

Somatic embryogenesis in *Abies alba* × *Abies alba* and *Abies alba* × *Abies nordmanniana* hybrids

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Abstract

Maturation and germination of somatic embryos of hybrids *A. alba* × *A. alba* and *A. alba* × *A. nordmanniana* were followed by protein analysis of single embryogenic -suspensor masses (ESM) and analysis of storage protein accumulation during somatic embryo development. Very important step was one week pre-cultivation of ESM on medium with polyethylene glycol (PEG) and abscisic acid (ABA). Low osmotic potential of maturation medium and addition of ABA supported development of somatic embryo. Also partial drying of somatic embryo during following three weeks was needed for its normal development. In spite of morphologically fully developed, the somatic embryos were not physiologically ready for germination at least in terms of storage protein accumulation.

Additional key words: fir, somatic embryo maturation, protein analysis.

Introduction

Somatic embryogenesis is one of the most promising method for conifer micropropagation. In the genus *Abies* the induction of somatic embryogenesis has been achieved both from the immature zygotic embryos in *Abies alba* (Schuller *et al.* 1989, Ostrolucká 1992), in *A. nordmanniana* (Norgaard and Krogstrup 1991) and from mature zygotic embryos in *A. alba* (Gebhardt *et al.* 1988, Hristoforoglu *et al.* 1995), *A. nordmanniana* (Norgaard *et al.* 1992), *A. fraseri* (Guevin *et al.* 1992) and *A. balsamea* (Guevin *et al.* 1993). More recently, plantlets of *A. balsamea* (Guevin *et al.* 1994) and *A. alba* (Hristoforoglu *et al.* 1995) have been regenerated from somatic embryos (SE), but the reports did not include information about the further growth of these plants.

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In extensive hybridization program concerning the firs, several promising hybrids have been obtained with the aim to obtain trees more resistant to rapidly changing conditions of environment (Kormuťák 1985). In connection with this program somatic embryogenesis from immature embryos of several hybrids has been induced (*A. alba* × *A. alba*, *A. alba* × *A. nordmanniana* - Gajdošová *et al.* 1995; *A. alba* × *A. cephalonica*, *A. alba* × *A. numidica* - Salajová *et al.* 1996), but no plantlets production has been reported until now.

In this study maturation and germination of somatic embryos of *A. alba* × *A. alba* and *A. alba* × *A. nordmanniana* is described followed by protein analysis of single embryogenic-suspensor masses (ESM) and analysis of storage protein accumulation during somatic embryo development.

Materials and methods

Induction and maturation of somatic embryos: Embryogenic-suspensor masses were established as described previously (Gajdošová *et al.* 1995). The cultures were initiated in 1991 and 1992 from immature zygotic embryos of *Abies alba* × *Abies alba* and *Abies alba* × *Abies nordmanniana* hybrids, cultured on Schenk and Hildebrandt (SH) medium with 1 mg dm⁻³ 6-benzylaminopurine (BAP, *Sigma B9395*) according Schuller *et al.* (1989). Embryogenic lines were maintained on proliferation SH medium supplemented with 0.5 mg dm⁻³ BAP, 1000 mg dm⁻³ casein hydrolysate, 500 mg dm⁻³ L-glutamine, and 50 mg dm⁻³ myo-inositol, in dim light at 24 °C. Cultures were subcultured biweekly onto fresh medium. The following lines were used in this study: *A. alba* × *A. alba* induced in 1991 (1, 2, 4, 7, 8, 9, 12, 19, 24, 25), 1992 (11/2, 12/2, 13/2, 15/2, 20/2), *A. alba* × *A. nordmanniana* induced in 1991 (42, 63), and 1992 (41/2, 42/2, 45/2, 48/2).

ESMs were transferred on maturation medium in plastic Petri dishes (Ø 90 mm) two weeks after the last subculture. Average mass of each ESM was 500 mg. SH maturation medium contained double concentration of SH medium vitamins, 100 mg dm⁻³ myo-inositol, 2000 mg dm⁻³ casein hydrolysate, 1000 mg dm⁻³ L-glutamine. Low osmotic potential was caused by lactose (*Ferak*) and polyethylene glycol (PEG 4000, *Merck 807490*). Maturation medium was gelled with 0.3 % (m/v) of phytigel (*Sigma P8169*) and autoclaved (with all media components) at 121 °C for 20 min.

During the first maturation step ESMs were cultured on medium containing 60 g dm⁻³ lactose, 10 g dm⁻³ PEG in presence or absence of 10 mg dm⁻³ abscisic acid (ABA, *Sigma A1049*). After one week cultivation the embryogenic lines were transferred to medium with 72 g dm⁻³ lactose, 10 g dm⁻³ sucrose (*Slavus*), 10 mg dm⁻³ ABA. In the second variant this medium was supplemented with 0.1 mg dm⁻³ IBA (*Sigma I5386*). Five embryogenic cell lines (ECL) were compared to find effect of IBA addition on somatic embryo maturation. Cultivation of ESM on maturation medium continued for seven weeks in dim light.

Mature somatic embryos were removed and placed in Petri dish (\varnothing 60 mm) and subjected to a high relative humidity treatment (Roberts *et al.* 1990b). Petri dish was opened and placed on moist filter paper in Petri dish (\varnothing 90 mm) which was sealed with parafilm. Somatic embryos in Petri dishes were cultured at 22 - 23 °C during three weeks. The last 48 h they were kept at 4 °C.

Somatic embryos germinated on liquid SH medium with 0.5 mg dm⁻³ gibberellic acid (GA₃, *Sigma G7645*) placed 4 d in the dark, later in the light. Seedlings were cultured on SH medium with half strength concentration of salts, 1 % (m/v) activated charcoal, 20 g dm⁻³ sucrose and 0.75 % (m/v) agar. The experiment was repeated twice with the same embryogenic lines.

Protein analysis: Samples (single ESMs and different stages of somatic embryo development) were ground in liquid nitrogen to a fine powder and extracted in 0.1 M Tris-HCl buffer (pH 8.5) containing 4 % (m/v) sodiumdodecylsulphate, 2 % 2-mercaptoethanol, 20 % glycerol and 10 µg cm⁻³ leupeptin (Hurkman and Tanaka 1986). Zygotic embryo protein extracts were used for comparison with protein composition of somatic embryos. The extracts were heated for 3 min at 100 °C and insoluble material was removed by centrifugation at 12 000 g for 15 min at 4 °C. The supernatants were diluted with sample buffer (Laemmli 1970) containing bromophenol blue as a tracking dye. Samples containing equal amounts of proteins were loaded to 12.5 % SDS PAGE. Gels were stained with Coomassie brilliant blue R-250 and analysed by scanning densitometry. Molecular mass standards (ISS) were used to calculate the molecular mass of proteins in individual bands.

Results and discussion

Low osmotic potential of medium with PEG, presence of ABA supported development of somatic embryos in presence or absence of IBA. The following responses were observed during maturation: differentiation of mature somatic embryos, precociously germinating somatic embryos, nodule and abnormal somatic embryo formation. IBA stimulated maturation of somatic embryos in some ECL and number of mature SE was different in individual genotypes, but expressive formation of normal developed SE was observed in line 45/2 on medium with IBA. Normal developed somatic embryos which looked like zygotic embryos were yellow -green with developed cotyledons and root tip.

The response of tested ECL to maturation treatment was following: *A. alba* × *A. alba* 1991 - in 30 % of ECL was observed somatic embryo maturation, in ECL from 1992 in 80 %. *A. alba* × *A. nordmanniana* 1991 - no maturation was obtained, maturation of somatic embryos was observed in 75 % of ECL from 1992. In spite of the fact, that no detailed analysis had been done, the dependence of somatic embryo maturation on genotype was apparent.

Very important step was one week cultivation on medium with PEG, where ABA was added. Two weeks or longer cultivation on this medium was not effective. Differences were observed later during cultivation on lactose contained media.

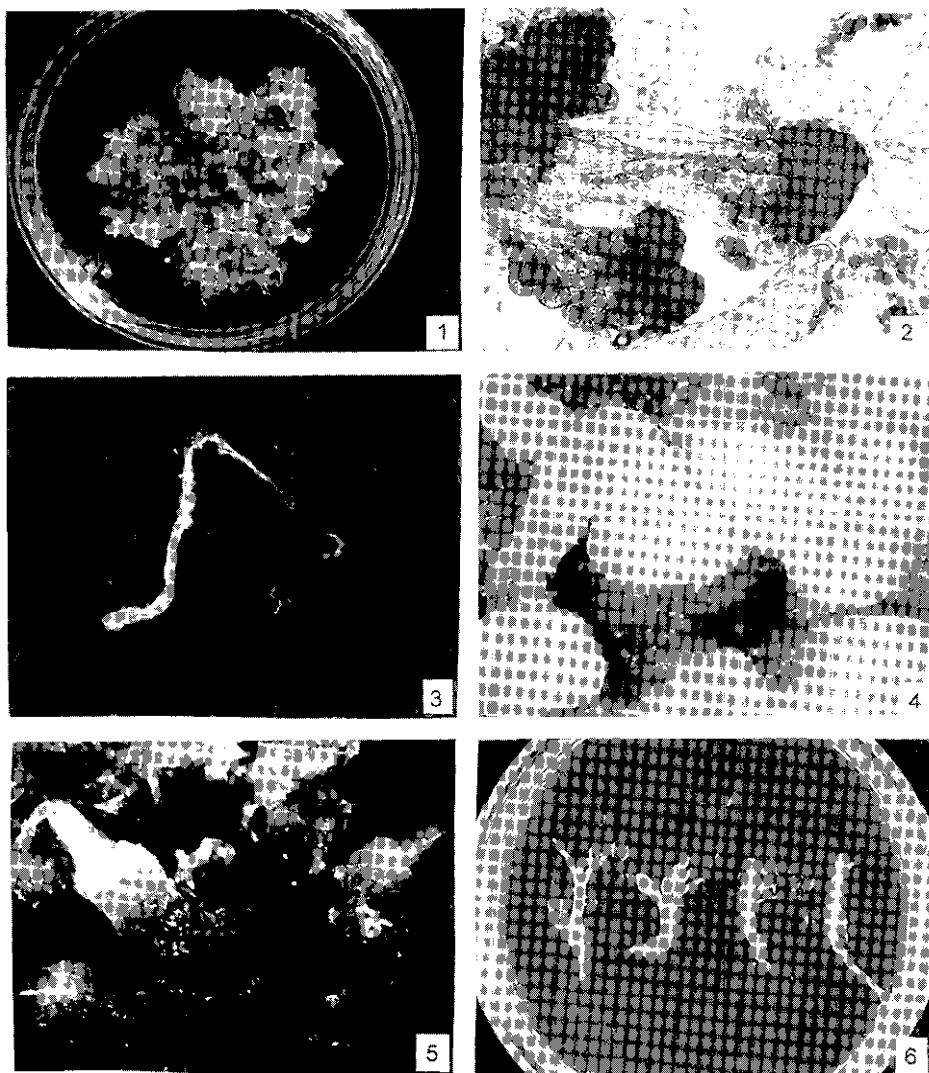


Fig. 1. Embryogenic suspensor mass (ESM) of interspecific hybrid *A. alba* \times *A. nordmanniana* on proliferation medium, stage 1.

Fig. 2. Clusters of embryogenic cells with elongated suspensor cells.

Fig. 3. Isolated young somatic embryo of *A. alba* \times *A. alba* from ESM.

Fig. 4. Developing somatic embryo in globular stage on maturation medium with lactose and ABA, stage 2.

Fig. 5. Somatic embryos with developing cotyledons before desiccation, stage 3

Fig. 6. Plantlets regenerated from somatic embryos.

Somatic embryos developed into globular stage but in cultures precultured two weeks or longer on medium with PEG and ABA they turned brown and died.

Dehydration of somatic embryos in high relative humidity treatment was needed for their normal development. Also in the absence of the partial drying treatment somatic embryos of white spruce enlarged in size, what was primarily due to vacuolisation of cells and the formation of large intercellular air spaces (Kong and Yeung 1992).

Mature somatic embryos developed further when transferred to medium lacking ABA. After two week cultivation, germinating embryos developed into plantlets with elongated hypocotyl, cotyledons and root. In general, shoots either with or without roots, had a tendency to form a resting buds what results in inhibition of following growth. Similar results were described in *Picea abies* (Jalonon and von Arnold 1991).

Embryogenic cell lines compared in total protein analysis were uniform irrespective of their morphology or embryogenic potential. The lack of correlation between the different morphological types of ESM and the protein profiles of individual callus lines indicates the absence of association between the corresponding traits at both the morphological and biochemical levels (Gajdošová *et al.* 1995). But a comparative study on isoenzyme composition of peroxidase, glutamate dehydrogenase and non-specific esterase in five embryogenic cell lines of *Abies alba* and two embryogenic lines of the hybrid combination *Abies alba* × *Abies nordmanniana* revealed a considerable variability between individual embryogenic lines what could be connected with different embryogenic capacity (Kormuťák and Vooková 1998).

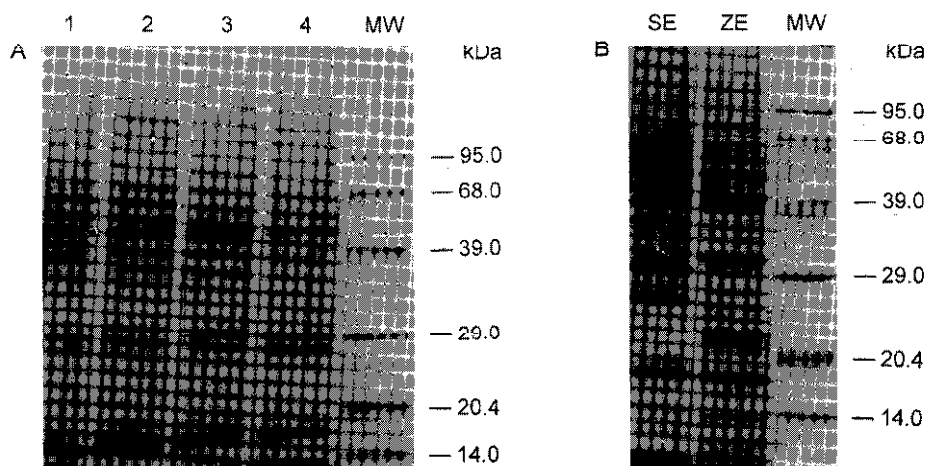


Fig. 7. SDS-PAGE profiles of proteins from *A. alba* × *A. alba*: A) ESM + maturing embryos at different stages of development: 1 - stage 1, 2 - stage 2, 3 - selected embryos at stage 3, 4 - mature somatic embryo after desiccation; B) Comparison of protein profiles of desiccated somatic embryo (SE) and zygotic embryo (ZE). MW - molecular mass standards.

Since the storage proteins are of the great importance for quality of somatic embryos and seedling establishment (Pitel *et al.* 1992, Hakman 1993), the protein accumulation during four different stages of somatic embryo development has been analysed for better understanding and regulation of maturation in this recalcitrant species. Stage 1 represents ESM on proliferation medium (Figs. 1 - 3), stage 2 - ESM with globular embryos after 1 week on medium with lactose, PEG and ABA (Fig. 4), stage 3 - fully developed cotyledonary embryos (Fig. 5) and stage 4 - somatic embryos after desiccation.

The SDS-PAGE analysis under reducing conditions showed similar protein pattern of stage 1 and stage 2. There are visible strong bands representing the proteins with molecular masses (M_r) of 48, 45, 17 kDa and faintly visible bands of M_r 38 and 30 kDa. In stage 3 the protein of M_r 30 kDa is less abundant like in stages 1 and 2, and an increase of the proteins with M_r 29 and 27 kDa is clearly visible, which accumulated during the desiccation period (stage 4). These bands were not detected in mature zygotic embryos. Strong protein bands of M_r 48, 45 and 17 kDa were detected in mature zygotic embryos of *A. alba* similarly as in all stages of somatic embryo development. In protein patterns of zygotic embryos there are some bands with M_r of 41, 33, 24, 22, 20 and 14 kDa which were faintly visible in the somatic embryos (Fig. 7).

During the development of *Abies alba* somatic embryos similar morphological stages as described for zygotic embryogenesis, were observed. At the end of maturation period morphologically ripe somatic embryos were obtained. However, germination process of these embryos rarely continued after a few days. Hakman *et al.* (1990) found a similar content of storage proteins in somatic and zygotic embryos of Norway spruce. Flinn *et al.* (1991) confirmed the same phenomenon in interior spruce. In our investigation we found different protein composition in zygotic and somatic embryos by SDS-PAGE analysis. In contrast to the protein pattern of somatic embryos proteins with M_r of 41, 33, 24 and 22 were found to be abundant in zygotic embryos. These proteins which belong to the major storage proteins in conifers (Roberts *et al.* 1990a, Flinn *et al.* 1991, Hakman 1993) were very slightly visible in obtained mature somatic embryos. Our results confirmed absence of 33 kDa range subunit and 22 kDa range subunit of the complex 55 kDa globulin storage protein as a unique feature of seed proteins from conifers. ESMs were cultured on medium with cytokinin during four years, which probably could influence somatic embryo development. The cytokinin treatment keeps the embryo in a germinationlike phase and it is therefore possible to postulate that some or all of the storage proteins are also involved in processes related to cell division in the hypocotyl and cotyledons. The cytokinin treatment of *Picea abies* zygotic embryos suppressed the synthesis of 14 proteins which are abundant during seedling development (Stabel *et al.* 1990). On the other hand, the most abundant proteins were 29 and 27 kDa in our somatic embryos which were not detected in zygotic embryos. The increased concentration of these two proteins was observed after maturation on lactose and ABA (stage 3) and intensively continued during desiccation period (stage 4). Central role of ABA and osmoticum in conifer embryogeny is the inhibition of cleavage polyembryony, maintenance of dormancy and inhibition of precocious

germination, embryo morphogenesis, desiccation tolerance and accumulation of storage reserves (Misra 1994). According to the literature osmotica and ABA have an additive effect on development of early stages of somatic embryos and represents an additional means to maximize globular embryo production. However, prolonged exposure of the cultures to medium with low osmotic potential inhibited the formation of cotyledonary embryos in interior spruce (Roberts 1991). The proteins of 29 and 27 kDa which were abundant in somatic embryos and were not detected in zygotic embryos are considered as stress proteins which are produced in consequence of improper type, concentration and treatment duration of osmoticum and continued during desiccation period.

The analysis of accumulation of storage protein showed that in spite of morphologically fully developed, somatic embryos were not physiologically ready for regular germination. Therefore, the further experiments will be focused on improving single steps of the maturation process.

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