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Somatic embryogenesis in European black pine (*Pinus nigra* Arn.)

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Abstract

Embryogenic callus was initiated from immature zygotic embryos of black pine on medium DCR supplemented with 2 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BAP. The diploid number of chromosomes confirmed the origin of callus from zygotic embryos. The callus was white, glossy, mucilaginous and contained somatic embryos consisted of an embryonic region with densely cytoplasmic cells and suspensor region with long vacuolated cells. Although somatic embryos with green cotyledons were recognisable after ABA treatment and subsequent transfer to growth-regulator free media whole plants have not yet been obtained.

Introduction

Conifers have been considered to be recalcitrant to the process of somatic embryogenesis for a long time. In recent years a great success has been achieved in somatic embryogenesis of several coniferous species. Somatic embryogenesis has been reported for Norway spruce (Hakman *et al.* 1985, Becwar *et al.* 1987, Lelu *et al.* 1987), white spruce (Hakman and Fowke 1987, Tremblay 1989), black spruce (Hakman and Fowke 1987), European larch (Nagmani and Bonga 1985), hybrid larch (Klimaszewska 1989), white pine (Finer *et al.*1989), loblolly pine (Becwar *et al.* 1990), sugar pine (Gupta and Durzan 1986), *Pinus caribaea* (Laine and David 1990) and European silver fir (Schuller *et al.* 1989). Immature zygotic embryos seemed to be the most suitable explants for induction of somatic embryogenesis although embryogenic callus initiation from mature zygotic embryos was described too (von Arnold 1987, Jain *et al.* 1988, Tremblay 1989). In appropriate regime plantlet development took place from somatic embryos (Hakman and von Arnold 1985, Klimaszewska 1989, Tremblay 1990).

As European black pine was included into hybridization programme and several promising hybrids were obtained (Kormuťák and Lanáková 1988) we attempt to propagate this species by tissue culture methods. We succeeded in the adventitious

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Abbreviations used: 2,4-D - 2,4-dichlorophenoxyacetic acid, BAP - 6-benzylaminopurine, NAA - naphthylacetic acid, KIN - kinetin, ABA - abscisic acid.

bud induction and axillary bud development (Salajová 1990). In the present work we focused on the initiation of embryogenic callus in *Pinus nigra* Arn. and subsequent somatic embryo development.

Material and methods

Plant material: The experiments were carried out during the years 1989 and 1990. The green cones were collected in July and August from open pollinated trees of *Pinus nigra* Arn. located in natural stands Drážovce (district Nitra). The cones were stored in plastid bags at 4 °C about two weeks. Before culture the seeds were removed from cones and treated with 10% H_2O_2 10 min and finally rinsed four times with sterile distilled water.

The following explants were cultured: megagametophytes that contained immature zygotic embryos and zygotic embryos in precotyledonary and cotyledonary stage of development.

Culture conditions: Several basal media were tested. In the year 1989, the media von Arnold and Eriksson (1981) were tested with 1 mg l^{-1} BAP and 2 mg l^{-1} NAA, and with 1 mg l^{-1} BAP and 5 mg l^{-1} 2,4-D. In 1990, two basal media were used: DCR medium (Gupta and Durzan 1985) supplemented with 500 mg l^{-1} casein hydrolysate, 50 mg l^{-1} glutamine, 2 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BAP, and 20 g l^{-1} saccharose (Klimaszewska 1989), and LM medium (Litvay *et al.* 1981) in full strength and half-strength of macro- and microelements containing 1000 mg l^{-1} casein hydrolysate, 500 mg l^{-1} glutamine, 2 mg l^{-1} BAP, 2 mg l^{-1} NAA, and 20 g l^{-1} saccharose. The media were solidified with 0.7% Agar-Agar (*Serva*). pH was adjusted with 1 M KOH to 5.8 before autoclaving at 120 °C 20 min and *ca.* 12 cm^3 of the medium was poured into plastic Petri plates. Eight to ten explants were cultured in each plate wrapped with *Parafilm* (Amer. Can. Comp., Greenwich, CT). The cultures were kept in darkness in a thermostat at 25 ± 1 °C. The explants were transferred to the fresh media at 2 - 3 week intervals.

In 1989, only embryos in cotyledonary stage of development were cultured, 336 explants in all experiments. In 1990, 1100 explants were cultured (465 megagametophytes, 550 isolated cotyledonary embryos and 85 precotyledonary embryos).

For further development of embryos the calli were cultured on the media with the addition of following growth regulators: 0.2 mg l^{-1} KIN and 0.1 mg l^{-1} ABA; 0.1 mg l^{-1} BAP and 0.1 mg l^{-1} 2,4-D; ABA 0.1, 1, 2, 5, 8 mg l^{-1}; KIN 0.1 mg l^{-1}. After 3 weeks of culture on these media the calli were transferred to the growth regulator-free media. The cultures were exposed to the light (16 h/day, 7 W m^{-2}) and temperature 25 ± 2 °C.

Histochemical observations: The embryogenic callus was stained with 2% acetocarmine. White mucilaginous calli stained red with acetocarmine and embryo heads and suspensor cells were clearly visible (Jain *et al.* 1988). In order to establish the chromosome number the callus was pretreated overnight with 0.2% colchicine, acid hydrolysed and stained for a few minutes in 1% acetic orcein. Squashed

preparations were examined under a light microscope and photographed. The chromosome counts were done in 100 callus cells.

Results and discussion

Almost all cultured explants in cotyledonary stage (1989) started to proliferate callus but no embryogenic callus development was achieved. The proliferated calli died in transfer procedures within 3 months.

Explants of megagametophytes with zygotic embryos and excised embryos in precotyledomary and cotyledonary stage of development were studied in 1990. Excised precotyledonary embryos were not suitable explants for callus induction. Regardless of culture media used this explants died shortly after placing onto medium.

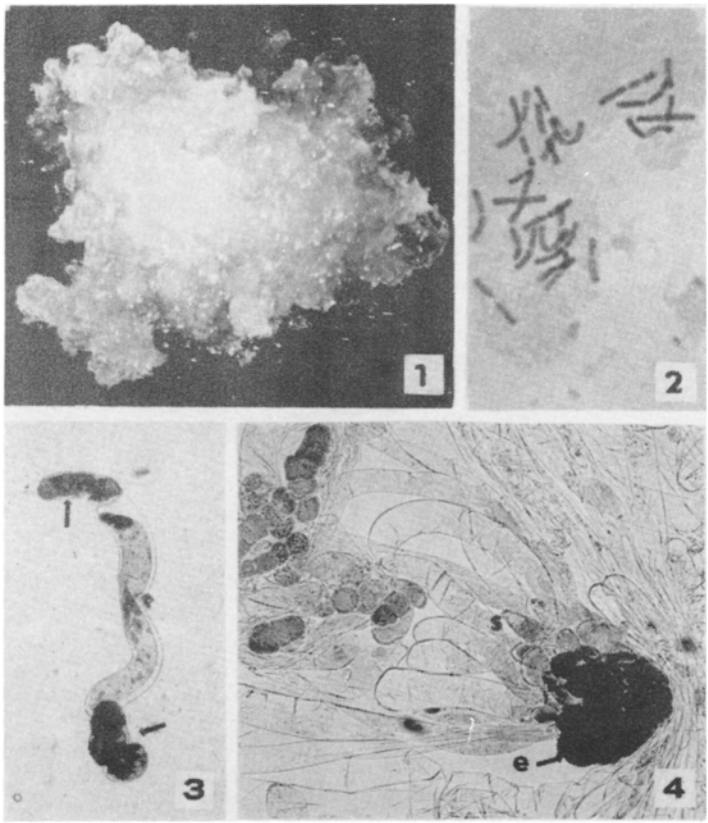

Fig. 1. Embryogenic callus of *Pinus nigra* after two weeks of transfer (× 5).
Fig. 2. Metaphase in cell of embryogenic callus showing $2n$=24 chromosomes (× 1 100).
Fig. 3. Two somatic proembryos shared a common suspensor (× 125).
Fig. 4. Somatic embryo consisted of embryonal region (e) and long vacuolated cells of suspensor (s) (× 300).

Cotylenonary embryos produced callus on all tested media at a frequency 80%, but without embryogenic capacity. The most vigorous callus was formed on medium 1/2 LM from collection August 9 and 21. The callus was white-yellowish, friable and required transfer onto fresh medium every two weeks, but remained still non-embryogenic.

The isolated megagametophytes produced callus of hard consistance which failed to develop on all tested media after the second transfer. Only on medium DCR from collection 3 July, the formed callus was embryogenic in nature at the frequency 2%. The callus was extruded from micropylar end of megagametophyte after 10-12 d of cultivation and closely resembled the white, mucilaginous embryogenic callus (Fig. 1) in other species of conifers (Hakman and von Arnold 1985). After transfer to light a change in the colour of calli was observable. The translucent white colour changed to slightly grey and no chlorophyll synthesis took place. For long-term maintenance of callus the basal medium DCR and half-strength LM medium were available.

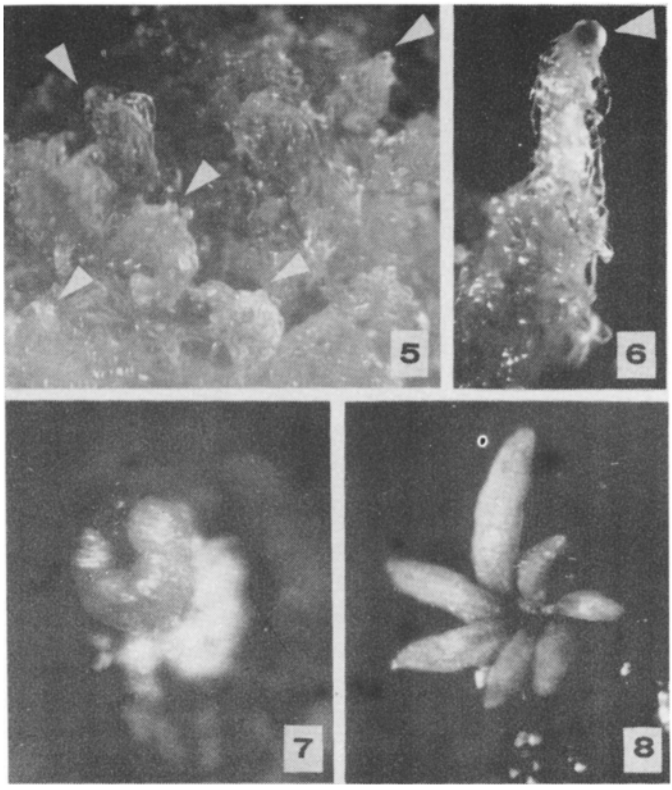

Fig. 5. Somatic embryos protruding from embryogenic callus after 2 weeks of culture on growth-regulator medium (× 12).

Fig. 6. A detailed view on somatic embryo at precotyledonary stage of development (× 32).

Fig. 7. The development of cotyledon primordia on the medium DCR lacking growth regulators (× 19).

Fig. 8. Somatic embryo with well developed cotyledons (×19).

To establish the origin of callus chromosome numbers were determined. The cells of embryogenic callus were diploid (Fig.2) at the frequency 97.2%, only 2.8% of cells being polyploid. According to this fact we supposed that the callus arose from diploid zygotic embryos. The immature zygotic embryos have been considered the superior explants for embryogenic callus initiation (Hakman *et al.* 1985, Laine and David 1990), but recently mature zygotic embryos were induced to produce embryogenic callus (Jain *et al.* 1988, Tremblay 1990).

Microscopic observations revealed the presence of somatic embryos in the white mucilaginous callus (Fig. 4). The somatic embryo consisted in this stage of an embryonic region with densely cytoplasmic cells and suspensor with prolonged highly vacuolated cells although the suspensor was not so precisely organised as in zygotic embryos (Hakman and Fowke 1987). The somatic embryos found in mucilaginous callus of *Pinus nigra* closely resembled the somatic embryos of other gymnosperms (Hakman *et al.* 1985, Klimaszewska 1989, Laine and David 1990). In some cases the developing somatic embryos shared a common suspensor because two embryo heads developed in opposite directions (Fig. 3). Jain *et al.* (1989) reported development of somatic proembryos with two heads on the same suspensor cells in *Pinus elliottii.*

The culture of embryogenic calli on media containing ABA and subsequent transfer to media without growth regulators led to the development of somatic embryos. After the ABA treatment numerous somatic embryos emerged from the callus (Fig. 5). The embryonic region showed smooth, glossy surface and was attached to a long translucent suspensor (Fig. 6). The embryonic segments continued to develop, turned green and gradually cotyledon primordia appeared (Fig. 7).The growth of embryos beyond this stage was only sporadical although somatic embryos bearing well developed green cotyledons were observable (Fig. 8). The exogenously supplied ABA seemed to be an important factor for somatic embryo development in *Pinus nigra.* In our experiments no embryo development was achieved without ABA treatment. Von Arnold and Hakman (1988) reported about essential role of ABA on somatic embryogenesis in *Picea abies.* ABA promoted maturation of somatic embryos and ABA treated somatic embryos resembled mature zygotic embryos in appearance and growth habit.

In the present work we demonstrated the establishment of an embryogenic callus from immature zygotic embryos and the development of somatic embryos after ABA treatment. Our experiments on initiation of embryonic callus performed during the summer 1991 were successful and embryogenic callus was obtained (Salajová, unpublished). These data confirmed our previous experimental results suggesting that the process of somatic embryogenesis in *Pinus nigra* was reproducible. Our current research is focused on the regeneration of plants from somatic embryos.

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