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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_2O_2. 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^{-1} benzylaminopurine and 2% sucrose. The medium was solidified with 0.3% Phytagel. 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 Kormuťák 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 Kormuťák 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 Kormuťák 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 mg l^{-1} abscisic acid (ABA) and 500 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* × *A. nordmanniana* was influenced by ABA. The addition of 20 mg l^{-1} ABA into the maturation medium was the most effective for maturation (Vooková and Kormuťák 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* × *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 Kormuťák 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* × *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 Kormuťák 2003, 2004). Exogenously applied myo-inositol (100 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 21–23 °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 transfered to a germination medium and cultured in the light (16-h photoperiod).

Table 1 Numbers (± 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 ±
A. cilicica	6 ± 1.5	16 ± 1.9	0	74.99	6.81
A. numidica	16 ± 4.9	26 ± 2.9	1 ± 0.7	85.45	4.11
A. concolor	–	61 ± 7.5	0	71.10	5.22
A. cilicica × *A. nordmanniana*	3 ± 1.3	45 ± 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 Kormuťák 2001) no significant differences were detected between MS and SH media. The addition of 1% activated charcoal or 0.05 mg l^{-1} 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 leguminlike 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

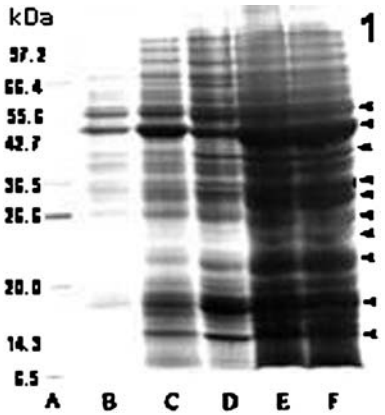

Fig. 1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (*SDS-PAGE*) profiles of soluble proteins during zygotic embryo development. *A* molecular size marker, *B* precotyledonary embryos, *C* early cotyledonary embryos, *D* advanced cotyledonary embryos, *E* morphologically differentiated embryos, *F* physiologically mature embryos

overwhelming majority of proteins during embryo development an abrupt increase in the amount of the 46-, 40-, 36-, 30-, 22- and 18-kDa proteins at the advanced cotyledonary stage was rather conspicuous (lane D). All these proteins dominate the soluble protein profile of mature embryos. Their identity as seed storage reserves was inferred from degradation of individual proteins during seed germination. Figure 2 illustrates that SDS-PAGE protein profiles of zygotic embryos are identical during the first 48 h of seed imbibition (lanes H, I). Profound changes appear only when the radicle emerges from a seed coat. The 24-kDa protein is depleted completely at this stage, while the proteins of 46, 36, 26 and 22 kDa are consumed only partially (lane J). During advanced germination (lane K) and at the seedling stage (lane L) the degradation of 46- and 36-kDa proteins is completed. The depletion of the 26- and 22-kDa proteins is also considerable but not complete. Their synthesis seems to be resumed at the seedling stage along with a strengthened synthesis of the 55-kDa protein and de novo synthesis of the 19-kDa protein (lane L). On the basis of the abundance criterion and degradation kinetics during germination, it seems reasonable to ascribe the storage reserve function to the 46-, 36-, 26-, 24- and 22-kDa proteins in silver fir zygotic embryos. This figure is very similar to that found for *Picea abies*, where three major seed storage proteins of 42, 33 and 22 kDa were distinguished by Stabel et al. (1990). Hakman et al. (1990) have in addition included among *Picea abies* storage proteins a 28-kDa protein. An essentially similar situation was also found in *Picea glauca* zygotic embryos with 43-, 33-, 22-, 18- and 16-kDa proteins dominating the SDS-PAGE profile and with less abundant 28- and 24-kDa proteins (Flinn et al. 1993). Recently, Klimaszewska et al. (2004) reported seed storage proteins in zygotic embryos of *Pinus strobus* involving soluble proteins with

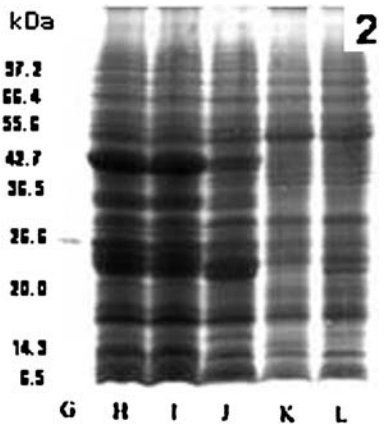

Fig. 2 SDS-PAGE profiles of soluble proteins during germination of seeds. *G* molecular size marker, *H* dormant embryos after 24-h imbibition, *I* dormant embryos after 48-h imbibition, *J* beginning of seed germination, *K* advanced seed germination, *L* seedlings

little deviating molecular mass ranges of 46.0–49.0, 38.2–40.0, 25–27, 27–29 and 22.5–23.5 kDa. With special reference to major storage proteins detected in *A. alba*, they seem to fall into these classes of proteins as well. According to Gifford (1988) and Gifford and Tolley (1989) this suggests that storage proteins may be conserved among the conifers, although the relative amount of different proteins differ among the species.

4
SDS-PAGE Protein Profile of *A. Alba* Somatic Embryos

As far as somatic embryos of silver fir are concerned, their SDS-PAGE protein profiles were comparable with the corresponding profiles of zygotic embryos. Among the proteins detected, the most abundant were those with molecular masses of 53, 46, 40, 36, 30, 28, 24, 20 and 18 kDa, respectively (Fig. 3). As an exception, the presence of the 53-kDa protein in somatic embryos may be mentioned instead of the 55-kDa protein detected in zygotic embryos. Also, the 14-kDa protein of somatic embryos was expressed less than the corresponding fraction of zygotic embryos.

An overwhelming majority of abundant proteins may be traced in developing somatic embryos. They are already weakly expressed in embryogenic callus (lane C) and become very distinct at the globular, torpedo and cotyledonary stages of somatic embryos (lanes D–F). The 46-kDa protein is an exception in this respect, exhibiting the highest concentration at the cotyledonary stage only (lane F). However, during desiccation of mature somatic embryos this protein is depleted preferentially (lane G). The same is also true

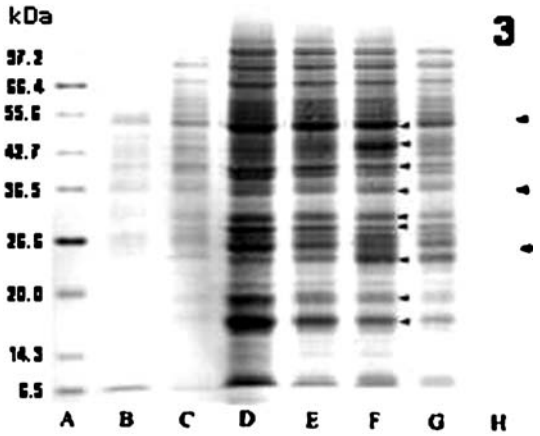

Fig. 3 SDS-PAGE profiles of soluble proteins during somatic embryogenesis. *A* molecular size marker, *B* non-embryogenic callus, *C* embryogenic callus, *D* globular stage, *E* torpedo stage, *F* cotyledonary stage, *G* mature somatic embryos after desiccation, *H* regenerated emblings

of the rest of the soluble proteins when at the stage of regenerated emblings nearly all proteins were consumed. Detectable amounts were found only in the case of 53-, 36- and 24-kDa proteins (lane H with arrows). Owing to the buffer-soluble nature of these proteins we assume they represent the soluble matrix proteins as quoted by Misra and Green (1990).

5 Peroxidase Activity in Developing Zygotic and Somatic Embryos of *A. Alba*

In contrast to soluble proteins the differences between zygotic and somatic embryos of silver fir in peroxidase activity are more evident. The enzyme was found to exhibit 3 times higher activity in mature somatic embryos than in dormant zygotic embryos (Table 2). No activity was detected in precotyledonary zygotic embryos. Starting with the early cotyledonary stage, a decline in peroxidase activity was registered throughout zygotic embryogenesis, and the situation was similar during somatic embryogenesis. However, peroxidase activity changed abruptly during two stages of somatic embryogenesis. The first stage was the transition of non-embryogenic to embryogenic callus, accompanied by a conspicuous decline in specific enzyme activity. The higher peroxidase activity in non-embryogenic callus is due to increased levels of phenolic substances in this tissue, some of which serve as substrates in peroxidase-catalysed reactions (Hrubcová et al. 1994). The second stage was that of regenerated emblings, which had 7 times higher peroxidase

Table 2 Changes in peroxidase activity during zygotic and somatic embryogenesis of silver fir (From Kormutak et al. 2003, with permission of Versalius University Medical Publisher in Cracow)

Developmental stage	Specific activity
Zygotic embryogenesis	
Precotyledonary	0
Early cotyledonary	0.25 ± 0.00
Advanced cotyledonary	0.20 ± 0.00
Morphologically difffenet embryos	0.16 ± 0.00
Physiologically mature embryos	0.08 ± 0.00
Somatic embryogenesis	
Non-embryogenic callus	3.15 ± 0.050
Embryogenic callus	0.93 ± 0.015
Precotyledonary	0.11 ± 0.005
Cotyledonary	0.11
Mature embryos	0.24
Regenerated emblings	1.83 ± 0.080

activity than mature somatic embryos. Obviously, this increase in enzyme activity is a part of metabolic events underlying embryo germination and progressive embling development. The higher metabolic potential of mature somatic embryos than that of mature zygotic embryos may probably be ascribed to the different levels of dormancy which seem to be lower in somatic embryos.

The changes outlined in peroxidase activity during zygotic embryogenesis were also paralleled by the changes in isoenzyme composition. The only exception were embryos at the precotyledonary stage lacking peroxidase activity but containing as many as seven to eight isoenzymes (Fig. 4, lane A). Starting with the early cotyledonary stage until mature embryos, the number of isoperoxidases followed closely the tendencies in peroxidase activity. The early and advanced cotyledonary embryos accordingly possessed the highest number of isoperoxidases visualized in the gels as eight intensively stained bands (lanes B, C). Also, morphologically differentiated and physiologically mature zygotic embryos with lowered peroxidase activity possessed very similar isoenzyme profiles consisting of five isoperoxidases (lanes D, E).

Like in zygotic embryos, a close correlation between peroxidase acitity and its isoenzyme composition has been observed during somatic embryogenesis as well. As shown in Fig. 5, the high enzyme activity of both embryogenic callus (lane A) and regenerated emblings (lane D) is also reflected by the enriched isoenzyme profiles involving nine to ten isoperoxidases as compared with six to seven isoperoxidases detected in precotyledonary (lane B) and cotyledonary (lane C) embryos. However, as a molecular marker, this enzyme

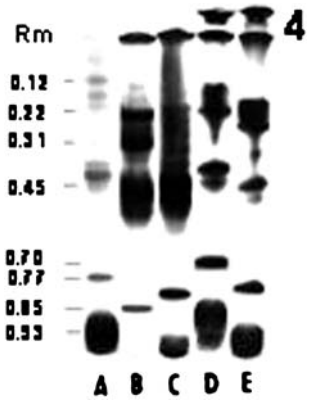

Fig. 4 Isoperoxidase composition of developing zygotic embryos. *A* precotyledonary embryos, *B* early cotyledonary embryos, *C* advanced cotyledonary embryos, *D* morphologically differentiated embryos, *E* physiologically mature embryos

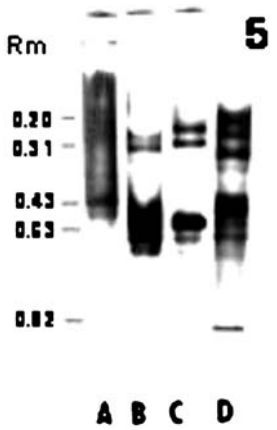

Fig. 5 Isoperoxidase composition of developing somatic embryos. *A* embryogenic callus, *B* torpedo stage, *C* cotyledonary stage, *D* regenerated emblings

seems to be more indicative of individual stages of zygotic embryogenesis than those of somatic embryogenesis.

6 Conclusions and Future Prospects

Emblings of *A. concolor*, *A. numidica*, *A. cilicica*, *A. alba* and *A. cilicica* × *A. nordmanniana* hybrid firs have been regenerated from immature zygotic embryos via somatic embryogenesis.

In spite of the postulated divergency of *Abies* storage proteins from other *Pinaceae*, the data presented indicate a high degree of similarity between soluble protein profiles of silver fir and the corresponding profiles of *Picea* and *Pinus* sp. Most probably, these proteins represent the soluble matrix proteins. The question whether insoluble proteins as the main constituent of the conifer storage reserves share the genus- or species-specific features remains to be answered. Additional experiments which will help to resolve this point are highly desirable. A high degree of homology has also been observed between zygotic and somatic embryogenesis of silver fir with respect to the major storage proteins represented by ten or nine fractions, respectively. The only difference observed so far was related to the dynamics of the 46-kDa protein synthesis. As the main component of the soluble protein profile this protein seems to be synthesized continuously during zygotic embryogenesis starting with the precotyledonary stage of embryo development. In contrast, during somatic embryo development its synthesis becomes conspicuous at the cotyledonary stage only. A remarkable feature of the somatic embryo soluble protein dynamics is their nearly complete depletion in regenerated emblings. This aspect of *Abies* somatic embryo development needs to be verified as well. The metabolic potential as revealed by peroxidase activity seems to be higher in developing zygotic embryos than in somatic ones; however, zygotic embryos after reaching maturity become enzymatically more quiescent than somatic embryos. Isoperoxidase composition can be discriminated more clearly between individual stages of zygotic embryo development than in somatic embryogenesis. In order to find out efficient molecular markers of *Abies* embryogeny, additional isoenzyme systems have to be involved in future comparative studies.

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References

Boulay MP, Gupta PK, Krogstrup P, Durzan DJ (1988) Development of somatic embryos from cell suspension cultures of Norway spruce (*Picea abies* karst). Plant Cell Rep 7:134–137

Cyrr DR, Webster FB, Roberts DR (1991) Biochemical events during germination and early growth of somatic embryos and seed of interior spruce (*Picea glauca engelmanii* complex). Seed Sci Res 1:91–97

Dong JZ, Dunstan DI (2000) Molecular biology of somatic embryogenesis in conifers. In: Jain SM, Minocha SC (eds) Molecular biology of woody plants. vol 1. Kluwer, Dordrecht, p 51–87

Flinn BS, Roberts DR, Taylor JEP (1991) Evaluation of somatic embryos of interior spruce. Characterization and developmental regulation of storage proteins. Physiol Plant 82:624–632

Flinn BS, Roberts DR, Newton CH, Webster FB, Taylor JEP (1993) Storage protein gene expression in zygotic and somatic embryos of interior spruce. Physiol Plant 89:719–730

Gajdošová A, Vooková B, Kormuťák A, Libiaková G, Doležel J (1995) Induction, protein composition and DNA ploidy level of embryogenic calli of silver fir and its hybrids. Biol Plant 37:169–176

Gaspar T, Kevers C, Penel C, Crèvecoeur M, Greppin H (1988) Biochemical characterization of normal and habituated sugarbeet calli. Relationship with anatomy, habituation and organogenesis. Potsdamer Forsch 57:21–30

Gifford DJ (1988) An electrophoretic analysis of the seed protein from *Pinus monticola* and eight other species of pine. Can J Bot 66:1808–1812

Gifford DJ, Tolley MC (1989) The seed proteins of white spruce and their mobilization following germination. Physiol Plant 77:254–261

Gresshoff PM, Doy CH (1972) Development and differentiation of haploid *Lycopersicum esculentum* (tomato). Planta 107:161–170

Guevin TG, Kirby EG (1997) Induction of embryogenesis in cultured mature zygotic embryos of *Abies fraseri* (Pursh) Poir. Plant Cell Tissue Org Cult 49:219–222

Guevin TG, Micah V, Kirby EG (1994) Somatic embryogenesis in cultured mature zygotic embryos of *Abies balsamea*. Plant Cell Tissue Org Cult 37:205–208

Hakman I (1993) Embryology in Norway spruce (*Picea abies*). An analysis of the composition of seed storage proteins and deposition of storage reserves during seed development and somatic embryogenesis. Physiol Plant 87:148–159

Hakman I, Stabel P, Engström P, Eriksson T (1990) Storage protein accumulation during zygotic and somatic embryo development in *Picea abies* (Norway spruce). Physiol Plant 80:441–445

Hřib J, Vooková B, Kormuťák A (1997) Biochemical differences between normal callus and embryogenic suspensor mass of silver fir. Biol Plant 39:507–513

Hristoforoglu K, Schmidt J, Bolhar-Nordenkamp H (1995) Development and germination of *Abies alba* somatic embryos. Plant Cell Tissue Org Cult 40:277–284

Hrubcová M, Cvikrová M, Eder J (1994) Peroxidase activities and content of phenolic acids in embryogenic and non-embryogenic alfalfa cell suspension cultures. Biol Plant 39:175–182

Jensen U, Lixue Ch (1991) *Abies* seed protein profile divergent from other *Pinaceae*. Taxon 40:435–440

Kormuťák A, Salaj T, Matúšová R, Vooková B (2003) Biochemistry of zygotic and somatic embryogenesis in silver fir (*Abies alba* Mill.). Acta Biol Cracoviensia, Ser Botan 45:59–62

Klimaszewska K, Morency F, Jones-Overton C, Cooke J (2004) Accumulation pattern and identification of seed storage proteins in zygotic embryos of *Pinus strobus* and in somatic embryos from different maturation treatments. Physiol Plant 121:682–690

Krajňáková J, Häggman H (1997) Regeneration of *Abies cephalonica* through somatic embryogenesis. In: Identification and control of phase changes in rejuvenation. COST 822, 4th Meeting of WG3, Nitra, Slovakia, 15–19 October 1997, pp 102–104

Misra S, Green MJ (1990) Developmental gene expression in conifer embryogenesis and germination. I. Seed proteins and protein body composition of mature embryo and the megagametophyte of white spruce (*Picea glauca* [Moench] Voss.). Plant Sci 68:163–173

Misra S, Attree SM, Leal J, Fowke LC (1993) Effect of abscisic acid, osmoticum, and desiccation on synthesis of storage proteins during the development of white spruce somatic embryos. Ann Bot 71:11–22

Mo LH, Egertsdotter U, von Arnold S (1996) Secretion of specific extracellular proteins by somatic embryos of *Picea abies* is depended on embryo morphology. Ann Bot 77:143–152

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Nørgaard JV (1997) Somatic embryo maturation and plant regeneration in *Abies nordmanniana* LK. Plant Sci 124:211–221

Nørgaard JV, Krogstrup P (1991) Cytokinin induced somatic embryogenesis from immature embryos of *Abies nordmanniana* LK. Plant Cell Rep 9:509–513

Nørgaard JV, Krogstrup P (1995) Somatic embryogenesis in *Abies* spp. In: Jain SM, Gupta PK, Newton RJ (eds) Somatic embryogenesis in woody plants, vol 3. Gymnosperms. Kluwer, Dordrecht, pp 341–355

Rajbhandari N, Stomp A-E (1997) Embryogenic callus induction in Fraser fir. Hort Sci 32:737–738

Roth R, Schmidt J (1998) Induction of embryogenic suspensor masses from cotyledons of zygotic seedlings of *Abies alba*. In: Symp. Zum Gedenken an die 100 Wiederkehr der Begründung der Gewebekultur durch Gottlieb Haberlandt, Vienna, 8–9 October 1998, p 45

Salajová T, Salaj J (2001) Somatic embryogenesis and plantlet regeneration from cotyledon explants isolated from emblings and seedlings of hybrid firs. J Plant Physiol 158:747–755

Salajová T, Salaj J (2003/2004) Somatic embryo formation on mature *Abies alba* × *Abies cephalonica* zygotic embryo explants. Biol Plant 47:7–11

Salajová T, Salaj J, Jásik J, Kormuťák A, Hakman I (1996) Embryogenic culture initiation and somatic embryo development in hybrid firs (*Abies alba* × *Abies cephalonica* and *Abies alba* × *Abies numidica*). Plant Cell Rep 15:527–530

Salaj T, Matúšová R, Salaj J (2004) The effect of carbohydrates and polyethylene glycol on somatic embryo maturation in hybrid fir *Abies alba* × *A. numidica*. Acta Biol Cracov Bot 46:159–167

Schenk RU, Hildebrandt AC (1972) Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. Can J For Res 50:199–204

Schuller A, Reuther G, Geier T (1989) Somatic embryogenesis from seed explants of *Abies alba*. Plant Cell Tissue Org Cult 17:53–58

Schuller A, Kirchner-Ness R, Reuther G (2000) Interaction of plant growth regulators and organic C and N components in the formation and maturation of *Abies alba* somatic embryos. Plant Cell Tiss Org Cult 60:23–31

Stabel P, Eriksson T, Engström P (1990) Changes in protein synthesis upon cytokinin-mediated adventitious bud induction and during seedling development in Norway spruce, *Picea abies*. Plant Physiol 92:1174–1183

Thorpe TA (2000) Somatic embryogenesis: morphogenesis, physiology, biochemistry and molecular biology. Korean J Plant Tissue Cult 27:245–258

Vooková B, Kormuťák A (2001) Effect of sucrose concentration, charcoal, and indole-3-butyric acid on germination of *Abies numidica* somatic embryos. Biol Plant 44:181–184

Vooková B, Kormuťák A (2002a) Effect of plant growth regulators treatment on proliferation and somatic embryo development of Algerian fir (*Abies numidica* De Lann.). Folia Oecolog 29:141–146

Vooková B, Kormuťák A (2002b) Some futures of somatic embryo maturation of Algerian fir. In Vitro Cell Dev Biol 38:549–561

Vooková B, Kormuťák A (2003) Plantlet regeneration in *Abies cilicica* and *Abies cilicica* × *Abies nordmanniana* hybrid via somatic embryogenesis. Turk J Bot 27:71–76
Vooková B, Kormuťák A (2004) Propagation of some *Abies* species by somatic embryogenesis. Acta Univ Latv Biol 676:257–260
Vooková B, Kormuťák A, Hřib J (2001) Effect of myo-inositol on somatic embryogenesis of *Abies numidica*. J Appl Bot 75:46–49
Vooková B, Matúšová R, Kormuťák A (2003) Secondary somatic embryogenesis in *Abies numidica* De Lann. Biol Plant 46:513–517
Zimmerman JL (1993) Somatic embryogenesis: A model for early development of higher plants. Plant Cell 5:1411–1423