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Secondary somatic embryogenesis in *Abies numidica*

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Abstract

The induction of secondary somatic embryogenesis in *Abies numidica* De Lann. was achieved. Precotyledonary, cotyledonary, and desiccated cotyledonary embryos were used as explants. Cotyledonary embryos before desiccation were the most suitable. The most beneficial was induction medium Schenk and Hildebrandt (SH) with 1 mg dm^{-3} thidiazuron and 1000 mg dm^{-3} myo-inositol. Initiation frequency was from 1 to 34 %. Maturation of somatic embryos was achieved on modified Murashige and Skoog medium supplemented with 40 g dm^{-3} maltose, 100 g dm^{-3} polyethylene glycol-4000 and 10 mg dm^{-3} abscisic acid. Mature somatic embryos after three weeks of desiccation germinated on SH medium with 10 g dm^{-3} charcoal and 10 g dm^{-3} sucrose.

Additional key words: Algerian fir, auxin, cytokinin, myo-inositol, protein analysis.

Introduction

Somatic embryogenesis in *Abies* was initiated from immature as well as from mature zygotic embryos (for review see Nørgaard and Krogstrup 1995). Roth and Schmidt (1998) initiated embryogenic culture from cotyledon explants of 5 to 10-d-old seedlings of *Abies alba*. Recently, Salajová and Salaj (2001) reported initiation of somatic embryogenesis from cotyledon explants isolated from hybrid firs seedlings and repetitive somatic embryogenesis from cotyledon explants of hybrid firs emblings. In other conifers, secondary somatic embryogenesis was reported in *Picea abies* (Mo *et al.* 1989), *Picea glauca* (Ellis *et al.* 1993), *P. glauca* × *engelmannii* complex (Eastman *et al.* 1991) and in *Larix* sp. (Lelu *et al.* 1994). Secondary somatic embryogenesis of *Larix laricina* was exploited in order to achieve genetic transformation (Klimaszewska *et al.* 1997). The method of secondary somatic embryogenesis is being used in our laboratory to improve the maturation potential of *A. numidica* lines that exhibit decline.

The aim of the work presented here was the development of efficient protocol for initiation of secondary somatic embryogenesis in *Abies numidica* De Lann. For this purpose, precotyledonary, cotyledonary and desiccated cotyledonary embryos were used as primary explants. Difference among the primary explants we tried to define by protein analysis. Four types of treatments were used in order to assess the most beneficial induction medium. In this report we described also somatic embryo maturation and regeneration of plants.

Materials and methods

Initiation of the first cycle embryogenic cultures: Embryogenic cultures were initiated from zygotic embryos explanted on Schenk and Hildebrandt (1972; SH) medium with 1 mg dm^{-3} benzylaminopurine (BAP) and developed on Murashige and Skoog (1962; MS) maturation medium. This medium contained 1/2 strength MS macro- and microelements, FeEDTA, and modified vitamins: 1 mg dm^{-3} nicotinic acid, 1 mg dm^{-3} thiamine

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Abbreviations: ABA - abscisic acid; BAP - benzylaminopurine; MI - myo-inositol; MS - Murashige and Skoog (1962) medium; NAA - naphthaleneacetic acid; SH - Schenk and Hildebrandt (1972) medium; TDZ - thidiazuron.

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HCl, 1 mg dm^{-3} pyridoxin HCl, 2 mg dm^{-3} glycine, 100 mg dm^{-3} myo-inositol (MI). The medium was supplemented with 40 g dm^{-3} maltose, 100 g dm^{-3} polyethylene glycol (PEG)-4000, 10 mg dm^{-3} abscisic acid (ABA), 500 mg dm^{-3} casein hydrolysate and 500 mg dm^{-3} L- glutamine.

Secondary somatic embryogenesis: Precotyledonary, cotyledonary and cotyledonary somatic embryos after 3 week desiccation were chosen as explants. To assess the most beneficial medium for initiation of secondary somatic embryogenesis, four types of treatments were used (Table 1). SH basal medium contained salts of SH media, 500 mg dm^{-3} L-glutamine, 1000 mg dm^{-3} casein hydrolysate, and 20 g dm^{-3} sucrose. Ten explants were incubated in each Petri dish containing 25 cm^{-3} of medium in the dark at 21 - 23 °C.

Development and germination of secondary somatic embryos: Secondary embryogenic tissue was cultured in the dark on MS maturation medium in 6-cm Petri dish. Prior to germination the isolated mature somatic embryos were undertaken to partial drying during 3 weeks in the dark at 21 - 23 °C. Desiccated somatic embryos germinated on SH medium containing 10 g dm^{-3} sucrose and 10 g dm^{-3} activated charcoal. They were cultured at 16-h photoperiod under irradiance of 110 μmol m^{-2} s^{-1}.

Protein extraction and electrophoresis: Proteins were extracted from precotyledonary, cotyledonary, and cotyledonary somatic embryos after desiccation treatment. The embryos were frozen in liquid nitrogen and ground to a fine powder. Proteins were extracted in 100 mM Tris-HCl extraction buffer (pH 8.5) containing 4 % (m/v) sodium dodecyl sulphate (SDS), 2 % (v/v) β-mercaptoethanol, 20 % (v/v) glycerol and 10 μg cm^{-3} leupeptin (Hurkman and Tanaka 1986). The crude homogenate was boiled in a water bath for 3 min and centrifuged at 14 000 *g* for 15 min at 4 °C. The protein content in the supernatant was determined using the Bradford method (Bradford 1976).

Electrophoretic separation of proteins was carried out under reducing conditions in 13 % acrylamide slab gels (SDS-PAGE) as described previously (Vooková *et al.* 1997/1998). The gel profiles of total proteins were photographed and analysed by *LabWorks*™ software *V 3.02* (Cambridge, UK). Molecular mass of proteins was estimated by co-electrophoresis with marker proteins (*ISS Protein MW Standards, Euprotech*, Vatick, MA, USA) ranging from 14 to 95 kDa.

Histological study: For histological investigation 10 μm sections of paraffin embedded somatic embryos with induced embryogenic tissue were stained with safranine and fast green (Pazourková 1986). Samples were analysed with a light microscope *NU 2* (*Carl Zeiss*, Jena, Germany). Somatic embryos present in secondary embryogenic tissue were observed using inverted microscope *Nikon TMS* (Tokyo, Japan).

Results and discussion

Storage proteins were shown to be indicative of the advanced stages of somatic embryo development (Hakman *et al.* 1990, Misra 1994). SDS-PAGE analysis showed similar but not the same protein pattern of *A. numidica* somatic embryos in three developmental stages (Fig. 1). The amounts of proteins representing the protein fraction with molecular mass of 61 and 63 kDa decreased during the development of somatic embryos. Their maturation was accompanied by the abundance of protein fraction with molecular masses in range of 16 - 17.5 kDa (referred to 16 kDa) and 43 kDa. Nearly the same proteins are present in mature seeds of the conifers, although the relative amount of the different proteins differ among the species. In *Picea abies,* 16 and 43 kDa proteins are considered to be embryo specific proteins and, they are accumulated during late embryogenesis (Stabel *et al.* 1990). The 16 kDa protein belongs to the major storage proteins in *Abies* sp. (Jensen and Lixue 1991). Storage protein of 28 kDa was present in nearly the same amount in embryos of all tested developmental stages. This protein was identified in *Picea abies* embryos as chlorophyll *a/b* binding protein (Stabel *et al.* 1990).

Somatic embryos of *A. numidica* produced new embryogenic tissue differently depending on the stage of development (Table 1). Cotyledonary embryos before desiccation were the most suitable explant for induction of secondary somatic embryos. Precotyledonary embryos were not able to produce embryogenic tissue. To date, researchers have found that competence of conifer zygotic embryo explants to form embryogenic cultures is dependent on their stage of development (Eastman *et al.* 1991). Also induction of embryogenic tissue in *A. alba* × *A. nordmanniana* zygotic embryos reached the highest frequency at the early cotyledonary stage, the fully differentiated embryos collected at the end of August lost this ability completely (Kormuťák *et al.* 1996).

In our experiment secondary embryogenic tissue extruded from somatic embryo hypocotyl (Fig. 2) similarly as was observed on mature zygotic embryos of *Abies* sp. (Nørgaard and Krogstrup 1995). Embryogenic structures appeared on the hypocotyl after 5 - 10 weeks of culture. Cell division activity in the epidermal and subepidermal layers of hypocotyl gave rise to numerous

nodular structures (Fig. 3 - 5). Subsequently, these nodules developed into typical somatic embryos. Similar developmental pattern was observed in cotyledons isolated from emblings and seedlings of hybrid firs (Salajová and Salaj 2001). Embryogenic tissue contained numerous embryos (Fig. 6), some of them underwent to cleavage multiplication. The studies on many gymnosperm species showed that such cleavage is a common way of early somatic embryo multiplication (Jásik *et al.* 1995, Havel and Durzan 1999).

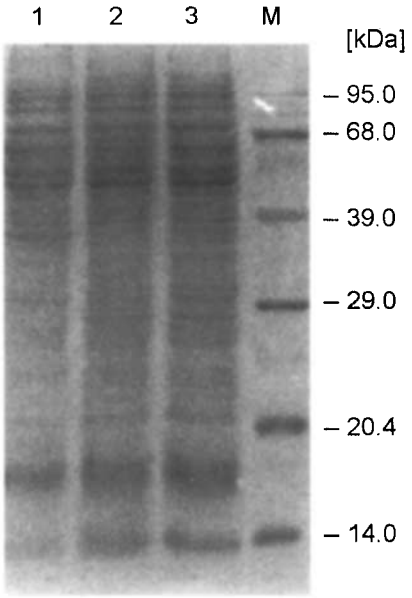

Fig. 1. SDS-PAGE of total proteins stained with Coomassie blue from different stages of *Abies numidica* somatic embryos development. 1 - precotyledonary stage, 2 - cotyledonary stage and 3 - cotyledonary stage after dessication. M - molecular mass standards.

For initiation of secondary somatic embryogenesis in *A. numidica*, SH medium containing TDZ was the best (Table 1). Nørgaard and Krogstrup (1991) found no difference between BAP and TDZ in their efficiency on induction of somatic embryogenesis from immature zygotic embryos of *A. nordmanniana.* TDZ was effective in forming of globular embryos *Cajanus cajan* (Mohan and Krishnamurthy 2002) but they developed into cotyledonary embryos rarely. Presence of mio-inositol in medium was inevitable for iniciation of secondary somatic embryogenesis. Induction percentage was significantly higher on media with 1000 mg dm^{-3} myo-inositol than on media with 100 mg dm^{-3}. Stimulation effect of myo-inositol in medium during somatic embryogenesis process was observed only in phases of somatic embryogenesis when it was applied together with cytokinin (Vooková *et al.* 2001). Our experiment extended these results about suitable concentration for initiation of secondary somatic embryogenesis.

Table 1. Induction of secondary somatic embryos [%] on different explants (PE - precotyledonary embryos, CE - cotyledonary embryos, DE desiccated embryos) on modified SH media with myo-inositol (MI) and growth regulators of different concentrations [mg dm^{-3}]. The number of explants 90 - 100.

Growth reg.	Conc.	MI	PE	CE	DE
BAP	1	0	0	0	0
BAP	1	100	0	2.0	0
BAP	1	1000	0	7.7	0
BAP	2	1000	0	8.9	2.2
TDZ	1	1000	0	34.0	8.0
BAP + NAA	1 + 1	1000	0	0	1.0

Initiation frequency was from 1 to 34 %. In general, higher levels of initiation were observed in somatic embryo explants of other conifers than in *Abies numidica.* Over 90 % of mature *Larix laricina* somatic embryos were capable of secondary somatic embryogenesis when placed on the induction medium (Klimaszewska *et al.* 1997). Initiation from mature somatic embryos of larch showed the highest frequency (83 %) of embryonal masses induction of all explant sources tested (Lelu *et al.* 1994).

Embryogenic lines of *Abies numidica* induced from somatic embryos matured and typical precotyledonary (Fig. 7) and cotyledonary stages were detected. Cotyledonary stage exhibited some morphological abnormalities (number and size of cotyledons, fused cotyledons, robust hypocotyl) which were usually observed not only in conifer but also in other plant species, *e.g.* pea (Griga 2002). The somatic embryos resembled in their morphology, the one from primary cultures. Although maturation potential was not quantified, there were apparent differences in the number of developing somatic embryos from different cell lines.

Mature somatic embryos after three weeks of desiccation were able to germinate on SH medium with charcoal and sucrose, root was observed between day 6 and 10. After 40 d of cultivation plantlets with cotyledons, hypocotyl and root were obtained (Fig. 8).

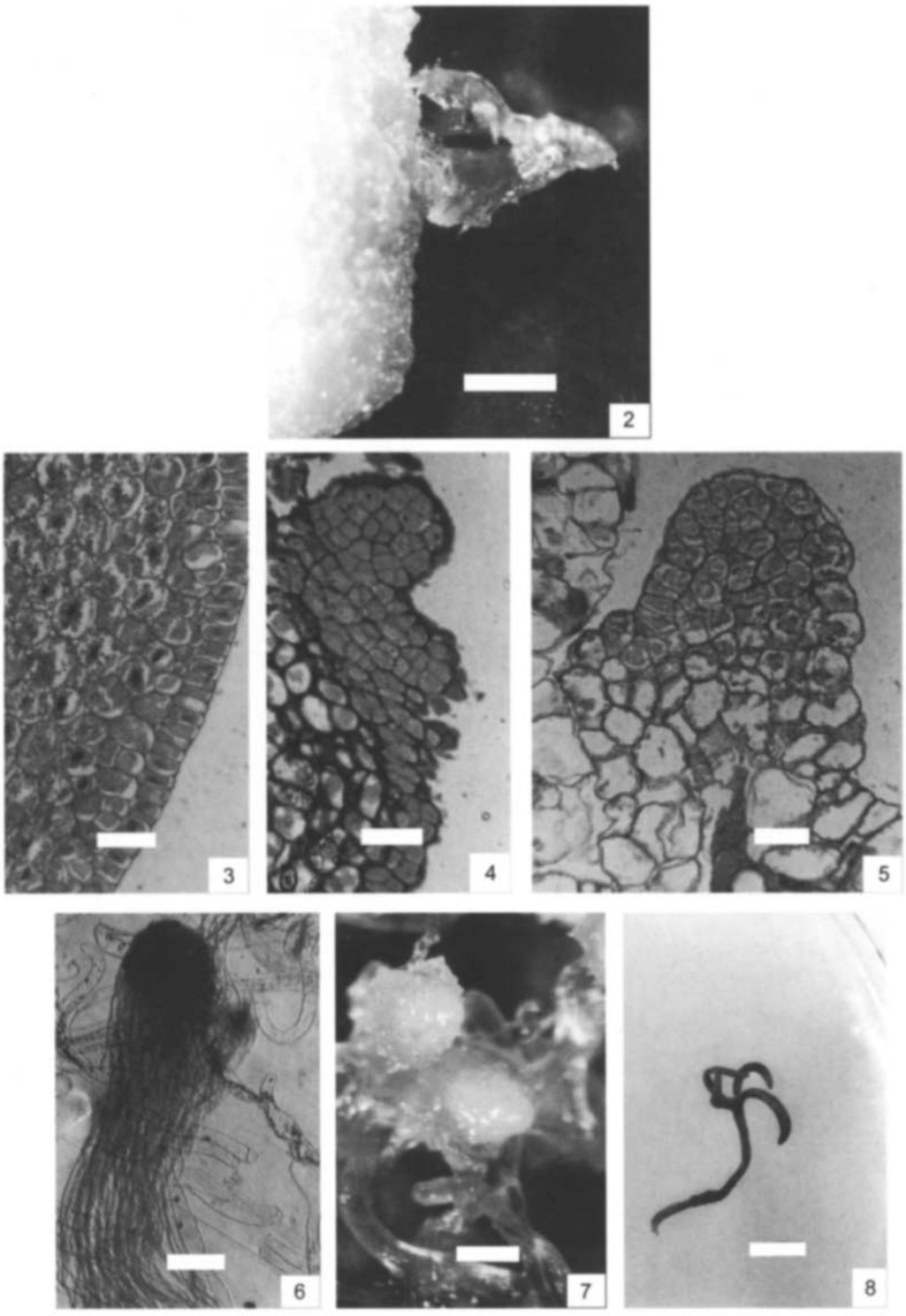

Fig. 2. Initiation of secondary embryogenic tissue on a hypocotyl of somatic embryo. *Bar* = 1 mm.
Fig. 3. Transversal section of intact hypocotyl of cotyledonary somatic embryo. *Bar* = 40 μm.
Fig. 4. Formation of the nodules on surface of somatic embryo hypocotyl – early developmental stage of somatic embryos. *Bar* = 70 μm.
Fig. 5. More developed somatic embryo with clear polarity. *Bar* = 50 μm.
Fig. 6. Typical somatic embryo present in secondary embryogenic tissue. *Bar* = 250 μm.
Fig. 7. Precotyledonary somatic embryos matured on MS maturation medium with maltose and ABA. *Bar* = 2.5 mm.
Fig. 8. Plantlet regenerated from secondary somatic tissue. *Bar* = 5 mm.

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