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Somatic embryo formation on mature *Abies alba* × *Abies cephalonica* zygotic embryo explants

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Abstract

Somatic embryogenesis was achieved from mature embryos excised from stored hybrid *Abies alba* × *Abies cephalonica* seeds. Embryogenic tissue formation occurred on SH medium supplemented with 1 mg dm^{-3} benzyladenine. The formation of embryogenic tissue was influenced by the time of storage of seeds. Initiation frequencies 27.2 - 29.0 % were obtained in embryos isolated from 6 month and 1 year stored seeds. Embryos excised from 4-year stored seeds showed no response. Embryogenic structures appeared on the surface of hypocotyl. They originated without previous callus formation. Embryogenic tissues were maintained in long-term cultures. After maturation treatment cotyledonary somatic embryos developed and germinated in small plantlets.

Additional key words: benzyladenine, conifers, hybrid fir, histology.

Introduction

Somatic embryogenesis is a progressive method for *in vitro* plant propagation and also a useful tool for theorethical studies of embryo development. The process has been reported for many conifers (Jain *et al.* 1995). In the genus *Abies* somatic embryogenesis was initiated from immature (Schuller *et al.* 1989, Norgaard and Krogstrup 1991, Krajňáková and Häggman 1997, Vooková *et al.* 1997/98) as well as from mature zygotic embryos (Hristoforoglu *et al.* 1995, Norgaard and Krogstrup 1995). The embryogenic tissue appeared on the suspensor of immature zygotic embryos (Salajová *et al.* 1996) or on the hypocotyl of mature zygotic embryos (Guevin *et al.* 1994). In our previous work we reported somatic embryogenesis in hybrid firs from immature zygotic embryos (Salajová *et al.* 1996). The disadvantage of using immature embryos is that they are available only during a short period of zygotic embryo maturation. Therefore, the use of mature embryos is more advantageous. Although preliminary results of somatic embryogenesis on hybrid *Abies alba* × *Abies cephalonica* mature zygotic embryos were reported earlier (Salajová and Salaj 1998), no detailed description of initiation, maturation and histological examination was given. The aim of the present work is to describe the initiation of somatic embryogenesis from mature zygotic embryos excised from stored seeds and to follow the origin of somatic embryos on explants. Maturation of somatic embryos and plantlet regeneration in embryogenic tissues derived from mature zygotic embryos have also been tested.

Materials and methods

Plants: Seeds were collected in September from *Abies alba* cones pollinated with the pollen of *Abies cephalonica.* Seeds were surface sterilized in 0.1 % $HgCl_2$ for 15 to 30 min, rinsed four times in sterile distilled water and imbibed 48 h in the last wash. Finally the embryos were carefully dissected and placed on

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Abbreviations: ABA - abscisic acid; BA - benzyladenine; PEG - polyethylene glycol.
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culture medium. In experiments on initiation, altogether 264 mature zygotic embryos were excised from seeds stored for 4 years, 1 year and 6 months.

Nutrient medium: For embryogenic tissue initiation, Schenk and Hildebrandt medium (1972) supplemented with 1 mg dm^{-3} benzyladenine (BA), 1000 mg dm^{-3} myo-inositol, 1000 mg dm^{-3} casein hydrolysate, 500 mg dm^{-3} glutamine, and 2 % sucrose was used. After autoclaving, the medium was poured into plastic Petri dishes. In each dish 6 - 7 embryos were cultured. The cultures were kept in darkness at temperature of 25 °C.

For the maturation of somatic embryos, small pieces of embryogenic tissues were placed on DCR basal medium (Gupta and Durzan 1985) supplemented with 10 mg dm^{-3} abscisic acid (ABA) and 7.5 % polyethylene glycol-4000 (PEG) for 6 - 8 weeks. After this period, the tissues with developing embryos were transferred to ABA- and PEG-free medium. This medium was also used for the germination.

Histological observation: The hypocotyls of mature zygotic embryos were cut into small pieces and fixed with 4 % glutaraldehyde in 0.1 M phosphate buffer and postfixed in buffered 1 % OsO_4, dehydrated in acetone series and subsequently embedded in *Durcupan ACM* (*Fluka*, Buchs, Switzerland). Semithin sections (2 - 3 μm) were prepared on *Ultratome III* (*LKB*, Bromma, Sweden) ultramicrotome and stained with 1 % toluidine blue. The samples were examined under an *Axioplan 2* (*Zeiss*, Jena, Germany) bright-light microscope.

Results and discussion

Initiation of somatic embryos: Isolated intact embryos placed on initiation medium showed pale green cotyledons and slightly swelled hypocotyl with intact epidermis after one week in culture. In the radicula area, non-embryogenic callus formation started at the same time. This callus was soft, watery and did not proliferate further but necrotised. Around the 20th day of culture, white embryogenic structures were observable. These structures arose from protuberances appeared on the hypocotyls (Figs. 1, 2). In contact with medium, they proliferated and gradually explants were overgrown with embryogenic tissue. When satisfactory amount of tissue was formed (after 4 to 6 weeks), it was separated and cultured as individual cell line. Occasionally, the embryogenic structures did not proliferate but necrotised, mainly in case when they did not contact the medium. The embryogenic tissue formation was accompanied by non-embryogenic callus formation and rarely by adventitious bud primordia development.

Embryos isolated from seeds stored for 6 months showed 27.2 % initiation frequencies, those isolated from 1 year-stored seeds 29 %, while embryos from seeds stored for 4 years were without response. The non-responsive zygotic embryo explants necrotised during the culture. The mature embryo-derived cell lines closely resembled the cell lines originating from immature zygotic embryos (Salajová *et al.* 1996) showing numerous early stage somatic embryos. From the initiated tissues, 6 cell lines were selected for the maintenance. All of them were fast growing, vigorous, with characteristic features of embryogenic cultures even after two years in maintenance.

Histological examinations have revealed the somatic embryos formed on hypocotyl explants directly without an intervening non-embryogenic phase. At early stage of development they were composed of meristematic embryonal part and long vacuolised suspensor cells (Fig. 3). The proliferation of these structures resulted in the formation of the embryogenic tissue maintained in long-term cultures (Fig. 4).

For *Abies*, cytokinin as sole growth regulator was sufficient to bring about this process in immature (Schuller *et al.* 1989, Norgaard and Krogstrup 1991) as well as in mature embryo explants (Hristoforoglu *et al.* 1995). Norgaard and Krogstrup (1991) observed deleterious effect of auxin on somatic embryo initiation and tissue proliferation in *Abies nordmanniana*. In *Abies balsamea*, the mature embryo-derived embryogenic cultures were proliferated and maintained on a medium containing also an auxin (Guevin *et al.* 1994). Our previous work showed that medium containing an auxin also yielded embryogenic tissue formation although the initiation frequency was lower (Salajová and Salaj 2001).

The initiation frequencies from mature zygotic embryo explants reached 3.5 % for *Abies balsamea* (Guevin *et al.* 1994) and 40 % for *Abies nordmanniana* (Norgaard and Krogstrup 1995) and *Abies alba* (Hristoforoglu *et al.* 1995). In comparison to latter mentioned species, hybrid fir mature somatic embryos showed lower initiation frequencies and their longer storage caused even loss of explants competence to form embryogenic tissue. In contrast, *Picea glauca* mature embryos formed embryogenic callus even after 11 years of storage, but the initiation frequencies decreased with increasing number of years of storage (Tremblay 1990).

Several studies have been performed to identify the origin of somatic embryos in conifer juvenile explants. In sectioned seedling material of *Picea abies*, somatic embryos were shown to originate from nodules differentiated in epidermal and subepidermal cell layers

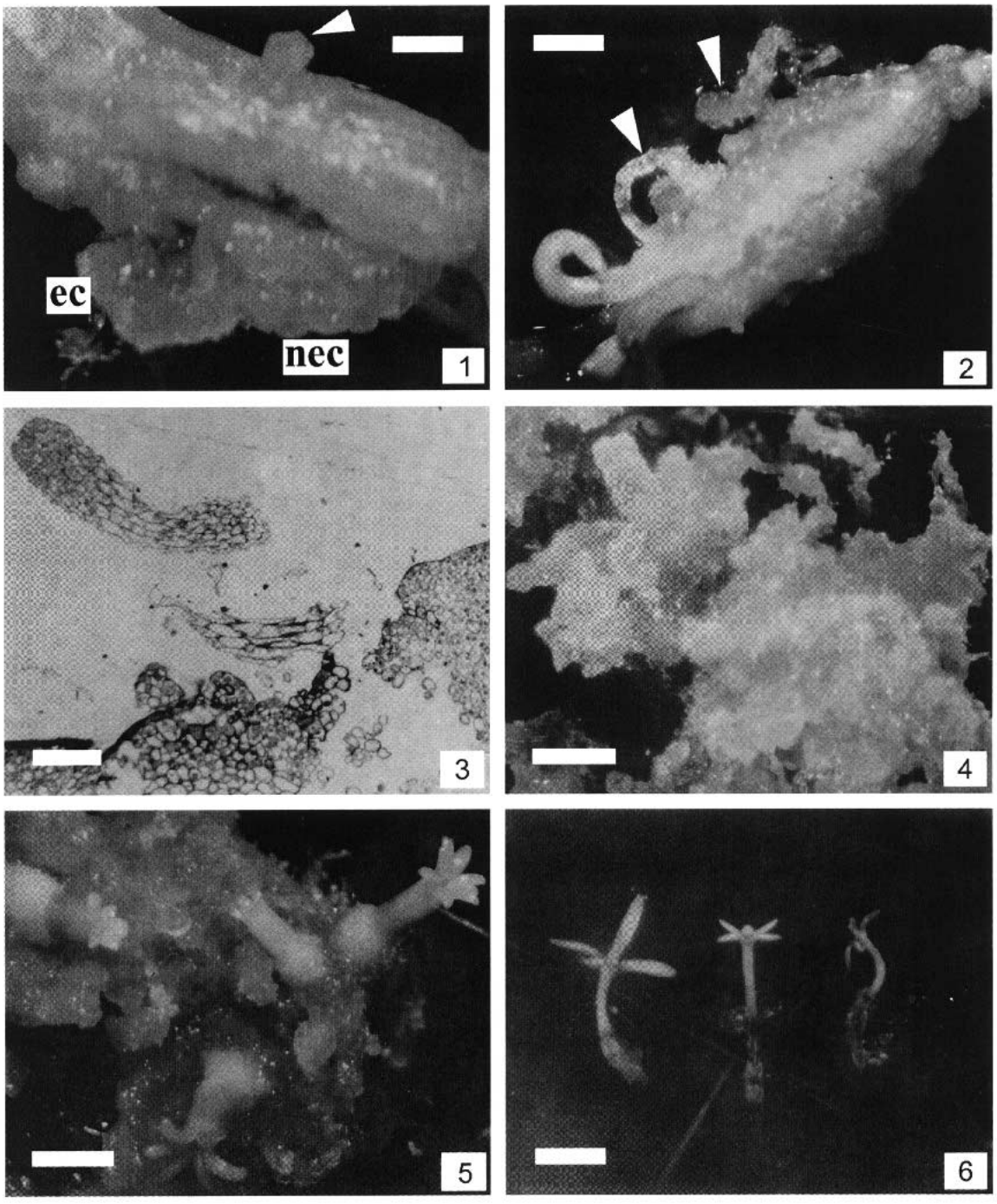

Fig. 1. Protuberance (*arrowhead*) observable on the hypocotyl of cultured zygotic embryo. Note the formation of both embryogenic (EC) and nonembryogenic callus (NEC) on the same explant (*bar* = 1 mm).
Fig. 2. Protrusion of single somatic embryos (*arrowheads*) from the hypocotyl of mature zygotic embryo (*bar* = 2 mm).
Fig. 3. A histological section through zygotic embryo (corresponding to Fig. 2) with single somatic embryo attached to the hypocotyl tissue *via* vacuolised suspensor (*bar* = 200 μm).
Fig. 4. White, mucilaginous embryogenic tissue derived from mature zygotic embryo explants (*bar* = 2.5 mm).
Fig. 5. Cotyledonary somatic embryos developed after maturation treatment (*bar* = 2.5 mm).
Fig. 6. Plantlets developed from mature somatic embryos (*bar* = 4 mm).

or in the cortex (Mo and Arnold 1991). Similar pattern has been observed in mature embryos of *Abies alba*. Periclinal division of epidermal and subepidermal cell of hypocotyl gave rise to globular structures clearly separated from other tissue. Subsequently these structures developed into typical somatic embryos (Zoglauer and Reuther 1996).

In immature embryos of Norway spruce and white spruce, the callus phase preceded somatic embryogenesis (Nagmani *et al.* 1987). Outer 2 - 4 cell layers of hypocotyl formed white, glossy, mucilaginous callus. Successively in callus somatic embryos differentiated. Similarly, in cotyledons and whole embryo explants of Norway spruce, somatic embryos arose from callus (putative embryogenic callus) formed from peripheral meristematic cells (Krogstrup 1986). Lelu *et al.* (1990) hypothetised the existence of two cell populations with embryogenic potential. One cell population could form embryogenic structures only by auxin or by high ratio of auxin to cytokinin. The other cell population required a cytokinin pretreatment before the treatment by auxin.

Maturation of somatic embryos: Culture of embryogenic cell lines on maturation medium resulted in somatic embryo development. After 4 - 5 weeks in culture, precotyledonary somatic embryos appeared on the embryogenic tissues and after an additional 3 weeks, cotyledonary somatic embryos were visible (Fig. 5). Although individual cell lines showed various responses to maturation treatment in terms of number of developing embryos (Fig. 7), the pattern of somatic embryo development was similar in all cell lines. Precotyledonary somatic embryos were formed in high number but their development towards the cotyledonary stage was supressed. Cell lines AC2, AC3, AC4, developed precotyledonary somatic embryos (66 to 73 per plate), but only 26.66 to 42.42 % of these were converted into the cotyledonary stage. In contrast, cell lines AC1 and AC5 formed lower number of precotyledonary embryos, but 63.82 to 66.66 % of them reached the cotyledonary stage. Cotyledonary embryos germinated and formed more or less developed small plantlets (Fig. 6).

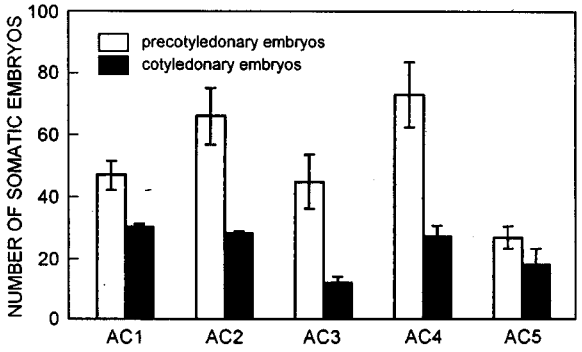

Fig. 7. Precotyledonary and cotyledonary somatic embryo development after 5 and 8 weeks of maturation treatment (mean number per plate containing 1 g of tissue is given).

Somatic embryo development in conifers is influenced by several factors. Abscisic acid is used to stimulate the maturation process and in many cases it is necessary to be combined by sucrose, mannitol or polyethylene glycol. The role of ABA is to stop the cleavage (Gupta and Durzan 1993), to stimulate storage reserve accumulation (Arnold and Hakman 1988) and to prevent precocious germination (Lelu *et al.* 1994). Genotype is another important factor as shown for *Larix* (Lelu *et al.* 1994), *Abies* (Norgaard 1997) and *Pinus* (Laine and David 1990).

Our results showed that it was possible to initiate somatic embryogenesis on mature zygotic embryo explants of hybrid fir. The somatic embryos formed directly on explants without an intervening callus phase. Their proliferation in contact with the medium resulted in the formation of embryogenic tissue maintained in long term cultures. After maturation treatment somatic embryos regenerated small plantlets.

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