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BRIEF COMMUNICATION

Cytokinin-mediated axillary shoot formation in *Pinus heldreichii*

D. STOJIČIĆ*[1] and S. BUDIMIR**

*Institute of Forestry, Kneza Višeslava 3, 11010 Belgrade, Serbia and Montenegro**
Institute for Biological Research "Siniša Stanković",
*29 novembra 142, 11060 Belgrade, Serbia and Montenegro***

Abstract

Shoot formation from seedling explants of *Pinus heldreichii* was induced by pulse treatment with benzyladenine at different concentration, followed by culture on medium lacking plant growth regulators. After treatment with 222 μM benzyladenine (BA) an average of 4.6 shoots per explant was obtained. Shoots, detached from explants, rooted with a frequency of about 10 %, and rooted plantlets were successfully transferred to *ex vitro* conditions.

Additional key words: benzyladenine, pine, plant regeneration, seedling explant

Pinus heldreichii is relic species endemic to the Balkan Peninsula and Southern Italy. This pine usually grows on steep and dry limestone cliffs and slopes of high mountain regions (Vidaković 1982). Although the tree grows slowly it could be an important species for afforestation because it is well adapted to environmental stresses such as low temperature and extreme drought. This pine species is also tolerant to air pollution.

Micropropagation of conifers is generally possible from embryos or seedlings explants. There are several methods for *in vitro* plant regeneration such as adventitious or axillary bud induction and somatic embryogenesis. The aim of this study was to elaborate methods for plant propagation of *Pinus heldreichii* by axillary shoots.

Seeds of *Pinus heldreichii* Christ. were collected from open-pollinated trees in a natural stand located on Lovćen Mountain (Serbia and Montenegro). After 24 h under running tap water, seeds were surface disinfected in 25 % sodium hypochlorite for 30 min, and rinsed three times with sterile destined water. Mature embryos were then excised from surrounding gametophytic tissue, and vertically placed into test tubes on the 1/4 strength MS (Murashige and Skoog 1962) culture medium supplemented with 2 % sucrose. On this medium embryos germinated. Four weeks after embryo germination radicle and part of the hypocotyl were cut off and explants consisting of stem apex, cotyledons and 2 cm long hypocotyl (hypocotylary explant) were utilized for the experiments. These explants were treated with solution containing 0, 4.40, 11.50, 22.50, 44.0, 222.0 or 444.0 μM benzyladenine (BA) and 0.1 % dimethyl sulfoxide for 1 h. After this pulse-treatment, explants were allowed to dry in a laminar flow hood on open-faced Petri dishes. Explants were then transferred on the Gresshoff and Doy (1972) medium (GD), as modified by Sommer *et al*. (1975), supplemented with 3 % sucrose, 0.5 % activated charcoal and 0.7 % agar (*Torlak*, Belgrade, Serbia and Montenegro). Axillary shoots formed on explants were then isolated and further cultured on the half strength GD medium with the same supplements. Six explants per bottle were used with at least four replicates per treatment. The pH of the media was adjusted to 5.7 prior autoclaving for 25 min at 115 °C. Cultures were maintained at temperature of 25 ± 2 °C, a 16-h photoperiod at a photosynthetic photon flux density of 47 μmol m^{-2} s^{-1} provided by white fluorescent tubes (*4500 K*, *Tesla*, Pančevo, Serbia and Montenegro, 65 W).

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Abbreviations: BA - benzyladenine; GD - Gresshoff and Doy medium; MS - Murashige and Skoog medium.
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[1] Author for correspondence; fax: (+381) 11 545 969, e-mail: stojicic@net.yu

Hypocotylary explants of *Pinus heldreichii* seedlings pulse-treated with solution of BA started to elongate after transfer to growth regulator-free medium. Elongation of explants was due to hypocotyl elongation rather than growth of shoot axis, which remained short especially in explants treated with higher BA concentrations. The first axillary buds were visible 2 weeks after BA pulse-treatment as swollen protrusions below the stem tip. In *P. heldreichii* culture the explants treated with 222 μM BA showed the highest frequency of axillary bud formation and bud forming capacity index (BFC) index (Table 1). Within 16 weeks in culture axillary buds elongate to shoots with average length of 4.75 to 8.09 mm. Concentrations of BA above 4.40 μM stimulated shoot elongation. Apart from well developed axillary shoots, unorganized mass of needles was also produced on explants treated with highest BA concentrations (222 and 444 μM), and these needles were frequently fasciated. Pospíšilová *et al*. (1999) reported that although conditions during *in vitro* culture could result in the formation of plantlets of abnormal morphology and physiology these abnormalities can be repaired after transfer to *ex vitro* conditions.

Table 1. Effects of BA concentrations on axillary shoot formation. Means ± SE, n = 60. Means in column followed by different letters are different according to Duncans' Multiple Range Test ($P \leq 0.05$), BFC index = (average number of buds per explant) × (% of explants forming buds) / 100.

BA [μM]	Number of shoots [explant^{-1}]	Shoot length [mm]	BFC index
0.00	-	-	-
4.40	0.20 ± 0.09 ab	4.75 ± 0.41 a	0.04
11.50	0.78 ± 0.25 ab	7.57 ± 0.87 b	0.03
22.50	1.25 ± 0.35 b	7.70 ± 0.92 b	0.96
44.00	3.25 ± 0.66 c	7.97 ± 0.49 b	2.44
222.00	4.57 ± 0.60 d	8.09 ± 0.36 b	4.07
444.00	2.78 ± 0.58 c	7.96 ± 0.87 b	2.09

Successful adventitious and axillary bud formation using pulse-treatment has been described for some other conifer species (Austin Burns *et al*. 1991, Goldfarb *et al*. 1991, Drake 1997). In *Pinus sylvestris* culture (Toribio and Pardos 1989) cytokinin influenced formation and number of buds per explant, however, bud development also occurred in the absence of added cytokinins. In *Pinus heldreichii* embryo culture (Stojičić *et al*. 1999) the frequency of adventitious buds was higher than frequency of axillary buds formation obtained upon liquid pulse (222 μM BA). Nevertheless, the axillary buds in this treatment developed more rapidly and were more vigorous.

Axillary shoots removed from the original explant (Fig. l*A*) and transferred to half strength growth regulator-free medium continued to elongate and occasionally produced new axillary shoots. After 4 weeks from out of 19 isolated shoots, roots were formed on about 10 %. Employing similar approach in *P. ellottii* seedling explant culture Austin Burns *et al*. (1991) reported rooting frequency less than 1 %. Rooted plantlets of *P. heldreichii* were transferred to pots in the greenhouse where they continued to grow as phenotypically normal plants (Fig. 1*B*).

In conclusion, explants from 30-d-old *P. heldreichii* seedlings were stimulated to develop axillary buds after BA pulse-treatment under *in vitro* conditions. As in the case with other pine species plant regeneration of *P. heldreichii* by axillary shoots could be the most reliable method for true-to type plant regeneration and large-scale micropropagation.

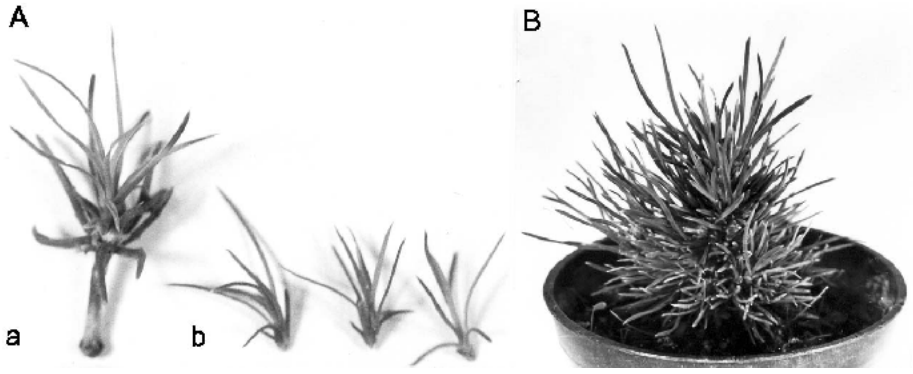

Fig. l. Micropropagation of *Pinus heldreichii* by axillary shoots. *A* - hypocotylary explant, 16 weeks after 222 μM BA pulse-treatment (*a*) and elongated shoots (*b*) detached from a single explant; *B* - regenerated plant.

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