DENDROBIOLOGY
2008, vol. 59, 31–40

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Embryogenic callus induction and differentiation in silver fir (*Abies alba* Mill.) tissue cultures

Received: 27 January 2008, Accepted: 23 April 2008

Abstract: The research was conducted on explants of silver fir (*Abies alba* Mill.) deriving from several forest districts in southern Poland. The study encompassed the influence of the origin of plant material, type of explants, kind of substances used for explants sterilization, PPM and the type of medium on the ability to form embryogenic callus and to develop somatic embryos in silver fir explants. From the plant material collected in three sites, 57 clones were obtained from mature zygotic embryos; this produced an embryogenesis frequency of 6%. Embryogenic callus was obtained with a diameter of 65–70 mm depending on the material origin. The best medium for development of callus inducted on embryos isolated from mature silver fir seeds was the SH medium. Somatic embryos were formed in a globular stadium (24 pieces) on this medium. The 10% solution of NaOCl (used for 15 minutes) turned out to be the most effective substance for seed sterilization.

Additional key words: embryogenic suspensor mass, somatic embryogenesis

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Introduction

Vegetative reproduction of trees using *in vitro* methods allows for a more effective propagation of individuals with the desired characteristics and permits for vegetative reproduction of these species, which are very difficult to propagate using traditional methods, like oaks, beech, yew and silver fir. In the years 1985–1990, studies on micro-propagation on trees from our climatic zone employing the method of somatic embryogenesis were started. The method of somatic embryogenesis, which gives the highest coefficient of propagation, is based at the forming somatic embryos without the fertilization process and the zygote stage. The individuals obtained using this method are either genetically identical to the maternal plant, or they display some somaclonal variation (Tautorus et al. 1991). The process of somatic embryogenesis can be started directly from the explant cells, or it can be preceded by the stage of embryogenic callus (embryogenic suspensor mass – ESM), which is initiated from the explant . This second way provides broad opportunities of using the embryogenic tissue or somatic embryos in biotechnological investigations (somaclonal variability, genetic transformations).

The first studies on the somatic embryogenesis of spruces were started in the mid 1980's on Norway spruce (Chalupa 1985; Hakman et al. 1985). On silver fir, investigations have been conducted since 1986 (Erdelsky and Barancok 1986), but only after several years Hristoforoglu et al. (1992) have obtained a high frequency (40%) of embryogenic callus and development of single seedlings. Research on silver fir was continued until 2001 (Braumüller et al. 2001; Szczygieł and Kowalczyk 2001). In Slovakia the investigations have been focused on the hybrids of silver fir

and other fir species (*Abies alba* × *Abies nordmanniana, Abies alba* × *Abies cephalonica*) (Vookova et al.1997/98; Salajova and Salaj 2001).

The main target of this researches was obtaining somatic embryos using the clonal propagation method, through the phase of embryogenic callus. In the case of silver fir, it is difficult to obtain embryogenic callus; therefore, the optimum cultivation conditions were supposed to be developed (selection of original explant, manner of disinfection, selection of medium and appropriate ratio of growth regulators contained therein). It made possible to use the embryogenic callus of the silver fir to the biotechnological researches (Nawrot – Chorabik 2008 [In press]).

Material and methods

Plant material

The megagametophytes, zygotic embryos and needles of silver fir *Abies alba* Mill. were used as explants. The immature embryos were collected in two sample plots, while the mature seeds were collected in eleven sample plots, distributed over seven forest districts in southern Poland (Table 1). The megagametophytes (in *Coniferae* original endosperm with immature zygotic embryo) were isolated from cones of various sizes, collected from mid-June until the end of July 2001 year. Zygotic embryos were obtained from seeds gathered in mid-October 2001 from one well-developed cone from each sample plot. The needles were collected from the current year shoots (Table 1).

Sterilization methods

Seeds and needles were rinsed in 70% ethanol and sterilised using hydrogen peroxide (H_2O_2) or sodium hypochlorite (NaOCl) or mercuric chloride ($HgCl_2$) (Table 1). In the experiment employing the explants taken from needles, the influence of PPM (Plant Preservative Mixture, which is a fungicide and bactericide) on culture sterility was also studied. In investigations on the effectiveness of the disinfecting agent and its influence on the process of callogenesis, various control combinations were used (Table 1).

Zygotic embryos isolated from mature seeds were most promising explants. The research on their regeneration in callus determined the impact of disinfecting agents and the impact of the type and consistency of media of callogenesis.

Media

In this study, the influence of the kind of media and their state of aggregation on the development of callus and formation of somatic embryos was examined. The three following kinds of modified media were used: MS (Murashige and Skoog 1962), SH (Schenk and Hildebrandt 1972), and MCM (Bormann and Jansson 1981), enriched in vitamins (MS: thiamine 2.5 mg × dm^{-3}; SH: thiamine 5.0, nicotic acid 5.0, pyridoxine 0.5 mg × dm^{-3}; MCM: thiamine 17, biotine 12.5, folic acid 11, pantothenic acid 5.0, pyridoxine 12.0 mg × dm^{-3}), casein hydrolysate (1.500 mg × dm^{-3}), solidified with phytagel (4.000 mg × dm^{-3}), with various concentrations of growth regulators (Table 1).

Embryogenic callus induction and somatic embryos development

Explants were put separately into test tubes. We used 100 explants in each combination, depending on the kind of the analysed plant material.

After two weeks, the sterile explants were transferred in groups of 5 onto Petri dishes with the same medium containing a doubled concentration of the growth regulators (callus proliferation). Callus was transferred to fresh media every two weeks. The cultures were kept in an air-conditioned room, in darkness, at a variable temperature of 24°C for sixteen hours and 20°C for eight hours. The percentage not infected of explants the frequency of the induction of callus and its size was determined. Additionally, using a magnifying glass and an interferometer microscope, the formation of embryogenic callus was observed, along with the development of somatic embryos (number of embryos and their developmental stage).

SH maturation medium contained additionally 20 μM × dm^{-3} ABA and 40 g × dm^{-3} maltoza (instead of the sucrose). Embryogenic callus growth on zygotic embryos of silver fir by means of somatic embryogenesis was presented as an outline (Fig. 1).

Statistical Analysis

In experiment 3 zygotic embryos isolated from mature seeds of silver fir were subject to analysis (Table 1). In order to check the influence of media on the callus frequency induction of each origin separately, significance test for two structure indexes was applied. The significance level was applied at $\alpha = 0.05$. The same test was applied to describe the influence of the type and concentration of the disinfecting agent on the callus frequency induction from the seeds of silver fir. To show the significance of differences between the callus mass bred on media of various consistency (solid, liquid), t-Student test was applied for dependent variables on the significance level of $\alpha = 0.05$. To compare the average frequency of callus between the SH medium and the MCM medium disregarding the origin, the t-Student test was also applied, whereas this test was preceded by transforming on the callus frequency induction (provided in percentage values) by means of Bliss transformation.

Table 1. Types of explants from silver fir *Abies alba*, their origin, type of sterilization, medium used and callus formation

Number of the experiment	Explants	Origin of the plant material (Forest district, forest compartment)	Kind of sterilization	Kind of medium*	Growth regulators** (callus initiation) [mg × dm^{-3}]	Callus formation
1	megagametophytes with immature embryo megagametophytes	Myslenice (155c)	10% H_2O_2 – 3 min.	MS	BA – 1.0 BA – 2.0	none
				SH	BA – 1.0 BA – 2.0	
2	as above	Krynica (34a)		SH	BA – 1.0 TDZ – 0.5	
				MCM	BA – 1.0 TDZ – 0.5	
3	zygotic embryos	Lesko (14a)	10% NaOCl – 15 min.	SH and MCM	SH BA – 1.0 MCM BA – 0.5	N
		Lesko (110g)				
		Lesko (37c)				
		Lesko (126b)				
4	as above	Rymanów (80g)				
		Kroscienko (92a)				
		Lopuszna (756)				
		Krynica (34a)				
		Zwierzyniec (139c,d)				
		Myslenice (155c)				
		Krynica (34a)	0.1%$HgCl_2$ – 10 min.			
		Zwierzyniec (139c,d)				
		Myslenice (155 c)				
		Krynica (34a)	0.4%$HgCl_2$ – 10 min.			
		Zwierzyniec (139c,d)				
		Myslenice (155 c)				
		Krynica (34a)	10% NaOCl – 15 min.		SH, BA – 1.0 SH, TDZ – 0.5 MCM, BA – 1.0 MCM,TDZ – 0.5	ESM
		Myslenice (155 c)				
		Kroscienko (92a)				
5	needles	Krynica (34a)	control	SH	BA – 1.0 without PPM with PPM (PPM – 0.5 cm^3× dm^{-3})	none
			5% NaOCl – 15 min.			
			10% NaOCl – 15 min.			
			3% H_2O_2 – 10 min.			
			10% H_2O_2 – 10 min.			
			5%chloramine – 15 min.			
			10%chloramine – 15min.			
			0,01%$HgCl_2$ – 5 min.			
			0,1%$HgCl_2$ – 5 min.			

*medium: MS – Murashige and Skoog (1962), SH – Schenk and Hildebrandt (1972), MCM – Bornman and Jansson (1981)
**BA (6-benzylaminopurine), TDZ (1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea), N – non-embryogenic callus, ESM – embryogenic callus (embryogenic suspensor mass)

No statistical analysis was conducted for megagametophytes and needles of silver fir due to lack of callus initiation.

Also in experiment 4 (Table 1) where embryogenic callus was obtained on zygotic embryos due to small frequency induction of callus, no statistical analysis was conducted (the sample was too small).

Results

Sterilization methods

A high percentage (95–100%) of not-infected cultures of the megagametophytes of *Abies alba* was obtained using 10% H_2O_2 (3 min.).

In respect to the sterilization of seeds from which the zygotic embryos were isolated, the best results

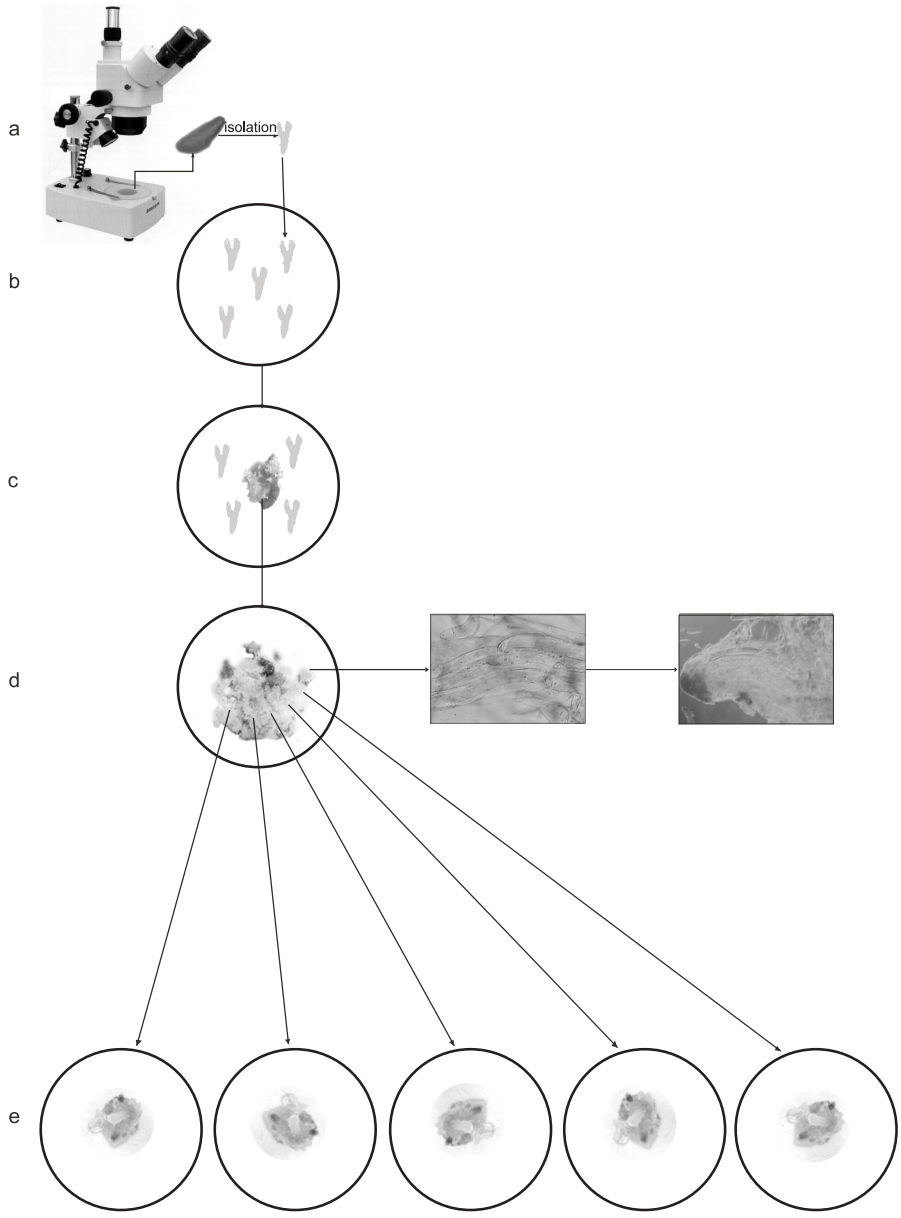

Fig. 1. The outline of the embryogenic callus growth on zygotic embryos of silver fir (*Abies alba* Mill.)
a – isolation of zygotic embryos of fir (2n chromosomes) in sterile conditions, under the binocular magnifying glass;
b – zygotic embryos placed on solidified medium on a Petri dish;
c – induction of embryogenic callus on some zygotic embryos;
d – zygotic embryos with proliferated embryogenic callus, passaged on a medium with doubled concentration of growth regulators – on each dish a separate callus, i.e. fir genotype (growing somatic embryos are visible on callus); at this stage microscopic photographs were taken where it is possible to see the embryogenic sphere – ESM, i.e. small cells with thick cytoplasm, whose nuclei are dyed red and long colourless suspensor cells;
e – each line of embryogenic callus divided into;
possibility of using the embryogenic callus line for research on somaclonal variability by PCR-RAPD method.

were obtained using 10% solution of NaOCl (15 min.) or 0.4% $HgCl_2$ (10 min.) – on average, 85% of sterile cultures. A much less effective agent of sterilization turned out to be the 0.1% $HgCl_2$ (10 min.) – on average, 70% of sterile cultures were obtained. The percentage of sterile cultures was not relative to the place of origin of seeds and ranged from 0 (Lesko – compartment 110 g) to 100% (Lopuszna) (experiment 3), (Table 2).

Media

Investigations concerning types of media (solid medium versus a liquid medium) proved that the solid medium was more conducive (medium to induce the callus formation from the mature zygotic embryos). On the liquid medium callus deteriorated faster, changed colour into brown and died out (Table 2). Moreover, comparing the two kinds of media (SH

Table 2. Percentage of sterile, regenerated and alive zygotic embryos isolated from seeds of *Abies alba* (sterilized in 10% NaOCl) and the mass of the non-embryogenic callus (experiment 3)

Origin (Forest district, forest compartment)	Medium	Sterile **[%]	Frequency of the non-embryogenic callus induction [%]				A live explants on solid or liquid medium [%]				Mass of callus [mg]			
		Days of the growing:												
		14	28	42	56	70	84		98		84		98	
1. Lesko (14a)	SH	60*	100*	100*	100*	100*	100	100	100	56	19.0	24.6	21.6	24.0
2. Lesko (110g)		0	0	0	0	0	0	0	0	0	0	0	0	0
3. Lesko (37c)		80*	82*	94*	94*	95*	100	100	78	56	21.6	18.3	23.6	18.3
4. Lesko (126b)		80	100	100*	100	100	100	100	92	58	27.6	27.0	30.0	24.0
5. Rymanów (80g)		70*	76	95*	96	98*	100	100	78	44	15.0	23.3	22.3	18.3
6. Kroscienko (92a)		17*	100	100	100	100	100	100	100	0	19.0	21.5	20.5	0
7. Lopuszna (756)		100	100	100	100*	100	100	100	93	53	14.3	16.3	18.0	14.3
8. LZD Krynica (34a)		57*	18	18*	29	29	100	100	67	0	16.6	13.6	19.3	0
9. Zwierzyniec (139c,d)		87	92	92	100	100	100	100	85	69	22.3	18.0	27.6	17.3
10. Myslenice (155c)		93	100	100	100	100	100	100	100	86	21.6	30.6	23.0	21.6
Average:		64.4	76.8	79.9	81.9	82.2*	90.0	90.0	79.3	42.2	17.7	19.3	20.6*	13.9*
1. Lesko (14a)	MCM	90	63	63	70	85	100	100	92	55	19.5	24.2	23.6	24.0
2. Lesko (110g)		0	0	0	0	0	0	0	0	0	0	0	0	0
3. Lesko (37c)		57	53	53	59	65	100	100	100	0	22.2	23.5	26.0	0
4. Lesko (126b)		80	100	82	100	100	100	100	100	45	28.6	20.3	35.6	20.3
5. Rymanów (80g)		97	76	76	83	86	100	100	100	50	26.0	26.3	27.6	20.6
6. Kroscienko (92a)		47	100	100	100	100	100	100	86	0	18.2	17.5	19.8	0
7. Lopuszna (756)		100	93	93	93	93	100	100	100	71	12.3	13.0	19.0	20.0
8. LZD Krynica (34a)		43	23	23	31	31	100	100	100	0	16.5	8.5	28.0	0
9. Zwierzyniec (139c,d)		87	100	100	100	100	100	100	100	38	16.3	30.6	21.0	25.6
10. Myslenice (155c)		90	100	100	100	100	100	100	100	77	20.6	31.3	23.6	24.0
Average:		69.1	70.8	70.0	73.6	76.0	90.0	90.0	87.8	33.6	18.0	19.5	22.4	13.4

Liquid medium (division of explants into solid and liquid media since the 84-th day of growing)

Average for

medium: SH – Schenk and Hildebrandt (1972), MCM – Bornman and Jansson (1981)

* statistically significant differences on the level of $\alpha = 0.05$ ** *in vitro* sterile cultures (non infected)

and MCM), it was noted that a higher frequency of non-embryogenic callus occurred on the SH with 1 mg × dm^{-3} BA as compared with the MCM medium (Table 3).

No matter what the developmental phase of megagametophytes isolated from cones on both plots was (Myslenice and Krynica), the explants did not lead to any callus initiation, despite the fact that various kinds of media were employed with various concentrations of growth regulators (experiments 1 and 2), (Table 1).

The investigations on mature zygotic embryos sterilized in 10% NaOCl (15 min.) (experiment 4) (Table 1) supported the earlier findings concerning the influence of the type of medium on the frequency of the callus induction. The best medium was SH with 1 mg × dm^{-3} BA (Table 1). On this medium, after 70 days of growth the embryogenic callus was obtained (Fig. 3a). The frequency of callus was 6%. The size on the embryogenic callus was surprisingly large (the mean length of callus from 3 localities was equal to 45 mm (Fig. 2). The embryogenic tissue looked like a white, mucilaginous mass with characteristic cells in which you can see pro-embryos (a clear zone of em-

Table 3. Effects of disinfecting agent and type of medium on the frequency formation of the non-embryogenic callus on the mature zygotic embryos of *Abies alba* (experiment 3)

Disinfecting agent/ Solid medium	Frequency formation of callus [%]		Statistically significant differences
	SH	MCM	
10 % NaOCl (15 min.)	76.8	70.8	none
0.1% $HgCl_2$ (10 min.)	83.3	76.3	none
0.4% $HgCl_2$ (10 min.)	40.0	27.6	none
average	66.7	58.2	yes

medium: SH – Schenk and Hildebrandt (1972), MCM – Bornman and Jansson (1981)

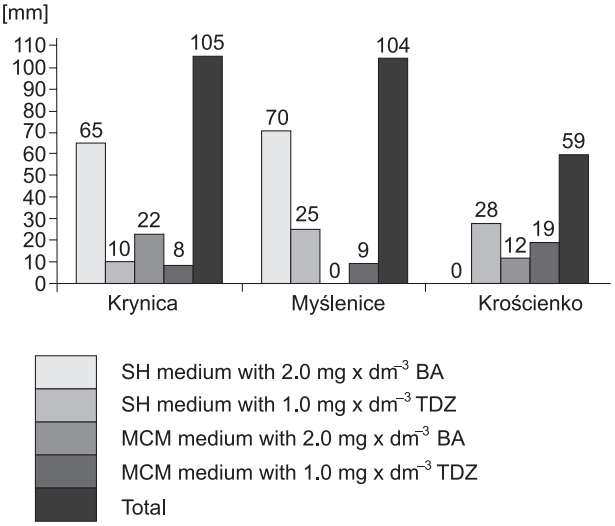

Fig. 2. Size of the embryogenic callus of silver fir after 70 days of culture (medium SH or MCM with doubled concentration of growth regulators – multiplication

bryogenic mass containing red coloured nuclei and long, colourless suspensor cells having large vacuoles and small nuclei) (Fig. 3). The obtained embryogenic callus from the Krynica and Myslenice plots achieved an impressive size (65 and 70 mm in diameter, Fig. 2).

Embryogenic callus induction and somatic embryos development

The remaining growing callus was divided into lines which formed clones. In total, from the seeds collected in two sample plots (Krynica and Myslenice) growth of 57 embryogenic line were obtained (Table 4, Fig. 3a).

On these clones, further investigations on the somaclonal variation in *Abies alba* were conducted employing the molecular markers RAPD (Nawrot-Chorabik 2008 [In review]).

In fragments of embryogenic callus which had not been subjected to freezing and thus not included in investigations of somaclonal variability, after passaging into the SH and MCM media with an admixture of ABA (20 μM × dm^{-3}), from 8 to 24 somatic embryos were obtained at the globular stage on each of the grown calluses (after 3 months of the cultivation) (Table 4, Fig. 3c).

Table 4. Effect of medium on the multiplication embryogenic callus of silver fir (*Abies alba* Mill.) and regeneration of somatic embryos* (experiment 4)

No.	Origin of seeds	Medium for embryogenic callus multiplication	Number of obtained lines	Number of somatic embryos*
1	Krynica compartment 34a	SH with 2.0 mg × dm^{-3} BA	18	24
2				
3		MCM with 2.0 mg × dm^{-3} BA	10	8
4	Myslenice, compartment 155c	SH with 2.0 mg × dm^{-3} BA	22	23
5				
6		SH with 1.0 mg × dm^{-3} TDZ	7	12
Total:			57	67

*the medium with ABA (20 μ × dm^{-3})

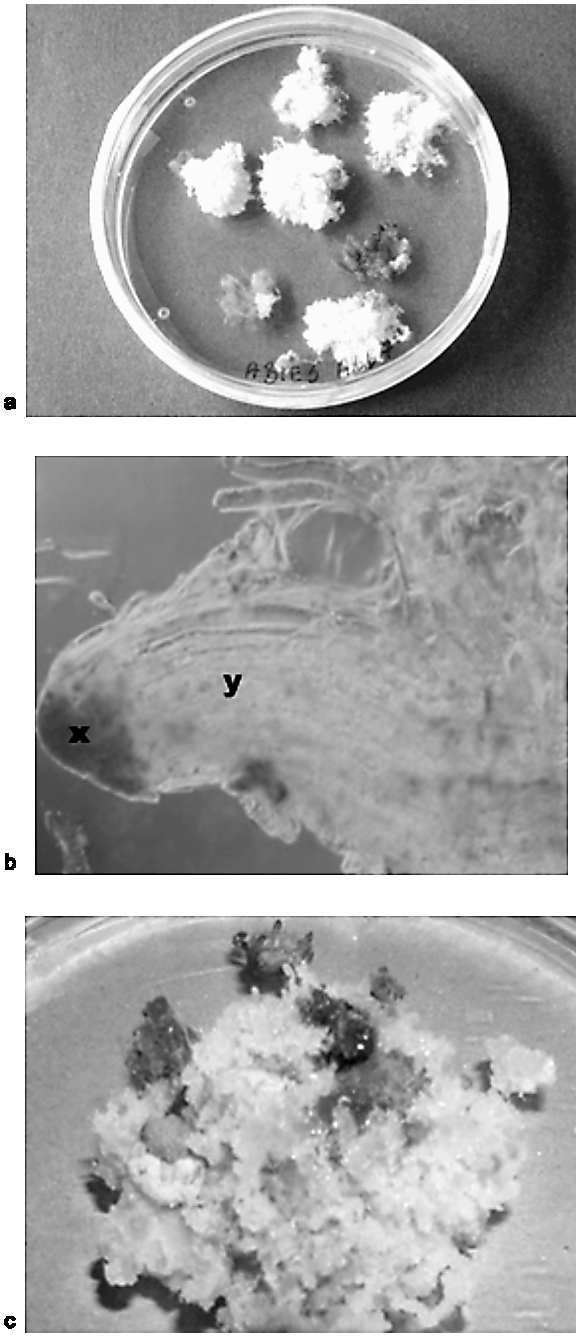

Fig. 3. Embryogenic callus and somatic pro-embryo of *Abies alba*

a – Embryogenic callus of *Abies alba* , initiated on SH medium with addition of BA (1.0 mg × dm^{-3})

b – Somatic pro-embryo in embryogenic callus *Abies alba*: × – proembryogenic region, y – suspensor cells

c – Embryogenic callus of *Abies alba* with developing somatic embryos growing on SH medium, with addition ABA (20 μM × dm^{-3})

Better development of the somatic embryos at the globular stage was found on the SH medium in comparison with MCM medium. Abscisic acid positively affected the maturation of somatic embryos.

On sterilized needles of *Abies alba* (experiment 5), no callus was obtained despite the fact that the percentage of non-infected needles was very high (95%) (Table 1). While examining the effect of PPM (0.5 $cm^3 \times dm^{-3}$) on the percentage of non-infected explants, it was found that this disinfecting agent did not have any significant influence on the percentage of sterile cultures obtained from silver fir needles (on average, 82% sterility was obtained on the medium with PPM as well as on the medium without it).

Statistical Analysis

On the basis of the significance test, comparing subsequent origins of seeds between media on the basis of principle "each with each" it was showed that the medium has an influence on the non-embryogenic callus of silver fir frequency induction (the exceptions were seeds of two origins: Zwierzyniec and Myslenice). Significant statistical differences were marked with a star (Table 2). Average callus frequency induction calculated from all origins on the SH medium was higher than the same value on the MCM medium (Table 2 – stars by average values) on each time of breeding. Frequency differences increased systematically in time. It was possible to show the statistical significance only on the 70th day of *in vitro* culture ($t = 2.287$; $df = 9$; $p = 0.0480$). The SH medium turned out to be better than MCM medium.

Statistically significant differences were also showed in the case of the comparing the mass of callus developing on solid or liquid medium (Table 2). On the solid medium, the callus mass was larger than on the liquid medium and the statistical differences visible on the 98th day of cultivation did not depend on the type of medium but on its consistency (SH medium $t = 2.789$; $df = 9$; $p = 0.211$, MCM medium: $t = 2.313$, $df = 9$, $p = 0.0460$) (t – value of student test; df – number of liberty steps; p – probability). Application of different surface disinfecting agents of the silver fir seeds have not had an effect on the callogenesis (lack of statistically significant differences). The results of statistical analysis were confirmed by data obtained from observations presented in percentage terms.

Discussion

Investigations concerning *in vitro* propagation of *Abies alba* employing the method of somatic embryogenesis have been conducted since the mid-nineteen eighties. Among the research centres dealing with this topic, an outstanding role has been played by the Institute of the Slovak Academy of Sciences in Nitra, where research was focused on hybrids from the genus *Abies* (Vookova et al. 1998), and the Austrian Research Centre in Seibersdorf (Hristoforoglu et al. 1992, 1995).

The origin of the plant material affects the results of *in vitro* propagation in respect to the number of non-infected cultures, the process of callus formation and the kind of callus produced. This work suggests that the induction of embryogenic or non-embryogenic callus is, to a large extent, determined by the origin of the plant (its genotype) (Tables 1, 2). The biochemical composition of the embryogenic tissue cultures differs from the non-embryogenic cultures. Embryogenic cultures produce less ethylene, contain lower concentrations of glutation and produce proteins at a quick rate. The embryogenic tissues achieved from explants of coniferous trees are species specific (in *Abies alba,* they are white with small, elongated cells, dense cytoplasm, large vacuoles and small nuclei).

The published data suggests that in silver fir, the immature zygotic embryos with endosperm (megagametophytes) (Schuller et al. 1989; Vookova et al. 1997/98) or mature zygotic embryos separated from seeds (Hristoforoglu et al. 1992, 1995; Braumûller et al. 2001) are usually used as a source of explants. In this investigation, embryogenic callus was obtained from mature zygotic embryos; this turned out to be advantageous in comparison with megagametophytes and with needles (lack of the callus induction). Similar results were achieved by Hristoforoglu et al. (1992, 1995), Jasik et al. (1999), Szczygieł and Kowalczyk (2001).

The type of substance used for the sterilization of explants is the another factor which is essential for the callus production. The researchers working on the micro-propagation of plants usually do not pay much attention to the effectiveness of sterilization of explants; they usually refer to the type of chemicals used for his purpose and the length of time they are applied. This does not allow for comparison of the results among various publications, especially taking into account the fact that various chemicals are being used for the sterilization of explants taken from gymnosperm plants. Earlier research conducted on *Abies alba*, as well as earlier experiments made by the author of this work (Nawrot-Chorabik 2007), indicated that the explants from *Abies alba* are being attacked by bacteria, fungi and slime-moulds, which is especially visible in the first two weeks of growing callus (Nawrot-Chorabik and Jankowiak 1999).

In most of the experiments conducted in Slovakia, the substance usually used for the sterilization of explants was mercuric chloride ($HgCl_2$) in a concentration of 0.1–0.2% (10–15 minutes) (Kormutak et al. 1996; Ditmar and Bies 1993). A relatively effective chemical, especially in the case of firs, was also a 10%

solution of sodium hypochlorite (NaOCl) applied for 10 minutes (Bajaj 1995). In the case of *Abies alba*, good results were obtained by soaking seeds in a 4% solution of chloramine and repeating this 4 or 5 times (Krajčakova and Miklovičova 1986). A substance also frequently used for sterilization is calcium hypochlorite ($Ca(OCl)_2$). However, in comparison with mercuric chloride, it was definitely less effective (Misson 1987).

The above-mentioned substances used for sterilisation of explants are more useful in the case of mature seeds. For megagametophytes with immature embryo, they can be too strong, which was shown in this study. The megagametophytes sterilised with a 10% solution of hydrogen peroxide for 3 minutes were 95–100% non-infected, but the substance used for sterilisation turned out to be harmful and could be one of the reasons for the lack of initiation of callogenesis (Table 1). Mature seeds were sterilised with two different chemicals (Table 1), of which the 10% solution of sodium hypochlorite was more advantageous, because it did not stop the initiation of callus (on isolated mature embryos, an embryogenic callus was formed). On the contrary, needles treated with four different sterilising substances showed in each case a high level (about 82%) of non-infected cultures, and adding PPM to the growth medium did not increase the level of culture sterility.

It is very difficult to obtain of the embryogenic cultures in the case of trees. After callus initiation, during the following divisions and passages of callus, part of the strains change colour into brown and die out, which was observed during investigations in *Abies alba* and in *Abies nordmanniana* (Norgaard et al. 1992). In the latter case, after six months of culture, only 21% of the strains continued the growth. The rest had died.

The process of initiation of embryogenic callus is substantially affected by the type of growth medium. Contrary to spruce and larch, which need for callus initiation the addition of auxine and cytokinins to the growth medium, in the case of silver fir, we need only to add cytokinins to obtain the embryogenic tissue (Schuller et al. 1989; Norgaard and Krogstrup 1991). This was also supported by the results of this study (experiment no. 4). In other works conducted on mature embryos and megagametophytes of various species representing the genus *Abies* (*A. alba, A. balsamea, A. nordmanniana and A. alba* × *A. alba)* in Austria, Germany, the Netherlands and Slovakia, the authors obtained the embryogenic callus and mature somatic embryos on the following growth media: MCM with 2.2 μM BA and 2.3 μM of kinetin (2.2 μM BA, i.e. 1.25 mg × dm^{-3} BA), (2.3 μM kinetin, i. e. 0.49 mg × dm^{-3} of kinetin) (Hristoforoglu et al. 1995), 1/2 MS and BLG (Norgaard 1997), BLG with TDZ, or BA or 2iP (Guevin et al. 1994), SH (Vookova et al. 1998).

In this study, the embryogenic callus was obtained from mature zygotic embryos coming from 3 sample plots (Krynica, Myslenice and Kroscienko), grown on a solid medium of SH and MCM with the addition of 1.0 mg × dm^{-3} BA or 0.5 mg × dm^{-3} TDZ (Table 1). Among the others, the MCM medium with cytokinine (BA) was tried, because it was characterized by a high frequency of ESM (40%) in the study by Hristoforoglu et al. (1995). However, in this case, the frequency of ESM was much lower. The embryogenic callus of *Abies alba* reproduced quickly, and after 70 days of growth, somatic embryos were produced (Fig. 3c), but, probably due to a weaker genotype of seeds than in the case of Hristoforoglu et al. (1995), they produced a smaller number of lines (Table 4).

The authors of other papers concerning micro – propagation of coniferous trees reported various percentages of ESM. Von Arnold (1987) obtained 8% of ESM in *Picea abies*, Hakman and Fowke (1987) obtained 5% of ESM initiation in *Picea mariana*. Szczygieł and Kowalczyk (2001), working on *Abies alba*, obtained – depending on the seed source – from 1.2 to 29.4% of callus initiation on mature embryos isolated from seeds kept in a freezing chamber. Norgaard and Krogstrup (1991) obtained in the earlier stages of callus development in *Abies nordmanniana* about 30% of embryogenic tissue, but in the later stages of callus development, only 1% of ESM remained. A similar phenomenon of parabolic initiation of embryogenic callus was found in this study – in the first stage of the *in vitro* culture, 6% of ESM initiation was recorded, then the embryogenic callus increased gradually, giving the highest percentage of ESM (8.5%) between the 28^{th} and 42^{nd} day of culture growth, but then the percentage of ESM declined (down to about 6%) and remained at the same level until the 70^{th} day of culture growth. Only after one year of growing *in vitro* cultures the callus showed a tendency of faster deterioration, and obtaining somatic embryos at that stage was much more difficult.

The facts presented above suggest that even while using similar methods of growing silver fir *in vitro*, the final ESM effect depends strongly on the genotype. In this study, despite the lower frequencies of initiation of embryogenic tissues than those obtained by Hristoforoglu et al. (1995), the influence of numerous factors upon callogenesis – i.e. seed origin, type of explants, the kind of substances used for sterilization and PPM, the type of growth medium – were examined. In the near future, this can allow for the improvement of methods of micropropagation in *Abies alba* and the ability to higher frequencies of ESM in this species.

In conclusion, it can be said that conducting somatic embryogenesis employing *in vitro* cultures is a difficult and labour – consuming task, but it has many advantages and opens many opportunities. It allows –

among the other advantoges – for the micropropagation of valuable genotypes and permits for further studies in the bio-technology of trees.

Acknowledgements

The author would like to express their special thanks to Prof. dr hab. Tadeusz Kowalski for the given valuable suggestions and help in doing microscopic photos.

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