

Micropropagation of Endangered and Decorative Species *Dianthus pinifolius* Sibth. et Sm.

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ABSTRACT

The aim of this study was to establish an efficient protocol for the *in vitro* propagation of the endangered, endemic and decorative species *Dianthus pinifolius* Sibth. et Sm. The effects of different concentrations of 6-benzylaminopurine (BAP) and naphthalenacetic acid (NAA), and different explant types (single-node cuttings, terminal buds and shoot cuttings) on shoot multiplication were examined on Murashige and Skoog (MS) and half-strength MS media. The best results were obtained for shoot cuttings on the MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L NAA, achieving a maximum rate of regeneration (100%) and a total of 15.4 newly-developed shoots per explant. The highest rooting rate (96.7%) was obtained on MS medium containing 1 mg/L NAA, while the acclimatization of the microplants obtained to *ex vitro* conditions was successful (88.9%).

Key words: pineleaf pink, *in vitro* culture, BAP, NAA, multiplication

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INTRODUCTION

The endemic species of the southeastern Balkans (Tomović et al. 2003) *Dianthus pinifolius* Sibth. et Sm. (Caryophyllaceae) is included in category V (vulnerable taxa) in Serbia and protected by law as an endangered plant species (Law on Environmental Protection, Rulebook on protected species 2010). Although, *D. pinifolius* can be found at several localities in Serbia, it is present in small numbers, occurring in its natural habitats (Tomović et al. 2003). This species grows on dry, brushy and grassy siliceous stony ground, and blooms in summer with purple or violet flowers (Gajić 1970; Randelović et al. 2008). Thanks to its bluish-green leaves, *D. pinifolius* is decorative throughout the vegetation period and particularly well-suited for cottage gardens. Due to its low-maintenance requirements, and the fact that it is a native species that fits in a natural environment without adverse impacts on the surrounding natural ecosystems, *D. pinifolius* is suitable for planting in Serbian mountain resorts, which is in accordance with the increasingly dominating trend of naturalistic garden design that involves wild plant species (Marković 2013).

Population of *D. pinifolius* in Mt Kopaonik is situated in protected area which was protected as a National Park by the proclamation of the Law on the Protection of the National Park Kopaonik (Official Gazette RS, 41/81, 44/93). To enable its *ex situ* and *in situ* conservation, we decided to propagate *D. pinifolius*. However, large amount of seeds could not be collected due to its threatened status.

As the most suitable method for rapid propagation of endangered taxa is micropropagation (Pence 1999), many studies have so far been published on the micropropagation of the endangered *Dianthus* species in the Balkans, mostly in Romania and Serbia. These studies include *D. petraeus* Waldst. & Kit. ssp. *noeanus* (Boiss.) Tutin, *D. giganteus* d'Urv. ssp. *croaticus* (Borbás) Tutin, *D. ciliatus* Guss. ssp. *dalmaticus* (Čelak.) Hayek (Radojević et al. 1997; 2006; 2010), and *D. serotinus* Waldst. et Kit. (Marković et al. 2007, 2013a), which were investigated in Serbia. Several species such as *D. callizonus* Schott & Kotschy (Holubiuc and Blindu 2006), *D.*

glacialis Haenke ssp. *gelidus* (Schott, Nym. et Kotschy) Tutin (Holubiuc et al. 2010a), *D. giganteus* d'Urv. subsp. *banaticus* (Heuff. Ex Griseb. & Schenk) Tutin (Pop and Pamfil 2011), *D. henteri* Heuff. ex Griseb. & Schenk (Cristea et al. 2010), *D. petraeus* Waldst. & Kit. ssp. *simonkaianus* (Péterfi) Tutin (Miclăuş et al. 2003), *D. pratensis* M. Bieb. subsp. *racovitzae* (Prodan) Tutin (Cristea 2010), *D. nardiformis* Janka (Holubiuc et al. 2009; 2010b) and *D. spiculifolius* Schur (Butiuc-Keul et al. 2001) were studied in Romania.

The above studies have mainly investigated the optimum concentration of phytohormones added to culture media, which differed depending on the species. As there were no published results regarding the micropropagation of *D. pinifolius*, the aim of this study was to establish a protocol for simple and rapid micropropagation of this endangered, endemic and decorative species.

MATERIAL AND METHODS

The seed capsules of *D. pinifolius* were collected from different plants randomly, on Mt. Kopaonik. The seeds were taken from capsules in the laboratory and surface disinfected by immersion in 4% NaOCl supplemented with 3 - 4 drops of the Tween 20 (Sigma) for 20 min. After rinsing three times in sterile distilled water, they were used for germination on hormone-free MS medium (Murashige and Skoog 1962). In all the experiments, the media used were MS basal medium and half-strength MS basal medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, and the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. The effects of plant growth regulators on shoot initiation and multiplication were studied at the multiplication stage. The culturing of explants (single-node cuttings, terminal buds and shoot cuttings with one node) was performed in 200 mL glass vessels (five explants/vessel) containing 25 mL of the medium (MS, or half-strength MS) supplemented with 0.1, 0.5, or 1.0 mg/L of BAP and 0.1, or 0.5 mg/L of NAA, while each experiment had three replications including 20 explants in each treatment. After 25 days in culture, both the number of shoots and nodes produced per explant and shoot and internode lengths were recorded. Basal MS or half strength MS media supplemented with NAA (0.05, 0.1,

0.5 mg/L) or without growth regulators were used to induce rooting. At the rooting stage, the shoots (10-35 mm long) were cultured in 720 mL glass vessels (15 explants/vessel), containing 100 mL of the medium. Thirty explants were cultured in each treatment, which was replicated three times. The percentage of shoots forming roots was quantified, as well as the number of roots per rooted shoot; root length was measured after 15 days. After the cultures were grown at $24 \pm 2^\circ\text{C}$, under a 16/8 h photoperiod and light intensity of $50 \mu\text{mol}/\text{m}^2$ provided by fluorescent tubes ("Tesla" – Pančevo). The 90 uniformly rooted plantlets were acclimatized in a 1:1 mixture of peat and sand, treated with a 1.5% solution of Previcur-N fungicide before use. During the first 15 days of acclimatization, the plants were covered with perforated plastic wrap to maintain high relative humidity, while ventilation was applied once daily for 5-10 min. Following the removal of the plastic wrap, the plants were grown for 10 more days before recording their survival rate.

The program Statgraphics, version 5.0 (STSC Inc. and Statistical Graphics Corporation, 1994-2000, USA) was used to statistically analyze the data, whereas the significance of differences between the mean values was determined by the analysis of variance (ANOVA, $p < 0.05$) and the method of least significant difference (LSD).

RESULTS AND DISCUSSION

Results showed that the *in vitro* culture was successfully established from the seeds, since the obtained germination rate of 92% was high compared to the values recorded for some other *Dianthus* species, including *D. deltooides*-58%

(Popović et al. 2008), *D. ciliatus* ssp. *dalmaticus* - 70%, *D. giganteus* ssp. *croaticus* - 42% (Radojević et al. 2010) and *D. henteri* - 75% (Cristea et al. 2010). Similarly, a high germination percentage was also recorded for *D. giganteiformis* ssp. *kladovanus* - 88% (Marković et al. 2006) and *D. serotinus* - 97% (Marković et al. 2007). Besides being affected by the species, germination rate can also be influenced by the sterilizing agent and duration of the treatment. The most commonly used NaOCl and H_2O_2 are oxidizing agents, which have also been reported to have a stimulatory effect on seed germination in some species. This is reflected in the partial degradation of the seed coat and solubilization and oxidation of the inhibitors of seed germination (Frank and Larson 1970; Miyoshi and Mii 1995; 1998; Lee et al. 2007). The germination of some *Dianthus* species could also be affected by light conditions, as Marcu et al. (2006) found that *D. pyrenaicus* germinated better in light - 90% than in darkness - 70%.

At the multiplication stage, the percentage of shoot regeneration (Table 1) was high on all the media tested, ranging between 91.7 and 100%, whereas the best results were obtained on the medium with 0.1 mg/L BAP and 0.1 mg/L NAA (100% - for all explant types). There were no records of vitrification, whereas the concentration of MS salts (MS or half-strength MS) did not affect the frequency of shoot regeneration. However, in some other *Dianthus* species, such as *D. serotinus*, the concentration of MS salts significantly affected shoot regeneration, showing better results on half-strength MS media (Marković et al. 2013a).

Table 1- Shoot regeneration on MS and half strength MS (1/2 MS) medium

BAP mg/L	NAA mg/L	single node cuttings (%)		terminal buds (%)		shoot cuttings (%)	
		1/2MS	MS	1/2MS	MS	1/2MS	MS
1.0	0.5	91.7 ^b	93.3 ^b	95.0 ^{ab}	95.0 ^a	93.3 ^b	95.0 ^a
1.0	0.1	95.0 ^{ab}	96.7 ^{ab}	93.3 ^{ab}	96.7 ^a	95.0 ^{ab}	95.0 ^a
0.5	0.5	95.0 ^{ab}	98.3 ^{ab}	95.0 ^{ab}	96.7 ^a	93.3 ^b	96.7 ^a
0.5	0.1	96.7 ^{ab}	96.7 ^{ab}	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a
0.1	0.1	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a	100.0 ^a

Note: The values followed by different letters are significantly different at the $P < 0.05$ level according to the LSD test.

Contrary to that, in this study the concentration of MS salts did not influence shoot regeneration, but it had an impact on the mean number of shoots and nodes per explant and better results

were achieved on MS media (Table 2), like in the case of the micropropagation of *D. Serotinus* (Marković et al. 2013a). Differences among explant types were also observed, and the shoot

cuttings had the highest number of newly developed shoots (15.4) and nodes (9.7) on the MS medium containing 0.5 mg/L BAP and 0.1 mg/L NAA. The concentration of BAP affected the mean number of shoots per explant, in the most cases causing better results on the media with 0.5 mg/L BAP than on the ones with 1.0 or 0.1 mg/L BAP. However, statistically significant difference was recorded only for shoot cuttings, and for terminal buds on MS medium. Similarly, a statistically significant influence of BAP concentration on the number of shoots was recorded for some other *Dianthus* species, including *D. deltooides*, *D. gratianopolitanus*, *D. giganteus banaticus*, *D. spiculifolius*, and *D. henteri* (Fraga et al. 2004; Marković et al. 2013b;

Pop and Pamfil 2011). However, its optimum concentration differed depending on the species. Fraga et al. (2004) found that cytokinin concentration significantly affected the mean number of nodes in *D. gratianopolitanus*, although that was not the case in this research, as the mean number of nodes did not change significantly with different BAP concentrations (Table 2) added to the media. Similarly, during the micropropagation of *Nepeta rtanjensis*, the mean number of nodes did not change significantly, when BAP concentration was increased from 0.05 mg/L to 1.0 mg/L (Mišić et al. 2005).

Table 2- The average number of shoots and nodes developed at the multiplication stage

BAP mg/L	NAA mg/L	single node cuttings		terminal buds		shoot cuttings	
		1/2MS	MS	1/2MS	MS	1/2MS	MS
No. of shoots per explant							
1.0	0.5	2.7 ^{bc}	6.5 ^a	5.3 ^{ab}	6.5 ^{ab}	7.1 ^{abc}	9.1 ^{abc}
1.0	0.1	2.5 ^{bc}	5.9 ^{ab}	4.8 ^{bc}	6.3 ^{ab}	6.7 ^{bc}	8.3 ^{bc}
0.5	0.5	3.0 ^{bc}	6.9 ^a	5.8 ^{ab}	6.5 ^{ab}	9.4 ^{ab}	12.1 ^{ab}
0.5	0.1	5.1 ^a	6.0 ^{ab}	5.9 ^{ab}	7.2 ^a	10.3 ^a	15.4 ^a
0.1	0.1	4.1 ^{ab}	4.6 ^{ab}	6.9 ^a	4.7 ^{bc}	6.0 ^{bc}	7.0 ^{bc}
No. of nodes per explant							
1.0	0.5	2.5 ^{ab}	7.1 ^a	3.2 ^a	6.8 ^{ab}	2.7 ^a	6.5 ^b
1.0	0.1	2.7 ^{ab}	3.4 ^c	1.5 ^b	5.2 ^{bc}	1.3 ^c	6.2 ^b
0.5	0.5	3.0 ^a	6.6 ^a	1.2 ^b	6.9 ^{ab}	1.5 ^c	9.5 ^a
0.5	0.1	1.0 ^c	6.2 ^{ab}	2.1 ^{ab}	8.5 ^a	1.3 ^c	9.7 ^a
0.1	0.1	1.4 ^c	6.0 ^{ab}	1.1 ^b	8.3 ^a	1.6 ^{bc}	8.2 ^{ab}

Note: The values followed by different letters are significantly different at the $P < 0.05$ level according to the LSD test.

In contrast to the results obtained for *D. gratianopolitanus*, whose internode length was significantly influenced by the type of explant (shoot tip or node explants) (Fraga et al. 2004), the average internode length in this study ranged from 2.0 to 4.2 mm and was unaffected by the explant type and the concentration of MS salts (Table 3). Although the impact of hormone concentration was not statistically significant in most cases, internodes were the longest on the medium supplemented with 0.1 mg/L BAP and 0.1 mg/L NAA (Table 3). Contrary to that, phytohormone concentration significantly influenced internode length during the micropropagation of *D. deltooides* and *D. gratianopolitanus* (Fraga et al. 2004; Marković et al. 2013b). Since their lengths were highly variable, the shoots were placed in three length categories

(less than 10 mm, 10-20 mm and more than 20 mm), while the number of shoots belonging to a certain length category was expressed as a percentage of the total number of shoots (Fig. 1). Evidently the concentration of hormones affected shoot length, as with an increase in NAA concentration and a decrease in BAP concentration the percentage of shoots shorter than 10 mm decreased, whereas the percentage of shoots longer than 20 mm increased. A higher percentage of long shoots (>20 mm) was recorded on MS medium than on half-strength MS medium (Fig. 1), which was similar to the response observed during the micropropagation of *D. deltooides*, *D. petraeus* ssp. *noeanus*, *D. superbus*, and *D. giganteiformis* ssp. *kladovanus* (Radojević et al. 1997; Mikulík 1999; Marković et al. 2006; 2013b).

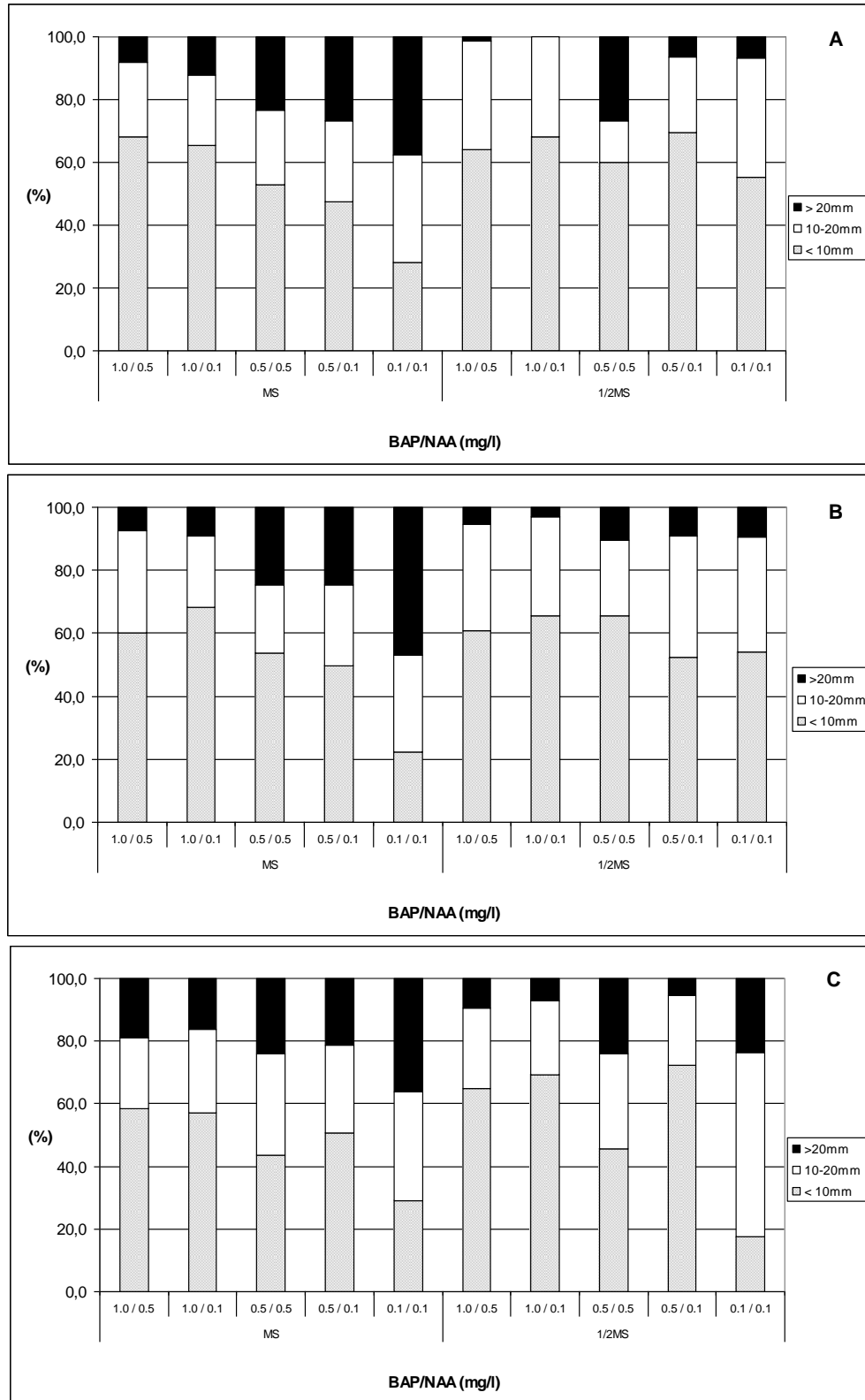


Figure 1- The effect of different concentrations of BAP and NAA on the length of shoots developed from single node cuttings (A), terminal buds (B) and shoot cuttings (C) on full-strength MS basal medium (MS) and half-strength MS basal medium (1/2MS).

However, the stimulatory effect of NAA on shoot elongation was missing in the case of the propagation of *D. henteri*, *D. giganteus banaticus* and *D. spiculifolius*. In these species, the longest shoots developed on the media supplemented with 1.0 or 2.0 mg/L BAP and 0.1 NAA, whereas the shortest ones appeared on the medium with 1.0 mg/L BAP and 0.5 mg/L NAA and in the control without plant hormones

(Popand Pamfil 2011). It is known that different cytokinins can affect shoot length differently, and Marcu et al. (2006) revealed twice as high shoot length in *D. pyrenaicus* on the medium containing 1.0 mg/L BAP compared to the one obtained on the medium containing 1.0 mg/L of kinetin, with the addition of the same NAA concentration (0.5 mg/L) in both cases.

Table 3- The average internode length of shoots developed at the multiplication stage

BAP mg/L	NAA mg/L	single node cuttings (mm)		terminal buds (mm)		shoot cuttings (mm)	
		1/2MS	MS	1/2MS	MS	1/2MS	MS
1.0	0.5	3.2 ^{ab}	3.0 ^{ab}	2.9 ^b	3.3 ^{ab}	3.1 ^b	3.0 ^b
1.0	0.1	2.3 ^b	2.2 ^b	2.3 ^{bc}	2.0 ^c	2.5 ^{bc}	2.2 ^{bc}
0.5	0.5	3.1 ^{ab}	3.1 ^{ab}	2.7 ^b	2.9 ^{ab}	2.7 ^{bc}	3.2 ^b
0.5	0.1	2.5 ^b	2.3 ^b	2.4 ^{bc}	2.6 ^b	2.4 ^c	2.4 ^{bc}
0.1	0.1	3.9 ^a	4.2 ^a	4.1 ^a	3.8 ^a	3.8 ^a	4.0 ^a

Note: The values followed by different letters are significantly different at the $P < 0.05$ level according to the LSD test.

Successful rooting, with a high 96.7% of rooted shoots, was recorded on the MS medium containing 1.0 mg/L NAA (Table 4). Similarly, a high rooting percentage has already been reported for other *Dianthus* species, including *D. superbus* ssp. *superbus* - 100%, *D. mainensis* - 100%, *D. deltoides* - 100%, *D. giganteiformis* ssp. *kladovanus* - 94%, and *D. petraeus* ssp. *noeanus* - 91% (Radojević et al. 1997; Mikulik 1999; Marković et al. 2006; 2013b; Erst et al. 2014). However, it was much lower for some other species, i.e. *D. gratianopolitanus* 'Spotti' - 80%, *D. gratianopolitanus* 'Frosty Fire' - 70% (Fraga et al. 2004) and *D. caryophyllus* (62 - 80%) (Radojević et al. 1990). The impact of the concentration of MS salts on rooting was obvious, since the rooting percentage was higher on MS media (88.3 - 96.7%) than on half-strength MS media (78.4-85.0%). The addition of NAA to the medium also showed a stimulative effect on rooting by increasing the rooting

percentage, but no effect on the number and length of roots (Table 4). The effect of the concentration of MS salts was also recorded for *D. mainensis* (Erst et al. 2014), but in that case, the rooting was higher on the half-strength MS medium (100%) than on the MS medium (64%). Although during the micropropagation of *D. serotinus*, the rooting rate was also higher on half-strength MS media, the mean number of roots per explant was higher on MS media (Marković et al. 2014). In this study, neither NAA concentration nor the concentration of MS salts had an impact on the mean number of roots, ranging from 9.8 to 16.8, which could be considered high compared to the mean number of roots of up to eight in *D. mainensis*, *D. petraeus* ssp. *noeanus*, *D. gratianopolitanus*, *D. ciliatus* ssp. *dalmaticus*, *D. giganteus* ssp. *croaticus* (Radojević et al. 1997; 2010; Fraga et al. 2004; Erst et al. 2014).

Table 4- The percentage of rooted shoots, the average number of roots and the mean length of the longest root per explant.

NAA (mg/L)	Medium	Rooting percentage (%)	No. of roots per explant	Mean length of the longest root (mm)
0.0	MS	88.3 ^{ab}	14.3 ^a	38.2 ^a
0.1	MS	86.7 ^{ab}	15.6 ^a	36.2 ^a
0.5	MS	95.0 ^a	15.1 ^a	31.5 ^a
1.0	MS	96.7 ^a	16.8 ^a	33.5 ^a
0.0	1/2MS	78.4 ^b	10.3 ^{ab}	22.1 ^b
0.1	1/2MS	78.4 ^b	9.8 ^{ab}	26.4 ^{ab}
0.5	1/2MS	83.4 ^b	11.8 ^{ab}	28.8 ^{ab}
1.0	1/2MS	85.0 ^{ab}	12.5 ^a	27.0 ^{ab}

Note: The values followed by different letters are significantly different at the $P < 0.05$ level according to the LSD test.

The obtained acclimatization rate of 88.9% can be regarded as satisfactory, being similar to the ones recorded for other *Dianthus* species, i.e., *D. mainensis* - 83%, *D. trifasciculatus* ssp. *parviflorus*, *D. arenarius* ssp. *bohemicus* - 85%, *D. gratianopolitanus* - over 99%, *D. petraeus* ssp. *noeanus* - 100%, and *D. deltoides* - 100% (Kováč 1995; Radojević et al. 1997; Fraga et al. 2004; Marković and Popović 2012; Holobiuc et al. 2013).

CONCLUSIONS

From the results it was concluded that the endangered and decorative species *D. pinifolius* could be successfully propagated using the protocol presented in this work. In the course of *D. pinifolius* micropropagation, MS medium should contain 0.5 mg/L BAP and 0.1 mg/L NAA, while the type of explant used should be shoot cuttings. In addition to that, MS medium supplemented with 0.1 mg/L NAA should be used for *in vitro* rooting, followed by the acclimatization of the rooted plantlets, which was successful in a 1: 1 mixture of peat and sand.

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REFERENCES

- Cristea V, Brummer AT, Jarda L, Miclăuș M. *In vitro* culture initiation and phytohormonal influence on *Dianthus henteri* – a Romanian endemic species. *Rom Biotech Lett. Suppl.* 2010; 15(1):25-33.
- Cristea V. Photoautotrophic *in vitro* culture of endemic and endangered *Dianthus* species from Romania. Cluj-Napoca: Todesco, 2010.
- Erst AA, Erst AS, Shaulo DN. *In vitro* Propagation of *Dianthus mainensis*, an Endemic Plant from the West Sayan (North Asia). *Taiwania.* 2014; 59(2): 106– 110
- Fraga M, Mertxe A, Ellul P, Borja M. Micropropagation of *Dianthus gratianopolitanus*. *Hort Sci.* 2004; 39 (4): 112-115.
- Frank AB, Larson KL. Influence of oxygen, sodium hypochlorite, and dehulling on germination of green needlegrass seed. *Crop Sci.* 1970; 10 (6): 679-682.
- Gajić M. Genus *Dianthus* L. In: Josifović M. Editor. Flora SR Srbije. Belgrade: SANU, 1970.
- Holobiuc I, Blîndu R. Improvement of the micropropagation and *in vitro* medium-term preservation of some rare *Dianthus* species. *Contrib Bot.* 2006; 41(2): 143 - 151.
- Holobiuc I, Blîndu R, Cristea V. Researches concerning *in vitro* conservation of the rare plant species *Dianthus nardiformis* Janka. *Biotechnol Biotech Eq.* 2009; 23(2): 221-224.
- Holobiuc I, Catana R, Cristea V. Researches concerning *in vitro* cultures optimization of the vulnerable species *Dianthus nardiformis* Janka. *Analele Univ Oradea - Fasc Biol.* 2010b; 17(1): 116-121.
- Holobiuc I, Mitoi M, Blîndu R, Helepciuc F. The establishment of an *in vitro* gene bank in *Dianthus spiculifolius* Schur. and *D. glacialis* ssp. *gelidus* (Schott Nym. et Kotschy) Tutin: II. Medium-term cultures characterization in minimal growth conditions. *Rom Biotech Lett.* 2010a; 15 (2): 5111-5119.
- Holobiuc I, Catană R, Voichiğă C, Helepciuc F. *In vitro* introduction of *Dianthus trifasciculatus* Kit ssp. *parviflorus* as *ex situ* preservation method. *Oltenia - Stud Comun Stiint Nat.* 2013; 29: 93-100.
- Kováč J. Micropropagation of *Dianthus arenarius* subsp. *bohemicus* - an endangered endemic from the Czech Republic. *Bot Gardens Microprop News.* 1995; 8: 106-108.
- Lee YI, Lu CF, Chung MC, Yeung EC, Lee N. Developmental changes in endogenous abscisic acid concentrations and asymbiotic seed germination of a terrestrial orchid, *Calanthe tricarinata* Lindl. *J Am Soc Hort Sci.* 2007; 132: 246-252.
- Marcu D, Cristea V, Butiuc-Keul A. Micropropagation of *Dianthus pyrenaicus* Pourr. - endemic species from Pyrenean Mountains. *Contrib Bot.* 2006; 41(2): 153-159.
- Marković M. New trends in landscaping and gardening as a method for preserving biodiversity. *Nature Conservation.* 2013; 63(1-2): 25-33.
- Marković M, Grbić M, Šindelić A. Possibility of micropropagation of *Dianthus giganteiformis* ssp. *kladovanus* (Degen) Soo by the method of proliferation of lateral shoots. *Bull Fac For.* 2006; 94: 171-179.
- Marković M, Grbić M, Skočajić D, Đukić M. Effect of explant type on the rooting and acclimatization of *Dianthus serotinus* Waldst. & Kit. *Bull Fac For.* 2014; 109: 125-136.
- Marković M, Grbić M, Skočajić D, Đunisijević-Bojović D. The effect of phytohormone balance on shoot multiplication and rooting of the

- species *Dianthus serotinus* Waldst. et Kit. *Bull Fac For.* 2007; 95: 83-94.
- Marković M, Grbić M, Djukić M. Micropropagation of the Endangered and Decorative Species *Dianthus serotinus* Waldst. et Kit. *Not Bot Horti Agrobo.* 2013a; 41(2): 1-8.
- Marković M, Popović M, Vilotić D. Micropropagation of *Dianthus deltoides* L. through shoot tip and nodal cuttings culture. *Archives of Biological Sciences.* 2013b; 65(1): 17-22.
- Marković M, Popović M. Effect of explant type, medium and soil mixture content on *Dianthus deltoides* L. rooting and acclimatization. *Bull Fac For.* 2012; 105: 117-126.
- Miclăuș M, Cristea V, Deliu C. Micropropagation on *Dianthus petraeus* W. et K. ssp. *simonkaianus* (Peterfi) Tutin. *Contrib Bot.* 2003; 38: 77-84.
- Mikulík J. Propagation of endangered plant species by tissue cultures. *Acta Univ Palack Olom Bio.* 1999; 37: 27-33.
- Mišić D, Ghalawenji N, Grubišić D, Konjević R. Micropropagation and Reintroduction of *Nepeta rtanjensis*, an Endemic and Critically Endangered Perennial of Serbia. *Phyton.* 2005; 45: 9-20.
- Miyoshi K, Mii M. Enhancement of seed germination and protocorm formation in *Calanthe discolor* (Orchidaceae) by NaOCl and polyphenol absorbent treatments. *Plant Tiss Cult Lett.* 1995; 12: 267-272.
- Miyoshi K, Mii M. Stimulatory effects of sodium and calcium hypochlorite, pre-chilling and cytokinins on the germination of *Cypripedium macranthos* seed *in vitro*. *Physiol Plantarum.* 1998; 102: 481-486.
- Murashige T, Skoog F. A revised medium for growth and bioassays with tobacco tissue culture. *Physiol Plantarum.* 1962; 15: 473-497.
- Pence VC. The application of biotechnology for the conservation of endangered plants, Chapter 15. In: Benson E.E. Editor. *Plant Conservation Biotechnology.* London: Taylor and Francis; 1999. p. 227-241.
- PopT, PamfilD. *In vitro* Preservation of Three Species of *Dianthus* from Romania. *Bull UASVM Hort.* 2011; 680: 414-422.
- Popović M, Grbić M, Marković M. Propagation of *Dianthus deltoides* L. by shoot culture. *Bull Fac For.* 2008; 97: 209-219.
- Radojević Lj, Čalić-Dragosavac D, Špirić J, Stevanović B, Stevanović V. *In vitro* culture of stem segments of *Dianthus ciliatus* ssp. *dalmaticus* and *D. giganteus* ssp. *croaticus* (Caryophyllaceae). *Bot Serbica.* 2010; 34(2): 153-161.
- Radojević Lj, Marinković N, Jevremović S. Vegetative *in vitro* propagation of *Dianthus petraeus* Waldst. et Kit. subsp. *noeanus* by meristem and stem segments culture. *Arch Biol Sci.* 1997; 31: 73-77.
- Radojević Lj, Špirić J, Stevanović B, Stevanović V. *In vitro* culture of several endemic species *Dianthus* in Balkan Peninsula. XXII International Symposium Section Ornamentals, 2006, Sanremo, Italy: September 11-15, 2006. p. 36.
- Randelović V, Zlatković B, Milosavljević V, Randelović N. The endemic flora of Bosilegrad surroundings (Krajište region) in SE Serbia. *Phytol Balcanica.* 2008; 14(3): 367 - 375.
- Tomović G, Randelović V, Niketić M, Vukojičić S, Zlatković B. New distribution data of some Pontic and submediterranean plant species in Serbia. *Arch Biol Sci.* 2003; 55 (1-2): 45-54.

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