Arch. Biol. Sci., Belgrade, 65 (1), 17-22, 2013 DOI:10.2298/ABS1301017M

MICROPROPAGATION OF *DIANTHUS DELTOIDES* L. THROUGH SHOOT TIP AND NODAL CUTTINGS CULTURE

MARIJA MARKOVIĆ, MARIJA POPOVIĆ and DRAGICA VILOTIĆ

Faculty of Forestry, University of Belgrade, 11000 Belgrade, Serbia

Abstract - Micropropagation (shoot tip and nodal cuttings culture) was used for the rapid propagation of the non-invasive, decorative, native plants of maiden pink (*Dianthus deltoides* L.) in order to preserve their genetic diversity. *In vitro* culture was successfully established on Murashige and Skoog medium (MS) using seeds as the initial material. In the shoot multiplication phase, the explants were cultured on MS medium supplemented with different concentrations of 6-benzylaminopurine (BAP) and naphthaleneacetic acid (NAA). The highest multiplication rate was achieved on a medium containing 0.1 mgL^{-1} of BAP and 0.1 mgL^{-1} of NAA. The rooting was successful on a hormone-free medium (100%), and the highest percentage of microplant acclimatization (97%) was recorded in a 4: 1 mixture of peat and sand.

Key words: Maiden pink, tissue culture, hyperhydricity, shoot regeneration, *Dianthus deltoides*

INTRODUCTION

Plants within the genus *Dianthus* are popular with many gardeners. *D. deltoides* is a spreading plant, growing to 30 cm in height. The flowers are scented and the plants bloom in late spring and summer. Single blooms appear in shades of pink and rose, although cultivars are available in salmon, white and crimson. These plants make perfect borders or edgings where a semi-trailing plant is needed. *D. deltoides* is not invasive and it can easily be controlled when planted around the outer edges of landscapes. These plants can also be used in rock gardens (Nau, 1996). For all these reasons, because of its low maintenance requirements, and the fact that it is a native species that fits into a natural environment without adverse impacts on the surrounding natural ecosystems, *D. deltoides* is suitable for planting in Serbian mountain resorts. The characteristics of *D. deltoides* are in accordance with the increasingly dominating trend of naturalistic garden design that involves wild plant species (Rappaport, 1993).

Seed propagation is a common method and cuttings can be taken during the spring or summer from non-flowering shoots (Nau, 1996). Tissue culture is used in *Dianthus* spp. propagation. So far, a number of papers have been published related to the regeneration of *D. caryophyllus* using different types of explants and methods (Roest and Bokelmann, 1981; Radojević et al., 1990; Nugent et al. 1991; van Altvorst et al., 1992; Nakano and Mii, 1992; Sato et al., 2000; Jain et al., 2001; Pareek and Kothari, 2003). Fraga et al. (2004) provided a protocol for the elimination of the viruses that pose a major problem in the production of the species *D. gratianopolitanus*. *D. plumarius* was also successfully propagated using a meristem culture (Montes et al., 1997). The species *D. barbatus* and *D. chinensis* were propagated using different *in vitro* methods including the use of hypocotyl culture, organogenesis of leaflets or organogenesis in the shoot tip, meristem or node culture (Jethwani and Kothari, 1993; Jethwani et al., 1994; Jethwani and Kothari, 1996). In addition, Pareek and Kothari (2003) provided a successful procedure for direct somatic embryogenesis for *D. barbatus* and

D. chinensis by growing leaf segments in an *in vitro* culture, and Pareek et al. (2004) propagated the two above-mentioned species by using shoot tip and nodal cuttings as explants. The species *D. deltoides* was micropropagated by shoot culture (Popović et al., 2008), but it is expected that using shoot tip and nodal cuttings could be more efficient and useful for the rapid multiplication of this species.

The objective of this study was to establish a micropropagation system for the rapid and efficient propagation of native *D. deltoides* plants while preserving their genetic diversity.

MATERIALS AND METHODS

Plant material and surface sterilization

Seed shells with *D. deltoides* seeds were picked from healthy, well-developed plants, at the locality of Mt. Goč. *D. deltoides* seeds were used to establish the culture *in vitro*. The seeds were surface-disinfected by immersion in a 70% ethanol solution for 30 s, followed by immersion in 2% NaOCl supplemented with 3-4 drops of Tween 20 (Sigma) preparation for 10 min before three rinses in sterile, distilled water.

Culture medium and conditions

MS (Murashige, Skoog 1962) basal medium supplemented with 3% sucrose and 0.8% agar was used in all experiments. Various growth regulators were added to the medium and the pH was adjusted to 5.8 before autoclaving at 121°C for 20 min. Explants (four per vessel) were cultured in 200 ml glass vessels containing 30 ml of the medium. The cultures were incubated at 24±2°C, under a 16/8 h photoperiod, with 50 μmol/m^{2}s light intensity provided by fluorescent tubes ("Tesla" – Pančevo). Germination *in vitro* was induced on a half strength MS medium without growth regulators.

Effects of plant growth regulators on shoot initiation and multiplication

Shoot tips (5 mm) and single nodal segments from 25-day-old seedlings were collected and cultured on a basal medium containing 0.1-3 mgL^{-1} of BAP (6-benzylaminopurine) and 0.1-1 mgL^{-1} of NAA (1-naphtalenacetic acid). Each experiment had five replicate cultures of four explants per treatment and was conducted three times. The number of shoots and nodes produced per explant, the shoot and internode length and the percentage of hyperhydric explants were recorded after 25 days in culture.

Root initiation

Four groups of explants were taken from the shoots that grew on a hormone-free medium: nodal cuttings, shoot-tip cuttings, shoots with (1-3) nodes (10-25 mm in length) and shoots with (4- 6) nodes (over 25 mm in length). Rooting was induced on basal MS medium without growth regulators or supplemented with NAA (0.05, 0.1, 0.5 mgL^{-1}). Each experiment had two replicate cultures of 15 explants per treatment and was conducted three times. The percentage of shoots forming roots was quantified, as well as the number of roots per rooted shoot, and the root length was measured after 15 days.

Transfer to soil

The rooted and unrooted shoots (with 3-4 nodes) were rinsed in water to remove all agar, and transplanted into three different substrate mixtures of peat and sand in 1:1, 2:1 and 4:1 ratios. Before use, the substrate was treated with a 1.5% solution of the preparation Previcur N. The plants were watered regularly and kept in shade for 15 days, covered with a plastic wrap to maintain high humidity. After this, the wrap was removed and the cultivation continued for another 15 days, which was followed by the transplant of these plants.

Statistical analysis

The obtained data were statistically analyzed using the program Statgraphics, version 5.0 (STSC Inc. and Statistical Graphics Corporation, 1994-2000, USA). 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

The seeds started to germinate as soon as a week after sowing, and after 25 days, 58% of the sown seeds had germinated. This value is relatively low compared to other *Dianthus* species, including *D. zeyheri* ssp. *natalensis* (80%), and *D. giganteiformis* subsp. *kladovanus* (88%) (Crouch and van Staden, 1993, Marković et al., 2006). Vitrification exceeding 40% was detected in the shoot-multiplication phase on more than half of the media (Table 1). One of the possible causes of vitrification could be the impact of BAP, especially at higher concentrations, as has already been proved for the species *D. caryophyllus* and *D. petraeus* (Radojević et al., 1997, Jain et al., 2001). The occurrence of vitrification was affected by the explant type as well. A higher percentage of vitrified shoots originated from shoot-tip cuttings than nodal cuttings, as in the species *D. gratianopolitanus* (Fraga et al., 2004).

Spontaneous rooting of the explants occurred on certain media, despite the presence of BAP. The percentage of rooted explants ranged from 18% (on a medium containing 1 mgL^{-1} of BAP and 0.1 mgL^{-1} of NAA) to 100% (on a medium containing 0.1 mgL^{-1} of BAP and 0.1 mgL^{-1} of NAA). In most cases the root system was poorly developed, usually displaying a small number of short roots.

The average number of shoots per explant varied, revealing a large overlap between homogenous groups (Table 2). The number of shoots was generally higher on the media with lower BAP concentrations (1 mgL^{-1} and lower) and it did not grow with the increasing concentration of BAP. In addition, the type of explants had no significant impact, as in the case of *D. gratianopolitanus* micropropagation (Fraga et al., 2004).

The shoots were divided into appropriate length categories, and their percentage in the appropriate category was determined. Most shoots on all media (60-100%) were up to 10 mm long. At lower BAP concentrations or increasing NAA concentrations at the same BAP concentration, the percentage of 21-30 mm-long shoots and shoots longer than 30 mm was increasing. The length of shoots was also affected by the type of explant. On the same medium, the shoots originating from nodal cuttings were shorter than the ones originating from shoot-tip cuttings. The average length of internodes ranged from 1.1 mm (shoots originating from nodal and shoot-tip explants on a medium containing 2 mgL^{-1} of BAP and 0.1 mgL^{-1} of NAA) to 4.5 mm (nodal explants on a medium containing 1 mgL^{-1} of BAP and 0.5 mgL^{-1} NAA), but on the majority of the media the average internode length ranged between 2 and 3 mm. The average internode length was affected by the concentration of added phytohormones, in the same way as the length of the shoots. However, the type of explant had no impact.

Only the nodes that could be used for subculture or those whose axillary buds did not develop into new shoots were considered in determining the number of nodes per explant (Table 2). The media with lower concentrations of phytohormones proved to be the most favourable. If all the measured parameters are taken into account (the proportion of normally developed explants, the number of shoots = potential shoot-tip cuttings, the number of nodes) the most favourable medium for the multiplication of both types of explants should contain 0.1 mgL^{-1} each of BAP and NAA.

The rooting of shoots *in vitro* was performed successfully. There were no unrooted explants on any media, and the root system in all types of explants on all nutrient media for rooting was well developed, displaying a large number of thin, branchy roots. A high percentage of rooting was also recorded in the carnations *D. superbus* ssp. *superbus* (100%) (Mikulik, 1999) and *D. petraeus* ssp. *noeanus* (91%) (Radojević et al., 1997). Somewhat lower values were recorded during the rooting of D. *arenarius* ssp. *bohemicus* (85%) (Kovac, 1995), *D. gratianopolitanus* 'Spotti' (80%) (Fraga et al. 2004), *D. gratianopolitanus* 'Frosty Fire' (70%) (Fraga et al., 2004), and *D.*

Table 1. State of explants after 25 days of cultivation in *in vitro* culture.

Explant type		Shoot-tip explants			Nodal explants		
Hormones (mgL^{-1})		Vitrified (%)	Normally developed (%)	Necrosed (%)	Vitrified (%)	Normally developed (%)	Necrosed (%)
BAP	NAA						
3	1	66.7^{e}	26.6^{f}	6.7^{bcd}	42.7^{e}	20.0^{f}	37.3^{ef}
3	0.5	83.3^{f}	6.7^{g}	10.0^{de}	46.7^{e}	8.3^{g}	45.0^{f}
2	1	26.6^{c}	66.7^{b}	6.7^{bcd}	11.7^{bcd}	75.0^{c}	13.3^{cd}
2	0.5	53.3^{d}	33.3^{ef}	13.4^{e}	41.7^{e}	25.0^{f}	33.3^{e}
2	0.1	51.7^{d}	48.3^{d}	0.0^{a}	68.3^{f}	20.0^{f}	11.7^{bc}
1	1	15.0^{ab}	85.0^{a}	0.0^{a}	48.3^{e}	48.3^{e}	3.4^{ab}
1	0.5	6.7^{a}	88.3^{a}	5.0^{abc}	13.3^{cd}	73.4^{c}	13.3^{cd}
1	0.1	46.7^{d}	43.3^{de}	$10,0^{cde}$	16.7^{d}	61.7^{d}	21.6^{d}
0.5	0.5	46.7^{d}	53.3^{cd}	0.0^{a}	0.0^{a}	90.0^{ab}	10.0^{bc}
0.5	0.1	31.7^{c}	61.6^{bc}	6.7^{bcd}	5.0^{ab}	81.7^{bc}	13.3^{bcd}
0.1	0.1	16.7^{b}	80.0^{a}	3.3^{ab}	6.7^{bc}	93.3^{a}	0.0^{a}

BAP - 6-benzylaminopurine, NAA - 1-naphtalenacetic acid; *means followed by the same letter do not differ significantly at P <0.05.

Table 2. Average number of shoots and nodes per explant.

Hormones (mgL^{-1})		Shoots per explant		Nodes per explant	
BAP	NAA	Shoot tip cuttings	Nodal cuttings	Shoot tip cuttings	Nodal cuttings
3	1	3.0^{e}	2.0^{e}	3.9^{c}	1.6^{fg}
3	0.5	2.8^{e}	1.7^{e}	1.7^{c}	1.9^{fg}
2	1	4.9^{de}	4.3^{de}	8.5^{b}	6.6^{bcd}
2	0.5	3.4^{e}	5.4^{bcd}	7.5^{b}	3.4^{def}
2	0.1	4.0^{e}	2.5^{e}	3.3^{c}	1.2^{g}
1	1	8.7^{a}	5.2^{cd}	9.5^{a}	5.2^{cd}
1	0.5	6.5^{bcd}	6.1^{bc}	3.5^{c}	3.1^{ef}
1	0.1	7.8^{ab}	9.4^{a}	8.1^{ab}	6.9^{ab}
0.5	0.5	7.1^{abc}	7.7^{ab}	9.3^{ab}	10.0^{a}
0.5	0.1	6.1^{cd}	4.8^{cd}	7.2^{b}	4.8^{de}
0.1	0.1	7.8^{ab}	7.2^{b}	7.1^{b}	5.8^{c}

BAP - 6-benzylaminopurine, NAA - 1-naphtalenacetic acid; *means followed by the same letter do not differ significantly at P < 0.05.

Table 3. Average number of roots per explant.

NAA (mgL^{-1})	Shoot tip cuttings	Nodal cuttings	Shoots with 1-3 nodes	Shoots with 4 or more nodes
0	12.0 a	15.4 a	17.5 a	16.2 a
0.5	17.2 a	13.7 a	18.8 a	12.2 a
0.1	12.4 a	12.1 a	17.5 a	15.8 a
0.05	17.3 a	15.2 a	13.3 a	15.3 a

caryophyllus (62-80%), depending on the cultivar (Radojević et al., 1990).

The average number of roots per explant was high on all media. It ranged between 12 and 18 roots,

and neither the origin from different explant type nor the impact of NAA proved to be statistically significant (Table 3). Similarly, during the rooting of *D. gratianopolitanus*, the supplementing of different concentrations of auxin - NAA, IBA and IAA (0.5 and 1 mgL^{-1}) did not significantly affect the number of formed roots (Fraga et al., 2004). In comparison to other *Dianthus* species, including *D. gratianopolitanus* and *D. petraeus* ssp. *noeanus* that on average developed 1-7 roots per explant (Radojević et al., 1997, Fraga et al., 2004), the average number of *D. deltoides* roots was high.

Root length was quite variable, and therefore the roots were grouped into the corresponding length categories, followed by the calculation of their percentage in each category (data not shown). Irrespective of the explant type or the medium, more than half of the roots were shorter than 10 mm, approximately one-third of them were 11 to 20 mm long, and less than 10% were longer than 20 mm. The explant type and medium composition had no effect on root length.

The percentage of rooting shoots *ex vitro* was significantly lower than the rooting of explants *in vitro*, ranging from 58% (peat:sand – 1:1) to 75% (peat:sand – 4:1). However, in the species *D. gratianopolitanus,* the rooting of shoots was significantly more successful in *ex vitro* (97%) than in *in vitro* (70 - 80%) conditions (Fraga et al., 2004). During this experiment, it was not possible to measure the length and number of roots, because of their delicacy and the possibility of tearing during plant extraction. Therefore, only the percentage of rooting shoots was determined.

In vitro rooted plants were successfully acclimatized (82-97%), which corresponds with the results obtained for other carnation species: *D. arenarius* ssp. *bohemicus* (85%), *D. gratianopolitanus* (over 99%) and *D. petraeus* ssp. *noeanus* (100%) (Kovac 1995; Radojević et al., 1997; Fraga et al., 2004). The impact of medium composition on the acclimatization is evident, and the percentage of acclimatized plants planted in the 1:1 peat and sand substrate mixture turned out to be the lowest (82.7%), while the ones planted in a 4:1 ratio mixture of peat and sand revealed the most successful acclimatization (97%). The impact of substrate composition is manifested during the acclimatization of other species as well (Kovac, 1995; Benson et al., 2000). The microplants were acclimatized during May and they were transferred to pots filled with a mixture of garden soil, sand, peat and fully decomposed farmyard manure in June. In late August, these plants were planted in outdoor conditions, where they successfully overwintered and bloomed the next year in June. The present study presents a protocol for the successful micropropagation of *D. deltoides* native plants.

Acknowledgments - This paper was supported by the project TR 31041 (Establishment of wood plantations intended for afforestation of Serbia) of the Ministry of Science and Technological Development of Serbia.

REFERENCES

Benson, E., Danaher, J.E., Pimbley, I.M., Anderson, C.T., Wake, J.E., Daley, S. and *L.K. Adams* (2000). In vitro propagation of *Primula scotica*: a rare Scottish plant. *Biodiversity and Conservation* **9**, 711-726.

Crouch, N.R. and *J. van Staden* (1993). *In vitro* culture of *Dianthus zeyheri subsp. natalensis*, a South African carnation. *Plant Cell Tissue and Organ Culture* **35**, 81-85.

Fraga, M., Mertxe, A., Ellul, P. and *M. Borja* (2004). Micropropagation of *Dianthus gratianopolitanus*, *HortScience* **39 (5),** 1083-1087.

Jain, A., Kantia, A. and *S.L. Kothari* (2001). *De novo* differentiation of shoot buds from leaf-callus of *Dianthus caryophyllus* L. and control of hyperhydricity. *Scientia Horticulturae* **87,** 319-326.

Jethwani, V. and S.L. Kothari (1993). Micropropagation of *Dianthus barbatus* and *D. chinensis* through cotyledonary node culture. *Plant Tissue Culture* **2,** 91-96.

Jethwani, V. and *S.L. Kothari* (1996). Phenylacetic acid induced organogenesis in cultured leaf segments of *Dianthus chinensis. Plant Cell Reports* **15,** 869-872.

Jethwani, V., Sharma, V.K. and *S.L. Kothari* (1994). Micropropagation of *Dianthus chinensis* and *Dianthus barbatus* through shoot tip culture. *Journal of Indian Botanical Society* **73,** 357-358.

Kovác, J. (1995). Micropropagation of *Dianthus arenarius* subsp. *bohemicus* - an endangered endemic from the Czech

Republic, *Botanic Gardens Micropropagation News* **8**, 106-108.

Marković, M., Grbić, M. and *A. Šindelić* (2006). Possibility of micropropagation of *Dianthus giganteiformis* ssp. *kladovanus* (Degen) Soo by the method of proliferation of lateral shoots. *Bulletin of the Faculty of Forestry* **94,** 171-180. DOI:10.2298/GSF0694171M

Mikulík, J. (1999). Propagation of endangered plant species by tissue cultures, *Acta Universitatis Palackianae Olomucensis, Biologica* **37,** 27-33.

Montes, S., Ramírez, L., Hernández, M.M., Santana, N. and *D.L. Martínez* (1997). Varietal micropropagation of carnation (*Dianthus caryophyllus* L. i *Dianthus plumarius* L.) through meristem culture. *Cultivos Tropicales* **18,** (1).

Murashige, T. and *F. Skoog* (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, **15**, 473–497.

Nakano, M. and *M. Mii* (1992). Protoplast culture and plant regeneration of several species in genus *Dianthus*. *Plant Cell Reports* **11**, 225-228.

Nau, J. (1996). Ball Perennial Manual: Propagation and Production, 193-196. Ball Publishing, Batavia, Illinois USA.

Nugent, G., Wardley, R.T. and *C. Lu* (1991). Plant regeneration from stem and petal of carnation (*Dianthus caryophyllus* L.). *Plant Cell Reports* **10**, 477-480.

Pareek, A., Kantia, A. and *S.L. Kothari* (2004). *In vitro* cloning of ornamental species of *Dianthus*. *Indian Journal of Biotechnology* **3**, 263-266.

Pareek, A. and *S.L. Kothari* (2003). Direct somatic embryogenesis and plant regeneration from leaf cultures of ornamental species of *Dianthus*. *Scientia Horticulturae* **98**, 449-459.

Popović M., Grbić M. and *M. Marković* (2008). Propagation of *Dianthus deltoides* L. by shoot culture. *Bulletin of the Faculty of Forestry* **97**, 209-220.

Radojević, Lj., Đorđević, N. and *J. Petrović* (1990). *In vitro* culture techniques for carnation breeding. *Acta Horticulturae* **280**, 163-168.

Radojević, Lj., Marinković, N. and *S. Jevremović* (1997). Vegetativno razmnožavanje u kulturi meristema i segmenata stabla *Dianthus petraeus* Waldst. et Kit. subsp. *noeanus*. *Glasnik instituta za botaniku i Botaničke bašte Univerziteta u Beogradu*, **31**, 73-77.

Roest, S. and *G.S. Bokelmann* (1981). Vegetative propagation of carnation *in vitro* through multiple shoot development. *Scientia Horticulturae* **14**, 357-366.

Sato, S., Katoh, N., Yoshi*d*a, *H., Iwai*, *S*. and *M. Hagimori* (2000). Production of doubled haploid plants of carnation (*Dianthus caryophyllus* L.) by pseudofertilized ovule culture. *Scientia Horticulturae* **83**, 301-310.

Rappaport, B. (1993). Green Landscaping: Greenacres. Landscaping with Native Plants. Available at: www.epa.gov/greenacres/weedlaws/JMLR.html (accessed December 29, 2011).

van Altvorst, A.C., Koehorst, H.J.J., Bruinsma, T., Jansen, J., Custers, J., De Jong, J. and *J.J.M. Dons* (1992). Adventitious shoot formation from in vitro leaf explants of carnation (*Dianthus caryophyllus* L.). *Scientia Horticulturae* **51**, 223-235.