Mature embryos	520.5 ± 48.3	1,972.5 ± 188.4	1,455.5 ± 135.2
Desiccation 2 weeks			

Fig. 1. Ivory-coloured torpedo stage embryos of Norway spruce after 5-weeks cultivation on maturation medium

D'Auzac 1992) and development of *Medicago sativa* globular pro-embryos (Cvikrová et al. 1999).

Analyses of PAs after 1 week of desiccation showed a marked decrease in all three PAs contents. At the 2nd week of desiccation, the radicle of embryos with well-developed cotyledons started changing its colour to the red. Mainly these embryos converted into plantlets (Fig. 2). During this interval of desiccation, a further decrease in Put and Spd occurred, whereas the contents of Spm significantly increased (Table 1). This increase could be interpreted as a non-specific response to desiccation stress. However, Spd could not represent the dominant polyamine in somatic embryos of conifers. Whereas the development of both somatic and zygotic embryos of *Pinus radiata* was characterized by a high level of Spd and its concentration positively correlated with the embryo development (Minocha et al. 1999), Put was the most abundant in the embryogenic suspension cultures of *Pinus taeda* (Silveira et al. 2004). High contents of Put were determined in the pro-embryogenic tissue of *Picea rubens*, while Spd was predominant during embryo development of this species (Minocha, Long 2004).

It was already mentioned above that the embryo maturation and low germination frequencies mean crucial obstacles for a broader use of somatic embryogenesis in forest practice. Since cellular polyamines are important for the growth and development of plant cells, the effect of improved nutrient composition of the culture media by addition of polyamines was studied in relation to plant regeneration ability. Exogenously supplied polyamines could positively influence the induction and somatic embryo development in less responsive plant genotypes. A stimulatory effect of putrescine and spermidine on the development of pro-embryogenic masses of *Cryptomeria japonica* was described (Nakagawa et al. 2006). Exogenous application of Spd in the proliferation stage of *Panax ginseng* somatic embryogenesis was proved to significantly increase the production

Fig. 2. The embryos with red coloured radicles which further developed and converted into plantlets after 2 weeks of desiccation phase

of embryos in cultures (Kevers et al. 2000). Favourable modification of cellular polyamine levels by addition of exogenous putrescine and spermidine led to the promotion of regeneration in poorly responding genotypes *Oryza sativa* (Shoeb et al. 2001). The studies of the possibility of improving the somatic embryogenesis in a less responsive hurst ecotype of Norway spruce by exogenous addition of polyamines will be the subject of our next experiments.

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Polyaminy v průběhu vývoje somatických embryí smrku ztepilého (*Picea abies* [L.])

ABSTRAKT: Volné polyaminy (putrescin, spermidin, spermin) byly stanovovány v průběhu vývoje somatických embryí smrku ztepilého metodou HPLC. Stanovení byla provedena v embryogenním pletivu rostoucím na proliferačním médiu po čtyřech týdnech, dále po druhém a pátém týdnu kultivace embryí na maturačním médiu a ve vyvinutých embryích po dvoutýdenní desikaci. Maturace somatických embryí po pátém týdnu byla provázena zvýšením koncentrace putrescinu (2,3krát) a spermidinu (3,2krát). Ve srovnání se zmíněnými polyaminy byly koncentrace sperminu významně nižší (4,3krát). Ve srovnání se zralými embryi po dvoutýdenní desikaci se koncentrace putrescinu snížila 5,4krát a spermidinu 2,2krát. Na základě výsledků se dá předpokládat, že lze pozitivně ovlivnit vývoj somatických embryí méně responzibilních genotypů suplementací polyaminů do živných médií.

Klíčová slova: smrk ztepilý; somatická embryogeneze; putrescin; spermidin; spermin

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