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GENETIC STABILITY DURING *IN VITRO* PROPAGATION OF *DIANTHUS SPICULIFOLIUS* SCHUR

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Abstract

Dianthus spiculifolius Schur is an endemic and endangered plant species from Romania with high ornamental value. SSR markers were used to investigate the genetic structure of *in situ* populations of this species and also for study the genetic stability of *in vitro* plants. The molecular studies showed that the two populations are similar, and there are no specific markers of one population. The genetic variability of plants from natural habitats was conserved during *in vitro* culture. Genetic differences between somaclones derived from the same individual were low, so these plants can be used for *ex situ* collections and for ornamental purpose.

Key words: alleles frequency, gene diversity, heterozygosity, plant growth regulators, somaclonal variability, SSR markers

INTRODUCTION

The concept of sustainable development implies the conservation of biodiversity both *in situ* and *ex situ*. Nowadays, *ex situ* conservation is an excellent alternative (Cristea and Denaeyer 2004). The *ex situ* conservation by *in vitro* collections is a viable complementary alternative of the *in situ* conservation strategies. In order to develop a proper conservation management it is very important to know the genetic variability of *in situ* populations. In the case of *in vitro* conservation, it is also necessary to evaluate the somaclonal variability (i.e., the variability induced by the *in vitro* conditions) and to avoid the genetic uniformity. Molecular techniques are valuable tools used in the analysis of genetic fidelity of micropropagated plants. Among these, the SSR (Simple Sequence Repeats or microsatellites) have been used to examine genetic stability of several micropropagated plants (Potter and Jones 1991, Schaal et al. 1991, Agarwal et al. 2008, Lopes et al. 2009, Bairu et al. 2011). SSR markers are able to identify certain variable fragments in the genome and the comparisons are usually done regarding the presence/absence of these fragments (Gostimsky et al. 2005, Gholizadeh et al. 2012).

D. spiculifolius Schur (Caryophyllaceae) is an important endemic plant species from Eastern Carpathians Mountains (Tutin 1964, Ciocârlan 2009). Different Red Lists from Romania and abroad describe this species as vulnerable (Dihoru and Dihoru 1994), rare (IUCN 2006, Oltean et al. 1994) or endangered (Sârbu et al. 2007). It is an ornamental species, having beautiful and scented flowers and has been extensively collected from its natural populations. This perennial species is caespitose, having numerous stems. The leaves are linear subulate, and the flowers have white or rosy petals, slashed from 1/3 to 1/2. It is growing on calcareous rocks, from mountain to alpine area (Prodan 1953).

In vitro culture was reported by different authors (Butiuc-Keul et al. 2001, Cristea et al. 2002, Holobiuc and Blîndu 2006) but there are no comparative molecular studies regarding the genetic structure of *in situ* populations of *D. spiculifolius*. For a proper *in vitro* collection it is very important to introduce and maintain *in vitro* many individuals from different populations, in order to avoid the genetic uniformity. There are no available data regarding genetic stability of this plant species after *in vitro* preservation.

The aim of this study was to evaluate the intra- and

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inter-population genetic variability on *in situ D. spiculifolius* plants and the genetic stability during *in vitro* propagation of this species.

MATERIAL AND METHODS

Plant material and in vitro culture

The plant material was collected from two distinct and spatially separated *in situ* populations of *D. spiculifolius*: population 1 (encoded D.s. 1) with 5 distinct individuals (1.1, 1.2, 1.3, 1.4, and 1.5), from ROSCI0027 Natura 2000 site, Hăşmaş Mountains (Romanian Oriental Carpathian, 46º44'27"N, 25º47'58" E) and population 2 (D.s. 2), with 5 distinct individuals (2.1, 2.2, 2.3, 2.4, and 2.5), from ROSCI0002 Natura 2000 site, Apuseni Natural Park, Vlădeasa Massif (Romanian Occidental Carpathian, N 46º35'45" N, 22º48'38"E). *In vitro* shoot tips with 2 nodes were used as initial explants. They were disinfected with 20% Domestos ((Unilever, United Kingdom) for 10 min. The basal medium (BM), for all variants contained Murashige and Skoog (1962) micro- and macroelements, 2.96 µM thiamine, 4.86 µM pyridoxine HCl, 8.12 µM nicotinic acid, 0.55 mM myo-inositol, 2% sucrose and 0.7% agar (w/v) (Duchefa Biochemie B. V., Netherlands). *In vitro* culture was initiated on the BM, supplemented with 4.44 µM 6-benzyladenine (BA) and 5.37 µM α-naphthaleneacetic acid (NAA) (V1 variant). After 30 days, micropropagated adventitious shoots were cultured on BM supplemented with 4.44 µM BA and 0.54 µM NAA (V2 variant). Moreover, four transfers at 60 days on V2 medium variant were performed to improve the multiplication ratio. Then, the plantlets were simultaneously transferred on V2, V3 (BA 0.44 µM and NAA 0.05 µM) and V4 (without PGRs) medium variants, for 60 days to improve the roots formation. The last 6 transfers before the molecular analysis, each after 60 days, were performed on V4 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).

SSR analysis

Genomic DNA was isolated from leaves using CTAB method described by Doyle and Doyle (1987). For SSR analysis plants from the two *in situ* populations were used, each with 5 individuals (previously mentioned) and plant material from *in vitro* culture after 2 years of culture. During the 2 years of *in vitro* culture some of the individuals died because of infection or necrosis. Molecular analyses were accomplished in 1/3 somaclones of each individual with normal growth. *In vitro* plants were annotated as follows: the individuals belonging to population D.s. 1 were encoded as 1.1v1, 1.1v2, 1.2v1, 1.2v2, 1.2v3, 1.4v1, 1.4v2, 1.5.v1 (the 3rd digit from each codification represents the somaclone's code) and the individuals belonging to population D.s. 2 were encoded 2.2v1, 2.2v2, 2.3v1, 2.3v2, 2.3v3, 2.4v1. A total of five SSR primer pairs - DCAMCRBSY, DCDIA30, DINCARACC, DINGSTA, DINMADSBOX (Smulders et al. 2000) – producing reproducible and clear bands ranging from 75 to 300 bp in size, both in plants from natural habitats and *in vitro* plants, were used. 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 the following program: 1. T = 94°C, 5 min; 2. T = 94°C, 45 s; 3, primer annealing at 55°C, 45 s; 4. elongation T = 72°C, 45 s; steps 2-4 were repeated 35 times. Five primers have been tested (Table 1). Amplicons were separated on 1.5% agarose gel, stained with 0.5 µg ml^{-1} ethidium bromide. At least two independent PCR amplifications were performed for each primer.

Data analysis

The experimental design for *in vitro* propagation consisted on two repetitions for each experiment and on 12 explants per each individual. The results were expressed as the average of replicates ± standard deviation (SD). Data were subjected to One-Way ANOVA

Table 1. Characteristics of primers used for DNA amplification.

Name	Acc. no. EMBL	Start of repeat	Repeat motif	Forward and reverse sequence
MS-DCAMCRBSY	Z18952	1369	(CAA)17	5'-CAACAATGACAACAACATCAG-3' 5'-TCT TCG ATTGTTGAAGCTAAG-3'
MS-DCDIA30	X59260	1010	(TA)7–1	5'-CACTGACGACACAGCTGATGT-3' 5'-ACTCGTCCAAACACAAACGAC-3'
MS-DINCARACC	M66619	0057	(TA)8	5'-GGTCTTAAT TT TGTCACTTT-3' 5'-AhCCCATCAAAGTACTCCAAAT-3'
MS-DINGSTA	L05915	5347	(T)23–1	5'-CACAAACCTGAAAGTACGATC-3' 5'-ACATTCGAGCCCTCATATAAG-3'
MS-DINMADSBOX	L40805	2695	(TA)7	5'-ACGAGTGTCCAGGATCG-3' 5'-CCCCTATTGCAAACTGC-3'

test (for testing 3 or more columns) with Tuckey`s Multiple Comparison post test, or to *t* tests (for testing 2 columns), at 95% confidence intervals. The statistical analysis was performed using GraphPad Prism (version 5.00 for Windows, GraphPad Software, San Diego). Several genetic diversity parameters of individuals belonging to the populations from natural habitats and *in vitro* plants were calculated according to Liu and Muse (2005). These included gene diversity (expected heterozygosity), observed heterozygosity (referred to as heterozygosity), and Polymorphism Information Content (PIC) value.

RESULTS AND DISCUSSIONS

In vitro propagation

The aseptic explants were between 57% and 70%, depending on individuals. The viability index after disinfection was between 99.3% and 100%.

The higher mean number of shoots was 23.6 and 49.9 for the Hăşmaş Mt. population (D.s. 1) and for the Vlădeasa Mt. population (D.s. 2) respectively, on V2 culture medium, with 4.44 µM BA and 0.54 µM NAA (Fig. 1 A), but on this culture medium the root formation was not obtained (Table 2). We consider that some of the somaclones were hypersensitive to cytokinins, and therefore, the rhizogenesis was not obtained and/or the hyperproliferation phenomenon appeared (Fig. 1 B). On the V3 and V4 variants of the medium, with low concentration of PGRs and without PGRs, respectively, the multiplication was reduced but formation of roots and root growth was achieved. The best rhizogenesis was recorded on medium without PGRs. Thus, mean number of roots was 11.1 in population D.s. 1 and 5.1 in population D.s. 2 (Table 2). It is obvious that population D.s. 1 possesses better rhizogenesis process, but weaker multiplication, compared to population D.s. 2. By subsequent transfers, the multiplication rate increased even on the medium without PGRs. After more transfers, during 60 days of culture, mean number of shoots was 28.8 in population D.s. 1 and 74.9 in population D.s. 2 on V2 medium (Cristea et al. 2013).

SSR analysis

All loci amplified well in nearly all individuals tested (Table 3). By amplification with DCAMCRBSY primer, 1-3 fragments were obtained in plants from natural habitats and 1-2 fragments in plants from *in vitro* culture. These fragments had 175, 150 and 100 bp (Table 4). The fragments of 175 and 150 bp were present only in some individuals from the two populations, but the fragment of 100 bp was present in all individuals, independently of population and culture conditions.

Amplification with DCDIA30 primer showed only one fragment of about 175 bp, which was present in all individuals from both populations but was missing in some individuals from *in vitro* plants like 1.1v1, 2.2v1, and 2.2v2.

The most polymorphic patterns were obtained by amplification with DINCARACC primer. Three fragments of about 250, 200, and 150 bp were observed both in plants from natural habitats and *in vitro* plants. The individuals belonging to population D.s. 1 showed all two fragments, while some of the individuals belonging to population D.s. 2 showed only the fragment of about 250 bp. *In vitro* plants showed only 1-2 fragments but the patterns were very polymorphic.

Amplification with DINGSTA primer showed two fragments having 250 and 175 bp. The fragment of 175 bp was missing in plants from natural habitats independently of population, but it was present in some plants from the *in vitro* culture.

By amplification with DINMADSBOX primer only one fragment of 130 bp was obtained which was present in all individuals except 2.2v1 and 2.2v2.

The highest allele frequency in the polymorphic loci (0.62) was observed for MS-DCAMCRBSY 100 pb allele and DINGSTA 250 pb allele (0.55), and the lowest (0.10) was obtained for MS-DCAMCRBSY 175 pb allele (Table 4). Two loci were monomorphic. The distribution power of each marker was estimated by the polymorphism information content (PIC) value. PIC value ranged from 0 for the markers generated for MS-DCDIA30 and DINMADSBOX loci (monomorphic loci) to 0.58 for the MS-INCARACC locus. The

Table 2. Effect of culture medium on the multiplication and rhizogenesis of *D. spiculifolius* binodal shoot tips, after 60 days of culture.

Population	D.s. 1			D.s. 2		
Culture medium	V2	V3	V4	V2	V3	V4
No of shoots	23.6 ± 7.5	9.3 ± 0.8*	3.4 ± 0.7**, ns	49.9 ± 12.1	19.7 ± 4.2 **	13.7 ± 4.8 **, ns
Rooted explants (%)	0.0 ± 0.0	77.8 ± 4.8***	86.1 ± 7.6***, ns	0.0 ± 0.0	66.7 ± 8.3***	61.1 ± 4.8***, ns
No of roots	0.0 ± 0.0	8.6 ± 0.6***	11.1 ± 1.2***, *	0.0 ± 0.0	4.6 ± 0.6 ***	5.1 ± 0.6 ***, ns

The values are mean ± SD. a) Statistical differences between the three culture medium variants: * Significant difference ($p < 0.05$); ** Highly significant difference ($p < 0.01$); *** Extremely significant difference ($p < 0.001$); First data on V3 and V4 represents the statistical differences from V2. Second data on V4 represents the statistical difference from V3. b) Statistical differences between D.s. 1 and D.s. 2 populations: ns - no statistical significance ($p > 0.05$), except the No of roots on V4 variant.

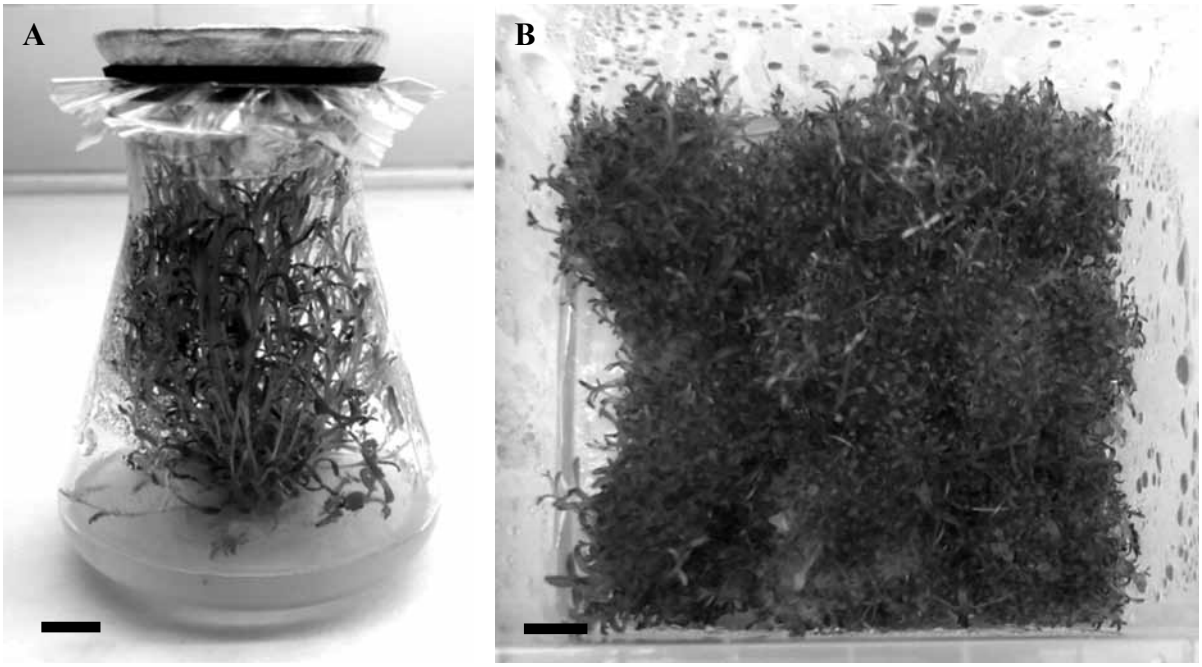

Fig. 1. *In vitro* propagation of *D. spiculifolius*. A) *In vitro* multiplication at individual D.s. 2.2., on V2 culture medium, with 4.44 μM BA and 0.54 μM NAA, 60 days of culture, B) Phenomenon of hyperproliferation at individual D.s. 2.2., 60 days of culture.

mean PIC value for all loci was 0.28. Markers with high PIC values such as MS-INCARACC and MS-DCAM-CRBSY could be effectively used in genetic diversity studies of *D. spiculifolius*. The highest genetic diversity and the proportion of the heterozygous individuals at a particular locus (observed heterozygosity) were also observed in these two loci (Table 5).

The analysis of genetic variability of *D. spiculifolius* individuals belonging to natural habitats and *in vitro* plants showed some changes concerning the absence/presence of certain fragments in some individuals. This could be explained by the somaclonal variation that could appear as a result of *in vitro* culture. Similar studies with *Dictyospermum ovalifolium* (Chandrika et al. 2008) also show the appearance of variability in micropropagated plants by the absence/presence of certain fragments, while in guava plants (Rai et al. 2012) the homogeneity of *in vitro* regenerated plants was shown by SSR and ISSR markers. Other molecular markers such as RAPD were also used to analyze the genetic stability of *Erigeron nanus* (Catană et al. 2010) or *D. caryophyllus* and *D. chinensis* (Kumawat et al. 2013), but SSR and ISSR markers are more informative, cost efficient, overcome hazards of radioactivity and require lesser amounts of DNA (Zietkiewicz et al. 1994, Bairu et al. 2011). These markers have been successfully used in analyzing the genetic fidelity of *in vitro* plants (Liu et al. 2011, Sharma et al. 2012). Genotyping of carnation varieties was accomplished with SSR markers (Smulders et al. 2003) and genetic diversity of *Dianthus* accessions was assessed using molecular marker such as SRAP and ISSR (Fu et al. 2008), but there are no previous data regarding genetic diversity of endangered and/or threatened *Dianthus* species and *in vitro* plants of these species.

Molecular analysis by SSR on *D. spiculifolius in*

Table 3. Fragments obtained by amplification with SSR primers.

D. spiculifolius populations	Number of fragments per population				
	MS-DCAMCRBSY	MS-DCDIA30	MS-DINCARACC	MS-DINGSTA	MS-DINMADSBOX
D.s. 1	1-3	1	1-4	2	1
D.s. 2	1-3	1	0-1	1-2	1
In vitro plants from D.s. 1	1-2	0-1	2-3	2	1
In vitro plants from D.s. 2	1-2	0-1	2-3	0-2	0-1

Table 4. Allele frequencies at 5 SSR loci.

Locus	Allele	Frequency
MS-DCAMCRBSY	100	0.625
MS-DCAMCRBSY	150	0.2750
MS-DCAMCRBSY	175	0.1000
MS-DCDIA30	175	1.0000
MS-DINCARACC	150	0.2632
MS-DINCARACC	200	0.3947
MS-DINCARACC	250	0.3421
MS-DINGSTA	175	0.4444
MS-DINGSTA	250	0.5556
MS-DINMADSBOX	150	1.0000

Table 5. Gene diversity, heterozygosity and PIC values of 5 SSR loci.

Locus	Gene diversity	Hetero-zygosity	PIC
MS-CAMCRBSY	0.5238	0.5500	0.4553
MS-DCDIA30	0.0000	0.0000	0.0000
MS-INCARACC	0.6579	0.5789	0.5836
MS-DINGSTA	0.4938	0.4444	0.3719
MS-DINMADSBOX	0.0000	0.0000	0.0000
Mean	0.3351	0.3147	0.2822

vitro propagated plants showed that the genetic variability of plants from natural habitats was conserved by *in vitro* culture and that the genetic differences between somaclones derived from the same individual are low. These differences could be explained by the specific culture conditions. It is known that several concentrations of PGRs and long term of *in vitro* culture could induce genetic mutations (Bordallo et al. 2004, Gaafar and Saker 2006, Singh et al. 2009).

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