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INTER-SIMPLE SEQUENCE REPEAT ANALYSIS OF GENETIC FIDELITY IN *IN VITRO* MICROPROPAGATED PLANTS OF THE ENDANGERED PLANT SPECIES *CENTAUREA DAVIDOVII* URUM. (ASTERACEAE)

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Abstract: The genus *Centaurea* s. l. (Asteraceae) is one of the richest in endemics in the Bulgarian flora, represented by more than 70 species. One of them, the endemic *Centaurea davidovii* Urum., occurs in several very fragmented populations in the floristic region of Balkan Mountains. Habitat destruction and low reproductive capacity of this species, mainly due to weak seed germination and damage of seeds by insects, necessitate its conservation both *in situ* and *ex situ*. An *ex situ* collection of *Centaurea davidovii* individuals has been established recently at the experimental field of the Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences. Plants had been obtained from a single seed using *in vitro* multiplication techniques. They were successfully acclimatized to the open-air field plot, bloomed and formed fertile seeds. True to type clonal fidelity is one of the most important prerequisites in the micropropagation of any plant species. A major problem encountered with the *in vitro* culture is the potential presence of somaclonal variation. Various factors, such as the *in vitro* process and its duration, nutritional conditions, *in vitro* stress, etc. occasionally appear to induce somaclonal variation. Hence, it becomes imperative to regularly check the genetic integrity of the *in vitro* regenerated plants in order to produce clonally uniform progeny. In the present study, the genetic stability of *in vitro* propagated *Centaurea davidovii* plants was tested for the first time using Inter Simple Sequence Repeat (ISSR) markers. The amplification products were monomorphic and no polymorphism was detected. Furthermore, ISSR analysis indicated the genetic integrity of *in vitro* propagated plants, as well as an identical banding pattern after more than ten passages of subculture. Our results showed that the regenerated plants are similar and therefore the developed micropropagation protocol for *in vitro* multiplication of *C. davidovii* is reliable and

represents a safe mode for production of true-to-type plants. Besides the establishment of the *ex situ* collection, planting of *in vitro* obtained plants in the wild populations has been designed aiming at their strengthening. This *in situ* measure should be conducted with strict respect to the genetic diversity conservation, which requires improvement of the seed germination rate and regeneration of plants of numerous normal genotypes.

INTRODUCTION

Centaurea davidovii is a Bulgarian endemic, represented nowadays by seven or eight very fragmented subpopulations, each with an area of occupancy of about 1-2 decares. The total number of individuals of this species does not exceed 1000. The plant populations are decreasing in size and the species is considered critically endangered, according to the IUCN criteria (Bancheva and Gorgorov, 2010).

The limited natural resources of *Centaurea davidovii*, as well as its low reproductive capacity require an urgent development of a long-term conservation program with multidisciplinary investigations on preservation and sustainable use of available genetic resources of this endangered plant species.

The use of biotechnological tools, such as *in situ* and *ex situ* conservation is a key strategy for long term conservation of endangered plant species (Henry, 2006). An *ex situ* collection of *Centaurea davidovii* individuals has been established recently at the Institute of Biodiversity and Ecosystem Research, BAS. The regenerated plants had normal vegetative and reproductive organs and intensive growth on the open experimental field. Their fertility has been manifested by mass flowering (**Fig. 1**) and formation of seeds which were able to germinate and to give rise to *in vitro* flowering plantlets (Gorgorov et al., 2015).

Fig. 1. *Ex situ* collection of *C. davidovii* established from *in vitro* multiplied plants. **A.** Acclimatized plants on the open field (May, 2014); **B**. Flowering stage (June, 2015)

True to type clonal fidelity is however one of the most important prerequisites in the micropropagation of any plant species (Chandrika et al., 2010). It is well known that somaclonal variation occurs ocasionally, depending on different factors, such as the species and the *in vitro* conditions, especially in case of callus derived plantlets (Nwauzoma and Jaja, 2013). On the other hand, the different methods of clonal propagation increase the genetic fidelity of the propagated plants, but also provoke genetic instability, stimulating DNA mutations of the regenerants (Bairu et al., 2006).

The present study was undertaken as a rapid screening for evaluation of the genetic stability of mature plants obtained by *in vitro* micropropagation of *Centaurea davidovii*, using the Inter Simple Sequence Repeat (ISSR) technique. This is the first molecular assessment of *C. davidovii*, as the first attempt for *in vitro* cultivation of this endemic plant species has been published recently. Molecular analyses of genetic diversity and population structure of this species are also contemplated as a part of other particular study.

MATERIALS AND METHODS

Plant material

An *ex situ* collection of *Centaurea davidovii* individuals has been established recently at the experimental field of the Institute of Biodiversity and Ecosystem Research, BAS. Plants were obtained from a single seed using *in vitro* multiplication techniques for direct and indirect regeneration, as previously described (Gorgorov et al., 2015). They were successfully acclimatized to the open-air field plot, bloomed and formed fertile seeds.

DNA extraction

Genomic DNA was extracted from leaf tissues (50 mg) of nine *Centaurea davidovii* plants from the *ex situ* collection, following the procedure described by Doyle and Doyle (1987). All DNA samples were quantified using NanoDrop® ND-1000 spectrophotometer (Thermo Fisher Scientific, USA).

Inter Simple Sequence Repeat Analysis

The following ten ISSR primers (Microsynth, Balgach, Switzerland) were used in the analysis of genetic integrity of nine randomly chosen *in vitro* propagated *Centaurea davidovii* plantlets:

ISSR 1: 5' - GAG AGA GAG AGA GAG AT - 3'
ISSR 2: 5' - GAG AGA GAG AGA GAG AA - 3'
ISSR 3: 5' - CAC ACA CAC ACA CAC AG - 3'
ISSR 4: 5' - ACA CAC ACA CAC ACA CT - 3'
ISSR 5: 5' - ACA CAC ACA CAC ACA CC - 3'
ISSR 6: 5' - ACA CAC ACA CAC ACA CG - 3'
ISSR 7: 5' - AGA GAG AGA GAG AGAGYT - 3'
ISSR 8: 5' - AGA GAG AGA GAG AGA GYC - 3'
ISSR 9: 5' - GAG AGA GAG AGA GAG AYG - 3'
ISSR 10: 5' - ACA CAC ACA CAC ACA CYT - 3'

The ISSR-PCR analyses followed Petrova et al. (2014) with minor modifications. All PCR reactions were performed in a volume of 25 µl (1X PCR buffer (GenetBio, Korea), 1U Taq DNA polymerase (GenetBio, Korea), 100 µM of each dNTP, 1 µM of each primer and 50 ng of extracted DNA). The PCR experiments in the present study were performed using a a Techne TC-5000 Thermal cycler at the following cycling conditions: initial denaturation at 95ºC for 5 min, followed by 35 cycles of amplification [45 s at 94ºC, 1 min at the annealing temperature (T_a is adjusted according to the T_m of each ISSR primer being utilized in the reaction), elongation at 72ºC for 2 min] and a final elongation at 72ºC for 5 min. The PCR products were analyzed on 2% agarose gel electrophoresis in 0.5X TBE buffer (1.5 h at 120 V). The gels were stained by Ethidium bromide (0.5 mg/ml in TBE). The DNA - profiles were visualized with a UV - transilluminator and further analyzed along with 100 bp DNA ladder size (Thermo Scientific, Vilnius, Lithuania) using a video image analyzer (BioImaging Systems, Cambridge, UK). The well resolved and consistently reproducible amplified DNA fragments as bands were scored with regards to their presence (1) or absence (0).

RESULTS AND DISCUSSION

True-to-type clonal fidelity is one of the most important prerequisites in the micropropagation of any plant species. Many studies have however shown that the major problems in the protocols for *in vitro* cultures are the potential presence of somaclonal variation among sub-clones of one parental line, as well as the loss of genetic stability caused by genetic and epigenetic variations in regenerants (Tiwari et al., 2010). High concentrations of different plant growth regulators may induce somaclonal variation in different development stages (Bairu et al., 2006). The genetic instability is however highly unacceptable, especially in cases, where the *in vitro* collection is directed to the conservation of germplasm of endangered and rare species (Mallón et al., 2008).

The molecular approaches are being used extensively for scrutinizing the genetic stability of *in vitro* raised plants. As DNA based markers are not influenced by environmental factors, they present the most effective way to screen the tissue culture induced variations (Peredo et al., 2009). Nowadays, DNA markers are particularly useful for assessing genetic stability in many micropropagated plants ranging from

economically valuable clones (Valladares et al., 2006) to endangered plant species (Guo et al., 2007). As such, they are very important when there is no prior knowledge of the genome under consideration (Agarwal et al., 2008).

Among the PCR - based marker technique, ISSR molecular markers offer an advantage of being considerably less expensive and less time-consuming, because of the reduced number of protocol steps required and the smaller amounts of DNA consumed. Furthermore, no prior genomic information is required for their use and they can detect a greater number of polymorphisms than RFLP or RAPD (Bornet et al., 2002; Ye et al., 2005; Seyedimoradi and Talebi, 2014; etc.). ISSR - system is considered suitable to detect variations among micropropagated plants since a simple sequence repeat targets the fast evolving hypervariable sequences (Parida et al., 2011).

So far, ISSR analysis has been successfully applied to study the genetic stability of micropropagated plantlets of different crops (Martins et al., 2004; Alizadeh et al., 2008; 2009; Tsvetkov et al., 2014; etc.). Due to the clonal origin of the plants from our *C. davidovii ex situ* collection, they have been expected to share identical genotypes with that of the initial seed.

In the present molecular study with nine randomly chosen micropropagated plants *C. davidovii*, along with the donor mother plant, all selected ISSR primers generated a unique set of amplification products ranging in size between 200 and 3000 bp, total of 115 bands within 200 and 3000 bp. The number of formed bands in each primer ranged from 8 to 15 (**Table 1**).

Table 1. Number and molecular weight range of bands generated as products of ISSR -PCR amplification with selected primers and DNA from *C. davidovii* individuals regenerated by *in vitro* culture.

ISSR primer	Annealing temperature (°C)	Total number of bands	Size range (bp)
ISSR 1	60	8	200-1000
ISSR 2	60	14	250-2650
ISSR 3	60	10	300-2500
ISSR 4	60	12	370-2750
ISSR 5	60	10	200-2500
ISSR 6	60	12	200-2500
ISSR 7	55	13	350-1550
ISSR 8	55	12	220-3000
ISSR 9	55	9	300-2700
ISSR 10	55	15	250-3000

The ISSR-profile of all analyzed regenerants was compared with that of the original mother plant of 'Kozyata Stena' chalet (**Fig. 2**).

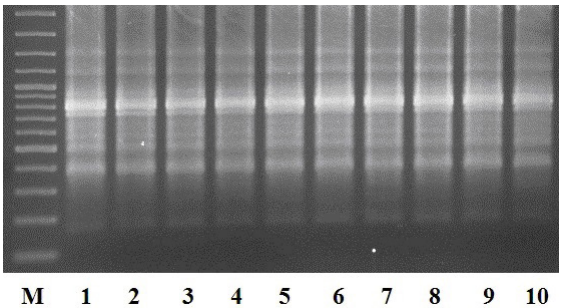

Fig. 2. Inter Simple Sequence Repeat (ISSR) amplification pattern obtained for mother plant (**10**) and daughter/micropropagated *C. davidovii* individuals (**1-9**) using primer ISSR7. **M**: 100bp DNA ladder

The obtained results showed that the ISSR-profile of all analyzed regenerants matches the profile of the original mother plant. The genetic stability of another species of genus *Centaurea, C. ragusina,* has been studied under the conditions of long-term *in vitro* culture (Radić et al., 2005). Authors reported polyploidy and aneuploidy concerning only single cells which did not affect the cytogenetic stability of the whole plants. The genetic integrity of the regenerated plants from the critically endangered Spain endemic *Centaurea ultreiae* has been demonstrated using Random Amplified Polymorphic DNA (RAPD) analysis, which did not reveal genomic alterations in any of the regenerated plants (Mallón et al., 2010). Here, we present that none of selected ISSR-primers showed polymorphism in the analyzed *Centaurea davidovii* plants which indicated the fidelity of regenerated plantlet population to the genotype of mother plant. Based on our findings, we suggest that the *in vitro* propagation did not cause variation in the genetic make-up of regenerated *C. davidovii* plantlets.

CONCLUSION

Since all ISSR-based bands were monomorphic and there were no variations detected in the micropropagated *C. davidovii* plants compared with the mother plant, indicating genetic stability among the clones, the previously developed micropropagation protocol could be carried out for a considerable length of time without much risk of genetic instability. However, no technique alone can completely guarantee the genetic fidelity of regenerated plants. Therefore, even if ISSR analysis showed no genomic alterations, as in the present study, this does not necessarily mean that there are none. Our finding has important implications

for „true-to-type“ micropropagation protocol for *C. davidovii,* which is crucial for urgent conservation of the species. However, further research needs to be undertaken to investigate the ploidy evaluation of *in vitro* regenerated plants in order to assure trueness-to-type.

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