

## Storage Proteins and Peroxidase Activity During Zygotic and Somatic Embryogenesis of Firs (*Abies* sp.)

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**Abstract** Somatic embryogenesis was initiated from immature embryos of *Abies concolor* (Gord. et Glend), *A. numidica* De Lann. and *A. cilicica* Carr., *A. alba* Mill. as well as in hybrid fir *A. cilicica* × *A. nordmanniana*. Soluble protein profiles and peroxidase activity were compared in developing zygotic and somatic embryos of silver fir (*A. alba* Mill.). On the basis of sodium dodecyl sulfate polyacrylamide gel electrophoresis of soluble proteins a high degree of homology was established between the two types of embryos. A higher peroxidase activity was registered throughout zygotic embryogenesis than during somatic embryo development but the opposite was true at the stage of mature embryos. Isoperoxidase composition reflected more efficiently the developmental stages of zygotic embryogenesis than those of somatic embryogenesis.

### 1

## Introduction

Somatic embryogenesis has become a major tool in the study of plant embryology, as it is possible in culture to manipulate cells of many plant species to produce somatic embryos in a process that is remarkably similar to zygotic embryogenesis (Thorpe 2000).

Induction of somatic embryogenesis in the genus *Abies* has been demonstrated in five pure species: *A. alba* (Hristoforoglu et al. 1995; Schuller et al. 2000), *A. nordmanniana* (Nørgaard and Krogstrup 1991, 1995), *A. balsamea* (Guevin et al. 1994), *A. fraseri* (Guevin and Kirby 1997; Rajbhandari and Stomp 1997) and *A. cephalonica* (Krajňáková and Häggman 1997).

In our laboratory, embryogenic cultures of hybrid firs have been derived from immature *A. alba* × *A. alba*, *A. alba* × *A. nordmanniana* (Gajdošová et al. 1995), *A. alba* × *A. cephalonica*, *A. alba* × *A. numidica* (Salajová et al. 1996) and mature *A. alba* × *A. cephalonica* zygotic embryos (Salajová and Salaj 2003/2004).

## 2

### Somatic Embryogenesis of *Abies* sp.

#### 2.1

##### Initiation of Embryogenic Tissue from Immature Zygotic Embryos

Embryogenic tissue was induced from immature embryos of *A. concolor* (Gord. et Glend), *A. numidica* De Lann. and *A. cilicica* Carr. derived from self-pollination as well as in hybrid fir from interspecific crosses *A. cilicica* × *A. nordmanniana*. Immature seeds were surface-sterilized for 10 min in 10% H<sub>2</sub>O<sub>2</sub>. Endosperms containing embryos (from July) or embryos after excision from the megagametophyte (from August) were plated on Schenk and Hildebrandt (SH) initiation medium (Schenk and Hildebrandt 1972) with 1 mg l<sup>-1</sup> benzylaminopurine and 2% sucrose. The medium was solidified with 0.3% Phytigel. The cultures were kept in the dark at 21–23 °C. After 4–8 weeks of explant cultivation, white, mucilaginous extrusions were observed from the micropylar end of the megagametophyte. Early zygotic embryos in megagametophytes, collected in early July, produced more readily embryogenic cultures. Embryogenic tissue of *A. concolor* was induced in 5.6% of explants, and of *A. numidica* in 6.8% of explants (Vooková and Kormut'ák 2004). In *A. cilicica*, the initiation of embryogenic tissue frequency ranged between 5.4 and 63.5%, and in *A. cilicica* × *A. nordmanniana* between 3.0 and 27.6% (Vooková and Kormut'ák 2003). For *Abies*, the cytokinin as a sole growth regulator was sufficient to induce somatic embryogenesis in immature (Schuller et al. 1989; Nørgaard and Krogstrup 1991) as well as in mature (Hristoforoglu et al. 1995) embryo explants.

#### 2.2

##### Proliferation of Embryogenic Cultures

Embryogenic tissue proliferated on SH initiation medium with supplement of 0.05% L-glutamine and 0.1% casein hydrolysate and were subcultured every 3 weeks. More than 90% of the responding explants developed embryogenic tissue within 1 month of culture. The embryogenic cultures in *Abies* sp. regardless of their different origin exhibited the common morphological features. It was found in our previous experiments (Hřib et al. 1997) that embryogenic tissue of *A. alba* shows many similarities with habituated non-organogenic sugar beet callus (Gaspar et al. 1988).

*A. numidica* embryogenic culture was used as a model for characterization of cell lines (Vooková and Kormut'ák 2002a). Embryogenic cell lines have been divided into two groups on the basis of morphology and growth characteristics of somatic embryos according to Mo et al. (1996). The cell line representing group B with undeveloped somatic embryos was stimulated to undergo maturation by treatment with plant growth regulators.

## 2.3

### Somatic Embryo Maturation and Germination

Somatic embryo maturation of *Abies* species and hybrid was achieved on modified Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 4% maltose, 10% polyethyleneglycol 4000 (PEG-4000),  $10 \text{ mg l}^{-1}$  abscisic acid (ABA) and  $500 \text{ mg l}^{-1}$  L-glutamine and casein hydrolysate.

Maturation of fir somatic embryos is promoted by ABA. ABA plays an important role in conifer embryogenesis. It inhibits cleavage polyembryony, allowing embryo singulation, its further development and maturation (Boulay et al. 1988). The production of cotyledonary somatic embryos in *A. cilicica* and *A. cilicica*  $\times$  *A. nordmanniana* was influenced by ABA. The addition of  $20 \text{ mg l}^{-1}$  ABA into the maturation medium was the most effective for maturation (Vooková and Kormut'ák 2003).

The literature data indicated that carbohydrates as a source of carbon or osmotica influenced somatic embryogenesis in *Abies*. Lactose and sorbitol favoured *A. alba* somatic embryo maturation up to an early cotyledonary stage (Schuller et al. 2000). Maltose gave a better maturation response and the addition of PEG-4000 to the medium promoted the maturation of somatic embryos in *A. nordmanniana* (Nørgaard 1997) and *A. alba*  $\times$  *A. numidica* (Salaj et al. 2004).

In *A. numidica*, the effect of subculture period and the concentration of PEG and maltose was confirmed on maturation of somatic embryo (Vooková and Kormut'ák 2002b). The maturation was promoted by PEG-4000, at 7.5 to 10%. Maltose (3 to 6%) significantly enhanced the yield of mature embryos. It seems that choice of the basal medium for somatic embryo maturation is also important. Embryogenic tissues of *A. cilicica*, *A. numidica*, *A. concolor* and *A. cilicica*  $\times$  *A. nordmanniana* hybrid were cultured on SH, Gresshoff and Doy (GD; Gresshoff and Doy 1972) and modified MS media. The tendency for better maturation on SH and MS media was common for all cultures tested (Table 1). GD medium was not suitable because maturation was slow and achieved only the precotyledonary stage of development (Vooková and Kormut'ák 2003, 2004). Exogenously applied myo-inositol ( $100 \text{ mg l}^{-1}$ ) influenced somatic embryogenesis of *A. numidica* although this process occurred on media with and without this compound (Vooková et al. 2001).

Prior to germination, isolated mature somatic embryos with four to six cotyledons were subjected to partial drying in the dark at  $21\text{--}23^\circ\text{C}$  for 3 weeks. Mature somatic embryos were placed in small Petri dishes (60-mm diameter). The Petri dish was open and placed on moist filter paper in a bigger Petri dish (90-mm diameter), which was sealed with Parafilm. Then desiccated mature somatic embryos were transferred to a germination medium and cultured in the light (16-h photoperiod).

**Table 1** Numbers ( $\pm$  standard error, *SE*) of cotyledonary somatic embryos of *Abies* species and hybrid (per gram of embryogenic tissue) matured on Schenk and Hildebrandt (*SH*), Murashige and Skoog (*MS*) and Gresshoff and Doy (*GD*) media, and germination frequency of somatic embryos on *SH* medium

Species/hybrid	SH	MS	GD	Germination (%)	SE $\pm$
<i>A. cilicica</i>	6 $\pm$ 1.5	16 $\pm$ 1.9	0	74.99	6.81
<i>A. numidica</i>	16 $\pm$ 4.9	26 $\pm$ 2.9	1 $\pm$ 0.7	85.45	4.11
<i>A. concolor</i>	–	61 $\pm$ 7.5	0	71.10	5.22
<i>A. cilicica</i> $\times$ <i>A. nordmanniana</i>	3 $\pm$ 1.3	45 $\pm$ 6.6	0	83.61	11.40

Media for germination are routinely used with sucrose in 2% concentration, and with (Nørgaard 1997) or without (Salajová et al. 1996; Guevin and Kirby 1997) activated charcoal. In our experiment (Vooková and Kormut'ák 2001) no significant differences were detected between *MS* and *SH* media. The addition of 1% activated charcoal or 0.05 mg l<sup>-1</sup> indole-3-butyric acid into both media had a positive influence on *A. numidica* embryo germination. A high rooting percentage (85%) was recorded on half *SH* medium with 1% sucrose and activated charcoal. It seems that this medium is widely applicable. We have used it successfully for germination of other *Abies* sp. and hybrid (Table 1). With increased sucrose concentration the germination was reduced.

### 3

#### Storage Proteins of Conifer Seeds

Comparative study of zygotic and somatic embryogenesis in conifers has shown that except for morphological similarity there exists a high degree of biochemical homology between zygotic and somatic embryos of conifers, especially with respect to their storage proteins (Hakman et al. 1990). Because of their accumulation during embryo development, the latter were reported to be excellent markers for comparison of zygotic and somatic embryo programmes (Flinn et al. 1993). On the basis of similarities of the protein molecular weight, the somatic embryos of *Picea glauca* (Flinn et al. 1991; Misra et al. 1993), *Picea abies* (Hakman 1993; Hakman et al. 1990) and *Pinus strobus* (Klimaszewska et al. 2004) were shown to contain the same storage proteins as the corresponding zygotic embryos. The greater biochemical similarity of somatic embryos to their zygotic counterparts is believed to improve the conversion of somatic embryos to plants (Klimaszewska et al. 2004). According to Cyrr et al. (1991) the criteria for obtaining high-quality somatic embryos include both the formation of storage reserves that are analogous to those of seed embryos and the absence of precocious germination. The authors pre-

sented evidence suggesting that differences between the performance of *Picea glauca* somatic and seed embryos during germination and early growth could be attributed to the differences in the kinetics of storage reserve utilization.

As far as the nature of conifer seed storage proteins is concerned, both insoluble crystalloids and soluble matrix proteins were identified (Misra and Green 1990). Insoluble proteins have molecular masses in their non-reduced form of 57 kDa, whereas in reduced form they migrate as three distinct groups of proteins in the molecular mass range of 42 kDa, 34.5–35 kDa and 22.5–23 kDa. The soluble fraction involves proteins in the molecular mass range of 27–30 kDa. In two of the three *Picea* species analysed the 34.5-kDa protein band was absent, indicating interspecific variation in quality of storage reserves (Misra and Green 1990). In *Pinus strobus*, the most abundant were the buffer-insoluble 11S globulins of molecular mass 59.6 kDa, which dissociate under reduced conditions to 38.2–40.0 and 22.5–23.5-kDa range polypeptides, and buffer soluble 7S vicilin-like proteins of molecular mass 46.0–49.0 kDa, which did not separate under reduced conditions. Other relatively abundant soluble proteins were in the ranges of 25–27 and 27–29 kDa (Klimaszewska et al. 2004). The *Abies* species lack 55 kDa  $\alpha\beta$ -dimer legumin-like proteins in their seeds and were reported to deviate conspicuously from *Cedrus*, *Larix*, *Picea* and *Pseudotsuga*. Other proteins are present in *Abies* seeds like in the remaining *Pinaceae*. Their soluble fraction involves 43-, 28- and 16-kDa proteins (Jensen and Lixue 1991).

Our data derived from comparison of the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein profiles of both zygotic and somatic embryos of silver fir (*A. alba* Mill.) indicate the presence of some additional proteins which meet the criteria of storage reserves. Their origin was traced from the cub-like embryo stage until germinating embryos during zygotic embryogenesis and from the non-embryogenic callus until regenerated emblings during somatic embryogenesis.

Figure 1 illustrates the dynamics of soluble protein synthesis during silver fir zygotic embryo development. At least ten major components along with numerous minor protein bands may be distinguished in the SDS-PAGE profile of mature embryos. The approximate molecular masses of the major proteins correspond to 55, 46, 40, 36, 30, 26, 24, 22, 18 and 14 kDa, respectively (Fig. 1, lane F with arrows). Their presence in embryos may be traced already at the precotyledonary stage (lane B). In particular, it is true of the 55- and 46-kDa proteins, which represent the prominent components of the soluble protein profile of young zygotic embryos. The only exception is the 24-kDa protein, whose synthesis seems to begin at the advanced cotyledonary stage only (lane D). We infer, this protein belongs to the category of Lea proteins that are synthesized during late embryogenesis and which are believed to prevent embryos from damage from desiccation and from precocious germination during somatic embryo development (Dong and Dunstan 2000; Zimmerman 1993). Within the context of a continuous synthesis of an



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