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Somatic embryogenesis: method for vegetative reproduction of conifers

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Abstract

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For *Pinus nigra* Arn. somatic embryogenesis has been initiated from immature zygotic embryos enclosed in megagametophytes. The initiated embryogenic tissues contain bipolar structures – somatic embryos consisted of meristematic embryonal part and long vacuolised suspensor cells. The embryogenic tissues/cultures are usually maintained on solid or liquid nutrient media. For long-term storage, recently the method of cryopreservation has been used to replace the time consuming regular transfers to nutrient media. The initiated cell lines represent individual genotypes and the structure of somatic embryos as well as their maturation is cell line dependent. The maturation of early somatic embryos occurs on media containing abscisic acid and osmotica. The process of somatic embryogenesis is completed by plantlet (somatic seedlings) regeneration.

Keywords

cryopreservation, *in vitro*, micropropagation, pine, somatic embryogenesis

Introduction

Somatic embryogenesis refers to the process in which somatic or non-sexual cells are induced to form bipolar embryos through a series of developmental steps similar in those occurring during *in vivo* embryogenesis (Stasolla et al., 2002). The initiated bipolar structures are capable of development producing cotyledonary stage somatic embryos that in appropriate conditions germinate and their development is completed by whole plants (somatic seedlings) regeneration. Owing to the fact through the developmental process of somatic embryogenesis large number of plants can be obtained in relatively short period of time, the method became an attractive tool for clonal propagation (Klimaszewska and Cyr, 2002).

For conifers somatic embryogenesis has been initiated for different species belonging to genera *Picea*, *Pinus*, *Abies*, *Pseudotsuga* and plantlet (somatic seedlings) regeneration has been achieved, but some problems still remain. For *Pinus* species the relatively low initiation frequencies represent serious problem and recently efforts have been made to optimize the initiation process. For *Pinus radiata* Hargreaves et al. (2009) obtained on average 55% initiation rates although the initiation was depending on families and collection time of explants. The initiation process can be enhanced through seed family screening, zygotic embryo staging as well as media adjustment (Montalbán et al., 2012). The development of early bipolar somatic embryos present in embryogenic tissues can be stimulated by using abscisic acid (ABA) combined with non-penetrating osmoticum as polyethylene glycol (Svobodová et al., 1999; Vooková and Kormuťák, 2009) or carbohydrates maltose and sucrose (Salajová et al., 1999; Lelu-Walter et al., 2008). The advantage of embryogenic tissues is their ability to regenerate after cryopreservation – storage in liquid nitrogen at –196 °C.

In our laboratory, somatic embryogenesis for *Pinus nigra* Arn. has been repeatedly initiated as well

as plantlets/somatic seedlings regeneration has been achieved. The aim of presented paper is to evaluate somatic embryogenesis for the mentioned species.

Material and methods

Plant material

In our experiments megagametophytes containing immature embryos have been used as explants. The green cones of *Pinus nigra* Arn. have been collected at the beginning of June (usually between 1 and 15). The cones were stored at 4 °C for several days, after washed in tap water and the immature seeds were dissected. Surface sterilization of seeds was done by 10% H_2O_2 for 10 min. and then four washings in sterile distilled water followed. Finally, the megagametophytes were excised and placed on the culture medium.

Culture media

For the initiation as well as maturation of somatic embryos in most of cases DCR medium (Gupta and Durzan, 1985) has been used. Other media as LV (Litvay et al., 1981) or LP (Quoirin and Lepoivre, 1977) were also tested. The media were supplemented with enzymatic caseinhydrolysate (500 mg l^{-1}), glutamine (50 mg l^{-1}) as well as myo-inositol (200 mg l^{-1}) and solidified with 0.3% gelrite (Ducheva). Plant growth regulators as 2,4-dichlorophenoxyacetic acid (2,4-D, 2 mg l^{-1}) and 6-benzyladenine (BA, 0.5 mg l^{-1}) have been incorporated into the nutrient media as well. Sucrose (2%) was used as carbohydrate source. Maturation occurred on DCR medium containing abscisic acid (25 mg l^{-1}) and 6–9% maltose or high concentration of Phytagel (1%). After appearing of cotyledonary somatic embryos the tissues were transferred to media without ABA. The germination medium contained activated charcoal (1%). The cultivation of explants occurred in dark at 23 °C (except the culture of regenerated somatic seedlings that were transferred to light (110 μM s^{-1}/day).

Cryopreservation of embryogenic tissues

For cryopreservation of embryogenic tissues the slow-freezing method has been used. In the experiments altogether 46 cell lines were included. On the 8th day of growth cycle 3.0 g of tissues was re-suspended in 9 ml of DCR medium containing 180 g l^{-1} sucrose. After 1 hour incubation in this medium gradually 15% DMSO was added to reach the final concentration 7.5%. Subsequently 1.8 ml of suspension was pipetted into cryovials and placed into the Mr. Frosty container. The samples were incubated in deep freezer (–80 °C) until the temperature in controlled cryovial reached –40 °C. Finally, the cryovials were plunged into liquid nitrogen and kept there for 1 hour to 1 year.

Thawing of tissues occurred at 40 °C in water bath. Following, the tissues were cultured on DCR medium as mentioned above in dark at 23 °C. Pretreated but not cryopreserved tissues were considered as control 1 (C1). Visual observations have been done in 3–4 days intervals. Growth analysis of tissues occurred three months after cryopreservation.

Microscopic observations

The structure of somatic embryos was investigated by light microscopy (Axioplan 2, Zeiss) using squash preparations and 2% acetocarmine staining.

Results and discussion

Initiation of embryogenic tissues

The production of embryogenic tissues has been observed approximately 3 to 5 weeks after placing the explants to the culture medium. The tissues were protruded from micropylar end of the megagametophyte (Fig. 1a), and reaching the size about 5 mm in diameter, they were separated from the explants and cultured individually as cell lines. The embryogenic tissues are of white color, translucent and relatively rapidly growing (Fig. 1b). Microscopic observations revealed the presence of bipolar structures – somatic embryos as the most important components of the tissues (Fig. 1c). The initiation frequencies reached values from 1.53% to 24.11% and changed from year to year. Plant growth regulation treatment as well as basal media formulations affected the initiation process. The highest initiation frequencies were obtained on medium DCR. The most important factor as we experienced is the developmental stage of zygotic embryos used as starting explants. For *Pinus nigra* the very early developmental stages – immature precotyledonary embryos gave the best initiation frequencies. As the maturation of zygotic embryos progressed the initiation frequencies dropped and finally the explants failed to produce embryogenic tissues. Although the bipolar structures are present in embryogenic tissues their structural organization is changing in dependence on the cell line. Relatively low initiation frequencies are characteristic for *Pinus* species and many attempts were done in order to achieve improvement. For *Pinus nigra* the developmental stage of original zygotic embryos is decisive and this phenomenon was also confirmed for *Pinus radiata* (Hargreaves et al., 2009; Montalbán et al., 2012), *Pinus pinaster* (Miguel et al., 2004), *Pinus sylvestris* (Keinonen-Mettälä et al., 1996).

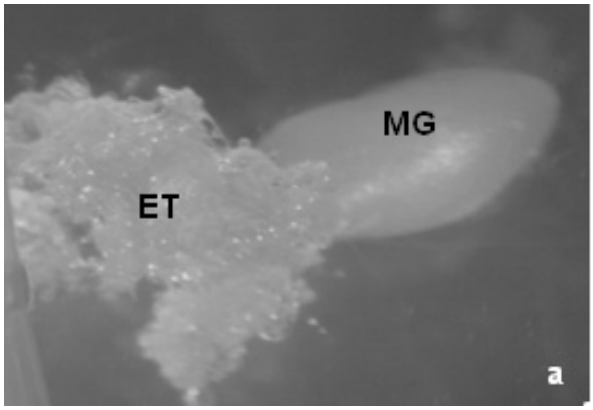

Fig. 1a. Initiation of embryogenic tissues (ET) on megagametophyte explants (MG).

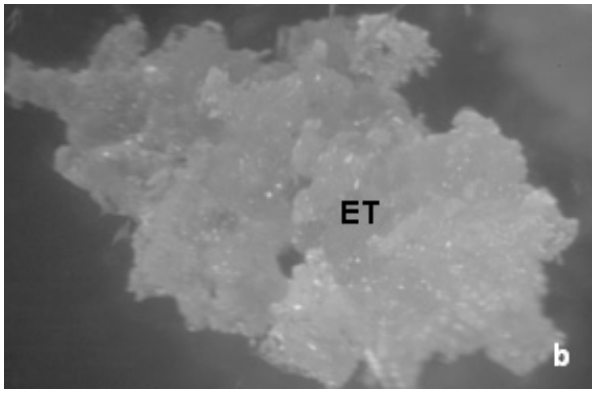

Fig. 1b. Proliferating embryogenic tissue 8 days after transfer to fresh medium.

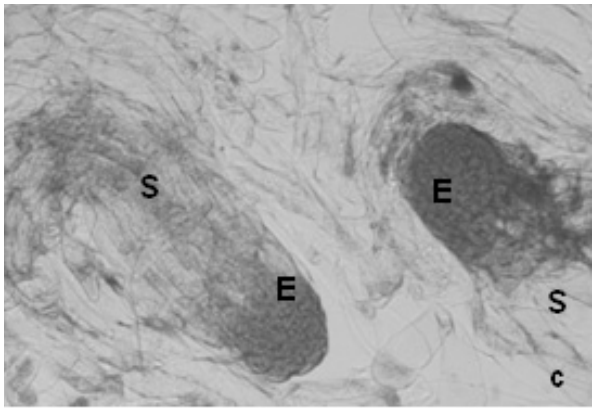

Fig. 1c. Bipolar somatic embryos composed of meristematic embryonal cells (E) and long vacuolised suspensor (S).

Maturation of somatic embryos

The early bipolar structures are capable of development and plantlets (somatic seedlings) production. Transfer of tissues from proliferation medium containing 2, 4-D and BA to maturation medium with ABA and maltose resulted in the development of early somatic embryos. Approximately around the fifth week of culture on maturation medium numerous precotyledonary somatic embryos appeared on the surface of embryogenic tissues. The suspensor was still present and connected the developing embryos to the tissue. Cotyledonary somatic embryos appeared around the eighth week of maturation (Fig. 1d). Their quantity was strongly cell line dependent. For germination somatic embryos at least with four cotyledons were selected. The germination occurred on ABA free medium and resulted in somatic seedling formation (Fig. 1e).The obtained plantlets were transferred to soil and survived five to six months.

During somatic embryo development (maturation) structural changes were visible in the developing somatic embryos. The most conspicuous features were differentiation of root meristem and later the procambium formation. The maturation of conifer somatic embryos is genotype dependent (Keinonen- Mettälä et al., 1996; Salajová et. al., 1999) and is influenced by composition of the maturation medium (Carneros et al., 2009; Montalbán et al., 2010).

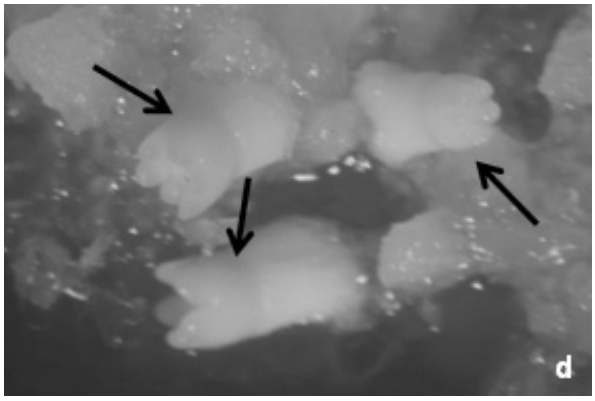

Fig. 1d. Cotyledonary somatic embryos (arrows) developed on the maturation medium.

Fig. 1e. Plantlets (somatic seedlings) regenerated from somatic embryos.

Cryopreservation of embryogenic tissues

Cryopreservation of embryogenic tissues enables their storage for long-term period. Out of 46 cell lines cryopreserved 35 survived the storage in liquid nitrogen. The regeneration of tissues after thawing (Fig. 1f) was dependent on cell line although the duration of storage in liquid nitrogen had no significant effect on the growth and behaviour of tissues. Our examinations also showed no correlation exists between the maturation ability and cryotolerance of cell lines. The use of cryopreservation method for long-term storage of conifer embryogenic tissues has been demonstrated for several species (Aronen et al., 1999; Vondráková et al., 2010).

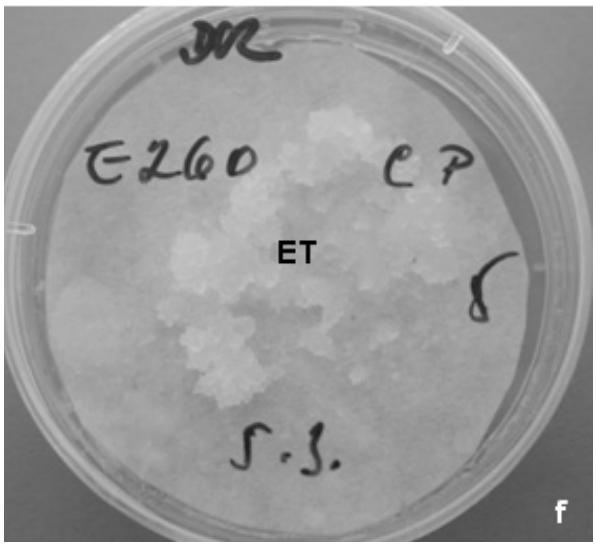

Fig. 1f. Re-growth of embryogenic tissue (ET) after cryopreservation.

The obtained results for *Pinus nigra* give evidence that it is possible to initiate embryogenic cell lines from immature zygotic embryos, but to obtain higher initiation frequencies the method/approach needs refinement. Although the maturation of somatic embryos is cell line dependent, cotyledonary somatic embryos were produced and somatic seedling regeneration occurred as well. Cryopreservation of embryogenic tissues using slow-freezing was also successful for the majority of tested cell lines.

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