



THE EFFECT OF CARBOHYDRATES AND POLYETHYLENE GLYCOL ON SOMATIC EMBRYO MATURATION IN HYBRID FIR *ABIES ALBA* × *ABIES NUMIDICA*

TERÉZIA SALAJ, RADOSLAVA MATÚŠOVÁ, AND JÁN SALAJ

*Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences,
Akademická 2, P.O. Box 39A, 950 07 Nitra 1, Slovak Republic*

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Embryogenic cell line AN 72 derived from immature hybrid fir *Abies alba* × *A. numidica* zygotic embryos was subjected to different maturation treatments. The effect of the carbohydrates sucrose, maltose and glucose (each at 3%, 6% and 9%) or PEG-4000 (5.0%, 7.5% and 10%) combined with different carbohydrate sources was tested. PEG-4000 stimulated somatic embryo maturation of hybrid fir. This stimulatory effect was dependent on the carbohydrate source used. Culture medium with maltose as carbohydrate source combined with PEG-4000 produced the highest number of cotyledonary somatic embryos. Carbohydrates supplied alone (mainly at 6% and 9%) exerted an unfavorable effect, increasing the frequency of abnormally shaped somatic embryos without regeneration capacity. The structural organization of morphologically well-developed cotyledonary somatic embryos was similar to that of zygotic embryos. In abnormal somatic embryos the shoot apical meristem and root meristem were very damaged. Electrophoretic separation of denatured proteins using SDS-PAGE showed differences in the accumulation of low molecular weight storage proteins in somatic embryos. Storage protein accumulation was dependent on the concentration of PEG-4000 and the carbohydrate source.

Key words: *Abies alba* × *A. numidica*, carbohydrates, embryogenesis, PEG, somatic embryos.

INTRODUCTION

At present, somatic embryogenesis is considered an efficient method of vegetative plant propagation in conifers. The system has many advantages, is important as a multiplication technique, and greatly contributes to theoretical studies of conifer embryology (Hakman, 1993) and conifer biology (Kong et al., 1999).

Somatic embryogenesis is a routine method in several *Picea* species, giving thousands of emblings used in forestry research and acceptable for commercial propagation (Grossnickle and Major, 1994; Tremblay et al., 1999). Recently, progress in somatic embryo maturation and plantlet regeneration has been reported in some *Pinus* species previously considered recalcitrant (Klimaszewska and Smith,

1997; Häggman et al., 1999; Lelu et al., 1999; Salajová et al., 1999).

In the genus *Abies*, somatic embryogenesis was first induced in *Abies alba* (Gebhardt, 1988; Schuller et al., 1989; Hristoforoglu et al., 1995). Later, somatic embryogenesis was reported for *A. nordmanniana* (Norgaard and Krogstrup, 1991), *A. cephalonica* (Krajňáková and Häggman, 1997), *A. balsamea* (Guevin et al., 1994) and hybrid firs (Gajdošová et al., 1995; Salajová et al., 1996). In these species, more or less well-developed plantlets have also been obtained; only Norgaard (1997) reported large-scale multiplication and transfer of emblings to soil. In hybrid firs we obtained small plantlets using abscisic acid (ABA) for maturation. Plantlet development was poor and the germination frequencies low (Salajová et al., 1996). In later studies, different

*e-mail: nrgtrtesa@savba.sk

embryogenic cell lines were tested for their maturation ability using ABA and polyethylene glycol (PEG) combined with sucrose as the carbon source (Jásik et al., 1999).

Data on the beneficial effect of carbohydrates on somatic embryo development have been published recently. Carbohydrates as a source of carbon or osmotica influenced somatic embryogenesis in both angiosperms (Canhoto and Cruz, 1994; Blanc et al., 1999) and gymnosperms. Maltose as a carbon source and osmoticum was effective in *Abies nordmanniana* (Norgaard, 1997), *Pinus elliotii* (Liao and Amerison, 1995) and *P. nigra* (Salajová et al., 1999). In *P. pinaster*, 6% sucrose combined with a high concentration of gellan gum helped the recovery of cotyledonary somatic embryos (Ramarosandratana et al., 2001). Somatic embryo maturation was improved after sucrose treatment in *P. strobus* (Finer et al., 1989), *Picea mariana* and *P. rubens* (Tremblay and Tremblay, 1991). Recent studies indicate the regulatory role of sucrose (allowed to hydrolyze) on somatic embryo maturation through stimulation of storage protein synthesis (Iraqi and Tremblay, 2001).

In this paper we studied the effect of sucrose, glucose and maltose alone or applied with PEG on somatic embryo development in hybrid fir. The internal organization and protein profiles of developing somatic embryos were also studied.

MATERIALS AND METHODS

PLANT MATERIAL

Embryogenic cultures of *Abies alba* × *Abies numidica* were initiated from immature zygotic embryos in 1991 (Salajová et al., 1996). The cultures were maintained on DCR proliferation medium (Gupta and Durzan, 1985) containing 6-benzyladenine (BA, 1 mg l⁻¹) by regular transfers every three weeks.

MATURATION OF SOMATIC EMBRYOS

Small pieces of embryogenic tissue were transferred from proliferation medium to maturation medium around day 8 of culture. The inoculum weight for the Petri dish was ~1.2 g.

DCR maturation medium (Gupta and Durzan, 1985) contained macro- and microelements and vitamins, and was supplemented with casein hydrolysate (500 mg l⁻¹), glutamine (500 mg l⁻¹), myo-inositol (100 mg l⁻¹) and abscisic acid (10 mg l⁻¹). The media were solidified with Phytigel (3 g l⁻¹).

The first series of experiments tested the effect of sucrose, glucose and maltose, each at 3%, 6% and 9% concentrations. The sugars were autoclaved separately and poured into cooled basal medium. In the second series of experiments, polyethylene glycol (PEG-4000) at 5%, 7.5% and 10% concentrations was combined with 3% sucrose, glucose or maltose as carbohydrate source. The media pH was adjusted to 5.8 before autoclaving.

The cultures grew on these media for 6–8 weeks and then were transferred to medium without ABA and PEG but containing 3% carbohydrate.

The experiments were done in two replicates, with 5 or 6 Petri dishes (9 cm diameter) per treatment in each experiment. The tissues were kept in the dark at 23°C.

DESICCATION AND GERMINATION

For partial desiccation, well-developed somatic embryos possessing at least 4 cotyledons were selected. After 3 weeks of desiccation the embryos were transferred to DCR germination basal medium containing 2% sucrose, glucose or maltose as carbohydrate source (according to the maturation treatment). The germination medium was supplemented with 1% activated charcoal. Desiccation and the first week of germination occurred in the dark.

HISTOLOGY

The tissue samples (well-developed and abnormal somatic embryos) were fixed in FAA, dehydrated in a butyl alcohol series and embedded in paraffin. Sections 8–10 µm thick were cut and mounted on glass slides. After deparaffinization in toluene the sections were stained with hematoxylin or safranin/fast green and examined with a Zeiss Axio-plan 2 bright-field light microscope.

EXTRACTION OF PROTEINS

Protein analyses used somatic embryos from treatments with 0%, 5%, 7.5% and 10% PEG combined with 3% carbohydrate (glucose, maltose or sucrose). The embryos were ground to a fine powder in liquid nitrogen. Proteins were extracted in 100 mM Tris-HCl extraction buffer (pH 8.5) containing 4% (w/v) sodium dodecyl sulphate (SDS), 2% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 10 µg ml⁻¹ leupeptin (Hurkman and Tanaka 1986). Extraction was carried out at 4°C. Samples were boiled for 3 min in a water bath. Insoluble material was removed by cen-

trifugation at 14,000 g for 15 min at 4°C. Protein content was determined by the method of Bradford (1976). BSA was the standard. The supernatant containing total proteins was applied for SDS-polyacrylamide gel electrophoretic separation (SDS-PAGE).

ELECTROPHORETIC SEPARATION OF PROTEINS

The SDS-PAGE procedure was based on the method described by Laemmli (1970) with minor modifications. Proteins were separated using 13% SDS polyacrylamide separating gels. In each lane, 15 µg of proteins were loaded. Following electrophoresis, proteins were visualized with Coomassie Brilliant Blue R 250. The gel profiles were photographed and analyzed with LabWorks™ Image Acquisition and Analysis Software, V 3.02 (UVP Ltd., Cambridge, UK). The molecular weight of proteins was estimated by co-electrophoresis with marker proteins (ISS Protein MW Standards) ranging from 14 to 95 kDa.

STATISTICAL ANALYSIS

Statistical evaluation was done according to Erdelský and Frić (1979).

RESULTS

EFFECT OF SUCROSE, GLUCOSE AND MALTOSE ON SOMATIC EMBRYO MATURATION

Sucrose, glucose and maltose supplied alone did not benefit somatic embryo maturation. Regardless of the concentrations tested (3%, 6% or 9%), high numbers of precotyledonary embryos appeared at 3–5 weeks of culture on maturation medium; their further development was determined by the concentrations of carbohydrate used. Sporadically, cotyledonary somatic embryos developed, mainly in the presence of 3% carbohydrate. Higher concentrations of carbohydrates resulted in degeneration and subsequent necrosis of precotyledonary somatic embryos. With glucose treatment (6% and 9%) the precotyledonary somatic embryos soon formed hard globules which did not develop further. Maltose treatments resulted in overproliferation of embryogenic tissue and degeneration of early-stage embryos. If later cotyledonary somatic embryos appeared they showed abnormal morphology as fused cotyledons, extremely swelled hypocotyl or shooty

somatic embryos without clear distinctions between the hypocotyl and cotyledons. Owing to the abnormal development, no plantlet regeneration occurred.

EFFECT OF PEG-4000 COMBINED WITH DIFFERENT CARBOHYDRATES

The presence of precotyledonary embryos peaked at around week 5 of culture. After this period, cotyledonary somatic embryos gradually appeared. For statistical analysis and comparison of treatments, only well-formed cotyledonary somatic embryos were counted (Figs. 1, 2).

Our results show that PEG stimulated somatic embryo maturation in hybrid fir (Tab. 1). The stimulatory effect of PEG was dependent on the carbohydrate source used. When PEG was applied with sucrose the stimulatory effect was apparent at higher concentrations. Treatment with 5% PEG and without PEG gave the same number of mature cotyledonary somatic embryos. Under treatment with PEG combined with glucose, all PEG concentrations significantly stimulated somatic embryo maturation, and the differences in maturation between PEG concentrations were not statistically significant. The stimulatory effect of PEG was marked when maltose served as the carbon source. Maltose without PEG gave only a few mature somatic embryos, which were taken for biochemical analysis and for this reason not subjected to desiccation and germination. With maltose, higher concentrations of PEG enhanced the production of mature somatic embryos much more than under a lower concentration (5%) or in the control. Treatment with PEG combined with maltose yielded the highest number of cotyledonary somatic embryos. Significantly lower numbers of cotyledonary somatic embryos developed when sucrose or glucose were used as the carbohydrate source.

The developing somatic embryos differed in their size and color depending on the carbohydrate and PEG treatments. Control somatic embryos in the presence of 3% sucrose or glucose were greenish and bulkier than in the PEG treatments. PEG treated somatic embryos were yellow. In maltose treatments (combined with PEG) the somatic embryos were mostly green in color. Although no quantitative analysis of size was done, the somatic embryos treated with PEG combined with maltose seemed smaller than those matured in the presence of PEG combined with sucrose or glucose.



Fig. 1. Cotyledonary somatic embryos of hybrid fir (treatment 10% PEG-4000 + 3% sucrose). Bar = 1 mm. **Fig. 2.** Single somatic embryo (detail from Fig. 1). Bar = 0.6 mm. **Fig. 3.** Emblings derived from somatic embryos (maturation treatment 10% PEG + 3% sucrose) after three weeks on germination medium. **Fig. 4.** Plantlets grown in soil after three to four months. (Petri dish diameter 8 cm).

GERMINATION OF SOMATIC EMBRYOS AND POST-GERMINATIVE GROWTH OF EMBLINGS

For germination, well-developed somatic embryos were selected. Before germination the somatic embryos were partially desiccated and transferred to germination medium. Hypocotyl and cotyledon elongation occurred after one week of culture, and the radicle protruded at 10–12 days. At day 21, most germinating embryos had 0.5–1 cm long radicles and green cotyledons (Fig. 3).

We found a correlation between maturation capacity and germination of somatic embryos. Treatments that stimulated embryo maturation also resulted in higher germination frequencies later

(Tab. 2), although the data on germination are based on different embryo numbers.

After 21 days, plantlets were transferred from Petri dishes to baby food jars. The transfer resulted in a transient decline of growth. The emblings stopped their growth, and after three weeks some of them started to elongate again. Survival of plantlets was affected by the different maturation treatments (Tab. 3). Emblings that developed from somatic embryos with better maturation treatments also showed higher survival rates. However, the plantlets from the glucose + PEG treatment grew very slowly and subsequently became necrotized. The surviving plantlets were transferred to soil (Fig. 4) for further growth.

TABLE 1. Effect of PEG-4000 and different carbohydrates on maturation of somatic embryos (means \pm SEs number per Petri dish and standard errors of mean are given)

Carbo- hydrate (3%)	PEG-4000 (%)			
	0.0	5.0	7.5	10
Sucrose	15 \pm 3.71	15 \pm 4.32	24 \pm 4.98	41 \pm 7.47
Maltose	4 \pm 1.04	35 \pm 10.31	156 \pm 28.58	144 \pm 12.16
Glucose	14 \pm 2.58	36 \pm 5.40	38 \pm 7.73	45 \pm 6.00

TABLE 2. Germination frequencies (%) of somatic embryos after different maturation treatments. Numbers of somatic embryos (SEs) are given in parenthesis

Carbohydrate (3%)	PEG-4000 (%)			
	0.0	5.0	7.5	10
Sucrose	41.27 (93)	42.34 (52)	71.35 (129)	68 (250)
Maltose	– –	51.74 (124)	76.76 (622)	78.92 (793)
Glucose	35.01 (42)	23.56 (54)	47.61 (141)	49.53 (154)

HISTOLOGICAL OBSERVATIONS

Well-developed cotyledonary somatic embryos (Fig. 5) with regular shape were characterized by internal organization similar to that of zygotic embryos. Both the apical and basal parts of somatic embryos were well differentiated. The apical part of the somatic embryo was formed by well-defined shoot apical meristem surrounded by the ring of the cotyledon primordia (Fig. 6). Characteristic of the basal part was the presence of distinct root meristem and a root cap with a well-established columella (Fig. 7).

Many abnormally developed structures appeared during somatic embryo maturation, so we also studied their anatomy. These structures had mostly fused cotyledons (Fig. 8) and an extremely swollen hypocotyl. In embryos with fused cotyledons the apical part was abnormally differentiated. These

embryos also showed disruption in the cortex or "budding" in the junction region connecting the radicle and hypocotyl (Fig. 9). Some of the embryos were extremely swollen in the basal region. In these embryos the root meristem was completely disorganized or absent (Fig. 10).

ELECTROPHORETIC PROFILES OF TOTAL PROTEINS

The protein pattern of somatic embryos after 3% carbohydrate and different PEG treatments was compared. One-dimensional SDS-PAGE analyses of total proteins showed that most of the major proteins of the well-developed somatic embryos were present in comparable amounts. Sucrose + PEG treatment resulted in increased accumulation of the 16 kDa protein fraction in the somatic embryos. The 16 kDa fraction increased gradually with the concentration of PEG in the culture media (Fig. 11a). The electrophoretic profiles of total proteins of somatic embryos grown on medium containing maltose + PEG showed no marked differences in accumulation of 16 kDa proteins in all treatments tested (Fig. 11b). We observed higher accumulation of 28.5 kDa proteins when the embryos were grown on medium with maltose without PEG. A similar pattern of accumulation of 16 kDa proteins was observed in treatments using glucose as the carbohydrate and different concentrations of PEG (Fig. 11c). In glucose and sucrose treatments combined with PEG, we also observed slightly higher accumulation of 43 kDa proteins.

DISCUSSION

Carbohydrates incorporated into the media impaired somatic embryo maturation: degeneration in early stages of development occurred. These results suggest that carbohydrates tested were insufficient as osmotic agents. In our experiments, somatic embryo maturation efficiency was significantly enhanced by polyethylene glycol. PEG has been applied for somatic embryo maturation in several

TABLE 3. Survival of emblings derived from somatic embryos subjected to different maturation treatments

	PEG-4000 (%) + 3% sucrose				PEG-4000 (%) + 3% maltose		
	0.0	5.0	7.5	10	5.0	7.5	10
Cultured emblings	70	52	85	181	30	271	185
Survival (%)	31.89	42.34	61.48	62.12	28.80	71.38	75.56

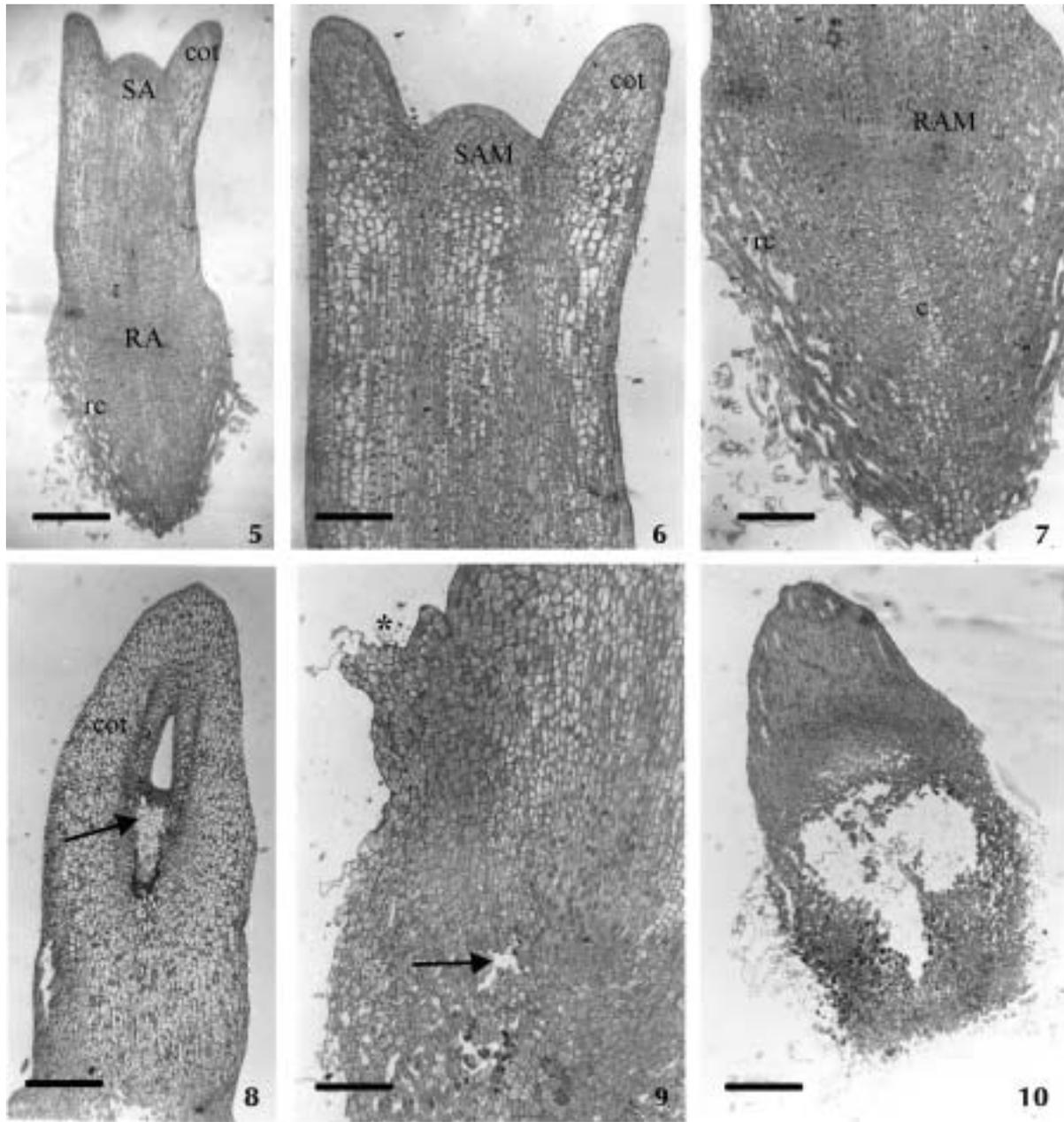


Fig. 5. Longitudinal section through normally developed cotyledonary somatic embryo. cot – cotyledon; RA – root apex; SA – shoot apex; rc – root cap. Bar = 0.3 mm. **Fig. 6.** Detail of apical part of somatic embryo, showing distinct apical dome/shoot apical meristem (SAM) and cotyledons (cot). Bar = 0.1 mm. **Fig. 7.** Somatic embryo with well-organized basal part: root apical meristem (RAM), columella (c) and root cap (rc). Bar = 0.1 mm. **Fig. 8.** Abnormally developed somatic embryo with fused cotyledons (cot) and disturbances in shoot apex (arrow). Bar = 0.2 mm. **Fig. 9.** Formation of bud-like structure (asterisk) in junction region of somatic embryo. Ruptures of root cap (arrow) in the vicinity of root apical meristem are also seen. Bar = 0.1 mm. **Fig. 10.** Abnormally developed somatic embryo lacking both shoot and root meristems, with very disorganized root part. Bar = 0.3 mm.

conifers including *Picea abies* (Find, 1997; Svobodová et al., 1999), *P. glauca* (Attree et al., 1991) and *Abies numidica* (Vooková and Kormuťák, 2001). PEG in concentrations of 5.0–7.5% tripled the ma-

turation frequency of white spruce somatic embryos (Attree et al., 1991). PEG is a non-penetrating osmoticum exerting water stress similar to natural drying (Attree and Fowke, 1993).

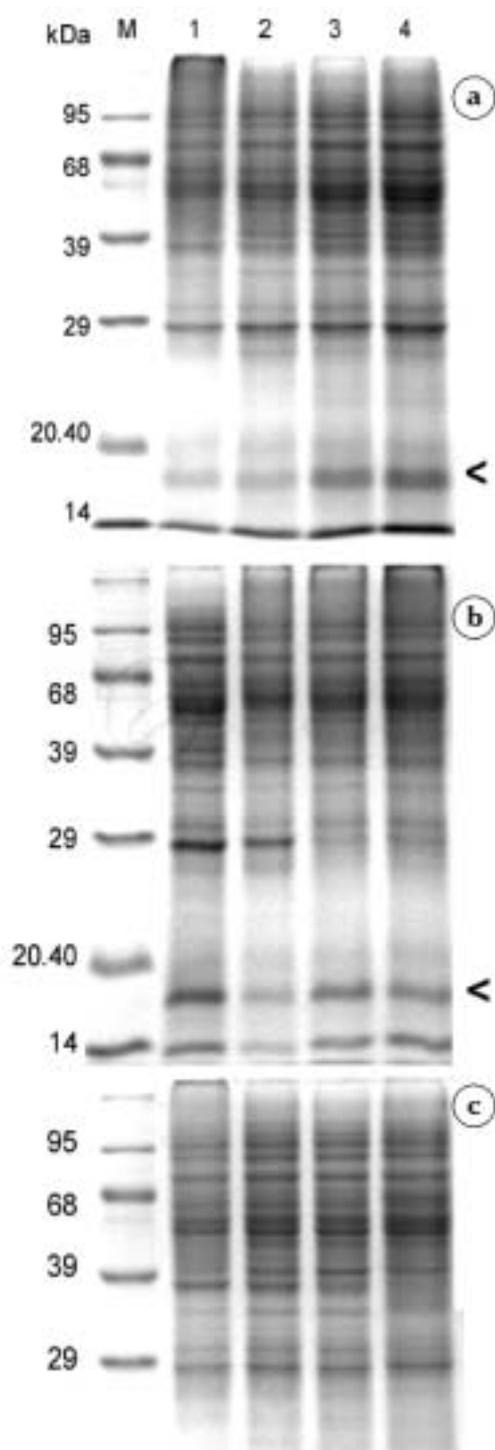


Fig. 11. SDS-PAGE profiles from cotyledonary somatic embryos of hybrid fir. (a) Treated with sucrose. M – molecular weight standards; 1 – 3% sucrose; 2 – 3% sucrose + 5% PEG-4000; 3 – 3% sucrose + 7.5% PEG; 4 – 3% sucrose + 10% PEG. (b) Treated with maltose. 1 – 3% maltose; 2 – 3% maltose + 5% PEG; 3 – 3% maltose + 7.5% PEG; 4 – 3% maltose + 10% PEG. (c) Treated with glucose. 1 – 3% glucose; 2 – 3% glucose + 5% PEG; 3 – 3% glucose + 7.5% PEG; 4 – 3% glucose + 10% PEG. Left: marker proteins and their MW.

In *Picea glauca*, polyethylene glycol positively affected storage protein accumulation. Somatic embryos matured in the presence of PEG contained thrice the protein content of the non-treated control. The spectrum of storage polypeptides resembled that of mature zygotic embryos (Misra et al., 1993). Storage protein synthesis regulation was at the post-transcriptional or transcriptional level, and the combined effect of PEG and ABA was necessary (Leal et al., 1995).

The anatomy of Norway spruce somatic embryos developing under the influence of PEG-4000 has been studied in detail recently (Svobodová et al., 1999). The optimal concentration (3.75% PEG) speeded the maturation process in terms of differentiation of tissues such as the root cap, cotyledons and apical dome. Only a few somatic embryos (13%) showed minor abnormalities. Increased expression of genes is known to be involved in the formation of the embryo body plan, and in the control of shoot and root apical meristems this phenomenon was detected after PEG treatment in white spruce somatic embryos (Stasolla et al., 2003), suggesting the effect of PEG on gene expression in developing somatic embryos.

The cited results clearly indicate the positive influence of PEG on the quantity and quality of conifer somatic embryos in these species. Polyethylene glycol was less effective in some other species, however, resulting in inferior somatic embryos or in their complete degeneration at an early developmental stage (Klimaszewska and Smith, 1997).

In *Picea abies* somatic embryos, Bozhkov and von Arnold (1998) found that PEG stimulated somatic embryo maturation but also produced pronounced alterations in somatic embryo morphology (irregular shape) and anatomy (intercellular spaces in pericolumn tissue, degradation of quiescent center). These changes led to lowered germination frequency and poor ex vitro growth of emblings. The negative effect of PEG disappeared when plantlets were transferred to the substrate.

In our study we observed different somatic embryo morphology under different treatments, although these findings are based on visual observations as mentioned earlier. These small differences in PEG-treated embryos did not result in lowered germination ability; PEG-treated somatic embryos showed higher germination frequencies. Somatic embryo maturation in the absence of PEG was poor, with only a few plantlets obtained. An interaction between PEG and carbohydrate treat-

ments, was noted but the stimulatory effect of PEG was clearly demonstrated. A similar relationship was confirmed in *Pinus taeda*. In this species, cotyledonary somatic embryos differentiated after treatment PEG + maltose, while PEG combined with sucrose gave poor results (Li et al., 1998). Blanc et al. (2002) enunciated a hypothesis concerning the effect of maltose: through slow hydrolysis of maltose, the endogenous hexose content in the medium is sustained at a low level, and this may be the biochemical element that triggers the orientation of metabolism towards somatic embryogenesis.

One-dimensional SDS-PAGE of total proteins showed the effect of PEG-4000 and 3% carbohydrate on accumulation of storage proteins, as well as on the protein composition of somatic embryos. The major storage proteins of zygotic embryos of *Abies* sp. are 43, 28 and 16 kDa proteins (Jensen and Lixue, 1991). The electrophoretic profiles of denatured proteins showed differences in the accumulation of low molecular weight storage proteins. The addition of PEG to the medium with 3% glucose or 3% sucrose affected the accumulation of the 16 kDa fraction. The concentrations of 43 and 28 kDa proteins were not higher than in the protein profile of zygotic embryos. It is well known that in many cases somatic embryos synthesize and accumulate seed-specific storage proteins in amounts much lower than or in proportions different from those of mature zygotic embryos (Sreedhar and Bewley, 1998; Vooková et al., 1998). Most of the major proteins were present equally in all embryos tested.

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REFERENCES

- ATTREE SM, MOORE D, SAWHNEY VK, and FOWKE LC. 1991. Enhanced maturation and desiccation tolerance of white spruce [*Picea glauca* (Moench) Voss] somatic embryos: Effects of a non-plasmolysing water stress and abscisic acid. *Annals of Botany* 68: 519–525.
- ATTREE SM, and FOWKE LC. 1993. Embryology of Gymnosperms: Advances in synthetic seed technology of conifers. *Plant Cell, Tissue and Organ Culture* 35: 1–35.
- BLANC G, MICHAUX-FERRIERE N, TEISSON C, LARDET L, and CARRON MP. 1999. Effects of carbohydrate addition on the induction of somatic embryogenesis in *Hevea brasiliensis*. *Plant Cell Tissue and Organ Culture* 59: 103–112.
- BLANC G, LARDET L, MARTIN A, JACOB JL, and CARRON MP. 2002. Differential carbohydrate metabolism conducts morphogenesis in embryogenic callus of *Hevea brasiliensis* (Müll. Arg.). *Journal of Experimental Botany* 53: 1453–1462.
- BOZKHOV P, and von ARNOLD S. 1998. Polyethylene glycol promotes maturation but inhibits further development of *Picea abies* somatic embryos. *Physiologia Plantarum* 104: 211–224.
- BRADFORD MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry* 72: 248–254.
- CANHOTO JM, and CRUZ GS. 1994. Improvement of somatic embryogenesis in *Feioja sellowiana* Berg. (Myrtaceae) by manipulation of culture media composition. *In Vitro Cellular and Developmental Biology - Plant* 30: 21–25.
- ERDELSKÝ K, and FRIČ F. 1979. *Praktikum a analytické metody vo fyziológii rastlin*. SPN, Bratislava 621.
- FIND J. 1997. Changes in endogenous ABA levels in developing somatic embryos of Norway spruce [*Picea abies* (L.) Karst.] in relation to maturation medium, desiccation and germination. *Plant Science* 128: 75–83.
- FINER JJ, KRIEBEL HB, and BECWAR MR. 1989. Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.). *Plant Cell Reports* 8: 203–206.
- GAJDOŠOVÁ A, VOOKOVÁ B, KORMUTÁK A, LIBIAKOVÁ G, and DOLEŽEL J. 1995. Induction, protein composition and DNA ploidy level of embryogenic calli of silver fir and its hybrids. *Biologia Plantarum* 37: 169–176.
- GEHARDT KH, WEISBERGER H, and FRÖHLICH HJ. 1988. *In vitro* germination and production of embryogenic callus from liquid suspension cultures of *Abies alba*. *International Conifer Tissue Culture Working Group*, poster No 17, Saskatoon, Canada.
- GROSSNICKLE SC, and MAJOR JE. 1994. Interior spruce seedlings compared with emblings produced from somatic embryogenesis. III. Physiological response and morphological development on a reforestation site. *Canadian Journal of Forest Research* 24: 1397–1407.
- GUEVIN TD, MICAH V, and KIRBY EG. 1994. Somatic embryogenesis in cultured mature embryos of *Abies balsamea*. *Plant Cell, Tissue and Organ Culture* 37: 205–208.
- GUPTA PK, and DURZAN DJ. 1985. Shoot multiplication from mature trees of Douglas fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). *Plant Cell Reports* 4: 177–179.
- HAKMAN I. 1993. Embryology in Norway spruce (*Picea abies*). An analysis of seed storage proteins and adaptation of storage reserves during seed development and somatic embryogenesis. *Physiologia Plantarum* 87: 148–159.
- HÄGGMAN H, JOKELA A, KRAJNAKOVA J, KAUPPI A, NIEMI K, and ARONEN T. 1999. Somatic embryogenesis of Scots pine: cold treatment and characteristics of explants affecting induction. *Journal of Experimental Botany* 50: 1769–1778.
- HRISTOFOROGLU K, SCHMIDT J, and BOLHAR-NORDENKAMP H. 1995. Development and germination of *Abies alba* somatic embryos. *Plant Cell, Tissue and Organ Culture* 40: 277–284.

- HURKMAN WI, and TANAKA CK. 1986. Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiology* 81: 802–806.
- IRAQI D, and TREMBLAY FM. 2001. The role of sucrose during maturation of black spruce (*Picea mariana*) and white spruce (*Picea glauca*) somatic embryos. *Physiologia Plantarum* 111: 381–388.
- JÁSIK J, SALAJOVÁ T, KORMUTÁK A, and SALAJ J. 1999. Somatic embryogenesis in hybrid firs. In: Jain SM, Gupta PK, Newton RJ [eds.], *Somatic embryogenesis in woody plants*, vol 4, 505–523. Kluwer Academic Publishers, Dordrecht.
- JENSEN U, and LIXUE C. 1991. *Abies* seed protein profile divergent from other Pinaceae. *Taxon* 40: 435–440.
- KLIMASZEWSKA K, and SMITH DR. 1997. Maturation of somatic embryos of *Pinus strobus* is promoted by a high concentration of gellan gum. *Physiologia Plantarum* 100: 949–957.
- KONG L, ATTREE SM, EVANS DE, BINAROVA P, YOUNG EC, and FOWKE LC. 1999. Somatic embryogenesis in white spruce: studies of embryo development and cell biology. In: Jain SM, Gupta PK, Newton RJ [eds.], *Somatic embryogenesis in woody plants*, vol. 3, 1–28. Kluwer Academic Publishers, Dordrecht.
- KRAJŇÁKOVÁ J, and HÄGGMAN H. 1997. Regeneration of *Abies cephalonica* through somatic embryogenesis. *Identification and control of phase changes in rejuvenation. COST 822 – 4th meeting of WG3*. Oct. 15–19, 102–104, Nitra, Slovakia.
- LAEMMLI UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.
- LEAL I, MISRA S, ATTREE SM, and FOWKE LC. 1995. Effect of abscisic acid, osmoticum and desiccation on 11S storage protein gene expression in somatic embryos of white spruce. *Plant Science* 106: 121–128.
- LELU A-M, BASTIEN C, DRUGEAULT A, GOUEZ M-L, and KLIMASZEWSKA K. 1999. Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without growth regulators. *Physiologia Plantarum* 105: 719–728.
- LI XY, HUANG FH, MURPHY JB, and GBUR EE. 1998. Polyethylene glycol and maltose enhance somatic embryo maturation in loblolly pine (*Pinus taeda* L.). *In Vitro Cellular and Developmental Biology – Plant* 34: 22–26.
- LIAO YK, and AMERSON VH. 1995. Slash pine (*Pinus elliottii* Engelm) somatic embryogenesis. II. Maturation of somatic embryos and plant regeneration. *New Forests* 10: 165–182.
- MISRA S, ATTREE SM, LEAL I, and FOWKE LC. 1993. Effect of abscisic acid, osmoticum, and desiccation on synthesis of storage proteins during the development of white spruce somatic embryos. *Annals of Botany* 71: 11–22.
- NORGAARD JV. 1997. Somatic embryo maturation and plantlet regeneration in *Abies nordmanniana* Lk. *Plant Science* 124: 211–221.
- NORGAARD JV, and KROGSTRUP P. 1991. Cytokinin-induced somatic embryogenesis from immature embryos of *Abies nordmanniana* Lk. *Plant Cell Reports* 9: 509–513.
- RAMAROSANDRATANA A, HARVENGT L, BOUVET A, CALVAYRAC R, and PAQUES M. 2001. Influence of the embryonal suspensor mass (ESM) sampling on development and proliferation of maritime pine somatic embryos. *Plant Science* 160: 473–479.
- SALAJOVÁ T, JÁSIK J, KORMUTÁK A, SALAJ J, and HAKMAN I. 1996. Embryogenic culture initiation and somatic embryo development in hybrid firs (*Abies alba* × *Abies cephalonica* and *Abies alba* × *Abies numidica*). *Plant Cell Reports* 15: 527–530.
- SALAJOVÁ T, SALAJ J, and KORMUTÁK A. 1999. Initiation of embryogenic tissues and plantlet regeneration from somatic embryos of *Pinus nigra* Arn. *Plant Science* 145: 33–40.
- SCHULLER A, REUTHER G, and GEIER T. 1989. Somatic embryogenesis from seed explants of *Abies alba*. *Plant Cell, Tissue and Organ Culture* 17: 53–58.
- SREEDHAR L, and BEWLEY JD. 1998. Nitrogen and sulfur-containing compounds enhance the synthesis of storage reserves in developing somatic embryos of alfalfa (*Medicago sativa* L.). *Plant Science* 134: 31–44.
- STASOLLA C, VAN ZYL L, EGERTSDOTTER U, CRAIG D, LIU W, and SEDEROFF RR. 2003. The effect of polyethylene glycol on gene expression of developing white spruce somatic embryos. *Plant Physiology* 131: 49–60.
- SVOBODOVÁ H, ALBRECHTOVÁ J, KUMSTÝŘOVÁ L, LIPAVSKÁ H, VÁGNER M, and VONDRÁKOVÁ Z. 1999. Somatic embryogenesis in Norway spruce: Anatomical study of embryo development and influence of polyethylene glycol on maturation process. *Plant Physiology and Biochemistry* 37: 209–221.
- TREMBLAY L, and TREMBLAY FM. 1991. Carbohydrate requirements for the development of black spruce [*Picea mariana* (Mill.) B.S.P] and red spruce (*P. rubens* Sarg.) somatic embryos. *Plant Cell, Tissue and Organ Culture* 27: 95–103.
- TREMBLAY L, LEVASSEUR C, and TREMBLAY F. 1999. Frequency of somaclonal variation in plants of black spruce (*Picea mariana*, Pinaceae) and white spruce (*P. glauca*, Pinaceae) derived from somatic embryogenesis and identification of some factors involved in genetic instability. *American Journal of Botany* 86: 1317–1381.
- VOOKOVÁ B, GAJDOŠOVÁ A, and MATUŠOVÁ R. 1998. Somatic embryogenesis in *Abies alba* × *Abies alba* and *Abies alba* × *Abies nordmanniana* hybrids. *Biologia Plantarum* 40: 523–530.
- VOOKOVÁ B, and KORMUTÁK A. 2001. Effect of sucrose concentration, charcoal, and indole-3-butyric acid on germination of *Abies numidica* somatic embryos. *Biologia Plantarum* 44: 181–184.