USING RAPD TECHNIQUES TO CHECK THE GENETIC STABILITY OF *Erigeron nanus* SCHUR REGENERANTS IN THE *EX SITU* CONSERVATION CONTEXT

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Abstract. In this study we intended to check the genetic stability of micropropagated shoots obtained through indirect organogenesis of a rare, Carpathian endemic *Erigeron nanus* Schur plant species using the RAPD techniques.

Our study is joining to the Convention on Biological Diversity's 2010 target, which consists in the reduction of the current rate of biodiversity loss, trying to check the genetic stability of micropropagated plants at *Erigeron nanus* Schur for the conservative purpose.

An *in vitro* propagation protocol through indirect organogenesis has been developed for multiplication of *Erigeron nanus* Schur and has been used continuously as an initial step for the production and maintenance of shoot cultures.

Reproducible RAPD patterns were obtained using 4 primers which showed monomorphic bands in the all samples tested. Despite of the pre-request conditions for somaclonal variation (indirect micropropagation pathway - via calli), duration of *in vitro* culture (more than 12 months), *in vitro* stress due to unnatural conditions, the tested primers showed any differences in the RAPD pattern of the regenerants.

Keywords: *Erigeron nanus* Schur, RAPD pattern, *in vitro* culture, indirect organogenesis

INTRODUCTION

In the last time, the biodiversity loss is one of the world's most pressing crises. A summary of overall changes in numbers of threatened plant species over recent years showed that the number of threatened species in all IUCN Red Lists from 1996 to the current Red List is increased from 909 to 1.577 for critically endangered, 1.197 to 2.316 for endangered and 3.222 to 4.607 for vulnerable category [47]. According to some authors, 14.5% from the total number of taxa from Romanian Flora are endangered [11].

The governments, scientific world and civil society have responded to this challenge by setting clear conservation targets to reduce the current rate of biodiversity loss, such as the Convention on Biological Diversity's 2010 target [42].

Regarding the priority in conservation according to taxonomic categories, the rare plant species have the highest priority induced by their restrictive area [20].

The genus *Erigeron* has a cosmopolitan distribution. Some species are used as: ornamental plants, as food for larvae of some *Lepidoptera* species and also like medicinal plants (*E. breviscapus, E. annuus*) [31].

Erigeron nanus Schur is a vulnerable/rare plant species for Romania [33, 34, 19], a charpatian endemit [9, 43] and is considered as a target species of European concern [35].

Numerous DNA-based markers can be used for screening genetic stability of tissue cultured plants [1, 21]. One of the most used techniques is Random Amplified Polymorphic DNA (RAPD). This technique require only small amount of DNA sample without involving radioactivity tests and are simpler as well as faster. RAPD has proven to be quite efficient in detecting genetic variations [44] and is used for phylogeny studies [3, 17, 18, 38, 39, 41].

In the recent years, the RAPD markers was used for genetic variability investigations in the plant species collections [4, 24, 26, 40] and also for detection of somaclonal variation of the *in vitro* regenerated plants [2], like *Populus deltoides* [36], *Begonia* [6], etc.

The present paper deals with investigations related to genetic stability of *Erigeron nanus* Schur regenerants obtained through indirect organogenesis. According to the authors knowledge this is first description into field of genetic stability of the regenerants obtained through *in vitro* cultures of the *Erigeron nanus* Schur.

MATERIALS AND METHODS

The plant material used for the initiation of the micropropagation protocol was represented by seeds, which were germinated aseptically. The seeds of *Erigeron nanus* Schur were collected from the natural habitat in 2006 from only one plant. The aseptic shoot cultures used in the present study were established as reported in an earlier study [5] and were maintained on the media containing Murashige and Skoog [30] basal salts (MS) supplemented with sucrose 30g l^{-1}, added with plant growth regulators like N^6-benzyladenine (BAP) 1 mg l^{-1}, 1-naphthalene acetic acid (NAA) 0.1 mg l^{-1} and kinetin 1 mg l^{-1}. The pH was adjusted to 5.8 before autoclaving at 121ºC for 20 min. Cultures were maintained at 25 ± 2ºC under a 16 hrs light / 8 hrs dark photoperiod. The *in vitro* cultures were established and maintained on the same media variant for more than 12 months with repetitive cultures at every 3 months.

The genomic DNA from the *Erigeron nanus* regenerants obtained through micropropagation was extracted after 12 months of *in vitro* culture. The DNA was extracted by using Genomic DNA Purification Kit, Fermentas following the manufacturer protocol. Quality and quantity of DNA preparations were checked by 0.8% Sigma agarose gel.

RAPD amplifications were performed routinely using PCR mixture (25 µl) which contained: 1X buffer, 0.2 mM dNTPs, 1.5 µM primer, 1U *Taq* DNA polymerase, 2.5 mM $MgCl_2$, 25 µl ADN. PCR was performed at initial denaturation at 94ºC for 3 minutes,

followed by 35 cycles of 1 minute denaturation at 94ºC, 1 minute annealing at 36ºC and 2 minutes extension at 72ºC with a final extension of 72ºC for 2 minutes using a Termocycler '*Gene Amp9600'* (Applied BioSistem). The PCR products obtained were separated on 1.2% agarose gel, stained with ethidium bromide (0.001%). Molecular size of the amplification products were estimated by using a GeneRuler 1Kb DNA Ladder Fermentas. The primers used are presented in the Table 1.

The DNA profiles were analyzed using GelQuest Ver 2.7.1. – a DNA Fingerprint Analysis Software, by SequentiX- Digital DNA Processing, Klein Raden, Germany [14]. To measure the similarity between individuals, the Jaccard coefficient [19] was used. The Jaccard similarity coefficient, *J*, is given by the formula:

$$J = \frac{M_{11}}{M_{01} + M_{10} + M_{11}}$$

where: M_{11} represents the total number of attributes where *x* and *y* both have a value of 1; M_{01} represents the total number of attributes where the attribute of *x* is 0 and the attribute of *y* is 1; M_{10} represents the total number of attributes where the attribute of *x* is 1 and the attribute of *y* is 0.

RESULTS

The plant material used for this experiment was obtained starting from a germinated seed. The indirect organogenesis, based on a previous protocol [5], was induced from leaves fragments inoculated on MS basal media added with N^6-benzyladenine (BAP) 1 mg l^{-1}, 1-naphthalene acetic acid (NAA) 0.1 mg l^{-1} and kinetin (1 mg l^{-1}). In the first month of *in vitro* culture, the explants changed the color from green to brown and started to degenerate (Fig. 1a, 1b). This process stopped after first subculture. The number of regenerats/explant was around 8. Being a rare plant species and because of the reduced number of the regenerants, the samples used for RAPD analysis was reduced (Fig. 1c).

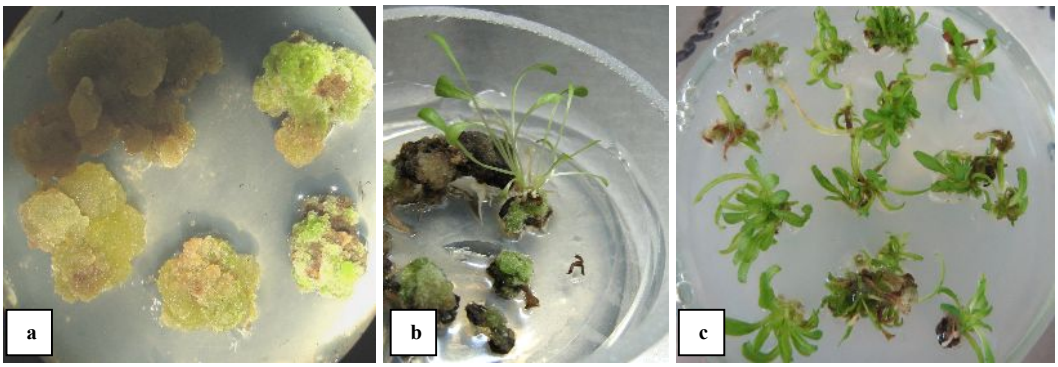

Figure 1. Indirect organogenesis and plant regeneration from leaf explant of *Erigeron nanus* Schur inoculated on the media MS added with BAP 1 mg l^{-1}, NAA 0.1 mg l^{-1} and kinetin 1 mg l^{-1}: **a)** calli formation, **b)** degenerated explant and regeneration, **c)** regenerants obtained through indirect organogenesis used for the evaluation of genetic stability.

In order to confirm the genetic fidelity, a comparison of RAPD patterns of the 7 regenerants obtained through indirect organogenesis (soma-clonal variation pre-requisites) from the same initial plant was carried out.

The DNA extraction was performing using Genomic DNA Purification Kit (Fermentas). Despite of the small quantity of fresh material (0.2 g), the DNA samples obtained was of a good quality and quantity (Fig. 2).

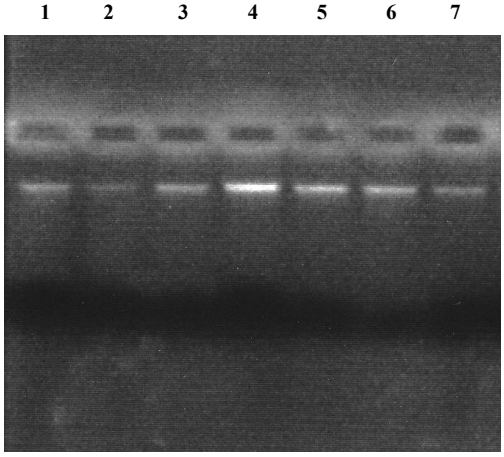

Figure 2. *Erigeron nanus* Schur genomic DNA**.** 1-**7** *Erigeron nanus* Schur regenerants.

A total of 39 reproducible bands with an average of 9.75 bands/ primer were obtained from the 4 primers used. The size of amplified fragments varied between 300 and 20.000 bp (Table 1). A distance matrix was constructed using the Jaccard (1901) similarity index (Table 2). The RAPD banding pattern showing

monomorphic bands obtained among the 7 clones regenerated through indirect organogenesis and maintained in the same condition for more than 1 year is shown in Fig. 3.

Table 1. Primers used for amplification of RAPD analysis.

Serial number	Name of primer	Primer sequence (5′–3′)	Total number of amplified fragments
1.	OPA-2	5'TGCCGAGCTG3'	13
2.	OPA-20	5'GTTGCGATCC3'	9
3.	OPG-14	5'GGATGAGACC3'	11
4.	PSG-2	5'GTTTCGCTCC3'	6
		Total	**39**

Table 2. Similarity index (Jaccard's coefficient) of the tested samples.

Sample	Sample						
	0	**1**	**2**	**3**	**4**	**5**	**6**
6	0.86486	0.78788	0.79412	0.91667	0.84211	0.79412	**0.00000**
5	0.76667	0.90625	0.80000	0.90323	0.85294	**0.00000**	
4	0.51852	0.94444	0.60714	0.72414	**0.00000**		
3	0.65385	1.00000	0.74074	**0.00000**			
2	0.76667	0.87097	**0.00000**				
1	0.90909	**0.00000**					
0	**0.00000**						

Range=0.51852-1.00000; Avarage=0.8078

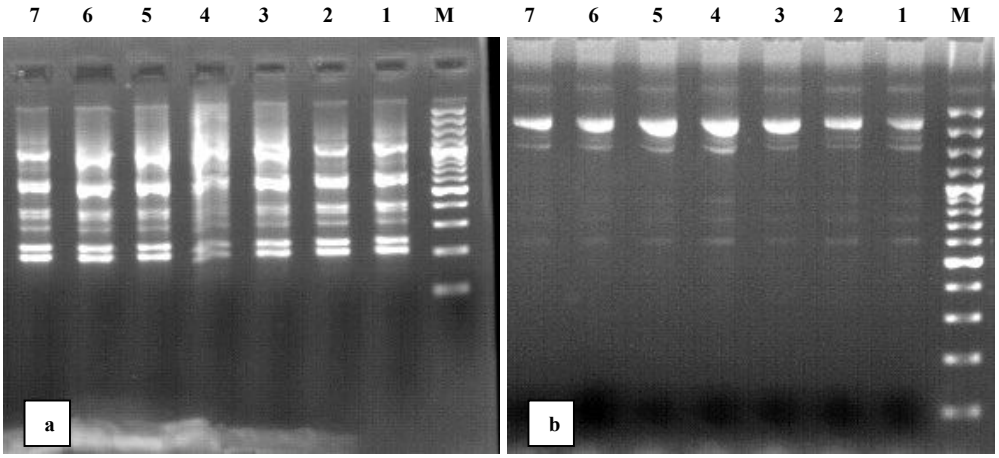

Figure 3. Molecular analysis of *Erigeron nanus* Schur *in vitro* regenerated through indirect organogenesis using RAPD. Electrophoretic analysis of DNA amplification with the primers OPG-14 (a) and PSG-2 (b). Lanes designated as M represents GeneRuler^{TM} 1 Kbp Plus DNA Ladder.

DISSCUSSION

The analysis of genetic stability at molecular level are essential in the *ex situ* conservation context. True-to-type clonal fidelity is one of the most important prerequisites in the micropropagation of plant species. It was recognized that alterations in auxin-cytokinin concentrations and their ratio, duration of *in vitro* culture, *in vitro* stress due to unnatural conditions and alteration of nutritional conditions together or independently are responsible for somaclonal variation [28]. The data concerning the genetic stability after *medium* and *long term* conservation of propagated species are restricted [16].

Several studies concerning the plant regeneration of *Erigeron* genus were done. Some of these studies are regarding the *in vitro* effects of some active compounds in *Erigeron canadensis* [10], *E. annuus* [25] and *Erigeron breviscapus Vant* [45]. Also, an efficient micropropagation system for *Erigeron breviscapus* (Vant.) Hand. Mazz., an important medicinal plant for heart disease, has been developed. The number of regenerated plants on the MS basal salts and vitamin medium containing 5 µM N^6-benzyladenine (BAP) and 5 µM 1-naphthalene acetic acid (NAA) was around 17 [8]. The same result concerning the necrosis of the callus was obtained by cultured under the light of the leaf explant of *E. breviscapus* [46].

Regarding the molecular studies concerning genetic variability of *Erigeron* genus, also were perform. The variability of *E. annuus* populations used RAPD techniques was analyzed [12]. Some studies concerning the biogeography and the phylogeny of *Erigeron* were also performed using ITS (internal transcribed spacer) [32].

Variation in the ability to produce RAPD fragments depended on the primer. In this case the total number of bands varied between 6 (PSG-2) and 13 (OPA-2) (Table 1).

A total of 273 bands (number of individuals X number of bands with all primers) were generated through RAPD techniques. The primers used generated monomorphic pattern across all 7 clones analyzed.

According to the similarity index by Jaccard's coefficient (Table 2), the range of the similarity varied between 0.51852 and 1.00000, with an average equal with 0.8078. The lowest similarity found was between the sample 0 and sample 4 with a value of 0.51852. The sample 1 and the sample 3 gave the highest ratio, 1.00000. This high similarity between samples indicated that there is a low variability between the studied regenerants.

Despite of the pre-request conditions for somaclonal variation [indirect micropropagation pathway (via calli)], duration of *in vitro* culture (more than 12 months), *in vitro* stress due to unnatural conditions, the tested primers showed any differences in the RAPD pattern of the *Erigeron nanus* regenerants with the same origin (Fig. 3).

Our results are concordant with the results of some authors whom did not find genetic changes in the *in vitro* conserved shoots of different plants like *Curcuma longa* [35], *Vetiveria zizanioides* Nash [29], *Pinus thunbergii* [15], *Drosera anglica* and *Drosera binata* [22], *Arachis retusa* [13], *Chlorophytum borivilianum* Santapau [37], chestnut root stock hybrids [7], etc.

The result obtained in our experiment suggest that *in vitro* micropropagation protocol established previously, despite of the indirect way, can be used for multiplication and conservation for more than 12 months of the rare *Erigeron nanus* Schur. with a low risk of somaclonal variation.

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