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Božena Vooková, Andrej Kormuťák

Study of *Abies* somatic embryogenesis and its application

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Abstract: This paper provides results of somatic embryogenesis study in our laboratory. General description of somatic embryogenesis (SE) induction, maturation of somatic embryos and plantlets regeneration of the *Abies* species, followed by a comparisons of some characteristics of zygotic and somatic embryos, seedlings and emblings (somatic seedlings). Own results are supplemented with some literature data. Also aplication of SE for improving of plantlet regeneration of elite fir trees from Dobroč primeval is described as well as initiation of the SE from seeds of incompatible crossings of firs where zygotic embryos abort usually several weeks after pollination.

Additional key words: fir, protein analysis, chlorophyll content, macro- and microelements, defence reactions, incompatible crossing

Address: B. Vooková, A. Kormuťák, Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademická 2, P.O. BOX 39A, 950 07 Nitra, Slovak Republic, e-mail: nrgrvook@savba.sk

Introduction

The discovery of conifer somatic embryogenesis (SE) and the subsequent development of SE protocols for a range of genera and species have opened new research opportunities to forest biotechnologists (Klimaszewska and Cyr 2002). The aim of most tree breeding programmes is to mass produce genetically superior clones or populations. Trees are mostly propaged sexually. Vegetative propagation, however is preferred because superior characteristics are maintained better than by sexual propagation. The most common method of vegetative propagation of conifers has been rooting of cuttings, but current research is not sufficiently developed (Ragonezy et al. 2010). Cuttings often fail to root properly and biggest problem in *Abies* is prevalent plagiotropic growth of shoots. Rooting problem is associated with tree maturation phase, an age-related developmental process that affects reproductive competence, morphology and growth rate. From this point of view SE is more promising aproach. This system offers the capability to produce unlimited numbers of high-valued plants but it also enables the use of genetic transformation to improve traits such as disease and insect resistance (Kim et al. 2009). SE is interresting for regeneration of *Abies* species for their tissue genetic stability in condition *in vitro* (Libiaková et al. 1995; Gajdošová et al. 1995), althoug organic supplement in the medium can affect this stability during long term cultivation (Roth et al. 1997). Also aplication of cryopreservation for embryogenic cells may case a risk for genetic fidelity (Aronen et al. 1999; Krajňáková et al. 2011).

Abies concerns 40–45 species growing in the Middle and South Europe, in Mediterranean, in East Asia, North Amerika and one species in Quatemala and in Mexico. First notice about initation of SE in *Abies* is from 1988. In *A. alba* Mill. embryogenic tissue has been initiated from immature zygotic embryos (Gebhardt et al. 1988). Somatic embryogenesis in *Abies* with limited success or succesfull regeneration was reported for ten pure species: *A. alba* Mill., *A. balsamea* Mill., *A. cephalonica* Loud., *A. cilicica* (Antoine et Kotschy) Carrière, *A. concolor* (Gordon) Lindl. ex Hildebr., *A. fraseri* (Pursh) Poir, *A. koreana* Wils., *A. nordmanniana* LK, *A. numidica* De Lann. and *A. lasiocarpa* (Hook.) Nutt. (for review see Vooková and Kormuták 2007). At present, 80 to 90% of *A. nordmanniana* embryos with normal morphology can be converted into plantlets, independently of their genotype (Zoglauer et al. 2012).

In our laboratory, besides of pure species also embryogenic cultures of interspecific hybrids 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; *A. cilicica* × *A. nordmanniana,* Vooková and Kormuťák 2003) and mature (*A. alba* × *A. cephalonica*, Salaj and Salaj 2003/2004) zygotic embryos. In the last time, SE was initiated from immature and mature embryos of *A. cilicica* × *A. cephalonica* in Czech Republic (Korecký and Vítámvás 2011).

The objective of this report is to present an overview of the *Abies* somatic embryogenesis research that has been carried out in our laboratory over several years.

Process of somatic embryogenesis in *Abies*

Somatic embryogenesis (SE), an asexual way of plant regeneration can be divided into four steps: initiation of embryogenic tissue contained somatic embryos from the primary explant, proliferation of embryogenic tissue (Fig. 1), maturation of somatic embryos (Fig. 2) and regeneration of plants from somatic embryos. Many of the experimental condition of SE can be generalised and used for the most *Abies* species but it would be optimizing for individual species (Vooková and Kormuťák 2004). Developmental stage of zygotic embryo used as explant is important for induction of somatic embryogenesis. In mostly cases, the initiation frequencies of embryogenic tissue were higher when zygotic embryo was in precotyledonary or early cotyledonary stage (Norgaard and Krogstrup 1991; Salajová et al. 1996; Vooková and Kormuták 2003; Kvaalen et al. 2005). Unlike other genera in the *Pinaceae*, *Abies* requires only cytokinin for induction of embryogenic tissue from zygotic embryos (Schuller et al. 1989; Nørgaard and Krogstrup 1991), although the embryogenic suspensor mass of *A. alba* proliferated on a medium supplemented by 2,4-D as well as on an auxin-free medium (Vondráková et al. 2011). To improve maturation process, the most beneficial was pre-culturing of embryogenic tissue on SH medium (Schenk and Hildebrandt 1972) without growth regulator for 2 weeks (Vooková et al. 2010). Maturation of fir somatic embryos is promoted by abscisic acid (ABA) and the presence of carbohydrates in the maturation medium. Maturation medium with 10 $mg \cdot l^{-1}$ ABA is the most frequently used for obtaining cotyledonary embryos (Nørgaard 1997; Salajová and Salaj 2003). The production of *A. cilicica* and *A. cilicica* × *A. nordmanniana* mature embryos was influenced by ABA concentration, 20 $mg \cdot l^{-1}$ was the most effective (Vooková and Kormuťák 2003). Maltose gave a better maturation response (Nawrot-Chorabik 2008) and the addition of polyethylene glycol-4000 (PEG) to maturation medium promoted

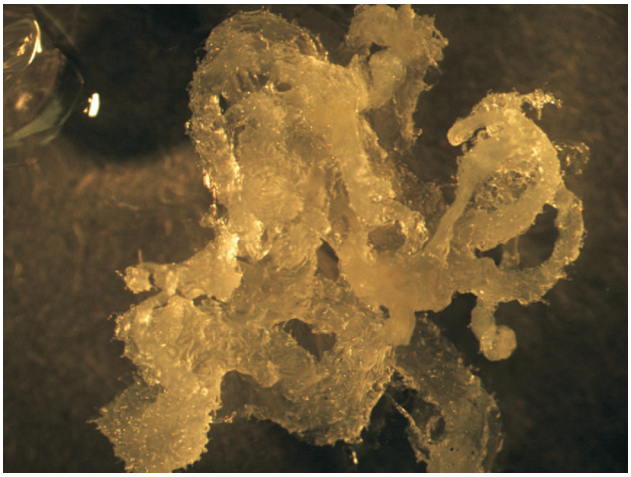

Fig. 1. Proliferation of embryogenic tissue

Fig. 2. Somatic embryo maturation

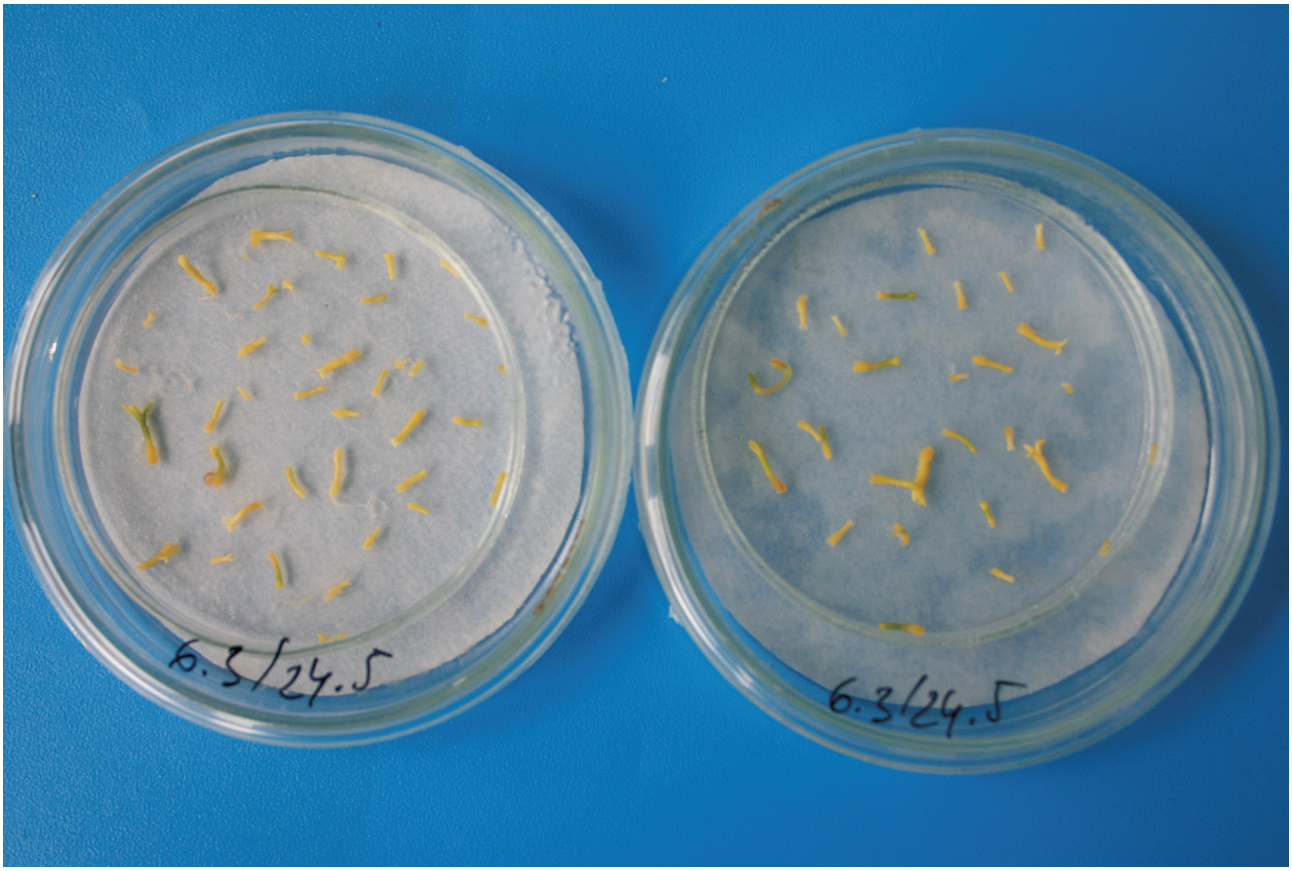

Fig. 3. Desiccation of somatic embryos

the maturation of somatic embryos (Nørgaard 1997; Salajová et al. 2004). Maturation of *A. numidica* somatic embryos was promoted by 7.5 – 10% PEG. Three to 6% maltose significantly enhanced the yield of mature embryos. The most effective maturation occured when embryogenic tissue was transferred to maturation medium after 14–21 d cultivation on proliferation medium (Vooková and Kormuťák 2002). The addition of IBA and PEG into the basal medium with ABA improved *A. alba* embryo development (Szczygiel and Kowalczyk 2001). Cultivation of *A. cephalonica* embryogenic tissue with the ectomycorrhiza fungi during the proliferation period reduced the proliferation but enhanced the subsequent embryo formation and maturation (Krajňáková et al. 2012).

Variables as a carbon source, ABA and osmotic agents has been used to increase germination and conversion rates of somatic embryos of *A. cephalonica* (Krajňáková et al. 2009). For germination, well-developed cotyledonary somatic embryos used to be selected and subjected to a partial desiccation treatment (Fig. 3) for three weeks (Nørgaard 1997; Vooková et al. 1998; Kvaalen et al. 2005). Media for germination are routinely used with sucrose in 2% concentration with (Nørgaard 1997) or without activated charcoal (Salajová et al. 1996; Guevin and Kirby 1997). The reduction of sucrose concentration to 1% had pos-

Fig. 4. Germination on half SH medium with 1% sucrose

itive influence on *A. numidica* embryo germination (Vooková and Kormuťák 2001).

The high rooting percentage (85%) was recorded on half SH medium with 1% sucrose and 1% activated charcoal (Fig. 4). It seems that this medium is widely applicable. We used it succesfully for germination of other *Abies* sp. and hybrids. Somatic seedlings (emblings) regeneration was in our laboratory succesful in: *A. numidica, A. cilicica, A. concolor, A. alba, A. cilicica* × *A. nordmanniana, A. nordmanniana* × *A. veitchii.*

The cornerstone of future research will be acclimatisation of somatic seedlings and field establishment of regenerants. A low percentage (less than 5%) of somatic germinants of *A. fraseri* survived following transplanting to soil however, most of them did not show further growth (Kim et al. 2009). In contrast to other genera, *Abies* seedlings go into dormancy in the cotyledonary stage even under long-day conditions. However, 2 to 3 months after acclimatisation dormancy can be broken using an artificial cold treatment for 6 weeks minimum at 5°C or lower. Although this treatment may speed up their growth, somatic seedlings develop slower and loose up to 1 year compared to plantlets from seeds (Zoglauer et al. 2012).

What differences are between somatic and zygotic embryos?

Somatic embryos of *Abies* morphologically resemble zygotic embryos (Fig. 5a, b). They were different in dry mass content, and some differences were found in storage protein synthesis as well as in enzyme activity (Kormuťák and Vooková 2001; Kormuťák et al. 2003, 2006). Precotyledonary, early cotyledonary and late cotyledonary stages of somatic embryogenesis were characterized by a substantially reduced peroxidase activity compared to callus tissues and regenerated plantlets. In vitro testing of defence reaction showed important differences between somatic and zygotic embryos (Vooková et al. 2012).

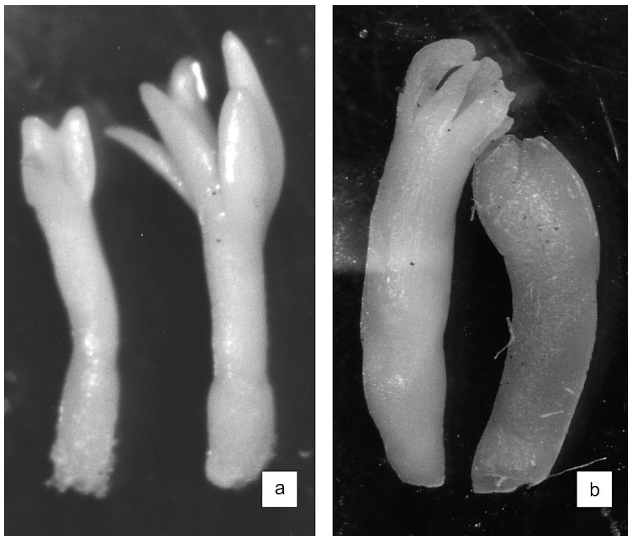

Fig. 5. Somatic (a) and zygotic (b) embryos

Dry mass content of zygotic and somatic embryos

Dry mass was determined by drying of 50 embryos per treatment at 80°C for 48 h. Zygotic embryos contained 66.3% ± 2.54 of dry mass. Desiccated somatic embryos contained only 23.6% ± 1.28 of dry mass.

Pullman et. al. (2003) compared *Pinus taeda* zygotic embryos at different stages of development and most advanced cotyledonary somatic embryos using several measures of embryo quality morphology (dry weight, germination performance, and gene expression). Zygotic embryos showed an increase in dry weight and a decreased percentage of water content as embryo stage advanced. Somatic embryos from genotypes with the most advanced development, resulting decreased dry weight per embryo. Somatic embryo morphology and dry weights were most similar to those of the zygotic embryos at stages 8–9.1. Somatic embryos grow approximately only halfway through the normal sequence of development and then prematurely discontinue growth.

Biochemistry of zygotic and somatic embryos

The formation of a mature embryo is characterized by the accumulation of LEA and storage proteins. The accumulation of these two types of proteins is controlled by ABA-dependent regulatory mechanisms (Dodeman et al. 1997). Altogether 9 major protein components with molecular masses of 14, 16, 22, 24, 27, 30, 35, 38 and 43 kDa along with numerous minor protein components were detected in zygotic embryos of *A. alba* and *A. concolor* based on soluble protein extraction procedure (Kormuťák and Vooková 2000, 2006). However, separated extraction of soluble and insoluble proteins revealed the presence of 4 additional soluble protein components in zygotic embryos of *A. numidica* with molecular masses of 97, 80, 55 and 6 kDa as well as 7 insoluble fractions of 57, 55, 42, 40, 30, 18 and 14 kDa size (Kormuťák et al. 2005). In the light of these findings, a species-specific profile of zygotic embryo proteins may be assumed in *Abies*. It is worth mentioning that the soluble protein profiles described above differ from those reported for *Abies* seed by Jensen and Lixue (1991) who were able to distinguish only 4 components in the entire female gametophyte – embryo complex.

As for somatic embryos of *A. concolor*, their soluble protein profiles resemble very closely those of zygotic

embryos. In contrast, soluble proteins of somatic embryos in *A. numidica* are similar but not identical with the corresponding profiles of zygotic embryos. In comparison with zygotic embryos the lack of 10, 24 and 34 kDa proteins was registered in mature somatic embryos of the species. Like in zygotic embryos, the 43 kDa protein is the most prominent component of the storage proteins in mature somatic embryos. Its abundance is apparent since the globular stage of somatic embryo formation. All the developmental stages are characterized by an identical protein pattern (Kormuťák et al. 2006).

Mature somatic embryos possessed three times higher peroxidase activity than the mature zygotic embryos. The reverse was true of the specific activity of esterase, which was higher in zygotic embryos than in somatic embryos (Kormuťák et al. 2003).

A wider picture about quality of somatic embryos we can found in literature data from other authors and species. In *Picea glauca*, it has been shown that mature somatic embryos have lower amounts of storage proteins and higher starch concentrations than zygotic embryos (Joy et al. 1991), whereas somatic embryos of *Pinus strobus* have similar or lower amounts of storage protein than zygotic embryos (Klimaszewska et al. 2004). Marked differences were observed in carbohydrate spectra between developing zygotic and maturing somatic embryos of *Picea abies* (Gösslová et al. 2001). The decrease in total carbohydrate as well as the accumulation of sucrose in later developing stages was a common features in both systems (Konrádová et al. 2002). The concentrations of total lipids exhibited marked variation during maturation of *Picea abies* somatic embryos, indicating the importance of lipid reserves during embryo development (Svobodová et al. 1999, Grigová et al. 2007). The accumulation of high levels of polyamines *in Picea abies* somatic embryos may be causally linked to their lower germinability than in zygotic embryos (Gemperlová et al. 2009). Endogenous levels of IAA decreased in the period of embryo development and increased again in the late maturation stage. This pattern was described during development of *Pinus sylvestris* zygotic embryos (Sanberg et al. 1987) and during early stages of somatic embryogenesis of *Picea abies* (Vágner et al. 1998).

In vitro defence reactions of somatic and zygotic embryos

Defence reactions of desiccated cotyledonary somatic embryos and mature zygotic embryos of *A. numidica* were tested *in vitro* by dual cultures with tester, basidiomycete *Phaeolus schweinitzii* (Vooková et al. 2012). The growth of mycelium in Petri dish alone and in presence of embryos was measured using a mm rule and compared. The measurements of fungal mycelium size were taken in 2-day intervals. In presence of somatic and zygotic embryos mycelial growth was inhibited. By this way both types of embryos expressed defence reactions. Greater defence reactions were observed in zygotic embryos relative to defence found in somatic embryos.

Comparisson of some zygotic and somatic seedling (embling) characteristics

Morfologically there are not differences between zygotic and somatic seedlings (Fig. 6a, b) They were different in dry mass and chlorophylls content as well as in concentration of essential elements. No qualitative differences were detected between the protein profiles of seedlings and emblings.

Accumulation of dry weight in seedlings and emblings

Accumulation of dry mass in seedlings and emblings during 50 d of culture was investigated (Table 1). Emblings contained nearly the same percentage of dry mass as seedlings after 14-d cultivation. It is interesting that dry mass of seedlings increased during 50-d of cultivation, while dry mass percentage of emblings was nearly the same during all days of cultivation.

In comparison with emblings, greater shoot and root dry weights of interior spruce sedlings was found also when they grew in soil (Grossnickle and Major 1994). But the emblings of *Picea abies* and *Pseudotsuga menziesii* grown in soil for field testing exhibited uniform growth and phenology within a clone, compared with seedlings (Gupta et al. 1996).

Fig. 6. Somatic (a) and zygotic (b) seedlings

Table 1. Growth of dry mass (in %) of seedlings and emblings over 50 days culturing on ½ SH medium with *myo*-inositol and IBA. n=5. Values followed by the same letters are not significantly different ($p \leq 0.05$)

Material/Days	14	20	30	40	50
Seedlings	11.17 ± 0.67a	17.50 ± 0.72b	18.88 ± 1.61b	19.71 ± 0.89b	22.20 ± 1.12b
Emblings	10.03 ± 0.32a	10.51 ± 1.02a	11.48 ± 0.66a	12.34 ± 0.37a	13.44 ± 0.25a

Chlorophyll content of cotyledons in seedlings and emblings

This analysis was perform from cotyledon material of 20 days old seedlings and emblings. Content of chlorophyll *a* and chlorophyll *b* in seedlings was higher than in emblings (Table 2). But relation of chlorophyll *a* / chlorophyll *b* was 2/1. This relation was usualy found also in cotyledons of some conifer species seedlings and leaves of higher plants (Šalgovičová and Hudák 2005).

Table 2. Chlorophyll content in mg per g of dry weight in cotyledons of 20 days seedlings and emblings. Means ± SE, n=6; Values followed by the same letters are not significantly different ($p \leq 0.05$)

Material	Chlorophyll *a*	Chlorophyll *b*	Relation *a/b*
Seedlings	0.6156 ± 0.05a	0.2882 ± 0.07c	2.13
Emblings	0.2700 ± 0.04b	0.1033 ± 0.02d	2.61

Concentrations of essential elements in seedlings and emblings

The content of macro- and microelements differed between seedlings and emblings. Higher micronutrients contents were found in seedlings, also Fe contents was higher in seedlings. All these results indicate higher physiological activity of seedlings. But C and N contents were higher in emblings as well as concentrations of K, Ca , Mg and Na.

Protein analysis of seedlings and emblings

No qualitative differences were detected between the protein profiles of seedlings and emblings. In both cases the electroforetic patterns consisted of 11 protein bands of comparable molecular size with major proteins positioned at 55.6 kDa and 26.6 kDa. The protein fraction of 43 kDa size which is considered to be the major storage protein in *Abies* (Jensen and Lixue 1991) was less abundant as contrasted with abundancy of 26 kDa protein which is another storage protein specific for *Abies*. It is obvious that with respect to these characteristics seedlings and emblings share the same quality. In comparison with other developmental stages of somatic embryos, a noticeable increase of the 55 kDa protein was observed in regenerated emblings (Kormuťák et al. 2006).

Utilize of the methods of somatic embryogenesis

Applications of this process include: clonal propagation of genetically uniform plant material, elimination of viruses, can be used in the regeneration of genetically transformed plants, provision of source tissue for genetic transformation, development of synthetic seed technology, metabolite production, also offers unique opportunities as a model system for learning to study embryology.

Our results confirm, that methods of SE process can be used for regeneration of elite fir individuals from primeval (Vooková and Kormuťák 2009). With more or less succes it is possible to obtained fir hybrids from in nature incompatible crossing, where zygotic embryos abort usually several weeks after pollination (Vooková and Kormuťák 2008).

Improved plantlet regeneration of *Abies alba* trees of Dobroč primeval

Somatic embryogenesis was initiated from immature zygotic embryos of *Abies alba* Mill. from open-pollinated families of 4 trees in Dobroč primeval. Totally, two from the primeval families (50%) responded to initiation condition. Initiation frequencies among families ranged in primeval 5.4–16.8%. Maturation ability was shown by 77.3% of the primeval cell lines. Mature cotyledonary embryos were converted into emblings. Regenerants were obtained from 9 cell lines of two Dobroč primeval families (trees).

In summary, SE seems to be convenient supplementary tool for in vitro conservation of primeval forest genetic resources and/or for preservation of elite trees of silver fir by micropropagation. The results of this study indicate that embryogenic potential of immature zygotic embryos is independent on stands but dependent on genotype. Also biochemically, no differences were found between somatic embryos derived from zygotic embryos of silver fir primeval stand and somatic embryos originating from the trees of managed stand.

Somatic embryogenesis in some hybrid firs from artifficial pollination

Methods of SE process can be used for recovering plants from sexual crosses where the majority of embryos cannot survive in vivo. It would be also applied more or less successfully for raising hybrids from a number of incompatible crosses. Embryo rescue has been carried out with a large number of species inluding tree species, but to our knowledge has never been ettempted to conifer.

The present study was aimed at initiation of the somatic embryogenesis from immature seeds of incompatible crossings of fir species: *A. concolor* × *A.weitchii, A. concolor* × *A. alba, A. concolor* × *A. pinsapo, A.nordmanniana* × *A. veitchii, A.nordmanniana* × *A. concolor, A. pinsapo* × *A. veitchii*, *A. pinsapo* × *A. concolor, A. alba* × *A. veitchii*. The number of explants available at the date of collection was limited by the number of developing megagametophytes in a cone. Later when embryo was not present any more in developing seeds and megagametophytes degenerated meanwhile, the seeds were too hard to remove their coats. The induction of embryogenic tissue was rather rare and occurred with frequencies of 0.64–1.6% in *A. nordmanniana* × *A. concolor,* 0.69–3.82% in *A. nordmanniana* × *A. veitchii,* 5.55% in *A. concolor* × *A. veitchii,* 0.64–1.60% in *A. nordmanniana* × *A. concolor* and 1.23% in *A. pinsapo* × *A. veitchii*.

Maturation and development of cotyledonary stage somatic embryos was achieved only in *A. nordmanniana* × *A. veitchii*. Ten cell lines response to maturation treatment. Maturation was observed also in two cell lines of *A. concolor* × *A. veitchii* but they formed only globule-shaped embryos. These cell lines represented group B with undeveloped somatic embryos (Mo et al. 1996; Salajová and Salaj 2005). After partial desiccation the mature embryos of *A. nordmanniana* × *A. veitchii* germinated to small plantlets. But not every line embryos respond to the germination treatment. This step was succesful in six cell lines.

Molecular evidence for the hybrid nature of *A. nordmanniana* × *A. veitchii* cross was based on the paternal inheritance of cpDNA. The presence of 550 bp restriction fragment of *A. veitchii* paternal tree in all the 10 cell lines with maturation response may be considered as evidence supporting their hybridity. But it was not possible to prove the hybrid nature of *A. concolor* × *A. veitchii* cell line. This cell line was of *A. concolor* instead of *A. veitchii* haplotype.

The results of this study indicated that biotechnology is now at a stage when its application in hybridological studies is becoming a reality opening a new perspective for obtaining exceptional hybridological material not only in horticulture but also in forestry.

Acknowledgements

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