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Somatic embryogenesis of the hybrid *Abies cilicica* × *Abies cephalonica*

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Abstract: The interspecific hybrid fir *Abies cilicica* × *Abies cephalonica* is based on native species *Abies cephalonica* (Loud.) and *Abies cilicica* (Ant. et Kotschy) Carr. Many variants of nutrient media have been tested. The medium BAP at a concentration of 1 mg·l^{-1}, 1,000 mg·l^{-1} myo-inositol, 500 mg·l^{-1} glutamine, and 1,000 mg·l^{-1} casein was found to be the most suitable initiation medium for somatic embryogenesis of this hybrid. Embryogenic suspensor mass was produced from immature and mature primary explants. The initiation frequency of ESM was dependent on the composition of nutrient medium, plant growth regulators, and time of seeds collection. The ESM initiation from immature seeds was higher in comparison with mature zygotic embryos, but the results were not significantly different. As the most suitable maturation medium was evaluated the medium based on Murashige and Skoog medium which contained 10 mg·l^{-1} abscisic acid, 100 g·l^{-1} PEG and 40 g·l^{-1} maltose (MM 2 medium).

Keywords: *in vitro* propagation; hybrid *Abies cilicica* × *Abies cephalonica*; embryogenic suspensor mass; ESM; initiation; proliferation

Somatic embryogenesis is one of the progressive methods of plant propagation *in vitro*. In some cases the somatic embryogenesis is favoured over other methods of vegetative propagation (von Arnold et al. 2002). This method is useful not only in practical forestry, but also it provides material for further theoretical investigations of the development process of a somatic embryo, the activity of proteins, examination of physiological processes and responses of plant material to a change in defined and controlled factors.

To initiate the process there must be a suitable concentration of phytohormones in the nutrient medium. Somatic embryogenesis of *Abies alba* (Mill.) was first described by Schuller et al. (1989). In the genus *Abies* the embryogenic tissue was initiated from immature as well as mature zygotic embryos.

Salajova et al. (1996) stated that immature embryos possessed an increased generating capacity of embryogenic mass (ESM) compared to mature embryos.

Salajová and Salaj (2001) attempted to induce the ESM directly from plant parts of hybrids of the genus *Abies*.

Differences in the requirements of particular culture conditions among the species of the genus *Abies* and its hybrids pose greater demands on an individual approach to the optimization of cultivation protocols. Nørgaard (1997) even mentioned that the particular genotype of plant material may be the most important factor determining plant regeneration.

This paper summarizes the results of the various stages of somatic embryogenesis in hybrid fir *Abies cilicica* × *Abies cephalonica*.

MATERIAL AND METHODS

The interspecific hybrid fir *Abies cilicica* × *Abies cephalonica* is based on native species *Abies cephalonica* (Loud.) and *Abies cilicica* (Ant. et

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Table 1. Labelling sources of primary explant

A	mature hybrid seed of *Abies cilicica* × *Abies cephalonica,* year of collection 2006
B	mature hybrid seed of *Abies cilicica* × *Abies cephalonica,* year of collection 2007
C	immature hybrid seed of *Abies cilicica* × *Abies cephalonica,* collected in the end of June 2008

Kotschy) Carr. Seed material obtained by controlled pollination comes from the hybridization of the seed orchard of interspecific hybrids of the second filial generation (♀: *Abies cilicica* × *Abies cephalonica* × ♂: *Abies cilicica* × *Abies cephalonica*) (Kobliha 2000).

Research was proceeded with mature and immature primary explants (Table 1). Mature seeds were collected at the time of their technological maturity.

General laboratory techniques for somatic embryogenesis were carried out according to Gamborg and Phillips (1995).

Sterilization of seed was done according to procedure published by Korecký and Vítámvás (2008).

Nutrient medium

Based on the positive experiences of many authors, e.g. Gajdošová et al. (1995), Vooková et al. (2003), Nawrot-Chorabik (2008), the initiation medium was chosen according to Schenk and Hildebrandt (1972) – also known as the SH medium.

The most frequently used carbohydrate in proliferation medium is sucrose (Hristoforoglu et al. 1995). In maturation media maltose often replaced sucrose. Vooková and Kormuťák (2004) used maltose as the saccharide component.

The different variants of SH medium were modified (Table 2). The pH of the culture medium was adjusted to the value of pH 5.8.

In our experiment development and maturation of somatic embryos was tested on 4 types of media (Table 3).

The basis of maturation media (MM) is the medium according to Murashige and Skoog (1962). These media are labelled as MM 1, MM 2 and MM 3. Medium MM 4 is the medium prepared according to Gupta and Durzan (1985). All media were solidified with 3 g·l^{-1} Phytagel.

Table 2. Composition of initiation and proliferation media (variable components only)

Variable component	Medium						
	SH 600	SH 601	SH 602	SH 603	SH 604	SH 605	SH 606
BAP (mg·l^{-1})	0	0.1	1	5	0	1	5
NAA (mg·l^{-1})	0	0	0	0	1	1	1
Myo-inositol (mg·l^{-1})	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Glutamine (mg·l^{-1})	500	200	200	500	500	500	500
Casein (mg·l^{-1})	500	500	500	1,000	1,000	1,000	1,000
Sucrose (g·l^{-1})	30	30	30	30	20	20	20
Agar (g·l^{-1})	6	6	6	6	0	0	0
Phytagel (g·l^{-1})	0	0	0	0	3	3	3
	SH 607	SH 611	SH 613	SH 614	SH 615	SH 616	SH 617
BAP (mg·l^{-1})	2	0,2	1	5	1.1	1	1
NAA (mg·l^{-1})	0	0,1	0	0	0	0	0
Myo-inositol (mg·l^{-1})	1,000	1,000	1,000	1,000	1,000	1,000	100
Glutamine (mg·l^{-1})	500	500	500	500	500	500	500
Casein (mg·l^{-1})	1,000	1,000	1,000	1,000	1,000	1,000	1,000
Sucrose (g·l^{-1})	30	30	25	25	20	20	20
Agar (g·l^{-1})	6	6	6	6	0	0	0
Phytagel (g·l^{-1})	0	0	0	0	3	3	3

Table 3. Composition of maturation media (variable components only)

Variable component	MS medium			GD medium MM 4
	MM 1	MM 2	MM 3	
ABA ($mg \cdot l^{-1}$)	10	10	20	10
PEG ($g \cdot l^{-1}$)	100	100	100	100
Myo-inositol ($mg \cdot l^{-1}$)	500	500	500	500
Glutamine ($mg \cdot l^{-1}$)	500	500	500	500
Casein ($mg \cdot l^{-1}$)	500	500	500	500
Sugar ($g \cdot l^{-1}$)	sucrose 40	maltose 40	sucrose 40	maltose 40

Media MM 1 and MM 2 differ in their saccharide components: MM 1 contains sucrose at a concentration of 40 $g \cdot l^{-1}$ while medium MM 2 contains maltose in the same amount. Medium MM 3 contains abscisic acid at a concentration of 20 $mg \cdot l^{-1}$, whereas in other media its concentration is 10 $mg \cdot l^{-1}$.

The experiment searching a convenient medium always started on the material which proved zero contamination. Experiments with different types of media were performed in 4 replications. In each series the experiment was established with 20 embryos.

Culture conditions

Explants were cultured in an air-conditioned culture room with a controlled heat and light regime. The initiation, proliferation and maturation phases of somatic embryogenesis were carried out in darkness at 24 ± 1°C.

Movement to a fresh nutrient medium took place depending on the stage of somatic embryogenesis in intervals of 2–8 weeks.

Morphological characteristics of somatic embryos

For the evaluation of qualitative and morphological characteristics, average somatic embryos from the maturation medium were selected. Dimensional measurement of embryos was done using graph paper.

Statistical method

For all collected and statistically analysed values the variance homogeneity test of binomial distribution was used (Hayter 1984).

Statistical analysis was carried out using the Statgraphics program. The hypothesis that the value of the i-th quantity of the characteristics has the same value as the parameter P of j-th quantity at a significance level $\alpha = 0.05$ was tested.

RESULTS

Optimization of initiation nutrient medium composition

The formation of embryonal suspensor mass (ESM) was observed within four to six weeks on initiation medium (Table 4).

The SH 600 medium was used only for control, it contained no plant hormones. It was therefore assumed that on this medium the embryogenic tissue would not begin to form. This assumption was verified on the medium without phytohormones; any ebryogenic tissue were created.

Table 4. Formation of ESM on initiation media

Medium	Formation of ESM (%)
SH 600	0
SH 601	0
SH 602	7.5
SH 603	2.5
SH 604	0
SH 605	5.0
SH 606	2.5
SH 607	0
SH 611	0
SH 613	1.25
SH 614	1.25
SH 615	5.0
SH 616	15

Table 5. Production of ESM – immature primary explant

Explant C	Initiation of ESM (%)
Serie 1	3.0 (16.8)
Serie 2	3.8 (21.1)
Total	3.5 (19.3)

Value in brackets – after taking into account a growth rate

The effect of BAP at the concentration of 0.1 mg·l^{-1} was shown insufficient, the ESM did not start to develop. The concentration 0.2 mg·l^{-1} BAP was also proved inadequate, even though in this medium the growth hormone NAA in the concentration of 0.1 mg·l^{-1} was also present.

The medium with the concentration of 1 mg·l^{-1} BAP, in comparison with the medium containing 5 mg·l^{-1} BAP showed above 5% higher production of embryogenic tissue.

The concentration of glutamine in the media SH 601 and SH 602 was 200 mg·l^{-1}. The content of this substance for all other media was 500 mg·l^{-1}. SH 602 and SH 603 were compared to show the suitability of ESM formation. It was concluded that different amount of glutamine in the nutrient medium did not have any influence on ESM initiation.

The SH 604 medium contained only the growth hormone NAA at a concentration of 1 mg·l^{-1}; the positive effect of the growth hormone NAA on the initiation phase of somatic embryogenesis could not be demonstrated because there was no tissue formation.

The SH 605 medium included the growth hormones BAP and NAA, both at concentrations of 1 mg·l^{-1}. The SH 605 medium had a 5% success of embryogenic tissue formation.

The SH 606 medium, which contained BAP at a concentration of 5 mg·l^{-1} and 1 mg·l^{-1}NAA reached 2.5% success in the formation of ESM.

The SH 614 medium, containing 5 mg·l^{-1} BAP showed 1.25% success in the formation of embryogenic suspensor mass.

The media SH 613, SH 615, and SH 616 were different from each other in the type of stiffening agent and concentration of the saccharide component. In the case of SH 613 medium the stiffening agent agarose was used in a concentration of 6 g·l^{-1}, the other media were reinforced with 3 g·l^{-1} Phytagel. The SH 613 medium contained the saccharide component in a concentration of 25 g·l^{-1}, the other two initiation media contained the saccharide component at the level of 20 g·l^{-1}.

The most suitable initiation medium was SH 616, which achieved 15% success in the formation of ESM.

The used statistical analysis shows significant differences in the formation of embryogenic tissue between the most successful variant, which achieved 15% success in the formation of ESM, and the variants with the success rate of 2.5% and less.

The formation of embryogenic tissue on tested initiation media (primary explants A and B)

The overall effectiveness of the methods of somatic embryogenesis using mature seeds as the primary explant (A and B), taking into account all tested media, is a 1.66% success rate of the formation of ESM. In the case if only SH 616 medium (the most appropriate medium) was taken into the success rate of ESM production was 15%.

Evaluation of ESM formation using immature primary explant C

All trials with immature primary explants were performed on the optimized SH 616 medium.

Table 6. Media number of produced somatic embryos

Labelling of ESM	Source of primary explant	Average number of formed somatic embryos			
		MM 1	MM 2	MM 3	MM 4
ESM 1	C	2.4	4.3	1.3	0.8
ESM 2	C	1.2	8.6	2.3	0.8
ESM 3	A	2.5	14.3	2.5	2.,0
ESM 4	B	1.3	22.0	0.3	0.3
Total		7.4	49.1	6.3	3.8
Fitness of media (%)		15.0	100.0	12.9	7.8

In the case of primary explant C, an average 3.5% success rate of embryogenic mass formation was reached. However, it must be taken into account that the primary explant was the whole unripe seed, and the zygotic embryos were not removed. Kobliha (2008) reported that the hybrid seed of *Abies cilicica* × *Abies cephalonica* had a growth rate of 18%. After taking into account this fact we got an average success rate of 19.3% in the formation of embryogenic mass (Table 5).

Comparison of embryogenic tissue from mature and immature primary explant

The optimized SH 616 medium reached a 15% success rate of embryogenic suspensor mass formation on the primary explant from mature seeds.

In the case of using immature zygotic embryos, the seed C on the SH 616 medium reached 19.3% formation of ESM. Based on the statistical analysis of these two results, it was found that the difference between successful initiations of embryogenic tissue from mature and immature primary explants was statistically significant.

Effect of media on somatic embryo development

The experiment with maturation media MM 1 to MM 4 was carried out on embryogenic tissue from all sources of the primary hybrid explants (A, B, C).

The production of mature somatic embryos was influenced by maturation medium. The highest average number of formed somatic embryos from all sources of primary explants was produced in the maturation medium MM 2.

The results of statistical comparison of different maturation media by methods of statistical analysis confirmed that the maturation medium MM 2 showed a significantly higher effect on the maturation of somatic embryos compared to all other tested media. Differences in the suitability of maturation media MM 1, MM 3 and MM 4 for the maturation of somatic embryos were not significant (Table 6). Characteristics of developed somatic embryos are summarized in Table 7.

DISCUSSION

For the initial phase of somatic embryogenesis the nutrient medium according to Shenk and Hildebrandt (1972) was selected.

The SH medium was tested on the basis of positive experiences of many authors (e.g. Gajdošová et al. 1995; Kulchetscki et al. 1995; Salajova et al. 1996; Vooková et al. 2003). Many variants of modifications, which consisted in changes in the content and concentration of phytohormones, carbohydrate components and other substances, were tested. A total number of the tested media was 13, the SH 600 medium was the control medium without any plant hormones.

The nutrient medium marked SH 616 was evaluated as the most appropriate initiation medium. This medium contained BAP in a concentration of 1 $mg \cdot l^{-1}$ and other variable components such as: 20 $g \cdot l^{-1}$ sucrose and 1,000 $mg \cdot l^{-1}$ myo-inositol.

The most variable component of tested media were plant hormones. Schuller et al. (2000) noted that in the genus *Abies* the initiation and proliferation of ESM could be achieved even if the medium contained cytokinin only, other conifers required auxin as the other component for ESM initiation. The same findings were also presented by Hřib et al. (1997): ESM was initiated on a medium with the addition of BAP in the absence of auxin. Nørgaard and Krogstrup (1991) argued that the presence of auxin in the nutrient medium inhibited initiation and proliferation phases of somatic embryogenesis. These findings correspond with our results. The media include auxin (NAA), showed a lower level of forming tissue mass.

Find et al. (2002) considered the natural production of auxin in seeds as one of the possible causes

Table 7. Average morphological characteristics of developed somatic embryos

Embryo	Medium	Length (mm)	Thickness (mm)	Remark
Zygotic	–	6	2	average length and thickness of normal embryo
Somatic	MM 1	4–6	1.5–3	similar length as a zygotic embryo, but thicker
Somatic	MM 2	< 5	1	generally smaller, but the proportion was the same as zygotic embryo, most similar to ZE
Somatic	MM 3 MM 4	3	2	very similar to each other, embryos achieved only a globular stage of development

of a failure of the maturation of somatic embryos on a maturation medium, this process slowed down or stopped the formation of ESM completely.

The composition of the initiation medium was optimized for the ripe seed material (zygotic embryos were excised from the seed). On this medium (SH 616) a 15% success rate of ESM formation was achieved. This optimized initiation medium was already used for the immature seed.

The effect of myo-inositol on the success of the proliferation of embryogenic mass was tested in the proliferation phase. Vooková et al. (2001) stated that the proliferation medium containing myo-inositol showed a greater increase in ESM formation compared with the proliferation medium without myo-inositol. This difference was significant after approximately three weeks of cultivation of embryogenic tissue on a nutrient medium. These findings did not correspond with our conclusions because higher production of ESM after myo-inositol addition into the proliferation medium was not observed.

Nørgaard (1997) tested the effect of the saccharide component used in the maturation phase of somatic embryogenesis. Tested carbohydrates were sucrose and maltose, the higher number of somatic embryos was produced on nutrient media containing maltose. Vooková and Kormuťák (2002), who used maltose as the carbohydrate component in nutrient media, reported a higher number of somatic embryos in comparison with the medium which contained sucrose.

Schuller and Reuther (1993) tested the effect of fructose, glucose, sucrose and galactose on the formation of somatic embryos. The observations of tissues showed a significant difference between the media with different carbohydrate components, the largest number of somatic embryos developed on maturation medium with the addition of lactose.

In our experiment as the most appropriate maturation medium for hybrid fir *Abies cilicica* × *Abies cephalonica* was estimated the medium with the addition of maltose.

Eight variants of maturation media were tested. The most appropriate maturation medium was MM 2 medium. This maturation medium contained 10 mg·l^{-1} abscisic acid, 100 g·l^{-1} PEG and 40 g·l^{-1} maltose at as the saccharide component. The MM 2 medium is based on the nutrient medium developed by Murashige and Skoog (1962).

The MM 4 medium, which is based on DCR medium by Gupta and Durzan (1985), showed the smallest fitness for the maturation of somatic embryogenesis.

Rajbhandari and Stomp (1997) mentioned June as the optimal time for the collection of primary explants. The highest percentage of embryogenic tissue was initiated on the material obtained in this period.

Kvaalen et al. (2005) conducted experiments with explants from immature seeds collected from late June to late August.

Guevin et al. (1994) and Guevin and Kirby (1997) initiated the formation of embryogenic tissue from mature zygotic embryos. Also Salaj and Salaj (2004) reported that they successfully obtained the embryogenic material of hybrid *Abies alba* × *Abies cephalonica* from a mature primary explant.

Gajdošová et al. (1995) obtained the best results with the induction of embryogenic mass from material collected in the second half of July. The same conclusions were also drawn by Jasik et al. (1999). Salajova et al. (1996) explained that megegametophytes which are not taken out by immature seed may serve as nurse tissue supplying some pant growth regulators foe embyogenic tissue initiation.

In our experiment the formation of embryogenic suspensor mass was initiated from mature and immature (collected at the end of June) primary explants. The statistical method of assessment revealed no significant differences in the percentage of successful ESM formation from these ontogenic different sources.

CONCLUSION

As the most suitable initiation medium was evaluated the SH medium with the addition of 1 mg·l^{-1} BAP, 1,000 mg·l^{-1} myo-inositol, 500 mg·l^{-1} glutamine, and 1,000 mg·l^{-1} casein (SH 616), ESM initiation frequency of 15% was achieved from mature embyos.

The SH 616 medium was optimized on the primary explant from mature seeds (zygotic embryos).

If immature zygotic embryos were used, the 19.3% success rate of ESM formation was reached on the SH 616 medium. Based on the statistical analysis of these two results it was concluded that the difference in the successful initiation of ESM from mature and immature primary explants was not statistically significant.

The most appropriate maturation medium for this interspecific hybrid has been found.

Another possibility for future studies of somatic embryogenesis in the hybrid fir *Abies cilicica* × *Abies cephalonica* is the induction of secondary somatic embryogenesis derived from somatic embryos. Research of this problem was already addressed in a team led by Vooková (2003) or Vooková and Kormuťák (2006).

Kormuťák et al. (2006) also studied the dynamics of proteins and proteomic zygotic and somatic embryos of the genus *Abies*, which is another possibility of continuing research in this field.

Another potential practical application is the possibility of commercialization of an optimized technique of somatic embryogenesis in the hybrid fir *Abies cilicica* × *Abies cephalonica.*

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