Arch. Biol. Sci., Belgrade, 59 (3), 199-202, 2007. DOI:10.2298/ABS0703199S

INDUCTION OF SOMATIC EMBRYOGENESIS IN *PINUS HELDREICHII* CULTURE

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Abstract – The potential for somatic embryogenesis in zygotic embryo and megagametophyte cultures of *Pinus heldreichii* was examined. Somatic embryogenesis was initiated from megagametophytes containing immature zygotic embryos at early stages of development. An induction frequency of up to 6.7% was obtained on Gresshoff and Doy medium in the presence of 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/l benzyladenine (BA). Formation and further proliferation of embryogenic tissue were achieved upon transfer of explants to a medium with reduced levels of growth regulators. Somatic embryos are being cultured for further development.

Key words: Conifers, *Pinus heldreichii,* somatic embryos, tissue culture

UDC 582.475 : 57.086.83 : 581.3

INTRODUCTION

Somatic embryogenesis is the development of embryos from somatic, non-sexual cells. This is accomplished through a series of developmental stages, most of which are similar to zygotic embryogenesis. Plant regeneration through somatic embryogenesis has been reported for many conifer species belonging to the genera *Pinus*, *Picea, Larix*, and *Abies* (S t a s o l l a et al., 2002). In general, the genus *Pinus* is considered to be recalcitrant to somatic embryogenesis. Embryogenic cultures were established in several pine species, but somatic embryo maturation and plantlet regeneration still remain problematical.

Pinus heldreichii (Bosnian pine) is a Tertiary relic species endemic on the high mountains in the Balkans and Southern Italy. It occurs on steep and dry limestone slopes, most often in pure stands (V i d a k o v i ć, 1982). *Pinus heldreichii* is a decorative, pyramidal-shaped tree that can grow to a height of approximately 30 m. Although the tree grows slowly, it could be important for afforestation, since it is well adapted to environmental stresses such as low temperature and extreme drought (J o v a n o v i ć, 1971).

Natural regeneration of *P. heldreichii* is from seeds, but years with abundant flowering occur infrequently. As vegetative propagation by the rooting of cuttings has been only partially successful, there is a need to develop an alternative propagation method. We previously reported plant regeneration of *P. heldreichii* through adventitious buds (S t o j i č i ć et al., 1999) and through axillary buds (S t o j i č i ć and B u d i m i r, 2004). The aim of this study was to define conditions for induction of somatic embryogenesis.

MATERIAL AND METHODS

Cones of *P. heldreichii* were collected from open-pollinated trees in a natural stand located on Lovćen Mountain (Montenegro) during July of 2003 and stored at 4ºC until use. Before the experiments, seeds were removed from cones, washed for 24 h under running tap water, surface disinfected in 20% sodium hypochlorite for 30 minutes, and rinsed three times with sterile distilled water. Isolated megagamethopytes and precotyledonary, early cotyledonary, and mature zygotic embryos were then placed on the culture medium. The basal culture medium was GD (G r e s s h o f f and D o y, 1972) medium as modified by S o m m e r et al. (1975).

In the first set of experiments for induction of somatic embryogenesis, cultures were grown on GD medium supplemented with 2 mg/l 2,4-D (2,4-dichlorophenoxy acetic acid) or NAA (α-naphthaleneacetic acid) and 0.5 mg/l BA (benzyladenine) for 5 or 14 days, then transferred to GD medium with five times lower concentration of growth regulators.

In the second set of experiments, cultures were grown on a medium designated as GD1, with nitrogen salts reduced to one half compared to the basal GD medium. The combination of growth regulators, their concentration, and the time of induction were the same as in the first set of experiments.

For proliferation and maintenance of embryogenic tissue, cultures were transferred to fresh GD medium supplemented with 0.2 mg/l 2,4-D and 0.05 mg/l BA every four weeks. The basal medium without growth regulators or GD with 3.2 mg/l ABA (abscisic acid) was used as the medium for maturation of somatic embryos.

All media were supplemented with 3% sucrose and 0.7% agar (Torlak, Belgrade). The pH of the media was adjusted to 5.7 prior to autoclaving for 25 min at 115ºC. Cultures were maintained at 25 ± 2ºC in darkness during the induction and proliferation stages, and under 16 h/8 h photoperiod at dim light during the maturation phase.

The embryogenic nature of proliferated tissue was determined by staining with 2% (w/v) acetocarmine, followed by 0.05% Evans blue. The preparations were examined under a Jenamed microscope from Carl Zeiss and photographed.

RESULTS

Four to ten weeks after planting, megagametophytes showed extrusions of mucilaginous tissue from the micropylar end. The extruded tissue mostly emerged as a proliferating cell clump (Fig. 1) around the micropyle, and then spread along the surface of the medium. Early stages of embryo development could be clearly observed in this tissue. The frequency of embryogenic tissue initiation was 6.7% for explants cultured on GD medium containing 2,4-D 2 mg/l and BA 0.5 mg/l for 5 days, after transfer to a medium with reduced concentrations of growth regulators (Table 1). Longer induction time (14 days) was less effective in induction of somatic embryogenesis. On a medium with nitrogen salts reduced to one half compared to the basal medium, 2/60 explants formed embryogenic tissue after 14 days of induction. In treatments where NAA was used instead of 2,4-D, somatic embryogenesis was not recorded.

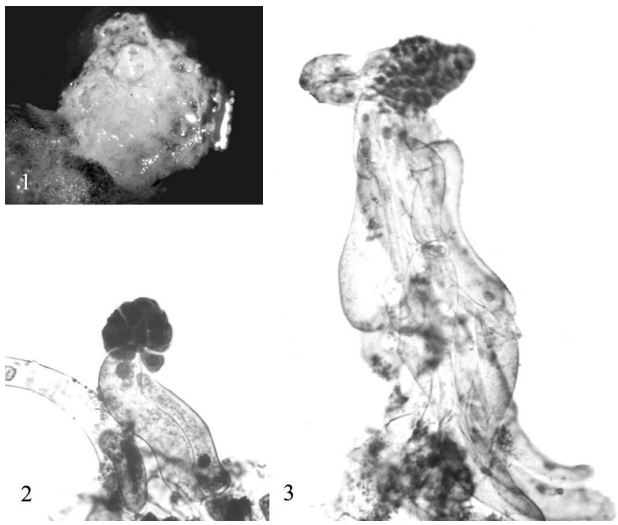
Table 1. Initiation of somatic embryogenesis in *Pinus heldreichii* megagamethophyte culture.

Culture medium*	Induction time (days)	Number of explants	Explants with non-embryogenic callus (%)	Explants with embryogenic tissue (%)
GD	5	30	53.3	6.7
GD	14	60	35.0	1.7
GD1	5	30	40.0	0
GD1	14	60	35.0	1.7

* Both GD and GD1 media were supplemented with 2,4-D (2 mg/l) and BA (0.5 mg/l)

Early cotyledonary and mature zygotic embryos when cultured started to proliferate, producing non-embryogenic callus tissue. This friable creamy-white callus gradually died in subsequent cultures. In precotyledonary zygotic embryo culture, extruded tissue consisted of up to eight embryos, which did not divide further and eventually turned brown.

For continuous somatic embryo proliferation and



Figs. 1-3. Somatic embryogenesis in *Pinus heldreichii.*
Fig. 1. Initiation of embryogenic cell mass around the micropyle of a megagamethophyte.
Fig. 2. Initial stage of somatic embryo formation. Note the apical, cytoplasm rich cells and translucent vacuolated suspensor cells.
Fig. 3. Immature filamentous somatic embryo with well developed apical dome and long secondary suspensor.

long-term culture maintenance, the embryogenic tissue formed on a megagametophyte was isolated and grown on a medium supplemented with 0.2 mg/l 2,4-D and 0.05 mg/l BA. On this medium, the tissues grew rapidly. Microscopic observation revealed numerous white, translucent, loosely packed immature embryos at different developmental stages. Apart from young embryos consisting of suspensor-subtended several meristematic cells (Fig. 2), embryos with a well developed meristematic dome and an elongated suspensor were frequently present (Fig. 3). With regular subculturing every four weeks, the embryogenic potential of embryogenic lines was maintained for several months. Occasionally, mucilaginous embryogenic cultures turned into non-embryogenic calli.

Upon transfer of embryogenic cultures to a medium without growth regulators or to one supplemented with 3.2 mg/l ABA, tissue turned opaque cream in color, culture growth was minimal, and the first stages of embryo maturation were observed only sporadically.

DISCUSSION

Various factors have been found to influence induction of somatic embryogenesis in conifers, one of them being the developmental stage of the zygotic embryo explant. Immature zygotic embryos served as an excellent explant source for somatic embryo initiation in a variety of conifer species. The optimum stage of immature zygotic embryo development for initiation of embryogenic tissue in *Picea* species is postcotyledonary (H a k m a n and v o n A r n o l d, 1985; H a k m a n and F o w k e, 1987), while that for *Pinus* species is precotyledonary (J o n e s et al., 1993; M i g u e l et al., 2004). There are only few reports of somatic embryogenesis in mature pine embryo culture (G a r i n et al., 1998; R a d o j e v i ć et al., 1999).

The best response in *Pinus heldreichii* was obtained when using immature zygotic embryos contained within a megagametophyte. The highest somatic embryogenesis initiation frequency in *P. heldreichii* was 6.7%. Similar results were obtained for *P. banksiana* (3.9%) (P a r k et al., 2006), *P. nigra* (8%) (S a l a j o v a et al., 1995), and *P. elliottii* (10%) (L i a o and A m e r s o n, 1995). However, in some other pine species, the best initiation rates were considerably higher and went up to 54.6% for *P. strobes* and 76.2% for *P. pinaster* (P a r k et al., 2006).

Modifications to medium components and culture conditions can also significantly affect induction of embryogenic tissue. Although the nitrogen level and composition are known to be important factors (T a u t o r u s et al., 1991), in *Pinus heldreichii* on embryogenic response was obtained both on media fully supplied with nitrogen salts and on media in which nitrogen salts were reduced. This result suggests that if explants are obtained at the right developmental stage, culture conditions are not crucial, although they may significantly enhance the frequency of initiation. The induction of nonembryogenic callus and embryogenic tissue under identical culture conditions can be attributed to the various cell populations that constitute gametophytic explants.

The maintenance medium supporting proliferation of embryogenic tissue was in most studies the same as for the initiation stage. In *Pinus heldreichii*, for embryogenic tissue formation and proliferation it was necessary to transfer explants to a medium with reduced levels of growth regulators. Similar results were also reported for *P. elliottii* (J a i n et al., 1989).

Embryogenic lines in *P. heldreichii* were established in a relatively short time, successfully maintained, and manipulated. These results suggest the possibility of developing an efficient method for rapid regeneration of plants that can be eventually used for reforestation. However, maturation of *P. heldreichii* somatic embryos is still problematical and requires further study in experiments involving variation of the time of maintenance on proliferation media and ABA-containing media, as well as the use of embryogenic lines from a range of different genotypes.

Acknowledgment – This work was supported by the Serbian Ministry of Science and Environment Protection, Grant No. 143026.

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ИНДУКЦИЈА СОМАТСКЕ ЕМБРИОГЕНЕЗЕ КОД *PINUS HELDREICHII* У КУЛТУРИ

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У култури изолованих зиготских ембриона и овула мунике (*Pinus heldreichii*) испитиван је ефекат хранљиве подлоге и регулатора растења на индукцију соматске ембриогенезе. Соматска ембриогенеза је индукована у култури овула које су садржавале ембрионе на раном ступњу развића. Највиша фреквенција индукције од 6.7% постигнута је када су овуле 5 дана гајене на Gresshoff и Doy (GD) хранљивој подлози у присуству 2,4-D 2 mg/l и BA 0.5 mg/l, а затим пренете на GD подлогу у којој је концентрација регулатора растења била 5 пута нижа. У циљу дуготрајног одржавања у култури, ембриогено ткиво је изоловано и гајено на GD подлози са 2,4-D 0.2 mg/l и BA 0.05 mg/l. Микроскопском анализом је утврђено да се ткиво састоји од бројних ембриона на раним ступњевима развића. За клоналну пропагацију ове значајне ендемореликтне четинарске врсте у култури *in vitro*, неопходна су даља истраживања са циљем повећања фреквенције индукције соматске ембриогенезе, као и фреквенције сазревања ембриона.