

Somatic embryogenesis in *Pinus nigra*: embryogenic tissue initiation, maturation and regeneration ability of established cell lines

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Abstract

The effect of plant growth regulators (PGR), 6-benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), α -naphthaleneacetic acid (NAA) and sugars (sucrose, maltose, glucose, fructose) on the initiation of somatic embryogenesis of *Pinus nigra* Arn. was investigated. Megagametophytes containing immature zygotic embryos have been used as explants. The experiments were done in the years 2000 and 2001. Higher initiation frequencies were obtained in 2001 when the zygotic embryos showed uniformity, being in the precotyledonary stage of development. Embryogenic tissue initiation occurred on all the media tested, including PGR-free medium. Relatively high initiation frequencies were obtained on media containing either NAA (9.09 %) or 2,4-D (7.14 %) alone. Somatic embryos were present as bipolar structures and showed differences in morphological features among cell lines. Plantlet regeneration occurred in cell lines containing bipolar somatic embryos composed of compact meristematic embryo "head" and suspensor organized into bundles.

Additional key words: pine, plant growth regulators, saccharides.

Introduction

Since the first report on somatic embryogenesis in conifers (Chalupa 1985, Hakman *et al.* 1985) the process has been recorded for many conifer species (Jain *et al.* 1995, 1999). High initiation frequencies were reported for *Picea* from immature (Hakman and von Arnold 1985, Lu and Thorpe 1987) as well as mature zygotic embryos (Tremblay 1990). In comparison with other conifers, *Pinus* species have shown lower initiation frequencies and survival of initiated cell lines. Somatic embryogenesis in pine was influenced by medium composition (Becwar *et al.* 1990), developmental stage of dominant zygotic embryo (Klimaszewska and Smith 1997, Miguel *et al.* 2000), explant type (Finer *et al.* 1989) and provenance/seed family (Garin *et al.* 1998).

Based on the morphology and maturation capacity of cell lines, Jalonen and von Arnold (1991) divided the somatic embryos of *Picea abies* into three groups (A1 - polar, A2 - solar and B - undeveloped). It is important to note that only the A group somatic embryos

appear capable of maturation and complete plantlet regeneration (Jalonen and von Arnold 1991). Categorization of stage 1 embryos according to morphological features has also been done for other *Pinus* species, as *P. caribaea* (Laine and David 1990), *P. pinaster* (Bercetche and Pacques 1995), *P. sylvestris* (Keinonen-Mettälä *et al.* 1996).

In *Pinus nigra*, somatic embryogenesis has been initiated from immature zygotic embryos. The initiation frequencies were lower in comparison with other conifer species and reduced by the maturation stage of zygotic embryos used as explants (Salajova *et al.* 1995). Somatic embryo maturation was stimulated by maltose combined with ABA and was cell line dependent (Salajova *et al.* 1999). We have also recorded the decreased regeneration ability of cell lines during the maintenance; some cell lines lost this regeneration capacity after longer culture period (unpublished results). These responses led us to initiate new embryogenic cell lines every year.

Received 19 November 2003, accepted 4 November 2004.

Abbreviations: BA - 6-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; NAA - α -naphthaleneacetic acid; PGR - plant growth regulators.

Acknowledgement: We highly appreciate the financial support from VEGA, Slovak Grant Agency, project No. 2/2089/22.

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In the last two years our main interest was focused on improvement of embryogenic tissue initiation and the selection of cell lines with high regeneration ability. For this purposes we used various concentrations and

combinations of plant growth regulators (PGRs) and sugars. The maturation capacity of established cell lines was also tested.

Materials and methods

The green cones were collected from open-pollinated trees of *Pinus nigra* Arn. They were stored in refrigerator at 4 °C for one week and after washing in tap water and dried with filter paper. Unripe seeds were removed and surface sterilized for 10 min with 10 % H₂O₂ and four times rinsed in sterile distilled water. Megagametophytes were excised and placed on culture medium. In each Petri plate (6 cm in diameter) eight explants were cultured.

Two experiments were done: in both, the basal medium was DCR (Gupta and Durzan 1985) supplemented with myo-inositol (200 mg dm⁻³), glutamine (50 mg dm⁻³), and casein hydrolysate (500 mg dm⁻³). Media with various concentration (ranging from 0 to 2 mg dm⁻³) and combination of 6-benzyladenine (BA), 2,4-dichlorophenoxyacetic acid (2,4-D), and α -naphtheneacetic acid (NAA) were tested. In this experiment we used sucrose as carbon source. Further we tested the effect of sugars. Sucrose, glucose, fructose and maltose (each at 2 %) were incorporated into the basal medium containing 2,4-D (2 mg dm⁻³) and BA (0.5 mg dm⁻³). The sugars were autoclaved separately and poured into cooled medium. The cultures were kept in darkness at 23 °C in both experiments.

Cytological observation was done three times, at 2 - 3 month intervals. All the established cell lines were cytologically investigated. Small pieces of tissue (5 pieces from different clumps of tissues in Petri plates) were stained with 2 % acetocarmine, squashed and examined using an *Axioplan 2* (Carl Zeiss, Jena,

Germany) light microscope.

For the maturation experiment, 30 cell lines were selected according to the morphology of somatic embryos. Seven or eight days following subculture, the embryogenic tissues were transferred to maturation medium. The basal medium was the same as for initiation, supplemented with abscisic acid (ABA, 25 mg dm⁻³) and maltose (9 %). The medium was gelled with *Phytigel* (0.4 %). The embryogenic tissue was placed in a thin layer on the filter paper on the surface of the gelled medium. The embryogenic tissues with filter paper were transferred to fresh maturation media four weeks after the beginning of the maturation experiment. After 8 weeks of culture the tissues with developing somatic embryos were transferred to media without ABA and with a lowered concentration of maltose (3 %).

Well-formed cotyledonary somatic embryos were selected for partial desiccation (2 - 3 weeks). Maturation as well as desiccation occurred in darkness. The germination medium was PGR-free and contained activated charcoal (1 %) as well as maltose (2 %).

When the germinating somatic embryos started to elongate they were transferred to light (irradiance of 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 14 h photoperiod) at temperature 23 °C. The developing emblings were grown in *Magenta* jars on basal medium containing maltose (2 %), activated charcoal (1 %) and no PGRs. The experiments were repeated twice, with 5 - 6 Petri plates for each cell line and treatment.

Results

Developmental stage of the zygotic embryo in the time collection: To check the developmental stage of the zygotic embryo, unripe seeds were excised and examined using a dissecting microscope. In year 2000, out of 45 seeds examined 6 (13.3 %) were without a megagametophyte; 24 megagametophytes contained zygotic embryos at the precotyledonary stage (53.3 %), 12 megagametophytes contained early cotyledonary zygotic embryos (26.2 %). Late cotyledonary embryos were found in 3 megagametophytes (6.6 %). In year 2001 all the examined zygotic embryos (45) were in the precotyledonary stage of development.

Explants placed on culture media were swollen after 1 week of culture. Around the 12 - 14 d of culture, the first signs of somatic embryo initiation were observed,

the suspensor protruded from the megagametophyte and somatic embryo proliferation was seen. At the 3rd week of culture the embryogenic tissue was clearly recorded as white tissue present on the already-observed suspensor, or protruding from the micropylar end of megagametophyte.

The majority of embryogenic tissues initiated between the 3rd and 6th week of culture. After 11 weeks of culture, the tissues were transferred to proliferation medium for long-term culture. Quantitative evaluation of initiation was done after 16 weeks after of culture.

In general, the initiation frequencies were low; of 1108 explants cultured, somatic embryo (SE) initiation (protruding of the white embryogenic tissue) was recorded for 34 explants (3.06 %). Altogether, 34 cell

lines were established for long-term maintenance. The majority of these cell lines survived (94.11 %), only 2 cell lines died after the 3rd transfer.

The concentration of PGR in the basal DCR medium was low, ranging for all PGRs 0 - 2 mg dm⁻³ (Table 1). Using 2,4-D, the most productive response was obtained with an equal mix with BA (5.68 %). Among the NAA treatments the highest initiation frequency was obtained with low NAA content (0.5 mg dm⁻³) combined with 2 mg dm⁻³ BA. Embryogenic tissue initiation occurred on media containing only one PGR, reaching values of 1.13 % for BA (1 mg dm⁻³), 1.25 % for 2,4-D (1 mg dm⁻³) and 5 % for NAA (1 mg dm⁻³); the latter is the most effective treatment among sole PGRs. Explants cultured on medium lacking PGRs showed no response.

Table 1. The effect of plant growth regulators (PGR) on the initiation frequency (IF) of embryogenic tissues from immature zygotic embryos from seeds collected in different years.

PGR	Conc. [mg dm ⁻³]	2000 number of explants	IF [%]	2001 number of explants	IF [%]
PGR-free	0	80	0.00	79	3.89
2,4-D	1.0	80	1.25	84	7.14
BA	1.0	88	1.13	83	3.61
2,4-D + BA	2.0 + 0.5	88	2.27	81	7.40
2,4-D + BA	0.5 + 2.0	88	4.54	99	1.01
2,4-D + BA	2.0 + 2.0	88	5.68	84	8.33
NAA	1.0	80	5.00	88	9.09
NAA + BA	2.0 + 0.5	84	1.19	77	6.49
NAA + BA	0.5 + 2.0	80	8.75	93	7.52
NAA + BA	2.0 + 2.0	88	2.27	102	5.88

Table 2. The effect of sugars (2 %) on the initiation frequency (IF) of embryogenic tissues from immature zygotic embryos from seeds collected in different years.

Sugar	2000 number of explants	IF [%]	2001 number of explants	IF [%]
Sucrose	88	2.27	87	7.40
Maltose	88	6.81	80	8.75
Glucose	88	1.13	63	1.59
Fructose	88	0.00	87	1.13

In 2001, the initiation frequencies were higher than in year 2000. In total, 61 cell lines have been initiated from 1101 cultured megagametophytes; the initiation frequency was 5.54 %. Initiation occurred on all the media tested, even without PGRs (Table 1). Surprisingly high initiation frequencies have been obtained on media with 2,4-D (7.14 %) or NAA (9.09 %) alone.

Testing the sugars effect, the culture media were

supplemented with 2,4-D (2 mg dm⁻³) and BA (0.5 mg dm⁻³) and contained sucrose, glucose, fructose, or maltose (each at 2 %). No embryogenic tissue initiation was observed on medium containing fructose. When we compared the effect of the other 3 sugars, maltose was more effective than sucrose or glucose and led to the highest somatic embryo initiation frequency (6.81 %). In the presence of glucose and sucrose the initiation frequencies significantly decreased, reaching values 2.27 % (sucrose) and 1.13 % (glucose). In 2001, among the sugars used, maltose and sucrose gave the best results. The media containing glucose or fructose were less effective for the initiation of embryogenic tissue (Table 2).

Initiation occurred mostly during the first 6 weeks of culture, and afterwards decreased significantly. The survival of cell lines in long-term cultures was 81.96 %. To determine the origin of somatic embryos, several megagametophytes showing extrusion of embryogenic tissue were opened and the zygotic embryos were examined microscopically. Small somatic embryos were visible close to the suspensor. We suppose they differentiated from the suspensor but their exact origin remains still unclear.

To reveal the morphological and cytological features of somatic embryos, the cell lines growing in long-term cultures were investigated using "squash" preparations. Microscope observations revealed differences in somatic embryo morphology among cell lines. Some of them contained well-developed somatic embryos. In these cell lines the somatic embryos were organized as bipolar structures. The embryonal part had a regular outline and consisted of tightly packed meristematic cells with attached long vacuolated suspensor cells. The cells of the suspensor were often arranged into bundles - group 1 somatic embryos (Fig. 1A). Other cell lines were characterised by the presence of less developed somatic embryos. In these cultures, the somatic embryos were also bipolar structures with the embryonal part organized as a loosely aggregated mass of meristematic cells. The long vacuolated suspensor cells were attached to the embryonal part without their organization into bundles - group 2 somatic embryos (Fig. 1B). A few cell lines contained small aggregates of meristematic cells intermingled with long, vacuolated suspensor-like cells. In these cultures, the occasionally observed few meristematic cells connected with 1 - 2 long vacuolated ones were the only structures resembling somatic embryos - group 3 somatic embryos (Fig. 1C). The somatic embryo morphology differed among cell lines, but within each cell line they were similar. Among established cell lines the cultures with the group 2 somatic embryos over-dominated. Well-organized somatic embryos were observed only in 9 cell lines out of the 92 investigated (including cell lines initiated in 1998). Similarly, the group 3 somatic embryos were present only in a few cell lines.

For the maturation experiments we selected 30 cell

lines characterized by the presence of somatic embryos with different morphology. Within one group the results were similar and therefore in Table 3 only data for 15 cell lines are given.

Mature somatic embryos with well-formed cotyledons (Fig. 1D) developed in cultures containing bipolar somatic embryos with well organized embryonal and suspensor parts (group 1 somatic embryos). After maturation and desiccation treatment these somatic embryos germinated (Fig. 1E) and regenerated plantlets (Fig. 1F).

In cultures containing less organized somatic embryos (group 2 somatic embryos), the development was restricted mainly to the precotyledonary stage.

Occasionally, later developmental stages were also observed, but in this case the developing somatic embryos showed morphological abnormalities such as fused cotyledons or precocious germination. Due to their abnormal appearance these embryos were not selected for desiccation. Unsatisfactory maturation occurred in cell lines with group 3 somatic embryos. In these cell lines only a few developing precotyledonary somatic embryos were observed: they never passed later developmental stages, so no plantlets were obtained.

Although all the “regenerating” cell lines produced mature cotyledonary somatic embryos we have noticed differences in their maturation capacity (Table 3).

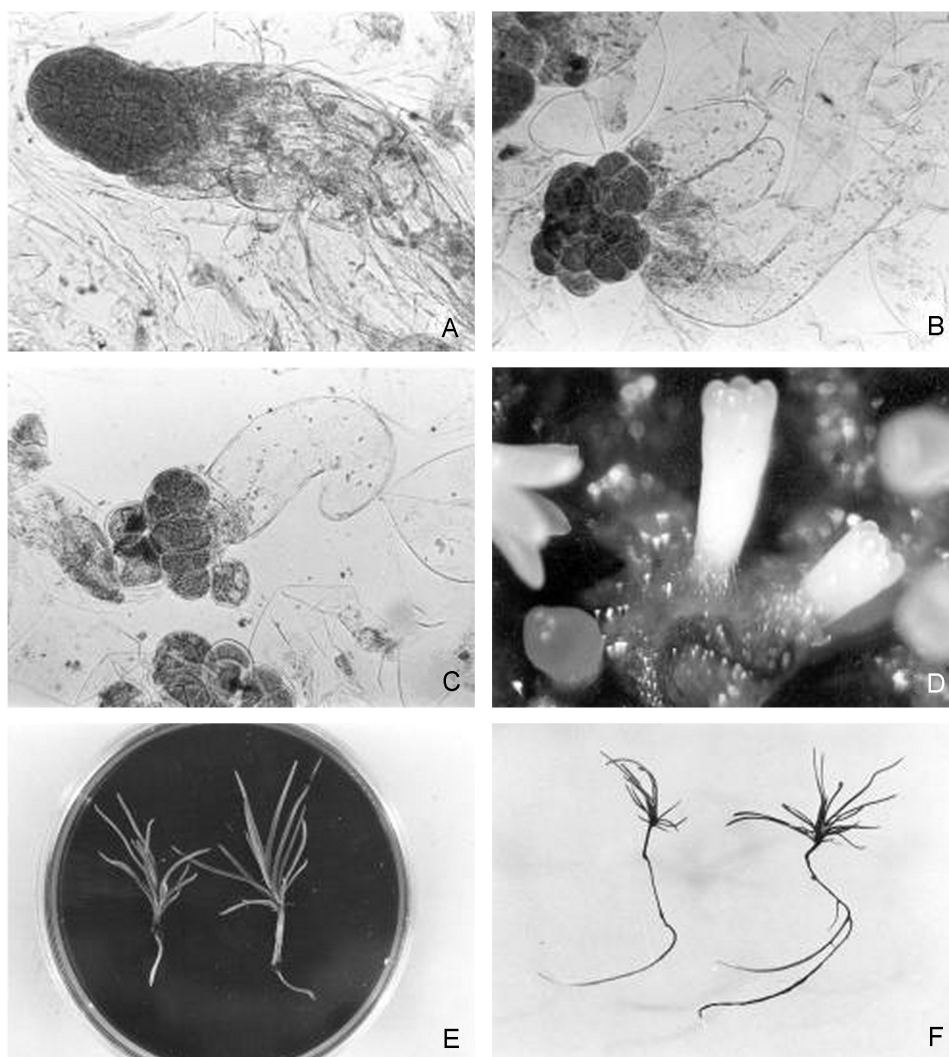


Fig. 1. A - Somatic embryo with tightly packed meristematic cells in embryonal part and long vacuolated cells arranged into bundles (categorized as group 1, cell line E50); B - somatic embryo with loosely packed meristematic cells and few vacuolated long suspensor cells (categorized as group 2, cell line E47); C - bipolar structure representing the less developed somatic embryos (categorized as group 3, cell line E49); D - cotyledonary somatic embryos developed on maturation medium (cell line E103); E - emblings developed after germination treatment (cell line E103); F - plantlets regenerated from somatic embryos (cell line E50).

Discussion

In the experiments with *P. nigra* the initiation frequencies were relatively low despite the inclusion of wide range of concentration and combination of PGRs or alteration of carbon source in the medium. We suppose the low initiation frequencies were a consequence of high variability in the developmental stage of the zygotic embryo at time of collection (year 2000). Higher initiation frequencies were obtained when immature zygotic embryo explants showed no variability (they were in the same precotyledonary developmental stage), although an optimized protocol has not been obtained. In *Pinus* species the developmental stage of zygotic embryos strongly influenced the initiation frequencies (Klimaszewska and Smith 1997, Häggman *et al.* 1999, Miguel *et al.* 2000). Low initiation frequencies can also be the result of open pollination and genetic differences among selected trees (Jones and Van Staden 1995).

Table 3. Somatic embryo maturation in different cell lines of *Pinus nigra* Arn. Mean numbers of developing somatic embryos calculated per 1 g of fresh mass inoculum \pm SE are given (* - cell lines categorized as group 1, ** - cell lines categorized as group 2, *** - cell lines categorized as group 3).

Cell line	Precotyledonary SE	Cotyledonary SE	Germination [%]
E 19***	5.0 \pm 1.32	-	-
E 27***	7.0 \pm 2.21	-	-
E 34**	78.0 \pm 16.31	abnormal	-
E 42*	59.0 \pm 10.39	33.0 \pm 7.95	41.90
E 43**	25.0 \pm 7.94	abnormal	-
E 47**	33.0 \pm 6.14	abnormal	-
E 49***	4.0 \pm 0.86	-	-
E 50*	80.0 \pm 12.89	50.0 \pm 9.32	39.95
E 52**	39.0 \pm 10.66	abnormal	-
E 57***	59.0 \pm 22.12	-	-
E 103*	122.0 \pm 10.73	54.0 \pm 3.99	47.86
E 104*	135.0 \pm 4.08	42.0 \pm 4.79	42.53
E 106**	32.0 \pm 6.47	abnormal	-
E 113**	24.0 \pm 3.03	abnormal	-
E 114**	51.0 \pm 10.93	abnormal	-

Somatic embryogenesis occurred on most media formulations containing PGRs including those with only one PGR. In *P. pinaster* and *P. sylvestris*, PGRs were not an absolute-requirement for somatic embryogenesis. The process occurred also on PGR-free medium with relatively high frequency, but 3 - 6 weeks delay was observed in their growth (Lelu *et al.* 1999). Klimaszewska *et al.* (2001) reported high initiation frequencies in *P. strobus* by using lower PGR concentration in the medium.

In recent years, several sources of evidence suggest the role of sugars for the initiation of somatic

embryogenesis and different species have given different responses, depending on the carbon source. In *Feijoa sellowiana*, sugar concentrations improve somatic embryogenesis both as a carbon source and as an osmotic regulator (Canhoto and Cruz 1994). In *P. nigra* maltose and sucrose were found to have beneficial effect on somatic embryo initiation.

Microscope observations showed significant differences in the morphology of somatic embryos from different cell lines. Differences in the structural features have also been observed in other *Pinus* species (Laine and David 1990, Keinonen-Mettälä *et al.* 1996). Bercethe and Paques (1995) categorized the *P. pinaster* embryogenic cultures based on the morphology of somatic embryos into 3 groups: distinguishing cultures with poorly-organized somatic embryos, well-organized polarized structures, and structures built by densely packed embryonic cell surrounded by long vacuolated suspensor cells. The causes are unknown and differences even occurred in lines derived from the same cone. Mo *et al.* (1996) supposed at least partly genetic differences based on the fact that mature somatic embryos of group A produced new cell lines with somatic embryos belonged to A group. This feature remains stable during long-term maintenance (Von Arnold *et al.* 1996) and did not change even after cryopreservation (Nörsgaard *et al.* 1993). Domon *et al.* (1994) have made a comparative study of extracellular proteins among phenotypes of one-stage somatic embryos of *P. caribaea*. Their results showed that different phenotypes of somatic embryos were characterized by qualitative and quantitative variations of extracellular proteins. In Norway spruce embryogenic cultures, the arabinogalactan proteins have been found to play an important role in the development of somatic embryos (Egertsdotter and von Arnold 1995). The proposed role of arabinogalactan proteins (AGPs) is for the generation of signal molecules of oligosaccharide nature (Domon *et al.* 2000).

In embryogenic cultures of *P. nigra*, the somatic embryo maturation showed interaction with cytological and morphological features of early stage somatic embryos. Only cultures containing well-formed somatic embryos (group 1) gave mature embryos capable of plantlet regeneration. Ramarosandratana *et al.* (1999) found the well-structured somatic embryos of *P. pinaster* were capable of maturation using a wide range of treatments, including also liquid medium. In our experiment the maturation medium included ABA and maltose because this combination gave the best results among all the tested treatments (Salajova *et al.* 1999).

Among all the long-term-maintained embryogenic cultures of *P. nigra*, the occurrence of cell lines with well-developed somatic embryos was infrequent. Cell lines with structurally less organized somatic embryos over-dominated. A similar phenomenon was described

for *P. sylvestris* embryogenic cultures. Out of 139 cell lines investigated only 10 were observed containing well structured somatic embryos (Keinonen-Mettälä *et al.* 1996).

In *P. nigra* embryogenic cultures the less organized somatic embryos developed abnormally and resulted no vigorous plantlets, therefore it would be particularly desirable to regulate their morphology and improve their maturation. According to the work of Sallandrouze *et al.* (1999), in *Cupressus sempervirens* adding bovine serum

albumine (BSA) to the culture medium yielded well-structured somatic embryos undergoing development and maturation. The authors conclude the possible role of BSA is the maintenance of cell cohesion in the embryogenic and suspension regions and, in turn, it is a prerequisite for development of well-formed somatic embryos. Another possibility by which to change the somatic embryo morphology, could be the use of maltose treatment for the initiation as well as the maintenance of embryogenic tissues (Gupta *et al.* 1997).

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