AN EFFICIENT IN VITRO PROPAGATION PROTOCOL OF DIANTHUS GIGANTEIFORMIS BORBAS SUBSP. KLADOVANUS (DEGEN) SOO

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Abstract: Dianthus giganteiformis subsp. kladovanus is an endemic, endangered, horticulturally appealing perennial plant that can be used for the revegetation of sand dunes of the Danube region. The appropriate method for its effective production is micropropagation. For this reason, the experiments were conducted in order to establish an efficient protocol for the micropropagation of this subspecies. The sterile culture was initiated from seeds collected in situ and the germination percentage was high (88%). According to the results obtained in this study, the multiplication phase should be performed on an MS medium enriched with 0.1 mg/L BAP and 0.1 mg/L NAA. The concentration of MS salts significantly influenced rooting, and higher rooting percentages were obtained on reduced MS media (91.7 - 95%) than on MS media (73.4 - 76.7%). The addition of NAA slightly increased rooting percentage (up to 95%). Obtained microplants were successfully acclimatized (83.3%) in a substrate composed of peat and sand (1: 1; v/v). Using the protocol presented in this paper, the efficient propagation of D. giganteiformis spp. kladovanus can be achieved for rapid plant production aimed at revegetation, biodiversity protection and floricultural production of this species.

Keywords: Dianthus giganteiformis ssp. kladovanus, in vitro, plant biotechnology, endangered species

INTRODUCTION

The large number of valuable protected sands, sand steppes and loess steppes are located in the Danube basin and they are highly sensitive to degradation (Jean-Vasile et al., 2013; Biserkov et al., 2015; Kadović et al., 2016). The protection or restoration of natural sands vegetation is needed to maintain sandy-steppes and their associated species richness (Biserkov et al., 2015; Edthofer & Samec, 2016). In Serbia, Kladovo sands is an important habitat, designated as an ecologically important area within the ecological network of the Republic of Serbia. The main negative impacts are surrounding forest plantations, transformation of natural habitats into agricultural lands, spreading of invasive species, grazing and other human activities (Edthofer & Samec, 2016). The conservation management of these areas includes active management to stop spreading of the invasive species and expansion of forest vegetation, followed by the restoration of natural vegetation and ex-situ protection of psammophytic endangered species (Šefferová Stanová et al., 2008; Biserkov et al., 2015; Edthofer & Samec, 2016).
**Dianthus giganteiformis** Borbas subsp. **kladovanus** (Degen) Soo (syn. *Dianthus pontederae* A. Kern. subsp. **kladovanus** (Degen) Stoj. & Stef.) (Jalas & Suominen, 1988; Ciocărlan, 2009) is an endemic subspecies of the Balkan Peninsula. In Serbia, it has the status of a critically endangered (CR) species (Diklić et al., 1999), and is covered by legal protection (Law on Environmental Protection, Rulebook on protected species, 2010). In the neighbouring countries Bulgaria and Romania, it has the status of a rare species, while in Bulgaria it is also protected by law (Petrova, 1997; Oprea, 2005; Petrova & Vladimirov, 2009). In Serbia, this species is found only in the vicinity of the village of Davidovac, in Kladovska Sands, where its subpopulation is estimated at 250 individuals with a decreasing tendency (Jalas & Suominen, 1988; Diklić et al., 1999).

*D. giganteiformis* subsp. **kladovanus** is a perennial psammophytic species which grows in sandy steppes and can be used as an ornamental on sandy soils. For this reason, Marković et al. (2006) conducted a preliminary research investigating the possibility of micropropagation of this taxon. However, their research included a small number of treatments only on a half-strength MS medium (Murashige & Skoog, 1962) containing only a single concentration of BAP (1 mg/L), while the multiplication index was low. Since micropropagation is an important method for *ex situ* and *in situ* conservation of endangered taxa (Fay, 1992; Pence, 1999), numerous research papers, investigating the possibilities of successful micropropagation of the endangered or endemic *Dianthus* spp. in the Balkans and Romania, have been published (Cristea, 2010; Jarda et al., 2011; Cristea et al., 2013a, 2013b, 2014; Tsoktouridis et al. 2013; Jarda et al., 2014; Marković et al., 2016). Micropropagation protocols obtained in those studies for more than 20 *Dianthus* taxa differ depending on the species or even a genotype of the same species. In order to establish a reliable and efficient protocol for the rapid micropropagation of *D. giganteiformis* subsp. **kladovanus**, we conducted a more detailed investigation of the effect of different media compositions on the multiplication and rooting of shoots on MS and half-strength MS medium. In this way, not only its *in situ* and *ex situ* conservation will be enabled but also the production of a large number of plants necessary for the restoration of sustainable natural habitats.

**MATERIAL AND METHOD**

The seeds were randomly collected from different plants in Kladovska Sands, brought to the laboratory and used for initiating the *in vitro* culture. The 4% NaOCl solution was used for the seeds surface disinfection, followed by rinsing three times in sterile distilled water. The seeds were placed to germinate in full day light conditions (16 h light / 8 h dark). In all experiments the MS medium or half-strength MS medium (Murashige & Skoog, 1962) with the addition of 3% (w/v) sucrose, 0.8% (w/v) agar and different concentrations of plant growth regulators were used. Germination was performed on a hormone-free MS medium.

At the multiplication stage, plant growth regulators Benzylaminopurine - BAP (0.1, 0.5 or 1.0 mg/L) and Naphthaleneacetic acid - NAA (0.1 or 0.5 mg/L) were added to the media (MS or half-strength MS). Three types of explants were used, including single-node cuttings (with 2 axillary buds), terminal buds (containing only apical bud and small part of stem bellow) and terminal shoot cuttings with one node (containing apical bud and one pair of axillary buds). The explants were incubated at 24 ± 2°C, under long day conditions (16/8 h photoperiod), under a light intensity of 50 µmol/m²s, for 25 days subculturating intervals. Each treatment was repeated three times with 20 explants of the same type. The following parameters were recorded: the number of shoots and nodes developed per each explant and shoot length. In order to avoid a high variability of data for a shoot length, the shoots were grouped into three categories (shorter than 10 mm, 10-20 mm and longer than 20 mm), and the number of shoots in each length category was expressed as a percentage of the total number of shoots.

The rooting of *in vitro* obtained microshoots (10-35 mm long) was performed on the media supplemented with NAA (0.05, 0.1, 0.5 mg/L) or without growth regulators. The experiments were repeated 3 times with 30 explants per treatment. The rooting percentage, the number of roots per microplant, and length of the longest root were determined after 15 days in culture. The uniformly rooted plantlets were treated with a fungicide (1.5% solution of Previcur-N) and acclimatized in a 1: 1 mixture of peat and sand for 25 days before
their survival rate was recorded. In order to maintain high relative humidity, during the first 15 days the microplants were covered with a transparent plastic.

The obtained data were subjected to statistical analysis using the program Statgraphics, version 5.0 (STSC Inc. USA). The percentage data were arcsine-transformed before statistical analysis. The analysis of variance (ANOVA, p < 0.05) and the method of least significant difference (LSD) were performed to determine the differences between the treatments.

RESULTS AND DISCUSSION

The seeds successfully germinated in the sterile culture with a high germination percentage (88%), which is an important factor for preserving a population variability after propagation. Although this value is lower than the germination rate achieved by *D. pinifolius* - 92% or *D. carthusi-anorum* - 95% (Marković et al. 2016; Muszyńska & Hanus-Fajerska, 2017), it is significantly higher than the germination percentage in vitro of some other *Dianthus* species, such as *D. glacialis* - 31%, *D. giganteus* ssp. *croaticus* - 42%, *D. ingoldbyi* - 65%, or *D. henteri* - 75% (Colombo et al., 2004; Radojević et al., 2010; Pop & Pamfil, 2011; Arda et al., 2016). According to the ISTA (International Rules for Seed Testing) rules for some commercially important *Dianthus* species, the pre-chilling treatment is recommended for dormancy breaking (ISTA, 2011). However, in our research the seeds were sown immediately after collection, without the cold pre-sowing treatment.

Perhaps this could be an explanation for the low germination percentage obtained with *Dianthus* spp. in the above mentioned studies. In addition, some authors found that a germination of certain *Dianthus* species can be considerably better in light than in a dark conditions (Marcu et al., 2006), although there were some other reports indicating that light conditions had no impact on germination of some *Dianthus* taxa (Kołodziejek et al., 2018). Similarly, there is no recommendation for conducting *Dianthus* germination in the light in the ISTA rules (ISTA, 2011). Besides light, the sterilizing agent and treatment duration can also affect germination rate (Miyoshi & Mii 1995; 1998; Lee et al., 2007), and thus the low germination rate of *D. giganteus* ssp. *croaticus* can be a result of the low NaOCl (1%) concentrations used for disinfection (Radojević et al., 2010). Similarly, the germination of *D. callizonus* was much higher in vitro (80%) than ex vitro (46%) (Catana et al., 2013). In some cases, gibberellic acid (GA3) added in growing medium, as an antagonist of ABA (Abscisic acid) which inhibited germination, positively influenced germination (Watkinson & Pill, 1998). For example, the germination rate of *D. henteri* was 100% on the medium with 100 mg/L GA3, while it was only 75% on the same medium without GA3 (Cristea et al., 2010).

The percentage of shoot regeneration at the multiplication stage was high, reaching over 93% in the majority of the media tested (Table 1). The concentration of MS salts (MS or half-strength MS) did not affect the frequency of shoot regeneration. On the other hand, there were some small differences in the percentage of regeneration depending on the explant type, since higher regeneration

| BAP mg/L | NAA mg/L | single node cuttings (%) | terminal buds (%) | shoot cuttings (%) |
|----------|----------|--------------------------|-------------------|-------------------|
|          |          | 1/2MS MS 1/2MS MS 1/2MS MS 1/2MS MS | |
| 1.0      | 0.5      | 83,3bc 91,7ab | 100,0a 90,0bc 96,7ab 93,3b | |
| 1.0      | 0.1      | 91,7ab 93,3ab | 93,3ab 93,3ab 95,0ab 95,0b | |
| 0.5      | 0.5      | 93,3ab 93,3ab | 98,3a 96,7ab 100,0a 100,0c | |
| 0.5      | 0.1      | 93,3ab 96,7a | 100,0a 100,0a 98,3ab 100,0c | |
| 0.1      | 0.1      | 98,3a 98,3a  | 100,0a 100,0a 100,0a 100,0a | |

Note: The values followed by different letters are significantly different according to the LSD test, at the P < 0.05 level.
rates were achieved with shoot cuttings compared to terminal buds and single-node cuttings. A similar result was reported for *D. serotinus*, whose regeneration rate of shoot cuttings was higher than the regeneration rate of single node cuttings, whereas the concentration of MS salts significantly affected the shoot regeneration of *D. serotinus*, showing better results on half-strength MS media (Marković et al., 2013).

The mean number of shoots regenerated per explant ranged between 2.6 and 4.6 (Table 2), but a statistically significant impact of a hormone or MS salt concentration on the number of shoots could not be observed. The mean number of shoots can be significantly different depending on the auxine type. Thus, during the micropropagation of *D. carthusianorum*, the number of shoots was only 1.7 on MS medium supplemented with 1 mg/L BAP and 0.2 mg/L NAA, but it was 8 times higher on the same medium with 0.2 mg/L IAA (Indole-3-acetic acid) instead of NAA (Muszyńska & Hanus-Fajerska, 2017). However, the mean number of nodes (Table 2) was considerably higher than in the preliminary research of this subspecies micropropagation (Marković et al., 2006). While the average number of nodes obtained in the research conducted by Marković et al. (2006) ranged between 3.5 and 7.6, on 50% of the media tested in this research, the average number of nodes exceeded 7.6, reaching 12.5 nodes regenerated per explant (Table 2).

Generally, the shoots were longer on media with lower concentrations of plant growth regulators, and more than 30% of them were longer than 20 mm on media with 0.1 mg/L BAP and 0.1 mg/L NAA, for all explant types (Figure 1). The impact of the concentration of MS salts is not significant, but generally longer shoots develop on MS media. The impact of the concentration of MS salts on shoot length was recorded for *D. serotinus* and *D. pinifolius* (Marković et al., 2013, 2016), with longer shoots developed on full strength MS media. Nevertheless, half strength MS media can have a favourable effect on shoot regeneration (Desilets et al. 1993; Daud et al. 2011; Marković et al. 2013).

Rooting on half strength MS media was successful, as the rooting percentage ranged from 91.7 - 95%, which corresponds with the preliminary results obtained by Marković et al. (2006), who recorded a rooting percentage of 94% on a hormone free half-strength MS medium. Contrary

### Table 2. The average number of shoots and nodes regenerated on different explants after 25 days of in vitro culturing

| BAP mg/L | NAA mg/L | single node cuttings | terminal buds | shoot cuttings |
|----------|----------|----------------------|--------------|---------------|
|          |          | MS 1/2MS             | MS 1/2MS     | MS 1/2MS      |
| 1        | 1        | 3.1<sup>ab</sup>     | 3.3<sup>ab</sup> | 3.2<sup>ab</sup> | 3.8<sup>ab</sup> | 4.1<sup>a</sup> | 3.8<sup>ab</sup> |
| 1        | 0.5      | 3.3<sup>ab</sup>     | 3.9<sup>ab</sup> | 3.4<sup>ab</sup> | 2.6<sup>ab</sup> | 3.6<sup>ab</sup> | 3.8<sup>ab</sup> |
| 0.5      | 0.5      | 2.9<sup>ab</sup>     | 4.4<sup>a</sup> | 4.6<sup>a</sup> | 2.8<sup>ab</sup> | 4.0<sup>a</sup> | 3.9<sup>ab</sup> |
| 0.5      | 0.1      | 3.7<sup>a</sup>      | 4.5<sup>a</sup> | 4.1<sup>ab</sup> | 4.2<sup>a</sup> | 3.7<sup>ab</sup> | 4.1<sup>ab</sup> |
| 0.1      | 0.1      | 3.6<sup>a</sup>      | 2.7<sup>ab</sup> | 2.8<sup>bc</sup> | 1.7<sup>abc</sup> | 3.7<sup>ab</sup> | 3.5<sup>bc</sup> |

Note: The values followed by different letters are significantly different according to the LSD test, at the P < 0.05 level.
Figure 1. The effect of different BAP and NAA concentrations added to MS and half-strength MS media on the length of *D. giganteiformis* spp. *kladovanus* shoots regenerated on single node cuttings (A), terminal buds (B), shoot cuttings (C)
to expectations, NAA concentration neither had an impact on the rooting rate nor on the number of roots per explant (Table 3). The addition of auxine to the medium generally promotes rooting, and sometimes high concentrations of auxines are necessary for the rooting of some *Dianthus* taxa (Salehi, 2006; Papafotiou & Stragas, 2009) or for a more efficient rooting (Tsoktouridis et al. 2013). On the other hand, there were also different reports on *Dianthus* spp. micropropagation, in which rooting was successful on a hormone-free medium, including *D. ciliatus*ssp. *dalmaticus*, *D. mainensis* and *D. spiculifolius* (Radojević et al., 2010; Cristea et al., 2013b; Erst et al. 2014).

The concentration of MS salts significantly influenced rooting, and the percentage was considerably higher on half strength MS media (table 3), which was also obtained for *D. mainensis* (Erst et al. 2014). However, during the micropropagation of *D. pinifolius*, the rooting percentage was higher on MS media than on half-strength MS media (Marković et al. 2016), and, in some cases (e.g. *D. nardiformis*), the concentration of MS salts (MS, 1/2MS or 1/4MS) had no significant effect on the percentage of rooting (Holobiuc et al. 2010). The addition of NAA did not influence the mean number of roots and root length (Table 3), like in the case of *D. pinifolius* (Marković et al. 2016) or *D. gratianopolitanus* (Fraga et al. 2004). On the other hand, the auxine type can considerably influence the rooting rate (Marcu et al. 2006).

The acclimatization rate obtained in this study is 83.3%, which can be considered satisfactory. Similar results were reported for other *Dianthus* taxa, including *D. mainensis* - 83%, *D. trifasciculatus* ssp. *parviflorus* - 85% and *D. pinifolius* - 88.9% (Holobiuc et al. 2013; Erst et al. 2014; Marković et al. 2016). A lower acclimatization percentage was obtained for *D. fruticosus* - 70% (Papafotiou & Stragas, 2009) but in some cases a higher acclimatization rate was achieved, e.g. by *D. petraeus*, 90-100% (Tsoktouridis et al. 2013) or *D. caryophyllus* cultivars - 90% (Salehi, 2006).

### CONCLUSIONS

The endangered and decorative subspecies *D. giganteiformis* ssp. *kladovanus* can be successfully propagated using the protocol presented in this research. The *in vitro* culture was established from seed collected from different plants in the same population, so the obtained results present the average response to the culture conditions. Although the multiplication phase should be performed on an MS medium containing 0.1 mg/L BAP and 0.1 mg/L NAA, *in vitro* rooting should be on a half-strength MS medium, while the addition of 0.5 mg/L NAA can have a favourable effect. Further, the rooted microplants can be successfully acclimatized in a 1:1 mixture of peat and sand. In this way, efficient *D. giganteiformis* ssp. *kladovanus* propagation can be achieved for both plant production aimed at biodiversity protection and the floricultural production of this species.

### Table 3. Rooting of shoots after 15 days of *in vitro* culturing

| NAA mg/L | MS salts | Rooting percentage (%) | No. of roots per explant | Mean length of the longest root (mm) |
|----------|----------|------------------------|--------------------------|-------------------------------------|
| 0.0      | MS       | 73,4<sup>b</sup>       | 3,2<sup>b</sup>          | 17,5<sup>ab</sup>                   |
| 0.05     |          | 73,4<sup>b</sup>       | 3,1<sup>b</sup>          | 16,2<sup>ab</sup>                   |
| 0.1      |          | 75,0<sup>b</sup>       | 3,8<sup>b</sup>          | 15,1<sup>b</sup>                   |
| 0.5      |          | 76,7<sup>b</sup>       | 4,6<sup>b</sup>          | 18,4<sup>a</sup>                   |
| 0.0      | 1/2MS    | 93,4<sup>a</sup>       | 8,9<sup>a</sup>          | 22,5<sup>a</sup>                   |
| 0.05     |          | 91,7<sup>a</sup>       | 9,1<sup>a</sup>          | 18,5<sup>a</sup>                   |
| 0.1      |          | 91,7<sup>a</sup>       | 9,6<sup>a</sup>          | 17,4<sup>ab</sup>                  |
| 0.5      |          | 95,0<sup>a</sup>       | 10,2<sup>a</sup>         | 19,0<sup>a</sup>                   |

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

Ard a H., S. Daya n, C. Kart a l, Guler N. (2016): In vitro conservation of critically endangered Dianthus ingoldbyi Turrill under slow growth conditions. Trakya Univ J Nat Sci. 17: (47-54)

Biserkov, V. (ed.). (2015): Red Data Book of Republic of Bulgaria, Vol. 3. Natural habitats, IBAS – BAS & MEW, Sofia. pp 145-14

Cat a na R., I. Holobri uc, Moldove a nu a n, M. (2013): In vitro seed germination in three rare taxa from the Romanian Carpathians Flora. Oltenia. Studii ş i comunicări. Ştiinţele Naturii, 29: (85–92)

Cio căran, V. (2009): Contributions To The Knowledge Of The Vascular Flora Of Romania. J. Plant Develop., 16: (25–28)

Colombo A., A.Castiglioni, A. Tosca, and C. Bonomi. (2004): Evaluation of germination capacity in Dianthus glacialis, an endangered alpine species. Abstracts, 27th ISTA Congress. Seed Symposium Budapest, Hungary May 17th – 19 th. p. 69.

Cristea V., A.T. Brummer, L. Jarda and M. Miclaus. (2010): In vitro culture initiation and phytohormonal influence on Dianthus henteri – a Romanian endemic species, Rom. Biotech Lett., Suppl. 15 (1): (25–33)

Cristea V., C. Crăciunaş, D. Marcu, M. Palada and A. Butiuc-Keul. (2014): Genetic stability during in vitro propagation of Dianthus spiculifolius Schurr. Propag Ornam Plants, 14 (1): (26–31)

Cristea V., L. Jarda and I. Holobiuc. (2013a): Ex situ conservation of three endemic and/or endangered Dianthus species. Not Bot Horti Agrobot Cluj Napoca, 41: (73–78).

Cristea V., M. Palada, L. Jarda and A. Butiuc-Keul. (2013b): Ex situ in vitro conservation of Dianthus spiculifolius, endangered and endemic plant species. Studia Universitatis Babeş-Bolyai Biologia 58 (1): (57–69)

Cristea, V. (2010): Photoautotrophic in vitro culture of endemic and endangered Dianthus species from Romania. Todeesco, Cluj-Napoca, 227 p. (in Romanian)

Daud N., R. Taha, N. Noor and H. Alimon. (2011): Provision of low cost media options for in vitro culture of Celosia sp. Afr. J Biotechnol., 10 (80): (18349–18355)

Desilets H., Y. Desjardins and R. Bélanger. (1993): Clonal propagation of Pelargonium x hortorum through tissue culture: Effects of salt dilution and growth regulator concentration. Can. J Plant Sci., 73: (871–878)

Diklić N., M. Niketić and G. Tomović. (1999): Dianthus giganteiformis Borbás subsp. kladovanus (Degen) Só o. The red data book of the flora of Serbia. Ministarstvo za životnu sredinu Republike Srbije, Biološki fakultet Univerziteta u Beogradu, Zavod za zaštitu prirode Republike Srbije, Beograd. pp. 249–251

Edthofer M., E. Samec. (2016): The restoration of wetland and grassland priority habitats in the Danube Basin Region. CEE web for Biodiversity. Budapest, Hungary. pp. 19-22

Erst A.A., A.S. Erst and D.N. Shaulo. (2014): In vitro propagation of Dianthus mainensis, an endemic plant from West Sayan (North Asia). Taiwania, 59 (2): (106–110)

Fay, M.F. (1992): Conservation of rare and endangered plants using in vitro methods. In Vitro Cell Dev Biol Plant. 28: (1–4)

Fraga M., A. Mertxe, P. Ellul and M. Borja. (2004): Micropropagation of Dianthus gratianopolitanus. Hort Sci., 39 (4): (112–115)

Holobiuc I., R. Catana and V. Cristea. (2010): Researches concerning in vitro cultures optimization of the vulnerable species Dianthus nardiformis Janka. Analele Universităţii din Oradea - Fascicula Biologie, 17 (1): (116–121)

Holobiuc I., R. Catana, C. Voichita and F. Helepeciu. (2013): In vitro introduction of Dianthus trifasciculatus KIT ssp. parviflorus as ex situ preservation method. Muzeul Olteniei Craiova. Oltenia. Studii şi comunicări. Ştiinţele Naturii, 29: (93–100)

ISTA (2011): International Rules for Seed Testing Edition 2011, The International Seed Testing Association (ISTA), Bassersdorf, Switzerland

Jalas J. and J. Suominen. (1988): Atlas Florae Europaeae. Distribution of Vascular Plants in Europe. 3. Caryophyllaceae. Cambridge University Press, Cambridge. p. 208

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Jardia L., A. Butiuc-Keul, M. Höhn, A. Pedryc and V. Cristea. (2014): *Ex situ* conservation of *Dianthus giganteus* d’Urv. subsp. *banaticus* (Heufl.) Tutin by *in vitro* culture and assessment of somaclonal variability by molecular markers. *Turk. J Biol.*, 38: (21–30)

Jean-Vasile A., T. Adrian, J. Subic and D. Dusanescu. (2013): Sustainable technologies, policies, and constraints in the green economy. AEEGT Book Series, IGI Global, Hershey, PA. p 157

Kadović R., V. Bohajar, V. Perović, S. Simić, M. Todosijević, S. Tošić, M. Andelić, D. Mlađan and U. Dovezenski. (2016): Land Sensitivity Analysis of Degradation using MEDALUS model: Case Study of Deliblato Sands, Serbia, *Arch Environ Prot.* 42(4): 114-124. doi: https://doi.org/10.1515/aep-2016-0045

Kolodziejek J., J. Patykowski and M. Wala. (2018): An experimental comparison of germination ecology and its implication for conservation of selected rare and endangered species of *Dianthus* (Caryophyllaceae). *Botany*. 96: 319–328

Law on Environmental Protection, Rulebook on protected species (2010): Official Gazette of the Republic of Serbia, No. 5/10, Belgrade, Serbia

Lee Y.I., C.F. Lu, M.C. Chung, E.C. Yeung and N. Lee (1998): Stimulatory effects of sodium and calcium hypochlorite, pre-chilling and cytokinins on the germination of *Cyripedium macranthos* seed *in vitro*. *Physiol. Plantarum*, 102: (481–486)

Murashige T. and F. Skoog. (1962): A revised medium for growth and bioassays with tobacco tissue culture. *Physiol. Plantarum* 15: (473–497)

Muszyńska, E. and E. Hanus-Fajerska. (2017): *In vitro* multiplication of *Dianthus carthusianorum* calamine ecotype with the aim to revegetate and stabilize polluted wastes. *Plant Cell Tiss Organ Cult.* 128: 631-640. https://doi.org/10.1007/s11240-016-1140-0

Oprea, A. (2005): Lista critică a plantelor vasculare din România. Iaşi: Edit. Univ. “Alexandru Ioan Cuza”, pp 668

Papafiotiou M, Stragas J. (2009): Seed germination and *in vitro* propagation of *Dianthus fruticosus* L. *Acta Hort.* 813: 481–484

Pence, V.C. (1999): The application of biotechnology for the conservation of endangered plants. In: Benson E.E. (ed) Plant Conservation Biotechnology, Taylor and Francis, London: Chapter 15, pp. 227–241

Petrova A. and V. Vladimirov. (2009): Red List of Bulgarian vascular plants. *Phytologia Balcanica* 15 (1): (63–94)

Petrova A. and V. Vladimirov. (2009): Red List of Bulgarian vascular plants. *Phytologia Balcanica* 15 (1): (63–94)

Pop T. and D. Pamfil. (2011): *In vitro* Preservation of Three Species of *Dianthus* from Romania. *Bulletin UASVM Hort.* 68(1): (414–422)

Radojević Lj., D. Ćalić-Dragosavac, J. Špirić, B. Stevanović and V. Stevanović. (2010): *In vitro* culture of stem segments of *Dianthus ciliatus* ssp. *dalmaticus* and *D. giganteus* ssp. *croaticus* (Caryophyllaceae). *Botanica Serbica*, 34 (2): (153–161)

Salehi, H. (2006): Can a general shoot proliferation and rooting medium be used for a 440 number of carnation cultivars? *Afr. J Biotechnol.*, 5: (25–30)
Šefferová Stanová V., Z. Vajda and M. Janák. (2008): Management of Natura 2000 habitats. 6260
*Pannonic sand steppes. European Commission
Tsoktouridis G., K. Grigoriadou, E. Doua, A. Nikolaidou, G. Menexes and E. Maloupa. (2013): *In vitro* shoot proliferation, rooting, and acclimatization of four diverse *Dianthus petraeus* W. et K. genotypes using TDZ, NAA, and IBA. *Propag Ornam Plants*, 13 (4): (181–188)
Watkinson J.I., Pill. W.G. (1998): Gibberellic acid and presowing chilling increase seed germination of indiangrass (*Sorghastrum nutans* (L.) Nash.), *HortScience*, 33(5): 849–851