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The effect of culture medium formulation on *Pinus nigra* somatic embryogenesis

Abstract: The potential of immature zygotic embryos to produce embryogenic tissue was tested using culture media differed in inorganic salt composition as well as plant growth regulator content. Explants cultured on DCR as well as MLV media gave the highest initiation percentages with maximum values of 8.8% (MLV) to 10.4% (DCR). Plant growth regulators content (standard or reduced) had no profound effect on embryogenic tissue initiation. Somatic embryo maturation as well as germination was dependent on the cell line and salt composition of the medium. Plantlet regeneration occurred in three cell lines out of five tested. During the maturation process profound changes occurred in the internal organisation of somatic embryos such differentiation of root meristem in precotyledonary somatic embryos and formation of provascular strands in cotyledonary somatic embryos.

Additional key words: European black pine, histology, conifers, plant growth regulators, somatic embryos

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Abbreviations: PGRs (plant growth regulators), 2, 4-D (2, 4-dichlorophenoxyacetic acid) BA (N6-benzyl adenine), ABA (abscisic acid)

Introduction

Somatic embryogenesis represents a powerful tool for vegetative clonal propagation of conifers as well as an experimental system for the study of conifer early development. Since the first report of initiation of somatic embryogenesis in Norway spruce (Hakman et al. 1985, Chalupa 1985) the process was described for many other conifer species (Klimaszewska and Cyr 2002).

The process of somatic embryogenesis in conifers comprises of initiation, multiplication (prolifera-tion), maturation, germination (Pullman et al. 2003) and each step has special requirements in order to obtain successful plant regeneration. The initiation of embryogenic tissues is a crucial step and, however, it has been reported for relatively large number of conifer species this step remains still problematic. Low initiation frequencies are often obtained, many desired seed sources are recalcitrant and tissue survival is often poor (Pullman et al. 2009). Many attempts have been done in order to improve the initiation of embryogenic tissues in conifers. The effort focused mainly on testing of nutrient medium com-

position and plant growth regulators (Ewald et al. 1995, Hazubska-Przybyl and Bojarczuk 2008), carbon sources (Salajova and Salaj 2005), vitamins (Pullman et al. 2006), medium salt composition (Lelu et al. 1994, Becwar et al. 1990, Miguel et al. 2004, Kim et al. 2009, Krajňáková et al. 2008), polyamines effect (Nakagawa et al. 2011) as well as environmental (Vookova and Kormutak 2009) and genetic effects (Cheliak and Klim·aszewska 1991, Niskanen et al. 2004, Yildirim et al. 2006) have been studied.

In *Pinus nigra* Arn. embryogenic tissues have been initiated (Salajova and Salaj 1992) and the effect of zygotic embryo developmental stage (Salajova et al. 1995), different concentration as well as combination of plant growth regulators and carbon source on the initiation process have been tested (Salajova and Salaj 2005). In several conifer species it was demonstrated the mineral composition of culture medium affected the initiation of somatic embryogenesis as well as somatic embryo maturation (Becwar et al. 1990, Klimaszewska et al. 2001). In *Pinus nigra* this factor has not been studied yet. Therefore, the objective of this work was to determine the effect of mineral composition of culture medium on the process of somatic embryogenesis in *Pinus nigra* Arn.

Material and methods

Plant material

Megagametophytes dissected from unripe seeds of *Pinus nigra* Arn. and containing immature zygotic embryos were used as explants.

The green cones were collected from open pollinated trees (at least from 12 trees depending on the cone yield in a given year) and stored in paper bags for several days (4 to 7) at 4°C. The cones were washed in tap water, blotted dry with a filter paper and the unripe seed were dissected and surface sterilized with 10% H_2O_2 for 10 min., afterwards they were rinsed four times with sterile distilled water. Finally, the megagametophytes were excised from seeds and placed on the culture media. Eight explants were cultured in each plastic Petri dish (6 cm in diameter) in the dark at 23°C.

Experiment 1

Culture medium composition

Four culture media differing in inorganic salt composition were tested: DCR (Gupta and Durzan 1985), LV (Litvay et al. 1981) with full or half concentration of macro- and microelements (MLV) and QP (Quoirin and Lepoivre 1977). The media were supplemented with glutamine 50 mg·l⁻¹, enzymatic casein hydrolysate 500 mg·l⁻¹, myo-inositol 200 mg·l⁻¹,

vitamins (thiamine-HCl 1 mg·l⁻¹, pyridoxine-HCl 0.5 mg·l⁻¹, nicotinic acid 0.5 mg·l⁻¹) glycine 2 mg·l⁻¹, sucrose (2%) and solidified with gellan gum (Gelrite ™, 0.3%). Plant growth regulators (PGRs) were used at a „standard" (9 μM 2, 4-D and 2.2 μM BA) as well as at reduced 2,4-D concentration (2.25 μM 2,4-D and 2.2 μM BA, media abbreviated as DCR-R, LV-R, MLV-R, QP-R). The pH of the medium was adjusted to 5.8 before autoclaving. The experiments were carried out for two consecutive years (2007, 2008) and in total 1856 megagametophytes were cultured.

Experiment 2

Based on 2007 and 2008 results in the following years (2009, 2010, 2011) DCR medium was chosen to study the effect of „standard" (9 μM 2, 4-D and 2.2 μM BA) or reduced (2.25 μM 2, 4-D and 2.2 μM BA) PGRs content in the medium. In these experiments (including years 2007 and 2008 for PGRs effect) altogether 3938 explants were cultured. The medium composition and culture conditions were the same as in Experiment 1.

Somatic embryo maturation

After 7–8 days of growth cycle the embryogenic tissues growing in clumps on proliferation medium were suspended in a culture medium without PGRs (see below) and the suspension containing approximately 150 to 200 mg of fresh mass was pipetted on the stacked filter paper discs to absorb the liquid. The upper filter paper disc with cells was then transferred to a maturation medium. The maturation media were the same as for initiation (DCR, LV, MLV, QP) with respect to macro-and microelements and were supplemented with 95 μM ABA (± abscisic acid, SIGMA), glutamine (50 mg·l⁻¹), enzymatic casein hydrolysate (500 mg·l⁻¹), vitamins (thiamine-HCl 1 mg·l⁻¹, pyridoxine-HCl 0.5 mg·l⁻¹, nicotinic acid 0.5 mg·l⁻¹), glycine 2 mg·l⁻¹, myoinositol 200 mg·l⁻¹ and maltose 6%. The media were solidified with 1% gellan gum. The tissues were cultured on maturation media for 8 weeks and afterwards the cotyledonary somatic embryos were selected for germination. The maturation of somatic embryos occurred in the dark at 23°C. After eight weeks of maturation the number of somatic embryos was counted using stereomicroscope and the maturation capacity was calculated as the number of cotyledonary somatic embryos in 1 g of fresh mass inoculum. In the case that no cotyledonary somatic embryos were obtained, the precotyledonary somatic embryos were counted.

For germination of somatic embryos the same media were used as for maturation (DCR, LV, MLV, QP with the same organic compounds) without ABA and supplemented with 2% maltose, activated charcoal (1%) and gellan gum (0.5%). Eight somatic

embryos were cultured per each Petri dish (6 cm in diameter).

The germination occurred in the dark for 10–12 days and afterwards the somatic embryos were transferred to the light. After 3 to 4 weeks the germinating embryos were transferred to the Magenta jars with the same medium as used for germination and cultured in light.

Statistical analysis

The data on initiation, maturation and germination were evaluated according to Erdelský and Frič (1979). Statistically mean differences were determined by Student´s test. The differences between treatments were calculated at $P \leq 0.05$ or $P \leq 0.01$.

Histological observation

Tissue samples (proliferating embryogenic tissues, precotyledonary and cotyledonary somatic embryos) were fixed in FAA for 4 hours, dehydrated in tertiary butyl alcohol series and embedded in paraffin. Sections were stained with safranine and fast green. For the investigation of early somatic embryo structure on proliferation medium small pieces of tissue were stained with acetocarmine (2%) and squashed. The samples were examined under Zeiss Axioplan 2 bright field microscope.

Results

Experiment 1

The megagametophyte explants started to extrude the embryogenic tissues from the micropyle after 2–3 weeks in culture. Extrusions (Fig. 1A) were observed with various frequencies and the initiation was considered to be successful when the tissues survived at least 6 weeks after extrusion.

The extruded tissues grew relatively fast, forming white, translucent, soft masses containing bipolar somatic embryos (Fig. 1B) and could be separated from the megagametophyte explants when reached approximately 5 mm in diameter. Most of the non-extruding explants necrotised and turned brown. Some of the megagametophytes formed white-yellowish non-embryogenic calli and we never observed conversion of such callus into embryogenic tissue.

The initiation frequencies calculated as the percentage of initiated tissues from the total number of explants varied depending on the culture medium composition (Table 1). The highest initiation frequencies in both years were obtained for DCR medium with reduced concentration of PGRs (2.25 μM 2, 4-D and 2.2 μM BA) and reached 10.3 and 10.4 %. The explants cultivation on modified LV medium (MLV) resulted in slightly lower initiation frequencies (7.3 to 8.8%). Similar results were obtained for DCR medium with „standard" concentration of PGRs and the differences between DCR and MLV media effect were statistically not significant. LV medium with full content of macro- and microelements gave poor results (2.1% in one year). The QP medium was not suitable for SE initiation because only one cell line was produced (0.74%). This cell line survived only six weeks and afterwards necrotised.

Experiment 2

To further study the effect of „standard" or reduced PGRs concentrations on somatic embryos initiation and growth, DCR was selected (Table 2) because in two consecutive years (2007, 2008) this medium supported the highest initiation frequencies. In 2007 the differences in initiation frequencies

Table 1. Initiation of somatic embryogenesis from immature zygotic embryos of *Pinus nigra* using different culture media. Initiation frequencies (IF) are expressed as % of explants producing embryogenic tissues per total number of cultured explants. Values represent means ± SE

| Medium | Years | | | |
| | 2007 | | 2008 | |
	IF	No. of initiated cell lines	IF	No. of initiated cell lines
DCR	5.2 ± 1.84[a]	5	9.6 ± 0.7[a]	13
DCR-R	10.4 ± 1.4[b]	10	10.3 ± 0.93[a]	14
LV	2.1 ± 1.4[a]	2	0.0[b]	0
LV-R	0.0	0	0.0[b]	0
MLV	8.3 ± 2.81[a,b]	8	7.4 ± 2.72[a]	10
MLV-R	7.3 ± 2.86[a,b]	7	8.8 ± 1.79[a]	12
QP	0.0	0	0.7 ± 0.73[b]	1
QP-R	0.0	0	0.0[b]	0

For each medium 96 explants were cultured in 2007 and 136 explants in 2008.
Data in columns displaying the same letters are not different at $P \leq 0.05$ or $P \leq 0.01$ within the given year
Media DCR, LV, MLV, QP were with 9 μM 2, 4-D and 2.2 μM BA
Media DCR-R, LV-R. MLV-R, QP-R were with 2.25 μM 2, 4-D and 2.2 μM BA
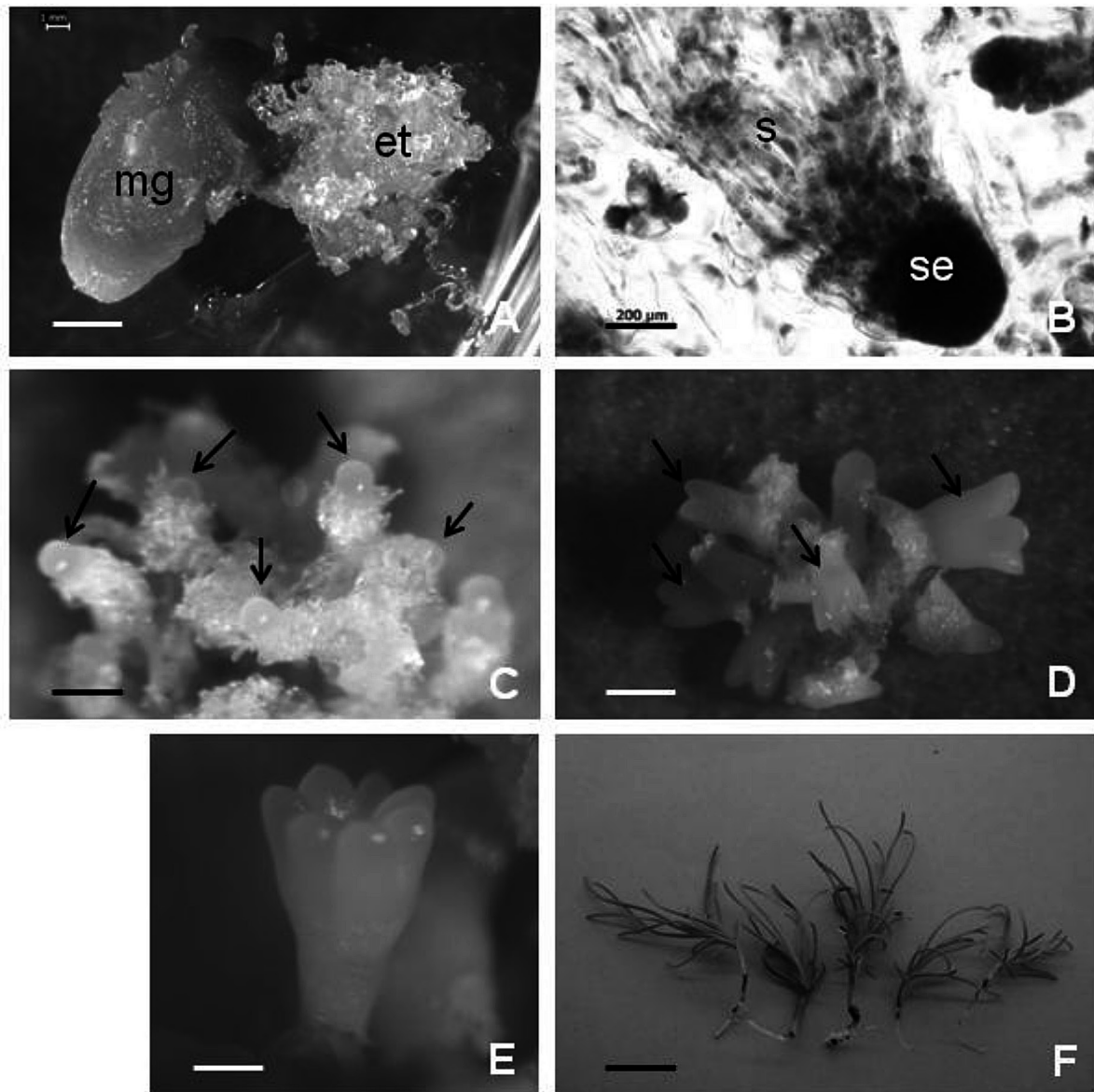
Fig. 1. Somatic embryogenesis in *Pinus nigra* Arn.
A – Embryogenic tissue (et) extrusion from micropylar end of the megagametophyte (mg). **B** – Bipolar somatic embryo (se) observed in embryogenic tissue on proliferation DCR medium (s – suspensor cells). **C** – Precotyledonary somatic embryos (after 5–6 weeks of development on maturation medium). **D** – Cotyledonary somatic embryos after 8 weeks of development. **E** – Details of cotyledonary somatic embryo. **F** – Regenerated somatic seedlings with cotyledons and roots. Bars: A – 2 mm, B, D – 200 μm, C, E – 100 μm, F – 1 cm.

were apparent and significant (P≤ 0.05), 5.2 % versus 10.4% in favour of reduced PGRs content but in subsequent years the differences were less apparent and the benefitial effect of DCR-R has not been confirmed for this period.

Somatic embryo maturation

After transfer of embryogenic tissues to maturation medium, somatic embryo development occurred and around the third week the first precotyledonary embryos could be distinguished. After approximately six weeks on maturation medium the precotyledonary somatic embryos were connected to the tissue

with apparent suspensor and the embryonal part was bullet-shaped, opaque and clearly distinguishable from the suspensor (Fig. 1C). Cotyledonary somatic embryos (Figs. 1D, 1E) appeared in high numbers in later period, mostly around the 8th week of cultivation. The cotyledonary somatic embryos germinated and converted into somatic seedlings (Fig. 1F).

Quantitative evaluation: Somatic embryo maturation was dependent on the cell line and medium composition (Table 3). Out of six tested cell lines two of them (E267, E306) produced only precotyledonary somatic embryos without further development. In the remaining cell lines somatic embryo develop-

Table 2. Initiation of embryogenic tissues in 2007–2011 on media DCR and DCR-R. Initiation frequencies (IF) are expressed as % of explants producing embryogenic tissues per total number of cultured explants. Values represent mean ± SE

| Years | Medium | | | |
| | DCR | | DCR-R | |
	IF	No. of initiated cell lines	IF	No. of initiated cell lines
2007	5.2 ± 1.84	5	10.4 ± 1.4	10
2008	9.6 ± 0.7[a]	13	10.3 ± 0.93[a]	14
2009	3.4 ± 1.15[a]	10	2.7 ± 1.43[a]	7
2010	1.6 ± 0.61[a]	13	0.7 ± 0.93[a]	7
2011	3.8 ± 0.87[a]	18	4.5 ± 1.13[a]	22
Mean	4.7 ± 1.32[a]		5.7 ± 1.97[a]	

Data in columns displaying the same letters are not different at $P \leq 0.05$ or $P \leq 0.01$ between two media in the given year
Number of cultured explants is 1827 for DCR and 2037 for DCR-R.
Medium DCR (9 μM 2, 4-D and 2.2 μM BA)
Medium DCR-R (2.25 μM 2, 4-D and 2.2 μM BA)

ment occurred and was dependent on the medium composition. In cell lines E262 cotyledonary somatic embryos developed and the highest number has been obtained on DCR medium. On MLV medium the number of cotyledonary somatic embryos was lower and the differences were statistically significant at P ≤ 0.05. Precotyledonary somatic embryos developed on LV and QP media but their further development was arrested. Similarly, in cell line E 323 media LV and QP gave very low number of cotyledonary somatic embryos and for this cell line media DCR or MLV were more successful. Cell lines E 326 and E 331 were characterised by high embryogenic capacity and as a result produced numerous cotyledonary somatic embryos. The differences in quantity of cotyledonary somatic embryos developed on various medium formulations were less apparent and statistically not significant for cell line E 326. In cell line E 331 medium QP gave the lowest yield of cotyledonary somatic embryos which was significantly different from other three media (P≤ 0.05).

The effect of media formulations on the somatic embryo germination was more apparent. Cotyledonary somatic embryos that developed on media DCR or MLV germinated in higher frequencies (Table 4).

Table 3. Maturation of somatic embryos on different culture media. The maturation capacity was calculated as number of somatic embryos per 1 g of fresh mass inoculum (pc – precotyledonary somatic embryos, cot – cotyledonary somatic embryos). Values represent means ± SE

| Embryogenic line | Maturation capacity | | | |
	DCR	MLV	LV	QP
E262	57 cot ± 7.49	25 cot ± 6.97	34 pc ± 5.26[a]	29 pc ± 7.53[a]
E267	33 pc ± 5.73[a]	26 pc ± 5.75[a]	0.0	0.0
E306	0.0	0.0	0.0	31 pc ± 7.35
E323	35 cot ±17.13[a]	37 cot ±26.19[a]	6 cot ± 4.85[a]	8 cot ± 8.00[a]
E326	203 cot ±25.30[a]	235 cot ± 54.59[a]	208 cot ±75.82[a]	170 cot ± 50.58[a]
E331	198 cot ±22.71[b]	136 cot ±18.11[a]	269 cot ±76.02[ab]	96 cot ± 27.28

Data in columns displaying the same letters are not different at $P \leq 0.05$ or $P \leq 0.01$ among media in the given cell line.

Table 4. Germination (%) of somatic embryos on different culture media. The germination percentages express the number of germinating embryos out of the total number of cotyledonary somatic embryos cultured on germination medium. Values represent means ± SE

| Embryogenic line | Germination % | | | |
	DCR	MLV	LV	QP
E262	30.7 ± 2.14[a] (52[SO])	24.5 ± 2.45[a](40[SO])	NT	NT
E267	NT	NT	NT	NT
E306	NT	NT	NT	NT
E323	NT	NT	NT	NT
E326	71.3 ± 8.34[a] (56[SO])	45.0 ± 8.53[a] (54[SO])	0.0 (40[SO])	0.0 (37[SO])
E331	55.0 ± 3.0[a] (50[SO])	60.3 ± 3.13[a] (58[SO])	25.4±8.4[b](48[SO])	15.5±5.57[b](37[SO])

Data in columns displaying the same letters are not different at $P \leq 0.05$ or $P \leq 0.01$ among media in the given cell line;
[SO] indicate the number of tested somatic embryos;
[NT] not tested (the yield of cotyledonary somatic embryos was very low, or no cotyledonary somatic embryos were obtained).
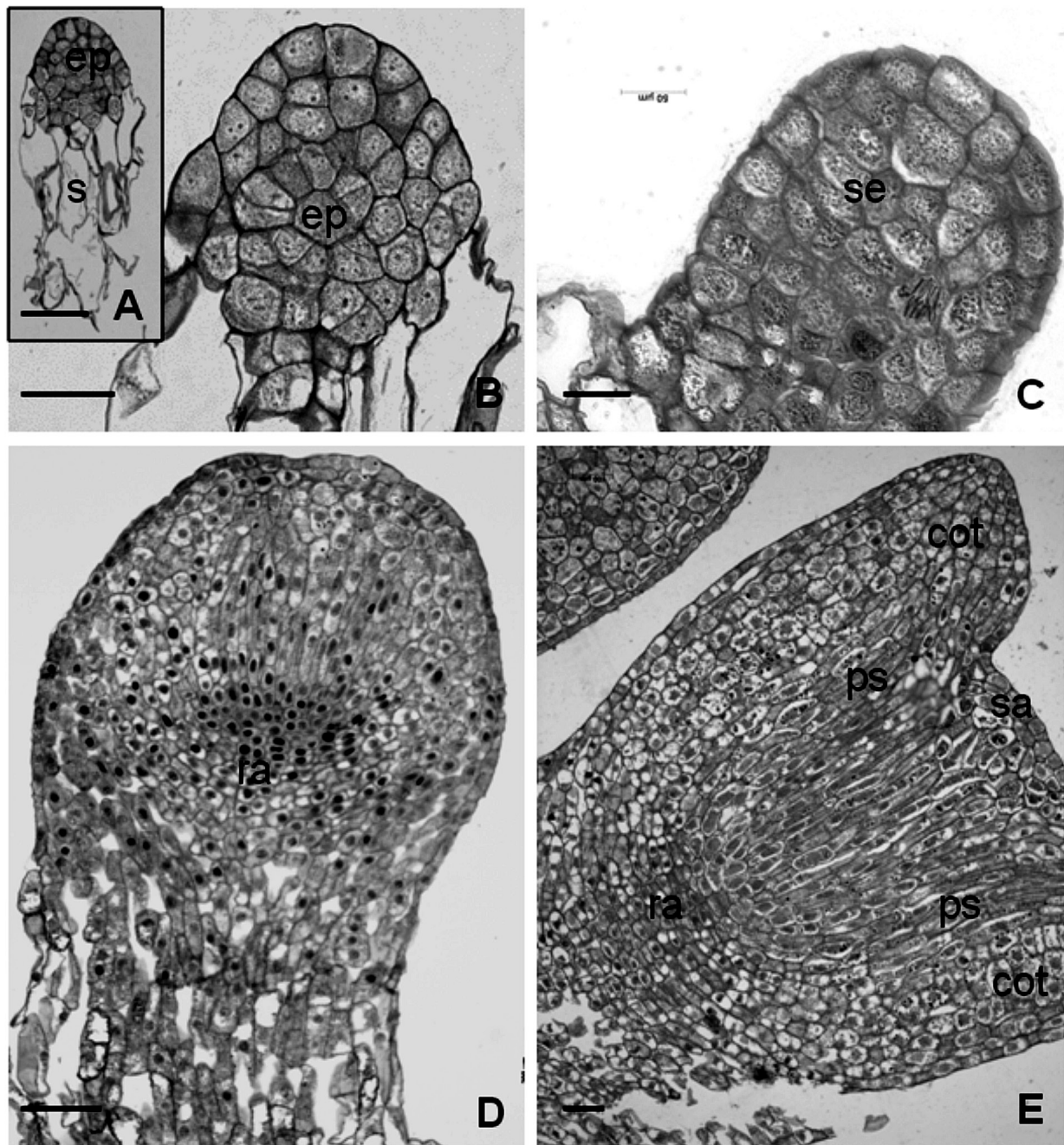
Fig. 2. The histology of somatic embryo development in *Pinus nigra* Arn.
A – Somatic embryo structure on the proliferation medium. The embryos are composed of the meristematic embryonal part (ep) subtended by long vacuolised cells of suspensor (s). **B** – Detailed view of meristematic cluster giving rise to somatic embryo. **C** – Early stage of somatic embryo with mitotic cells. **D** – Precotyledonary bipolar somatic embryo with differentiated root apical meristem (ra) aproximately after 6 weeks on a maturation medium. **E** – Mature somatic embryo with fully developed shoot (sa) and root (ra) apex, cotyledons (cot) and procambial strands (ps). Bars: A, D – 200 μm, B – 50 μm, C, E – 100 μm.

Histology of the somatic embryo development

For the histological study somatic embryos in proliferation as well as precotyledonary and cotyledonary stages of development were used and their internal structure was compared.

The somatic embryos in proliferation stage were bipolar structures with highly meristematic embryonal part as well as long vacuolised suspensor (Figs 2A, B) and mitotic cells were also present (Fig. 2C).

The precotyledonary somatic embryos (approximately 6 weeks on maturation medium) were con-

nected to the tissue with apparent suspensor and the embryonal part consisting of meristematic cells was bullet-shaped, opaque and clearly distinguishable from a suspensor. At this stage of development – in comparison with somatic embryos on the proliferation medium, some changes were observable in the organization of embryonal part, and the most apparent feature was differentiation of root meristem (Fig. 2D). Cotyledon primordia were not yet present. In cotyledonary somatic embryos (Fig. 2E) both the shoot and root apicies were already developed. The most conspicuous feature of cotyledonary somatic embryos was the differentiation of the procambium (Fig. 2E).

Discussion

We have evaluated the somatic embryos initiation frequencies using media with different inorganic salt composition. The results suggest profound effect of medium salt composition on embryogenic tissue initiation, and the results from for all the used media were reproducible in two consecutive years. The different intitation frequencies suggest that the embryogenic tissue initiation can be optimised by modification of medium compositon and in some cases the initiation could also be extended to recalcitrant genotypes (Becwar et al. 1990). For the most of conifer species the presence of PGRs in culture medium is emphasized. Recently it was found that lower PGR content in medium than routinely used, had beneficial effect on the initiation process. For *Pinus strobus* decrease of PGR concentration in medium (2, 4-D from 9.5 μM to 2.2 μM and 4.5 μM BA to 2.2 μM) led to increase of initiation frequencies from 20% to 53% (Klimaszewska et al. 2001). In our experiments for *Pinus nigra*, by reducing the PGR content in the medium we obtained increase in the initiation frequencies for DCR medium in 2007 but experiments in the following years did not confirm the beneficial effect of lowered PGR concentration. For *Pinus nigra*, similarly as for other *Pinus* species (Becwar et al. 1990, Miguel et al. 2004, Klimaszewska et al. 2001, Carneros et al. 2009) the stage specific response of zygotic embryo explants to somatic embryogenesis initiation was observed and seemed to be an important factor. In our previous experiments we have used different concentration and combination of PGRs (2, 4-D, BA, NAA) for the initiation of somatic embryogenesis. Apart from PGRs content the initiation frequencies were higher when zygotic embryos were in the precotyledonary developmental stage (Salajova and Salaj 2005). In *Araucaria angustifolia* the initiation frequencies were affected by the developmental stage of explants, and the presence or absence of PGRs as well as basal salt composition of the medium did not

influence the somatic embryo induction frequencies, although in the maintenance phase cell browning occurred on media free of PGRs (dos Santos et al. 2002).

The initiation frequencies obtained for *Pinus nigra* (maximum 10.3 and 10.4%) in the present experiments are lower in comparison with initiation frequencies for *Pinus radiata* with 55% initiation frequency on average (Hargreaves et al. 2009) or 20-53% for *Pinus strobus* (Klimaszewska et al. 2001). The initiation of somatic embryogenesis in conifers is under strong genetic control as has been mentioned earlier in the text (Niskanen et al. 2004). Depending on the family, in *Pinus sylvestris* the initiation frequencies varied from 0 to 26.7% (Aronen et al. 2009). Similarly in *Pinus armandii* across nine open-pollinated families, initiation frequency ranged from 0 to 20% with an average of 1.5% (Maruyama et al. 2007). In our experiments, the megagametophytes with immature zygotic embryos were excised from open pollinated seeds, so the exact genetic background is unknown. The influence of genetic factors cannot be excluded for *Pinus nigra* because despite of adjustment of other factors such as zygotic embryo developmental stage (Salajová et al. 1995), PGRs and carbohydrates effect (Salajová and Salaj 2005) as well as basal medium formulation (present results) have resulted in initiation frequencies up to a maximum of 24.1% (Salajová et al. 1999).

The development of bipolar early conifer somatic embryos into precotyledonary and cotyledonary developmental stages is influenced by several factors. Abscisic acid belongs to PGRs with stimulatory effect on somatic embryo maturation (Vágner et al. 1998). ABA-treated somatic embryos accumulate storage reserves and develop in a similar manner as their zygotic counterparts in seeds (von Arnold and Hakman 1988). Abscisic acid also influenced the quantity of produced somatic embryos in *Pinus strobus*. Higher concentration of ABA (120 μM) in culture medium resulted in significantly higher number of mature somatic embryos (Klimaszewska and Smith 1997). In *Pinus sylvestris* the effect of ABA concentration (80 μM versus 120 μM) was significant in the presence of 0.2 M sucrose. Using lower concentration of sucrose (0.1 M) no differences in the two ABA concentration effect were observed (Lelu-Walter et al. 2008). The ABA concentration for maturation of *Pinus nigra* somatic embryos was tested in our previous experiments. Lower concentrations (0.38 μM, 3.8 μM, 19 μM and 38 μM) were not effective and the maturation did not result in somatic seedling regeneration (Salajova et al. 1995). Somatic seedlings regeneration occurred using 95 μM ABA (Salajova et al. 1999). Somatic embryo maturation is also stimulated by high concentration of gellan gum in the presence of ABA. In *Pinus strobus*, medium with high gel strength (1% gellan gum) had beneficial effect on somatic embryo

maturation in the four tested cell lines. However, the mature somatic embryos had lower water potential and germinated at higher frequency (Klimaszewska and Smith 1997). In *Picea abies* (Svobodová et al. 1999 and *Abies* hybrids (Salaj et al. 2004) polyethylene glycol improved somatic embryo maturation.

In this work we observed the effect of a basal medium formulation on maturation of somatic embryos. Quantitative differences in maturation yields were found and MLV or DCR media were superior with one exception (E331 produced the highest number of cotyledonary embryos on LV) over LV or QP media. On the other hand, the maturation capacity was not always in accordance with germination ability of somatic embryos. Somatic embryos produced on LV medium (269 per 1g of fresh weight) showed low germination ability. Somatic embryo maturation in *Pinus nigra* was also cell line dependent and large differences were observed among cell lines. Out of six cell lines tested in two of them (E267, E306) the somatic embryo development was restricted to the precotyledonary stage. The cell line-dependent somatic embryo maturation is a well known phenomenon in *Pinus nigra* (Salajova and Salaj 2005) and other conifer species (Jones and van Staden 2001, El Meskaoui and Tremblay 2001, Montalbán et al. 2010) and could be the determining factor for somatic seedlings regeneration (Ramarosandratana et al. 2001). Recently, Robinson et al. (2009) proposed a model predicting the regenerative capability of conifer embryogenic cultures by metabolomics. Variation in culture regenerative capacity was closely linked to the physiological transition of cultures from the proliferation phase to the maturation phase of development.

Acknowledgement

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