Morphological changes of *Hyoscyamus niger* calli resulting from somaclonal variation using ISSR markers

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 Received: 04.09.2020/Accepted: 12.09.2020

ABSTRACT

To illustrate the genetic variation in two in vitro culture samples of the medicinal plant *Hyoscyamus niger* including new (one-month old) and old (one-year old) calli versus fresh leaves, the inter simple sequence repeat (ISSR) marker was used. By using 10 primers, 6.49 per cent polymorphism was found in three studied populations. The frequency of somatic variation tended to increase with the increasing subculture time. Analysis of molecular variance (AMOVA) revealed the occurrence of 68 per cent of the variation among populations. UPGMA tree discriminated samples in two major groups and principal component analysis (PCoA) confirmed clustering. The first major cluster included old callus and the second involved new callus and fresh leaves. The high potential of *H niger* along with its high growth rate and abundance of secondary metabolites can make it a suitable species for plant breeding programmes to introduce a cultivar along with the programme's goals.

Keywords: *Hyoscyamus niger*; somaclonal variation; ISSR marker; successive subculture; cluster analysis

INTRODUCTION

Hyoscyamus niger L (Solanaceae) is a widespread weedy species that can be grown in large areas of the Irano-Turanian region and is one of the most prominent types of the natural vegetation of these areas ((Bahmanzadegan et al 2009, Li et al 2011). The economic importance of this plant is due to its valuable alkaloids with the therapeutic properties including antispasmodic activities and soothing and calming properties and the sedative to calm nerves and reduction in anxiety therapies as reported by Li et al (2011) and Sengupta et al (2011).

During in vitro culture several factors such as the propagation methods, genotype and explants, concentration of plant growth regulators, frequency as well as the duration of subcultures etc can cause genetic changes in tissue-cultured cells which in some cases can produce a different phenotype from their mother plants (Ahmed et al 2012, Peng et al 2015). The occurrence of somaclonal variation in tissue culture could be an appropriate source for generating new clones or variants with desirable traits (Bennici et al 2004, Bairu et al 2011).

For analysis of somaclonal variation in the in vitro micropropagated plants, molecular assays are valuable tools. Presently several molecular assays are available for detecting sequence variation in plants and somaclones. Hence ISSR markers are highly polymorphic; they are practical in studies on genetic diversity and somaclonal variations (Noormohammadi et al 2011, El-Shawaf et al 2016).

The present study considers evaluating the somaclonal variation between leaves and the new and old calluses of *H niger* to understand any possible genetic reasons behind successive subcultures.

MATERIAL and METHODS

Plant preparation and callus induction

H niger seeds were collected from the Khalkhal-Asalem region of Ardebil Province, Iran at the end of June of 2017. *H niger* seeds were sterilized by the conventional method and were implemented using NaOCl solution as described by Shen et al (2007) and then were placed in MS hormone-free medium and kept in dark. Germination occurred after 7 days (germination rate 85%) and emerging seedlings were cut and put in MS medium containing 10^{-5} mol/l kinetin and 10^{-6} mol/l 2, 4-D. The fresh calli were obtained after 14 days. The fresh calli were subcultured for one month and one year each 15 days (two and 24 times subcultures respectively).

DNA extraction and ISSR assay

The whole genomic DNA was extracted from fresh *H niger* leaves, fresh callus (one-month old) and old callus (one-year old) by using the modified CTAB method to obtain high quality intact DNA (Murray and Thompson 1980). The quality of DNA was confirmed by running on a 0.8 per cent agarose gel.

For ISSR assay, ten primers commercialized by the University of British Columbia were selected for further study based on their production of reproducible, clear and polymorphic bands (Table 1). PCR reactions were performed in a 25 µl volume containing 10 mM Tris-HCl buffer at pH 8, 50 mMKCl, 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 programme: 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 per cent agarose gel following ethidium bromide staining. Fragment size was estimated by using a 100 base pairs (bp) molecular size ladder (Fermentas, Germany). To check the reproducibility, PCR amplification with each primer was repeated three times. The consistently reproducible, clear and well-resolved bands were scored as present (1) or absent (0) form a binary matrix.

Statistical analysis

Data were evaluated as a mean ± standard deviation of three replicates. To cluster analysis, the dendrogram of amplified PCR bands based on the absences/presence of bands was done by UPGMA. Molecular variance (AMOVA) was also analyzed using GenAlEX 6.4 to determine genetic diversity within the populations of studied species.

RESULTS

ISSR assessment

To investigate somaclonal variation derived from the successive subculture, three populations were selected and were subjected to ISSR analysis. A total number of 77 clear DNA loci were detected through the ISSR analysis using 10 primers. All fragments ranged from 200 bp to 2,800 bp. The data given in Table 2 show that the primer (GA)8T(807) with 0 bands and the (AGC)5GA primer with 13 bands produced the highest and lowest bands respectively. The ISSR primer of AGC5GG150 bonding patterns is illustrated in Fig 1; specific bands were also marked with white arrow.

Table 1. ISSR primers used for genetic viability assessment in the study

Row	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

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

ISSR loci	S (bp)	U	Na	I
(GA)8T (807)	-	0	0	0
(TC)8C (823)	350-600	7	2	0.31
(AG)8YT (849)	250-550	9	1	0
(AGC)5GG	200-2,300	10	2.3	0.22
(AGC)5GC	300-650	6	1	0
(AGC)5GT	220-1,100	9	2	0.22
(AGC)5GA	300-2,800	13	2.3	0.45
(GA)9C	250-550	5	1	0
(GA)9T	300-1,100	9	3	1.49
(GA)9A	250-800	9	1	0
Total	-	77	-	-

S= Fragments ranges, U= Number of bands of each primer, Na= Number of different alleles, I= Shannon's information index= -1*[p*Ln (p) + q*Ln(q)]

Table 3. Summary of genetic variability among *H niger* populations analyzed using 10 ISSR primers

Population		N	Na	Ne	I	He	UHe	Percentage of polymorphic loci
Population 1: fresh leaves	Mean	3.000	0.935	1.041	0.036	0.024	0.029	6.49
	SE	0.000	0.050	0.020	0.016	0.011	0.013	
Population 2: new callus	Mean	3.000	0.974	1.053	0.049	0.032	0.039	9.09
	SE	0.000	0.052	0.021	0.018	0.012	0.014	
Population 3: old callus	Mean	3.000	0.909	1.023	0.021	0.014	0.017	3.90
	SE	0.000	0.046	0.015	0.012	0.008	0.010	
All populations	Mean	3.000	0.939	1.039	0.035	0.024	0.028	6.49
	SE	0.000	0.028	0.011	0.009	0.006	0.007	

Na= Number of different alleles, Ne= Number 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, UHe= Unbiased expected heterozygosity= [2N/(2N-1)]*He

According to the results of Table 3, the average percentage of polymorphic loci in all populations was low (6.49%). The observed and effective numbers of alleles (Na and Ne) were 0.939 and 1.039 respectively. Shannon's information index value (I) was 0.035. The genetic diversity of Pop 2 (new calli) (PPB= 9.09%, Na= 0.974, Ne= 1.053, He= 0.032, UHe= 0.039 and I = 0.049) was higher than other populations.

Cluster analysis

The dendrogram divided three populations into two main groups. The first cluster belonged to the old callus and the second cluster consisted of two separate sub-clusters belonging to fresh calli and plant leaves (Fig 2).

Analysis of molecular variance

Analysis of molecular variance (AMOVA) revealed 68 per cent variation among populations and 32 per cent within the populations (Table 4). The PCoA diagram showed that the first principal coordinate accounted for 94.41 per cent of the total variation and the second principal separate other populations of *H niger* (2 and 3) (5.58%).

DISCUSSION

Hyoscyamus plants have great potential for easily obvious callus induction from different parts of the plants (Ibrahim et al 2009). This ability makes it a candidate plant for in vitro cell culture to reach the valuable secondary metabolites or other plant breeding and genetic engineering purposes (Tapingkae et al 2012). The noteworthy point is the genetic stability of the *Hyoscyamus* in vitro culture programmes. Genetic instability may affect the plant in vitro survival in the successive subculture as well as the production of secondary metabolites. In the recent study, with increasing the number of subcultures, genetic variation

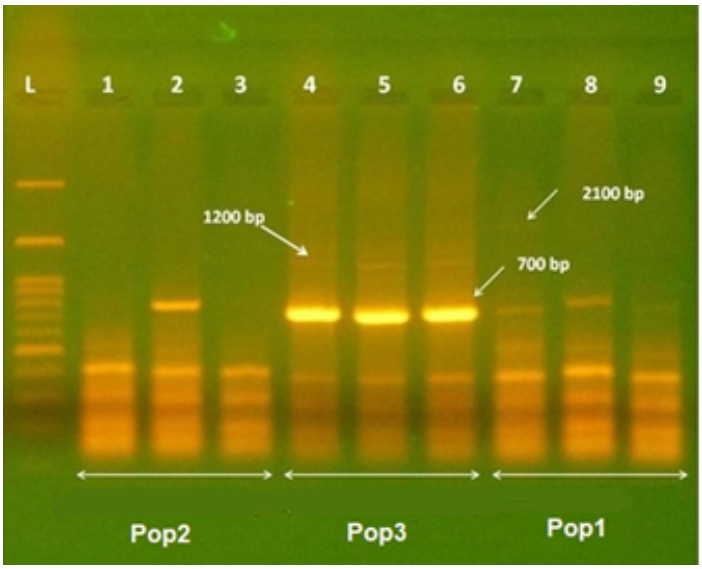

Fig 1. ISSR specific band patterns of the primer AGC5GG150 in three populations of *H niger*, (Population 1: fresh leaves, Population 2: new callus, Population 3: old callus)

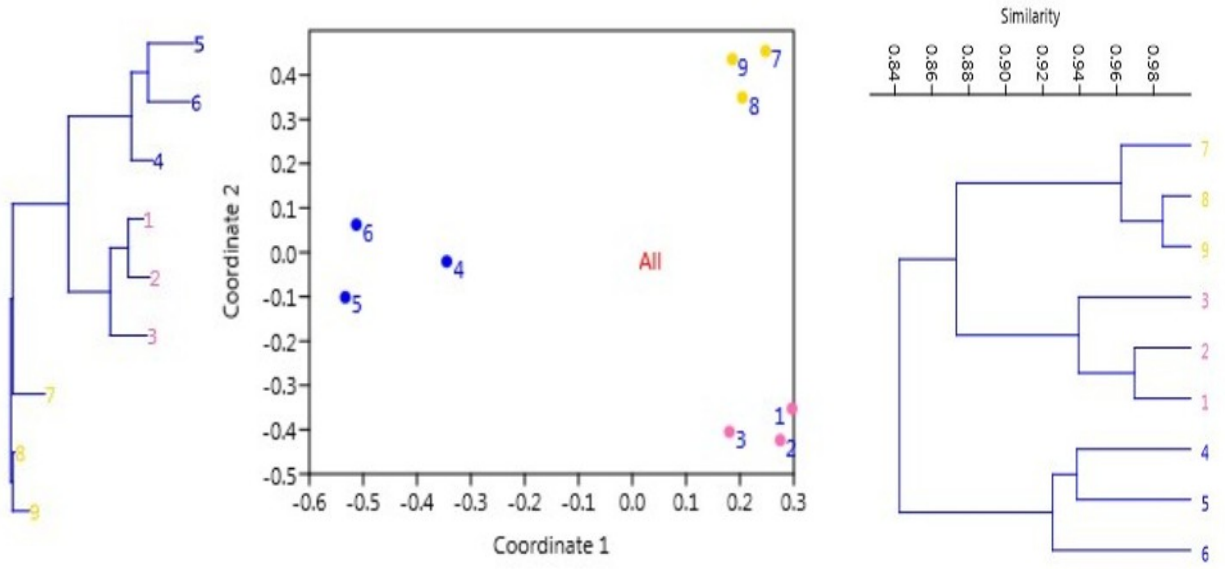

Fig 2. UPGMA dendrogram (right), NJ DICE (left) and PCOA (middle) constructed using genetic similarity analysis based on molecular profiles revealed by ISSR markers (1-3: New callus, 4-6: Old callus and 7-9: Fresh leaves of *H niger*)

Table 4. Analysis of molecular variance (AMOVA) summary for 3 populations of *H niger* accessions

SOV	df	SS	MS	EV	% EV	GD	P (rand >= data)
Among pops (AP)	2	24.222	12.111	3.481	68*	PhiPT= 0.676	0.001
Within pops (WP)	6	10.000	1.667	1.667	32	-	-
Total	8	34.222	-	5.148	100	-	-

df= Degrees of freedom, SS= Sum of square, MS= Mean sum of square, EV= Estimated variance, % EV= Percentage of variation, GD= Genetic differentiation, P (rand ≥data)= Probability for PhiPT based on standard permutation across the full data set, PhiPT= AP/(WP + AP)= AP/TOT (*P <0/001)

increases but the most polymorphism percentage has appeared in new callus. This can be due to plant growth regulators used for callus induction. Plant hormones are mutagenic and high somaclonal variation was reported in the use of PGRs (Besher et al 2014). Especially it was reported that 2, 4-D and kinetin as a hormone combination induce relative high somaclonal variation in regenerated cotton plants via somatic embryogenesis (Jin et al 2008). In a study Bello-Bello et al (2014) detected that variation was higher in callus than the other tissues of *Capsicum chinense* and it was probably because callus cultures were more exposed to 2, 4-D than other tissues. Similarly Jain (2001) reported that cultures exposed to 2, 4-D for a longer period accumulate more mutations. Different types of somaclonal variations also have been reported in the in vitro cultures maintained for a long time (Halder and Jha 2020).

Many publications believe that the chance of somaclonal variation increases with increasing the number of subcultures (Bairu et al 2011). Peng et al (2015) showed that frequency of somatic variation of the in vitro culture of *Tetrastigma hemsleyanum* was increased with the increasing subculture time in the early stage of subculture. They resulted in supplying true-to-true seedlings, germplasm conservation and further genetic transformation. Subculture frequency of callus should be controlled within 4 times to obtain clonally identical plantlets (Peng et al 2015). Peyvandi et al (2009) found the highest polymorphic RAPD bands between regenerated *Olea europea* in the first subculture and seventh subculture. They reported that micropropagation affected both the genetic and physiology of studied plants.

The Shannon index can vary from zero to one and has an opposite ratio to genetic diversity. The index value in this research has the lowest amount in the old callus (0.021) that shows more genetic variation in this population. Similarily heterozygosity and polymorphism percentage highest in new callus resulted in processes objected to tissues for callus induction.

CONCLUSION

Based on the results, genetic differences occurred during continuous subcultures of *H niger* in vitro. Since the in vitro cultures are multiplied for the long term, it will be necessary to evaluate the deviation from the primary genetic content after regular intervals or subculture times.

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