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In vitro Preservation of Three Species of *Dianthus* from Romania

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Abstract. Nowadays, the plant biodiversity is threatened and its conservation represents a priority at the international level. Conservation strategies are based on the management of wild populations in nature, but they require additional methods of *ex situ* conservation which helps preserve plants. The experiments in this article focused on testing the *in vitro* reactivity and adventitious rooting of three rare species of *Dianthus* from Romania (*Dianthus giganteus banaticus*, *Dianthus spiculifolius* and *Dianthus henteri*), which are part of the Red List of Romania's Flora. The *in vitro* cultures were initiated from stem fragments and seeds belonging to the *Dianthus* species, which were sterilized using different sterilizing agents. Seeds were cultivated on a simple MS with IBA and BAP. In the present study, five different culture media, supplemented with auxin and cytokinin, were tested for the explants, to observe the *in vitro* behavior of *Dianthus* species in terms of proliferation and rooting. The contamination and germination rates were determinated. The best germination rate was achieved by *D. giganteus banaticus*. The number of shoots and roots, the length of shoots, initiated for explants varied according to the concentration of growth regulator in the medium. The plants had a good *in vitro* behavior, but the adventitious rooting was weak, which represents a limiting factor in the acclimatization of plants. Plant micropropagation and conservation may also contribute to maintain natural populations through reintroducing the preserved material in the original habitat.

Keywords: *in vitro* propagation, *Dianthus giganteus banaticus*, *Dianthus spiculifolius*, *Dianthus henteri*, adventitious rooting

INTRODUCTION

The number of species threatened currently with extinction exceeds the available resources for conservation and the situation seems to worsen rapidly (Ehrlich, 1994; Myers, 1996; Pimm *et al*., 1995, Wilson 1992). The current status of Earth is characterized by an unprecedented overexploitation of natural resources and an associated decrease of global biodiversity (Foley *et al*., 2005). The main causes of diversity loss are climate changes and changing the purpose of land use, nitrogen deposition, biotic exchange and increased concentration of carbon dioxide, resulting in habitat modification (Foley *et al.*, 2005, Laurance *et al.*, 2001, Sala *et al.*, 2000). Ecological restoration involves assisting the recovery of an ecosystem that has been degraded, damaged or destroyed, usually as a result of human activities (Sala *et al.*, 2000). Restoration actions are being implemented worldwide (Clewell and Aronson, 2007), supported by global policy commitments, such as the Convention on Biological Diversity.

For the successful micropropagation of rare, endemic and endangered species, the plant material used to initiate *in vitro* cultures plays the most important role (Sarasan *et al.*, 2006). The individuals used for initiating *in vitro* cultures should have well-established origin and a well preserved genetic variability in the population (Fay, 1992). The germplasm of threatened plant species is nowadays conserved through the help of significant advances

achieved in biotechnology. The new developed techniques offer new options and facilitate preservation of *in vitro* cultures, seeds, pollen, embryos etc. The advantages of *in vitro* techniques and storage methods consist in enabling the establishment of extensive collection using minimum space. These collections allow continuous supply of valuable material, wild population recovery, molecular investigations, ecological studies or economic uses.

Dianthus is a genus of 300 species of flowering plants in the *Caryophyllaceae* family and it has a lot of rare, endemic or endangered species, some of them present in the Romanian territory.

MATERIALS AND METHODS

The biological material used for this experiment was represented by three rare species of *Dianthus* listed in the Red List of Romania's Flora (Oprea, 2005): *Dianthus spiculifolius*, *Dianthus giganteus banaticus*, *Dianthus henteri*. The aim of the study was represented by the *ex situ* conservation of *Dianthus* species, using *in vitro* methods involving collecting the plant material, aseptic tissue culture initiation, testing the *in vitro* reactivity of plants and the development of protocols for plant conservation.

In order to collect plant material for *ex situ* conservation, sites of Natura 2000 network (http://www.natura2000.ro/) were selected, where the species of *Dianthus* could be found. Plants of *D. spiculifolius* (Fig. 1) were located in Cheile Turzii Natural Reservation (Cluj county) using GPS and for each plant the location, the morphological characterization and plant associations were registered. Viable plants were transferred and acclimatized in the Agrobotanical Garden of USAMV Cluj-Napoca for the establishment of *ex situ* collections. *D. henteri* and *D. giganteus banaticus* were purchased with support from the Agrobotanical Garden of U.S.A.M.V. Cluj-Napoca (http://agricultura.usamvcj.ro/structura/gradina-botanica/gradina-agrobotanica/view), both whole plants and seeds.

Firstly, for *in vitro* culture initiation, stem fragments with a single node and growing tips were detached and sterilized. Secondly, seeds belonging to these species were collected (Fig. 2) and were used to initiate *in vitro* cultures.

Fig. 1. *Dianthus spiculifolius* plants from Cheile Turzii natural reservation

Fig. 2. Harvested seeds a) *Dianthus henteri;* b) *Dianthus spiculifolius;* c) *Dianthus giganteus banaticus*

The sterilization procedures of the plant material initially consisted in washing the explants with running tap water for two hours, according to data taken from literature, then immersing them in ethyl alcohol 70^0 for 30 seconds, followed by disinfection. Several

different methods of sterilizing agents have been used to fight against the contamination of the *in vitro* cultures. Tab. 1 presents different sterilizing agents for *Dianthus* explants, their concentrations and action time. Regardless of the method of sterilization used to sterilize the explants, the procedure was followed by three washes with sterile distilled water using sterile containers and tools in a laminar air flow hood.

Tab. 1

Sterilizing agents used for sterilizing explants of *Dianthus*

No.	Sterilizing agent	Concentration	Time (min)	Note
1.	mercuric chloride	0,10%	7	Se1
2.	sodium dichloroisocyanurate (SDIC)	1%	7	Se2
3.	Domestos	2,40%	5	Se3
	mercuric chloride	1%	5	
4.	mercuric chloride	0,10%	2x5	Se4

Different sterilization procedures were also used for the seeds of *Dianthus*. Firstly, the seeds were stored in gauze bags and were washed for 2 hours in running tap water and then were cleaned with the sterilizing agents shown in Tab. 2. Finally, *Dianthus* seeds were washed three times in sterile distilled water in abundance, using sterile containers and tools in a laminar air flow hood.

Tab. 2

Sterilizing agents used for sterilizing seeds of *Dianthus*

No.	Sterilizing agent	Concentration	Time (min)	Note
1.	mercuric chloride	0,10%	2 x 5	Ss1
2.	sodium dichloroisocyanurate (SDIC)	1,0%	5	Ss2
3.	Domestos	2,40%	10	Ss3

Tab. 3

Murashige-Skoog (1962) culture media

	Ingredients	Concentration in normal solution
Macroelements mg/l	NH_4NO_3	1 650
	KNO_3	1 900
	$CaCl_2$	440
	$MgSO_4$	370
	KH_2PO_4	170
Microelements mg/l	H_3BO_3	6,2
	$MnSO_4 . 4 H_2O$	22,3
	$ZnSO_4 . 7 H_2O$	8,6
	$Na_2MoO_4 . 2 H_2O$	0,25
	$CuSO_4 . 5 H_2O$	0,025
	$CoCl_2 . 6 H_2O$	0,025
	KI	0,83
Vitamins µg/l	Nicotinic acid	0,5
	Pyridoxine HCl	0,5
	Thiamine HCl	0,1
	Mezoinozytol	100

The success of *in vitro* propagation is largely influenced by the nutritional composition of the media, which obviously must correspond to the vital needs of the tissue in order to compensate for endogenous factors present in different proportions in the tissue cells (Casas *et al.*, 2010). For *in vitro* plant regeneration of *Dianthus*, several types of culture media were tested, with macro and microelements, according to MS formula (Murashige and Skoog, 1962), presented in Tab. 3, supplemented with vitamins. pH was adjusted to 5.8. Sucrose (30g/l) was added as carbon source and the culture media was solidified with 8 g/l agar (Duchefa Biochemie BV, http://www.duchefa.com/). Media used for *in vitro* germination of *Dianthus* seeds was MS supplemented with IBA 0.5 mg/l and BAP 1 mg/l. Five different culture media were used based on simple MS for apical and nodal explants for *in vitro* regeneration. These media variants are shown in Fig. 3, having different ratios between auxin and cytokinin (MSV1-MSV4), where MS media was used as control (MSV5).

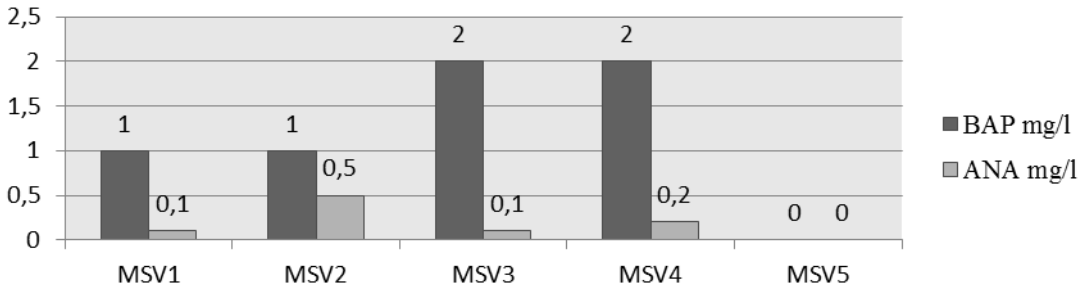

Fig. 3. Media variants used for *in vitro* culture initiated from explants
Note: BAP=6-Benzylaminopurine, NAA=1-naphthaleneacetic acid

The inoculation of the *in vitro* cultures was performed in laminar flow hood (Whitfield, 1966), in total aseptic conditions to avoid contamination. Previously sterilized seeds were inoculated two/tube/ten repetitions for each of the three species of *Dianthus*. When initiating cultures from explants, three nodal fragments/recipient/ten repetitions/species for each culture media variant were inoculated. To assess the effectiveness of sterilization procedures, the contamination rate was determined (number of explants or seeds infected/total number of inoculumx100) at the end of the germination process.

The *in vitro* cultures obtained from uninodal fragments of stem were kept in the growth chamber at a temperature of 25^0C, an illumination of 2000 lux and 16 hours light/8 hours dark (Cristea *et al.*, 2010). The *Dianthus* seeds were kept in the darkness at the same temperature until the germination process was activated. At the end of the first passage of 30 days and after 120 days, the number of new-born shoots on the initial inoculum and the average length of shoots were calculated. In addition to these strategies to quantify the results, observations about the degree of *in vitro* rooting were also made.

RESULTS AND DISCUSSION

In the case of the studied *Dianthus* species, the sterilization of explants detached from the mature plants was difficult to perform because of the high degree of contamination with fungus (Leifert and Cassells, 2001), which proved to be resistant to the tested sterilizing agents. The first contamination occurred at the beginning of the seventh day, with rates between 20-30 %, reaching 35.2 to 64.7% during the first month of culture. In figure 4 a) we can see that the lowest rate of culture contamination was recorded for sterilizing agent Se3, represented by mercuric chloride and Domestos and the highest rate for sodium

dichloroisocyanurate (Se2). In terms of initiating tissue cultures, the problem of contamination is an important step to overcome (Holobiuc *et al.*, 2009; Holobiuc *et al.*, 2010), it can cause loss of valuable biological resources and lead to high economic losses (Mirjalili *et al.*, 2005). It is therefore necessary to improve sterilization methods and test new reagents, which could provide a lower degree of contamination. The use of complex methods of sterilization, prolonging the sterilization treatment (Sivasithamparam *et al.*, 2002) allowed us to obtain viable and sterile inoculus for most individuals belonging to three species of *Dianthus*.

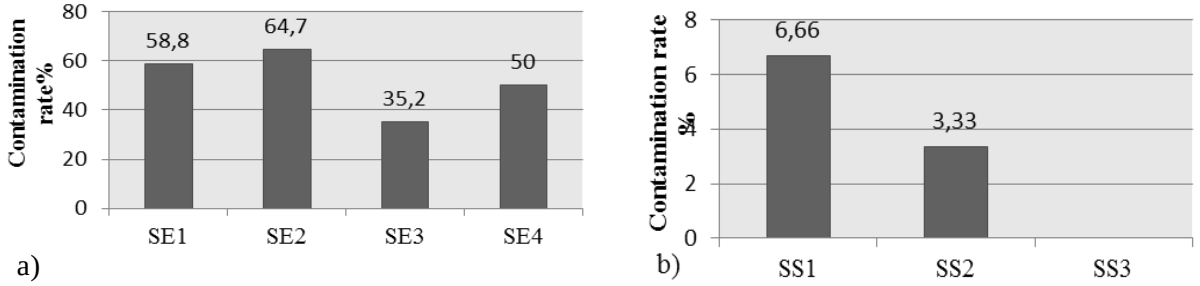

Fig. 4. a) Contamination rate of *Dianthus* explants depending on the sterilizing method b) Contamination rate of *Dianthus* seeds depending on the sterilizing method

Seed sterilization efficiency was better than explants sterilization and the degree of contamination was considerably lower, ranging between 0% and 6.66% (Fig. 4 b). In the case of the sterilizing commercial agent Domestos (http://www.snell.co.nz/images/pdf/Domestos%20Sheet.pdf), the degree of contamination was 0% and the worst results were obtained when mercuric chloride was used. Following the results, we conclude that *in vitro* cultures initiated from seeds of *Dianthus* have been protected from contaminants, this method is more appropriate to use for the initiation of aseptic cultures (Cristea *et al.*, 2006; Holobiuc *et al.*, 2004-2005). Tubes with *Dianthus* seeds were kept in the dark until germination was activated and then were transferred in the growth chamber under the following conditions: light intensity of 30µmol/m^2/sec, temperature of 25±1^{0}C, 16 hours light/8 hours dark. The first seeds germinated after 72 hours and belonged to *D. giganteus banaticus* (Fig. 5 a I), followed by *D. spiculifolius* and *D. henteri* (Fig. 5 a II) five days after inoculation. At the end of the 30 days interval the rate of seed germination was determinated. The best results were obtained for *D. giganteus banaticus* with a germination rate of 83.33%. The other species had lower germination values, 70.83% for *D. spiculifolius*, respectively 52.5% for *D. henteri* (Fig. 5 b).

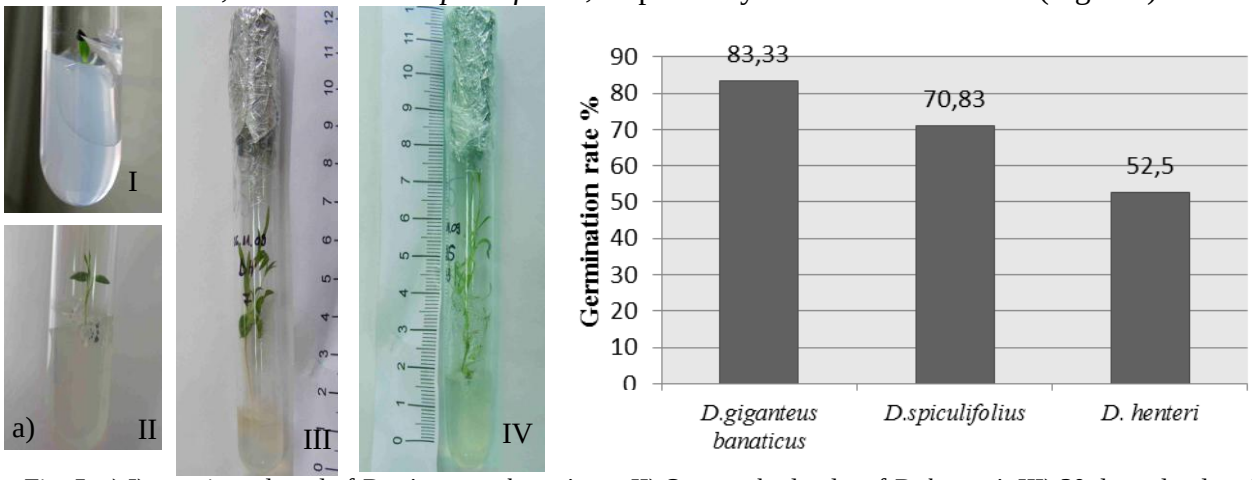

Fig. 5. a) I) germinated seed of *D. giganteus banaticus;* II) One week plantlet of *D. henteri;* III) 30 days plantlet of *D. henteri* ; IV) 30 days plantlet of *D. spiculifolius;* b) Germination rate of *Dianthus* seeds

Dianthus species had a pretty good *in vitro* reactivity, the way of regenerating explants was direct morphogenesis (Holobiuc *et al.*, 2010). There were differences in the *in vitro* behavior of cultures regarding the degree of regeneration, growth of shoots, rooting and vitrification, depending on the culture media used. The best results of regeneration rate were obtained on MSV1 culture media, which had an auxin/cytokinin ratio of 1/10 for all *Dianthus* species (Fig. 6). Among the analyzed species, *D. giganteus banaticus* recorded the highest regeneration on MSV1 after 30 days, with an average of 12 shoots/explant. *D. spiculifolius* recorded a lower number of shoots, 7.8, and *D. henteri* had only 6.7 shoots/inoculum after 30 days. Comparing the results on MSV1 (1 mg/l BAP, 0.1 mg/l NAA) and MSV4 (2 mg/l BAP and 0.2 mg/l NAA), which had the same auxin/cytokinin ratio, the differences between the three species were maintained on both media, the average values on MSV4 were slightly lower than on MSV5 (Fig. 6 a). On both media, the adventitious rooting was weak and callus was formed at the base of explants (Tab. 4). Cytokinin dominance favored the formation of shoots, a process that originates in the lateral meristems of explants.

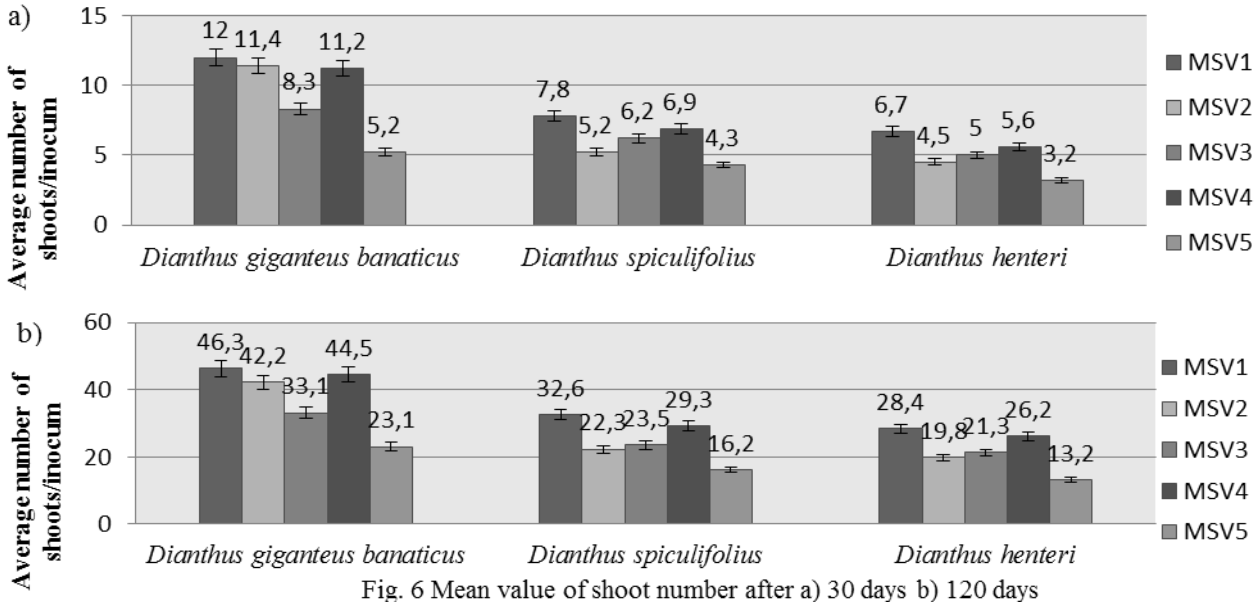

Fig. 6 Mean value of shoot number after a) 30 days b) 120 days

Tab. 4

Rooting observations depending on the media used

D. giganteus banaticus	MSV1	Random adventitious roots, callus
	MSV2	Adventitious roots at 40% of the plants
	MSV3	No roots
	MSV4	Random adventitious roots, callus
	MSV5	No roots
D. spiculifolius	MSV1	Random adventitious roots, callus
	MSV2	Adventitious roots at 30% of the plants
	MSV3	Few roots
	MSV4	Random adventitious roots, callus
	MSV5	No roots
D. henteri	MSV1	Random adventitious roots, callus
	MSV2	Adventitious roots at 20% of the plants
	MSV3	No roots
	MSV4	Random adventitious roots, callus
	MSV5	No roots

The efficiency to form new shoots on MSV4 (1/10 auxin/cytokinin) and MSV3 (1/20 auxin/cytokinin) was comparable at *D. spiculifolius* and *D. henteri*, while at *D.giganteus banaticus* the 1/20 ratio had a negative effect on the formation of new shoots, with an average of 8.3 shoots/explant. Decreasing the ratio from 1/10 to 1/20 was benefic for the species and no callus was formed. It was found that increasing the concentration of auxins (auxin/cytokinin increased to ½) in MSV2 did not affect the efficiency of forming new shoots, only slightly at *D. gigantheus banaticus*, which had an average of 11.4 shoots/explant. A positive effect on adventitious root formation manifested only *D. spiculifolius*, *D. henteri* did not register any improvement. The results highlight the different sensitivity of the species to auxins and raise the problem of media adaptation to specific requirements of *Dianthus* for the rooting process (Cristea *et al.*, 2010). Conversely, the high efficiency of shoots formation regardless of the media, show that sensitivity to cytokinins and auxins (Ma, 2008) does not seem to be restrictive (Vadassery *et al.*, 2008). Of all media tested, the lowest proliferation rate was obtained on simple MS, the average number of shoots ranged from 3.2 for *D. henteri* to 5.2 for *D. giganteus banaticus* (Fig. 6 a).

Long *in vitro* passages (up to 120 days) did not significantly affect the intensity of proliferation, the number of new shoots after 120 days, compared with the first period of 30 days, increased proportional for all species, on all media (Fig. 6 b). *D. giganteus banaticus* had the highest rate of multiplication on MSV1. The results show that these *Dianthus* species support long *in vitro* culture passages of time and could be preserved as a living collection. After completing the 120-day interval, comparing the media with simple MS, they presented significant positive differences and *D. henteri* presented the lowest rate of proliferation.

The growth of shoots was slightly stimulated by higher concentrations of cytokinin (MSV3 and MSV1) and inhibited by the growth of auxin/cytokinin ratio to ½ (MSV2) (Fig. 7). Internode length of newly formed shoots was constant, around 1 cm, for the three species of *Dianthus* and the shoot length was affected mainly by the number of nodes. Experiments showed the number of shoots did not affect the number of internodes and shoot length, only over a certain density of shoots in culture vessel, which generated a competition for nutrients. The highest average values of shoot length were recorded on MSV3 media, which had a auxin/cytokinin ratio of 1/20, followed by MSV4 (1/10) and MSV1 (1/10). *D. henteri* had no significant differences in the length of shoots on MSV5 and MSV2, the two media registered similar results for *D. spiculifolius* (Fig. 8 a).

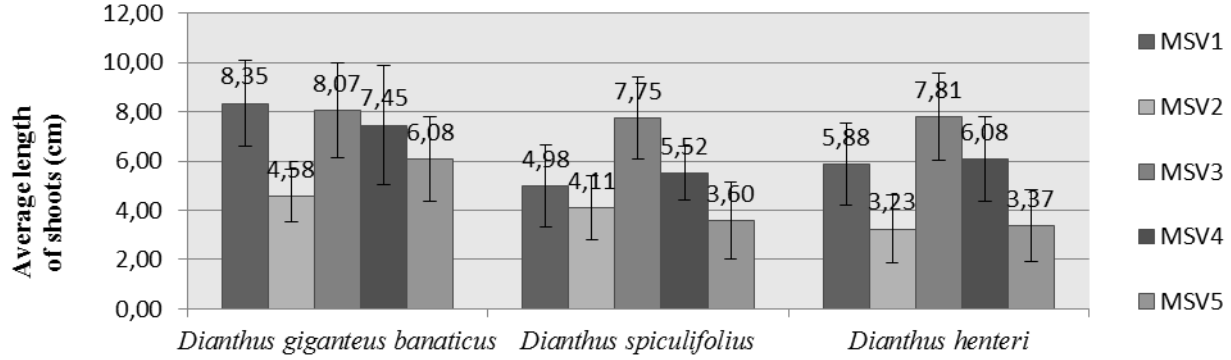

Fig. 7. Mean value of shoot lenght after 120 days

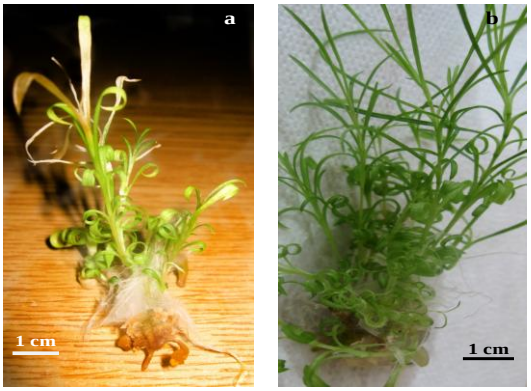

Fig. 8. Plants after 120 days *in vitro* culture; a) *Dianthus spiculifolius;* b) *Dianthus giganteus banaticus*

CONCLUSIONS

Although *in vitro* techniques for germplasm conservation are spreading, science needs to achieve notable progress in terms of the protocols tested to ensure more stable results. Effective protocols for propagation of endangered plants allow the supply of valuable material, as it was demonstrated by Cassells *et al.* (1999) and Choi *et al*. (2002). The value of plant biodiversity must be recognized and its conservation should be a priority focused particularly on the development of *in vitro* techniques, sterilization, rooting and acclimatization of the plant material. The successful rooting of explants and acclimatization of newly formed plantlets are fundamental for *in vitro* cultures of rare, endemic or endangered species. Rooting is considered a major problem of recalcitrant species, it is a difficult process and without it the viability of new plants is very low. Although *in vitro* rooting of micro cuttings is a common practice, this process faces a number of problems, in the present study most of explants failed to root, the transition zone between root and stem had an abnormal configuration and the vascular connections were poorly formed. The few roots formed did not present absorbing hairs and died shortly after the transplantation, leading to cessation of plant growth.

The constant effort to totally fight against the *in vitro* problems and the understanding of the genetic mechanisms that control the adventitious roots, a base component of *in vitro* rooting, could provide better results for the future and overcome the difficulties we face today.

REFERENCES

1. Casas, J.L., E. Olmos and A. Piqueras (2010). In Vitro Propagation of Carnation (*Dianthus caryophyllus* L.). Protocols For In Vitro Propagation Of Ornamental Plants. SM Jain and Ochatt SJ. 589**:**109-116.

2. Cassells, A, C. Walsh, M. Belin, M. Cambornac, J. Robin and C. Lubrano (1999). Establishment of a plantation from micropropagated *Arnica chamissonis* a pharmaceutical substitute for the endangered A. montana. Plant Cell, Tissue and Organ Culture 56(2):139-144.

3. Choi, Lee, Kim Han Jeong and Ko (2002). Mass production of Siberian ginseng plantlets through large-scale tank culture of somatic embryos. Plant Cell Reports 21(1):24-28.

4. Clewell, A.F. and J. Aronson (2007). Ecological Restoration:Principles, Values, and Structure of an Emerging Profession Washington, DC, Island Press.

5. Cristea, V., A. Brummer, L. Jarda and M. Miclăuș (2010). *In Vitro* Culture Initiation and Phytohormonal Influence on *Dianthus henteri* - a Romanian Endemic Species. Romanian Biotechnological Letters 15(1): 25-33.

6. Ehrlich, P.R. (1994). Energy use and biodiversity loss. Phil. Trans. R. Soc. Lond 344: 99-104.

7. Fay, M. (1992). Conservation of rare and endangered plants using *in vitro* methods. In Vitro Cellular & Developmental Biology - Plant 28(1): 1-4.

8. Foley, J.A., R. DeFries, G.P. Asner, C. Barford, G. Bonan, S.R. Carpenter, F.S. Chapin, M.T. Coe, G.C. Daily, H.K. Gibbs, J.H. Helkowski, T. Holloway, E.A. Howard, C.J. Kucharik, C. Monfreda, J.A. Patz, I.C. Prentice, N. Ramankutty and P.K. Snyder (2005). Global Consequences of Land Use. Science 309(5734): 570-574.

9. Holobiuc I, R. Blindu and V. Cristea (2009). Researches Concerning *In Vitro* Conservation of the Rare Plant Species *Dianthus nardiformis* Janka. Biotechnol. and Biotechnol. 23: 221-224.

10. Holobiuc I, R. Cătană and V. Cristea (2010). Researches Concerning *In Vitro* Cultures Optimization of the Vulnerable Species *Dianthus nardiformis* Janka. Analele Universității din Oradea.

11. Laurance, W.F., M.A. Cochrane, S. Bergen, P.M. Fearnside, P. Delamonica, C. Barber, S. D'Angelo and T. Fernandes (2001). Environment. The future of the Brazilian Amazon. Science 291(5503): 438-439.

12. Leifert, C. and A. Cassells (2001). Microbial hazards in plant tissue and cell cultures. In Vitro Cellular & Developmental Biology - Plant 37(2): 133-138.

13. Myers, N. (1996). Two key challenges for biodiversity: discontinuities and synergisms Biodiversity Cons. 5: 1025-1034.

14. Mirjalili, A., E. Parmoor, S. Moradi Bidhendi and B. Sarkari (2005). Microbial contamination of cell cultures: A 2 years study. Biologicals 33(2): 81-85.

15. Murashige, T. and F. Skoog (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum 15(3): 473-497.

16. Oprea, A. Ed. (2005). Lista critică a plantelor vasculare din România. Iaşi., Editura Universitaţii „Al.I.Cuza".

17. Pimm, S.L., G.J. Russell, J.L. Gittleman and T.M. Brooks (1995). The future of biodiversity. Science 269: 347-350

18. Sala. O.E., F.S. Chapin, J.J. Armesto, E. Berlow, J. Bloomfield, R. Dirzo, E. Huber-Sanwald, L.F. Huenneke, R.B. Jackson, A. Kinzig, R. Leemans, D.M. Lodge, H.A. Mooney, M. Oesterheld, N.L. Poff, M.T. Sykes, B.H. Walker, M. Walker and D.H. Wall (2000). Global biodiversity scenarios for the year 2100. Science 287(5459): 1770-1774.

19. Sarasan, V., R. Cripps, M. Ramsay, C. Atherton, M. McMichen, G. Prendergast and J. Rowntree (2006). Conservation *In vitro* of threatened plants—Progress in the past decade. In Vitro Cellular & Developmental Biology - Plant 42(3): 206-214.

20. Sivasithamparam, K., K.V. Dixon and R.L. Barret, Eds. (2002). Microorganisms In Plant Conservation and Biodiversity. Netherlands, Kluwer Academic Publishers.

21. Whitfield, W.J. (1966). LAMINAR FLOW AIR HOOD APPARATUS. Other Information: Orig. Receipt Date: 31-DEC-67**:** Medium: X.

22. Wilson, E.O., Ed. (1992). The Diversity of Life. Cambridge, Massachusetts.