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Micropropagation of the Endangered and Decorative Specie *Dianthus serotinus* Waldst. et Kit.

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Abstract

During past decades, great attention has been paid to propagation of endangered plant taxa in order to preserve biodiversity. The aim of this study was to optimize a protocol for *in vitro* propagation of the critically endangered and decorative species *Dianthus serotinus* Waldst. et Kit. The effects of different concentration of MS salt (Murashige and Skoog) of the culture, medium pH and different carbohydrates (sucrose, glucose, and fructose) on shoot multiplication were examined. The best results were obtained on half-strength MS (Murashige and Skoog) medium, whose pH was 5.8, with sucrose supplied at a concentration of 3%, when shoots with 1-2 nodes or shoot tips (with terminal buds only) were used as explants. The shoots were rooted (76.7%) on half-strength MS medium containing 0.5 mg·L^{-1} NAA (1-naphthaleneacetic acid). The obtained plantlets were successfully acclimatized (89%) in a 4:1 mixture of peat and sand and they flowered the following year. Presented protocol enables successful *in vitro* propagation of *D. serotinus*.

Keywords: acclimatization, fructose, glucose, *in vitro* culture, pH value, sucrose

Introduction

Dianthus serotinus Waldst. et Kit. (Caryophyllaceae) is an endemic Pannonian species included in category V (vulnerable taxa) of the IUCN Red List of endangered species. It is under legal protection and has the status of extremely endangered species in Serbia (Law on Environmental Protection, Rulebook on protected species, 2010). This species is found in small numbers only locally in the area of Subotica - Horgoš Sands, where it is threatened by the expansion of agricultural land and afforestation (Boža, 1999). *D. serotinus* is a decorative plant with bluish-green leaves and white flowers, which have a mild scent and a threadlike-rimmed corolla. This endangered species blooms all summer long and grows on poor sandy soils. It is drought tolerant, which makes it suitable for use as an ornamental in dry-stone constructions, rock and cottage gardens (Gajić, 1986; Boža, 1999).

To enable its ex situ and in situ conservation, we decided to propagate *D. serotinus* using the micropropagation method, which is a convenient method for rapid and efficient propagation of endangered species (Pence, 1999). Marković *et al.* (2007) established a sterile *D. serotinus* culture and investigated the effect of different concentrations of phytohormones on the multiplication and rooting of shoots of this species. However, their work examined the effect of phytohormones in a small number of treatments, and the acclimatization of the plantlets obtained was not performed. Considering that *D. serotinus* grows on humus-poor sand with pH ranging from 7.0 to 8.1 in natural habitat (Boža, 1999) and that pH medium in previous research was 5.8 (Marković *et al.*, 2007) we needed to improve protocol for regeneration and multiplication of this plant species.

Beside different concentrations of phytohormones that affect the development of in vitro cultures, important components are carbohydrates as sources of energy and carbon and osmotic agents that regulate the uptake of other culture constituents. In addition, the concentration and type of a carbohydrate in a medium can affect organogenesis and the development of an *in vitro* culture (Mohamed and Alsadon, 2010; Nowak *et al.*, 2004; Todorović *et al.*, 2006; Thorpe *et al.*, 2008). Although sucrose is the most widely used carbohydrate and the cheapest one (Thorpe *et al.*, 2008), other carbohydrates, mainly glucose and fructose, had a better effect on the development of in vitro cultures of some species, i.e. seven species of the genus *Alnus* (Tremblay and Lalonde, 1984; Barghchi, 1988), *Bougainvillea* 'San Diego Red' (Steffen *et al.*, 1988), *Castanea sativa* Mill., *C. crenata* Siebold et Zucc. (Chauvin and Salesses, 1988), *Nepeta rtanjensis* Diklić et Milojević (Mišić *et al.*, 2005b), *Corylus avelana* L. (Yu and Reed, 1993), *Fagus* spp. (Cuenca and Vieitez, 2000). Therefore, variations of carbohydrate components in a growth medium are inevitably taken into consideration in the optimization of protocols for *in vitro* propagation of certain plant species.

A large number of studies have been published on the micropropagation of endangered and/or endemic *Dianthus* species in the Balkans. They include the studies of endemic endangered species of the Czech Republic *D. arenarius* L. ssp. *bohemicus* (Novák) O. Schwarz (Kovác, 1995) and *D. superbus* L. ssp. *superbus* Domin (Mikulík,

1999), the endemic species of the Balkan Peninsula *D. petraeus* Waldst. & Kit. ssp. *noeanus* (Boiss.) Tutin (Radojević *et al*., 1997), *D. giganteus* d'Urv. ssp. *croaticus* (Borbás) Tutin and *D. ciliatus* Guss. ssp. *dalmaticus* (Čelak.) Hayek (Radojević *et al*., 2006, Radojević *et al*., 2010), the endemic and endangered species of Romania *D. spiculifolius* Schur (Butiuc-Keul *et al*., 2001), *D. petraeus* Waldst. & Kit. ssp. *simonkaianus* (Péterfi) Tutin (Micläuş *et al*., 2003), *D. callizonus* Schott & Kotschy (Holobiuc and Blindu, 2006), *D. nardiformis* Janka (Holobiuc *et al*., 2009, 2010a), *D. pratensis* M. Bieb. subsp. *racovitzae* (Prodan) Tutin (Cristea, 2010), *D. henteri* Heuff. ex Griseb. & Schenk (Cristea *et al*., 2010), *D. glacialis* Haenke ssp. *gelidus* (Schott, Nym. et Kotschy) Tutin (Holobiuc *et al*., 2010b), *D. giganteus* d'Urv. subsp. *banaticus* (Heuff. ex Griseb. & Schenk) Tutin (Pop and Pamfil, 2011), in Greece *D. fruticosus* L. (Papafotiou and Stragas, 2009) and many other studies. Also, to date, many studies regarding the optimization of the in vitro protocol of important decorative *Dianthus* taxa, including *D. chinensis* L., *D. barbatus* L. had been published (Jethwani and Kothari, 1993; Jethwani *et al*., 1994; Pareek *et al*., 2004). All mentioned studies investigated the optimal concentration of phytohormones added to MS culture media or less often half-strength MS media that differed depending on the species (Murashige and Skoog, 1962). The pH level was adjusted to 5.8 before autoclaving and 2% or even more often 3% sucrose was used as a source of carbohydrates. However, none of these papers investigated the effect of pH or different concentrations of different carbohydrates on the in vitro development of *Dianthus* spp. cultures. Taking all this into account, the aim of our research was to perform a detailed and complete study of the improvement of a protocol for the micropropagation and acclimatization of *D. serotinus*. This investigation included examining of the impact of culture medium pH, various carbohydrates and the development of in vitro cultures on the media with reduced concentrations of MS salts.

Materials and methods

The *in vitro* culture was established from seeds collected at the locality of Subotica - Horgoš Sands, in the vicinity of Palić. The seeds were surface disinfected by immersion in 4% NaOCl (sodium hypochlorite) supplemented with 2-3 drops of the Tween 20 for 20 minutes before three rinses in sterile, distilled water according to the procedure described by Marković *et al*. (2007). Three weeks later, nodal and shoot tip cuttings (with terminal buds only) were cut from the seedlings. These cuttings were placed on a multiplication culture medium containing Murashige and Skoog (MS) salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.8% agar and 0.5 mg · L^{-1} BAP (6-benzylaminopurine) and 0.5 mg · L^{-1} NAA (1-naphtalenacetic acid), as recommended by Marković *et al*. (2007) to obtain the material of sufficient quantity to set up the next experiment. Before the experiment setup, the shoots were cultured for 2 weeks on MS basal culture medium without phytohormones.

In all experiments three different types of explants (shoot tip cuttings with terminal buds only, single node cuttings with axillary buds only and shoots with 1-2 nodes) were placed on either solid MS or half-strength MS culture medium containing 0.8% agar, supplemented with 0.5 mg · L^{-1} BAP and 0.5 mg · L^{-1} NAA. The effect of culture medium pH supplemented with 3% sucrose on shoot induction and multiplication of *D. serotinus* was investigated. In this experiment the pH was adjusted before autoclaving the culture medium (20 min at 120 °C) to the four graded values (5.8, 6.8, 7.8 and 8.8). In the second experiment we investigated the effect of graded concentration (1%, 3%, 5% and 7%) of three different carbohydrate source (sucrose, glucose and fructose) on shoot induction and multiplication of *D. serotinus*. Considering that high concentration of sugar decreased culture medium pH (Thorpe *et al*., 2008), in this experiment the pH value of all tested media was set to 6.3 before autoclaving. During the multiplication stage, the culture medium was poured into glass vessels of 5 × 5 × 13 cm dimensions. The vessels contained 25 mL of the culture medium and 5 explants of the same type were placed in each of them. The experiments were repeated 3 times with 20 explants per treatment. After 25 days of cultivation, the following parameters were determined: the number of shoots per explant, the number of nodes and shoot length.

During the rooting stage, the shoots (with 1-3 nodes) were placed on basal medium (MS or half-strength MS) supplemented with NAA (0.0-0.5 mg · L^{-1}). The shoots were placed in glass containers of 8 x 8 x 15 cm dimensions, with 100 mL of medium and 15 shoots were placed in each of them. The experiments were repeated 3 times with 30 explants per treatment. After 15 days, the percentage of rooted plants, the number of roots and the length of the longest root per explant were determined.

During the acclimatization stage, the rooted plantlets were planted in three different mixtures of culture substrates, including (1:1) and (4:1) mixtures of peat and sand, and a (2:2:2:1) mixture of peat, sand, garden soil and burned farmyard manure. Before use, the soil was treated with a 1.5% solution of Previcur-N fungicide. Each treatment consisted of three replications with 30 plantlets. The planted plants were covered with perforated plastic wrap to maintain high relative humidity during the first 15 days of acclimatization. Ventilation was applied once daily for 5-10 minutes, after which the plastic wrap was removed. The plants were grown for 10 more days, and after that, their survival rate was recorded.

Plant cultures for all *in vitro* experiments were grown in a growth room under a 16/8-h (day/night) photoperiod and a photon flux rate of 50 μmol · m^{-2} · s^{-1} at 25±2°C in the light of fluorescent white pipes.

Statistical data processing was performed using an appropriate application for statistical analyses (Statgraphics, version 5.0). The significance of differences between the means was determined by the analysis of variance (ANOVA, p <0.05) and the least significant difference (LSD) test.

Results and discussion

Effect of the medium pH on shoot development

The *in vitro* culture was successfully established according to the procedure described by Marković *et al*. (2007). The concentration of MS salts (MS or half-strength MS) and culture medium pH, affected the frequency of shoot regeneration on the media of different pH levels, whereas this frequency was unaffected by explant type (Tab. 1). Contrary to expectations, regardless of the concentration of mineral salts, the shoot regeneration on explants was high on nutrition media with low pH. The difference between parameters obtained on the media of 5.8 and 6.8 pH levels was generally not statistically significant. On the media with a pH level of 7.8, the frequency of regeneration significantly decreased, whereas almost none of the explants developed on alkaline media with a pH level of 8.8 so the growth parameters couldn't be measured. In addition, the frequency of regeneration was higher on half-strength MS media (from 60.5 to 98.9%) compared to MS media (from 35.3 to 73.5%). However, the shoots on MS media were better developed, containing a higher average number of shoots per explant (Tab. 2). A high frequency of regeneration on half-strength MS media was achieved in the species *D. nardiformis* (Holobiuc *et al*., 2010a). However, it is not clear whether the lower concentrations of salts were more favourable for this species because the MS and half-strength MS media were supplemented with different concentrations and types of hormones.

Tab. 1. Frequency of shoot regeneration on different explants cultured on graded pH media after 25 days *in vitro*

pH value	Medium	Single node cuttings (%)	Shoot tips (%)	Shoots (%)
5.8	MS_0	58.9 ± 9.1 ^{abc}	65.5 ± 8.8 ^{c}	73.5 ± 9.1 ^{b}
6.8	MS_0	50.2 ± 8.0 ^{bc}	68.3 ± 9.1 ^{c}	72.1 ± 8.2 ^{b}
7.8	MS_0	35.3 ± 7.6 ^{c}	30.2 ± 7.2 ^{d}	38.2 ±8.6 ^{c}
8.8	MS_0	0.0 ± 0.0 ^{d}	3.3 ± 1.7 ^{e}	1.7 ± 1.7 ^{d}
5.8	1/2 MS_0	70.3 ± 8.3 ^{a}	98.9 ± 1.1 ^{a}	97.3 ± 2.7 ^{a}
6.8	1/2 MS_0	65.2 ±7.5^{ab}	90.8 ± 8.3 ^{ab}	92.5 ± 6.7 ^{a}
7.8	1/2 MS_0	60.5 ± 7.5 ^{ab}	81.5 ± 6.9 ^{b}	73.8 ±7.1 ^{b}

MS_0- basal medium containing MS mineral salts; 1/2 MS_0 - basal medium containing half strength MS mineral salts;
Note: Values followed by different letters are significantly different at the P < 0.05 level according to the least significant difference test

In determining the average number of nodes per explant, only the nodes that could be used for the next subculture, i.e. those whose axillary buds did not develop into new shoots were taken into account as potential nodal cuttings for the next subculture, and their number varied, although it could be observed that their number was generally high on media with low pH (Tab. 2).

Tab. 2. The average number of shoots and shoot nodes regenerated on different explants cultured on graded pH media after 25 days *in vitro*

pH value	Single node cuttings		Shoot tips		Shoots	
	MS_0	1/2MS_0	MS_0	1/2MS_0	MS_0	1/2MS_0
No. of shoots per explant						
5.8	10.1 ± 1.1 ^{a}	4.5 ± 0.7 ^{a}	8.8 ± 0.7 ^{ab}	7.8 ± 0.6 ^{b}	10.7 ± 0.6 ^{ab}	7.5 ± 0.3 ^{ab}
6.8	5.8 ± 0.8 ^{b}	5.3 ± 0.7 ^{ab}	11.5 ± 0.6 ^{b}	7.2 ± 0.5 ^{b}	11.5 ± 0.3 ^{a}	5.8 ± 0.4 ^{b}
7.8	6.0 ± 1.2 ^{b}	5.8 ± 1.0 ^{b}	6.9 ± 0.6 ^{a}	4.6 ± 0.3 ^{a}	9.3 ± 0.3 ^{b}	8.2 ± 0.3 ^{a}
No. of nodes per explant						
5.8	11.1 ± 0.8 ^{a}	7.1 ± 0.7 ^{ab}	12.3 ± 1.2 ^{ab}	15.1 ± 1.3 ^{a}	11.2 ± 1.2 ^{ab}	13.5 ± 1.0 ^{a}
6.8	4.6 ± 0.4 ^{b}	7.9 ± 0.7 ^{ab}	14.9 ± 1.0 ^{a}	11.9 ± 0.9 ^{ab}	14.5 ± 1.2 ^{a}	10.5 ± 0.8 ^{ab}
7.8	6.1 ± 1.1 ^{ab}	8.9 ± 0.3 ^{a}	7.1 ± 0.9 ^{b}	9.2 ± 1.1 ^{b}	8.1 ± 0.6 ^{b}	9.3 ± 0.7 ^{b}

MS_0- basal medium containing MS mineral salts; 1/2 MS_0 - basal medium containing half strength MS mineral salts;
Note: Values followed by different letters are significantly different at the P < 0.05 level according to the least significant difference test.

Because of the high level of variability in shoot length, the shoots were placed in different length categories (less than 10 mm, 10-20 mm and more than 20 mm) and the number of shoots belonging to a certain length category was expressed as a percentage of the total number of shoots. Generally, the shoots were longer on MS basal media. In some cases (nodal explants on media with 5.8 and 6.8 pH levels), over 40% of the shoots were longer than 20 mm, while in the case of half-strength MS media, the percentage of shoots longer than 20 mm did not exceed 20% on all media, regardless of the culture medium pH and explant type (Fig. 1). The importance of medium pH for in vitro development of plants is already known (Leifert *et al*., 1992; Harbage and Stimart, 1996). Nevertheless, in *Maranta leuconeura* cv. Kerchoviana different pH levels of a culture medium had no significant effect on the development of cultures, and there were no statistically significant differences in either rooting percentage or the average number of leaves per shoot and shoot length. Only the differences in the number of shoots per explant were observed (Ebrahim and Ibrahim, 2000). However, Bhatia and Ashwath (2005) showed that a change in medium pH did not significantly affect the number of shoots formed per explant of *Lycopersicum esculentum* cv. Red Coat, although it had an effect on their length. In addition, Ostrolucka *et al*. (2004) revealed that a lower pH level of 5.0 was

more favourable than pH 5.5 for acidophilic *Vaccinium corymbosum* 'Duke'. Additionally, a research on the effect of pH on axillary shoot proliferation in *Vaccinium vitis-idaea* L. showed that the optimum pH ranged from 4.0 to 5.5, depending on the cultivar (Ostrolucka *et al*., 2010). On the other hand, the research of Mišić *et al*. (2005a) showed that the high pH levels of 7.0 - 7.2 were the most suitable for the proliferation of lateral buds and the rooting of shoots of the calciphilic species *Nepeta rtanjensis*. However, contrary to expectations, our research showed that pH levels ranging from 5.8 - 6.8 were the most suitable for the species *D. serotinus*, while at higher pH levels the development of shoots in in vitro culture significantly decreased. A possible explanation for this phenomenon may lie in the specific conditions of in vitro culture and the fact that the adoption of mineral salts from culture medium, and thus the growth of plants, is influenced by not only the concentration of salts, but also pH, temperature, and the biochemical or physiological status of the plant tissues (George and de Klerk, 2008). Also, specific conditions of in vitro culture have a different influence on growth and development of the species belonging to distinct plant families with different metabolism and the response of *D. serotinus* perhaps was depending on interaction of mentioned factors, in different way than other studied species. Therefore, we can assume that a higher pH value would be better in case of a different composition of mineral components of the culture medium. This was confirmed by the data listed in Tab. 1 showing that better results were obtained on half-strength MS media with pH 7.8 than on MS media with pH values of 5.8 and 6.8 for the same type of explant.

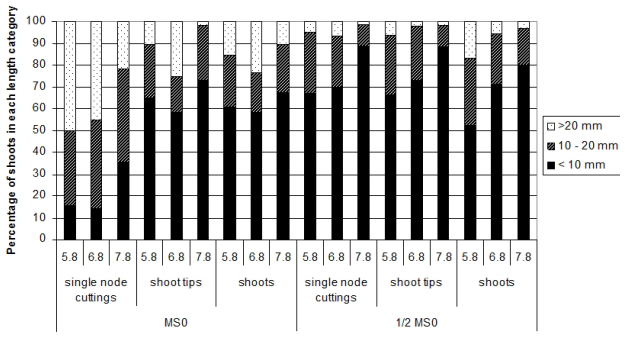

Fig. 1. The effect of graded medium pH on length of *D. serotinus* shoots regenerated on different explants on full-strength MS basal medium (MS_0) and half-strength MS basal medium ($1/2MS_0$)

The effect of carbon source on shoot regeneration

Generally, sucrose had the most favourable effect on the regeneration of explants. Glucose had the second most favourable effect, while the lowest frequency of regeneration was recorded on media containing fructose (Tab. 3). When explants were cultured on half-strength basal media, a low frequency of regeneration occurred only on nutrition media with high concentrations of glucose or fructose (7%). However, the frequency of regeneration was significantly lower on MS basal media. It was also affected by explant type, showing a higher regeneration rate of shoots than in the case of shoot tips and single node cuttings. Similarly, the highest average number of shoots was formed on MS basal medium with sucrose (from 6.2-nodal cuttings to 9.5-shoot tip cuttings). The average number of shoots was lower on media containing glucose and fructose, and generally, the best results were obtained if the concentration of these sugars was 3% (Fig. 2). Similar results were obtained by observing the average number of nodes that can be used for the next subculture, the MS basal media with sucrose supplied at a concentration of 3%, were the best, while the media with glucose and fructose, and half-strength MS medium with sucrose yielded poorer results (Fig. 3). As far as shoot length is concerned, regardless of the type and concentration of sugar, significantly longer shoots were formed on MS basal media. The percentage of shoots longer than 20 mm was generally 50% or higher on media containing glucose and sucrose, whereas on half-strength MS basal media it usually did not exceed 10%, reaching 32% only on the medium with 1% glucose (Fig. 4). In the examination of the effect of concentration and type of carbohydrate on the *in vitro* growth and organogenesis of the species *Rindera umbellata*, the highest frequency of adventitious buds formation on the stem explants was reached on medium with 0.06M (≈ 2%) sucrose, but the highest values of fresh and dry weight were obtained on medium with 0.1M (≈ 3.4%) sucrose (Perić *et al*., 2012). In contrast, glucose provided optimum growth and morphogenesis of the shoots of *Nepeta rtanjensis* (Mišić *et al*., 2005b). During the shoot multiplication of the species *Fagus sylvatica* and *F. orientalis* on media containing (3-4%) glucose, the number of shoots per explant was higher than on media containing sucrose or fructose. However, the effect of different sugars on shoot length varied depending on the clone of *F. orientalis* cultivated (Cuenca and Vieitez, 2000). Similarly, during the micropropagation of different cultivars and clones of *Vaccinium vitis-idaea*, optimum effects of carbohydrate type and concentration differed (Debnath, 2005). Considering that sucrose is the most commonly used carbohydrate (Thorpe *et al*., 2008), the results obtained in our research could be expected ones. Nevertheless, taking into account above-mentioned reports of better effect of other carbohydrates (e.g. glycose) on in vitro growth and development, and different response of different plants species and even the clones to variation of type and concentration of carbohydrates, we could assume that optimum results during in vitro culture of some other *Dianthus* species possibly would be achieved with carbohydrate other than sucrose.

Tab. 3. Frequency of shoot regeneration on different explants cultured on media supplemented with different carbohydrate source and graded concentration after 25 days *in vitro*

Type of sugar	Concentration	Single node cuttings (%)		Shoot tips (%)		Shoots (%)	
		MS_0	1/2 MS_0	MS_0	1/2 MS_0	MS_0	1/2 MS_0
sucrose	1%	85.0 ± 11.2 ^{a}	91.6 ± 5.7 ^{a}	68.4 ± 7.2 ^{b}	96.7 ± 2.1 ^{a}	88.3 ± 6.4 ^{a}	95.0 ± 1.8 ^{a}
sucrose	3%	73.3 ± 6.5 ^{abc}	90.0 ± 5.6 ^{a}	90.0 ± 5.6 ^{a}	98.3 ± 1.7 ^{a}	90.0 ± 4.1 ^{a}	96.7 ± 2.0 ^{a}
sucrose	5%	70.0 ± 6.8 ^{abc}	90.0 ± 5.6 ^{a}	81.7 ± 8.3 ^{ab}	96.7 ± 1.1 ^{a}	90.0 ± 2.3 ^{a}	96.7 ± 2.7 ^{a}
sucrose	7%	83.3 ± 5.8 ^{ab}	88.3 ± 8.1 ^{ab}	73.3 ± 8.3 ^{ab}	91.7 ± 5.3 ^{a}	86.7 ± 5.1 ^{a}	95.0 ± 2.4 ^{a}
glucose	1%	21.7 ± 4.3 ^{c}	98.3 ± 1.7 ^{a}	36.6 ± 10.5 ^{bc}	98.3 ± 1.7 ^{a}	60.0 ± 5.1 ^{ab}	98.3 ± 1.7 ^{a}
glucose	3%	18.3 ± 5.0 ^{c}	98.3 ± 1.7 ^{a}	65.0 ± 9.1 ^{b}	96.7 ± 2.4 ^{a}	85.0 ± 4.8 ^{ab}	98.3 ± 0.8 ^{a}
glucose	5%	16.7 ± 5.2 ^{cd}	95.0 ± 3.3 ^{a}	51.6 ± 8.9 ^{bc}	96.7 ± 2.4 ^{a}	56.7 ± 8.2 ^{b}	98.3 ± 0.8 ^{a}
glucose	7%	6.7 ± 3.3 ^{de}	91.7 ± 4.1 ^{a}	11.7 ± 4.6 ^{cd}	80.0 ± 5.6 ^{a}	15.0 ± 3.3 ^{bc}	95.0 ± 2.4 ^{a}
fructose	1%	11.7 ± 3.7 ^{cd}	96.7 ± 3.3 ^{a}	35.0 ± 5.2 ^{cd}	98.3 ± 0.8 ^{a}	18.3 ± 2.6 ^{bc}	96.7 ± 1.8 ^{a}
fructose	3%	48.3 ± 7.1 ^{bc}	96.7 ± 3.3 ^{a}	48.3 ± 5.7 ^{c}	95.0 ± 2.3 ^{a}	41.7 ± 5.8 ^{bc}	93.3 ± 2.3 ^{a}
fructose	5%	25.0 ± 6.2 ^{c}	80.0 ± 8.8 ^{ab}	20.0 ± 5.1 ^{cd}	70.0 ± 5.4 ^{b}	28.3 ± 5.1 ^{bc}	80.0 ± 7.1 ^{ab}
fructose	7%	3.3 ± 1.7 ^{de}	65.0 ± 7.4 ^{b}	5.0 ± 2.7 ^{d}	61.7 ± 6.7 ^{b}	23.4 ± 4.5 ^{bc}	76.7 ± 7.3 ^{b}

MS_0- basal medium containing MS mineral salts; 1/2 MS_0 - basal medium containing half strength MS mineral salts;
Note: Values followed by different letters are significantly different at the P < 0.05 level according to the least significant difference test

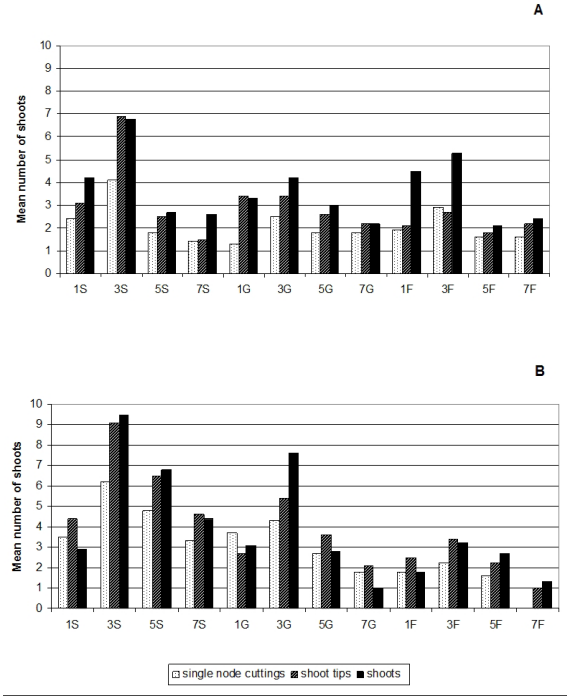

Fig. 2. The effect of graded concentration of different carbohydrate source on number of regenerated *D. serotinus* shoots on full-strength MS basal medium (B) and half-strength MS basal medium (A).

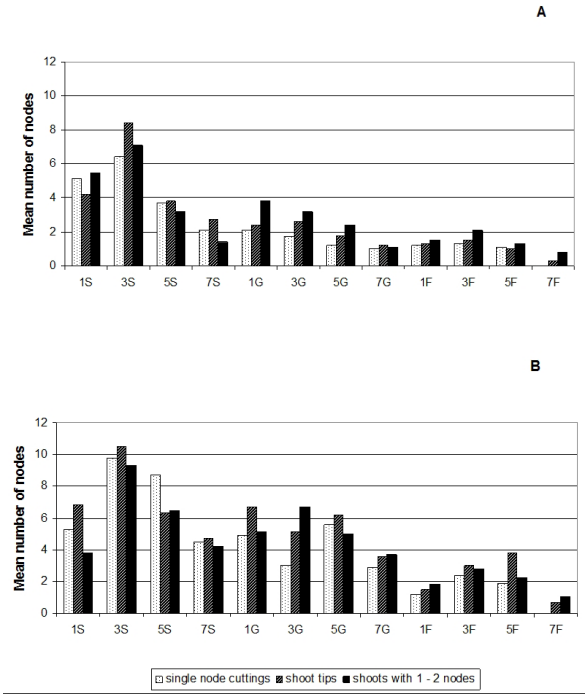

Fig. 3. The effect of graded concentration of different carbohydrate source on number of *D. serotinus* shoot nodes regenerated on full-strength MS basal medium (B) and half-strength MS basal medium (A)

(Figs. 2 and 3: the letters indicate different carbohydrate source and numbers indicate graded concentrations of different carbohydrate source; S-sucrose, G-glucose, F-fructose; 1 – 1%, 3 – 3%, 5 – 5%, 7 – 7%)

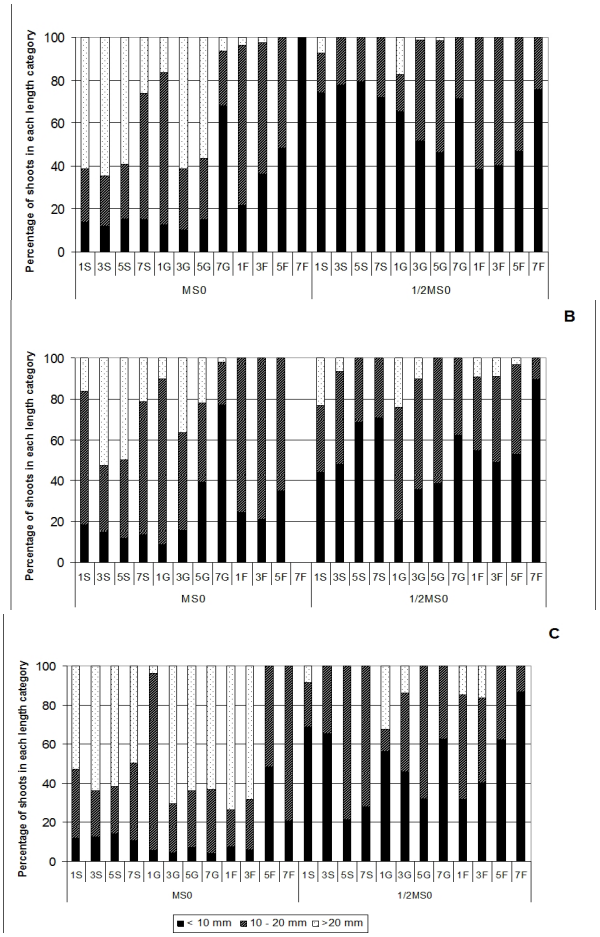

Fig. 4. The effect of graded concentration of different carbohydrate source on length of *D. serotinus* shoots regenerated on shoot tips (A), single node cuttings (B) and shoots (C)

(The letters indicate different carbohydrate source and numbers indicate graded concentrations of different carbohydrate source; S-sucrose, G-glucose, F-fructose; 1 – 1%, 3 – 3%, 5 – 5%, 7 – 7%; MS_0-full-strength MS basal medium, $1/2MS_0$ - half-strength MS basal medium)

Rooting and acclimatization

The rooting of shoots was the most successful on half-strength MS medium with 0.5 mg·L^{-1} NAA (76.7%), while the percentage of rooted shoots on MS medium with the same concentration of NAA was slightly lower (68.3%), which corresponds to the results previously described by Marković *et al.* (2007). The concentration of MS salts (MS or half-strength MS) had no significant effect on the percentage of rooting when media with the same concentration of NAA were compared. Similarly, during the propagation of *D. nardiformis* the rooting on MS media with half-strength MS or even 1/4 MS did not significantly affect the quality of rooting (Holobiuc *et al.*, 2010a).

Significantly poorer results were obtained on other media (Tab. 4), characterized by a lower concentration of NAA or a lack of hormones. The rooting percentage was lower than 50%, and the average number of roots per explant was almost halved (Tab. 4). The acclimatization of plantlets was the most successful (89%) in a 4:1 mixture of peat and sand, the least successful acclimatization of plantlets was recorded in a mixture of peat, sand, garden soil and burned farmyard manure (80%), and the acclimatized plants flowered the following year. When compared with other carnation species the rooting (68.3%) and acclimatization (89%) of *D. serotinus* obtained in this study can be considered satisfactory. Considerably better results in terms of rooting (100%) and acclimatization (97%) were obtained in the species *D. deltoides* L. (Markovic *et al.*, 2013), and the rooting of *D. superbus* ssp. *superbus* was successful (100%), although the acclimatization was relatively low (61.5%) (Mikulík, 1999). However, in some studies of other carnation species the rooting percentage was significantly lower (*D. henteri* - 20%, *D. spiculifolius* - 30%, *D. giganteus banaticus* - 40%) (Pop and Pamfil, 2011).

Tab. 4. Percentage of rooted shoots, average number of roots and the mean length of the longest root per explant

NAA (mg·L^{-1})	Medium	Rooting percentage (%)	No. of roots per explant	Mean length of the longest root (mm)
0	MS_0	28.3 ± 6.1 ^{cd}	6.7 ± 1.2 ^{bc}	16.8 ± 2.6 ^{a}
0.05	MS_0	23.3 ± 6.5 ^{d}	7.2 ± 1.0 ^{bc}	17.1 ± 2.0 ^{a}
0.1	MS_0	38.3 ± 5.5 ^{c}	13.0 ± 3.5 ^{b}	14.8 ± 2.1 ^{a}
0.5	MS_0	68.3 ± 7.2 ^{ab}	21.1 ± 3.1 ^{a}	14.5 ± 1.7 ^{a}
0	1/2 MS_0	35.0 ± 7.1 ^{c}	5.6 ± 1.3 ^{c}	15.5 ± 2.7 ^{a}
0.05	1/2 MS_0	36.7 ± 6.8 ^{c}	5.8 ± 1.8 ^{c}	16.2 ± 2.3 ^{a}
0.1	1/2 MS_0	48.4 ± 4.9 ^{bc}	6.3 ± 1.2 ^{bc}	11.3 ± 1.8 ^{ab}
0.5	1/2 MS_0	76.7 ± 7.2 ^{a}	10.5 ± 1.8 ^{b}	12.8 ± 1.5 ^{ab}

MS_0- basal medium containing MS mineral salts; 1/2 MS_0 - basal medium containing half strength MS mineral salts;
Note: Values followed by different letters are significantly different at the $P < 0.05$ level according to the least significant difference test

Conclusion

Taking the obtained results into account, it can be concluded that micropropagation of the endangered species *D. serotinus* should be performed on half-strength MS medium with a pH level ranging from 5.8 to 6.8. The most favourable sugar as carbon source is sucrose supplied at a concentration of 3%, and shoots with 1-2 nodes should be used as explants, although satisfactory results have also been achieved by using shoot tips. This research provide complete protocol for successful in vitro propagation of the critically endangered species *D. serotinus*, and its results could be useful for both activities aimed at biodiversity protection in sandy regions of Pannonian Basin and floricultural production of this species.

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