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BOZENA VOOKOVA

ANDREJ KORMUTAK

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Research Article

Plantlet Regeneration in *Abies cilicica* Carr. and *Abies cilicica* x *Abies nordmanniana* Hybrid via Somatic Embryogenesis

Božena VOOKOVÁ, Andrej KORMUŤÁK
Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Nitra, Slovak Republic

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Abstract: Somatic embryogenesis was initiated from immature zygotic embryos of *Abies cilicica* Carr. and its hybrid *A. cilicica* x *A. nordmanniana*. Schenk and Hildebrandt medium (SH) supplemented with 5 µM benzylaminopurine was used as the initiation medium. In *A. cilicica*, the initiation of embryonal suspensor mass (ESM) frequency ranged from 5.4 to 63.5%, and 28.6% of these cell lines formed mature somatic embryos. In *A. cilicica* x *A. nordmanniana*, from 3 to 27.6% of zygotic embryos formed ESM, and maturation of somatic embryos was observed in 34.8% lines. For somatic embryo maturation, Murashige, Skoog and SH media supplemented with 4% maltose and 10% polyethylene glycol-4000 were used. For maturation, 80 µM abscisic acid was most effective. After three weeks of partial desiccation, mature embryos germinated on SH medium with 1% sucrose and 1% activated charcoal, and plantlets with cotyledons, hypocotyls and radicles were obtained.

Key Words: Conifers, maturation, germination, plantlet regeneration

Introduction

The diversity and the extent of the world´s forests are declining, yet the demand for wood worldwide is expected to double in the 21st century. To accommodate this demand, the productivity of the remaining forest lands will have to be increased, while other areas are set aside for conservation. Advances in biotechnology will accelerate tree improvement. In particular, somatic embryogenesis offers new ways for faster multiplication of high-value clones for reforestation, which will help in the race to increase forest productivity (Gupta et al., 1996).

One important coniferous species in Central Europe is *Abies alba* Mill. This species is sensitive to drought and other environmental stresses, and is one of the most damaged tree species (Krehan, 1989). The rescue of *A. alba* may be possible by means of intra- and interspecific hybridization in order to extend its genetic variability. *Abies cilicica* Carr. is a fast growing species whose natural distribution is in Asia Minor (Bozkuş, 1987). Because of its fast growth, the species is recommended for introduction to the climatic conditions in Slovakia (Tokár, 1973). However, as pointed out by Lapin (1973), of no less importance is the ability of introduced species to intercross with other species of a particular region. In our field studies on artificial hybridization, the *A. cilicica* x *A. nordmanniana* hybrid form appeared very promising.

The induction of somatic embryogenesis in the Abies Mill. genus has been demonstrated in four pure species, *A. alba*, *A. nordmanniana*, *A. fraseri* (Pursch) Poir. and *A. balsamea* (L.) Mill., (for review see Norgaard and Krogstrup, 1995) and several hybrids, *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). Despite our knowledge of the somatic embryogenesis of *Abies* sp., reports on plant regeneration are rare (Guevin et al., 1994; Hristoforoglu et al., 1995; Norgaard, 1997; Salajová & Salaj, 2001).

The objective of this research was to investigate the possibility of somatic embryogenesis initiation, somatic embryo maturation and plantlet regeneration in *A. cilicica* and its hybrid.

Materials and Methods

An artificial pollination experiment was carried out in Arboretum Mlyňany, Slovakia, using one mother tree of cilician fir (*Abies cilicica* Carr.) and one father tree of Caucasus fir [*Abies nordmanniana* (Stev.) Spach]. Female flowers of *A. cilicica* were isolated before opening their scales using paper bags as isolators. Artificial pollination of female flowers was performed at the stage of their maximal receptivity at the beginning of May, using freshly collected pollen of *A. nordmanniana*. Except for the

interspecific controlled pollination, a small portion of female flowers were self-pollinated, serving as a control for the interspecific crossing *A. cilicica* x *A. nordmanniana*. The isolators were removed from female flowers after pollination.

Cones containing immature seeds of *A. cilicica* Carr. from self-pollination as well as from the interspecific crosses *A. cilicica* x *A. nordmanniana* were collected at regular intervals during July–August 1997. The availability of explants was limited by the number of developing megagametophytes in a cone (Table I). Immature seeds were surface-sterilized for 10 min in 10% H_2O_2. Endosperms containing embryos (see Fig. 1) (from July 8 to July 24) or embryos after excision from the megagametophyte (from August 5 to August 26) were plated on SH initiation medium (Schenk & Hildebrandt, 1972) with 5 µM benzylaminopurine (BA) and 2% sucrose. The medium was solidified with 0.3% Phytagel. All media components were autoclaved at 121°C for 20 min. The cultures were kept in darkness at 21-23 °C. Embryonal suspensor mass (ESM) proliferated on a medium with 0.05% L-glutamine (GL) supplement and 0.1% casein hydrolysate (CH) and were subcultured every three weeks.

Maturation

To determine whether embryogenic cell lines respond to maturation treatment, all induced cell lines *A. cilicica* (42 lines) and *A. cilicica* x *A. nordmanniana* (23 lines) were subjected to maturation treatment.

Pieces with an approximate weight of 500 mg ESM were transferred to 90 mm plastic Petri dishes containing maturation medium in darkness at 21-23 °C. Petri dishes were sealed with polyethylene film. Two types of treatment were used for somatic embryo maturation:

Fig. 1. Megagametophytes containing immature zygotic embryos plated on initiation medium.

1) modified SH medium used in previous experiments (Vooková et al., 1977/1998), where in the first step ESM was cultured on a medium containing 6% lactose, 10% polyethylene glycol-4000 (PEG-4000) and 40 µM (±) cis-trans-abscisic acid (ABA). After one week of cultivation, ESM was transferred to a medium with 7.2% lactose, 1% sucrose and 40 µM ABA. Media were supplemented with 0.05% GL and 0.01% CH and solidified with 0.3% Phytagel.

2) medium contained basal salts and vitamins of SH medium, 3% maltose, 10% PEG-4000, 0.05% GL, 40 µM ABA, 0.1% CH and 3% Phytagel.

To assess the most beneficial medium for somatic embryo maturation, three cell lines of both *A. cilicica* (50, 91, 98) and *A. cilicica* x *A. cephalonica* (102, 106, 145) were cultured on SH, GD (Gresshoff & Doy, 1972) and modified MS (Murashige & Skoog, 1962) media. SH and GD media contained original macro- and micro-elements, FeEDTA and vitamins. The MS medium contained 1/2

Table 1. Initiation percentage of embryogenic tissue from immature zygotic embryos. The number of explants is in brackets.

	Collection dates				
	July 8	July 15	July 24	August 5	August 26
Explant	Megagametophytes containing immature embryos			Immature embryos	
Species					
A. cilicica	63.5 (75)	11.9 (94)	5.4 (149)	0 (0)	5.7 (88)
A. cilicica x *A. nordmanniana*	-	6.5 (108)	3.0 (108)	27.6 (105)	0 (101)

strength MS macro and original micro-elements and FeEDTA, modified vitamins; 5.5 µM nicotic acid, 3 µM thiamine HCl, 4.9 µM pyridoxin HCl, 13.3 µM glycine and 0.01% *myo*-inositol. All media contained 4% maltose, 10% PEG-4000, CH and GI in 0.5% concentration and 0.3% Phytagel.

The effect of ABA on somatic embryo maturation of selected cell lines was determined by subculture of ESM onto MS maturation medium containing 40 and 80 µM (±) ABA.

In all maturation treatments, ABA was co-autoclaved together with other substances in the media. During maturation cultures were maintained at 21-23 °C. The experiment consisted of 10 replicate plastic plates (Ø 60 mm), each containing ESM of approximately 300 mg.

Germination and plantlet regeneration

After SE maturation on the most beneficial modified MS medium, cotyledonary embryos of the same cell lines used for maturation treatment were used in the germination experiment. Prior to germination, the somatic embryos were isolated, placed in Petri dishes (Ø 60 mm) and subjected to partial desiccation as follows: the Petri dish was open and placed on moist filter paper in a Petri dish (Ø 90 mm), which was sealed with parafilm. Somatic embryos in Petri dishes were cultured in darkness at 22-25 °C for three weeks.

After partial desiccation, mature somatic embryos (with at least four cotyledons) were transferred to germination medium and cultured in the light (16 h photoperiods) at 21-23 °C. The standard medium for germination was SH medium containing 1/2 concentration of basal salts, SH vitamins, 0.01% *myo*-inositol, 1% sucrose and 1% activated charcoal (Darco G 60). The medium was gelled with 0.3% Phytagel. Six replications of 10 embryos were cultivated in an erlenmayer flask with 50 ml medium per treatment under a light intensity of 110 $\mu mol.m^{-2}\ s^{-1}$ for 16 h per day. Germination percentages were evaluated after 40 days of cultivation.

Statistical evaluation of the data was carried out using Student's t-test.

Results and Discussion

Within four to six weeks on initiation medium, the formation of white glossy and mucilaginous ESM was observed from the megagametophytes at the micropilar end (Fig. 2). Immature zygotic embryos of *A. cilicica* showed a relative high frequency formation of ESM ranging from 5.4 (July 24–August 26) to 63.5% (July 8). In *A. cilicica* x *Abies nordmanniana* this was from 3 (July 24) to 27.6% (August 5) (Table I). To our knowledge, the 63.5% frequency of ESM formation in *Abies* has not been achieved elsewhere. Until then the highest induction frequency, 44.6%, had been reported in the *A. alba* x *A. numidica* hybrid (Salajová et al., 1996).

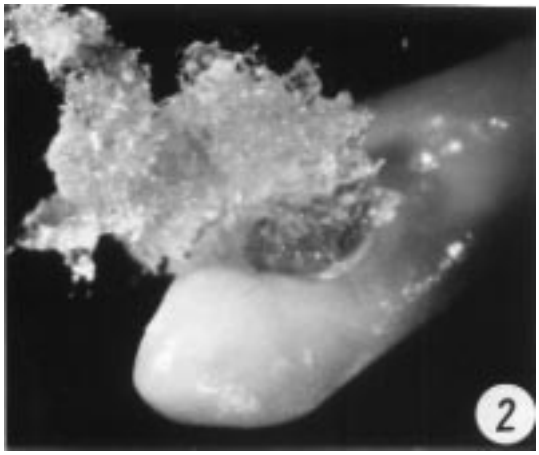

Fig. 2. Initiation of embryogenic tissue after three weeks in culture.

The different tendency was observed in maturation experiments in which 42 cell lines of *A. cilicica* and 23 lines of *A. cilicica* x *A. nordmanniana* were tested (Table 2). Somatic embryos in the cotyledonary stage of development were observed in 28.6% of *A. cilicica* and 34.8% of *A. cilicica* x *A. nordmanniana* cell lines. Somatic embryo maturation was observed in both with lactose and maltose media. It was noted that maturation on medium with lactose gave a higher frequency of globular (Fig. 3) and torpedo-shaped embryos than maltose, but further embryo development was aberrant (abnormal). The duration of maturation treatment was 8-10 weeks. Mature somatic embryos obtained on medium with maltose were yellow to green with cotyledons (1-6) and hypocotyls (Fig. 4). Histological observation of these embryos showed differentiation of the radicula meristem (Fig. 5).

The cell lines differed in their response to the three maturation media. The number of globular and mature cotyledonary embryos per g of ESM was different in individual cell lines (Table 3). The tendency for better

Table 2. Response of tested cell lines on maturation treatment. SE = somatic embryos.

Species	Number of tested lines	Number of cell lines (%) forming globular SE	cotyledonary SE
A. cilicica	42	30 (71.4)	12 (28.6)
A. cilicica x *A. nordmanniana*	23	15 (65.2)	8 (34.8)

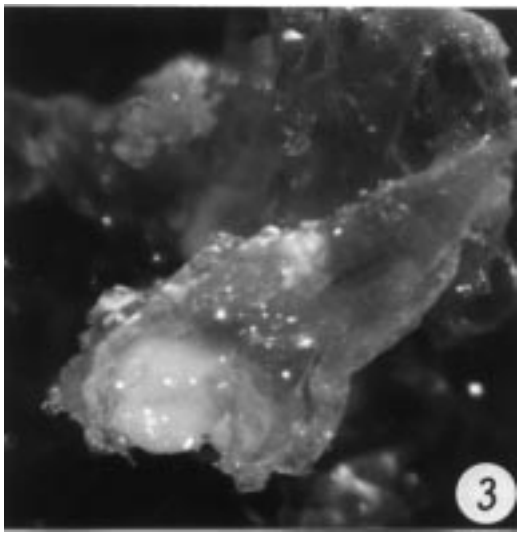

Fig. 3. Developing somatic embryo at the globular stage after three weeks on maturation medium with maltose.

maturation on SH and MS media was general for *A. cilicica* and the *A. cilicica* x *A. nordmanniana* hybrid, but mature embryos on SH medium showed more morphological abnormalities than those on MS medium. GD medium was not suitable because maturation was slow and achieved only a globular stage of development.

The production of cotyledonary somatic embryos was influenced by the ABA concentration (Table 4). The addition of 80 µM ABA (in comparison to 40 µM ABA)

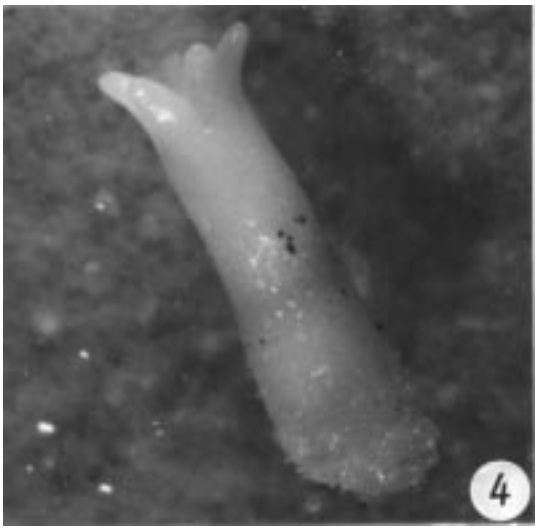

Fig. 4. Cotyledonary somatic embryo after eight weeks on medium with maltose.

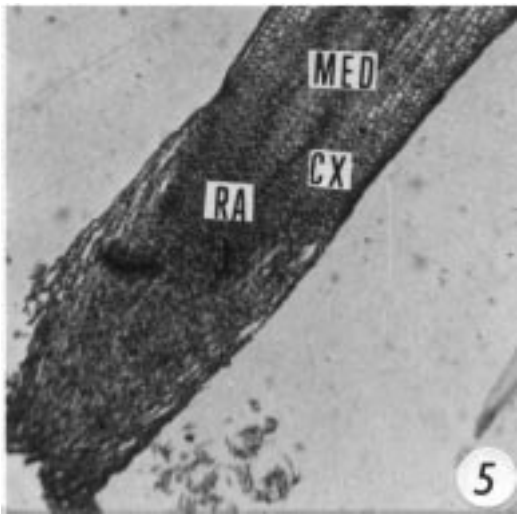

Fig. 5. Longitudinal section of the radicula-end of the cotyledonary embryo before partial desiccation. RA-root apex, MED-medula, CX-cortex.

Cell line	SH		MS		GD	
	Globular embryos	Cotyled. embryos	Globular embryos	Cotyled. embryos	Globular embryos	Cotyled. embryos
A. cilicica						
50	97 ± 13.7	6 ± 1.5	212 ± 12.5	16 ± 1.9	69 ± 9.0	0
91	12 ± 1. 1	3 ± 1.3	7 ± 3.2	3 ± 0.5	0	0
98	40 ± 12.3	12 ± 2.7	23 ± 7.1	6 ± 1.5	29 ± 5.2	0
A. cilicica x *A. nordmanniana*						
102	116 ± 12.6	3 ± 1.3	152 ± 15.3	45 ± 6.6	49 ± 8.5	0
106	44 ± 13.4	3 ± 0.8	71 ± 11.4	9 ± 1.2	11 ± 3.9	0
145	63 ± 3.5	8 ± 1.8	99 ± 11.3	8 ± 1.4	64 ± 10.6	0

Table 3. The number (± SE) of somatic embryos per g of ESM matured on SH, MS and GD media.

Cell line	Globular somatic embryos		Cotyledonary somatic embryos	
	40 µM ABA	80 µM ABA	40 µM ABA	80 µM ABA
A. cilicica				
50	103 ± 9.2	145 ± 17.9	20 ± 0.3	40 ± 1.1
91	7 ± 3.2	24 ± 8.7	3 ± 0.5	18 ± 2.4
98	11 ± 4.2	23 ± 5.3	5 ± 0.6	9 ± 0.8
A. cilicica x *A. nordmanniana*				
102	152 ± 15.3	45 ± 6.6	3 ± 0.6	6 ± 0.4
106	71 ± 11.4	96 ± 10.3	9 ± 1.2	26 ± 0.7
145	99 ± 11.3	29 ± 8.7	8 ± 1.4	27 ± 0.7

Table 4. The effect of ABA concentration on the maturation of somatic embryos cultured on MS medium. The mean number ± SE calculated per g of ESM.

into maturation medium had a very significant ($P < 0.01$) influence on embryo maturation in *A. cilicica* and *A. cilicica* x *A. nordmanniana* cell lines. The production of mature SE was significantly or very significantly different among the cell lines. However, differences in the production of these embryos between cell lines 106 and 145 as well as between cell lines 98 and 102 were not significant. All these results indicate somatic embryo maturation dependent on genotype (cell line) rather than on differences between species. Maturation of somatic embryos of *A. alba* was not observed on media lacking ABA. However, culture on ABA resulted in maturation (Hristoforoglu et al., 1995). Schuller and Reuther (1995) observed that in comparison with the pronounced carbohydrate effect ABA at low concentration (3.78 µM) proved to be of less importance in the maturation of *A. alba* somatic embryos. Exogenous ABA was shown to be necessary for further *Picea abies* (L.) H. Karst. proembryo development where 5-40 µM ABA triggered further development of somatic embryo proembryos. After the administration of ABA, endogenous ABA quickly rose from a very low level, and the increase was dependent on exogenous ABA concentration (Vágner et al., 1998). Maximum numbers of cotyledonary stage *A. fraseri* somatic embryos were observed at 80 µM ABA (Guevin, 1997).

Selected somatic embryos with four to six cotyledons were subjected to partial desiccation. After three weeks of partial desiccation they were germinated readily on medium containing activated charcoal. Mature embryos developed into plantlets with green cotyledons, red hypocotyls and white radicles (Fig. 6). The germination percentage was different among the cell lines but the differences were not significant (Table 5). In *A. alba* 75% of the embryos developed roots (Hristoforoglu et al., 1995) and 62% germination was obtained in *A. nordmanniana* (Norgaard, 1997). In *A. balsamea* somatic embryos germinated at a frequency of 86.6% (Guevin et al., 1994). However, embryos of these species germinated on different germination media in different culture conditions.

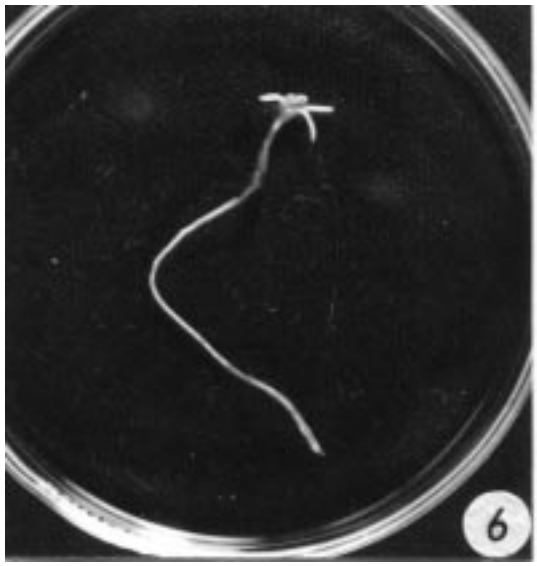

Fig. 6. A plantlet with developed cotyledons, hypocotyl and radicula.

Table 5. Germination of somatic embryos of tested cell lines on germination medium. Means ± SE.

Cell line	Germination (%)
A. cilicica	
50	74.99 ± 6.81
91	94.42 ± 2.71
98	92.13 ± 3.95
A. cilicica x *A. nordmanniana*	
102	99.60 ± 1.03
106	83.61 ± 11.39
145	98.33 ± 1.18

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