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IN VITRO ZYGOTIC EMBRYO CULTURE OF *PINUS PEUCE* GRIS.: OPTIMIZATION OF CULTURE CONDITIONS AFFECTING GERMINATION AND EARLY SEEDLING GROWTH

DRAGANA STOJIČIĆ[1], DUŠICA JANOŠEVIĆ[2], BRANKA UZELAC[3], V. ČOKEŠA[4] and SNEŽANA BUDIMIR[3]

[1] *Faculty of Science and Mathematics, University of Niš,* 18000 Niš, Serbia
[2] *Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade,* 11000 Belgrade, Serbia
[3] *Institute for Biological Research "Siniša Stanković", University of Belgrade,* 11060 Belgrade, Serbia
[4] *Institute of Forestry,* 11000 Belgrade, Serbia

Abstract - This study reports a protocol for the germination and early seedling growth of *Pinus peuce* Gris. using zygotic embryo culture. In order to overcome seed dormancy and optimize organogenesis, the effect of nutritional, plant growth regulatory and physical factors on *in vitro* germination and growth of isolated mature zygotic embryos of *P. peuce* were investigated.

Key words: Embryogenesis, Macedonian pine, organogenesis, seed germination, Serbia

INTRODUCTION

Pinus peuce Gris. (Macedonian pine) is a Tertiary relic species endemic to the Balkan Peninsula. This pine usually grows on high mountains, at altitudes between 600 and 2200 m, on slopes on siliceous soils and rarely on carbonate soils (Vidaković, 1982). The tree is ornamental and up to 25 m tall. Macedonian pine is tolerant to winter cold and wind exposure and is therefore recommended as a melioration tree suitable for planting on degraded and devastated soils (Janković, 1991). In natural stands, *P. peuce* trees begin producing cones at about the age of six years. Seeds are dormant and need chilling treatment of about six months to germinate. Non-stratified seeds have an extremely low germination rate (about 0.1%), which is partly due to the hard seed coat that acts as a physical barrier, and due to low (as low as 12%) and extremely variable seed viability (Nikolić, 2005). Because of the limited area of *P. peuce* natural distribution, variable abundance of seed production and low ability for natural reproduction, the implementation of conservation measures for this economically important forest plantation species is required.

Zygotic embryo culture has been shown to play an important role in rapid *in vitro* propagation of endangered forest tree species to overcome physical and biotic interference (Rambabu et al., 2006). The few *in vitro* culture studies with isolated mature zygotic embryos of conifers have indicated the importance of the megagametophyte to the embryo during early seedling growth (David et al., 1995). The method of substituting the megagametophyte with nutrients, plant growth regulators and physical factors is increasingly applied for successful breaking of seed dormancy, hence shortening the life cycle and allowing the rapid multiplication and conservation of endangered conifer species or species with extremely low germination rate. Therefore this study was con-

ducted to evaluate the requirements for *in vitro* germination and growth of Macedonian pine zygotic embryos, in order to optimize organogenesis using mature zygotic embryos as the starting explants.

MATERIALS AND METHODS

Plant material and culture of isolated embryos

Cones of *Pinus peuce* Gris. were collected from open pollinated trees in a seed orchard located on Mučanj Mountain (Serbia). Prior to the experiments, the seeds were removed from the cones, washed under running tap water for 24 h and surface sterilized with 25% (v/v) sodium hypochlorite for 25 min, followed by rinsing three times with sterile double distilled water. Mature zygotic embryos were then aseptically excised from the surrounding gametophytic tissue and, if not mentioned otherwise, placed horizontally on the surface of the culture medium (20 ml medium per 9-cm Petri dish).

Culture media and conditions

The basal medium was GD (Gresshoff and Doy, 1972) culture medium as modified by Sommer et al. (1975), supplemented with 3% (w/v) sucrose and 500 mg l^{-1} casein enzymatic hydrolyzate and solidified with 0.7% (w/v) agar (Torlak, Belgrade). The medium pH was adjusted to 5.7 prior to autoclaving at 115°C for 25 min. All cultures were maintained at 25 ± 2°C and, if not mentioned otherwise, incubated under cool white fluorescent light (16 h photoperiod; 47 μmol m^{-2} s^{-1}).

Experimental procedures

Isolated zygotic embryos were cultured under a range of different media variations and culture conditions. For each treatment, a total of 36 embryos was used and the experimental trials were repeated twice. There were three replicates per treatment, each consisting of six embryos.

The effects of five different medium strengths were tested: 0, 0.25, 0.5, 1 (basal medium) and 2 times that of GD. Other components were the same as in basal GD medium.

To examine the effects of different plant growth regulators, basal GD medium was supplemented with either one of the following growth regulators, at five concentrations each: benzyladenine (BA; 0, 0.06, 0.11, 0.22 or 0.44 μM), kinetin (KIN; 0, 0.12, 0.23, 0.46, 0.93 μM), gibberellic acid (GA_3; 0, 0.14, 0.29, 0.58, 1.15 μM), α-naphthalene acetic acid (NAA; 0, 0.07, 0.13, 0.27, 0.54 μM), or indole-3-butyric acid (IBA; 0, 0.06, 0.12, 0.25, 0.49 μM).

In order to determine the effect of medium composition on embryo germination and seedling growth, two different treatments were compared: 0.7% (w/v) agar-gelled medium and liquid medium.

To examine the effects of embryo orientation, explants were placed either horizontally on the surface of the medium, or vertically immersed into the medium, with either the radicle end or cotyledons (leaving the hypocotyl and radicle end free).

To investigate the influence of light, three different treatments were compared in this study: continuous (24-h) light, a 16-h photoperiod and continuous (24-h) darkness.

Data collection and statistical analysis

Embryos were scored as germinated if they exhibited root elongation. Germination percentages and the growth of embryos (measured as the root and whole seedling length) were scored at 7-day intervals up to day 28 (4 weeks). The dry weight of the 4-week old plantlets was recorded after drying the seedlings at 70°C for 24 h.

Germination percentages were arcsine transformed before analysis. The data of the 28-day scoring, obtained from two repeated experiments, were averaged and statistically analyzed, and differences were tested for significance using ANOVA Multiple range test at the significance level of $p \leq 0.05$.

RESULTS

In preliminary trials it was found that the majority of *P. peuce* zygotic embryos, when devoid of seed coat, testa and endosperm, and placed on culture medium, readily germinated already after 24 h (about 80% germinated after only one week in culture) without stratification.

Nutritional factors

The influence of medium strength on *P. peuce* zygotic embryo germination and development is shown in Table 1. There were no significant differences in germination percentages among the treatments ($P>0.05$). However, embryos grown on medium without salts and vitamins developed into physiologically weakened and malformed seedlings (Fig. 1A). Of the salt concentrations tested, half-strength as well as basal medium proved to be the most efficient in stimulating root and whole seedling growth. The seedlings grown on basal medium were more than twice as long (32.4 mm) as those grown on the control (0GD) medium (14.6 mm), while the dry weight of the seedlings grown on basal medium was nearly three times greater than that of the control (Table 1). In addition, the best morphological characteristics of seedlings were obtained on basal (Fig. 1B) and half-strength medium.

Plant growth regulators

The germination percentages obtained at tested concentrations of various plant growth regulators were similar, indicating an insignificant effect of PGR level on zygotic embryo germination (Table 2). However, in the presence of GA_3, germination was synchronous so that almost all embryos germinated after only one week in culture, with 100% germination rate reached at lower GA_3 levels (0.14-0.29 μM). In addition, root growth was slightly promoted at 0.29 μM GA_3 (Fig. 1C), while the highest seedling dry weight was obtained in the presence of GA_3 at concentrations from 0.29 μM and higher. Cytokinins, even at low concentrations, affected embryo growth, leading eventually to callus formation in the hypocotyl region and inhibiting root development (Fig 1D). However, shoot development was not affected by either BA or KIN. In the presence of 0.44 μM BA or 0.93 μM KIN occasionally a few adventitious buds developed at the tip of cotyledons and rarely in the base of cotyledons (Fig. 1D). Germinated embryos grown in the presence of auxins (NAA or IBA) developed long, crinkled cotyledons, and short, swollen hypocotyls on which the callus tissue was formed. NAA at all levels tested hindered root growth.

Physical factors

Germination and embryo growth were also affected by medium composition (agar-gelled vs. liquid medium). The frequency of embryo germination was higher on agar-solidified medium comparing to liquid medium, which greatly suppressed root growth (Table 3). Seedling length and dry weight were also significantly greater on agar-gelled medium compared to the liquid one.

Embryo orientation had a significant effect on both germination rate and growth parameters. The embryos with their radicle end immersed in the culture medium showed 100% germination and the best morphological characteristics, compared to both horizontally placed embryos and those growing with their cotyledons immersed into the culture medium (Table 3).

Germination of isolated embryos was independent of light, in contrast to embryo growth that appeared to be influenced by different light treatments (Table 3). Complete darkness inhibited root growth but did not affect hypocotyl growth. Also, the dry weight of the dark-grown seedlings was lower compared to those grown in light, irrespective of the light regime (Table 3).

DISCUSSION

The seeds of *Pinus peuce* are dormant, the germination rate of non-stratified seeds being about 0.1% under natural conditions (Nikolić, 2005). The dorman-

Table 1. Effect of nutritional factors on the germination and growth of isolated zygotic embryos of *Pinus peuce* Gris. after 28 days of culture

Medium strength	Percentage (%) of germinated embryos	Seedling root length (mm)	Whole seedling length (mm)	Seedling dry weight (mg)
0 GD	69.44 ± 10.92[a]	6.16 ± 0.42[a]	14.64 ± 0.67[a]	5.64 ± 0.35[a]
0.25 GD	77.78 ± 7.02[a]	11.86 ± 1.45[ab]	22.29 ± 1.91[b]	10.00 ± 0.37[b]
0.5 GD	80.56 ± 9.03[a]	16.10 ± 2.25[bc]	28.17 ± 2.61[bc]	13.69 ± 1.11[c]
1 GD	83.33 ± 10.21[a]	19.10 ± 3.14[c]	32.43 ± 3.70[c]	14.77 ± 1.32[c]
2 GD	77.78 ± 3.94[a]	11.36 ± 1.44[ab]	23.39 ± 1.56[b]	14.00 ± 1.02[c]

[a] The values are means ± standard error of two repeated experiments, each with 18 embryos per treatment. Means in the column followed by the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Table 2. Effect of plant growth regulators on the germination and growth of isolated embryos of *Pinus peuce* Gris. after 28 days of culture

Plant growth regulators (PGRs) (μM)	Percentage (%) of germinated embryos	Seedling root length (mm)	Whole seedling length (mm)	Seedling dry weight (mg)
0 (no PGRs)	83.33 ± 4.30[abc]	20.03 ± 1.35[hi]	36.23 ± 1.60[ijk]	23.20 ± 1.26[f]
BA				
0.06	77.78 ± 5.56[ab]	10.89 ± 0.64[cdef]	20.36 ± 0.79[cde]	16.00 ± 0.85[b]
0.11	75.00 ± 7.14[a]	11.19 ± 1.33[cdef]	20.11 ± 1.55[cde]	22.78 ± 1.80[ef]
0.22	75.00 ± 7.14[a]	9.33 ± 0.44[abcd]	16.59 ± 0.73[bcd]	15.70 ± 1.10[ab]
0.44	72.00 ± 8.24[a]	8.77 ± 0.52[abcd]	16.38 ± 0.76[bcd]	17.35 ± 1.30[bcd]
KIN				
0.12	80.56 ± 6.69[ab]	8.17 ± 0.53[abc]	15.28 ± 0.67[abc]	15.48 ± 0.86[ab]
0.23	77.78 ± 8.24[ab]	11.82 ± 1.47[cdef]	21.50 ± 1.53[def]	21.11 ± 1.18[cdef]
0.46	75.00 ± 7.14[a]	11.70 ± 1.50[cdef]	19.74 ± 1.45[cde]	21.26 ± 1.32[def]
0.93	77.78 ± 8.24[ab]	10.29 ± 0.68[bcde]	18.18 ± 0.70[cd]	17.96 ± 1.06[bcd]
GA_3				
0.14	100.00 ± 0.00[c]	15.81 ± 1.55[fgh]	28.06 ± 1.67[ghi]	20.72 ± 0.71[cdef]
0.29	100.00 ± 0.00[c]	24.58 ± 2.99[i]	40.50 ± 3.06[k]	29.97 ± 1.42[g]
0.58	94.44 ± 3.51[bc]	19.41 ± 3.53[h]	33.82 ± 3.65[j]	29.97 ± 1.88[g]
1.15	94.44 ± 3.51[bc]	18.12 ± 2.46[gh]	32.29 ± 2.74[ij]	27.50 ± 1.11[g]
NAA				
0.07	77.78 ± 7.03[ab]	5.29 ± 0.29[ab]	11.39 ± 0.43[ab]	11.93 ± 0.84[a]
0.13	83.33 ± 4.30[abc]	5.50 ± 0.37[ab]	16.27 ± 0.55[bcd]	28.40 ± 2.14[g]
0.27	77.78 ± 5.56[ab]	4.82 ± 0.20[a]	11.39 ± 0.43[ab]	22.25 ± 1.37[ef]
0.54	77.78 ± 3.51[ab]	4.36 ± 0.16[a]	10.04 ± 0.32[a]	30.29 ± 1.58[g]
IBA				
0.06	83.33 ± 6.09[abc]	8.90 ± 0.65[abcd]	19.07 ± 0.71[cd]	16.97 ± 1.25[bc]
0.12	86.11 ± 5.12[abc]	14.84 ± 1.69[efgh]	26.77 ± 1.83[fgh]	18.74 ± 1.76[bcde]
0.25	80.66 ± 5.12[ab]	19.10 ± 2.26[h]	31.62 ± 2.41[hij]	23.07 ± 1.26[f]
0.49	83.33 ± 4.30[abc]	13.77 ± 1.02[defg]	25.20 ± 1.26[efg]	18.10 ± 1.18[bcd]

[a] The values are means ± standard error of two repeated experiments, each with 18 embryos per treatment. Means in the column followed by the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Table 3. Effect of physical factors on the germination and growth of isolated embryos of *Pinus peuce* Gris. after 28 days of culture

Physical factors	Percentage (%) of germinated embryos	Seedling root length (mm)	Whole seedling length (mm)	Seedling dry weight (mg)
Medium composition				
0.7% agar	83.33 ± 4.30[b]	20.03 ± 1.35[b]	36.23 ± 1.60[b]	23.20 ± 1.26[b]
Liquid	69.44 ± 5.14[a]	10.14 ± 1.42[a]	20.28 ±1.58 [a]	16.38 ± 1.22[a]
Embryo orientation				
Horizontal	83.33 ± 4.30[b]	20.03 ± 1.35[b]	36.23 ± 1.60[b]	23.20 ± 1.26[b]
Radicle end in medium	100.00 ± 0.00[c]	24.06 ± 1.77[c]	39.56 ± 2.88[c]	29.08 ± 1.66[c]
Cotyledons in medium	69.44 ± 4.56[a]	17.36 ± 2.18[a]	32.40 ± 1.78[a]	20.56 ± 1.23[a]
Light				
24-h light	83.33 ± 4.67[a]	23.06 ± 1.82[c]	37.89 ± 1.73[b]	22.58 ± 1.34[b]
16-h light	83.33 ± 4.30[a]	20.03 ± 1.35[b]	36.23 ± 1.60[b]	23.20 ± 1.26[b]
24-h darkness	77.78 ± 4.17[a]	14.14 ± 2.24[a]	33.66 ± 1.46[a]	15.93 ± 0.98[a]

[a] The values are means ± standard error of two repeated experiments, each with 18 embryos per treatment. Means within the column of each factor followed by the same letter are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

cy of *P. peuce* seeds is probably caused by the hard seed coat which acts as a physical barrier to water uptake. In addition, Djordjeva et al. (1969) showed that the viability of *P. peuce* seeds is highly variable, ranging from 15-78%, since 5-20% of the seeds was found to contain no embryos, while in 12-60% they were poorly developed. The stratification of about six months has to be performed in order to obtain a higher rate of seed germination (up to 75%). In the present study we demonstrated that up to 100% of *P. peuce* isolated zygotic embryos can germinate and develop into healthy looking seedlings under optimal *in vitro* conditions.

The results of our previous study showed that in *P. heldreichii* zygotic embryo culture, GD medium was superior to both LP (von Arnold and Eriksson, 1981) medium and MS (Murashige and Skoog, 1962) medium, in terms of embryo survival *in vitro* (Stojičić et al., 1999). Preliminary trials confirmed this was also the case for *P. peuce* embryos. GD medium strength only slightly affected the germination percentage of *P. peuce* isolated embryos, but the concentrations of salts and vitamins in the medium were critical for regular seedling development. The best results for the tested parameters (germination, root length, whole seedling length and seedling dry weight) were obtained when 1GD medium was used. Similar results were obtained for zygotic embryos of *P. heldreichii* (Stojičić et al., 2008). The effect of the medium type and/or its strength on zygotic embryo germination and development was also shown for zygotic embryos of endangered tree species, such as *Givotia rottleriformis* (Rambabu et al., 2006) and *Boswellia serrata* (Ghorpade et al., 2010).

Being an essential component of the culture medium as a source of energy and for maintaining its osmotic potential, carbohydrates may be critical for both embryo development and their morphogenic response *in vitro*. Isolated zygotic embryos of *Pinus radiata* failed to germinate on the medium without any carbohydrates, whereas among several carbon sources tested, maltose was much inferior to sucrose, glucose or fructose at similar concentrations (Lin and Leung, 2002). *P. peuce* embryos could germinate in the absence of carbohydrates in the medium in a fairly high percentage, but under these conditions seedlings were somewhat abnormal. Sucrose applied at 3% was superior to glucose, fructose or maltose at similar concentrations in promoting embryo germination and further development (Stojičić et al., 2009). Based on these findings, 3% sucrose was se-

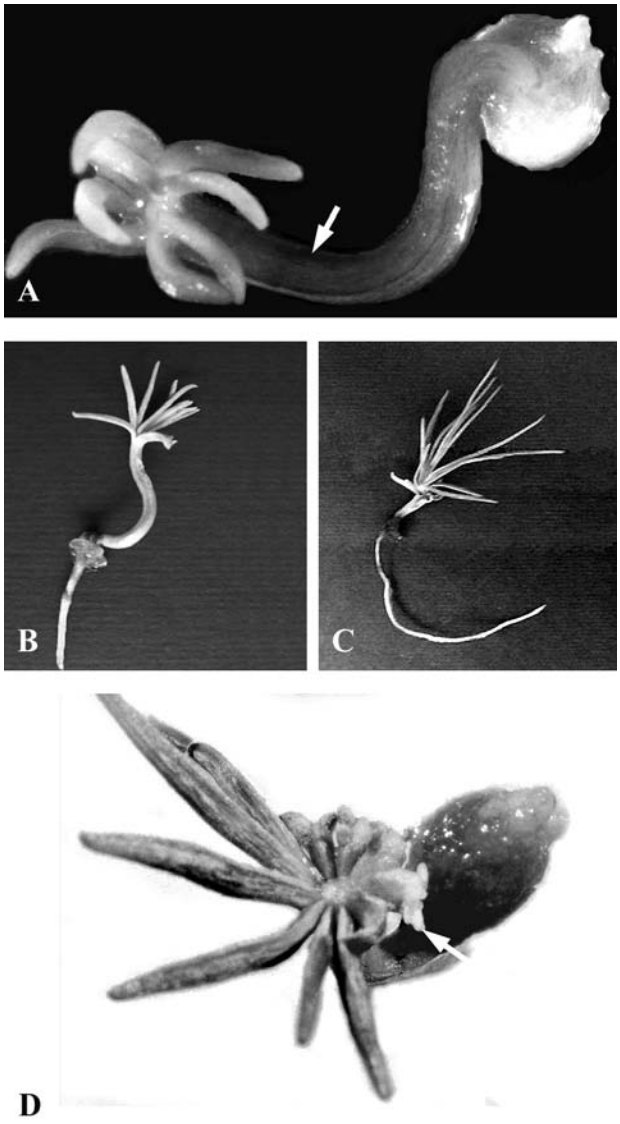

Fig. 1 *Pinus peuce* zygotic embryo development under different *in vitro* culture conditions. **A** Malformed seedling developed on medium without mineral salts and vitamins (0GD), supplemented with 3% sucrose, after 7 days of culture. Note anthocyanin accumulation along the hypocotyl, *arrow*. **B** Healthy looking seedling developed on full-strength GD medium (1GD) supplemented with 3% sucrose, after 28 days of culture. **C** Root elongation in seedling grown on 1GD medium supplemented with 3% sucrose and 0.29 μM GA_3, after 28 days of culture. **D** Callus formation in the hypocotyl region and inhibition of root development in a seedling cultured for 28 days on 1GD medium, supplemented with 3% sucrose and 0.44 μM BA. Note numerous adventitious buds (*arrow*) developing at the tip of cotyledons.

lected as a primary carbohydrate source for further experiments in *P. peuce* embryo culture.

In *P. peuce* zygotic embryo culture, among the growth regulators tested, only GA_3 was shown to increase the germination percentage and stimulate embryo development. Gibberellic acid was also shown to have a stimulatory effect on embryo germination and seedling growth in *P. radiata* (Lin and Leung, 2002) and some other woody species such as coconut (Pech et al., 2007). The growth of *P. peuce* embryos was adverse in the presence of all levels of cytokinins, whether BA, KIN, auxins, or NAA or IBA. Zygotic embryos of wild *Musa acuminata* (Asif et al., 2001) as well as *Prunus armeniaca* (Yildirim et al., 2007) germinated readily in the presence of BA.

In this study, the role of several physical factors in the germination and growth of isolated zygotic embryos of *P. peuce* was examined: medium composition, light regime and embryo orientation during sowing. Isolated embryos readily germinated and had a better growth performance on agar-solidified medium than in liquid medium. In *P. radiata*, the addition of a gelling agent to a medium increased the germination capacity, but seedlings grew significantly better in a liquid medium (Lin and Leung, 2002). Zygotic embryo germination of *P. peuce* was stimulated by light irrespective of photoperiod duration, but the best root growth was obtained under continuous light. Lin and Leung (2002) indicated that in *P. radiata* light was important for the integrated growth of isolated embryos. Although embryo germination was independent of light, different light treatments significantly influenced their further growth. Cotyledon development was best in the 16-h photoperiod, while continuous light was most effective for the seedling root growth. According to Yildirim et al. (2007), in *P. armeniaca* germination was not influenced by light or complete darkness, whilst the mean shoot length was significantly higher in dark-grown embryos, although plantlets developing under these conditions were tiny and unhealthy.

In conclusion, this study reports a simple, efficient and cost-effective protocol for the rapid and

synchronous production of *P. peuce* seedlings directly from seeds. Zygotic embryo culture increased the germination rate to ≥ 80% without using any pretreatments. The highest germination percentage and the most vigorous subsequent seedling growth of Macedonian pine were obtained when mature zygotic embryos were cultured with their radicle end immersed in the full-strength agar-solidified GD medium, supplemented with 3% (w/v) sucrose and 0.29 μM GA_3, and grown under continuous light. The results presented herein could be used for the successful breaking of *P. peuce* seed dormancy, thereby allowing a rapid and synchronous plant production and conservation of this endangered conifer species.

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