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BRIEF COMMUNICATION

Genetic variability in regenerated plants of *Ungernia victoris*

O.M. BUBLYK, I.O. ANDREEV*, K.V. SPIRIDONOVA and V.A. KUNAKH

Institute of Molecular Biology and Genetics, NAS of Ukraine, Acad. Zabolotny st. 150, Kyiv, 03680, Ukraine

Abstract

To determine the suitability of micropropagation techniques developed for conserving rare medicinal herb *Ungernia victoris* we estimated the genetic fidelity of plants produced through direct regeneration from the bulb scale segments and organogenesis from long-term callus culture. Average value of the Jaccard's distances between explant-derived regenerants and maternal plants calculated from RAPD data was 0.5 %, while that of estimated between callus-derived regenerants and maternal cell line was 4.2 %; average distances between the objects among the explant-derived and callus-derived regenerants were 0.7 % and 2.5 %, respectively. The data obtained suggest that conditions for *in vitro* culture applied in this work provide relatively high genetic stability of the species upon the direct regeneration *in vitro* and regeneration from the long-term cultured callus.

Additional key words: micropropagation, RAPD-analysis, somaclonal variation, tissue culture.

The use of the biotechnological approaches for accelerated propagation is one of the effective ways for conserving plant resources, and such techniques are developed to date for many plant species (Rani and Raina 2000, Rout *et al.* 2000, Kushnir and Sarnatska 2005). It is generally recognized that cultivation *in vitro* may generate genetic variation. To ascertain the genetic fidelity of plants regenerated *in vitro* (microclones) molecular genetic techniques are used. Random amplified polymorphic DNA - polymerase chain reaction (RAPD-PCR) technique allowing quickly scanning overall genome, being inexpensive and conservative in the use of genomic DNA, appeared to be the most widespread approach in such studies (Gupta and Varshney 1999, Gostimsky *et al.* 2005).

Ungernia victoris (*Amaryllidaceae*) is endemic plant of Tadjikistan and Uzbekistan. Preparations of isoquinoline alkaloids and biologically active polysaccharides from this plant are used for medical purposes (Khamidkhodzhaev 1982). Active exploitation of natural resources causes necessity to search the way of rapid propagation and conservation of the species. To date techniques have been developed for micropropagation of *U. victoris* both through direct regeneration from bulb scale segments and *via* regeneration from callus. The micropropagation would allow to produce up to one million bulblets within 1 year (Kunakh *et al*. 2008).

To determine the suitability of the *in vitro* propagation techniques developed for conserving *U. victoris* gene pool, we compare the genetic fidelity of the plants derived through direct regeneration from the bulb scale segments and indirect regeneration from long-term cultured callus using RAPD markers.

Twenty two plants of *Ungernia victoris* Vved. *ex* Artjushenko regenerated *in vitro* directly from bulb scale segments and 11 plants obtained *via* organogenesis from the long-term callus culture were analyzed. Additionally we included in analysis the maternal plants collected at the southern slopes of the Gissar Range (Tadjikistan) and six callus cell lines, derived from a single plant, including line, which was used for induction of organogenesis.

For direct regeneration bulb scale segments were cultured on 5C3N media supplemented with 0.5 mg dm^{-3} 1-naphtalene acetic acid (NAA) and 0.02 mg dm^{-3} kinetin, and 5C01 media supplemented with 2 mg dm^{-3} NAA and 1 mg dm^{-3} kinetin with mineral content according to Vollosovich *et al.* (1979). Complete composition of nutrient media and maintenance

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Abbreviations: NAA - 1-naphtalene acetic acid; PCR - polymerase chain reaction; RAPD - random amplified polymorphic DNA.
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* Corresponding author; fax: (+38) 044 526 07 59, e-mail: i.o.andreev@imbg.org.ua

conditions were described previously (Kunakh *et al.* 2008). For regeneration *via* organogenesis we used one of the six cell lines (line No 3), which were obtained from bulb scale segments around 7 years before the experiment and analyzed earlier (Kunakh *et al.* 2007, Bublyk *et al.* 2008a). Regeneration was induced on the medium 5C3N. Regenerants were grown *in vitro* for 0.5 to 2 years before analysis.

Nuclear DNA from bulbs of maternal and regenerated plants and callus tissues was isolated by standard sodiumdodecyl sulphate (SDS) method (Draper *et al*. 1988). RAPD-analysis was performed with 14 primers previously found to demonstrate somaclonal variability in *U. victoris* cell lines (Bublyk *et al.* 2008a,b). Amplification was performed in a *Tertsyk* four channel thermocycler (*DNK Tekhnologiya*, Moscow, Russia). Reaction mixture of 0.02 cm^3 contained 1× KCl-based PCR-buffer with 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U Taq polymerase (*Fermentas*, Vilnius, Lithuania), 0.6 μM primer and 20 ng analyzed DNA. For DNA amplification the following parameters were used: 94 °C/2 min; 5 cycles of 94 °C/30 s, 36 °C/30 s, 72 °C/40 s; then 35 cycles of 94 °C/20 s, 36 °C/20 s, 72 °C/40 s and final elongation at 72 °C/2.5 min. Each reaction was done in duplicate. Amplification products were separated in 1.7 % agarose gel (*Sigma*, St. Louis, USA) in 1× TBE buffer. After visualization of DNA with ethidium bromide the gels were photographed in UV-radiation.

To quantify the genetic polymorphism the RAPD-PCR amplification patterns were recorded as a binary matrix, in which presence or absence of fragments with the similar size as well as their significant differences in the fluorescence were scored as 1 or 0, respectively. Only clear reproducible bands were included in the analysis. The resulting binary matrix was used as the input for a computer program *FAMD* (Schluter and Harris 2006) for calculation of Jaccard's genetic distances (D_j = 1 - Jaccard's similarity coefficient) and creation the genetic similarity dendrogram with unweighted pair group method with arithmetic means (UPGMA).

We studied 22 plants obtained by direct regeneration, including four derived from one plant of *U. victoris* (group 1), and 18 from the other one (group 2). In addition we analysed both explant-donor plants. All explant-derived regenerants from the first group appeared morphologically normal. Five regenerants from the other group showed minor morphological anomalies such as thickened roots or thickened and malformed bulb scales. The regenerants from the group 1 were produced on medium 5C3N while the plants from the group 2 were regenerated and maintained on medium 5C01.

A total of 153 amplicons were scored for regenerants and maternal plant from the group 1 (Table 1). The number of amplified products per primer was on average about 10 and their size ranged from 280 to 1990 bp. Significant increase in the fluorescence of two polymorphic fragments was observed for two regenerants compared to the maternal plant (Fig. 1*A*). Jaccard's genetic distances between plant and explant-derived regenerants was 0 % or 1.3 % (on average 0.3 %), average value for the genetic distances between regenerants was 0.9 %.

A total of 152 RAPD-fragments were scored for regenerants and maternal plant from the group 2 (Table 1). Six fragments produced with four primers were polymorphic in this group. Four of them were lost in one regenerated plant (Fig. 1*B*), the remaining two showed quantitative variation – their fluorescence significantly rose in four other regenerated plants. No polymorphism of RAPD markers was observed between the remaining 13 regenerated plants *versus* the maternal plant.

Jaccard's genetic distances between the explant-donor plant and regenerated plants calculated from the RAPD data were 0 - 2.7 %, and those of estimated between the individual regenerants were below 4.0 %. Average value of genetic distances between maternal plant and explant-derived plants was 0.5 %, and that between the regenerated plants was 0.8 %. About 68 % of regenerated plants in both groups revealed no difference compared to RAPD patterns of the maternal plant.

We did not reveal appreciable difference in the level of variation between the explant-derived regenerants of *U. victoris* generated on two nutrient media with different phytohormone composition. Thus both nutrient media

Table 1. The number of all and polymorphic amplicons in two groups of explant-derived regenerants.

Primer	Nucleotide sequence (5'- 3')	Number of all/polymorphic amplicons		Primer	Nucleotide sequence (5'- 3')	Number of all/polymorphic amplicons	
		group 1	group 2			group 1	group 2
A04	AATCGGGCTG	13/0	11/2	A19	CAAACGTCGG	9/0	16/0
A05	AGGGGTCTTG	14/0	12/0	B03	CATCCCCCTG	9/0	6/0
A08	GTGACGTAGG	10/0	12/0	B05	TGCGCCCTTC	16/1	12/1
A09	GGGTAACGCC	12/0	10/0	B06	TGCTCTGCCC	6/1	10/1
A10	GTGATCGCAG	7/0	13/2	B08	GTCCACACGG	12/0	7/0
A11	CAATCGCCGT	11/0	10/0	B10	CTGCTGGGAC	11/0	10/0
A16	AGCCAGCGAA	13/0	13/0	total	number	153/2	152/6
A17	GACCGCTTGT	10/0	10/0		percentage	100/1.3	100/3.9

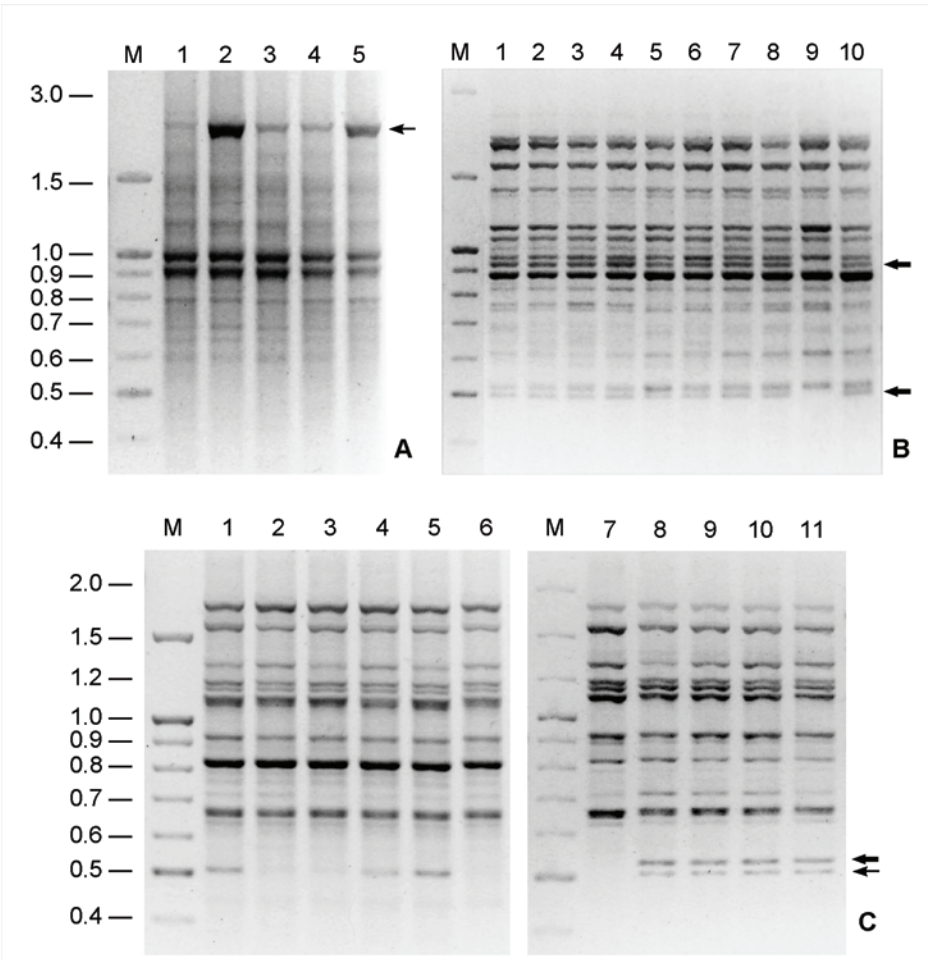

Fig. 1. The RAPD patterns generated from explant donor plants, regenerants and callus culture of *U. victoris*. *A*: 1 - maternal plant, 2 to 5 - explant-derived regenerants from group 1 (primer B05). *B*: 1 - maternal plant, 2 to 10 - explant-derived regenerants from group 2 (primer A04). *C*: 1 to 6 - callus cell lines Nos. 2, 3, 5, 6, 7 and 9a; 7 - line No. 3, 8 to 11 - callus-derived regenerants (primer A11); M - molecular mass marker. Bands with quantitative and qualitative polymorphism are indicated with *thin* and *bold arrows*, respectively.

may be used for obtaining genetically uniform material. However, the nutrient medium 5C3N was more effective in terms of induction of regeneration (data not shown).

We also performed the genetic analysis of 11 plants obtained by organogenesis from *U. victoris* callus subcultured for a period of about 7 years. All examined regenerants were the individual bulbs or bulb groups with normal morphology. Since the lack of the maternal plant's DNA we included in the analysis maternal cell line and additional five lines all derived from a single plant. Along with the difference from the original line we determined the divergence of the lines that allowed us to estimate the genetic changes resulting from tissue culture process.

A total of 152 fragments were scored for six cell lines and callus-derived regenerants (Table 2). Nine bands (5.9 %) were variable within the group of cell lines. Most of them, namely seven, showed quantitative variation (*i.e.*, reproducible differences in the fluorescence intensity). Genetic differences between regenerants and maternal callus cell line demonstrated 14 amplicons (9.2 %; Table 2), four of which displayed significant changes in fluorescence (Fig. 1*C*). One of the variable bands lacking within the RAPD patterns of the cell lines was observed in each regenerated plant, another four occurred only in some of the regenerants. The rest of the bands which differentiate regenerants and maternal cell line displayed variability both in plants and in cluster of cell lines.

Differences between regenerated plants were observed for 11 amplicons (7.2 %). Among them three fragments displayed quantitative variability. Singletons, fragments present or absent in a single plant, were represented by 5 fragments: four absence singletons and one presence singleton. The last one was also detected in four of six cell lines with the exception of the maternal line (No 3).

Jaccard's genetic distances varied from 1.4 to 4.9 %

(average 2.9 %) within the cluster of callus lines; from 0 to 6.2 % (average 2.5 %) in the cluster of callus-derived regenerants; and from 1.4 to 7.0 % (average 4.2 %) between regenerants and maternal callus line.

From the genetic distance matrix the dendrogram was constructed using the UPGMA to depict the genetic relations between the cell lines and plants regenerated from the cell line No 3 (Fig. 2). Cluster analysis revealed a clear differentiation between two types of examined objects: all regenerants with only two exceptions (R5 and R8) formed a cluster apart from callus cell lines. Regenerant R5 belonged to cluster of cell lines and plant R8 occupied the distant position on the dendrogram. In whole, however, the regenerants displayed greater relation within the cluster than the cell lines.

Studies on the somaclonal variation in *U. victoris* regenerated plants suggest that the previously developed techniques (Kunakh *et al.* 2008) may ensure the genetic stability of plant material produced through direct regeneration and *via* organogenesis from long-term callus culture. Most of the explant-derived regenerants show no changes in RAPD patterns when compared with maternal plants. At the same time, all regenerants obtained *via* organogenesis from the long-term callus culture had the differences from the maternal cell line. The results indicate the increase of divergence from original genotype and heterogeneity of regenerants derived from callus culture versus plants regenerated directly from explant tissues. The major reason for rising of the somaclonal variation level upon the indirect regeneration may be increase in the genetic heterogeneity of callus cells due to accumulation of genomic changes during long-term culture *in vitro*. Published data also show that the direct regeneration from the explant tissues might ensure the low genetic variation. RAPD-analysis of the regenerated plants from bulb scale segments of *Lilium* sp. demonstrated lack of genetic changes after 0.5 and 1.5 years of culture (Varshney *et al.* 2001). *Capparis decidua* plants obtained from leaves *via* adventitious shoot multiplication were genetically stable as assessed by RAPD markers (Tyagi *et al.* 2010). Flow cytometry, RAPD and SSR analysis revealed no genetic variation in the *Solanum melongena* plants regenerated through organogenesis from cotyledons (Xing *et al.* 2010). Micropropagated plantlets of *Platanus acerifolia* showed the high genetic similarity ranged from 91 to 100 %, after more than 8 years of axillary branch multiplication *in vitro* (Huang *et al*. 2009). Multiple shoot culture of *Pisum*

Table 2. The number of all and polymorphic RAPD products from callus-derived regenerants and callus cell lines of *U. victoris.* Numbers of amplicons of various types are presented in the following order: all/all polymorphic/ polymorphic within a batch of the cell lines/polymorphic between regenerants and cell line No. 3/polymorphic within the group of regenerants.

Primer	Number of amplicons	Primer	Number of amplicons
A04	11/2/1/1/1	A19	10/1/1/0/0
A05	14/2/1/2/2	B03	7/0/0/0/0
A08	10/3/1/3/3	B05	17/2/1/1/1
A09	11/1/1/0/0	B06	6/1/1/1/0
A10	8/1/0/1/1	B08	10/0/0/0/0
A11	12/2/1/2/0	B10	12/1/0/1/1
A16	15/2/1/2/2	total number	152/18/9/14/11
A17	9/0/0/0/0	percentage	100/11.8/5.9/9.2/7.2

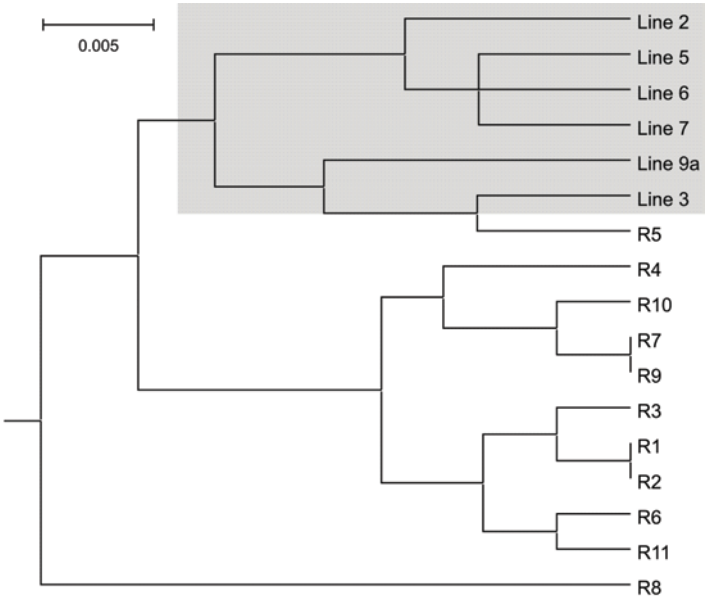

Fig. 2. UPGMA dendrogram based on genetic distance data for callus cell lines (2 - 9) and regenerants (R1 - 11) obtained from line No 3. The batch of callus cell lines is *shaded in grey*.

sativum maintained over even longer period (for 24 years) did not show any major alterations in primary genomic DNA structure and no DNA methylation (Smykal *et al.* 2007).

At the same time, regeneration through organogenesis from callus tissues, especially long-term cultured, are less effective for preserving the original genotype (Rani and Raina 2000). For example, the genetic changes were detected in plants regenerated from the callus in *Lycopersicon esculentum* (Soniya *et al.* 2001), *Zea maize* (Osipova *et al.* 2003), *Iris pseudacorus* (Kozyrenko *et al.* 2004), *Codonopsis lanceolata* (Guo *et al.* 2006) and *Saussurea involucrata* (Yuan *et al.* 2009).

Genetic analysis of plants obtained from the long-term cultured callus revealed in each regenerant or in some of them amplicons of identical size which were lacking within the RAPD patterns of maternal callus cell line and in some cases in all other lines. Such non-random genetic changes may result from the occurrence of the regions within *U. victoris* genome that show increased susceptibility to variation in culture *in vitro*. Existence of such regions was suggested earlier in *Secale cereale* (Linacero *et al.* 2000, De la Puente *et al.* 2008), *Arabidopsis thaliana* (Polanco and Ruiz 2002), *Allium sativum* (Al-Zahim *et al.* 1999), *Iris pseudacorus* (Kozyrenko *et al.* 2004), *Allium cepa* (Bohanec *et al.* 1995) and *Eucalyptus globulus* (Mo *et al.* 2009). Simultaneous occurrence of the same novel band in several regenerants may also results from their origin from the callus composed of cells bearing specific mutation. Such hypothesis was proposed by different authors to explain non-random genetic modifications in regenerated plants of *Lycopersicon esculentum* (Soniya *et al.* 2001), *Pisum sativum* (Kuznetsova *et al.* 2006), *Zea mais* (Osipova *et al.* 2001) and *Secale cereale* (Linacero *et al.* 2000). On the other hand it seems possible that only cells with specific characteristics have sufficient regeneration capacity. It is known that cultured cells carrying significant genetic alterations exhibit limited morphogenetic potential and viability and fail to regenerate normal plants or lose the regeneration capacity at all. There are published data suggesting that regenerated plants exhibit lower rate or complete absence of chromosome number variability and chromosomal mutations (Gupta 1998, Limanton-Grevet *et al.* 2000, Kozyrenko *et al.* 2004) as well as variation by the microsatellite loci (Wilhelm *et al.* 2005) typical for callus tissues. Grouping most of the regenerants and all cell lines of *U. victoris* into two distinct clusters on the dendrogram may suggest the occurrence of organogenesis in specific cell pool. After all, uniform alterations in RAPD patterns found in *U. victoris* regenerated plants may reflect the regular genome rearrangements apparently occurring in the course of cell differentiation during regeneration (Arnholdt-Schmitt 2001, Kunakh 2005).

In conclusion, this is the first report of RAPD markers application for assessment of genetic variability among regenerants of *U. victoris* produced through direct regeneration as well as *via* organogenesis from long-term callus culture. The micropropagation of *U. victoris* through direct regeneration ensure the high genetic fidelity of the produced plants and may be used for accelerated propagation of this rare medicinal herb and conservation of its natural resources. Furthermore, callus tissues even upon long-term culturing, display low level of the genetic variation and suggest another effective way for long-term germplasm preserving and reproducing of *U. victoris* plants.

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