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In Situ Genetic Variability and Micropropagation of *Cerastium banaticum* (Rochel) Heuff. (Caryophyllaceae) – a Rare and Endemic Species from Romania

Victoria Cristea[1,2], Enikő Besenyei[3,4], Liliana Jarda[1*], Anca Farkas[2,5], Delia Marcu[6], Doina Clapa[7], Adela Halmagyi[8*] and Anca Butiuc-Keul[2,5*]

[1]*'Alexandru Borza' Botanical Garden, Babeş-Bolyai University, Republicii 42, 400015 Cluj-Napoca, Romania*

[2]*Laboratory of Plant Biology, Genetics, Microbiology and Biotechnology, Center of Systems Biology, Biodiversity and Bioresources, Babeş-Bolyai University, Kogălniceanu 1, 400084 Cluj-Napoca, Romania*

[3]*Plant Genetic Engineering Group, Faculty of Biology and Geology, Babeş-Bolyai University, Clinicilor 5–7, 400006 Cluj-Napoca, Romania*

[4]*Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, 38124 Braunschweig, Germany*

[5]*Department of Molecular Biology and Biotechnology, Faculty of Biology and Geology, Babeş-Bolyai University, Kogălniceanu 1, 400084 Cluj-Napoca, Romania*

[6]*'Ana Aslan' Technical College, Decebal 41, 400124 Cluj-Napoca, Romania*

[7]*University of Agricultural Sciences and Veterinary Medicine, Calea Mănăştur 3–5, 400372 Cluj-Napoca, Romania*

[8]*Institute of Biological Research branch of NIRDBS, Republicii 48, 400015 Cluj-Napoca, Romania*

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Rare and endemic plant species represent important components of plant biodiversity which require protection to ensure their sustainable conservation. *Cerastium banaticum* (Rochel) Heuff. is such an endemic and rare species from Romania, for which the genetic variability of two natural populations was studied by SSR markers. Shannon's information index revealed low levels of genetic diversity in both populations (I = 0.296). As the first attempt in a conservation program a reproducible micropropagation protocol was established starting from seeds, followed by multiplication, rooting, and *ex vitro* acclimatization. Among the various plant growth regulators tested the highest multiplication coefficient was achieved on a culture medium with 0.5 mg L^{-1} 6-furfurylaminopurine (K) and 1 mg L^{-1} α-naphthaleneacetic acid (NAA). On this PGRs concentration a number of 26.6 shoots/individual explant with a mean length of 7.9 cm for new generated shoots was registered. The highest number of roots/individual initiated shoot was 2.6 and it was recorded on a culture medium with 0.5 mg L^{-1} 2-isopentyl-adenine (2iP) and 0.1 mg L^{-1} NAA. The outdoor acclimatization was successfully performed in a specially designed rocky area in the 'Alexandru Borza' Botanical Garden, Cluj-Napoca (Romania).

Keywords: acclimatization, biodiversity conservation, plant growth regulators, SSR markers

* Corresponding author, email: adela.halmagyi@icbcluj.ro; liliana.jarda@ubbcluj.ro

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INTRODUCTION

Nowadays, as response to the alarming increase of plant species with different degrees of endangerment, considerable attention is given to the conservation of biodiversity. *In situ* and *ex situ* conservation strategies are complementary approaches to maintain plant biodiversity (Primack et al., 2008; Butiuc-Keul, 2014). According to the International Union for Conservation of Nature (IUCN) Red List, there are approximately 33400 plant species threatened with extinction (Maunder et al., 2004).

Cerastium is a genus with a complex systematics (Berglund et al., 2004) comprising 400 perennial, annual, herbaceous, or rarely slightly woody species (http://www.theplantlist.org) with cosmopolitan distribution in temperate and cold regions, especially at high elevations with the center of diversity in Eurasia (Dequan and Morton, 2001). Most species have a restricted distribution being endemic in several regions. In Romania there are 14 *Cerastium* species which can be found at different altitudes (Ciocârlan, 2009). Some of the species are rare or vulnerable, and some of them have ornamental value (Dihoru and Dihoru, 1994; Oltean et al., 1994).

Cerastium banaticum (Rochel) Heuff. (Caryophyllaceae family) is endemic for the Balkans (Flora Europaea), and has a special phytogeographic value being considered a rare species since its discovery (Rochel, 1828). In Romania, it can be found only in the south-west region, sporadically of oak and beech rocky areas (Sârbu et al., 2013). This species was considered as rare in two Romanian Red Lists (Dihoru and Dihoru, 1994; Oltean et al., 1994), while Oprea (2005) cites it as a rare species which may become threatened in the future. The plant has two types of stems, one is radicant, caespitose and repent, while the other one is floriferous, reaching approximately 40 cm in height. The inflorescence shows wide pubescent silver bracts with white petals blooming from May to August (Prodan, 1953).

In vitro techniques are reliable alternatives not only for the conservation of endangered and/or endemic species for further reintroduction programs (Holobiuc et al., 2009; Marriott and Sarasan, 2010; Johnson et al., 2012) but also for the revegetation and stabilization of polluted wastes (Muszyńska and Hanus-Fajerska, 2017; Muszyńska et al., 2018). Both, the development of appropriate conservation programs for plant species and the preservation of their natural variability require thorough genetic variability studies in natural populations (Halmagyi and Butiuc-Keul, 2007). Molecular markers as microsatellites or Simple Sequence Repeats (SSR) are valuable tools for such investigations because they are cost efficient and require low amounts of DNA (Zietkiewicz et al., 1994). Moreover, SSR markers are codominant and despite their locus specificity in several cases some primers could be used for different species within the same family (Smulders et al., 2000, 2003; López-Vinyallonga et al., 2012; Zhang et al., 2013; Müller et al., 2015). There are only few studies on genetic variability by molecular markers in populations of other Caryophyllaceae species from Romania (Cristea et al., 2014; Jarda et al., 2014), whereas some studies investigated the chromosome number in *C. banaticum* (Boşcaiu et al., 1996, 1999) or referred to the *in vitro* multiplication of *C.* transsilvanicum – another endemic and rare species from Romania (Holobiuc et al., 2004–2005).

Considering that in the last decades the protection and conservation of rare and endangered plant species has become an issue of concern, the major goals of this research were: (a) to study the genetic variability in natural populations by SSR markers for an accurate assessment of genetic diversity and the relationship between individuals having as final aim the preservation of natural variation, and (b) to establish an optimized and reproducible protocol for micropropagation (including culture initiation, multiplication, plant regeneration, rooting) and *ex vitro* acclimatization of *C. banaticum*. This study represents the first report offering a comprehensive propagation protocol for *C. banaticum* through tissue culture which would be useful for future cultivation of this species to increase its accessibility.

MATERIALS AND METHODS

PLANT MATERIAL FOR MOLECULAR ANALYSIS

The plant material was collected from two populations (ten individuals from each population) of *C. banaticum*, both of them located in Natura 2000 sites, ROSCI0031-National Park Cheile Nerei Beuşniţa (population 1: Coronini 44°41'07.70" N 22°41'10.04" E, 251 m altitude) and ROSCI0206-National Park Porţile de Fier (population 2: Cheile Minişului 44°57'49.56" N 21°59'14.11" E, 295 m altitude).

SSR ANALYSIS

Genomic DNA was isolated from leaves using the CTAB method (Doyle and Doyle, 1987). For SSR analysis a total of 30 primer pairs were used as follows: MS-DCAMCRBSY, MS-DCDIA30, MS-DINCARACC, MS-DINGSTA, MS-DINMADSBOX (Smulders et al., 2000), Silaca1, 3, 7, 13, 18, 44 (Müller et al., 2015), Sle2, 4, 6, 9, 10, 11, 12,

14, 17, 23, 27 (López-Vinyallonga et al., 2012), and MPC9, 29, 31, 95, 113, 125, 227 (Zhang et al., 2013). The characteristics of the primers used for DNA amplification are presented in Table 1. PCR amplifications were performed in a 0.2 ml tube containing 2 mM $MgCl_2$, 1 μM of each primer, 200 μM of each dNTP, 1.5 U of Taq (Fermentas) and 25 ng of genomic DNA in a final volume of 25 μl. DNA amplification was performed according to the following program: 1) T = 94°C, 4 min; 2) T = 94°C, 30 s; 3) primer annealing at 50°C, 45 s; 4) elongation T = 72°C, 40 s; steps 2–4 were repeated 10 times; 5) T = 94°C, 30 s; 6) primer annealing at 56°C, 45 s; 7) elongation T = 72°C, 40 s; steps 5–7 were repeated 30 times; 8) final elongation T = 72°C, 5 min. Amplicons were separated on 1.5% agarose gel, stained with 0.5 μg mL^{-1} ethidium bromide. At least 2 independent PCR amplifications were performed for each primer.

SEED GERMINATION

In vitro culture of *C. banaticum* was initiated from seeds collected from population 2 (Cheile Minişului) located in the Natura 2000 site mentioned above. Surface sterilization of seeds was achieved as follows: immersion in 20% commercial Domestos (5% active chlorine) for 3 minutes followed by short rinsing in 10% H_2O_2 and thoroughly (five times) washing with sterile distilled water. For *in vitro* culture initiation the basal Murashige and Skoog (1962) (MS) supplemented with 0.5 mg L^{-1} 6-benzylaminopurine

TABLE 1. Characteristics of primers used for DNA amplification.

Primer	Forward and reverse sequence 5'-3'	Primer	Forward and reverse sequence 5'-3'
MS-DCAMCRBSY	F: CAACAATGACAACAACATCAG R: TCT TCG ATTGTTGAAGCTAAG	Sle9	F: AGATCCATTGGGCAAAAATG R: GGTAGCGAGAGAGACGATGG
MS-DCDIA30	F: CACTGACGACACAGCTGATGT R: ACTCGTCCAAACACAAACGAC	Sle10	F: CTAATCACCCGCGTTTCAAT R: GATCTTCTTCCGGCATTTGA
MS-DINCARACC	F: GGTCTTAAT TT TGTCACTTT' R: ACCCATCAAAGTACTCCAAAT	Sle11	F: TGTGGCTGCCTCAATGATTA R: GCTTCTCTGCTTTCAGGAACA
MS-DINGSTA	F: CACAAACCTGAAAGTACGATC R: ACATTCGAGCCCTCATATAAG	Sle12	F: CGTTCCTTCACCTCCACATT R: ATTCATGGCGGAGGTATGAG
MS-DINMADSBOX	F: ACGAGTGTCCAGGATCG R: CCCCTATTGCAAACTGC	Sle14	F: CACCACTCCACATCCTTCCT R: CTTTCAATCCTCTCCGCATC
Silaca1	F: TCTTATCATTTCCAACCTAGACGG R: TCGAACAAGGCAACCCAAC	Sle17	F: CCCCTTTTCTTCTCCTCCAA R: CACCAGTTCCTGCACAAAAC
Silaca3	F: GCGGATCTTGCTTGTGACG R: TTTCTACTAGTGCCCGCAG	Sle23	F: TGGCGATCAAGCTTTTCTCT R: GGAAATTGGGGAGATTAGGG
Silaca7	F: GGGGTCAATGTCATCAACATGAG R: AGAGAGTATGGTAGGTGGGG	Sle27	F: CTTTCAATGCCAGGCTCTTC R: GACGAGTGCGATCATCTTCA
Silaca13	F: GGGGTCAATGTCATCAACATGAG R: GAAGTAAGTCCATGTCCGCC	MPC9	F: CCTTACCTCCAACATCC R: CTCACGCATTCCTCAAAC
Silaca18	F: ACAAGTCGGATCAAGTGTTGG R: GCTCAACAGACCGGAATGC	MPC31	F: GTGGCAGTGTTAGAGGAC R: GCTGGAAAGTGAGGGATG
Silaca44	F: AGTAGTTATACAAGTGGTGGTGG R: TCCTCTATGAACTCGCTGCC	MPC95	F: GAAATAGTCGCAAATGG R: GCCTCGCTGTCTACCTCT
Sle2	F: GCCGGAATTCCTTTTTGTTC R: ATGGTTCGATCAACCTCGTC	MPC113	F: GGACCTTCTTCAAACTCAC R: ACGCCTCCAGACGACATAG
Sle4	F: GAAGGAGCTGGATGAACTCG R: TTTACTTAATGATCCCATAATTCCA	MPC125	F: AGCATTTCACGCATTCC R: ACCTCCGACAACCACGAC
Sle6	F: GAAACATTTCCCCACTCACAA R: AACCGGGTCACGTATTTCAG	MPC227	F: GATGACCCTTACCTCCAAC R: CACCATAAACCCTACTACT

(BAP), 0.1 α-naphthaleneacetic acid (NAA), 20 g L^{-1} sucrose, and 7 g L^{-1} agar was used (VG medium) (Table 2). The pH was adjusted to 5.7 before autoclaving at 121°C for 20 minutes. Seeds inoculated for germination as well as the *in vitro* cultures were maintained at 25 ± 2°C with a 16 h photoperiod and a light intensity of 90 μmol m^2 s^{-1} photosynthetic photon flux density (PPFD) provided by cool white fluorescent tubes. The seeds were individually germinated in glass jars (25 ml) in dark (30 seeds) at 25 ± 2°C and light (30 seeds) conditions as mentioned above. The efficiency of the sterilization protocol was assessed after 16 days, while the germination rate after 68 days.

TABLE 2. Culture media composition used for seed germination, initial multiplication (VG), and for multiplication and rooting (V1–V6). As control a medium without PGRs was used (V0).

Culture medium variants	PGRs (mg L^{-1})				
	Cytokinins			Auxins	
	BAP	K	2iP	IAA	NAA
VG	0.5	–	–	–	0.1
V0	–	–	–	–	–
V1	–	0.5	–	–	0.1
V2	–	1	–	–	0.1
V3	–	–	0.5	–	0.1
V4	–	–	1	–	0.1
V5	–	0.5	–	–	1
V6	–	0.5	–	1	–

MULTIPLICATION AND RHIZOGENESIS

To ensure an experimental stock of *in vitro* shoots, segments with two nodes (approximately 2.0 cm in length) from the median part of the initiated shoots were transferred to fresh VG medium four times successively at 45-day intervals. Several culture medium variants with different combinations and concentrations of plant growth regulators (PGRs) including cyokinins [BAP, 2-isopentyladenine (2iP), 6-furfurylaminopurine (K)], and auxins [indole-3-acetic acid (IAA), NAA] were tested for multiplication and rooting (Table 2). For multiplication and rooting shoot segments with two nodes (ten for each culture medium variant) from the above mentioned stock were transferred to the six culture medium variants (V1-V6). A culture medium without PGRs (V0) was used as control (Table 2). After 90 days from the transfer the following parameters were assessed: (a) multiplication coefficient (expressed as the mean number of shoots/individual explant), (b) mean length of newly generated shoots/individual explant, (c) mean number of roots/individual shoot, and (d) mean length of roots.

EX VITRO ACCLIMATIZATION

Plants showing a high multiplication coefficient and rhizogenesis were used for *ex vitro* acclimatization. Micropropagated plants with well-developed roots were acclimatized first in laboratory conditions (similar to those mentioned for the growth of *in vitro* cultures) in pearl stone, knowing its capacity to maintain water and to stimulate adventitious root development. Before transfer the roots were washed under tap water to remove the agar. For the first three weeks the pots were covered with transparent covers to maintain high humidity. After 45 days the plants (shoots with a length of approximately 10 cm and well-developed roots) were transferred to soil in greenhouse conditions for 4 months. In April, the plants from the greenhouse were planted in a specially designed rocky area in the 'Alexandru Borza' Botanical Garden, Cluj-Napoca.

DATA ANALYSIS

Several genetic diversity parameters were calculated for each locus and population using GENALEX6.5 program (Peakall and Smouse, 2006, 2012). The number of alleles per species (arithmetic mean across loci), the number of alleles with a frequency higher than 5%, the effective number of alleles, the number of private alleles and the number of locally common alleles occurring in less than 50% of the populations were explored. Principal coordinate analysis (PCoA) used to explore multivariate relationships among inter-individual genetic distances within and among populations was also performed with GENALEX6.5 program. Genetic relationships between 20 *C. banaticum* individuals from 2 populations revealed by SSR markers, based on Neighbor-joining (Perrier et al., 2003) and Jaccard similarity coefficient (Jaccard, 1908) were accomplished with DARwin 6.0.12 software.

Micropropagation data were analyzed using the GraphPad Prism software (version 6.01 for Windows). The entire set of experiments was repeated twice with three replicates for each tested parameter and culture medium variant. The multiplication coefficient was considered as the mean number of newly generated shoots per individual explant. The results are expressed as means ± standard deviation (SD). The statistical significance was calculated by One-way ANOVA with Tukey's post-test at 5% probability level.

RESULTS

GENETIC VARIABILITY IN NATURAL POPULATIONS

A total of 64 alleles were identified after amplification with SSR primer pairs in population 1 and 67 alleles in population 2. All alleles had frequencies equal or higher than 5% in both populations. There were 5 private alleles in population 1 and 8 in population 2 (Table 3). A total of 22 alleles were obtained with MS primers in population 1 and 23 alleles in population 2. The most polymorphic loci were MS-DCDIA30 where 9 alleles were identified in population 1 and 10 alleles in population 2 and MS-DCAMCRBSY with 5 alleles in population 1 and 7 alleles in population 2. The other MS primers revealed 1–3 alleles per population. The primers Silaca showed a total of 9 alleles per population 1 and 10 alleles per population 2, but only 1 allele in each locus, except the primer Silaca 44 with 2 alleles per population and Silaca 18, that showed 3 and 4 alleles per population. The Sle primers showed 16 alleles in both populations. Some of these primers showed 2 or 3 alleles which detected low differences between the populations. Other 17 alleles were detected in population 1 with the primers MPC and 18 alleles in population 2. Regarding the polymorfism over loci and populations, 53.47% of loci were polymorphic in both populations. The mean number of alleles is was 1.444 in the populations, and the effective alleles were similar in both populations with the mean value of 1.347. Shannon's information index revealed low levels of genetic diversity in both populations (I = 0.296) and the genetic diversity was also correlated with Shannon index (h = 0.2). All parameters were similar in the two populations although, higher values were obtained in population 2 (Table 4). A Neighbor-Joining tree showed the relationships of taxa (Fig. 1). Thus, 3 different clades could be observed, most of the individuals belonging to population 1 are clustered in the first clade and the individuals of population 2 are clustered together in the second clade, except the individuals 11, 12 and 19 that are clustered together with the individuals from population 1. There is a third clade that contains the individuals

TABLE 3. Number of alleles at SSR loci.

Locus	No. of alleles		Locus	No. of alleles	
	Population 1	Population 2		Population 1	Population 2
MS-DCAMCRBSY	5	7	Sle11	2	2
MS-DCDIA30	9	10	Sle12	1	1
MS-DINCARACC	3	2	Sle14	1	1
MS-DINGSTA	2	1	Sle17	1	1
MS-DINMADSBOX	3	3	Sle23	2	2
Silaca1	1	1	Sle27	1	1
Silaca3	1	1	MPC9	3	3
Silaca7	1	1	MPC29	1	1
Silaca13	1	1	MPC31	1	1
Silaca18	3	4	MPC95	3	3
Silaca44	2	2	MPC113	1	1
Sle2	1	1	MPC120	2	2
Sle4	1	1	MPC125	4	5
Sle6	3	3	MPC227	2	2
Sle9	1	1			
Sle10	2	2	Total alleles	64	67
No. alleles freq. ≥ 5%	64	67	No. private alleles	5	8

TABLE 4. Polymorphism parameters over loci and populations.

Population	Polymorphic loci (%)	Na	Ne	I	h
1	50.00	1.389 ± 0.080	1.325 ± 0.046	0.274 ± 0.035	0.185 ± 0.024
2	56.94	1.50 ± 0.074	1.369 ± 0.045	0.317 ± 0.035	0.214 ± 0.024
Total	53.47 ± 3.47	1.444 ± 0.055	1.347 ± 0.032	0.296 ± 0.025	0.200 ± 0.017

Na = No. of alleles; Ne = No. of effective alleles = $1 / (p^2 + q^2)$; I = Shannon's information index = -1 x (p x Ln(p) + q x Ln(q)); h = Diversity = $1 - (p^2 + q^2)$.

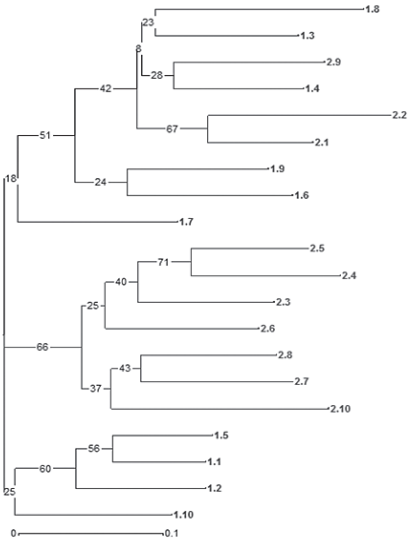

Fig. 1. Dendrogram of genetic relationships between *C. banaticum* individuals revealed by SSR markers based on Neighbor-Joining and Jaccard's algorithm. Numbers at the nodes indicate bootstrap values (30000 replications; individuals 1.1–1.10 belong to population 1; individuals 2.1–2.10 belong to population 2).

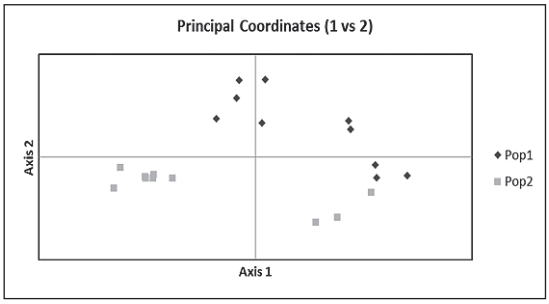

Fig. 2. A two dimensional plot of the PCoA analysis of SSR markers showing the clustering of two *C. banaticum* populations. The first and second coordinates account for 22.41% and 17.84%, respectively, of total variation.

1, 2 and 6 from population 1, and the individual 10 is clustered separately but close to the third clade. This distribution was confirmed by principal coordinate analysis (PCoA). Consistent with the low levels of genetic diversity, a low variety of allelic profiles was observed in both populations (Fig. 2).

INITIATION OF *IN VITRO* CULTURE, MICROPROPAGATION, AND *EX VITRO* ACCLIMATIZATION

After 16 days from inoculation of seeds on germination medium (VG) the low infection percentage (5%) proved the efficiency of the applied sterilization protocol. After 68 days in culture the germination rate in the dark was 87%, compared to 40% germination under light conditions. Moreover, after this period necrosis of seedlings occurred (13% in the dark and 20% in the light). Culture medium containing K in combination with NAA led to a significant increase in the number of shoots/individual explant (22.5 shoots on V1, 18.7 shoots on V2, and 26.6 shoots on V5), compared to the control medium (9.2 shoots/individual explant) (Table 2, Fig. 3a). On the contrary, a lower multiplication coefficient (11 and respectively 8.3 shoots/individual explant) was obtained on medium with 2iP and NAA (V3 and V4) (Table 2). The length of shoots of the rooted plants on V5 medium was 7.9 cm, while on medium V4 the length of shoots was only 3.5 cm, compared to V0 medium (6.4 cm) (Table 2, Fig. 3b).

The highest mean number (2.6) of roots/individual initiated shoot was achieved on V3 medium. Beside NAA, this medium also contains 2iP, which led to an increase in the length of roots (2.4 cm), although the longest roots (2.8) were obtained on V1 medium, while on V0 the mean number of roots/individual initiated shoot was 2.2 and the mean length of roots was 0.9 cm (Table 2, Fig. 4a, b).

Figure 5a-c shows *in vitro* grown *C. banaticum* plants on various culture media which were removed from the culture vessels before the transfer to pearl stone for later *ex vitro* acclimatization. The acclimatization was successfully performed first in pearl stone (Fig. 6a), then in

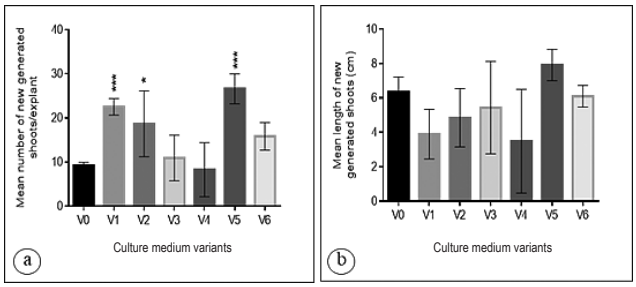

Fig. 3. Effects of PGRs on the mean number of newly generated shoots/individual explant (**a**), and the mean length of newly generated shoots (**b**) 90 days after inoculation. The bars represent means ± SD (Standard deviation). * $p < 0.05$; *** $p < 0.001$.

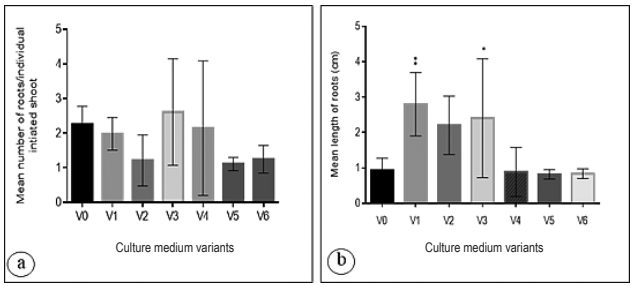

Fig. 4. Effects of PGRs on the mean number of roots/individual initiated shoot (**a**), and on the mean length of roots/individual initiated shoot (**b**) 90 days after inoculation. The bars represent means ± SD (Standard deviation). * $p < 0.05$; ** $p < 0.01$.

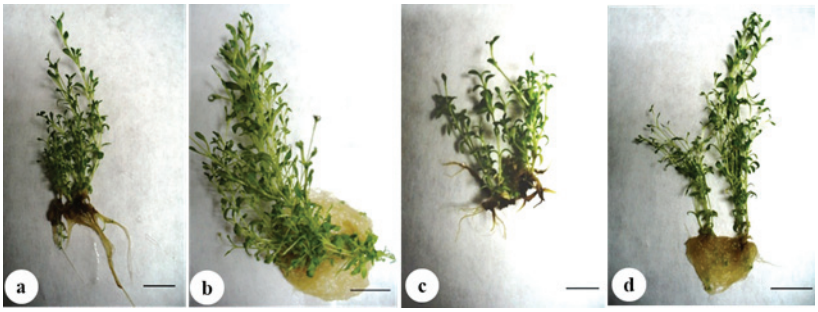

Fig. 5. *C. banaticum* plants developed *in vitro* on different culture medium variants 90 days after inoculation. (**a**) V0 without PGRs. (**b**) V2 with 0.1 mg L^{-1} NAA and 1 mg L^{-1} K. (**c**) V5 with 1 mg L^{-1} NAA and 0.5 mg L^{-1} K. (**d**) V6 with 1 mg L^{-1} IAA and 0.5 mg L^{-1} K. Scale bars: 1 cm.

Fig. 6. *Ex vitro* acclimatization of *C. banaticum*: (**a**) on pearl stone in laboratory 45 days after removal from *in vitro* growth conditions. (**b**) in soil in greenhouse conditions. (**c**) outdoor (in June) in the rocky area of 'Alexandru Borza' Botanical Garden. Scale bars: 1 cm.

soil in greenhouse conditions (Fig. 6b) and finally the plants were cultivated the rocky area in the 'Alexandru Borza' Botanical Garden in Cluj-Napoca (Fig. 6c), where other endemic and/or threatened plants are conserved as well. The percentage of the plants acclimatized to greenhouse conditions was 91%. Until now, to the best of our knowledge, there are no reports on outdoor acclimatization of *in vitro* micropropagated *C. banaticum* or other *Cerastium* species.

DISCUSSION

Analysis of genetic varibility in the population of endangered or endemic plants is important for proper development of conservation strategies because it has been used as an indicator of extinction risks (Gaudeul et al., 2000). Some studies suggest that rare species are more likely to be self-compatible with low genetic diversity when compared to closely related common species despite large populations with a high number of individuals due to the clonal propagation or bottlenecks (Hamrick et al., 1991; Lavergne et al., 2004). Other studies have found that several endemic species can exhibit both outcrossing and relatively high levels of genetic diversity (Ægisdóttir et al., 2009; Jabis et al., 2011). There are also some alpine endemic species with small populations and selfcompatibility that can further decrease the genetic variability by a reduced gene flow between individuals (Cole, 2003). The patterns of variation within and among populations of mountain species have not been clearly described so far. Low genetic diversity could be explained by clonal propagation of many plant species grown in severe environmental conditions that usually produce rapid but spatially limited spread genotypes. Along an altitudinal gradient the vegetative multiplication is increased because reproduction by seeds may be hampered by the harsh alpine conditions (Gabel et al., 2016). There are just few data about the genetic diversity of *C. banaticum* group from Romania (Boşcaiu et al., 1996, 1999), but there are no data regarding the estimation of genetic polymorphism within and between populations based on molecular markers. Our study revealed low genetic polymorphism in two populations of *C. banaticum*, both of them located in Natura 2000 sites from Romania.

Most of the primers tested in this study showed polymorphic patterns. The most polymorphic patterns were generated with the MS primers previously tested for molecular characterization of *Dianthus* species (Smulders et al., 2000, 2003). The Silaca primers previously used for analysis of genetic variability in the populations of *Silene acaulis* (Müller et al., 2015), Sle primers used for genetic characterization of *Silene sennenii* (López-Vinyallonga et al., 2012) and MPC primers used for *Silene conoidea* (Zhang et al., 2013) generated a low number of polymorphic alleles, but they are useful for molecular characterization, especially for polyploid plants, such as *Cerastium* species. It is known that polyploidy is considered a major mechanism of evolution and diversification (Roulin et al., 2013). In spite of the polymorphic patterns generated with these primers, we found low genetic diversity in both populations that could be explained by the small size of populations due to habitat fragmentation and also the vegetative multiplication in alpine conditions, which is common in Caryophyllaceae species. Our results are in accordance with previously published data in Scottish (Westergaard et al., 2008) and East Central European alpine plants (Szczecińska et al., 2016).

Previous studies on other endemic and/or endangered Caryophyllaceae species from Romania and Europe highlighted that micropropagation is favored by a balanced concentration of PGRs (Cristea et al., 2006; Papafotiou and Stragas, 2009; Muszyńska and Hanus-Fajerska, 2017). The multiplication coefficient for *C. banaticum* (26.6 new developed shoots/individual explant on V5 culture medium variant) obtained in our study is higher than the previously reported for *C. transsilvanicum* (15–20 regenerants/explant) on culture medium with 0.1 mg L^{-1} BAP, 0.05 mg L^{-1} NAA, and 0.25 mg L^{-1} glutamine (Holobiuc et al., 2004–2005). It is known that in the selection of an appropriate culture medium, beside the phytohormonal ratio, also the concentration of each PGR is essential to achieve high rates of shoot multiplication. In the case of *C. banaticum* the culture medium with 0.5 mg L^{-1} 2iP and 0.1 mg L^{-1} NAA (V3) led to unsatisfactory results, while for *Dianthus fruticosus* the same medium led to high multiplication rates (Papafotiou and Stragas, 2009). It is worth mentioning that on the control medium (V0), the number of newly generated shoots was lower but their elongation was relatively good. In contrast, on V5 medium both parameters showed the highest values. Thus, on V5 medium the number of newly generated shoots/explants was 2.9 times higher, while the length of newly generated shoots was 1.3 times higher than on the control medium.

Regarding rooting in *C. banaticum*, it was ascertained that V3 culture medium led to a number of 2.6 roots, compared to 2.2 roots on V0 medium (Fig. 4a). It was observed that low concentrations of NAA in combination with K and 2 iP (V1, V2, and V3) had a stimulating effect on the mean length of roots/individual initiated shoots with 2.8 cm, 2.2 cm, and 2.4 cm, respectively, compared to 0.9 cm for roots on V0 culture medium (Fig. 4b). Culture media V5 and V6 (Table 2) with high auxin concentrations led to a decrease in the mean length of roots, compared to the medium devoid of any PGRs (V0) (Fig. 4b). The fact that lower concentrations of PGRs led to higher rhizogenesis is essential in the context of micropropagation because it was reported that low concentration of PGRs could prevent the occurrence of somaclonal variability (Bairu et al., 2011). There is no evidence for *in vitro* rooting of any *Cerastium* species. In other Caryophyllaceae species the number of roots/

explant varied according to the used PGRs and their concentration. For example, for *Dianthus spiculifolius* 11 roots/explant (Cristea et al., 2014), for *D. henteri* 8.5 roots/explant (Cristea et al., 2010), and for *D. gratianopolitanus* 14 roots/explant (Cristea et al., 2006) were reported. Similar results regarding *in vitro* multiplication and rhizogenesis were reported for *D. carthusianorum* cultured on medium with the same PGRs (BAP, 2iP, IAA, NAA) (Muszyńska and Hanus-Fajerska, 2017).

CONCLUSIONS

The current study demonstrates that the genetic diversity is low in both *Cerastium* populations with a higher diversity in population 2 from Cheile Minişului. At the same time, the *in vitro* culture of *C. banaticum* was successfully induced from seeds with high germination rate. Among the tested PGRs the combination between 0.5 mg L^{-1} K and 1 mg L^{-1} NAA (V5) led to the highest multiplication coefficient, while rooting was stimulated on a culture medium with 0.5 mg L^{-1} K and 0.1 mg L^{-1} NAA (V3). Generally, this species shows a good tolerance towards *in vitro* culture conditions. The *ex vitro* acclimatization in pearl stone followed by outdoor planting of rooted plants could represent the first step in a more complex program contributing to the conservation of this rare and endemic species. Based on the obtained results we could estimate the genetic variability in natural population and could develop a proper micropropagation method which subsequently allowed *ex vitro* acclimatization. This strategy could be further applied to other Caryophyllaceae species.

AUTHORS' CONTRIBUTIONS

Study conception and design – VC, DC, AB; performing experiments – VC, EB, LJ, AF, AB; analysis of data – DM, AB; drafting of manuscript – VC, EB, LJ, DM, AB, AH; reading and final approval of the version submitted to be published – all authors.

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