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Optimization of In Vitro Regeneration of *Pinus peuce* (Gris.)

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Abstract: *Pinus peuce* (Macedonian pine) is considered a valuable ornamental tree that is frequently planted in parks and gardens, especially in Western Europe. This endemic pine is one of the most valuable conifer species in its native range, which currently consists of only two disjunct populations restricted to small mountainous areas of the Balkans and is listed as a near-threatened species. The reproduction of Macedonian pine by seed is limited, so in vitro propagation methods have emerged as a promising tool for large-scale propagation. The objective of this study was to develop an improved system for the micropropagation of *P. peuce* from juvenile plant material using a short-term liquid cytokinin pulse. For that, explants derived from 4-week-old seedlings were pulse-treated with different concentrations of N^6-benzyladenine (BA) for 1 or 2 h to stimulate the induction of axillary buds. The highest axillary shoot formation was achieved with 222 µM BA pulse treatment, with an average number of ~six shoots per explant. Elongated shoots (≥10 mm) were detached from the explants and pulse-treated with 0.27 or 1.08 mM α-naphthaleneacetic acid (NAA) or 0.25 or 0.98 mM indole-3-butyric acid (IBA) for 1 or 2 h. IBA was more effective than NAA and led to a maximum rooting percentage (up to 40%) and the highest number of acclimatized plants (15–20%). Rooted plants were successfully transferred to ex vitro conditions.

Keywords: cytokinin pulse treatment; axillary bud induction; Macedonian pine; micropropagation

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1. Introduction

Pinus peuce (Gris.), or Macedonian pine, was first discovered in 1939 by the German botanist August Grisebach on Mount Baba in Macedonia as a new species of five-needled pine that had not previously been recorded on the Balkan Peninsula [1]. *P. peuce* is phylogenetically isolated among the Balkan species of the genus *Pinus* and belongs to the subgenus *Strobus* according to the classification proposed by Gernandt et al. [2].

The Macedonian pine is a Tertiary relict and an endemic species of the Balkan Peninsula. Its current range is restricted to small mountainous areas of the Balkans between the northern latitudes of 41° and 43° and consists of two disjunct populations separated by the valley of the Vardar River [3,4]. The species is classified by Farjon [5] and IUCN [6] as "Near Threatened" under the criteria B2a.

P. peuce occurs on some of the highest Mediterranean and sub-Mediterranean mountainous regions of North Macedonia, Serbia, Montenegro, Bulgaria, Albania and Greece at altitudes ranging from 800–900 m to 2300–2400 m, with the optimum usually being 1600–1900 m [3]. This pine species favors cold mountain climates and high air humidity, occurring mainly on silicate soils and less frequently on carbonate ones. In its native range, it grows on soils usually poor in nutrients and derived from acid materials, but it can also be found on serpentine [7]. However, it is highly adaptable to different ecological sites and can therefore be found in a wide altitudinal range [8].

The Macedonian pine is one of the most valuable conifer species in its native range, where it mostly forms pure stands, but it can also form mixed stands with other conifers (Bosnian pine, black pine, fir) and deciduous species (beech). In the past, Macedonian pine forests used to form a spacious and powerful forest belt in the Balkans; however, this belt has been reduced to a limited, natural present-day range due to the negative anthropogenic impact [9]. The most extensive stands in its native range are found on north- and northwest-facing slopes [10]. The tree grows from the lower border of the submountain belt to the upper border of the subalpine forest belt [3,11]. This exceptional adaptability to harsh mountain climate conditions makes the species highly useful for afforestation at high altitudes and for protection against erosion [3,11].

P. peuce is considered a valuable, 35–40 m tall ornamental tree that is frequently planted in parks and gardens, particularly in Europe [4,10]. The durable wood of the tree is highly valued, and its resin is used in the chemical, optical and pharmaceutical industries, while the local populations use it to cure wounds, stomach diseases and other ailments [3]. Despite its slow juvenile growth, its basal area increment may later exceed that of other pines. Macedonian pine could be an important species for afforestation, as it appears to be less susceptible to biotic and abiotic hazards and is highly stable due to its deeply penetrating lateral root system, which renders it valuable for watershed and avalanche protection [3,4].

The natural reproduction of *P. peuce* by seeds starts in 5–6-year-old trees and is limited, as cones are formed every 2–4 years, while seeds exhibit low germination rates (~0.1%) unless stratified. The seeds of the Macedonian pine are deeply dormant and require chilling treatment at 3–5 °C for an average of 30 weeks to overcome dormancy [12,13]. The hard seed coat covered with resin appears to be an efficient barrier to water entry into the seed, which is necessary for the onset of germination, and a mechanical barrier to root emergence. The seeds of *P. peuce* are also characterized by undifferentiated or underdeveloped embryos [14].

In vitro propagation methods can help to overcome the problems encountered with classical methods of reproduction. Since the first report on the plantlet regeneration by organogenesis from mature zygotic embryos of *Pinus palustris* [15], considerable progress has been made in exploring opportunities for the use of in vitro vegetative propagation of conifers in forestry. Clonal propagation by somatic embryogenesis or organogenesis is still difficult in many conifer species and is often limited to the use of juvenile explants [16]. Another possibility is regeneration by propagating or promoting the axillary bud development of existing meristems. In *Pinus* species, axillary shoots arise from pre-existing quiescent meristems located adaxial to juvenile needles, in the axils of cotyledons, in the normally dormant short shoots surrounded by needle primordia, and adaxial to scale leaves (cataphylls) in older plant material [17–19].

Shoot proliferation via axillary meristems has several advantages over the de novo organogenic route. Besides rapidity and its time- and space-saving nature, the method of clonal propagation through axillary shoot formation is less prone to the risk of genetic instability [18]. Axillary shoot induction is a promising method for *P. peuce* reproduction in order to restore its former range and increase forest resilience through species diversification. The aim of the present study was to develop an improved system for the shoot propagation of this horticulturally valuable and potentially profitable pine species via axillary meristems from mature zygotic embryos using a short-term liquid cytokinin pulse.

2. Materials and Methods

2.1. Explant Source, Culture Medium and Culture Conditions

Cones of *Pinus peuce* (Gris.) were collected from open-pollinated trees in a seed orchard located on Mučanj Mountain (Serbia) (Figure 1). Prior to the experiments, the seeds were removed from the cones, washed under running tap water for 24 h, surface-disinfected with 25% (*v*/*v*) commercial bleach, "Snežnik" (Panonija AD, Pančevo, Serbia), in sterile deionized water (1% (*w*/*v*) sodium hypochlorite) for 25 min and rinsed three times with sterile distilled water. Seed coats were then removed, and the mature embryos were

excised from the surrounding gametophytic tissue and placed vertically in test tubes on 1/4-strength Murashige and Skoog (MS) culture medium [20] supplemented with 2% (*w*/*v*) sucrose and solidified with 0.7% (*w*/*v*) agar (Torlak, Belgrade, Serbia). The pH of the medium was adjusted to 5.7 with 0.1 N NaOH before autoclaving at 114 °C for 25 min. The cultures were maintained under cool white fluorescent tubes with a photon flux density of 45 µmol m^{-2} s^{-1} and a 16 h photoperiod at 25 ± 2 °C.

Germinated embryos produced seedlings with upright hypocotyls, elongated cotyledons and brown radicles (Figure 2A). Four weeks after embryo germination, the radicle and part of the hypocotyl were removed to obtain 1.5–2.0 cm long explants (consisting of the stem apex, the cotyledons and the apical part of the hypocotyl) for further experiments.

Figure 1. *Pinus peuce*. (**A**) Adult trees of the Macedonian pine in a seed orchard on the Mučanj Mountain (Serbia). (**B**) Uppermost branches of cone-bearing Macedonian pine tree. (**C**) Immature (*white arrow*) and mature (*black arrow*) cones collected from open-pollinated *P. peuce* trees. (**D**) Seeds isolated from mature cones containing mature zygotic embryos that were used as initial explants.

2.2. Axillary Shoot Induction

Explants were transferred to 100 mL Erlenmeyer flasks containing 6-benzyladenine (BA) purchased from Sigma-Aldrich (Saint Louis, MO, USA) and dissolved in 0.1% (*v*/*v*) dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Saint Louis, MO, USA) at the following concentrations: 4.4, 11.5, 22.5, 44.0, 220.0 and 440.0 µM. Control explants were treated with 0.1% (*v*/*v*) DMSO aqueous solution. Control and BA pulse-treated explants were agitated on a rotary shaker at 100 rpm for 1 h or 2 h at 25 °C. After cytokinin pulse treatment, the explants were deposited on a sheet of sterile filter paper placed inside an empty sterile Petri dish and left to dry in a laminar flow hood. After drying, the basal ends of the explants were immersed in Gresshoff and Doy culture medium [21], as modified by Sommer et al. [15] (GDmS medium), supplemented with 3% (*w*/*v*) sucrose, 0.5% (*w*/*v*) activated charcoal (Duchefa Biochemie BV, Haarlem, The Netherlands) and solidified with 0.7% (*w*/*v*) agar in 100-mL Erlenmeyer flasks. Explants were subcultured at 4-week intervals on a fresh medium of the same composition and grown for a total of 12 weeks, under the same light and temperature regime as used for culture initiation.

After 12 weeks of cultivation on a medium without plant growth regulators (PGRs), the number of explants developing shoots, the number of axillary shoots per explant, the shoot length and the number of shoots longer than 10 mm were recorded.

2.3. In Vitro Rooting

In order to induce adventitious rooting, a total of 207 elongated axillary shoots (≥10 mm) were isolated and transferred to 100 mL Erlenmeyer flasks containing auxins: α-naphthaleneacetic acid (NAA) at the concentrations of 0.27 or 1.08 mM or indole-3-butyric

acid (IBA) at the concentrations of 0.25 or 0.98 mM, both purchased from Sigma-Aldrich (Saint Louis, MO, USA) and dissolved in 0.1% (*v*/*v*) DMSO. The control shoots were treated with 0.1% (*v*/*v*) DMSO aqueous solution. The control shoots and the auxin-pulse-treated shoots were agitated on a rotary shaker at 100 rpm for 1 h or 2 h at 25 °C. After auxin pulse treatment, the shoots were deposited on a sheet of sterile filter paper placed inside an empty sterile Petri dish and left to dry in a laminar flow hood. After drying, the shoots were transferred to test tubes containing 10 mL of half-strength GDmS medium, supplemented with 2% (*w*/*v*) sucrose and solidified with 0.7% (*w*/*v*) agar. Auxin-pulse-treated shoot cultures were maintained under the same environmental conditions as used in previous experiments for at least eight weeks.

2.4. Acclimatization

The rooted shoots were removed from the test tubes, washed with water to remove adhering culture medium and transferred to plastic pots with the substrate Substral® (Evergreen Garden Care Deutschland GmbH, Meinz, Germany), and then enclosed in a thin, transparent plastic cover to reduce water loss and maintained in a greenhouse with high humidity (70–80%). The plantlets were gradually acclimatized over a 4-week period by removing the thin cover daily at increasing intervals until final acclimatization. Plant survival was assessed after 16 weeks.

2.5. Experimental Design and Data Analysis

The axillary shoot induction experiment was conducted in a completely randomized block design. Five replicates, each consisting of six explants, were used per treatment (n = 30). The frequency of axillary shoot formation and the number of axillary shoots per explant were recorded with the aid of a Leica Wild M3Z stereomicroscope (Leica, Wetzlar, Germany) after 12 weeks of culture. To take into account both the mean number and frequency of axillary shoot formation, the bud-forming capacity (BFC) index was calculated as follows:

$$\text{BFC} = (\text{mean number of buds per explant}) \times (\%\ \text{of explants forming buds})/100$$

For the root induction experiment, ten or eleven replicates were used per treatment, each consisting of a single axillary shoot immersed vertically into the medium, and the experiment was repeated (n = 20 or 21).

Percentage data were subjected to angular transformation prior to analysis. Statistical analyses were performed using Statistica 10 software (StatSoft, Hamburg, Germany). Data were subjected to standard analysis of variance (ANOVA), and means were separated using Duncan's test at a confidence level of $p < 0.05$. All results are presented as means ± standard errors.

3. Results and Discussion

BA-pulse-treated explants of *P. peuce* started to elongate after being transferred to a PGR-free medium, which was due to elongation of the hypocotyl rather than the shoot axis. Hypocotyl elongation was inversely proportional to the BA concentration used. In explants treated with higher BA concentrations, the shoot axes either failed to elongate or only elongated to a very small extent.

Two weeks after BA pulse treatment of the explants and their subsequent transfer to PGR-free medium, the first axillary buds were discerned as swollen protrusions beneath the stem tip of the explants. These axillary buds eventually elongated into axillary shoots. Both the 1 and 2 h control treatments exhibited a high frequency of responsive explants but a relatively low number of shoots per explant, which is why their BFC indices were the lowest (Table 1). The percentage of explants forming shoots in any of the BA pulse treatments did not differ significantly from that of the respective controls. However, for both BA pulse treatment durations, a significant increase in the number of shoots per explant was recorded for BA concentrations higher than 11.5 μM compared to the controls.

The highest number of axillary shoots and the maximum BFC index were obtained with 222 µM BA pulse treatment, regardless of the treatment duration (Table 1).

Table 1. Effects of BA concentrations and exposure time on axillary shoot formation.

Treatment Duration	BA (µM)	Explants Forming Shoots (%)	Number of Shoots per Explant	Shoot Length (mm)	BFC Index	Number of Shoots ≥ 10 mm
1 h	-	90.00 ± 5.77 ^{a}	0.22 ± 0.10 ^{a}	5.17 ± 0.40 ^{a}	0.19	0
	4.4	83.33 ± 3.33 ^{a}	0.40 ± 0.14 ^{a}	8.00 ± 0.58 ^{bc}	0.33	2
	11.5	80.00 ± 5.77 ^{a}	0.88 ± 0.22 ^{a}	8.29 ± 0.39 ^{bc}	0.70	5
	22.5	86.67 ± 3.33 ^{a}	1.65 ± 0.25 ^{b}	**9.12 ± 0.32 ^{c}**	1.43	21
	44.0	83.33 ± 8.82 ^{a}	4.52 ± 0.34 ^{d}	**9.57 ± 0.30 ^{c}**	3.77	53
	222.0	80.00 ± 5.77 ^{a}	**5.50 ± 0.40 ^{e}**	6.69 ± 0.24 ^{ab}	**4.40**	19
	444.0	76.67 ± 6.67 ^{a}	3.17 ± 0.31 ^{c}	5.34 ± 0.50 ^{a}	2.43	6
2 h	-	83.33 ± 3.33 ^{a}	0.28 ± 0.11 ^{a}	4.43 ± 0.62 ^{a}	0.23	0
	4.4	86.67 ± 3.33 ^{a}	0.65 ± 0.18 ^{a}	7.12 ± 0.41 ^{c}	0.63	0
	11.5	83.33 ± 8.82 ^{a}	0.76 ± 0.19 ^{a}	7.84 ± 0.62 ^{c}	0.63	2
	22.5	80.00 ± 5.77 ^{a}	2.13 ± 0.33 ^{b}	**9.78 ± 0.36 ^{d}**	1.70	24
	44.0	83.33 ± 3.33 ^{a}	4.92 ± 0.36 ^{c}	**10.11 ± 0.28 ^{d}**	4.10	**63**
	222.0	83.33 ± 6.67 ^{a}	**5.96 ± 0.37 ^{d}**	6.85 ± 0.21 ^{bc}	**4.97**	17
	444.0	80.00 ± 5.77 ^{a}	2.63 ± 0.26 ^{b}	5.41 ± 0.31 ^{ab}	2.10	0

Values represent means ± SE, n = 30. Different letters within columns indicate significant differences between treatments according to Duncan's Multiple Range Test ($p \leq 0.05$). Maximum values are in bold.

For all treatments, the number of axillary shoots developed per explant was lower than the number of adventitious shoots previously reported to develop from cytokinin-induced adventitious buds of the Macedonian pine [22]. However, the axillary shoots were more vigorous and developed more rapidly, so the axillary buds elongated to shoots with average lengths of 9.57 mm (1 h pulse treatment) and 10.11 mm (2 h pulse treatment) within 12 weeks. Interestingly, BA stimulated shoot elongation at all but the highest concentrations tested, but even then, it had no inhibitory effect on shoot elongation (Table 1). However, explants treated with the highest BA concentrations (222 and 444 µM) produced a bundle of needles that were occasionally fasciated. In vitro culture conditions are often reported to lead to the formation of plantlets with abnormal morphology and physiology, but these abnormalities can be recovered after transfer to ex vitro conditions [23].

Axillary shoot multiplication is generally considered more difficult in conifers than in angiosperm trees [24]. Although conifers are particularly recalcitrant to clonal propagation, successful adventitious regeneration and axillary shoot multiplication have been described for a number of conifer species [25–32]. The main mechanism determining axillary meristem growth is the balance between cytokinin and auxin. One way to influence this balance is the addition of cytokinins or cytokinin-like PGRs to the tissue culture medium [32,33]. An alternative method of cytokinin application in bud induction and shoot formation is cytokinin administration via liquid pulse treatment [34]. A number of studies have reported improved organogenesis using the liquid pulse method [34–36]. The effect of liquid BA pulse treatment on the development of axillary shoots has also been described in several *Pinus* species [37,38], in which formed axillary shoots developed more rapidly and were more vigorous than their adventitious counterparts [27,37].

Shoots longer than 10 mm were used for the rooting experiment (Figure 2B). A small proportion (5%) of the non-pulsed shoots formed roots when maintained on a PGR-free medium for 8 weeks. IBA was more effective than NAA and resulted in a maximum rooting percentage and the highest number of acclimatized plants (Table 2). The IBA pulse significantly improved rooting compared to the control, regardless of the duration of the pulse treatment. NAA at either concentration did not significantly affect rooting percentage for any treatment duration.

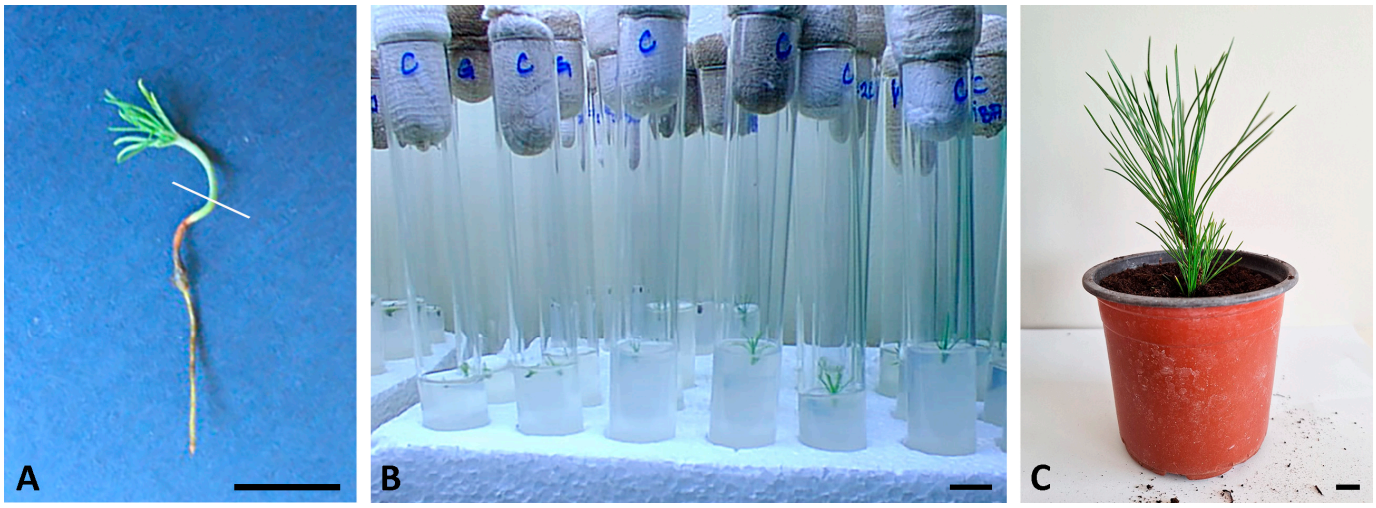

Figure 2. Overview of micropropagation of *Pinus peuce* by axillary shoots. (**A**) Four-week-old seedling, from which the radicle and part of the hypocotyl were removed (*white incision line*) to obtain explants for cytokinin pulse treatment. (**B**) Elongated axillary shoots detached from explants immediately after auxin pulse treatment and transfer to PGR-free medium. (**C**) *P. peuce* plant produced with the established micropropagation protocol after 21 months of acclimatization. Scale bar = 1 cm.

Table 2. Effects of auxin concentrations and exposure time on rooting and acclimatization effectiveness.

Treatment Duration	Auxins (mM)		Number of Shoots per Treatment	Rooting Percentage (%)	Number of Acclimatized Plants
	NAA	IBA			
1 h	-	-	20	5.00 ± 2.07 ^{a}	1
	0.27	-	21	19.05 ± 2.93 ^{abc}	2
	1.08	-	21	19.05 ± 2.93 ^{abc}	1
	-	0.25	21	33.33 ± 3.16 ^{bcd}	**4**
	-	0.98	20	**40.00 ± 2.15 ^{d}**	3
2 h	-	-	20	5.00 ± 2.07 ^{a}	1
	0.27	-	21	14.29 ± 2.93 ^{ab}	1
	1.08	-	21	23.81 ± 2.67 ^{abcd}	2
	-	0.25	21	**38.10 ± 2.67 ^{cd}**	**4**
	-	0.98	21	28.57 ± 2.07 ^{bcd}	**4**

Values represent means ± SE, $n = 20$. Different letters within columns indicate significant differences between treatments according to Duncan's Multiple Range Test ($p \leq 0.05$). Maximum values are in bold.

The formation of adventitious roots is a critical step in vegetative propagation that is essential for achieving an economically efficient production system as required in clonal forestry [39]. This complex developmental process, which is controlled by many environmental and endogenous factors, is considered a difficult step in conifers. The use of exogenous auxin is usually required for the induction of adventitious roots in difficult-to-root species. In most conifers, including *Pinus* spp., IBA and NAA are the most commonly used auxins [40]. However, the effectiveness of auxin pulses varies among species. A similar approach in the seedling explant culture of *Pinus elliottii* resulted in a very low rooting frequency (less than 1%) [37]. Humánez et al. [30] obtained similar rooting percentages in maritime pine *Pinus pinaster* shoots pulse-treated with significantly higher auxin concentrations (NAA 25 µM + IBA 4.4 µM) and a longer exposure time (48 h) at 4 °C before their transfer to potting mix in the greenhouse, whereas the application of auxin pulses before the transfer to a PGR-free medium or the presence of NAA (1 µM) in the rooting medium induced much lower rooting percentages (less than 15%).

Rooted plantlets of *P. peuce* were transferred to pots in the greenhouse, where they continued to grow as phenotypically normal plants (Figure 2C). Plants derived from IBA-

pulse treated explants had higher acclimatization rates (15–20%) than those treated with NAA pulse (Table 2). Similar results were obtained in *P. elliottii* plantlets, where plants obtained on media containing IBA or IBA + NAA had higher acclimatization percentages than those obtained on NAA-containing media [41].

4. Conclusions

The results of this research demonstrate that short-term liquid cytokinin pulse treatment and a PGR-free medium provide a starting point for developing an effective method for axillary shoot proliferation in *P. peuce* that is suitable for true-to-type plant regeneration and large-scale micropropagation for the sustainable management of its unique ecosystem. The effectiveness of auxin pulses in adventitious root formation requires further optimization, possibly by including a cold pretreatment followed by ex vitro rooting, which could lead to a higher survival rate of acclimatized plants.

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