NATURA MONTENEGRINA, Podgorica, 8(2):63-71

GERMINATION AND GROWTH OF ISOLATED ZYGOTIC EMBRYOS OF *PINUS HELDREICHII* AND *PINUS PEUCE*

Dragana STOJIČIĆ[1], Snežana BUDIMIR[2], Dušica JANOŠEVIĆ[3]

[1]Institute of Forestry, 11010 Belgrade, Serbia, stojicic@net.yu
[2]Siniša Stanković Institute for Biological Research, University of Belgrade, 11060 Belgrade, Serbia, budimir@ibiss.bg.ac.yu
[3]Institute of Botany Faculty of Biology University of Belgrade, 11000 Belgrade, Serbia

Key words:
Pinus heldreichii,
Pinus peuce,
embryo culture,
seed germination.

SYNOPSIS

Bosnian pine *Pinus heldreichii* (Christ.) and Macedonian pine *Pinus peuce* (Gris.) are Tertiary relics and endemics of the Balkan Penninsula. The cultivation of isolated zygotic embryos *in vitro* under different conditions defines the factors which regulate embryo germination outside the maternal tissue, and also avoids seed dormancy. For the study of the effects of nutritional factors on the embryo germination and growth, isolated mature zygotic embryos of *Pinus heldreichii* and *Pinus peuce* were grown four weeks on the Gresshoff and Doy (GD) medium supplemented with sucrose, glucose, fructose or maltose. All tested carbohydrates at applied concentrations stimulated embryo growth, but only 3% sucrose increased embryo germination comparing to control, while maltose had an inhibitory effect.

Ključne riječi:
Pinus heldreichii,
Pinus peuce,
kultura embriona,
klijanje semena.

SINOPSIS

KLIJANJE I RASTENJE IZOLOVANIH ZIGOTSKIH EMBRIONA PINUS HELDREICHII i PINUS PEUCE

Munika *Pinus heldreichii* (Christ.) i molika *Pinus peuce* (Gris.) su tercijarni relikti i endemiti Balkanskog poluostrva. Gajenjem izolovanih zigotskih embriona *in vitro* definisani su faktori koji regulišu klijanje embriona izvan materinskog tkiva, ali je takođe prevaziđena dormancija semena. U cilju proučavanja uticaja nutritivnih faktora na klijanje embriona i njihovo rastenje, izolovani zreli zigotski embrioni munike i molike su gajeni četiri nedelje na hranljivoj podlozi Gresshoff and Doy (GD), kojoj je dodavana saharoza, maltoza, glukoza ili fruktoza. Svi testirani ugljeni hidrati i njihove korišćene koncentracije su stimulisali rastenje embriona, ali je samo 3% saharoza pospješila klijanje embriona u poređenju sa kontrolom. Maltoza je imala inhibitoran efekat.

INTRODUCTION

Bosnian pine *Pinus heldreichii* Christ. and Macedonian pine, *Pinus peuce* Gris. are Tertiary relics and endemics and among the most significant species of the Balkan Penninsula dendroflora. These two pines usually grow in the mountains influenced by Mediterranean climate, i.e. on Prokletije, Koritnik, Šarplanina, Ostrovica, Kodža-Balkan, Pelister and others. It spreads up to 2000 m even beyond that altitude (JANKOVIĆ, 1991). Bosnian pine grows on steep and dry limestone slopes, most often in pure stands, while Macedonian pine grows on slopes on siliceous soils, rarely on carbonate soils (VIDAKOVIĆ, 1982).

Bosnian pine tree is ornamental, up to 30 m high, with a pyramidal habit. Mature bark is ash gray. Needles are two in fascicle, 4-10 cm long. Cones are 5-10 cm long, they are dark blue-purple before maturation, turning brown 16-18 months after pollination. Seeds are brown 6-7mm long.

Macedonian pine tree is 35-40 m high, bark on young trees is smooth silvery grey, later becoming darker and rough. Leaves are 5 per fascicle, 4-10 cm long, cones are pendulous 9-20 cm long, cylindric. Seeds are grey-brown, shed as soon as cones mature in October, 12-14 months after pollination.

Both coniferous species possess numerous remarkable characteristics, such as ecological tolerance and frost resistance that make them very valuable for forest tree breeding (MITCHELL, 1996). *Pinus heldreichii* is tolerant of drought and saline conditions. *Pinus peuce* is very tolerant of winter cold and also of wind exposure. From the ecological point of view, both species are recommended as melioration trees suitable for planting on degraded and devastated soils. They are also a popular ornamental trees in parks and large gardens. Because of their limited area of natural distribution, both species require special attention and implementation of measures for their conversion (JANKOVIĆ, 1991).

Bosnian pine and Macedonian pine seeds are dormant in natural conditions, as well as the seeds of majority of *Pinus* species. To be able to germinate, Bosnian pine seeds need to be stratified for six weeks, and Macedonian pine seeds for six months in cool-moist conditions (STILINOVIĆ, 1985). As stratification is a complex process, which increases the seedling production costs, efforts are made to ensure a fast, uniform and synchronized seed germination (COOKE & GIFFORD, 2002). Abundance of seed production varies greatly not only from year to year, but also within different trees and cones (ĐORĐEVA et al. 1972; MIČEV, 1972). Embryo culture *in vitro* is an increasingly applied method for the successful breaking of dormancy and shortening of the plant growth cycle.

P. heldreichii and *P. peuce* are usually propagated by seeds, since the rooting of cuttings has been only partially successful. However, variable seed production may complicate operational reforestation and tree improvement efforts. Therefore, the methods of *in vitro* culture can be used as a significant contribution to conventional methods of vegetative propagation of both coniferous species.

We have previously reported plant regeneration of *P. heldreichii* through adventitious buds (STOJIČIĆ et al., 1999) and through axillary buds (STOJIČIĆ & BUDIMIR, 2004), also the induction of somatic embryogenesis in culture of this pine (STOJIČIĆ et al., 2007). According to obtained results, micro-propagation and somatic embryogenesis have a potential use in the propagation of *P. heldreichii* but further improvement of the methods is required. Culture of isolated zygotic embryos could be used in gaining an insight into requirements for plant conversion from the embryo.

The aim of this study was to investigate the possibility for cultivation of isolated zygotic embryos *in vitro*, under different conditions, also to define the factors which regulate embryo germination and growth of isolated mature zygotic embryos outside the maternal tissue.

SUBJECT AND METHOD OF RESEARCH

Cones of *Pinus heldreichii* were harvested from open-pollinated trees on Lovćen Mountain (Montenegro) during October 2004. Cones of *P. peuce* were harvested from open-pollinated trees located on Mučanj Mountain during October 2003 and 2004. Seeds were extracted from the cones and kept at 4 °C. After surface-sterilization in 20% sodium hypohlorite for 30 minutes, seeds were rinsed three times with sterile distilled water, and then seed coats were removed. The embryos were aseptically isolated from megagametophyte and placed horizontally on the surface of the GD (GRESSHOFF & DOY, 1972) medium as modified by SOMMER et al. (1975).

For the study of embryo germination, embryos were grown 4 weeks on the GD medium supplemented with 0.7% agar (Torlak, Belgrade) and 0, 1, 2, 3, 4, or 5% sucrose, glucose, fructose or maltose. The pH of the media was adjust to 5.7 prior to autoclaving at 115 ºC for 25 min. Cultures were maintained at 25 ± 2 ºC on 16h/8h photoperiod.

There were two replicates per treatment, each consisting of 18 embryos, total 36 embryos for each treatment. Embryos were scored as germinated if they exhibited root and shoot elongation. The plantlets dry weight was recorded after drying at 70 ºC for 24h. Data collected from experiments were calculated and differences were tested for significance using ANOVA, and Duncans' multiple range test at lavel of significance $p \leq 0.05$.

RESULTS WITH DISCUSSION

The embryos of *Pinus heldreichii* and *Pinus peuce* contain the store of nutrient substances which are essential for the beginning of the germination process, but further embryo development requires the nutrients stored in the female gametophyte, i.e. the seed “endosperm”. If the endosperm is removed, the transport of nutrients from the endosperm to the embryo is made impossible. In that case, the embryos take the necessary nutrients from culture media on which they are grown. By varying the composition of culture media, the development process can be directed in the desired direction, and the factors which control plant embryo growth and development can be defined.

Bosnian pine and Macedonian pine seeds used in this research were one-year old, and they were stored at the temperature of 4ºC till the day of testing. On the appropriate culture media, the embryos of both species, with the removed testa and endosperm, germinated *in vitro* after 24 hours without any previous treatments. The culture medium composition optimal for Bosnian pine and Macedonian pine embryo germination and seedling growth is determined empirically. The study parameters are: the percentage of germinated embryos, length of seedlings and their fresh and dry mass.

Carbohydrates were not indispensable for Bosnian pine and Macedonian pine embryo germination. Still *in vitro,* the seedlings become mixotrophic and, in addition to photosynthesis, they absorbed the necessary energy for different processes by the assimilation of carbohydrates from culture media. Bosnian pine embryos (72 %) and Macedonian pine embryos (67 %) germinated on the media without carbon source (figure 1, 2). These seedlings were etiolated, physiologically weakened, but all of them succeeded in surviving for four weeks in such culture conditions. This finding is different from that of other studies. The isolated mature embryo of *Pinus radiata* could not germinate on the medium without any carbohydrate (LIN & LEUNG, 2002).

Sucrose was superior in promoting Bosnian pine and Macedonian pine embryo germination comparing to glucose, fructose and maltose. The germination frequency was increased with all sucrose concentrations (1−5%) comparing the control. Glucose and fructose had no effect on germination, while the media with maltose inhibited the process (figure 1, 2). Results similar with these were obtained in *Pinus radiata*, where sucrose, glucose and maltose serve as carbon source for isolated embryos cultured *in vitro*, while maltose was found to be inferior to these sugars in promoting germination and embryo growth (LIN & LEUNG, 2002).

The absence of carbohydrates in the medium caused slower seedling growth of both species. Medium with sucrose, maltose, glucose and fructose stimulated embryo growth, especially 3% sucrose, so that at day 28 seedlings were more than two times longer then on control medium (Table 1, 2).

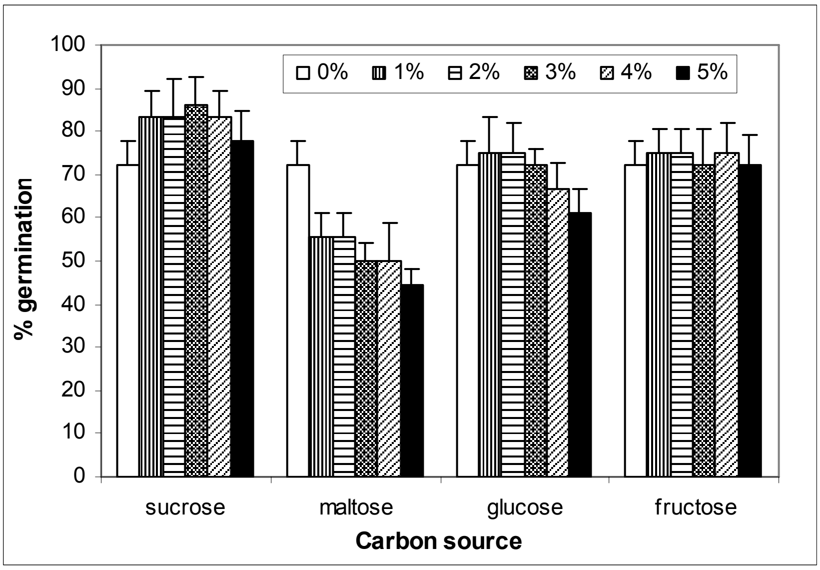

Figure 1. Effect of carbon source and concentration on mean germination percentage of isolated embryos of *Pinus heldreichii*

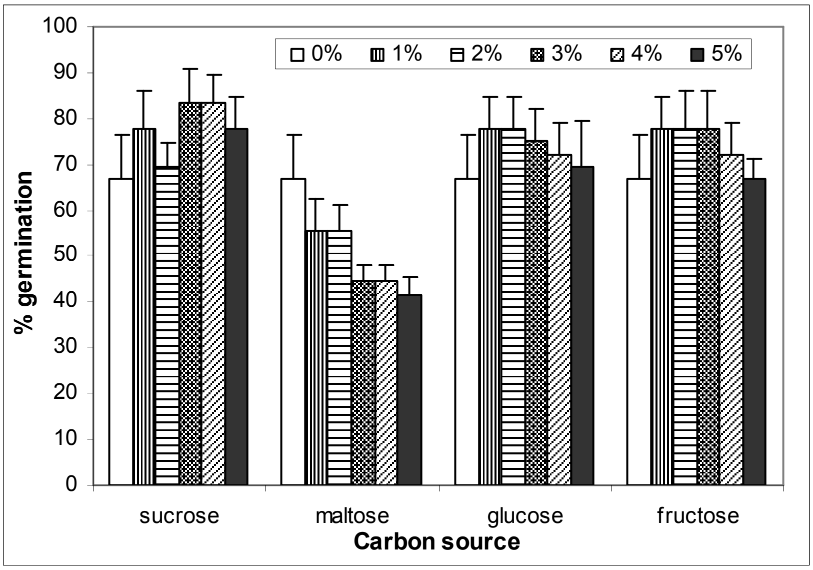

Figure 2. Effect of carbon source and concentration on mean germination percentage of isolated embryos of *Pinus peuce*

The media with high concentrations of maltose were watery and viscous, so the embryos were soaked in the medium. In such conditions, the inhibited exchange of gases caused the necrosis of embryo and, later on, of the seedling tissue. The high osmotic pressure in the medium and the difficult water absorption brought about the lower cell division frequency and the poorer cell growth, which resulted in the reduced explant growth.

The embryo germination, length of plantlet, as well as fresh and dry weight, were greater when embryos were cultured on medium supplemented with sucrose compared to the media with maltose, glucose or fructose (Table 1, 2). Maximum fresh and dry weights were obtained when Bosnian pine and Macedonian pine embryos were cultured on medium supplemented with 3, 4, 5% sucrose.

Table 1. Effect of carbon source on the growth of plantlets of *Pinus heldreichii*

Carbon source	Plantlets lenght (mm)	Fresh weight (mg)	Dry weight (mg)
Controles	19,00 ± 1,45^{a}	11,62 ± 1,51^{a}	4,69 ± 0,37^{a}
Sucrose (%)			
1	29,80 ± 2,00^{b}	21,27 ± 1,16^{a}	9,20 ± 1,01^{ab}
2	33,33 ± 3,58^{bc}	39,20 ± 5,29^{b}	11,27 ± 0,91^{b}
3	**42,77 ± 5,08^{c}**	74,06 ± 6,86^{c}	31,90 ± 3,74^{d}
4	39,93 ± 3,77^{bc}	73,20 ± 3,69^{c}	31,03 ± 1,21^{c}
5	30,07 ± 3,91^{b}	74,79 ± 2,04^{c}	30,71 ± 1,19^{c}
Maltose (%)			
1	27,15 ± 1,18^{d}	22,05 ± 1,55^{b}	9,45 ± 0,74^{b}
2	25,65 ± 0,83^{cd}	22,75 ± 2,07^{b}	10,05 ± 0,65^{b}
3	25,28 ± 0,88^{cd}	21,83 ± 1,22^{b}	9,61 ± 0,44^{b}
4	23,17 ± 0,84^{bc}	22,72 ± 1,33^{b}	9,77 ± 0,35^{b}
5	20,75 ± 0,56^{ab}	23,63 ± 1,40^{c}	10,44 ± 0,39^{b}
Glucose (%)			
1	29,00 ± 2,37^{ab}	21,22 ± 1,01^{b}	10,30 ± 0,36^{b}
2	35,22 ± 4,66^{b}	22,89 ± 0,92^{b}	10,93 ± 0,35^{b}
3	27,38 ± 3,41^{ab}	20,38 ± 0,76^{b}	9,54 ± 0,29^{b}
4	30,96 ± 4,51^{b}	22,24 ± 0,74^{b}	10,80 ± 0,39^{b}
5	29,42 ± 4,21^{ab}	20,40 ± 0,82^{b}	9,96 ± 0,33^{b}
Fructose (%)			
1	29,56 ± 1,59^{b}	20,27 ± 1,00^{b}	9,28 ± 0,35^{b}
2	34,26 ± 2,94^{b}	20,00 ± 0,92^{b}	9,91 ± 0,35^{b}
3	34,96 ± 2,09^{b}	21,19 ± 0,76^{b}	10,21 ± 0,29^{b}
4	30,74 ± 1,84^{b}	21,89 ± 0,77^{b}	11,00 ± 0,39^{b}
5	29,85 ± 1,73^{b}	22,00 ± 0,83^{b}	10,90 ± 0,33^{b}

Means in the column followed by different letters are different according to Duncans' Multiple Range Test ($p \leq 0.05$)

Table 2. Effect of carbon source on the growth of plantlets of *Pinus peuce*

Carbon source	Plantlets lenght (mm)	Fresh weight (mg)	Dry weight (mg)
Controles	24,33 ± 1,31[a]	33,92 ± 2,52[a]	5,25 ± 0,46[a]
Sucrose (%)			
1	32,50 ± 0,96[b]	50,89 ± 3,12[b]	8,00 ± 0,50[a]
2	36,44 ± 1,84[bc]	50,60 ± 3,80[b]	14,76 ± 1,27[b]
3	**42,97 ± 4,02**[c]	54,13 ± 3,14[b]	14,77 ± 1,32[b]
4	33,53 ± 2,88[b]	47,50 ± 2,63[b]	14,70 ± 1,26[b]
5	31,86 ± 1,96[b]	52,11 ± 3,17[b]	13,68 ± 1,21[b]
Maltose (%)			
1	34,20 ± 1,13[c]	35,50 ± 2,60[a]	9,85 ± 0,58[b]
2	30,40 ± 1,15[b]	36,30 ± 2,28[a]	11,65 ± 0,58[ab]
3	29,88 ± 1,55[b]	39,39 ± 3,11[a]	14,06 ± 0,99[c]
4	22,44 ± 0,59[a]	40,63 ± 4,50[a]	13,13 ± 1,25[c]
5	21,60 ± 0,99[a]	38,33 ± 3,84[a]	13,87 ± 1,23[c]
Glucose (%)			
1	34,43 ± 0,96[c]	38,82 ± 2,07[a]	8,82 ± 0,39[b]
2	29,36 ± 1,84[b]	39,89 ± 1,78[a]	11,68 ± 0,69[d]
3	27,48 ± 4,02[ab]	34,07 ± 2,25[a]	10,70 ± 0,58[cd]
4	24,08 ± 2,88[a]	33,35 ± 1,89[a]	9,58 ± 0,37[bc]
5	24,24 ± 1,96[a]	34,20 ± 2,09[a]	10,72 ± 0,43[cd]
Fructose (%)			
1	33,67 ± 1,35[c]	44,13 ± 2,32[b]	12,12 ± 0,39[b]
2	30,42 ± 1,68[c]	42,81 ± 2,40[b]	11,68 ± 0,69[b]
3	27,74 ± 2,02[b]	39,05 ± 2,27[b]	12,00 ± 0,58[b]
4	23,48 ± 2,35[a]	34,67 ± 2,10[a]	6,28 ± 0,37[a]
5	22,97 ± 2,21[a]	34,15 ± 2,12[a]	7,08 ± 0,43[a]

Means in the column followed by different letters are different according to Duncans' Multiple Range Test ($p \leq 0.05$)

CONCLUSION

It was concluded that isolated zygotic embryos of *P. heldreichii* and *P. peuce* germinated readily under *in vitro* conditions after only one week in culture. Results obtained in this study showed that embryos could germinate and grow on the medium without carbohydrates. The highest germination percentage and the best morphological characteristics of the seedlings were obtained on basal, GD medium supplemented with 3% sucrose.

Embryo culture *in vitro* could be increasingly applied for rapid and synchronous production of healthy seedlings of elite or selected genotypes with low seed production. Since the culture of isolated zygotic embryos can gain an insight into the requirements for successful plant conversion from the somatic embryo, our further work is aimed at defining the conditions for *P. heldreichii* and *P. peuce* somatic embryo maturation and conversion into plantlets, given the knowledge of the factors influencing zygotic embryo development.

Acknowledgment – This work was supported by the Ministry of Science of the Republic of Serbia, Grant No. 20052.

REFERENCES

COOKE, J., GIFFORD, D. 2002: Loblolly Pine Seed Dormancy: Constraints to Germination. - *Kluwer Academic Publishers*, *New Forests,* 23: 239-256.

ĐORĐEVA, M., STAMENKOV, M.& GUDEVSKI, A. 1972: Neke osobine semena i šišarki munike (*Pinus heldreichii* Christ.) pojedinih nalazišta Makedonije i Kosova. - *Simpozijum o munici, Zbornik radova,* 115-121.

GRESHOFF, P.M. & DOY, C.H. 1972: Development and Differentiation of Haploid *Lycopersicon esculentum* (tomato). *Planta 107*: 161-170.

JANKOVIĆ, M. 1991: On the Urgent Need to Plant a Constant Belt of the Endemorelict Balkan Pines *Pinus heldreichii* and *Pinus peuce* in the Mountains of Serbia and Yugoslavia. - *Ecology 26*: 61-67.

LIN, X. & LEUNG, D.W.M. 2002: Culture of Isolated Zygotic Embryos of *Pinus radiata* D.Don. Part I: Factors Influencing *in vitro* Germination and Growth of Isolated Eembryos. *In Vitro Cellular & Developmental Biology, Plant* 38: 191-197.

MIČEV, B. 1972: Istraživanja šišarki i semena munike. - *Simpozijum o munici, Zbornik radova*, 241-247.

MITCHELL, A. 1996: *Trees of Britain.* London. Harper Collins.

SOMMER, H.E., BROWN, C.L. & KORMANIK, P.P. 1975: Differentiation of Plantlets in Longleaf Pine (*Pinus palustris* Mill.) Tissue Culture *in vitro*. - *Botanical Gazette,* 136: 196-200.

STILINOVIĆ, S. 1985: Semenarstvo šumskog i ukrasnog drveća i žbunja. - Univerzitetski udžbenik, *OOUR Institut za šumarstvo Šumarskog fakulteta*, Beograd.

STOJIČIĆ, D. & BUDIMIR, S. 2004: Cytokinin-mediated Axillary Shoot Formation in *Pinus heldreichii. - Biologia Plantarum,* 48, 477-479.

STOJIČIĆ, D., BUDIMIR, S. & ĆULAFIĆ, LJ. 1999: Micropropagation of *Pinus heldreichii*. *Plant Cell Tissue and Organ Culture 59*: 147-150.

STOJIČIĆ, D., UZELAC, B., JANOŠEVIĆ, D., ĆULAFIĆ, LJ. & BUDIMIR, S. 2007: Induction of Somatic Embryogenesis in *Pinus heldreichii* Culture. - *Archives of Biological Sciences,* 59, 199-202.

VIDAKOVIĆ, M. 1982: Conifers. Morphology and Variability (in Serbocroat). - *Yugoslav Academy of Science and Arts*, Zagreb, pp.428-435.

Received: 3. October 2008.