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BRIEF COMMUNICATION

Micropropagation of *Pinus peuce*

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Abstract

In *Pinus peuce* zygotic embryo culture grown on Gresshoff and Doy (1972; GD) basal medium, 2.22 μM benzyladenine (BA) was superior in promoting adventitious bud induction during 4 weeks comparing to kinetin or BA + kinetin. Shoot elongation was achieved on half-strength GD medium devoid of plant growth regulators and containing activated charcoal. Pulse treatment with 1 mM indole-3-butyric acid (IBA) for 2 h, followed by transfer to half-strength GD medium, produced the most efficient rooting. Rooted shoots were transplanted to the greenhouse and plantlets continued to grow and developed into phenotypically normal plants. Up to 10 plants per explant can be obtained within 36 weeks from culture initiation.

Additional key words: adventitious buds, benzyladenine, conifers, Macedonian pine, micropropagation

Macedonian pine, *Pinus peuce,* is a tertiary relict species endemic to the Balkan Peninsula. This pine usually grows on high mountains, at altitudes between 800 and 2300 m, on slopes on siliceous soils and rarely on carbonate soils (Vidaković 1982). The tree is ornamental, 35 - 40 m tall. Macedonian pine is tolerant to winter cold and wind exposure and is recommended as a tree suitable for planting on degraded and devastated soils. Because of the limited area of its natural distribution, this species requires special attention and implementation of measures for its conservation (Janković 1991).

Plant regeneration systems developed for conifer species typically employ embryonic tissues as starting material (Stange *et al*. 1999, Zhang *et al*. 2006, Vooková *et al.* 2010). Optimization of factors influencing each step of the micropropagation procedure is necessary for successful regeneration through adventitious bud induction (Kaul 1990). This study was conducted with the aim to manage plant regeneration of *Pinus peuce via* adventitious buds. To our knowledge this is the first report of plant regeneration *in vitro* of this valuable conifer tree.

Cones of *Pinus peuce* Gris. were collected from open pollinated trees in a seed orchard located on Mučanj mountain (Serbia). Prior to the experiments, seeds were removed from cones, washed under running tap water for 24 h, surface disinfected in 25 % sodium hypochlorite for 25 min, and rinsed three times with sterile distilled water. Mature embryos were then excised from surrounding gametophytic tissue and grown on basal Gresshoff and Doy (1972; GD) culture medium as modified by Sommer *et al*. (1975). Ten embryos were placed horizontally in each 90 mm plastic Petri dish filled with 25 cm^3 of the medium. Experiments were repeated twice to give

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Abbreviations: AC - activated charcoal; BA - benzyladenine; GD - Gresshoff and Doy medium; IBA - indole-3-butyric acid; NAA - α-naphtaleneacetic acid.
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a total of 60 explants for each treatment. Unless stated otherwise, the medium was supplemented with 3 % sucrose and solidified with 0.7 % agar (*Torlak*, Belgrade, Serbia). The pH of the media was adjusted to 5.7 prior to autoclaving for 25 min at 115 °C, and cultures were maintained at 25 ± 2 °C under 16-h photoperiod with irradiance of 47 μmol m^{-2} s^{-1}. Auxin pulse-treated shoot cultures were maintained under the same conditions.

For adventitious bud induction the effect of BA (2.22, 4.40, 11.10 or 22.20 μM) or kinetin (2.32, or 4.60 μM) was tested. Apart from these, the combinations of 1.11 μM BA + 1.16 μM kinetin or 2.22 μM BA + 2.32 μM kinetin were also examined.

After bud induction, the explants were transferred to half-strength GD medium without growth regulators and supplemented with 2 % sucrose and 0.5 % activated charcoal (AC) to promote bud elongation. For root induction, elongated shoots were isolated, pulse-treated with 1 mM solution of α-naphtaleneacetic acid (NAA) or indole-3-butyric acid (IBA) for 2 or 5 h, and then transferred to 0.5 GD supplemented with 2 % sucrose. For each treatment 20 shoots were used, and experiments were repeated twice.

A large number of adventitious buds mostly formed on the swollen region between cotyledons and hypocotyl during fourth week of culturing on cytokinin treated embryos. When explants were transferred onto medium lacking plant growth regulators and containing activated charcoal, adventitious bud elongation was stimulated. Under these conditions buds also emerged along the cotyledons. The optimal concentration of BA for bud induction was 2.22 μM when the treatment lasted 4 weeks (Table 1). Higher BA concentrations (11.10 and 22.20 μM) in the medium after 4-week treatment led to development of stunted and clustered buds. On the other hand average shoot length increased when shorter induction time, 1 - 3 weeks, was applied (Table 1). Explants cultured on kinetin and BA medium as well as explants cultured in the presence of kinetin alone developed fewer buds than those cultured over the same time period on media with BA (Table 1).

A greater number of elongated shoots were obtained when explants, with each successive transfer to the fresh medium containing AC (0.5 %), were divided into smaller pieces.

After 16 weeks isolated shoots treated with 1 mM NAA or IBA pulse for 2 or 5 h and then transferred to 0.5 GD produced roots. Pulse treatment with IBA was preferable alternative over NAA both in terms of shoot survival and root induction. Up to 33.33 % of rhizogenesis was achieved in 67.5 % of survived 2-h pulse IBA-treated shoots. Rooted plantlets with few needles and well developed roots were transferred to pots, and continued to grow as phenotypicaly normal plants (Fig. 1).

In *Pinus peuce* embryo culture, BA was superior in promoting adventitious bud induction comparing to kinetin or combination of BA and kinetin. In *P. massoniana* embryo culture and in *P. pinea* cotyledon culture, BA also exerted stronger bud-inducing effect than any other cytokinin tested (Zhang *et al*. 2006, Alonso *et al*. 2006). Similar cytokinin specificity of explants for multiple bud induction was found in shoot apex culture of *P. roxburghii* Sarg (Kalia *et al.* 2007).

In *Pinus peuce* culture as in *Pinus pinea* cotyledon culture (Alonso *et al*. 2006) number of buds was highest

Table 1. Effect of cytokinin treatment on adventitious bud induction in *P. peuce* zygotic embryo culture. Explants were grown on GD induction medium and then transferred to the bud elongation medium for 4 weeks. Shoot length was measured 16 weeks after culture establishment. Means ± SE, n = 60. Means in the column followed by different letters are different according to Duncan multiple range test ($P \leq 0.05$).

Time [weeks]	Cytokinin	[μM]	Number of buds [explant^{-1}]	Shoot length [mm]
1	BA	2.22	2.12±0.76^{a}	5.21±0.76^{bc}
		4.40	2.85±0.72^{a}	5.04±0.70^{bc}
		11.10	2.88±0.95^{a}	5.75±0.94^{c}
		22.20	3.73±0.91^{a}	5.23±0.91^{bc}
2		2.22	4.32±0.99^{b}	5.66±0.69^{c}
		4.40	4.87±1.03^{b}	5.13±0.89^{bc}
		11.10	4.40±0.95^{b}	4.77±0.91^{b}
		22.20	4.64±0.91^{b}	4.78±0.91^{b}
3		2.22	9.03±0.95^{d}	4.67±0.74^{b}
		4.40	10.10±1.11^{d}	4.13±0.86^{b}
		11.10	6.17±0.74^{c}	3.76±0.85^{ab}
		22.20	7.11±0.86^{c}	3.80±0.91^{ab}
4		2.22	15.12±0.99^{f}	3.77±0.69^{ab}
		4.40	12.95±1.03^{e}	3.03±0.78^{a}
		11.10	10.40±0.95^{d}	2.75±0.80^{a}
		22.20	10.10±0.91^{d}	2.32±0.80^{a}
4	KIN	2.32	5.32±0.65^{c}	-
		4.60	5.46±0.86^{c}	-
4	BA+KIN	1.11+1.16	5.12±0.85^{c}	-
		2.22+2.32	5.33±0.83^{c}	-

Fig. 1. *Pinus peuce* plantlet (14 months old) regenerated *via* adventive organogenesis.

after longer exposure time, but longer exposure time influenced callus formation and affected bud elongation. In *P. massoniana* high BA concentrations (exceeding 4.4 μM) had a negative influence on shoot elongation, while the number of hyperhydric shoots increased (Zhang *et a*l. 2006).

To promote bud elongation, medium without plant growth regulators (Stojičić *et al.* 1999, Sul and Korban 2004) or medium with lower level of cytokinin with auxin (Tang *et al*. 2004, Zhang *et al*. 2006) or auxin alone (Chalupa 1989) was usually applied. In *Pinus radiata* culture Sul and Korban (2004) have achieved successful shoot elongation by further decreasing the strength of medium used for bud induction, while Stange *et al.* (1999) have used medium with lowered sucrose content for shoot elongation. For Macedonian pine adventitious bud elongation, half-strength plant growth regulator-free GD medium supplemented with 2 % sucrose and 0.5 % AC was applied. Significance of AC in different aspects of tissue culture was recently reviewed by Thomas (2008). In *Pinus heldreichii* shoot apex culture, 0.5 % AC was added to prevent exudation from the explant (Stojičić and Budimir 2004) and in zygotic embryo culture to promote shoot elongation during micropropagation (Stojičić *et al*. 1999).

The low efficiency of rooting remains a bottleneck for conifer propagation, thus reducing the possibilities of applying these techniques for large scale micropropagation. In *Pinus peuce* after pulse treatment with auxins up to 10 plants per explant were obtained within 36 weeks from culture initiation. IBA exerted stronger effect on isolated shoot survival and frequency of rooting than NAA. In *Pinus massoniana* IBA also had stronger effect than NAA in promoting root induction as well as for subsequent root growth (Zhang *et al*. 2006).

In conclusion, this study shows that multiplication of *Pinus peuce* seed material could be obtained through *de novo* organogenesis by adventitious buds formation. However, for increasing the effectiveness of existing propagation methods, improved protocols for rooting of adventitious shoots are needed.

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