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*Original scientific papers*

# ADVENTITIOUS BUD INDUCTION IN *Pinus heldreichii* Christ. SEEDLING EXPLANT CULTURE

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By using tissue culture methods multiple shoot regeneration was achieved from seedling explants of *Pinus heldreichii* in the presence of 1 mg/l benzyladenine. The age of seedlings was found to be important for bud induction and shoot elongation. Histological events associated with bud primordium formation were also examined.

*Key words:* Conifers, histology, organogenesis, *Pinus heldreichii*, seedling explant, tissue culture

## INTRODUCTION

*P. heldreichii* is a Tertiary relic species, endemic to the Balkan Peninsula and southern Italy. This pine together with *P. mugo* and *P. uncinata* is one of the high-elevation pines of the Mediterranean basin. It occurs on steep and dry limestone cliffs and slopes, most often in pure stands (VIDAKOVIĆ 1982). The tree is ornamental, up to 30 m high, with pyramidal crown. *P. heldreichii* forest belt is very degraded in the Balkans (JANKOVIĆ 1991). Although the tree grows slowly, it

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could be important for afforestation in subalpine belt regions, since it is well adapted to environmental stresses such as low temperature and extreme drought (JOVANOVIĆ 1971).

*P. heldreichii* is usually propagated by seeds. However, seed production in natural stands is limited, and there is a need to develop an alternative propagation method. Micropropagation of conifers is generally possible from embryos or seedling explants. There are several methods for *in vitro* plant regeneration such as adventitious and axillary bud induction or somatic embryogenesis. Micropropagation of *Pinus heldreichii* from mature zygotic explants has previously been achieved (STOJIČIĆ *et al.* 1999). The aim of this study was to analyze the competence of seedling explants for adventitious bud induction. The study is important in view of the need to characterize relationship between explant age and organogenic potential for the formation of structures capable of organized growth and final development into seedlings.

## MATERIAL AND METHODS

Plant material: Seeds of *P. heldreichii* were collected from open-pollinated trees in a natural stand located on Lovćen Mountain. After 24 h under running tap water, seeds were surface disinfected in 20% sodium hypochlorite for 30 minutes, and rinsed three times with sterile distilled water. Mature embryos were then excised from surrounding gametophytic tissue and placed horizontally onto the medium.

For embryo germination half-strength GD (Gresshoff and Doy 1972) medium as modified by Sommer et al. (1975), supplemented with 2% sucrose and 0.7% agar (Torlak, Belgrade) was used. Embryos were cultured for 3, 6, or 9 days on this medium. Seedlings from which necrotic radicle was cut of (seedling explant) were then transferred to the induction GD media supplemented with 1 mg/l BA, 3% sucrose and 0.7% agar. Following 4 weeks on the bud induction medium, the explants were transferred to the same half-strength, growth regulator-free medium supplemented with 2% sucrose and 0.5% activated charcoal (AC). The pH of the media was adjusted to 5.7 prior to autoclaving for 25 minutes at 115 °C. Experiments were repeated three times to give a total of 30 explants for each treatment.

Cultures were maintained at 25±2 °C under a 16-h photoperiod at a photosynthetic photon flux density of 47 mmol $m^{-2}$ $s^{-1}$ provided by white fluorescent tubes (Tesla, Pančevo, 65W, 4500K).

For paraffin sections organogenic tissue was fixed in formalin:acetic acid:ethanol (10:5:85), dehydrated in graded ethanol series and embedded in paraffin wax at 57 °C. Sections 5 mm thick were stained with haematoxylin. For semi-thin sections material was fixed in 3% phosphate-buffered glutaraldehyde, pH 7.2 for 2h and postfixed in 2% $OsO_4$ for 2 h. Samples were dehydrated in graded ethanol and embedded in Epon. Sections 1 mm thick were stained with methylene blue. Sections were observed and photographed under Jenamed, Carl Zeiss photomicroscope.

## RESULTS AND DISCUSSION

Isolated white embryos, 5-6 mm in size, placed on GD half-strength medium for 3, 6 and 9 days elongated slightly and the cotyledons and hypocotyl became green. The root development was poor and at the site of radicle emergence the small amount of slimy-necrotic callus developed. After transfer to the BA containing induction medium, seedling explants started to swell along cotyledons and upper hypocotyl region. The first well developed adventitious buds were generally localized at upper hypocotyl region, then they spread out over the cotyledon surface in zygotic and 3-day-old seedling explants. Bud formation in 6 and 9-day-old seedling explants occurred mainly on hypocotyl segments closest to the shoot apical meristem.

Seedling explants showed different abilities to survive and different organogenic responses after 8 weeks in culture (Table 1). The culture survival rate decreased with increase of the seedling age. Although mean number of buds was not significantly different among seedling explants, multiplication rate (BFC index) was lower for older explants. Also buds formed on 6 and 9-day-old explants generally failed to elongate into shoots.

*Table 1. - The effect of explant age on bud formation*

| Explant age (days) | N° of explants | Explant survival (%) | Explants with buds (%) | Average N° of buds per explant | BFC index |
|---|---|---|---|---|---|
| 3 | 30 | 70.0 | 90.5 | 6.7[a] | 6.0 |
| 6 | 30 | 50.0 | 66.7 | 5.1[a] | 3.4 |
| 9 | 30 | 36.7 | 72.7 | 4.3[a] | 3.0 |

Means in the column followed by same letters are not different according to Duncans' Multiple Range Test ($p \leq 0.05$).
BFC index = (Average No. of buds per explant) x (% of explants forming buds) / 100

Histological analyzes showed that buds on the cotyledons were formed directly from epidermal end subepidermal cells (Fig 1). Various mitotic divisions were observed spreading out in this superficial area of cotyledons resulting in formation of distinct meristematic regions. These meristemoids gave rise to apical domes and subsequently developed into adventitious buds. In the hypocotyl region cell proliferation first resulted in callus tissue formation. Within the callus tissue two kinds of cell division patterns were observed. Organized cell divisions led to the bud primordia formation (Fig 2), while divisions in various planes resulted in growth of undifferentiated tissue. Process of bud formation and development was asynchronous and various stages of bud development could be observed in all types of explants.

Lower morphogenetic capacity of *P. heldreichii* seedling explants compared to that of zygotic embryos (STOJIČIĆ *et al.* 1999) is in agreement with

results obtained for *Pinus sylvestris* (TORIBIO and PARDOS 1989), *Pinus strobus* (KAUL 1990) and *Pinus pinaster* (CALIXITO and PAIS 1997). In *P. heldreichii* culture bud elongation in older explants was arrested, while in *P.* sylvestrys the elongation of neoformed buds was better in older explants (TORIBIO and PARDOS 1989). Different bud-forming capacity in explants of different ages seems to be the result of morphological and biochemical characteristics relating explant age. The accumulation of wax covering surface of older cotyledons as well as increase in thickness of cell wall and vacuolation may prevent uptake of compounds from the medium, especially cytokinins, thus limiting the formation of meristematic tissue (AITKEN-CHRISTIE *et al.* 1985).

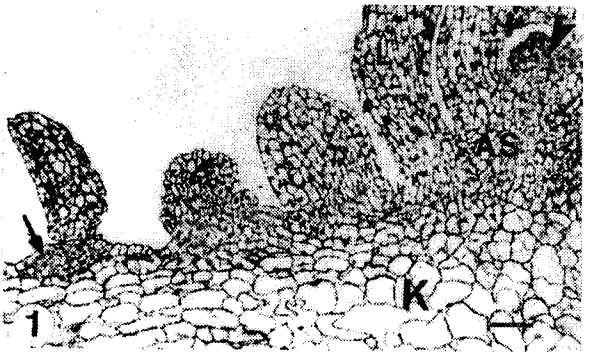


Fig. 1. - Longitudinal section through cotyledon (K). Note the meristematic domes (arrow) protruding away from the cotyledon surface and adventitious shoots (AS) with organized apical meristem (arrowhead), leaf primordia and developed leaves (L), bar = 100 μm


Fig. 2. - Section through proliferated hypocotyl segment. Note the adventitious bud primordia (arrow) arising from callus tissue (C), bar = 100 μm

In conclusion our results showed the possibility of adventitious bud induction in seedling explants of *Pinus heldreichii*. Defining the competence of explants for adventitious bud production together with further optimization of micropropagation methods for older explants could be an important step in vegetative propagation of superior genotypes for reforestation.

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# INDUKCIJA ADVENTIVNIH PUPOLJAKA U KULTURI KLIJANACA *Pinus heldreichii* Christ.

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I z v o d

Regeneracija izdanaka u kulturi klijanaca *Pinus hrldreichii* postignta je u prisustvu 1 mg/l benziladenina, korišćenjem metode kulture tkiva. Nađeno je da starost eksplantata utiče na proces indukcije i razvića adventivnih pupoljaka. Razviće adventivnih pupoljaka je histološki analizirano.

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