SOMATIC EMBRYOGENESIS OF SELECTED SPRUCE SPECIES (*PICEA ABIES*, *P. OMORIKA*, *P. PUNGENS* 'GLAUCA' AND *P. BREWERIANA*)

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ABSTRACT

Somatic embryogenesis was studied in four spruce species (*Picea abies*, *P. omorika*, *P. pungens* 'Glauca' and *P. breweriana*) to determine if this method can be used for in vitro propagation of coniferous trees. The highest frequency of initiation of embryogenic tissue was obtained when mature zygotic embryos were used as explants. It ranged then from 10.8% (*P. breweriana*) to 23.75% (*P. omorika* and *P. pungens* 'Glauca'). The frequency of embryogenic tissue initiation was strongly affected by medium composition, i.e. addition of appropriate auxins (2,4-D, NAA, Picloram) and sucrose concentration (10-20 $g \cdot l^{-1}$). A lower frequency was obtained in *Picea omorika* (10%) when megagametophytes (endosperms with immature zygotic embryos) were used as explants. No embryogenic tissue was produced from hypocotyls, cotyledons and needles. A satisfactory frequency was achieved with the use of somatic embryos of *Picea abies* (30%). The proliferation of embryogenic cell lines of spruces was affected by medium type. The experiments resulted in production of somatic plantlets of *P. abies* and *P. omorika*. This enables the application of this method of spruce micropropagation for genetic and breeding research or for nursery production.

KEY WORDS: *Picea*, embryogenic tissue, media, growth regulators, Picloram, ABA, somatic embryos.

INTRODUCTION

In spite of numerous studies on somatic embryogenesis, conducted in recent years in various laboratories all over the world, it is still not easy to produce somatic embryos in vitro, especially in coniferous tree species. The success of somatic embryogenesis depends on many factors, starting from physicochemical conditions of culture, through explant type and age, and ending with the genetically conditioned potential of explants to produce embryogenic tissue (John et al. 1995; Park et al. 1993, 1994; Yeung and Stasolla 2000). Micropropagation by means of somatic embryogenesis is very beneficial, mainly due to the high regeneration rate and possibility to produce, within a short period, an unlimited number of plantlets from a small amount of initial material (Gupta et al. 1993). This method is attractive also because the process of somatic embryo production can be automated, thanks to the use of bioreactors, which may substantially reduce the cost of the produced plants (Ibaraki and Kurata 2001; Jain and Ishii 2003). Somatic embryogenesis can be used for seed production to generate high-quality sowing material with the use of artificial seeds (Bach 2001).

Research on somatic embryogenesis has enabled its great improvement, so that it is now used for propagation of some broadleaves, including forest trees and fruit trees, e.g. *Fagus sylvatica* (Jorgensen 1988), *Quercus robur* (Chalupa 1990), *Q. suber* (Bueno et al. 1992), or *Tilia cordata* (Kärkönen 2000). In the mid-1960s, the first embryo-like structures were described, which were obtained from explants of the coniferous tree *Thuja orientalis* (Konar and Oberoi 1965; after Zenkteler 1984). Since then, the possibility of somatic embryogenesis was investigated in many coniferous tree species, including some spruces species, mainly because of their importance in forestry (Park et al. 1993; Adams et al. 1994; John et al. 1995). Somatic embryos of spruce, capable of further development into normal plants, were first produced independently by Chalupa (1985) as well as Hakman and von Arnold (1985), from embryogenic tissues initiated from immature zygotic embryos of *Picea abies*.

So far, research on somatic embryogenesis of coniferous trees has focused mainly on the methods of reaching complete somatic embryogenesis (from initiation of this process till cultivation of somatic plantlets and their acclimation in vivo) and on improving the efficiency of the whole

process. Such an approach to the problem of somatic embryogenesis in coniferous trees was understandable, and the positive results achieved at each stage of culture are now the basis for more advanced studies of this process, already at the molecular level (Stasolla et al. 2002; Bishop-Hurley et al. 2003; Silveira et al. 2004; Lippert et al. 2005; Peńa and Séguin 2001; Klimaszewska et al. 2003).

At the Institute of Dendrology in Kórnik, research on somatic embryogenesis was initiated in selected spruce species, which because of their high ornamental value could then be quickly propagated on a large scale in our nursery (Hazubska and Szczygieł 2003). In this study we aimed to develop a method for regeneration of somatic embryos of four spruce species (*Picea abies*, *P. omorika*, *P. pungens* 'Glauca' and *P. breweriana*) from embryogenic tissues induced from various explants.

MATERIALS AND METHODS

Plant material

Explants were taken from seeds of *Picea abies* L. Karst (place of origin: Augustów Forest District, Poland, as a model species), *P. omorika* (Paničić) Purk. (Kórnik Arboretum, Poland), *P. pungens* 'Glauca' Beissn. (Byczkowscy nursery in Kostrzyń, Poland, and Kórnik Arboretum) and *P. breweriana* S. Wats. (Oregon, USA). Seeds of the studied spruce species were stored in a cooling chamber at 4°C for 2-10 years.

At the stage of initiation of embryogenic tissue, we analysed the influence of various factors (explant type and age, date of seed collection, and medium composition) on the potential for embryogenic tissue initiation by explants of selected spruce species. In our experiments we used the following explant types: mature zygotic embryos (*P. abies*, *P. omorika*, *P. pungens* 'Glauca', *P. breweriana*), megagametophytes with immature zygotic embryos at the pre-globular stage (*Picea omorika*, *P. pungens* 'Glauca'), cotyledons and hypocotyls of several-week-old zygotic seedlings and needles from several-month-old zygotic seedlings (*P. omorika*, *P. pungens* 'Glauca', *P. breweriana*), and somatic embryos (*P. abies*, *P. omorika*). Mature zygotic embryos were taken from seeds which after harvest had been stored at 4°C.

Megagametophytes with immature zygotic embryos were isolated from seeds collected in the Kórnik Arboretum. The cones were collected every week from late May till late June in 2003-2004.

Spruce seeds were sterilized with 33% H_2O_2 with 2 drops of Tween 20.

Somatic embryos of *P. abies* originated from one embryogenic line and *P. omorika* from three lines cultured in vitro on BM-3 medium (Gupta and Durzan 1986) with 10, 20, 40 or 60 µM ABA (abscisic acid) and 1 µM IBA (idole-3-butyric acid).

Induction of embryogenic tissue

In this study, two basal media were tested: BM-3 (Gupta and Durzan 1986) and LM (Litvay et al. 1985), either full-strength or half-strength. The explants were cultured on three variants of the media: (a) full-strength BM-3 and LM; (b) 1/2 BM-3 and 1/2 LM (with 1/2 of full concentrations of media); and (c) 1/2 macro BM-3 and 1/2 macro LM (with 1/2 macro and full concentrations of micronutrients). The media were supplemented with 9 µM 2,4-D, 4.5 µM BA, 5 $g \cdot l^{-1}$ sucrose and were solidified with Phytagel (4 $g \cdot l^{-1}$). The pH of the medium was stabilized at the level 5.8. The explants were incubated at 24±1°C at darkness.

Among the studied medium variants, we selected for further experiments BM-3 and 1/2 LM media, as they proved to be the most useful for embryogenic tissue initiation in all the studied spruce species. We analysed the influence of various auxins on embryogenic tissue initiation from mature zygotic embryos. We tested the following combinations of growth regulators: (a) 9 µM 2,4-D (dichlorophenoxyacetic acid) and 4.5 µM BA (6-benzyladenine); (b) 9 µM NAA (1-naphthaleneacetic acid) and 4.5 µM BA; and (c) 9 µM Picloram (4-amino-3,5,6-trichloropicolinic acid) and 4.5 µM BA.

Also effects of various concentrations of sucrose in BM-3 medium on the potential to regenerate embryogenic tissue from mature zygotic embryos of the spruce species were studied. The applied media contained growth regulators: 9 µM 2,4-D, 4.5 µM BA. Sucrose was added at three concentrations: 10, 20 or 30 $g \cdot l^{-1}$.

Megagametophytes with immature zygotic embryos of *P. omorika* and *P. pungens* 'Glauca' were cultured on 1/2 LM with addition of 9 µM 2,4-D, 4.5 µM BA and sucrose (5 $g \cdot l^{-1}$). Hypocotyls, cotyledons and needles from zygotic seedlings were cultured on BM-3 and 1/2 LM media with addition of 9 µM 2,4-D (or NAA or Picloram) and 4.5 µM BA and sucrose (20 $g \cdot l^{-1}$).

We used as explants also somatic embryos of *P. abies* and *P. omorika* from embryogenic tissues initiated from mature zygotic embryos. Somatic embryos were cultured of BM-3 medium with 9 µM 2,4-D and 4.5 µM BA. The medium was supplemented with sucrose (10 $g \cdot l^{-1}$).

Proliferation of embryogenic tissue

The induced embryogenic tissues of selected spruce species were proliferated in darkness at the temperature of 24±1°C in media BM-3 and 1/2 LM, supplemented with 2,4-D (9 µM) and BA (2.25 µM). From the induced embryogenic lines of the studied spruce species, permanently proliferating lines were produced only for *Picea abies* and *P. omorika*. We analysed the influence of two media (BM-3 and 1/2 LM) on weight increments of embryogenic tissue in *P. abies* and *P. omorika*.

Maturation of somatic embryos

At the stage of maturation of somatic embryos of *Picea abies* and *P. omorika* (embryogenic lines I, II and III, each achieved from individual zygotic embryo), we tested the influence of various concentrations of abscisic acid (10--60 µM ABA) on the total number of embryos that were regenerated from embryogenic tissue (at the globular and cotyledonary stage) and on the number of embryos in the cotyledonary stage.

Maturation of somatic embryos was conducted for 5 weeks at 24±1°C at the light intensity of 7.5 $W \cdot m^{-2}$ (40-W fluorescent mercury lamps) with 16-h photoperiod.

Germination and conversion of somatic embryos

Somatic embryos of *P. abies* and *P. omorika* (lines II and III) at the cotyledonary stage, were isolated and placed on a germination medium developed by Margar (Margar 1977, after Bercetche 1988/1989), supplemented with su-

crose (10 g/l). They were cultured for 2 weeks in darkness, at 24±1°C, and for the next 2 weeks in light.

To achieve conversion of somatic embryos into plantlets, properly germinating embryos were transferred to 1/2 MS medium (Murashige and Skoog 1962) without growth hormones, for 2 months. During that period, we estimated the survival rate of the plantlets and evaluated their development, by measuring the length of their radicles and hypocotyls.

Acclimation of somatic plantlets to ex vitro conditions

Somatic plantlets of *P. abies* and *P. omorika* (line II) with properly developed radicles and hypocotyls, were directly transferred into pots (filled with a substrate composed of peat and soil from a spruce stand in the Experimental Forest 'Zwierzyniec' near Kórnik), at a ratio of 1:1. The plantlets were watered with fluid 1/2 MS medium and with 0.25% solution of the fungicide Previcur. Somatic plantlets were cultured at a reduced light intensity and elevated humidity, but in the course of their growth, light intensity was gradually increased.

Statistical analysis

The statistical analysis of data collected at the stage of embryogenic tissue initiation was performed by JMP 4.02 software on the basis of χ^2 test, at the significance level of p=0.05. The ANOVA/MANOVA program of the Statistica 5.1 PL package was used to assess differences between the compared variants at the stages of embryogenic tissue proliferation, maturation and germination of somatic embryos. One-way ANOVA based on the Tukey test was performed at the significance level of p=0.05.

RESULTS AND DISCUSSION

Induction of embryogenic tissue

Changes in concentrations of components of basal BM-3 medium generally did not affect the frequency of embryogenic tissue induction in the studied spruce species (Table 1). In the case of BM-3 medium and its modified forms, the highest proportion of embryos with embryogenic tissue was recorded for *P. abies* in 1/2 BM-3 medium (with 1/2 of full concentrations of nutrients), while for *P. omorika* and *P. pungens* 'Glauca' in the full-strength medium (Table 1, Fig. 1A). Explants of *P. breweriana* produced embryogenic tissue only in 1/2 macro BM-3 (with 1/2 of full concentrations of macronutrients).

Similarly, changes in concentrations of nutrients in basal LM medium did not contribute significantly to improved frequency of embryogenic tissue initiation in *P. abies* and *P. breweriana* (Table 2). The highest frequency of embryogenic tissue initiation for *P. abies* was recorded in media with lowered concentrations of macro and/or micronutrients (1/2 LM and 1/2 macro LM), while for *P. breweriana* in 1/2 macro LM (Table 2). Significantly higher frequencies of embryogenic tissue initiation were recorded for *P. omorika* in 1/2 macro LM, while for *P. pungens* 'Glauca' in 1/2 LM medium (Table 2).

So far, embryogenic tissue initiation in spruces has been conducted in full-strength media (Kolevska-Pletikapić et al. 1995; Vujicić and Budimir 1995) or half-strength media (Bellarosa et al. 1992; Ramarosandratana and Van Staden 2003). The effectiveness of initiation of somatic embryogenesis in spruces is determined mainly by selection of a proper medium. For example, Kolevska-Pletikapić et al. (1995) found that mature zygotic embryos of *P. omorika* regenerated embryogenic tissue with the highest frequency (47.8%) in LP medium, in MS medium embryogenic tissue was regenerated by only 2% of explants, while explants cultured in BLG medium did not produce any embryogenic tissue. Similarly, Afele et al. (1992), who tested half-strength media of three types (LM, LP, and BLG), recorded the highest frequency of embryogenic tissue initiation from mature zygotic embryos of *P. pungens* in 1/2 LM medium (13%). A much lower frequency of embryogenic tissue initiation was observed in 1/2 LP medium (1.79%), while explants cultured on 1/2 BLG medium did not regenerate any embryogenic tissue.

In our experiments, the influence of three auxins and cytokinin (BA) on the frequency of embryogenic tissue initiation from zygotic embryos was compared on BM-3 and 1/2 LM media. The most favourable influence on explants of *P. abies*, *P. omorika* and *P. pungens* 'Glauca' was exerted by Picloram in 1/2 LM medium (Table 3). The highest frequency of tissue initiation was recorded for *P. omorika*, *P. pungens* 'Glauca' (23.75% each) and for *P. abies* (11.25%). Mature zygotic embryos of *P. breweriana* regenerated embryogenic tissue only in 1/2 LM medium containing 2,4-D (Table 3). However, the differences were not statistically significant. A significant difference was observed for explants of *P. pungens* 'Glauca' in BM-3 medium, where the highest frequency of embryogenic tissue initiation (17.57%) was observed in the presence of 2,4-D (Table 3). After the experiment, the proportion of zygotic embryos capable of embryogenic tissue regeneration was higher if the tested auxins and cytokinin were added to 1/2 LM medium, as compared to hormonal variants in BM-3 medium. For embryogenic tissue initiation in spruces it is necessary to add both an auxin and a cytokinin to the medium (von

TABLE 1. Embryogenic tissue initiation from mature zygotic embryos of studied spruce species in BM-3 medium and its variants.

Medium	Zygotic embryos with embryogenic tissue (%)			
	Picea abies	*P. omorika*	*P. pungens* 'Glauca'	*P. breweriana*
BM-3	7.1	8.8	6.3	0.0
1/2 BM-3	7.5	3.8	0.0	0.0
1/2 macro BM-3	3.8	5.0	5.7	2.5
χ^2	1.27	1.91	7.1	4.08
P	0.5300	0.3852	0.0289	0.1301

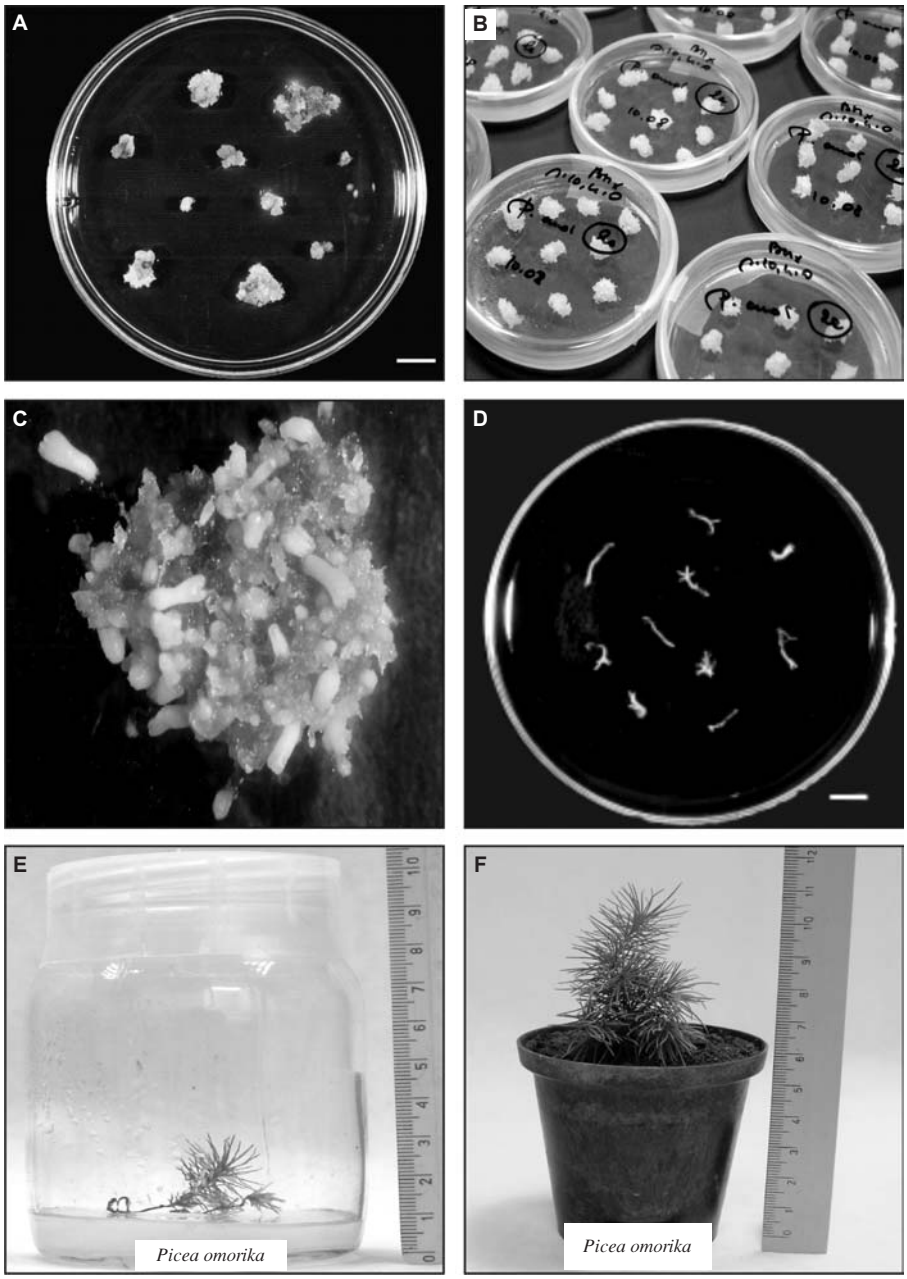

Fig. 1. Somatic embrygenesis induction and somatic seedlings development of *Picea omorika*. A – embryogenic tissue on the explant after one-month treatment with 9 µM 2,4-D and 4.5 µM BA, growing on the BM-3 medium. Scale bar represents 10 mm; B – Embryogenic tissue growing on the proliferation medium BM-3 containing 9 µM 2,4-D and 2.25 µM BA; C – the somatic embryos developed on the maturation medium BM-3 with 20 µM ABA; D – mature somatic embryos capable of roots and hypocotyls forming. Scale bar represents 10 mm; E – conversion somatic embryos into somatic seedlings on the 1/2 MS medium; F – acclimation of somatic plantlets to ex vitro conditions.

Arnold 1987; von Arnold et al. 1995). By contrast, in firs (*Abies* spp.) only a cytokinins must be added to initiate embryogenic tissue (Gajdosova et al. 1995; Salajova et al. 1998). The cytokinin most frequently used for initiation of somatic embryogenesis is BA (Chalupa 1985; Bozhkov and von Arnold 1998).

Among auxins, the most frequently used are 2,4-D (Bozhkov and von Arnold 1998; Filonova et al. 2000) and NAA (Afele et al. 1992; Ramarosandratana and Van Staden 2003). Less often, Picloram is applied (Joy et al. 1991; Park et al. 1993), as it is believed to be a less effective auxin than those mentioned above. Park et al. (1993) found that after addition of 2,4-D or Picloram, there was no significant difference in frequency of embryogenic tissue initiation in *P. glauca*. In our study we observed a tendency to initiate embryogenic tissue at a higher frequency by explants of *P. abies*, *P. omorika* and *P. pungens* 'Glauca' when Picloram was added to 1/2 LM medium, as compared to media supplemented with 2,4-D or NAA.

Sucrose concentration in BM-3 medium affected the frequency of embryogenic tissue initiation more strongly in *P. abies*, *P. pungens* 'Glauca' and *P. breweriana* than in *P. omorika* (Table 4). The highest number of zygotic embryos with embryogenic tissue was obtained in *P. pungens* 'Glauca' (16%) and *P. breweriana* (10.8%), when sucrose at a concentration of 10 $g \cdot l^{-1}$ was added. In the case of *P. abies* and *P. omorika*, the highest frequencies of explants with embryogenic tissue (15.3% and 6.7%, respectively) were recorded in media with sucrose at a concentration of 20 $g \cdot l^{-1}$. Research on induction of somatic embryogenesis showed that an important role in activation of this process is played by sucrose concentration in the medium (von Arnold and Hakman 1986; Mala 1991). Von Arnold and Hakman (1986) observed the highest frequency of explants with embryogenic tissue in *P. abies* when sucrose at a concentration of 10 $g \cdot l^{-1}$ was added to the medium. Results of our investigations were similar in *P. pungens* 'Glauca' and *P. breweriana*. However, in *P. abies* and *P. omorika* sligh-

TABLE 2. Embryogenic tissue initiation from mature zygotic embryos of studied spruce species in LM medium and its variants.

Medium	Zygotic embryos with embryogenic tissue (%)			
	Picea abies	*P. omorika*	*P. pungens* 'Glauca'	*P. breweriana*
LM	17.5	6.3	12.0	2.5
1/2 LM	20.0	11.3	23.75	6.7
1/2 macro LM	20.0	22.5	4.3	7.5
χ^2	0.22	9.5	12.80	2.40
P	0.8969	0.0087*	0.0017*	0.3000

* statistically significant

TABLE 3. Embryogenic tissue initiation from mature zygotic embryos of studied spruce species in BM-3 and 1/2 LM media supplemented with various auxins and 4.5 µM BA.

Species	Medium	Auxin (9 µM)	Zygotic embryos with embryogenic tissue (%)	χ^2 *P*
Picea abies	BM-3	2,4-D	6.25	0.70
		NAA	3.75	0.7065
		Picloram	3.85	
	1/2 LM	2,4-D	6.25	1.27
		NAA	8.75	0.5303
		Picloram	**11.25**	
P. omorika	BM-3	2,4-D	2.50	2.80
		NAA	0.0	0.2469
		Picloram	1.25	
	1/2 LM	2,4-D	21.25	0.14
		NAA	22.50	0.9308
		Picloram	**23.75**	
P. pungens 'Glauca'	BM-3	2,4-D	17.57	20.61
		NAA	1.33	<0.0001*
		Picloram	1.25	
	1/2 LM	2,4-D	21.25	3.79
		NAA	12.50	0.1503
		Picloram	**23.75**	
P. breweriana	BM-3	2,4-D	0.0	0
		NAA	0.0	0
		Picloram	0.0	
	1/2 LM	2,4-D	1.25	2.18
		NAA	0.0	0.3362
		Picloram	0.0	

* statistically significant

TABLE 4. Embryogenic tissue initiation from mature zygotic embryos of studied spruce species in BM-3 medium with various concentrations of sucrose.

Sucrose concentration ($g \cdot l^{-1}$)	Zygotic embryos with embryogenic tissue (%)			
	Picea abies	*P. omorika*	*P. pungens* 'Glauca'	*P. breweriana*
10	13.3	3.8	16.0	10.8
20	15.3	6.7	6.7	1.3
30	6.7	1.4	3.3	0.0
χ^2	6.41	4.88	16.06	27.31
P	0.0405*	0.0870	0.0003*	<0.0001*

* statistically significant

tly higher values were noted if sucrose concentration reached 20 $g \cdot l^{-1}$.

The type of explants influenced the potential for embryogenic tissue initiation. In most of our experiments, mature zygotic embryos were used for embryogenic tissue initiation. They were isolated from seeds stored for a long time at 4°C. After application of this explant type, the frequencies of embryogenic tissue initiation in the different va-

TABLE 5. Embryogenic tissue initiation from megagametophytes with immature zygotic embryos of *P. omorika* and *P. pungens* 'Glauca' cultured in 1/2 LM medium.

Collection date	No. of cultured megagametophytes of *P. omorika*	Explants with embryogenic tissue (%)	No. of cultured megagametophytes of *P. pungens* 'Glauca'	Explants with embryogenic tissue (%)
28.05.03	100	0.0	84	0.0
4.06.03	80	0.0	79	0.0
11.06.03	70	0.0	14	0.0
18.06.03	78	0.0	10	0.0
25.06.03	60	0.0	12	0.0
2.07.03	42	2.4	–	–
24.05.04	78	0.0	42	0.0
31.05.04	80	0.0	29	0.0
7.06.04	70	0.0	74	0.0
14.06.04	77	2.6	55	0.0
21.06.04	78	1.3	57	0.0
28.06.04	80	10.0	80	0.0

riants reached up to 23.75% for *P. omorika* and *P. pungens* 'Glauca' (Table 3), 20% for *Picea abies* (Table 2) and 10.8% for *P. breweriana* (Table 4). The relatively low frequencies of embryogenic tissue initiation from mature zygotic embryos might result from long-term storage at low temperatures. Our results are similar to those reported by Park et al. (1993) for explants of *P. glauca* extracted from seeds stored for some time at a low temperature.

For embryogenic tissue initiation, usually mature and/or immature zygotic embryos are used (Gupta and Grob 1995; Ramarosandratana and Van Staden 2003). Von Arnold (1987) and Mala (1991) obtained embryogenic tissue initiation from 50% and 40-60% mature zygotic embryos of *Picea abies*, respectively. Ewald (1995) recorded only 1--6.6% of explants with embryogenic tissue in that spruce species. According to published data, a much higher frequency of embryogenic tissue initiation was reached when immature zygotic embryos were applied as sources of explants. For example, Hakman and von Arnold (1985) obtained embryogenic tissue from 95% of explants in *P. abies*. Slightly lower frequencies of embryogenic tissue initiation for *P. abies* (64-74%) were reported by Mala (1991). There are also some reports about lower frequencies of immature zygotic embryos capable of producing embryogenic tissue, e.g. Ewald (1995) produced embryogenic tissue from 0.6-5.9% of explants of *P. abies*. In general for initiation of somatic embryogenesis it is necessary to use immature zygotic embryos, which are a very efficient sources of explants.

In the conducted experiments, embryogenic tissue from megagametophytes with immature zygotic embryos at the pre-globular stage was observed only in *P. omorika*, and its frequency ranged from 1.3 to 10% (Table 5). The frequency of embryogenic tissue initiation in *P. omorika* was higher for mature zygotic embryos (23.75%). Immature zygotic embryos of *Picea pungens* 'Glauca' did not produce embryogenic tissue. In practice, explants from mature zygotic embryos are more useful, as they can be extracted from seeds stored in seed banks. This option enables continuous access to the source of explants throughout the year. By contrast, immature zygotic embryos can be collected only in the period before they reach the cotyledonary stage. Their collection is even more difficult if weather conditions are unfavourable or the cone yield is low in the given year.

Another source of embryogenic cultures can be somatic embryos (secondary explants), resulting from somatic embryogenesis. In earlier studies, no somaclonal variation was found in plant material produced by means of somatic embryogenesis (Isabel et al. 1993; Nkongolo and Klimaszewska 1995; Harvength et al. 2001). Thus somatic embryos can be also applied for secondary initiation of embryogenic tissues. Embryogenic tissue initiation from somatic embryos of *P. abies* has already been reported, e.g. by Gjuleva and von Arnold (1999). In our studies, embryogenic tissue initiation from somatic embryos was successful in *P. abies* and *P. omorika*. The frequency of embryogenic tissue initiation from somatic embryos in *P. abies* reached 30% and was higher than from mature zygotic embryos (20%). For *P. omorika*, the frequency of embryogenic tissue initiation from somatic embryos varied from 2.73% to 11.9% (Tables 6 and 7). Somatic embryos (primary and secondary) can be very good sources of explants for new embryogenic cultures. Plants produced by means of somatic embryogenesis are very good donors of explants, because their tissues are physiologically younger than zygotic embryos. The potential for embryogenic tissue initiation by explants decreases with age (Ruaud 1993; Ruaud et al. 1992; Harvength et al. 2001).

Ruaud et al. (1992) used as sources of explants some needles of one-year-old somatic and zygotic seedlings of *P. abies*. A higher frequency of embryogenic tissue initiation was achieved by those authors from the former type of explants (80%), while only 10% of needles of zygotic seedlings were able to regenerate embryogenic tissue. Budimir and Vujicić (1992) as well as Vujicić and Budimir (1995)

TABLE 6. Potential for initiation of secondary embryogenic tissue from somatic embryos of *Picea abies* and two lines of *P. omorika* in BM-3 medium with 2.4-D (9 µM), BA (4.5 µM) and sucrose (10 $g \cdot l^{-1}$).

Species, line	Somatic embryos with embryogenic tissue (%)
Picea abies	30.0
P. omorika I	3.3
P. omorika II	4.4
χ^2	35.44
P	<0.0001*

* statistically significant

TABLE 7. Secondary embryogenic tissue initiation from somatic embryos of *Picea omorika* (line III) in BM-3 medium with various concentrations of ABA.

Explant origin – maturation medium with ABA (µM)	No. of cultured explants	Frequency of secondary embryogenic tissue initiation (%)	χ^2 P
10	210	11.90	10.97
20	70	4.29	0.0119*
40	10	0.0	
60	110	2.73	

* statistically significant

initiated embryogenic tissue from 9.6% of shoot apices with cotyledons from 10-21-day-old zygotic embryos of *P. omorika*. The culture of physiologically older explants, coming from 2- or 4-week-old and several-month-old zygotic seedlings of *P. omorika*, *P. pungens* 'Glauca' and *P. breweriana*, did not give positive results of embryogenic tissue initiation in our experiments.

Proliferation of embryogenic tissue

Embryogenic tissues of individual spruce species, initiated from mature zygotic embryos, varied in survival rate and potential for further proliferation. Our experiments with the four spruce species showed that embryogenic tissues of *P. abies* are characterized by the highest survival rate, a high viability and long-term growth (Szczygieł et al. 2007). After 24 months of *in vitro* culture, 9.1% of embryogenic tissues in *P. abies* and only 0.5% in *P. omorika* were still proliferating (Fig. 1B). Survival rates of lines of *P. pungens* 'Glauca' and *P. breweriana* in the first 6 months of culture reached 14.3% and 5.7%, respectively. After this period the obtained lines of both spruce species died. A characteristic feature of individual embryogenic lines is their varied growth dynamics. Salopek et al. (1997) succeeded to initiate a much higher number of embryogenic lines of *P. omorika* (127), but only 10 of them proliferated. In turn, Tramisak-Milaković et al. (1999) observed proliferation of embryogenic tissue in 87% of mature zygotic embryos of *P. omorika*, but within two years many of them died. A report by Mo and von Arnold (1991) shows that only 5-15% of embryogenic lines of *P. abies* continued their growth through about two years. Our results confirm the observations made by other authors.

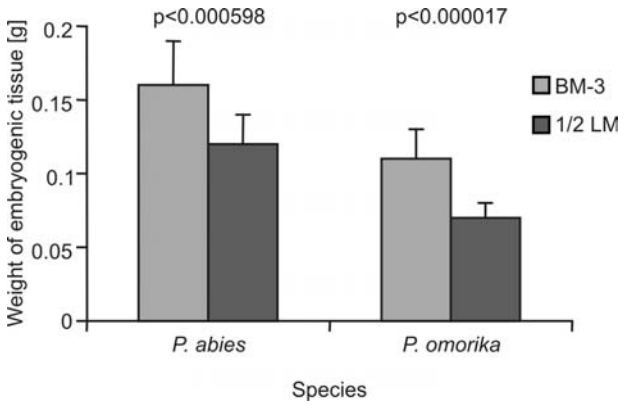

Fig. 2. Embryogenic tissue initiation in *Picea abies* and *P. omorika* (line III), cultured on both media. Figure legends: BM-3 medium (Gupta and Durzan 1986) and 1/2 LM medium (Litvay et al. 1985), containing 1/2 of nutrient concentrations of LM medium.

Proliferation of embryogenic tissues is conducted in darkness on solid or fluid media. The study shows that a prolonged period of passaging contributes to changes in the embryogenic potential of tissues: a reduced potential for generation of somatic embryos, and disturbances in their structure (Stasolla et al. 2002; Stasolla and Yeung 2003; Lelu-Walter et al. 2006). Embryogenic tissues are usually proliferated on the same medium as that used for embryogenic tissue initiation (Salajova et al. 1998) or with somewhat modified growth regulator concentrations (Kolevska-Pletikapić et al. 1995; Stasolla et al. 2002), which are usually slightly lowered. Proper proliferation of embryogenic tissue enables generation of sufficient amounts of material for further research. Our experiments showed that the type of medium used for proliferation exerted a significant influence on weight increments of embryogenic tissue in *P. abies* and in one of the lines of *P. omorika* (III). Considering the two compared media (BM-3 and 1/2 LM) a higher weight increment of embryogenic tissue was recorded on BM-3 in both species. Norway spruce (*P. abies*) was distinguished by the highest weight increments of embryogenic tissue on both media (Fig. 2).

Maturation of somatic embryos

Our earlier studies of the influence of ABA concentration on maturation of somatic embryos of *P. abies* and *P. omorika* (lines I and II) showed that ABA level can affect the total number of somatic embryos regenerated from 1 g of embryogenic tissue and can also influence their further development, reflected in the number of produced cotyledonary embryos (Hazubska and Szczygieł 2003). In further studies of another line of *P. omorika* (line III), ABA level did not have any significant effect on the total number of somatic embryos (Fig. 1C). The highest number of somatic embryos (490 per 1 g of tissue fresh weight) was produced by embryogenic tissue grown with 60 µM ABA (Table 8). A slightly lower frequency (421 somatic embryos per 1 g of tissue fresh weight) was recorded for embryogenic tis-

TABLE 8. Development of secondary somatic embryos of *Picea omorika* line III, cultured in medium BM-3 with various ABA concentrations. Mean values marked with the same letters are not significantly different at $P<0.05$.

ABA concentration (µM)	Total number of embryos*	Number of cotyledonary embryos*
10	421 a*	14 b
20	352 a	4 a
40	246 a	1 a
60	490 a	3 a

* per 1 g of fresh weight of embryogenic tissue

TABLE 9. Germination rate and further growth of somatic embryos of *P. abies* and *P. omorika* (lines II and III), after embryo maturation in BM-3 medium with various ABA concentrations, in week 4 of culture. Mean values marked with the same letters are not significantly different at P<0.05.

Species	ABA level during maturation (µM)	Initial no. of cultured embryos	Embryos with radicles (%)	Embryos with hypocotyls (%)	Mean radicle length (mm)	Mean hypocotyl length (mm)
Picea abies	20	40	5	100	0.13 a	6.23 b
	40	40	10	92.5	0.30 a	3.03 a
	60	40	2.5	97.5	0.03 a	3.08 a
P. omorika line II	20	40	30	100	0.53 a	4.50 a
	40	40	0	100	0.00 a	4.83 a
	60	40	10	100	0.28 a	4.85 a
P. omorika line III	10	80	100	100	3.9	4.7
	20	30	100	66.7	3.1	2.3
	40	10	100	70	2.5	1.8
	60	20	100	100	3.7	3.2

sue cultured on a medium supplemented with 10 µM ABA. Its concentration in the medium for maturation of somatic embryos affected the number of cotyledonary somatic embryos. Addition of 10 µM ABA to the medium contributed to an increase in the number of cotyledonary somatic embryos (Table 8). Higher concentrations of ABA inhibited the development of cotyledonary embryos.

ABA plays an important role in the embryogenesis of coniferous trees. Kong et al. (1997, 1999) found that developing seeds have higher levels of endogenous ABA than somatic embryos. In seeds, the major source of ABA for developing embryos is the megagametophyte (Kong et al. 1997). In micropropagation of coniferous trees, an elimination of auxins and cytokinins from the medium and addition of ABA as well as an increase in osmotic pressure at the stage of maturation of somatic embryos, are necessary for regeneration of cotyledonary embryos. Many researchers found that addition of exogenous ABA to the medium in a suitable concentration affects the number of mature somatic embryos in various species of coniferous trees (Norgaard 1997; Label and Lelu 2000; Percy et al. 2000; Stasolla et al. 2002; Hogberg et al. 2003). Reactions of embryogenic tissues of coniferous trees to ABA are highly varied. Its concentrations must be properly matched to plant species and genotype (Stasolla and Yeung 2003). Harry and Thorpe (1991) showed that a high concentration of exogenous ABA (40 µM) is necessary for proper development of somatic embryos in *P. rubens*. They also found that ABA at this concentration prevented precocious conversion of somatic embryos of this species. In turn, Attree et al. (1990) reported on the growth of somatic embryos of *P. glauca* and *P. mariana* at a low concentration of ABA (12 µM).

Germination and conversion of somatic embryos

As reported by Park et al. (1998), the genetic background does not exert any strong influence on germination of somatic embryos. However, many researchers emphasize the effect of earlier treatment, at the stage of embryo maturation, e.g. addition of ABA or PEG (polyethylene glycol) to the medium, on their subsequent potential for germination and further growth (Bozhkov and von Arnold 1998; Hogberg et al. 2001). Because of this, at the stage of germination and conversion of somatic embryos into plantlets, we studied the influence of ABA concentration during embryo maturation, on their further development. Somatic embryos of *P. abies* and *P. omorika* (line II) developed radicles and hypocotyls asynchronously, irrespective of ABA concentration during earlier culture (Table 9, Fig. 1D)). Somatic embryos of *P. omorika* (line III) developed much better, so the frequency of radicles was similar to that of hypocotyls. The earlier application of various ABA concentrations in BM-3 medium did not affect significantly the development of radicles of somatic embryos of *P. abies*, which in week 4 of culture reached average lengths ranging from 0.03 to 0.30 mm (Table 9). A significant correlation was observed between hypocotyl length and ABA concentration in the medium for maturation of the embryos. The highest hypocotyl growth rate was recorded in embryos cultured on media with 20 µM ABA. In those embryos, the mean hypocotyl length in week 4 reached 6.23 mm. In somatic embryos of *P. omorika* (line II), we observed no significant effect of ABA concentration during the earlier stage of culture, on the subsequent development of radicles and hypocotyls during germination. The maximum mean radicle length after 4 weeks of culture amounted to 0.53 mm for the somatic embryos that matured on media with 20 µM ABA. The somatic embryos of *P. omorika* (line III) that matured on media with 10 µM ABA, had the longest hypocotyls and radicles, as compared with the embryos cultured on media with higher ABA concentrations (Table 9).

After 2 months of culture on Margar's medium, to achieve conversion of somatic embryos into plantlets (Fig. 1E), the highest survival and growth rates were observed in plantlets of *P. abies* and *P. omorika* (line II), cultured earlier as embryos at higher ABA concentrations: 40 and 60 µM (Table 10). In contrast, in somatic plantlets of *Picea omorika* (line III), hypocotyls and parts of radicles did not show further growth, and the plantlets died (Table 10).

In previous studies of somatic embryogenesis in coniferous trees, the regeneration rate was relatively low: 10-20% (Becwar 1993). The highest germination rate (35%) was recorded by von Arnold and Hakman (1988) for somatic embryos of *P. abies*. Good results of germination were also achieved by means of partial drying of cotyledonary embryos at a relatively high humidity (Roberts et al. 1990).

The high germination rate of somatic embryos, and their later acclimation, depends on embryo quality and genotype (Timmis 1998; Hogberg et al. 2001). According to Park (2002), genetic effects at the germination stage are the lowest

TABLE 10. Conversion rate of somatic embryos of *P. abies* and *P. omorika* (line II) into somatic plantlets, after 2 months of culture on 1/2 MS medium.

Species	ABA level during maturation (µM)	Survival rate (%)	Embryos with hypocotyls >1 cm (%)	Embryos with radicles >0.5 cm (%)
Picea abies	20	40	20	40
	40	75	25	75
χ^2		1.10	0.03	1.10
p		0.2937	0.8577	0.2937
P. omorika	20	16.6	16.6	0
line II	60	50	25	25
χ^2		1.78	0.14	3.2
p		0.1824	0.7115	0.0736
P. omorika	10	0	0	13.8
line III	20	0	0	0
	40	0	0	0
	60	0	0	35.0

(3%). In comparison, according to that author, genetic effects reach 69% at the stage of embryogenic tissue initiation.

Acclimation of somatic plantlets to ex vitro conditions

The transfer of somatic plantlets to ex vitro conditions is associated with a strong stress. In comparison with normal seedlings, somatic plantlets grow less quickly at the early stage of growth in natural conditions (Grossnickle and Sutton 1999). The plantlets require a higher humidity and a lower light intensity during acclimation (Grossnickle et al. 1996).

In this study we succeeded, after ca. 12 months of ex vitro culture, to produce well-developed somatic plantlets of *P. abies* and *P. omorika*, capable of further growth in natural conditions (Fig. 1F).

During the acclimation of somatic plantlets to *ex vitro* conditions, a thorough success is possible in suitable environmental conditions (high humidity, light, temperature) and under proper care (shading, aeration, protection against pests and diseases).

CONCLUSIONS

In our study, the most effective explant type, in respect of embryogenic tissue regeneration in all four spruce species, were zygotic embryos, extracted from cold-stored seeds. The reduction of concentrations of macro- and micronutrients in some medium variants did not have any significant effect on the level of embryogenic tissue regeneration from mature zygotic embryos. However, we found that the frequency of embryogenic tissue initiation may be affected by the applied auxin type and sucrose concentration in the medium. Good results of embryogenic tissue initiation in three spruce species (*P. abies*, *P. omorika*, *P. pungens* 'Glauca') were achieved when 9 µM Picloram (an auxin) was added to the media. Hitherto, Picloram was rarely applied in somatic embryogenesis of coniferous trees. Medium variant had, in comparison with initiation of somatic embryogenesis, a strong effect on the proliferation rate of embryogenic tissues of *P. abies* and *P. omorika*. The maturation and further growth of somatic embryos and plantlets of these two spruce species may depend on ABA concentration at the stage of embryo regeneration.

Our results indicate that somatic embryogenesis is useful for spruce propagation *in vitro*. Coniferous tree species are particularly difficult plant materials in respect of micropropagation and subsequent acclimation to natural conditions, but our results show that this method can be used for large-scale propagation of conifers. The results may be an introduction to further, more detailed investigations into the processes that control somatic embryogenesis in spruces.

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