STUDIA UBB BIOLOGIA, LVIII, 1, 2013 (p. 57-69)

EX SITU IN VITRO CONSERVATION OF *DIANTHUS SPICULIFOLIUS*, ENDANGERED AND ENDEMIC PLANT SPECIES

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SUMMARY. The preservation of endangered and/or threatened plant species by biotechnological methods as *in vitro* culture is a complementary alternative of in situ conservation. Several individuals of *Dianthus spiculifolius* Schur from two distinct and spatially separated populations, from Hăşmaş and Vlădeasa Mountains (Romania) were preserved by *in vitro* culture. Following several stages of *in vitro* culture as culture establishment, stabilization, stable culture multiplication, *in vitro* rhizogenesis and outdoor acclimatization, an optimal protocol for conservation and acclimatization was achieved. Four culture media variants were used for different culture stages. During the stable culture multiplication stage, after 60 days of culture, a maximum of 104.8 new shoots/uninodal explant for the Vlădeasa Mt. population and 24.9 new shoots/explant for the Hăşmaş Mt. population were obtained. Nodes explants, horiozontally inoculated generated a very high number of shoots (maximum 345.8 shoots). The rhizogenesis ratio was 10 roots/explant in population from Hăşmaş Mt. and 5.3 in population from Vlădeasa Mt. The vitroplants were successfully acclimatized and they were cultivated in a special outdoor rocky area.

Keywords: acclimatization, endangered, endemic plant, explant type, micropropagation.

Introduction

Besides the different advantages of *in vitro* culture, the *ex situ* conservation by *in vitro* collection, also offers the possibility to generate biological material either for in situ repopulation programs, or collections in botanical gardens, international exchanges or fundamental and applied studies. Thus, the pressure of collecting plants

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from the natural populations is avoided. *In vitro* collections also have a social impact, serving to raise awareness among non-scientists about the *ex situ* conservation of plants.

Nowadays, the prioritization of endemic and/or threatened species and their natural habitats is a crucial point of conservation actions, particularly in areas with rich endemic floras (Bacchetta *et al*., 2012). Target 8 of 2011-2020 Global Strategy of Plant Conservation required having "At least 75% of threatened plant species in *ex situ* collections, preferably in the country of origin, and at least 20% available for recovery and restoration programs". The Red Book of Vascular Plants of Romania mentions that among the 3795 existing vascular plants taxa, 548 (14.4%) are endangered (Dihoru and Negrean, 2009). The number of the endangered plant taxa preserved *in vitro* in Romania is only 52 (less than 1.4%) (Cristea *et al*., 2006; Păunescu, 2009; Holobiuc *et al*., 2009; Cristea 2010, Cristea *et al*., 2010). Lately, in this context, an increasing importance is given to *in vitro* conservation of endemic and/or endangered species all over the world (Papafotiou and Stragas, 2009; Radojević *et al*., 2010; Gorgorov *et al*., 2011; Kumaria *et al.*, 2012, etc) including Romania (Holobiuc *et al*., 2009; Cristea *et al*., 2010; Jarda *et. al*, 2011; Coste *et al*., 2012).

D. spiculifolius Schur (*Caryophyllaceae*) is a phytogeographically and sozologically important species. It is endemic for the Eastern Carpathian Mountains (Tutin, 1964; Ciocârlan, 2009) and in Romania it is considered as Rare (Oltean *et al*., 1994; IUCN, 1997), Vulnerable (Dihoru and Dihoru, 1994; Sârbu et al., 2003) or Endangered (Sârbu *et al*., 2007). It is a caespitose perennial species, 10-30 cm height, with white, rarely pink scented flowers, that appear in June-August. This species is growing in the mountain and alpine belt, on skeletal, calcareous rocks (Ciocârlan, 2009). Because of its caespitose aspect, nice and scented flowers this species has ornamental importance as well.

There are some studies on *D. spiculifolius* that refer to *in vitro* introduction and multiplication (Zăpârţan, 1995; Butiuc-Keul *et al*., 2001; Cristea *et al*., 2002; Pop and Pamfil, 2011) but, some data are unclear or contradictory and do not permit replication of them. When initiating an *in vitro* collection it is very important to introduce and maintain *in vitro* more individuals from different populations, to avoid the genetic uniformity. There are no previous studies about the reaction of different individuals from natural populations to *in vitro* culture.

The aims of this study were to establish an optimal and high-frequency multiple micropropagation protocol, delineating more stages of *in vitro* culture, outdoor acclimatization of individuals from different populations of *D. spiculifolius*.

Materials and methods

Plant material and culture medium

The plant material originates from two distinct and spatially separated in situ *D. spiculifolius* populations: population 1 (encoded Ds 1) with 3 individuals (Ds 1.1; Ds 1.2; Ds 1.3), from ROSCI0027 Natura 2000 site, Hăşmaş Mountains (Romanian

Oriental Carpathian) and population 2 (Ds 2), with 3 individuals (Ds 2.1; Ds 2.2; Ds 2.3), from ROSCI0002 Natura 2000 site, Vlădeasa Mountains (Romanian Occidental Carpathian). All the collected individuals were planted in a special rocky area in the Alexandru Borza Botanical Garden from Cluj-Napoca, dedicated to the *ex situ* outdoor collection of endemic/endangered *Dianthus* taxa from Romania (Cristea *et. al*., 2013). *in vitro* culture was induced from different explants prelevated from these individuals.

Culture media containing MS macro - and microelements and FeEDTA (Murashige and Skoog, 1962), supplemented with thiamine 2.96 μM, pyridoxine HCl 4.86 μM, nicotinic acid 8.12 μM, myo-inozitol 0.55 mM, sucrose 2% and agar 0.7% [w/v] were used. Four different variants of culture media were studied: V1 - initiation medium (with 6-benzyladenine (BA) 4.44 μM and α-naphtaleneacetic acid (NAA) 5.37 μM); V2 - multiplication medium (with BA 4.44 μM and NAA 0.54 μM) and 2 rhizogenesis media: V3 - with reduced PGRs (plant growth regulators) concentration (BA 0.44 μM and NAA 0.05 μM) and V4 without PGRs.

Culture establishment and stabilization

In vitro culture was initiated from explants consisting of apices with 2-3 nodes from young shoots. We used the following variants for sterilization: (a) 20% Domestos (Unilever, United Kingdom) for 10 min; (b) 0.2% mercuric chloride for 10 min and (c) 10% Domestos for 5 min plus 0.2% mercuric chloride for 5 min. Then, the explants were washed stirring well, 5 times with sterile water. The explants were inoculated on V1 medium. All *in vitro* cultures were grown at 22 ± 2°C, under 16 h photoperiod (cool-white fluorescent lights, 50 μmol × s^{-1} × m^{-2} PPFD). The sterilization and survival index and the multiplication of the explants were monitored after 30 days from inoculation. Then, the entire cluster of shoots formed from an explant was transferred on fresh medium V2, two times, at 30 days. After a period of 90 days, the following parameters were scored: the multiplication rate, the length of shoots and the number of internodes. To study the variability during *in vitro* culture, the length variation of the 17 new shoots generated by a single individual (e.g., Ds 1.3) was also recorded.

Stable culture multiplication

For an optimal multiplication and a proper maintenance of a living collection we studied 3 different methods of multiplication, comparatively, using 3 different types of inocula, cultivated in Steri Vent culture containers (Duchefa Biochemicals, Haarlem, The Netherlands): a) binodal apices; b) uninodal fragments, from 2nd and 3rd nodes; c) 5 to 7 nodes fragment (under the apex), horizontally positioned. V2 culture media were used. The number of shoots generated/explant during a 60 days subculture was recorded.

In vitro rhizogenesis and acclimatization

Binodal apices from stable culture were inoculated on V3 and V4 culture media to induce or to improve the roots generation. At the same time, other explants were *in vitro* cultured in sterile wet sand or perlite instead of agar-containing medium. After 60 days from inoculation the plantlets were quantified and then transferred for ex vitro acclimatization on 2 types of substrate: perlite and perlite + sterile soil (1/1). The plantlets were soaked with a MS 1/2 macroelements solution (Murashige and Skoog, 1962) and maintained under high humidity the first week. Then, the atmospheric humidity was reduced progressively. After 3 weeks the plants were transferred in soil, in the greenhouse and then were weaned in shelter for 1 month. In the spring the plants were transferred on a special rocky area created for the vitroplants collection of the Alexandru Borza Botanical Garden from Cluj-Napoca.

Statistical analysis

The *in vitro* experimental design consisted of 12 replicates per each individual and the experiments were repeated twice. The results were expressed as the average of replicates ± standard deviation (SD). Data were subjected to One-Way ANOVA test (for testing 3 or more columns) or to the t tests (for testing 2 columns), with a 95% confidence intervals. In case of significant differences following the ANOVA test, Tukey`s test was used in order to determine the significance of the differences between the average values at $P < 0.05$ [$P < 0.001$ = extremely significant (***); P 0.001 to 0.01 = very significant (**); P 0.01 to 0.05 = significant (*); $P \geq 0.05$ = no statistical significance (ns)]. The statistical analysis was performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego, USA).

Results

Culture establishment and stabilization

The best results regarding the explants sterilization were obtained using the $HgCl_2$: 88% sterile inocula for (b) sterilization variant and 82% for variant (c). The viability index after sterilization can be considered as high as: 96% for (c) sterilization variant and 100% for variant (b). After 30 days from inoculation, the explants generated an average of 4 new shoots, with or without roots (Table 1 and Fig. 1). During the second and third subculture the proliferation increased, up to 74.3 shoots/initial explant, after 90 days. The results for the length of the generated shoots by a single individual (e.g., Ds 1.3) are presented in Fig. 2. It was observed that the length of shoots ranged between 9.3 cm and 40.0 cm, the average length being 21.3 cm.

Table 1.

D. spiculifolius explants evolution during the initiation stage (at 30 days of culture) and the stabilization stage (after another 60 days); on V1 culture medium supplemented with BA 4.44 μM and NAA 5.37 μM.

D. spiculifolius population:	Ds 1	Ds 2
Average no. of new shoots/explant after 30 days	4.8 ± 1.6 ^{ns}	3.2 ± 1.8 ^{ns}
Average no. of new shoots/explant after 90 days	25.9 ± 5.1 ^{ns}	74.3 ± 11.1 ^{ns}
Average length of shoots after 90 days (cm)	12.3 ± 3.0 ^{ns}	9.5 ± 3.3 ^{ns}
Average no. of internodes/shoot after 90 days	9.7 ± 2.1 *	8.9 ± 2.5 *

The results are presented as the mean ± SD.

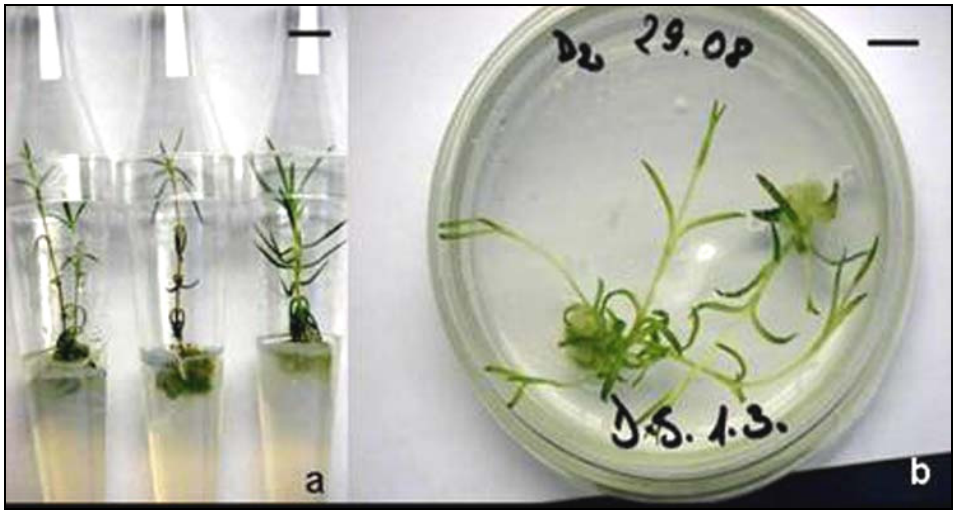

Figure 1. *D. spiculifolius in vitro* culture generated by binodal apices sampled from young shoot, in a: DeVit tubes (Duchefa Biochemicals, Neetherland) and b: Petri dishes. Culture on V1 medium, supplemented with BA 4.44 μM and NAA 5.37 μM. Bars represent 1 cm.

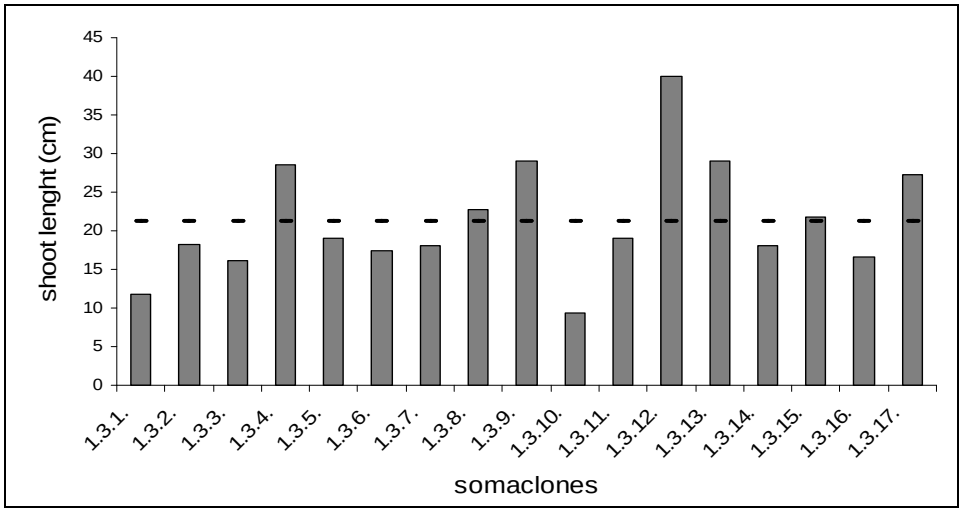

Figure 2. Variation of shoots length to the somaclones of Ds 1.3 individual, on V1 culture medium supplemented with BA 4.44 μM and NAA 5.37 μM. The dashed line represents the average value (21.3 cm) of the somaclones' length. ANOVA test: ns

Stable culture multiplication

After 60 days of culture on medium V2, the multiplication rate obtained for binodal apices and uninodal fragment is comparable (Table 2). The better results obtained for plurinodal horizontal inocula can be explained by the large number (5-7) of nodes/explant. Population Ds 2 shows a higher multiplication rate compared with Ds 1 for all the 3 types of explants: 2.6 times higher multiplication rate at binodal apices, 4.6 times higher multiplication at the uninodal fragments and 4 times higher multiplication at the multinodes fragments horizontally cultured. Different stage of *in vitro* culture of binodal apices and of 5-7 nodes horizontally cultured inocula are presented in Fig. 3.

Tabel 2.

Influence of explant type on the number of regenerated shoots, during the stable culture stage, at different individuals of the two *D. spiculifolius* populations, on V2 medium, supplemented with BA 4.44 μM and NAA 0.54 μM; results after 60 days of culture.

Population	Individuals	No. of regenerated shoots/explant		
		bA	uE	5-7n
Ds 1	Ds 1.1.	34.8 ± 4.3	23.1 ± 5.8	43.8 ± 6.3
	Ds 1.2.	41.7 ± 7.1	24.9 ± 4.4	69.5 ± 8.4
	Ds 1.3.	9.8 ± 1.3	4.9 ± 1.4	34.0 ± 3.6
average Ds 1		28.8 **	17.6 **	49.1 **

Population	Individuals	No. of regenerated shoots/explant		
		bA	uE	5-7n
Ds 2	Ds 2.1.	60.4 ± 5.3	57.1 ± 16.4	112.6 ± 20.9
	Ds 2.2.	98.7 ± 14.3	104.8 ± 15.3	345.8 ± 45.6
	Ds 2.3.	65.8 ± 18.4	78.9 ± 11.8	139 ± 26.8
average Ds 2		74.9 **	80.3 **	199.2 **

bA = binodal apices explant. uE = uninodal explant. 5-7n = 5 - 7 nodes explant horizontally inoculated. Data are presented as the mean ± SD. ANOVA test: between the 3 type of explant ns; t test: interpopulational variability: Ds 1 vs Ds 2 **; intrapopulational variability: Ds 1.1 vs Ds 1.2 and Ds 1.3 ns; Ds 1.2 vs Ds 1.3 *; between Ds 2.1, 2.2 and 2.3 ns.

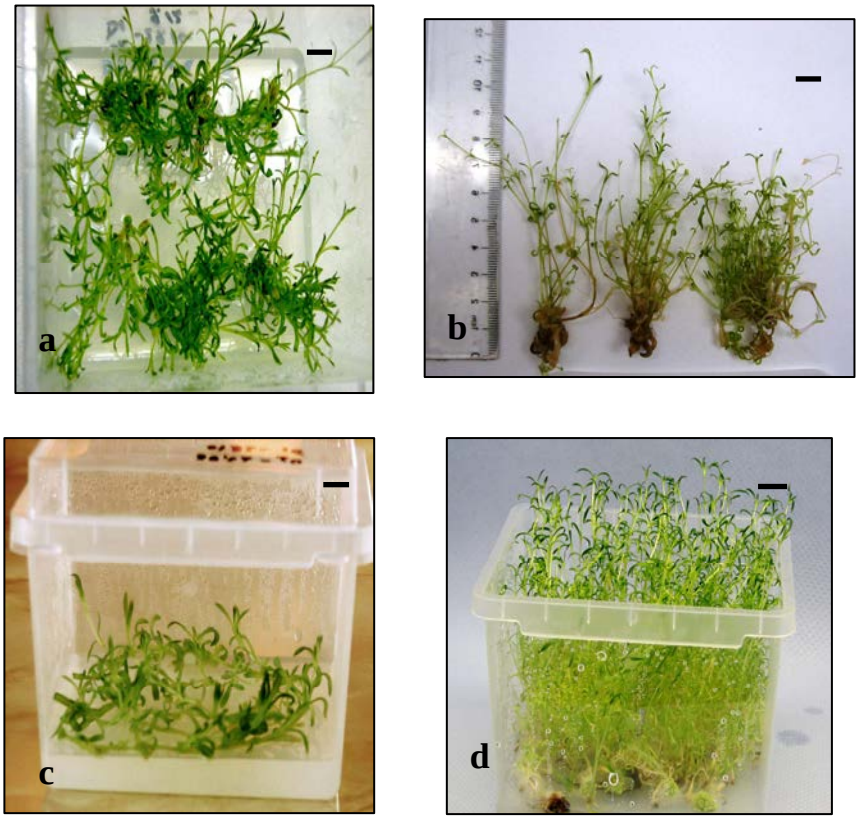

Figure 3. *D. spiculifolius* stable culture multiplication, on V2 medium supplemented with BA 4.44 µM and NAA 0.54 µM. a, b: binodal apices, of individual Ds 1.3, 60 days of culture; c, d: 5-7 nodes horizontally cultured inocula of individual Ds 1.2; c: 10 days; d: 60 days of culture. Bars represent 1 cm.

Rhizogenesis and acclimatization

The main characteristic of *D. spiculifolius* was the weak influence of culture medium composition on its growth parameters (Tables 3). There are no significant differences between the results obtained on V3 - with PGRs - and V4 - without PGRs - culture media. The number of roots/explant (between 4.7 and 15.8), their length (between 1.3 and 5 cm) and their vigor recommending them as suitable for transplanting regenerated plants in *ex situ* conditions. The vitroplants acclimatized on perlite (Fig. 4 a) had the best root system development, comparative to that developed in perlite+sterile soil (1/1). After 3 weeks of ex vitro culture in the laboratory, the root system was well developed. The efficiency of ex vitro survival and development was increased from about 50% to about 70% when the rhizogenesis was induced *in vitro* in sand or in perlite instead of agar-containing culture medium. After the green house and shelter acclimatization (Fig. 4 b), the plants were cultivated (Fig. 4 c) on a second special rocky area dedicated to different micropropagated species (Fig. 4 d).

Table 3.

Influence of the culture media on the rhizogenesis of *D. spiculifolius* binodal apices development. V3 = BA 0.44 μM and NAA 0.05 μM; V4 = without PGRs; after 60 days of culture.

Culture media	V3			V4		
Population individual	LS	NoR	LR	LS	NoR	LR
Ds 1.1.	8.8 ± 0.6	8.1 ± 0.4	3.1 ± 0.2	11.7 ± 0.3	8.2 ± 0.1	4.1 ± 0.1
Ds 1.2.	15.0 ± 0.1	15.8 ± 0.7	5.0 ± 0.2	15.1 ± 0.3	15.7 ± 0.6	4.6 ± 0.2
Ds 1.3.	11.8 ± 0.4	6.2 ± 0.3	2.8 ± 0.1	11.8 ± 0.3	6.3 ± 0.2	2.8 ± 0.1
average Ds 1	11.9 ^{ns}	10.0 ^{ns}	3.6 ^{ns}	12.9 ^{ns}	10.1 ^{ns}	3.8 ^{ns}
Ds 2.1.	11.2 ± 0.4	5.6 ± 0.4	1.7 ± 0.4	10.0 ± 0.1	5.9 ± 0.1	2.4 ± 0.3
Ds 2.2.	10.1 ± 0.9	4.7 ± 0.1	1.3 ± 0.1	10.5 ± 0.2	5.0 ± 0.3	2.8 ± 0.2
Ds 2.3.	12.3 ± 0.1	4.7 ± 0.4	1.5 ± 0.2	11.1 ± 1.0	5.0 ± 0.2	2.8 ± 0.1
average Ds 2	11.2 ^{ns}	5 ^{ns}	1.5 ^{ns}	10.8 ^{ns}	5.3 ^{ns}	2.7 ^{ns}

LS = length of shoots/explant (cm). NoR = no. of roots/explant. LR = length of roots/explant (cm). Data are presented as the mean ± SD. t test: V3 culture medium vs V4 ns; Anova test: intrapopulational variability: only individual 1.2 vs 1.3 *; between the other individuals there are not significantly differences.

Figure 4. a: *D. spiculifolius* acclimatization in perlite soaked with mineral nutrient solution before transplanting plants in soil. b: *D. spiculifolius* plants transferred in soil, after green house and shelter acclimatization; c: *D. spiculifolius* plants obtained by *in vitro* culture, after their outdoor transfer; d: special rocky area of Alexandru Borza Botanical Garden, dedicated to different endemic/endangered micropropagated species, at the time of planting. Bars represent 1 cm.

Discussion

Culture establishment and stabilization

The results regarding *in vitro* culture initiation of D. *spiculifolius* are better, when compared to that obtained for other endemic/endangered Romanian *Dianthus* species (Miclăuş *et al.*, 2003; Cristea *et al.*, 2009; Holobiuc *et al.*, 2009). They can be explained by the lower infestation degree of the explants and by using combinations of different disinfectant agents. Very good results for *in vitro* culture initiation are expected when seeds are used (Cristea *et al.*, 2010).

Considering the multiplication rate during the initiation stage, comparative results were obtain in the case of other Romanian endemic and/or endangered *Dianthus* species: *D. petraeus* ssp. *simonkaianus* (Miclăuş *et al*., 2003), *D. glacialis* ssp. *gelidus* (Cristea *et al*., 2006), *D. henteri* (Cristea *et al*., 2010), *D. giganteus* ssp. *banaticus* (Cristea *et al*., 2006; Jarda *et al*., 2011) and also for other sozologically important European *Dianthus* species, like *D. giganteus* ssp. *croaticus* (Prolic *et al*., 2002), *D. gratianopolitanus* (Fraga *et al*., 2004), *D. pyrenaicus* (Marcu *et al*., 2006), *D. giganteus*, *D. alpinus*, *D. ferrugineus* and *D. gallicus* (Cristea *et al*., 2006). Statistical analysis reveals that in this stage the number of shoots/explants and their length, after 90 days of culture, show no significant differences between the 2 populations (Table 1). However, the number of internodes/shoot reveals differences between the two populations. The average length of internodes was about 1 cm, as well as the initial vegetative shoot fragments used as explants source for *in vitro* culture establishment. This is important because, unlike other species, *in vitro* culture of *D. spiculifolius* does not cause significant morphological differences compared to in situ plants. More or less similar results regarding the regeneration capacity of this species were reported by some authors, but they didn't specify the type of inoculum (nodal or apical, position of nodes on the stem), the number of nodes of the inoculum, or the period of culture (Holobiuc *et al*., 2004-2005; Pop and Pamfil 2011).

This species show a large variability regarding the length of the somaclones generated by one individual (Ds 1.3), as can be observed in Fig. 2. To date, there are no results regarding comparative studies about *in vitro* reaction of more populations or more individuals.

Stable culture multiplication

Analyzing the data presented in Table 2, we conclude that, in this stage, significant differences appear between the responses of the 2 populations. Likewise, there were also differences between the different individuals of the same population. All the 3 explants types have provided a satisfactory multiplication, however rooting was often poor (Fig. 3). Comparing the 3 variants, the advantages and limitations of each one of them emerge: (i) the binodal apices are uniform explants recommended to be used in experiments to test different culture media and growing conditions; (ii) the uninodal fragments offer the possibility to highlight potential somaclonal variation that is phenotypically expressed, as a consequence of sensitivity differences to cytokine; (iii) plurinodal fragments horizontally cultured offer practical advantages, being easy to handle; at each node, a number of shoots occur that are easy to separate and simultaneously develop adventives roots, enabling early transplanting in soil for acclimatization. Previous studies performed on the *in vitro* multiplication of those species, after more transfers, revealed more or less similar results: a multiplication rate of 30-40 new shoots/explant, after 45-115 days of culture, on a medium with 1/10 auxins/cytokinins ratio (Cristea *et al*., 2002; Holobiuc *et al*., 2004-2005).

Rhizogenesis and acclimatization

On both studied culture media the root formation was appropriate for acclimatization. The obtained results lead to the idea that there is no need of PGRs for rhizogenesis induction in case of this species. This is very important, knowing the fact that PGRs are one of the factors that can induce somaclonal variability (Bairu et al. 2011). Even if some authors (Pop and Pamfil, 2011) have obtained only random adventitious roots for the same PGRs and the same PGRs balance, in our experiments, the rhizogenesis was medium or appropriate, not only on the media without PGRs, but even if the PGRs balance was in the detriment of auxins. For other endemic and/or endangered *Dianthus* species (Kovác, 1995; Miclăuş *et al*., 2003; Marcu *et al*., 2006; Jarda *et al*., 2011) the number of *in vitro* generated roots was similar to that reported in the present paper. The outdoor existence of acclimated vitroplants in a botanical garden is even more important because none of the previous studies on this species was followed by outdoor acclimatization after *in vitro* culture.

Conclusions

A high frequency multiple micropropagation protocol having several stages was obtained for endangered and endemic plant species *Dianthus spiculifolius*. Both multiplication and rhizogenesis were optimal on culture media studied. Vitroplants can be easily acclimatized and grown in outdoors conditions. All three types of explamts could be used for preservation depending on the aim of experiments. There are no significant differences between the reactions of the individuals belonging to the two populations in the initiating stage of *in vitro* culture, but significant differences were observed in the stage of stable culture multiplication. The individual belonging to Vlădeasa Mt. population showed a rate of multiplication of 2.6-4 times higher than the individual from Hăşmaş population. The are no significant differences regarding the reaction to *in vitro* culture of the individuals belonging to the same population.

Acknowledgements. This study has been partially supported by 31-008/2007-PNCDI2-Parteneriate-CNMP project from the Romanian Ministry of Education and Research. The authors are also thankful to Dr. Gheorghe Coldea and Tudor Ursu for their support in collecting the plant material from in situ and to Delia Marcu for her support in processing the statistical data.

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