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Evaluation of somaclonal variation of *Arnebia pulchra* (Boraginaceae) calli versus seeds using ISSR markers

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ABSTRACT

This is the first report on the analysis ISSR markers for assessing the genetic variation in *Arnebia pulchra.* As the rate of cell division and propagation in *A. pulchra* is very low, the achievement of biomass in this valuable plant requires techniques and strategies that can guarantee the survival of the callus. The present study was performed to investigate genetic variation in two *in vitro* culture samples of the medicinal plant *A. pulchra* including new (one month old) and old (four years old) calli versus seeds by Inter Simple Sequence Repeat (ISSR) marker. In total, 79 bands were produced by 10 ISSR primers. The total expected heterozygosity (He) was calculated 0.028 and the greatest value was contributed to seeds samples (0.054) followed by old and new calli, respectively. The maximum values of different alleles (*Na*), effective alleles (*Ne*) and Shannon's index were also observed in seeds. Cluster analysis was performed in the form of dendogram to indicate the genetic stability of the samples. UPGMA tree discriminated samples in two major groups and principal component analysis (PCoA) confirmed the clustering result. The first major cluster included seeds and the second involved new and old calli. Although it was thought that the morphological changes of *A. pulchra* could be due to the somaclonal variation caused by successive subcultures, however, the results indicated that the new and old calli were clustered in the same group. Therefore, it can be concluded that the morphological changes of the old and new calli could be due to environmental parameters or epigenetic changes but not directly due to somaclonal variation.

Key words: Cluster analysis, ISSR markers, Somaclonal variation, Successive subculture, Tissue culture.

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INTRODUCTION

Arnebia pulchra (Boraginaceae) is particularly favored because of the red content of its root known as shikalkin (a red content of shikonin and alkanin) (Haghbeen *et al.,* 2006). The pigment is used as a dye in food additives and cosmetics. It has also been used extensively to treat burns, ulcers, and gynecological inflammations in traditional medicine (Hosseini *et al.,* 2018).

A. pulchra grows in alpine region, Asia (especially in Iran), Himalaya and North Africa and is propagated by seeds (Aliasl *et al.,* 2014). Special growing conditions (height, humidity, etc.) and hard seeds crust of *A. pulchra*, makes it hard to propagation. *A. pulchra* is currently being overexploited; it is ranked among critically endangered species.

As the rate of cell division and propagation in *A. pulchra* is very low, the achievement of biomass in this valuable plant requires techniques and strategies that can guarantee the survival of the callus. Tissue culture techniques are capable of offering quicker and consistent pathways of large-scale multiplication of genetically uniform clonal planting material within a short period (Nei, 1978).

A. pulchra calli, due to the high amount of phenol compounds is exposed to intense oxidation and in consecutive subcultures, they show severe morphological changes that can even lead to necrosis. Hence, it is recommended that if the *in vitro* cultures are multiplied for a long period, it will be necessary to assess the genetic fidelity after a regular interval or subculture times (Peng *et al.,* 2015).

Somaclonal variation is considered one of the key obstacles to developing micropropagation procedures, particularly in terms of large-scale commercial operations in which the genetic and agronomic traits from selected individuals should be maintained strictly (Bennici *et al.,* 2004). Normally, somaclonal variation is by definition, the phenotypic and genotypic variation in plants and calli through cell culture procedures. These variations have genetic bases, i.e., transmitted through meiosis and are not typically reversible (Bairu *et al.,* 2011). The somaclonal loci mutations of *in vitro* culture of plant cells, tissues or organs are possibly the most frequently reported ones. During *in vitro* culture, the frequency of variation is determined by several factors including the propagation methods, genotype, nature of tissue used as starting material, type and concentration of growth regulators and number as well as the duration of subcultures (Krishna *et al*., 2016).

Studies on somaclonal variation are valuable firstly to control and regulate genetic variations or suppress them to prevent producing undesirable genetically variable plants. Secondly, they are useful tools for breeders for producing genetic variability, which could cause genetic improvement in plants (Rajan, and Singh, 2021). The quality control in plant tissue culture, transgenic plant production and the introduction of variants is dependent on identifying the likely somaclonal variants at an early stage of development (Noormohammadi *et al.,* 2014). Molecular techniques are valuable tools for analyzing the genetic fidelity of *in vitro* micro propagated plants. At the molecular level, variations in tissue culture-derived plants are due to changes in chromosome number or structure, or more subtle changes in the DNAs because of the high specificity of DNA (Gostimsky *et al*., 2005). Molecular markers can recognize a particular fragment of DNA sequence, which is linked with a part of the genome and comparisons are usually made based on the presence or absence of a DNA band (Yadav *et al.,* 2013). Among the various molecular markers, Inter-Simple Sequence Repeat molecular marker (ISSR) does not require any preceding information about the genome. Moreover, they are also simple, fast, effective, and reproducible in comparison with other molecular markers (Ahmed *et al.,* 2012). These features make them favorable for the present study.

The present study considers evaluating the somaclonal variation between new and old calluses of *A. pulchra* in comparison with seeds, as control, to understand any possible genetic reasons behind calli degeneration in successive subcultures.

MATERIALS AND METHODS

Callus induction

Seeds of *A. pulchra* were taken from Almas Mountains (2500-3000 m) at Khalkhal-Asalem region in Ardebil province, Iran, at the end of June of 2017. Seeds of each bush were put separately to dry at room temperature and then kept at 4˚C for further analysis (Ezati *et al.,* 2015). *A. pulchra* seeds were sterilized by the conventional method using NaOCl solution (Shen *et al.,* 2007).

The seeds were then placed in hormone-free solid MS medium and kept in the dark. Germinated seedlings were observed after 23 days (germination rate: 10%). Young seedlings were maintained under a 16-8 h light-dark photoperiod at 25 °C and light intensity of 500 lux for two weeks to grow. To obtain callus, the whole seedlings (two weeks old, 1.5-2 cm) were transferred

onto MS medium containing 10^{-5} M kinetin and 10^{-6} M 2,4-D (Merck, Germany) and kept at 25 °C in the dark. The new calli were obtained after about 28 days and the samples were subcultured in the same medium every three weeks.

DNA extraction and ISSR analysis

The whole genomic DNA of three samples including seeds, new calli (one-month-year-old with one subculture) and the old calli (four years old with about 48 subcultures, every one month) was extracted using the modified CTAB method (De la Rosa *et al.,* 2002). Approximately 200 mg of each sample tissue was ground in liquid nitrogen and added to 500 µl of extraction buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 0.2% β-mercaptoethanol, pH 8.0) and incubated at 62 °C for 20 min. The aqueous phase was extracted with 500 µl of chloroform:isoamyl alcohol (24:1) and centrifuged for 15 min at 11000 rpm. The extraction was repeated and 0.9 ml 96% cold ethanol was added to aqueous solution and left at -20 °C for 30 min to precipitate the nucleic acid. The samples were then centrifuged for 15 min at 10000 rpm. The precipitated nucleic acid was washed with 1.0 ml 70% cold ethanol, left to air dry, resuspended in 100 µl TE buffer (pH 8.0) and then treated with 1.0 µl RNase (10 mg/ml, Fermentas, Germany) for 30 min at 37 °C. The quality of DNA wsa tested by running on a 0.8% agarose gel and then quantified spectrophotometically at A_{260} and A_{280} nm. Each sample was diluted to 100 ng/µl in TE buffer (pH 8.0) and stored at -20 °C.

Ten different ISSR primers (Table 1) were used commercialized by UBC (the University of British Columbia) used by Zhao *et al*. (2008). The primers were obtained as lyophilized powders. Polymerase chain reactions (PCR) were performed in a 25 µL volume containing 10 mM Tris-HCl buffer pH 8.0; 50 mM KCl; 1.5 mM $MgCl_2$; 0.2 mM of each dNTP; 0.2 µM of a single primer; 20 ng genomic DNA and 1.0 unit of *Taq* DNA polymerase (Boiron, Germany). The Amplification reactions were conducted in a T100 thermocycler (BIORAD, USA) with the following thermal program: 5 min initial denaturation at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C. The reaction was completed by a final extension step of 10 min at 72 °C.

Amplification products were visualized by running on 1% agarose gels in 0.5 X TBE buffer systems, followed by ethidium bromide (0.5 µg mL^{-1}) staining. A 100 base pairs (bp) molecular size ladder (Fermentas, Germany) was applied for estimating the size of the generated fragments. By repeating PCR reactions as well as running on the gel for three times, the amplified DNA fragments were tested in terms of reproducibility. Reproducible bands of each locus were scored as binary present (1) or absent (0) and data matrices ISSR loci were assembled for further analysis. Analysis of Molecular Variance (AMOVA) was carried out to show the significant genetic differences between populations as well as among individuals of each population using GenAlex 6.4. UPGMA (Unweighted Paired Group using Arithmetic Average) and Neighbor- Joining (NJ) clustering based on different similarity matrices as well as ordination plot based on Principal Coordinate Analysis (PCO) were applied for grouping of the samples. POPGENE software was used to calculate genetic parameters such as Ne (number of effective alleles), Shannon index, percentage of polymorphism, He (expected heterozygosity), etc. for ISSR markers.

Table 1. Name and sequences of the primers used in ISSR analysis of callus and seeds of *A. pulchra*.

No.	ISSR primer name	Sequence
1	807	(GA)8T
2	811	(GA)8C
3	834	(AG)8YT
4	(AGC)5GG	(AGC)5GG
5	(AGC)5GC	(AGC)5GC
6	(AGC)5GT	(AGC)5GT
7	(AGC)5GA	(AGC)5GA
8	(GA)9C	(GA)9C
9	(GA)9T	(GA)9T
10	(GA)9A	(GA)9A

RESULTS

As shown in Figure 1, the morphological features of callus after sub-culturing for three months (four subcultures) varied from the primary callus slightly. All of them had a firm texture, irregular shapes and yellow to light brown color. But as time went by, the calli color and texture changed in the old calli.

All the 10 ISSR primers produced reproducible bands ranging from 250-2700 bp. In total, 10 ISSR loci produced 79 bands with an average 7.9 per primer. The greatest number of alleles was related to (AGC)5GG primer and the primer (AG)8YT (834) showed the lowest number of alleles. Primer (AGC)5GG had also the highest Shannon index. The whole numbers of different alleles and effective alleles weres 33 and 90, respectively, and the primer (AGC)5GT had the highest value for each of these parameters. The lowest number of effective alleles followed by the

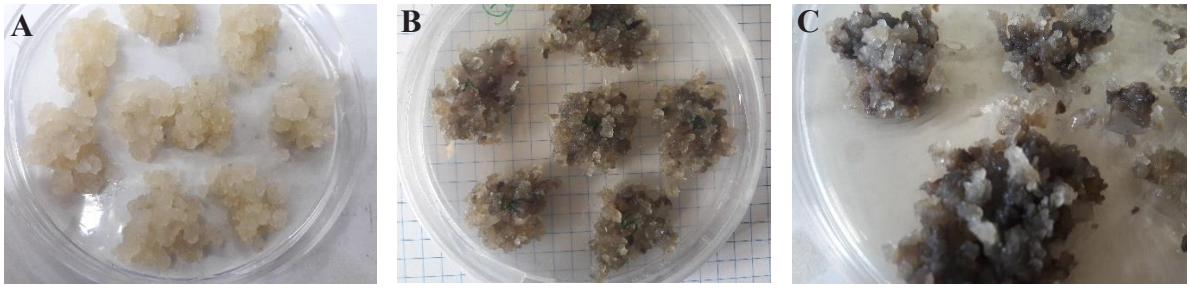

Figure 1. *A. pulchra* callus deterioration process over time after successive subcultures; **A:** the new fresh callus, **B:** callus after 30 days and one subculture, **C:** callus after 120 days and four subcultures.

Table 2. Genetic parameters of ISSR loci studied in three populations of *A. pulchra.*

ISSR loci	S (bp)	U	Na	Ne	I
(GA)8T (807)	350-550	6	3	6.428	0.477
(GA)8C (811)	300-900	8	1	8	0
(AG)8YT (834)	250-500	4	0	0	0
(AGC)5GG	200-2700	14	9	14.712	1.907
(AGC)5GC	250-1200	7	2	9	0
(AGC)5GT	300-2200	8	10	17.000	0.000
(AGC)5GA	250-2000	13	4	14	0
(GA)9C	250-950	6	1	6.000	0.000
(GA)9T	250-750	5	2	7.000	0.000
(GA)9A	300-800	8	1	8.000	0.000
Total	-	79	33	90	

U: No. of alleles, Na: No. of different alleles, Ne: No. of effective alleles=1/(p^2+q^2), I: Shannon's information index= -1*(p*Ln(p)+q*Ln(q)).

Table 3. Mean and SE over Loci for each population of *A. pulchra* ISSR marker study.

Pop		N	Na	Ne	I	He	P (%)
Pop=old calluses	Mean	3.000	0.838	1.027	0.030	0.019	6.25
	SE	0.000	0.057	0.012	0.013	0.008	
Pop2=new calluses	Mean	3.000	0.750	1.017	0.014	0.010	2.50
	SE	0.000	0.055	0.013	0.010	0.007	
Pop3=seeds	Mean	3.000	1.050	1.090	0.082	0.054	15.00
	SE	0.000	0.056	0.027	0.022	0.015	
Total	Mean	3.000	0.879	1.045	0.042	0.028	7.92
	SE	0.000	0.033	0.011	0.009	0.006	3.70

Na: No. of different alleles, Ne: No. of effective alleles=1/(p^2+q^2), I: Shannon's information index=-1*(p*Ln(p)+q*Ln(q)), He: Expected heterozygosity=2*p*q, %P: Percentage of polymorphic loci, Where for diploid binary data and assuming Hardy-Weinberg equilibrium, q=(1-Band Freq.)^0.5 and p=1-q.

lowest Shannon index occurred in (AG)8YT (834) loci. (Table 2). The total expected heterozygosity (He) was calculated 0.028 and the highest value was related to seeds samples (0.054), and it was followed by the highest numbers of different and effective alleles and Shannon index. The average polymorphism in all populations was found to be 7.92%. The maximum number of effective alleles, Shannon's index, and heterozygosity were observed in population three (seeds) and the whole heterozygosity in all populations was 0.028% (Table 3). The AMOVA showed the level of genetic diversity within and among populations of *A. pulchra* as 27% and 73%, respectively (Table 4). Figure 2 shows the polymorphism band pattern of one

Table 4. Summary AMOVA table in ISSR study of three populations of *A. pulchra*.

Source	df	SS	MS	Est. Var.	(%)
Among pops	2	39.78	19.89	5.89	73
Within pops	6	13.33	2.22	2.22	27
Total	8	53.11		8.11	100
Stat	Value	P(rand>=data)			
PhiPT	0.726	0.050			

Probability, P(rand>=data), for PhiPT is based on permutation across the full data set, PhiPT: AP/(WP+AP)=AP/TOT.
Key: AP: Est. var. among pops, WP: Est. var. within pops.

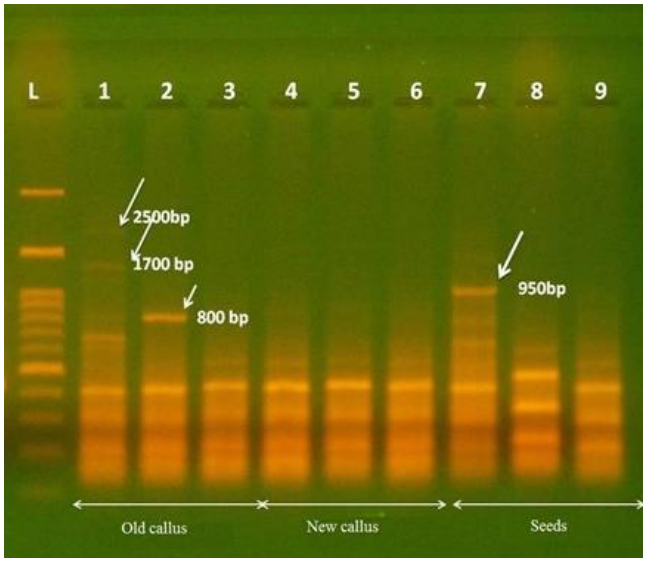

Figure 2. ISSR polymorphism band patterns of old callus (1-3), new callus (4-6) and seeds (7-9) of *A. pulchra* by (AGC)5GG primer.

of the primers ((AGC)5GG) used in this study in three populations.

In order to ensure and confirm the results, different clustering methods based on ISSR data showed two main groups by UPGMA, PCoA analysis using Jaccard's coefficient of similarity and NJ DICE method. The first major cluster included seeds and the second major cluster included the old and new calli (Figure 3).

DISCUSSION

This is the first report on applying the molecular analysis for assessing the genetic variation of *A. pulchra* using ISSR markers. Although it was thought that the morphological changes of *A. pulchra* may be due to somaclonal variation during the successive subculturing, however, the results indicated that the old and new calli were clustered in the same group. Different clusters of seeds and callus in this study maybe due to the genetic variation existing between seeds and two calli samples and less likely, due to the stresses caused by callus induction.

During the starting points of an *in vitro* culture, the genetic variations could be observed because of the accumulation of mutation factors such as stresses induced by biochemicals or other nutritional conditions (Bairu *et al.,* 2011). However, tissue tolerance will be enhanced with extending the culture time, thus, the somatic variation frequency will be approximately constant. Similarly, in another study, the somatic variation frequency increased with increasing subculture times in the early stages of subcultures; then, it remained comparatively stable following a peak (Peng *et al.,* 2015).

A very common reason in the case of different clusters between seeds and calli could be attributed to the use of plant growth regulators in the media (Azizi *et al.,* 2020). The primary events, controlled by exogenously applied plant growth regulators (PGRs) that initiate morphogenesis via cell-cycle disturbance might induce variability (Asadi *et al.,* 2021). Moreover, PGRs selectively increase the rate of division in cells that are not genetically normal. Thus, the relative levels of both auxins and cytokinin can affect the genetic composition of a cell population. Moreover, the synthetic auxin 2,4-D, which is regularly used in callus and cell culture media, is usually linked to genetic abnormalities such as polyploidy and the stimulated DNA synthesis leads to likely en do reduplication (Jin *et al.,* 2008). Jin *et al*. (2018) used molecular markers to study somaclonal variation in cotton. The result indicated that the combination of two PGRs 2,4-D and kinetin generated high variabilities (Jin *et al.,* 2008).

It is believed that the potentials of somaclonal variations increase in tissue culture-regenerates with increasing passages of subcultures (Bairu *et al.,* 2006; Mohanty *et al.,* 2008; Bairu *et al.,* 2011; Devi *et al.,*

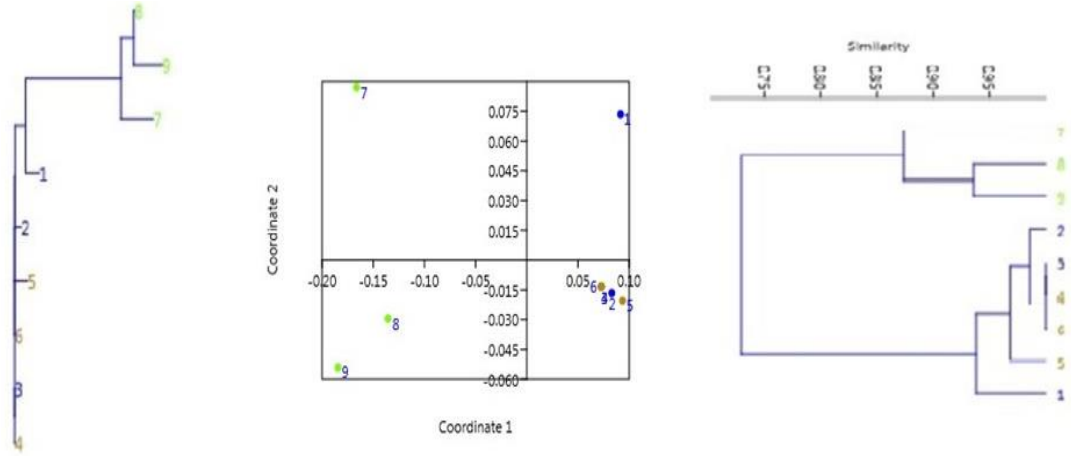

Figure 3. UPGMA dendrogram (left), NJ DICE (right) and PCOA (middle) constructed using genetic similarity analysis based on molecular profiles revealed by ISSR markers. (1-3: Old callus, 4-6: New callus and 7-9: Seeds of *A. pulchra*).

2014; Hrahsel *et al.,* 2014; Armijos-Gonzalez *et al*., 2021). However, some studies revealed conflicting results in terms of the impact of the culture duration on somaclonal variation, including this study (Razani *et al.,* 2020; Erişen *et al.*, 2020; Tikendra *et al.,* 2021). For example, multiple shoot culture of pea maintained over a long period (24 years) remained genetically unchanged and was similar to the original genotype (Smýkal *et al.,* 2007). The absence of genetic variation was also reported following a long culture period (17 months) in micro propagated fennel implying a likely genotype effect (Bennici *et al.,* 2004).

The increase of the variant rate as a function of the length of the culture period and reports of different variant rates among lines cultured for the same lengths of time under identical culture conditions are two seemingly puzzling experimental characteristics repeatedly observed in tissue culture (Podwyszynska, 2005). To shed light on this problem as well as the variant rate evolution in tissue culture, Cote *et al*. (2001) suggested a statistical model to predict the theoretical mutation rate with the number of multiplication cycles as the primary parameter. Two main conclusions were drawn from the model: a variant rate increase was expected as an exponential function of the number of multiplication cycles and secondly, following a given number of multiplication cycles, variable off-type percentages were expected (Côte *et al.,* 2001). The statistical approach is useful to shed light on the experimental features frequently reported in tissue culture, but the model is restricted in its applicability due to the complexity of biological systems as approved by Bennici *et al*. (2004).

The highest percentage of polymorphism and Shannon index as a measure of genetic diversity observed in this study was related to the seeds of *A. pulchra* which is a cross-pollinated plant. Cross-pollinated species are essentially explained by more genetic variation (Goodarzi *et al.,* 2015). The polymorphism observed between new and old calluses was not enough to divide two populations into separate groups but maybe the utilization of other markers or approaches can more accurately categorize them. The level of expected heterozygosity (He=0.028) was not sufficiently high in comparison to previous reports which emphasizes low genetic diversity and mutation rate in successive subcultures (Monfared *et al.,* 2018). The Shannon index can vary from zero to one and has an opposite ratio to genetic diversity. The Shannon index in this research was 0.042 and zero in about 8 out of 10 primers used and it was relatively high in comparison to other ISSR assay studies in Boraginaceae family (Bairu *et al.,* 2006; Noormohammadi *et al.,* 2014).

CONCLUSION

Although it was thought that the morphological changes of *A. pulchra* may be due to somaclonal variation caused by successive subcultures however, the results indicated that the old and new calli are clustered in the same group. Therefore, it can be concluded that the morphological changes of the old and new calli could be due to environmental parameters or epigenetic changes but not directly due to somaclonal variation. As we conclude in this research, morphological changes are not a good guide for the judgment on characteristics of the *in vitro* culture materials and other guidelines such as enzymatic, molecular, or other factor specifications, should be used. The authors suggest that the additional

information (additional markers or other approaches) is also needed to conclude on the genetic stability of this material.

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Disclaimer

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Competing interests

Authors have declared that no competing interests exist.

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