

Abies Biotechnology - Research and Development of Tissue Culture Techniques for Vegetative Propagation

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ABSTRACT

The genus *Abies* represents a large group of coniferous species growing in different parts of the world. This review is a compilation of the available literature referring to *Abies in vitro* cell, tissue and organ culture, micropropagation, somatic embryogenesis and transformation. Our data presented within this context cover such aspects of *Abies* biotechnology as initiation of callus, plantlet regeneration via axillary and adventitious buds development and subsequent rooting. Somatic embryogenesis has been regarded as a model for large-scale propagation of tree species through out the world. Also in *Abies*, the best results have been achieved using technics of somatic embryogenesis. The factors affecting induction of embryogenic tissue, somatic embryo maturation and germination are discussed. Emphasis has been given on comparison of soluble and insoluble protein profiles and enzyme activity during zygotic and somatic embryogenesis. Recent experiments concern cryopreservation of embryogenic cultures, genetic transformation and regeneration of transgenic plants.

Keywords: biochemical aspects, cryopreservation, organogenesis, somatic embryogenesis, transformation

Abbreviations: ABA, abscisic acid; BAP, 6-benzylaminopurine; GD medium, Gresshoff and Doy (1972) MCM medium, Bornman and Jansson 1981; MI, myo-inositol; MS medium, Murashige and Skoog (1962); NAA, α -naphthaleneacetic acid; PEG, polyethylene glycol-4000; SE, somatic embryogenesis; SH medium, Schenk and Hildebrandt (1972); TDZ, thidiazuron

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INTRODUCTION

The genus *Abies* includes about 40 species of trees widely scattered through the forests of North and Central America, Europe, Asia and Northern Africa (Harlow and Harrar 1958). Trees growing in southern latitudes are usually restricted to the upper slopes of mountains, those in boreal forests are largely confined to regions of relatively low elevations. With special references to the Mediterranean firs, they represent a group of species that are closely related genetically and which occupy disconnected and sometimes limited areas around the Mediterranean and in the central Europe. Their natural ranges may be found at the 400 m altitude already up to 2400 m. These may suffer from severe summer drought but receive abundant precipitation during the autumn and spring. They can be found on calcareous or non-calcareous soils, but develop best on deeper acid soils with high water reserves. In relation to a possible increase in drought linked to a temperature increase, there is a great risk that the present ranges of the Mediterranean firs will

decrease in the lowest zones of their range, but also in other zones characterized by southerly aspects and shallow soils. There is also the risk of a possible increase in late frost damages and water stress effects. The replacement of more hydrophilous *Abies* species with those of higher aridity index seems to be necessary in case of climate change (Ausencac 2002). Artificial pollination approach based on interspecific hybridization of the Mediterranean firs is looked upon as a powerful means of extending their genetic variability and subsequent increasing of their adaptive potential (Greguss 1988). In combination with somatic embryogenesis it may provide solution for the large-scale propagation of these hybrids.

IN VITRO STUDIES, MICROPROPAGATION

Callus culture

The first study to induce callus culture in the genus *Abies* was published 73 years ago (Gautheret 1934). Cambial ex-

plants of *A. pectinata* were cultured on Knop's medium with 1% glucose and 2% mannitol and formed callus cells which were described cytologically. Later, using medium supplemented with growth regulators, callus was initiated from the cambium and parenchyma of the branches of *A. sibirica*, *A. nephrolepis* mature trees (Bytchenkova 1963) and the cortex of one-year old shoots of *A. grandis* mature trees (Harvey and Grashman 1969). But callus cultures survived only for a few months. Data from several years cultivated *Abies* sp. calli were published much later. Callus cultures of *A. alba* and *A. concolor* × *A. grandis* were obtained from embryos as well as from hypocotyl and radicle segments of seedlings on SH medium (Schenk and Hildebrandt 1972) supplemented by casein hydrolysate (1000 mg/l), glutamine (500 mg/l) and with 1 mg/l each of 6-benzylaminopurine (BAP) and α -naphthaleneacetic acid (NAA) (Gajdošová and Vooková 1991). Explants were cultured in the dark or on the fluorescent light ($110 \mu\text{mol m}^{-2} \text{s}^{-1}$ and 16 h photoperiod) at $25 \pm 0.5^\circ\text{C}$. Formation of green, yellow-green and pink calli with white areas of callus was observed when explants were cultured on the light. Cell cultured in the dark were yellow-white with more intensive growth. Recently, Tang and Newton (2005) published differentiation of adventitious shoots from *A. fraseri* and *A. nordmanniana* callus cultures but they could not succeed in regenerating plants.

Culture of vegetative buds

During the 1970s, research began to focus on morphogenesis and organogenesis *in vitro*. In 1973, Chalupa and Durzan studied growth and development of dormant buds of *A. balsamea* (15-20 years old) and some other conifer species *in vitro*. Modified Risser and White medium (1964) with 109 mg/l ammonium chloride promoted growth, while with L-asparagine and L-glutamine in concentration 280, 560 and 1120 g/l not. Indole-3-acetic acid (IAA), NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), β -naphthoxyacetic acid (NOAA), gibberellic acid (GA_3) and kinetin at 0.1, 1.0, 10, 100 ppm did not stimulate growth. Bonga (1977) used terminal vegetative buds of adult trees of *A. balsamea* (15-20 years old) in his experiment. Embryonic shoots (buds without bud scales) were soaked in water as well as water with 1000 mg/l indole-butyric acid (IBA), N-dimethylaminosuccinamic acid (Alar-85), or 1-phenyl-3-methyl-5-pyrazolone (PPZ) singly or in combination with 100 mg/l caffeic acid. The soaked embryonic shoots formed new apical buds, adventitious dwarf needles or shoots, and root- and embryo-like structures on nutrient medium. The effects of season, forcing and cold storage on morphogenesis in *in vitro* cultures of *A. balsamea* vegetative shoots was observed by Bonga (1981). Koblíha *et al.* (1991) cultured vegetative buds of hybrid *Abies* (*Abies alba* × *A. alba*, *A. cephalonica* × *A. cephalonica*, *A. cephalonica* × *A. cilicica*) on WPM medium (Lloyd and McCown 1980) with BAP and NAA each at concentration 0.02 mg/l. Buds developed into shoots, but rooting was not achieved. Donor plant, genotype and physiological state on growth and vitality of plants were observed to have an effect.

All authors cultured vegetative buds on the light (continuous or 16h/d light at $110\text{-}180 \mu\text{mol m}^{-2} \text{s}^{-1}$) at temperature $21\text{-}25^\circ\text{C}$. The effect of these condition on growth and development of buds was not studied.

Embryonic explants

Only limited information is available on organogenesis in the genus *Abies*. Initiation of adventitious buds was observed by Erdelský and Barančok (1986). They cultured mature zygotic embryos of *A. alba* *in vitro* on MS medium (Murashige and Skoog 1962) with 1 or 2 mg/l BAP and 0.01 mg/l NAA. Adventitious buds surrounded by callus were formed on embryos but their development stopped. Zygmont and Schwarz (1987) initiated induction of adventitious buds on *A. fraseri* zygotic embryos. *In vitro* propagation of *A. fra-*

seri from embryonic explants was published by Saravitz and Blazich (1991). Adventitious buds developed on hypocotyls and on hypocotyls of cotyledon-hypocotyl explants of germinating seeds, but rarely on cotyledons. Elongated adventitious shoots were placed on media containing 0, 5, 10, 20, or 40 mg/l IBA. Forty-eight percent of shoots treated with 40 mg/l IBA rooted. Acclimation of the plantlets was unsuccessful. Histology of *in vitro* adventitious bud development was studied by Saravitz and Blazich (1993). In 1995, Kulchetski *et al.* described a protocol for the *in vitro* propagation of *A. amabilis*. Over 60% of cotyledonary explants from 5-day-old germinating embryos formed adventitious shoots cultured on SH medium with 2.2 mg/l BAP followed by another 7 days on SH medium containing 2 mg each of BAP and zeatin. Shoot multiplication was unsuccessful, but 17% rooting was obtained. Bud induction and shoot formation was successful on cotyledons of *A. sibirica* seedlings (Momot 1988).

In our laboratory, adventitious shoots have been induced on embryos and on the cotyledons of 7 d-old seedlings of the hybrid *A. concolor* × *A. grandis* (Vooková *et al.* 1989; Vooková and Gajdošová 1992). The most efficient cytokinin for adventitious bud induction was BAP at 5 mg/l. But further bud development was successful when BAP was used in combination with 0.01 mg/l NAA. Shoot growth was achieved on basal medium to which 14 mg/l spermidine was added.

Somatic embryogenesis

Somatic embryogenesis (SE) seems to be a very convenient method for mass multiplication of conifers, including the genus *Abies*. The technology to initiate, maintain, and develop somatic embryos and emblings (regenerants from somatic embryos) of conifer species via SE is now well established. SE in *Abies* with limited success or successful regeneration was reported for nine pure species: *A. alba*, *A. balsamea*, *A. cephalonica*, *A. cilicica*, *A. concolor*, *A. fraseri*, *A. nordmanniana*, *A. numidica* and *A. lasiocarpa*.

The first report about the initiation of SE in *Abies* dates back to 1988. In *A. alba* embryogenic tissue was initiated on SH medium contained 2% sucrose with 1 mg/l BAP from immature zygotic embryos (Gebhardt *et al.* 1988). To promote somatic embryo maturation, the influence of different media components on different developmental stages was studied and the formation of late cotyledonary embryos was achieved (Schuller *et al.* 2000). The most successful was maturation on SH medium contained lactose (6.8%) + sucrose (9.9%) with BAP (0.2 mg/l) and ABA (1 mg/l) Advanced maturation was achieved using SH pre-maturation medium. Also mature zygotic embryos of *A. alba* yielded embryogenic cultures from which plantlets could be regenerated (Hristoforoglu *et al.* 1995). Modified MCM medium (Bornman and Jansson 1981) containing 5 mg/l ABA in combination with 3.8% maltose was used for maturation of somatic embryos. Plantlets with a root developed on MCM medium with 1% sucrose and 1.8 % maltose. Zoglauer and Reuther (1996) compared the effect of different PGR treatments on the initiation of SE on mature embryos of *A. alba*. Embryogenic suspension cultures of *A. alba* were established using embryogenic tissue originating from zygotic embryos in immature seed explants and cultured on SH liquid medium with 0.2 mg/l BAP and 2.3% lactose. The protoplasts isolated from suspension culture were immobilized in alginate layers. The formation of proembryos succeeded when subcultured with modified SH liquid medium. Light, enhanced sugar concentration to 3.3% and addition of 1 mg/l ABA led to formation of green torpedo-shaped somatic embryos after 6-8 weeks from protoplast isolation (Hartman *et al.* 1992). In 1998 Roth and Schmidt initiated embryogenic cultures from cotyledon explants of 5 to 10-d-old zygotic seedlings of *A. alba*, but no plantlet regeneration occurred.

A. nordmanniana regenerants were obtained when embryogenic tissue was induced from immature (Nørgaard

and Krogstrup 1991) and mature (Nørgaard and Krogstrup 1995) zygotic embryos. Embryogenic tissue and plantlet regeneration has been achieved in *A. cephalonica* by culture of megagametophytes containing immature zygotic embryos (Krajňáková and Haggman 1997).

SE was also studied in American species of *Abies*. In *A. balsamea*, SE was initiated from mature zygotic embryos. Somatic embryos were readily germinated on medium lacking plant growth regulators (Guevin *et al.* 1994). Initiation and limited proliferation of *A. fraseri* embryogenic tissue was achieved using immature zygotic embryos (Rajbhandari and Stomp 1997). Induction of SE from mature zygotic embryos of *A. fraseri* was reported by Guevin and Kirby (1997) who could obtain mature and germinate somatic embryos at a low percentage (1%). Immature zygotic embryos have been used for initiation of SE in *A. concolor* resulting in 71.1% plantlet regeneration (Vooková and Kormuťák 2004).

In our laboratory, regenerants of *A. numidica* (Vooková and Kormuťák 2002) and *A. cilicica* (Vooková and Kormuťák 2003) were obtained from embryogenic tissue initiated from immature zygotic embryos. In 2005, Kvaalen *et al.* published a study on SE initiated from immature zygotic embryos of subalpine fir *A. lasiocarpa*. The study resulted in a proficient system for production of plants from somatic embryos.

Embryogenic cultures of hybrid firs have been derived from immature (*A. alba* x *A. alba*, *A. alba* x *A. nordmanniana*, Gajdošová *et al.* 1995; *A. alba* x *A. cephalonica*, *A. alba* x *A. numidica*, Salajová *et al.* 1996; *A. cilicica* x *A. nordmanniana*, Vooková and Kormuťák 2003) and mature (*A. alba* x *A. cephalonica*, Salajová and Salaj 2003/2004) zygotic embryos. After maturation on medium with 10 or 20 mg/l ABA cotyledonary embryos developed and germinated into small plantlets. Embryogenic tissue was also initiated on cotyledon explants dissected from seedlings of hybrid fir *A. alba* x *A. cephalonica* (Salajová and Salaj 2001).

Most of the experimental conditions favouring SE can be generalised and used for most *Abies* species but sometimes optimization for individual species is necessary (Vooková and Kormuťák 2004). A schematic illustration of the SE pathway in *Abies* based on our experiences and results is given in Fig. 1. Developmental stage of zygotic embryo used as an explant is important for the induction of SE. In most cases, the initiation frequencies of embryogenic tissue were higher when zygotic embryos were in the precotyledonary or early cotyledonary stage. For induction of SE, proliferation of embryogenic tissue, somatic embryo maturation and desiccation the cultures are incubated in darkness at temperature 21-23°C (Nørgaard and Krogstrup 1991; Salajová *et al.* 1996; Vooková and Kormuťák 2003; Kvaalen *et al.* 2005). Unlike other genera of the *Pinaceae*, *Abies* requires only cytokinin for induction of embryogenic tissue from zygotic embryos (Schuller *et al.* 1989; Nørgaard and Krogstrup 1991). The culture media did not significantly affect initiation frequencies of *A. lasiocarpa* embryogenic tissue, but subsequent growth and culture survival was dependent on the culture medium. Large loss of cultures on the SH medium was possibly related to the lower concentration of one or more microelements or low concentration of Ni (Kvaalen *et al.* 2005). Organic supplement (1000 mg/l casein and 500 mg/l glutamine) improved the proliferation rate as well as the maturation and vitality of somatic embryos. Culture of *A. balsamea* showed high rates (doubling time of 4.1 days) of proliferation on medium containing 1 mg/l BAP combined with 1.86 mg/l NAA (Guevin *et al.* 1994). A low sucrose concentration (1%) in the proliferation SH medium also favoured the formation of cotyledonary somatic embryos of *A. alba* in the maturation SH medium with ABA supplemented with 6.8% lactose and 0.9% sucrose (Schuller *et al.* 2000). Maturation of fir somatic embryos is promoted by both ABA and carbohydrates in the maturation medium. Maturation medium with 10 mg/l ABA is the most fre-

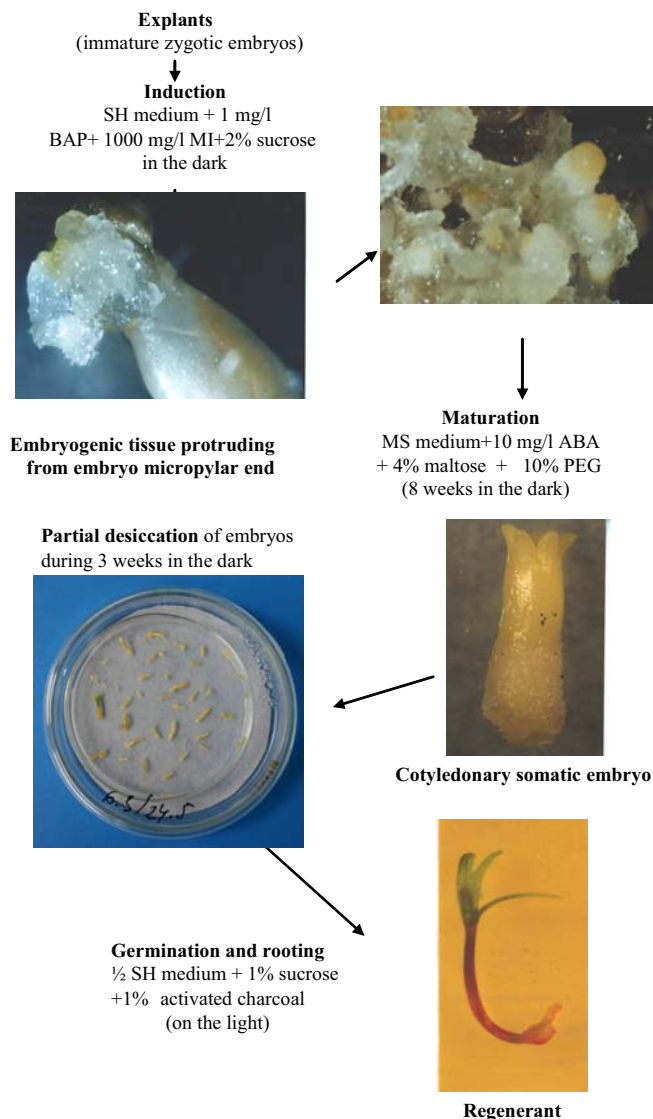


Fig. 1 Scheme of the somatic embryogenesis pathway for *Abies*.

quently used for obtaining cotyledonary embryos (Nørgaard, 1997; Salajová and Salaj 2003/2004). The production of *A. cilicica* and *A. cilicica* x *A. nordmanniana* mature embryos was influenced by ABA concentration, 20 mg/l being the most effective (Vooková and Kormuťák 2003). Lactose and sorbitol, both at concentration 6.8% favoured somatic embryo maturation up to an early cotyledonary stage (Schuller *et al.* 2000). A few *A. alba* hybrid plantlets were regenerated after maturation on medium with 6% lactose (Vooková *et al.* 1997/1998). *A. alba* plantlets with a root, primary needles and terminal bud development could form on maturation medium with 3.8% maltose after preculture of embryogenic tissue on basal medium with 1% sucrose and 1.8% maltose during 1 week (Hristoforoglu *et al.* 1995). 3.4-4.3% maltose gave a better maturation response and the addition of 7.5% polyethylene glycol-4000 (PEG) to maturation medium promoted the maturation of somatic embryos in *A. nordmanniana* and *A. alba* x *A. numidica* (Nørgaard 1997; Salaj *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 (from 3 embryos in control treatment to 23 embryos per g of embryogenic tissue). The most effective maturation occurred when embryogenic tissue was transferred to maturation medium after 14-21 d cultivation on proliferation medium (Vooková and Kormuťák 2002). It seems that the choice of basal medium for somatic embryo maturation is also important. Embryogenic tissues of *A. cilicica*, *A. numidica*, *A. concolor* and *A. cilicica* x *A.*

nordmanniana hybrid were cultured on SH, modified MS and Gresshoff and Doy (1972) maturation media with 4% maltose and 10% PEG. The tendency for better maturation on SH and modified MS medium was general for all tested cultures (Vooková and Kormuřák 2003). The relative concentration of most macroelements and microelements in GD medium is lower than in other media. One of the most beneficial MS medium was enriched with vitamins. Contrary, in *P. taeda*, the yield of cotyledonary embryos was increased when the concentration of B, K and Ca were reduced and concentration of Fe was increased (Pullman *et al.* 2003).

Exogenously applied *myo*-inositol (MI, 100 mg/l) influenced somatic embryogenesis of *A. numidica* although this process occurred on media with and without MI. Maturation on medium containing MI negatively affected somatic embryo germination (Vooková *et al.* 2001). Omitting MI in maturation medium resulted in 68.3% germination of somatic embryos. Maturation and germination on media with MI decreased the yield of regenerants to 30%. The results of Find *et al.* (2002) suggest that endogenously produced auxin may be one reason for low or failing maturation of embryogenic cultures of Nordmanns fir, but also imply that auxin may play a critical role for proper development of cotyledons during later stages of embryo maturation.

Prior to germination, well-developed cotyledonary somatic embryos were selected and subjected to a partial desiccation treatment at high relative humidity according to Roberts *et al.* (1990) for three weeks at 24°C in the dark. Embryos were maintained in open glass petri dish (Ø 60 mm) which was placed on a moist filter paper (with 4 ml water per dish) in other petri dish (Ø 90 mm) (Vooková *et al.* 1997/1998). Kvaalen *et al.* (2005) used Petri dishes with two compartments, one compartment was filled with solidified MgSO₄ and embryos were placed in the other compartment. Media for germination are routinely used with sucrose at 2% with (Nørgaard 1997) or without 1% activated charcoal (Guevin and Kirby 1997). Sixty one percent of *A. alba* plantlets with roots and primary needles developed on basal medium when a combination of 1% sucrose and 2% maltose was provided as a carbon source (Hristoforoglu *et al.* 1995). Results of Nørgaard (1997) indicated that charcoal but not the gelling agent was important for radicle protrusion. The reduction of sucrose concentration to 1% had a positive influence on *A. numidica* embryo germination (Vooková and Kormuřák 2001). The high rooting percentage (85%) was recorded on half SH medium with 1% sucrose and 1% activated charcoal and cultivation on the fluorescent light (110 µmol m⁻² s⁻¹ and 16 h photoperiod) at 25 ± 2°C. It seems that this medium and culture condition are widely applicable, and we used it successfully for germination of other *Abies* sp. and hybrids as well. Different culture conditions were used during germination but effect was not very expressive. *A. alba* somatic embryos were gradually adapted to increasing light levels and than they germinated 4 weeks at fluorescent light (70 µmol m⁻² s⁻¹) and temperature 24°C (Hristoforoglu *et al.* 1995). Incubation of germinated *A. lasiocarpa* embryos was under red light intensity 30 µmol m⁻² s⁻¹ at 22°C (Kvaalen *et al.* 2005). Plants with epicotyls were transferred to pots containing mixture of peat and perlite (2:1, v/v). Micropropagated plantlets of Nordmanns fir, required a period of low temperatures and short days before growth was resumed (Nørgaard 1997).

Secondary somatic embryogenesis

Salajová and Salaj (2001) reported repetitive SE from cotyledon explants of hybrid firs (*A. alba* x *A. cephalonica*, *A. alba* x *A. numidica*) emblings. They observed a similar developmental pattern of somatic embryos as in the primary process. The induction of secondary SE in *A. numidica* was achieved when cotyledonary and desiccated cotyledonary embryos were used as explants (Vooková *et al.* 2003). Pre-

cotyledonary embryos were not able to produce embryogenic tissue. Cotyledonary embryos before desiccation were the most suitable. The most beneficial was SH induction medium with 1 mg/l TDZ (thidiazuron) and 1000 mg/l MI. Initiation frequency ranged from 1 to 34%. Efficiency of this method for improving repetitive SE and plant recovery of Algerian fir was investigated by evaluating the induction frequency, maturation capacity and germination (Vooková and Kormuřák 2006).

Biochemical quality of somatic embryos

A comparative study on zygotic and somatic embryogenesis in conifers has shown that except for morphological similarity there exists also a high degree of biochemical homology between zygotic and somatic embryos (Hakman *et al.* 1990). In particular, it is true of the storage proteins exhibiting similar developmental patterns in both types of embryos (Flinn *et al.* 1991; Hakman 1993). The greater biochemical similarity of somatic embryos with their zygotic counterparts is believed to improve the conversion of somatic embryos to plants (Klimaszewska *et al.* 2004). However, in spite of this generally acknowledged qualitative similarity, the quantitative differences in storage proteins are also postulated to exist between zygotic and somatic embryos. Bornman *et al.* (2001) have for example revealed significantly reduced content of proteins in a dry mass of *Picea abies* somatic embryos as compared with the zygotic embryo-megagametophyte complex. This may be caused by the cellular organization of somatic embryos which were shown to differ in this respect from zygotic embryos of *P. abies* (Salajová *et al.* 1996; Bornman *et al.* 2001). At the enzyme level, considerable differences in peroxidase, esterase and invertase activities were observed in *Picea mariana*, *P. abies* and *A. alba* indicating different metabolic potentials of their zygotic and somatic embryos (Iraqi and Tremblay 2001; Konrádová *et al.* 2002; Kormuřák and Vooková 2006). All these findings refer predominantly to the genus *Picea*, to a lesser degree also to the genera *Pseudotsuga* and *Cupressus*. As far as the genus *Abies* is concerned, we have characterized the processes of zygotic and somatic embryogenesis in terms of storage protein dynamics and enzyme activity in *A. alba*, *A. concolor* and *A. numidica*.

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 female gametophyte and embryos of *A. alba* and *A. concolor* based on soluble protein extraction procedure (Kormuřák 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 (Kormuřák *et al.* 2005). In the light of these findings, a species-specific profile of zygotic embryo proteins may be assumed in *Abies*. Paralleled cytological investigations revealed a dramatic increase of protein synthesis at the stage of cellularized female gametophyte and in embryos with differentiated cotyledons. The nutritive function of individual fractions has been suggested according to their rapid degradation upon the onset of germination. All 9 proteins of interest were found to be mobilized in the female gametophytes of *A. concolor* sharing the function of storage proteins. The same was true of the protein components of *A. concolor* embryos. The only exceptions were the 24 kDa protein of the female gametophyte and the 16 kDa protein of embryos which were synthesized at a constant rate during the early stages of seed germination. It is worth mentioning that these 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 whose individual components may be traced as early as the non-embryogenic callus. The deviating nature of soluble protein profile of regenerated emblings is due to either partial or incomplete depletion of the corresponding storage reserves during somatic embryo germination. A noticeable increase of the 55 kDa protein has however been observed in regenerated emblings of *A. concolor* indicating the renewal of biosynthetic processes in developing emblings. In *A. numidica* this aspect of embling development is even more conspicuous as evidenced by the enhanced synthesis of the new proteins of 26 and 55 kDa (Kormuťák *et al.* 2006; Vooková and Kormuťák 2006).

At the enzyme level, a higher peroxidase activity is registered in both somatic embryos and regenerated emblings of *A. alba* than in dormant and germinating zygotic embryos of the species reflecting profound differences in metabolic activities of the compared embryo types.

Specific activity of the enzyme ranged during early cotyledonary stage of zygotic embryos until physiologically mature embryos between 0.08 and 0.25 units as compared with the 0.11–1.83 level of peroxidase activity detected during somatic embryogenesis of the species. A higher metabolic potential of mature somatic embryos was ascribed to the different levels of dormancy which seem to be lower in somatic embryos than in zygotic embryos (Kormuťák and Vooková 2006).

In comparison with proteins and isoenzymes, the DNA-based molecular markers have not been applied in *Abies* in vitro cultures yet. Among the conifers, the extent of somaclonal variation using RAPD and RFLP has been assessed in tissue cultures and primary regenerants of 3 *Picea* species and 2 *Larix* species only (Fourré 2000). Intracloonal polymorphism was observed during or after *in vitro* culture of *Picea abies* (Chandelier *et al.* 1996), *Picea glauca* (Isabel *et al.* 1996) and *Larix* species (de Verno *et al.* 1994). In *Pinus taeda* the quality of mature somatic embryos was examined using gene expression at the mRNA level and with respect to some morphological and physiological parameters (Pullmann *et al.* 2003).

In conifers this class of molecular markers is preferentially used in scoring the genetic variation at the species and population levels. Using PCR-RFLP analysis of the chloroplast DNA (cpDNA) Parducci and Szmidt (1999) found the species-diagnostic markers for the four of the ten investigated Mediterranean and North American species of *Abies*. The same approach has also been applied by Isoda *et al.* (2000) in the phylogenetic study on 3 Mediterranean, 6 North American and 9 Asian species of firs. The sequence analysis of the 42-kb inversion region of cpDNA revealed polymorphism among 5 *Abies* species native to Japan (Tsumura *et al.* 2000). At the population level, it was the finding of variation in the intergenic spacer region of the *trnS* and *psbC* genes of the cpDNA in *Abies alba* individuals which opened the way for analysis of the species population structure across its habitat (Ziegenhagen and Fladung 1997). All these findings enhanced considerably the breeding of firs, identification and control of the origin of forest reproductive material (Ziegenhagen *et al.* 2005).

Cryopreservation

Over the past decades, plant cryopreservation technologies have been evolving rapidly, opening the door to the possibility of long-term storage of valuable genetic resources

of many crop and forest species (Panis and Lambardi 2005). Embryogenic cultures are cryopreserved to maintain their juvenility and regeneration ability. Variation among *A. nordmanniana* cell lines to survive freezing has been documented by Nørgaard *et al.* (1993). Embryogenic cultures were cryopreserved after preculture in 7.3% sorbitol and pretreatment with 5% dimethyl sulfoxide (DMSO). Only a few meristematic cells in the embryo heads survived. Following an initial lag-phase, growth resumed and complete embryos were formed by surviving cells. There was apparent relation between cryotolerance and good regeneration ability of embryogenic masses. Only one of five genotypes resumed growth at same rate as before cryopreservation. Misson *et al.* (2006) developed simplified cryoconservation technique for *A. nordmanniana* somatic embryos. Chemical treatment (sucrose and sucrose/DMSO/cold +4°C) did not affect the growth of the lines tested. The cold-treated embryos of the reference lines were able to regenerate after this treatment. Aronen *et al.* (1999) applied cryopreservation for the embryogenic cultures of open-pollinated *A. cephalonica* and tested the genetic fidelity to the cryopreserved cell lines using random amplified polymorphic DNAs i.e. RAPD markers. DMSO, two mixtures of polyethylene glycol, glucose and DMSO (PGD I or PGD II) used as cryopreservant combined with slow cooling and fast thawing, proved to be applicable for embryogenic tissue. The best results, measured as survival percentage and mean growth ratio were achieved using PGD I treatment. Their results show that cryoprotectants may cause a risk for genetic fidelity of plant material and thus, its continual monitoring is of importance. The RAPD assay performed revealed considerable genetic variation in background variation in the DMSO treated but non-frozen samples, i.e. 16.8% of the produced RAPD profiles showed intracloonal variation while background variation was seen in 1.7% of the control amplifications.

Genetic stability of *in vitro* cultures

To be genetically stable is very important for successful mass cloning. But some cases of somaclonal variation have been reported for embryogenic cultures of *Larix* sp. (de Verno *et al.* 1994), *Picea abies* (Fourré 1985) and *Picea glauca* (Isabel *et al.* 1995).

Karyological analysis of 5-10 month cultured callus of *A. concolor* x *A. grandis* induced from seeds of *A. alba* and *A. concolor* x *A. grandis* on MS medium containing 2 mg/l BAP and 2 mg/l NAA showed that cells contained predominantly diploid chromosome number. Karyological changes occurred in a maximum of 10.8% of mitotic cells. The most frequent change was polyploidy. Polyploid cells occurred isolated, the tissue showed no tendency towards polyploidization. Less frequently observed deviation were anaphases with lagged chromosome and haploidy (Gajdošová and Vooková 1991). Stable diploid chromosome number was observed also in 3-year old calli of *A. alba*, *A. concolor* x *A. grandis* and *A. grandis* x *A. concolor* and 1-year old calli *A. grandis*, *A. concolor* obtained from hypocotyl segments cultured on SH medium with 1 mg/l BAP and 1 mg/l NAA (Libiaková and Gajdošová 1993). Karyological changes ranged from 2 to 7%. The deviations which occurred in mitotic cells were polyploidy and haploidy. The tissues showed no tendency towards polyploidization. The genotype and the age of calli did not influence the changes in chromosome number. The extent of somaclonal variability in adventitious and axillary shoots regenerated from mature embryos and cotyledons of seedling and in 3-year old calli of *A. concolor* x *A. grandis* was studied (Libiaková *et al.* 1995). The results of karyological analysis demonstrated remarkable stability of regenerated shoots mainly of axillary shoots where no deviations from the normal number were found. Among the cells observed in adventitious shoots only 1.4% cells were tetraploid. Stable diploid (2n = 24) chromosome number was found in 97% of dividing cells. These results were confirmed also by

Table 1 Summary of various studies conducted on transformation of *Abies* sp.

Author	Species/explant or culture	Strain, used plasmid/method used	Marker genes	Selective agent concentration	Transformation frequency	Detection method of transgene
Gajdošová et al. 1993	<i>A. concolor</i> x <i>A. grandis</i> <i>A. alba</i> /hypocotyls of seedlings	<i>A. tumefaciens</i> strain A281 infection		cephotaxime/ 500 mg/l	25.7% in <i>A. concolor</i> x <i>A. grandis</i> , 4.3% in <i>A. alba</i>	opine analysis (Ottenand Schilperoot 1978)
Jens et al. 2005	<i>A. nordmanniana</i> / embryogenic tissue	pCW122 bombardment	<i>uidA</i>	geneticin/ 15 mg/l	4.7%	histochemical assay (Jefferson 1987)/ELISA test/southern hybridization
Salaj et al. 2007	<i>A. alba</i> x <i>A. numidica</i> , <i>A. alba</i> x <i>A. cephalonica</i>	<i>A. tumefaciens</i> strain AGL0/ cocultivation	<i>uidA</i>	geneticin/ 10 mg/l		histochemical assay (Jefferson 1987)/PCR analysis

flow cytometry analysis of nuclear DNA content.

Embryogenic cultures of *Abies* sp. seems to be genetic stable but organic supplement in the medium can affect this stability during long term cultivation. Genetic stability of embryogenic cultures of *A. alba* induced from immature zygotic embryos was documented by Schuller et al. (1989). Chromosome counts in 25 young somatic embryos of *A. alba* revealed all as being diploid ($2n=2x=24$). The flow cytometric analysis of single embryogenic lines suggests that no changes in ploidy levels occurred during induction and 2 years' cultivation of *A. alba* embryogenic tissue initiated from immature zygotic embryos on SH medium with 1 mg/l BAP supplemented with 1000 mg/l casein hydrolysate and 500 mg/l l-glutamine (Gajdošová et al. 1995). Karyological studies of 6-year old embryogenic cell line of *A. alba* obtained from mature zygotic embryo revealed that all cells cultivated 3 years on medium without organic nitrogen, then 3 years with supplement of organic nitrogen (500 or 1000 mg/l casein hydrolysate and 500 mg/l L-glutamine) were trisomic and morphological changes such as malformation of the suspensor cells and loss of maturation capacity occurred (Roth et al. 1997). Florescent-banding methods and comparison with aneuploid cell line showed that the additional chromosome belonged to the group of long, metacentric chromosomes of *A. alba* without secondary constriction. Those cells cultured on medium not supplemented with organic nitrogen retained stable chromosome number of $2n=24$. The emergence of aneuploidy within one cell line could be consequence of high selection pressure caused by the different culture conditions.

Genetic transformation

Genetic transformation can be defined as a controlled introduction of exogenous genetic material into the nuclear or cytoplasmic genome of an organism in stable and inheritable manner (Diouf 2003). Summary of various studies conducted on transformation of *Abies* sp. is recorded in **Table 1**. Seventeen-day old seedlings of *A. alba* and *A. concolor* x *A. grandis* were inoculated by *Agrobacterium tumefaciens* strain A281 which contains TI plasmid (T-DNA) from wild strain *A. tumefaciens* Bo 542 with chromosome background of strain C 58 (Gajdošová et al. 1993). Infected seedling were cultured on MS medium with 500 mg/l cefotaxime to prevent growth of bacterium. Tumors formed on hypocotyls of 25.7% of hybrid fir and 4.3% of silver fir. After 4 months of culture the tumors were separated from plants and cultured on MS medium without growth regulators. Some of the tumors necrotized but 9.9% tumors from *A. concolor* x *A. grandis* and 1.4% from *A. alba* showed intensive growth on medium without growth regulators during 2 years. The presence of agropine in tumors was proved by opine analysis according to Otten and Schilperoot (1978). In recent years the embryogenic tissue of several conifer species have been included in genetic transformation experiments. Stable genetic transformation of *A. nordmanniana* embryogenic cultures was achieved using Biolistic® transformation followed by regeneration of transgenic plants (Find et al. 2005). Selection of the transgenic tissue was based on the antibiotic resistance induced by the neomycin phosphotransferase II gene (*nptII*) in combination with anti-

biotic geneticin. Six transclones were recovered from a total of 215 bombardments. Expression of the β -glucuronidase gene (*uidA*) was confirmed by histological analysis. Both genes were still expressed in the embryogenic tissue after 5 yr of *in vitro* culture and mature somatic embryos and plants produced from these cultures. The integration of *nptA* was confirmed by Southern hybridization after 5 yr of culture. After 5 yr of growth, *uidA* was still expressed in needles from transformed trees. Complete silencing of *npt II* was not observed in embryogenic tissue from any of the six transclones after 5 yr cultivation.

Genetic transformation of embryogenic tissues of hybrid firs by 48 h co-cultivation with *A. tumefaciens* strain AGL0 was presented by Salaj et al. (2007). Expression of *uidA* gene in the embryogenic tissues of *A. alba* x *A. cephalonica* and *A. alba* x *A. numidica* was achieved. Plasmid the binary vector pTS2 was used in experiments, which is binary vector pBinPLUS. Histochemical GUS assay conducted according to Jefferson (1987) and PCR analysis were used for determination of expression. Mature cotyledonary somatic embryos were GUS-positive and regeneration of plantlets was achieved.

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