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Ex situ Conservation of Three Endemic and/or Endangered *Dianthus* Species

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Abstract

Within the current context of declining biodiversity, the botanical gardens play an essential role in its conservation. *Dianthus callizonus*, *D. glacialis* ssp. *gelidus* and *D. spiculifolius* are the species that we seek to preserve in "Alexandru Borza" Botanical Garden of Cluj-Napoca (Romania). Several replicates were collected for each taxon from different populations in order to avoid the genetic uniformity. The material collected from the natural sites, was planted on a rockery, specially designed for this collection in the Botanical Garden. At the time of planting, each individual was sampled for setting up an *in vitro* collection and further biochemical and molecular analyses. In case of *ex situ* outdoor conservation of the three *Dianthus* species, 80.6% of the individuals collected in the field survived during the first year but the percentage decreased drastically after four years. In the case of *in situ* collected individuals, as well as in the case of *in vitro* individuals, *D. spiculifolius* had the best ability to acclimatize in the Botanical Garden, and *D. callizonus* presented the lowest number of surviving individuals. The *ex vitro* acclimatization of the plantlets had 80% efficiency at 10°C, using three different substrates: soil and pearl stone mix 1/1, soil and sand mix 1/1 and pearl stone. All the three species are preserved *in vitro,* whereas the plantlets are acclimatized outdoors. *Ex situ* conservation of these species will have a positive impact on the biodiversity conservation.

Keywords: *Dianthus callizonus*, *Dianthus glacialis* ssp. *gelidus, Dianthus spiculifolius*, from *vitro* plantlets acclimatization, Natura 2000 sites

Introduction

In recent years, global biodiversity declined drastically, according to the 2006 IUCN Red List: out of the 40,177 species assessed, 16,119 are now listed as threatened with extinction (IUCN, 2006). In addition, the IUCN listed 8,321 plant species (out of the 11,824 evaluated) as threatened in 2004 (Baillie *et al*., 2004). An efficient approach for plant conservation is achieved by combining *in situ* and *ex situ* conservation methods with the main goal of maintaining an optimum/high genetic diversity for the chosen species. For *in situ* conservation a large network has been established in Europe (i.e., NATURA 2000), comprising over 26,000 protected areas covering all the Member States (around 850,000 km^2, representing more than 20% of total EU territory) (EAP, 2011).

Ex situ conservation consists of a complex of various complementary proceedings, carried out in a different ecological environment than the characteristic one for the target species (Cristea and Denaeyer, 2004). These authors include thematic collections (working collections, active or basic collections), gene banks or *in situ* conservatories, in their envisioned conservation system. The botanical gardens represent traditional centers for the *ex situ* conservation of plant material as living collections. They successfully contribute to *ex situ* conservation of a high number of endemic and/or endangered plant species using combined methods: classical outdoor or greenhouses cultivation, seeds banks and *in vitro* collections. A survey indicated that out of 119 European botanical gardens in 29 European countries, 105 are cultivating 308 of the 573 threatened plant species listed by the Bern Convention. The survey identified 25 botanical gardens in 14 countries undertaking 51 conservation projects focused on 27 Bern listed species (Maunder *et al*., 2001). Besides the studies of flora and phytosociology (Puşcas *et al.*, 2012), there is permanent concern for the enrichment of endemic and/or threatened outdoor plant collections (Hentea *et al.*, 2004; Şuteu *et al*., 2004), in "Alexandru Borza" Botanical Garden of Cluj-Napoca (Romania).

The aim of this study is the creation of an outdoor *ex situ* conservation for 3 alpine or subalpine *Dianthus* species, endemic to the South-Eastern Carpathians (Coldea *et al*., 2009; Hurdu *et al*., 2012): *D. callizonus* Schott & Kotschy, *D. glacialis* Haenke ssp. *gelidus* (Schott, Nyman & Kotschy) Tutin and *D. spiculifolius* Schur. We've embarked on this project knowing that the Romanian botanical gardens only have a few endemic and/or threatened *Dianthus* species. Some studies using *in vitro* culture for these species have been done already in the lab (Butiuc-Keul *et al*., 2001; Cristea *et al*., 2002; 2006; Holobiuc and Blîndu, 2006; Zăpârţan, 2001), but the plant material came mostly from various collections, rather than their natural sites. Still, the *ex situ* outdoor conservation aspects for many species with sozological importance have not been tackled yet. Also, systematic studies regarding *ex*

vitro acclimatization of sozologically important species of Romania are lacking. Due to an accelerated degradation of natural habitats, including the subalpine and alpine ones, the existence of these collections will have a significant impact for future repopulations.

Material and methods

The studied species

D. callizonus is one the most emblematic Carpathian species, being strictly localized in the Piatra Craiului Mountains (Ciocârlan, 2000; Prodan, 1953). Old botanical literature reported the species also from Bucegi Mountains (Haret, 1938; Negrean, 1979; Negrean and Oltean, 1989; Simonkai, 1886), but the occurrence in that massif was not confirmed by subsequent explorations (Dihoru and Negrean, 2009). *D. callizonus* grows on alpine and subalpine calciphilous grasslands (Ciocârlan, 2000; Mihăilescu, 2003). This is a perennial species, with a 10-20 cm single-flowered (seldom 2-5) unbranched stem. The basal leaves are shorter than those on the stem and often form rosettes. The lanceolate leaves are much wider at the base. The flowers, which are very beautiful, have a carmine colour, the lamina of the petals being spotted in its upper part (Prodan, 1953). The National Red Lists (Sârbu, 2003) and Dihoru and Negrean (2009) indicated the species as being *vulnerable* or *with lower risk*. Seven individuals were sampled from a single population in Piatra Craiului Mountains (Fig. 1):

- Population 1 - Piatra Craiului Mountains (Piatra Craiului Mică), NATURA 2000 Site ROSCI0194, alt. 1626 m a.s.l. (45° 28' 21" N, 25°11'30" E).

D. glacialis ssp. *gelidus* is endemic to the Eastern and Southern Carpathians (Romania), with a sporadic distribution in the alpine belt of these mountains. The largest and most abundant populations are in the Bucegi, Făgăraş and Rodna Mountains (Romania) (Mihai Puşcaş, personal communication). It grows mainly in alpine primary herbaceous communities developed on bedrocks rich in carbonate rocks. This perennial, caespitose species has short stems (less then 10 cm) with solitary pedicellate flowers. The leaves are wider towards the tip. The petal's lamina is dark-rosy (Ciocârlan, 2000; Prodan, 1953). *D. glacialis* ssp. *gelidus* is mentioned in the Romanian Red Lists as *vulnerable* (Sârbu, 2003). The taxon was sampled from 3 different populations (5 individuals per sample) (Fig. 1):

- Population 1 - collected from Bucegi Mountains (Omu Peak), NATURA 2000 Site ROSCI0013, alt. 2400 m a.s.l. (45°22'51" N, 25°30'40" E);

- Population 2 - collected from Făgăraş Mountains (Bâlea Lake), NATURA 2000 Site ROSCI0122, alt. 2160 m a.s.l. (45°31'40" N, 24°44'26" E);

- Population 3 - collected from Bucegi Mountains (Obârşia), NATURA 2000 Site ROSCI0013, alt. 2417 m a.s.l. (45°22'50" N, 25°30'39" E).

D. spiculifolius has a broader distribution area in South-Eastern Carpathians, present in Romania (Apuseni Mountains, Southern and Eastern Carpathians), as well as in the Ukrainian Carpathians (Ciocârlan, 2000; Prodan, 1953 Tutin, 1964). It grows mainly on calcareous rocky outcrops and cliffs especially in the mountain to subalpine belt (Coldea, 1991). This perennial, caespitose species, is shorter then 20 cm, having numerous stems, each bearing 2-5 pairs of leaves and 1 (2) white or pink flowers. (Ciocârlan, 2000; Prodan, 1953). This species is mentioned in the Romanian Red List as vulnerable (Sârbu, 2003). *D. spiculifolius* was sampled from 2 different populations (4-5 individuals from each) (Fig. 1):

- Population 1 - collected from Hăşmaş Mountains (Bicaz Gorges), NATURA 2000 Site ROSCI0027, alt. 1668 m a.s.l. (46°44'27"N, 25°47'58" E);

- Population 2 - collected from Apuseni Natural Park, Vlădeasa Massif (Pietrele Albe), NATURA 2000 Site ROSCI0002, alt. 1050 m a.s.l. (N 46°35'45" N, 22°48'38"E).

Ex situ outdoor acclimatization of wild plant species

We set up a new rockery in the "Alexandru Borza" Botanical Garden from Cluj-Napoca (Romania), for the *ex situ* collection. The soil in the pockets was rich in limestone rock crumbs since the 3 species are calciphilous. At the same time, we used the original soil ball from the sampling spot, for planting. Individuals of the same population were planted in the same pocket, with space between them. Three-five individuals were planted in a pocket, according to its size. Initially the planted individuals have been shaded with a special net for a faster acclimation. During the four wintering periods, the rockery didn't have any special protection, considering that the species are alpine or subalpine. The planted material was monitored for four years (2008 - 2012) recording the number of generated shoots each year and the number of flowering stems/each individual. The seed were collected and kept at 4°C.

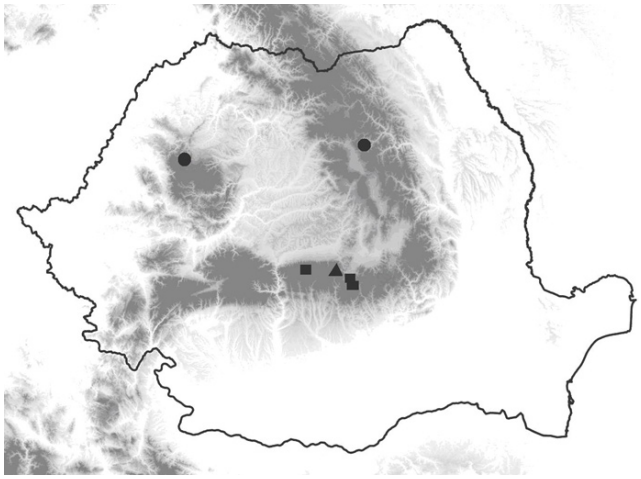

Fig. 1. Location of the studied populations of *D. callizonus* (▲), *D. glacialis* ssp. *gelidus* (■) and *D. spiculifolius* (●) in Romania

Ex situ outdoor acclimatization of plantlets

Leaf fragments from each wild individual were sampled and stored for *in vitro* culture, further biochemical and molecular analyses. Three experimental variants were used for the acclimatization of plantlets generated *in vitro*: soil and pearl stone mix 1/1, soil and sand mix 1/1 and pearl stone. The plantlets were kept at 25°C and 10°C, in parallel, for the first 4 weeks. The atmospheric humidity was high, close to 80%, in the first 10 days and gradually decreased. The culture substrates were watered daily with MS solution of macro and microelements ¼ diluted (Murashige-Skoog, 1962). The plantlets generated *in vitro* were acclimatised and planted outdoor, on a secondary rockery, different from the one used for plants brought from nature.

Statistical analysis

Using statistical analysis tools we compared the intra- and interpopulational variability of the plants collected in the wild and adapted to the conditions in the Botanical Garden. The individuals of each population and the populations of each species were compared. GraphPad Prism, version 5.00 for Windows, was used. One-Way ANOVA test (for testing 3 or more columns) or the t tests (for testing 2 columns), with a 95% confidence intervals was used. When ANOVA test showed significant differences, Tukey's post test was used in order to determine the significance of the differences between the average values at $p < 0.05$.

Results and discussion

Ex situ outdoor acclimatization of wild plant species

The first collected individuals of the namely *D. callizonus*, *D. glacialis* ssp. *gelidus* and *D. spiculifolius,* planted in June - August 2008, generated new shoots over the summer. Many plants, brought with buds or flowers from their natural sites, produced flowers and fertile seeds. During the observations, after one year 80.6% of the individuals were viable (Fig. 2, 3a, 4a and 5a).

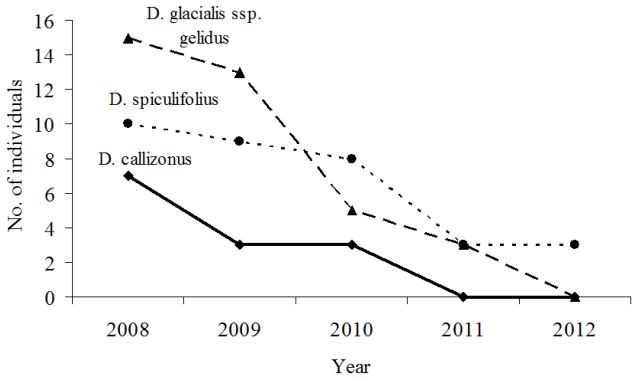

Fig. 2. *D. callizonus, D. glacialis* ssp. *gelidus* and *D. spiculifolius* 4 years evolution in the "Alexandru Borza" Botanical Garden of Cluj-Napoca (Romania). Anova test results: there are no significant differences between the different populations of the same species

As shown in Fig. 2, the studied species had a different evolution during the four years of monitoring. The best results were those of *D. spiculifolius*, which in the third year presented approximately the same number of shoots as in the first year and a greater number of flowering stems (Tab. 1 and 2). However, only three of the ten planted individuals were acclimatized in the case of this species. *D. glacialis* ssp. *gelidus* lived only for three years under the conditions of the Botanical Garden, and even though in the first and second year they bloomed, no flowering stems formed in the third year (Tab. 2). *D. callizonus* suffered the most as a result of transplantation in the Botanical Garden conditions, living and blooming only for one year (Tab. 1 and 2).

About two dozen individuals of all studied species (five individuals of *D. callizonus*, 12 individuals of *D. glacialis* ssp. *gelidus*, and 5 individuals of *D. spiculifolius*), bloomed

Tab. 1. Multiplication of *D. callizonus, D. glacialis* ssp. *gelidus* and *D. spiculifolius,* during four years, in the "Alexandru Borza" Botanical Garden of Cluj-Napoca (Romania)

Species	2009		2010		2011		2012	
	NoS/Sp	ANoS/I	NoS/Sp	ANoS/I	NoS/Sp	ANoS/I	NoS/Sp	ANoS/I
D. callizonus	27*	9.00 ± 3.46*	0	0	0	0	0	0
D. glacialis ssp. *gelidus*	113 ns	8.69 ± 4.11 ns	69 ns	16.50 ± 1.73 ns	45 ns	15 ± 7.00 ns	0	0
D. spiculifolius	100 ns	12.25 ± 8.01 ns	250 ns	29.83 ± 9.47 ns	135 ns	45± 7.00 ns	53 ns	24.33 ± 8.50 ns

NoS/Sp = Total number of shoots/species. ANoS/I = average number of shoots/individuals; the data in the 2nd column of each year are presented as mean ± SD. * p 0.01 to 0.05; ns $p > 0.05$ no statistical significance

Tab. 2. Number of flowering stem of *D. callizonus, D. glacialis* ssp. *gelidus* and *D. spiculifolius,* during four years, in the "Alexandru Borza" Botanical Garden of Cluj-Napoca (Romania)

Species	2009		2010		2011		2012	
	NoS/Sp	ANoS/I	NoS/Sp	ANoS/I	NoS/Sp	ANoS/I	NoS/Sp	ANoS/I
D. callizonus	13*	4.33± 1.52*	0	0	0	0	0	0
D. glacialis ssp. *gelidus*	66 ns	5.33 ± 3.52 ns	80 ns	19.25 ± 6.65 ns	0	0	0	0
D. spiculifolius	29 ns	2.50 ± 1.22 ns	164 ns	19.00 ± 12.01 ns	21 ns	32.66 ± 7.50 ns	26 ns	12.00 ± 4.69 ns

NoS/Sp = Total number of shoots/species. ANoS/I = average number of shoots/individuals; the data in the 2nd column of each year are presented as mean ± SD. * p 0.01 to 0.05; ns $p > 0.05$ no statistical significance

and set seed, after the first year of culture in the Botanical Garden. However, after four years of observation only *D. spiculifolius* did so, whereas the other 2 species didn't survive in the Botanical Garden.

The statistical analyzes reveal a reduced variability among the different individuals or populations regarding their acclimatization ability. *D. callizonus* had only one individual whose response was different from the rest. For *D. glacialis* ssp *gelidus*, the intrapopulational variability was low and there was no interpopulational variability. In case of *D. spiculifolius* there is no intra or interpopulation variability.

The results above illustrate an evolution below expectations for the evolution of plant material collected in the wild and transplanted in the "Alexandru Borza" Botanical Garden of Cluj-Napoca (Romania). Two of the species - *D. callizonus* and *D. glacialis* ssp. *gelidus* - did not succeed in getting acclimatized. That could be due to the fact that the first species is a subalpine one, whereas the latter is alpine, being hekistotherm (adapted to lower temperatures) by nature (Ciocârlan, 2000). The rokery where they were planted in the Botanical garden is at 402 m a.s.l. (which corresponds to the zone of oak forests, possibly beech). These climatic conditions are too divergent from those found *in situ* and could explain the constant decline of the individuals from the two species. The third species, which managed to get acclimatized, *D. spiculifolius*, is mainly found at altitudes corresponding to beech forests, all the way to the subalpine level (Ciocârlan, 2000). Therefore it grows in conditions that are more or less similar to those of the Botanical Garden. Another problem could be that the individuals brought to the Botanical Garden lack the phytosociological conditions from the wild. Hence the need for collecting surrounding species in the future, in a wider soil block, if conservation of endemic or endangered species is sought under these conditions. An alternative would be to collect seeds from *in situ* locations and later grow them *ex situ*, as recommended by other authors (Mikatadze-Pantsulaia *et al.*, 2012). Similar to our study, these authors studied more endemic and protected species in Georgia first by *in situ* observation and then conserving them by seed bank and seedling production for the purpose of *in situ* restoration. More or less similar studies on *ex situ* conservation were carried out for other endemic or endangered *Dianthus* species. *D. diutinus* Kit. individuals (endemic for Hungary and for the E of former Yugoslavia) were grown from seeds for the repopulation of their natural sites at the University of Szeged. The generative state of the individuals could be reached within one year. Plants grown in pots by *ex situ* propagation were out-planted in their natural site where the number of the individuals was critically low (Németh *et. al*., 2011). The ecology and population dynamics of *Dianthus armeria* L., spread in Western, Central and Southern Europe but included in English Nature's Species Recovery Programme was investigated at the University of Sussex, and research into the seed biology and germination ecology were carried out (Garnnet, 2004).

Ex situ outdoor acclimatization of plantlets

At present, *in vitro* collections for the studied species are available. The *ex vitro* acclimatization experiments of the *in vitro* generated plantlets had a good efficiency (80%), especially at 10°C, in all of the experimental variants and with all the species. The plantlets were cultivated on another rockery to differentiate between the 2 types of individuals (wild vs. *in vitro* culture) (Fig. 3b, 4b and 5b).

In the case of outdoor acclimatization of plantlets, *D. spiculifolius* also had the best multiplication rate, 136.4% after 2 years from planting while *D. callizonus* reached 125% (Tab. 3). The number of *D. glacialis* ssp. *gelidus* plants decreased by 33.3% after 2 years of outdoor culture.

The already published studies regarding the *in vitro* multiplication of the analyzed species (Butiuc-Keul *et al*., 2001; Cristea *et al*., 2002, 2006; Holobiuc and Blîndu, 2006) do not contain details on the *ex situ* acclimatization. The results presented here on the *ex situ* acclimatization of *in vitro* generated plantlets meet the expectations, having a superior efficiency. We have to stress out that the evolution had by the *in vitro* generated plantlets outdoor is supperior to that of plants collected *in situ* and transplanted in the Botanical Garden. Because of that we consider *in vitro* multiplication as being the best suited technique for endemic and/or threatened species in botanical gardens. Plus, this has the advantage of avoiding the negative impact of collecting species from their natural habitat. In order to avoid possible genetic changes induced by the *in vitro* culture, these plants must be genetically certified. Therefore we already performed SSR molecular studies on *D. spiculifolius* in order to monitor the somaclonal variability (submitted data), and we are currently working on the other two species (*D. callizonus* and *D. glacialis* ssp. *gelidus*).

Our present results on *ex vitro* acclimatization are in accord to those reported by other authors (Gorgorov *et al.*, 2011) who micropropagated three endemic and rare *Alchemilla* species, adapted them *ex vitro* and then transferred the surviving plants to a mountaneous environment. This study also reports that only two of the three species could be acclimatized, with a reduced rate due to low humidity stress. However, the adaptation efficiency in natural habitat conditions afterwards, was high.

Tab. 3. Outdoor aclimatization of plantlets, in "Alexandru Borza" Botanical Garden of Cluj-Napoca (Romania)

Species	No. of shoots			No. of flowering stems		
	2010	2011	2012	2010	2011	2012
D. callizonus	4	18	5	0	2	0
D. glacialis ssp. *gelidus*	6	4	4	0	2	2
D. spiculifolius	44	48	60	0	12	26

Fig. 3. Outdoor *ex situ* collection of *D. callizonus* on the rocky area in „Alexandru Borza" Botanical Garden (Romania); a. plants collected from *situ* in 2008, after 11 months; b. outdoor cultivated plantlets

Fig. 4. Outdoor *ex situ* collection of *D. glacialis* ssp. *gelidus*, on the rocky area in „Alexandru Borza" Botanical Garden (Romania); a. plants collected from *situ* in 2008, after 10 months; b. outdoor cultivated plantlets

Fig. 5. Outdoor *ex situ* collection of *D. spiculifolius*, on the rocky area in „Alexandru Borza" Botanical Garden (Romania); a. plants collected from *situ* in 2008, after 1 year; b. outdoor cultivated plantlets

Conclusions

In the course of four years of observations on cultivated endemic and endangered species of *D. callizonus, D. glacialis* ssp. *gelidus and D. spiculifolius* in "Alexandru Borza" Botanical Garden of Cluj-Napoca (Romania) that have been collected *in situ* and replanted, only the last one managed to survive acclimatization/adaptation. However, in the case of plantlets generated by *in vitro* culture they adapt and survive much better to outdoor conditions.

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