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Assessment of Genetic Fidelity of *In vitro* Micropropagated Plants of *Glycyrrhiza glabra* L. using Random Amplified Polymorphic DNA Technique (RAPD)

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Abstract: Random amplified polymorphic DNA (RAPD) markers were employed to determine the genetic fidelity of *Glycyrrhiza glabra* L. plantlets multiplied through *in vitro* micropropagation technique. Twenty RAPD primers were screened, of which 16 primers generated a total of 605 clear, distinct and reproducible bands. Out of 605, 429 bands (70.91%) were monomorphic and 176 (29.09%) were polymorphic. The similarity values amongst the aforesaid plants varied from 0.788 to 1.000. A UPGMA dendrogram was constructed to show the genetic similarity among 11 plants (10 micropropagated and 1 mother plant), the analysis revealed 98 % similarity among them. The present study exhibited successful application of the RAPD marker technique for molecular profiling and assessment of the genetic fidelity of micropropagated plants of *Glycyrrhiza glabra* L.

Introduction

Glycyrrhiza glabra L., a member of the family Fabaceae, is a genus of perennial herb and shrubs distributed in the subtropical and warm temperate regions of the world, especially in the Mediterranean countries and China (Anonymous, 1985). *Glycyrrhiza glabra* L., commonly known as liquorice or sweet wood in English, Jothi-madh, Mulhatti in Hindi, Yashti-madhuh, Madhuka in Sanskrit, Jashtimadhu, Jaishbomodhu in Bengali, Atimadhuranu, Yashtimadhukam in Telugu, Jethimadhu in Gujarati and Atimaduram in Tamil (Chopra *et al.* 2002). Licorice extracts and their principal component, glycyrrhizin, has extensive use in food, tobacco products, snuffand, and in traditional and herbal medicine. It is cultivated for its rhizomes that contain the compound glycyrrhizin, which is 50 times sweeter than sugar. It is cultivated in the Mediterranean basin of Africa, in southern Europe and in India (The Indian pharmaceutical codex, 1953., African pharmacopoeia, 1985., Ghazanfar, 1994., Chin *et al*., 1992). In India it is widely cultivated in Punjab and sub-Himalayan tracts (Dhuke *et al*., 2002), Baramulla, Srinagar, Jammu, Dehradun, Delhi and South India (Meena *et*. *al*., 2010).

Several studies on *in vitro* micropropagation have been reported for this plant previously. A stepwise protocol for the micro-propagation of *Glycyrrhiza glabra* L. on a simple minimal medium using shoot tip and nodal explants was reported by Thengane *et al*. (1998) where very high multiplication rates with a healthy root system were obtained. High frequency regeneration of *Glycyrrhiza glabra* L. was obtained, using young leaf and shoot tips explants via callus-mediated shoot regeneration (Sharma *et al.* 2010). Recently, Yadav and Singh (2012), Shrivastava *et al.* (2013) and (Badkhane *et al*., 2016) also reported micropropagation studies on this species.

An *in vitro* micropropagation technique for any plant provides an alternative means of plant propagation and also a tool for crop improvement (Vasil,

1988). Somaclonal variation may be exhibited in plantlets derived from *in vitro* culture (Larking and Scowcraft, 1981). *In vitro* micropropagation technique is very useful to produce true to type clones and one of the most important pre-requisites in the micropropagation of crop species is to check and assess the genetic stability of micropropagated plants. Maintaining genetic similarity is one of the major concerns in tissue culture techniques. The techniques, such as karyotyping and isoenzyme profiling, can also be used to assess the genetic similarity of tissue culture plants, but that has limitations (Chelageri and Babu, 2012). Among the different types of molecular markers techniques available, the RAPD marker-based technique is very useful to assess the genetic diversity, because of its simplicity and relatively low cost compared to other molecular markers studies (William *et al*. 1990). The present study confirms the genetic fidelity of the DNA of 11 regenerated plants compared to the DNA of the donor mother plant of *Glycyrrhiza glabra* L.

MATERIALS AND METHODS

Micropropagation

The nodal segments explants of *Glycyrrhiza glabra* L. were inoculated in MS medium (Murashige and Skoog, 1962) supplemented with 6-benzylaminopurine (BA) 2.0 mg/l for culture establishment. After the 4th week and successful establishment, nodal and intermodal explants derived from established *in vitro* cultures were re-inoculated on MS medium supplemented with 6-benzylaminopurine (BA) 2.0 mg/l + α-naphthalene acetic acid (NAA) 0.50 mg/l for proliferation and multiplication.

For callus induction *in vitro* nodal explants were cultured on MS media supplemented with 2.0 mg/l 6-benzylaminopurine (BA) and 0.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D). The callus induced from the *in vitro* cultures was further used as an explant for somatic embryogenesis on MS medium supplemented with BA (0.5 mg/l) and kinetin (KT) (0.5 mg/l). The resultant embryos were

collected from regeneration cultures and inoculated on the germination treatments. The best results were obtained on half-strength MS medium augmented with 2.00 to 3.00 mg/l BA. The healthy rooted plantlets were washed with running tap water to make them agar-free and transplanted to plastic cups containing soil, sand and compost (1:1:1) for hardening. The plantlets were kept in a poly chamber for acclimation. Established plantlets were transplanted in an open field under natural conditions.

Genomic DNA isolation

For RAPD analysis, fresh young leaf samples were collected from the micropropagated plants along with a single mother plant for screening their genetic similarity. DNA extraction methodologies of Doyle & Doyle (1987), Doyle & Doyle (1990), with some modifications, were used for the isolation of genomic DNA. After purification, the quality and quantity of DNA was inspected by both gel electrophoresis and spectrometric assays using a spectrophotometer (Thermo Scientific – Nano Drop, 1000. 3.70).

RAPD-PCR amplification

Molecular characterization of regenerated plants

Genetic fidelity of *in vitro* raised clones was tested using RAPD markers. For this purpose, 10 *in vitro* raised, hardened plants were chosen randomly from the population and compared with the mother plant from which the explants were taken. Total genomic DNA was isolated from 100 mg of fresh leaves in each category, following the Cetyl-trimethyl ammonium bromide (CTAB) method as described by (Doyle and Doyle, 1987, 1990) with some modifications.

Twenty RAPD primers (Operon Technologies Inc., Germany) were used for initial screening. PCR amplifications were carried out in a total volume of 25 µl PCR mixture containing 2 µl (25–30 ng) of genomic DNA. The reaction buffer consisted of 2.5 µl of PCR buffer, 0.5 µl dNTPs (10 mM each of dATP, dGTP, dTTP and dCTP), 3 µl primer, 0.5 µl DNA Taq polymerase and 17.82 µl water. PCR amplification was

performed in a DNA thermal cycler (ProFlex PCR System), which was programmed for initial DNA denaturation at 94^{o}C for 3 minutes, followed by 40 cycles of 1 min denaturation at 94^{o}C, 1 minute annealing and 1 minute extension at 72^{o}C, with a final extension at 72^{o}C for 5 min. Amplified products were resolved by electrophoresis on 1% agarose gel in tris-borate EDTA (TBE) buffer stained with 0.5 g/ml ethidium bromide and photographs were taken by using the gel documentation system (Ultra-Violet Products Ltd., Cambridge UK).

Data scoring and analysis

Visible, well-resolved fragments in the size range of 100 base pair (bp) to 2.5 kilobases (kb) were manually scored. Each band was treated as a marker. The scoring of bands was done on the basis of their presence ('1') or absence ('0') in the gel. The genetic associations were evaluated by calculating the Jaccard's similarity coefficient for pair-wise comparisons based on the proportion of shared bands produced by the primers. The similarity matrix was subjected to the cluster analysis of Unweighted Pair Group Method with Arithmetic Averages (UPGMA) and a dendrogram was generated by using PAST 3 software.

Results and Discussion

RAPD amplification

Out of 20 RAPD primers (10 mer), OPJ (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10) and OPC (1, 2, 3, 4, 5, 6, 7, 8, 13 and 14) series examined, 16 primers (OPJ- 4, 5, 6, 7, 8, 9 and 10 and in OPC-1, 2, 3, 4, 6, 7, 8, 13 and 14) produced quality amplification products and generated a total of 605 scorable bands, ranging from 250 to 2000 bps.

Based on results in primer screening, a particular primer was selected to carry out RAPD fingerprinting of the 10 randomly selected *in vitro* regenerated plants and mother plant. A total of 605 bands were generated (an average of 37.81 bands per primer) out of which 429 (70.91%) were

monomorphic and 176 (29.09%) were polymorphic bands. The maximum numbers of bands (5) were obtained with the marker (OPJ-04). The number of bands in the selected primers varied from 1 (OPJ-08, OPC-04, 07, OPJ-10, OPC-02, 03, OPC-08) to 5 (OPC-06), with an average of 2.0 bands per RAPD primer (Table-1, 2 Fig.2 - 3).

Molecular tools are more reliable than phenotypic observations for evaluating tissue culture-induced variations (Leroy *et al*., 2000). In plants regenerated via somatic embryogenesis, the quality of somatic embryos determines the production of true-to-type plants. Many authors have reported that dedifferentiation of plant tissues leads to genetic modifications (Taylor *et al*., 1995; Hashmi *et al*., 1997; Rani *et al*., 2000), but on the contrary, several reports also confirmed the genetic integrity of tissue culture-derived plants (Dale *et al*., 1981; Haydu and Vasil, 1981; Hanna *et al*., 1989; Jayanthi and Mandal, 2001; Gagliardi *et al*., 2004).

Many factors are known to be associated with the occurrence of somaclonal variations which affect the genetic fidelity of tissue culture plantlets, particularly when they are maintained for a prolonged duration. Molecular markers suitable for generating DNA profiles have proved to be an effective tool in assessing the genetic stability of regenerated plants (Chittora *et al*., 2015). A wide variety of PCR-based markers, such as simple sequence repeats SSRs) (Litt and Lutty, 1989), random amplified polymorphic DNA (RAPD; Williams *et al*., 1993), restriction fragment length

Polymorphism (RFLP: Bostein *et al*., 1980), Inter simple sequence repeats (ISSR; Zietiewicz *et al*., 1994) and amplified fragment length polymorphism (AFLP; Vos *et al*., 1995) have been used for assessment of genetic stability of regenerated plantlets.

PCR-based random amplified polymorphic DNA (RAPD) has been widely used to reveal DNA-based polymorphism and it is well suited to DNA fingerprinting (Santos *et al*., 1994; Thormann *et al*., 1994). RAPD markers are amplification products of anonymous DNA sequences using single,

short and arbitrary oligonucleotide primers, and thus do not require prior knowledge of a DNA sequence (Karp *et al*., 1997, Bardakci 2001).

In the present study, only 16 primers produced reproducible and scorable bands out of 20 selected RAPD 10-mer primers used for the initial screening. These 16 selected RAPD primers gave rise to a total of 605 scorable bands ranging from 250 to 2000 bp. Out of a total of 605 bands that were generated, 429 (70.91%) were monomorphic and 176 (29.09%) were polymorphic in all micropropagated plants as compared to the donor mother plant. As mentioned above, there were 29.09% polymorphic bands that showed genetic modification.

A dendrogram constructed based on Jaccard's similarity matrix, followed by UPGMA-based clustering analysis, showed 10 micro propagated plants, forming a major cluster along with the mother at 84% similarity level (Fig. 1). From the dendrogram, it was observed that the genotypes were grouped into three main clusters. Cluster I consists of three tissue-cultured progenies T8, T10 and T6, exhibiting the highest similarity to the mother plant. Mother plant M showed 90%, 92% and 90% similarity with T10, T8 and T6, respectively. Cluster II consists of three cultured progenies T1, T5 and T9. The similarity matrix ranged from 93% between T1 and T9 to 95% between T1 and T5 (Table 3). Cluster III involves T4 and T7 exhibiting self genetic resemblance and join at similarity 92 % level. The other two cultured-progenies T2 and T3 remain independent not exhibiting much similarity to other tissue cultured-progenies or the mother plant and are hence considered as molecular off-types.

Many investigators have reported genetic instability of several micropropagated plants, viz., *Allium sativum* L. (Al-Zahim *et al*., 1999) using five varieties, 50 polymorphic bands were obtained from a total of 7903 bands and only 0.63% polymorphism was observed. Yang *et al.*

(1999) analyzed somaclonal variation in cultured cells of rice. Somaclonal variation was found to increase with culture age. More than 50 polymorphic fragments were identified with the four primers tested. The variation at DNA level has occurred in *in vitro* culture of turmeric while used 14 primers, when separated on non-denaturing polyacrylamide gels, showed 38 novel bands. About 51 bands present in the control were absent in the regenerates. Total of 231 bands of which 16.5% showed polymorphism Salvi*et al*. (2001). In *Curcuma amada* Roxb. Prakash *et al*., (2004) reported 103 scorable bands from 10 primers, including 9 polymorphic bands (8.7% polymorphisms), which were absent in the control. Liu and Yand (2012) obtained a total of 2356 scorable bands from the full combination of primers and plantlets and only 39 (1.65%) were polymorphic across the plantlets. RAPD analysis of *Justicia tranquebariensis* L.F. regenerated plantlets revealed 46.15% polymorphism between the mother plant and tissue culture-generated plants Raji *et al*. (2014).

The findings of RAPD data of the present study thus demonstrated that RAPD analysis can be applied to assess the genetic fidelity of micropropagated plants of *Glycrrhiza glabra* L. This method might be useful for monitoring the stability of *in vitro* germplasm collections and cryopreserved material. It is evident from the above results that 29.62% polymorphism or genetic modification has been recorded between the mother plant and tissue culture generated plants. As per the literature cited above, different tissue cultured plants exhibit different levels of genetic polymorphism in different plant species as compared to mother plants -e.g. 0.63% in *Allium sativum* L. (Al-Zahim *et al*., 1999); 16.5% polymorphism in *Curcuma longa* Linn. (Salvi *et al*., 2001); 8.7% polymorphisms in *Curcuma amada* Roxb. (Prakash *et al*., 2004), Liu and Yand (2012) reported (1.65%) were polymorphic in *Psidium guajava* across the plantlets. Raji*et al*. (2014) recently reported that RAPD analysis of *Justicia tranquebariensis* L.F. regenerated plantlets revealed that 46.15%

polymorphism exists between the mother plant and tissue culture generated plants.

The micro propagation especially of medicinal plants is undertaken to ensure genetically true clonal multiplication of elite strains that have superior production of active principles especially the chemical constituents. Any lowering of quantity or quality is detrimental to the whole idea of plant multiplication by tissue culture technique where as an increase in the production of the active

principle is a welcome improvement. Both conditions are possible in tissue culture technology due to soma clonal variations. If these results are compared with the traditional breeding methods of sexual reproduction, the possibility of such changes also exists due to genetic recombination and mutations which are responsible for genetic variations. In such cases the progeny is a mixture of elite and other strains, whereas the tissue-cultured plants are clones. Hence, it is essential to further analyze the plants for the quantity and quality of occurrence of active principles.

Conclusion:

The study successfully demonstrated that Random Amplified Polymorphic DNA (RAPD) markers are effective tools for assessing the genetic fidelity of in vitro micropropagated *Glycyrrhiza glabra* L. High genetic similarity (up to 98%) between micropropagated plantlets and the mother plant confirms the clonal stability and reliability of the propagation protocol, ensuring true-to-type plant production for sustainable medicinal plant cultivation.

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Table 1. Summary of the RAPD profile and number of scored amplification products for the 20 primers used in the genetic analysis of micropropagated plants of *Glycyrrhiza glabra* L.

Primer Name	Sequence	Number of monomorphic bands	Number of Polymorphic band	Total number of bands	Size range (bp)
OPJ-01	CCCGGCATAA	-	-	-	-
OPJ-02	CCCGTTGGGA	-	-	-	-
OPJ-03	TCTCCGCTTG	-	-	-	-
OPJ-04	CCGAACACGG	5	0	5	250-1500
OPJ-05	CTCCATGGGG	3	1	3	250-1000

OPJ-06	TCGTTCCGCA	3	0	3	250-1500
OPJ-07	CCTCTCGACA	3	0	3	250-1000
OPJ-08	CATACCGTGG	3	0	3	250-1500
OPJ-09	TGAGCCTCAC	3	0	3	500-1500
OPJ-10	AAGCCCGAGG	2	2	4	500-2000
OPC-01	TTCGAGCCAG	3	0	3	100-2000
OPC-02	GTGAGGCGTC	2	2	4	250-1000
OPC-03	GGGGGTCTTT	2	2	4	500-1500
OPC-04	CCGCATCTAC	3	1	4	250-750
OPC-05	GATGACCGCC	-	-	-	-
OPC-06	GAACGGACTC	0	5	5	250-1000
OPC-07	GTCCCGACGA	1	1	2	500-1000
OPC-08	TGGACCGGTG	2	2	4	250-1500
OPC-13	AAGCCTCGTC	2	0	2	750-1500
OPC-14	TGCGTGCTTG	2	0	2	750-2000
Total		39	16	55	

Table 2 Summary showing RAPD amplified products obtained from *in vitro* raised plants of *Glycyrrhiza glabra* L.

Total number of primers used	20
Number of polymorphic primers	8
Number of monomorphic primers	8
Total number of scorable bands amplified by primers	605
Total number of polymorphic bands identified	176
Total number of monomorphic bands identified	429
Size range of amplified products	250-2000 bp
Average number of polymorphic bands per primer	2
Average number of bands per monomorphic primer	4.875
Percentage of total polymorphic bands	70.91 %
Percentage of total monomorphic bands	29.09 %

Table 3 Jaccard's similarity index between different individuals of *Glycyrrhiza glabra* L.

	M	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
M	1.00										
	0.90										
T1	4	1.00									
	0.86	0.84									
T2	8	3	1.00								
	0.81	0.89	0.78								
T3	5	8	8	1.00							
	0.85	0.86	0.90	0.84							
T4	2	3	0	3	1.00						
	0.94	0.95	0.84	0.86	0.86						
T5	2	9	6	3	5	1.00					
	0.90	0.88	0.92	0.82	0.94	0.88					
T6	6	2	0	7	0	5	1.00				

	0.88	0.90	0.86	0.84	0.92	0.90	0.90				
T7	9	2	5	6	2	4	4	1.00			
	0.92	0.90	0.90	0.84	0.88	0.90	0.94	0.88			
T8	5	2	2	6	5	4	1	7	1.00		
	0.88	0.93	0.86	0.84	0.92	0.94	0.90	0.92	0.92		
T9	7	9	3	3	0	0	2	2	2	1.00	
T1	0.90	0.92	0.88	0.86	0.90	0.92	0.96	0.90	0.98	0.94	1.0
0	6	0	2	3	2	2	0	4	0	0	0

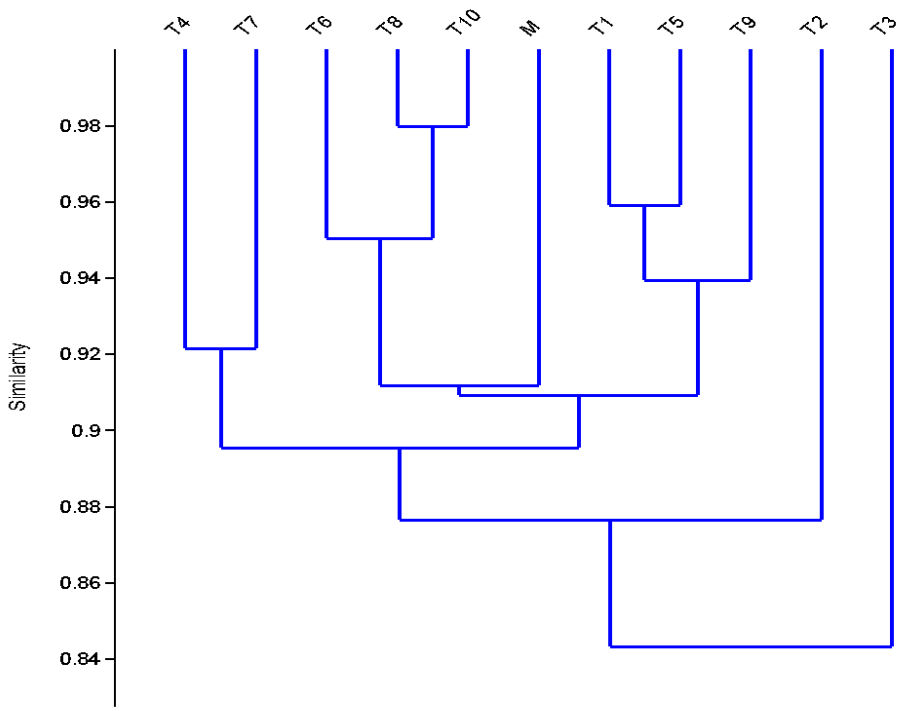
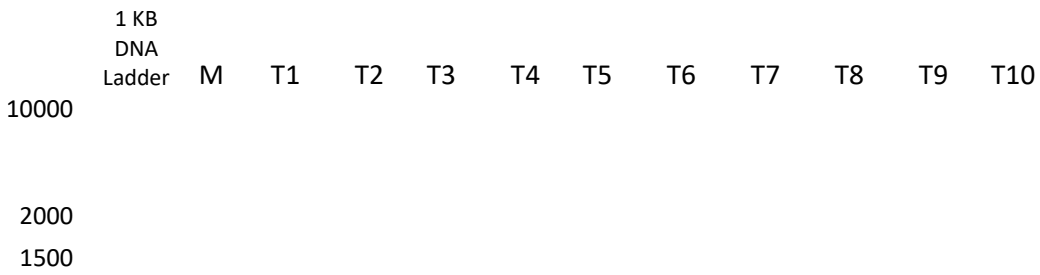
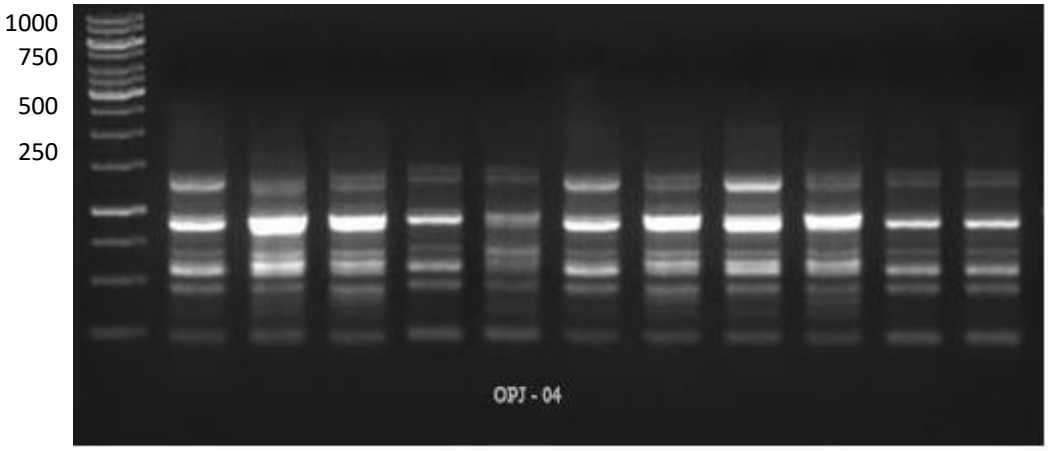


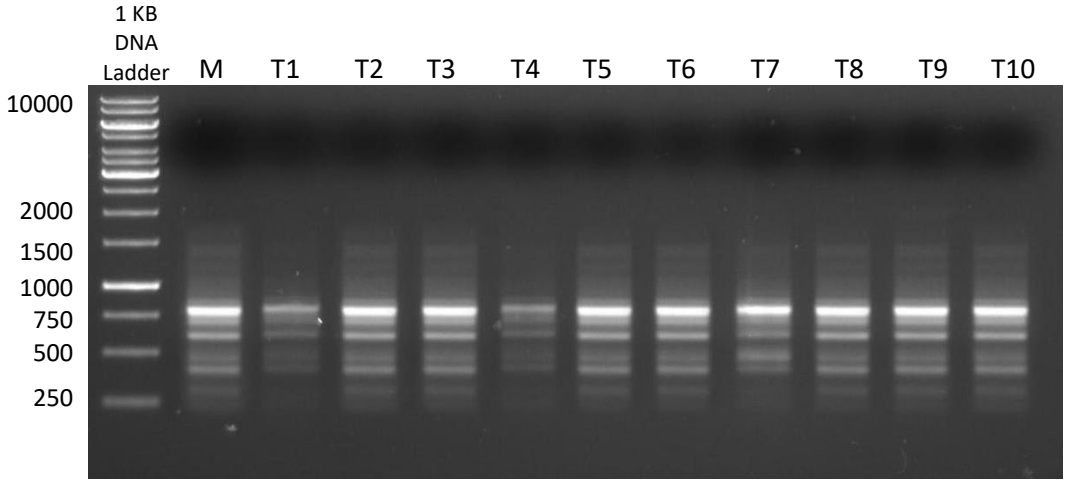
Fig. 1 Dendrogram of 10 hardening plants (T1-T10) and donor mother plant (M) of *Glycyrrhiza glabra* L. generated by UPGMA cluster analysis.





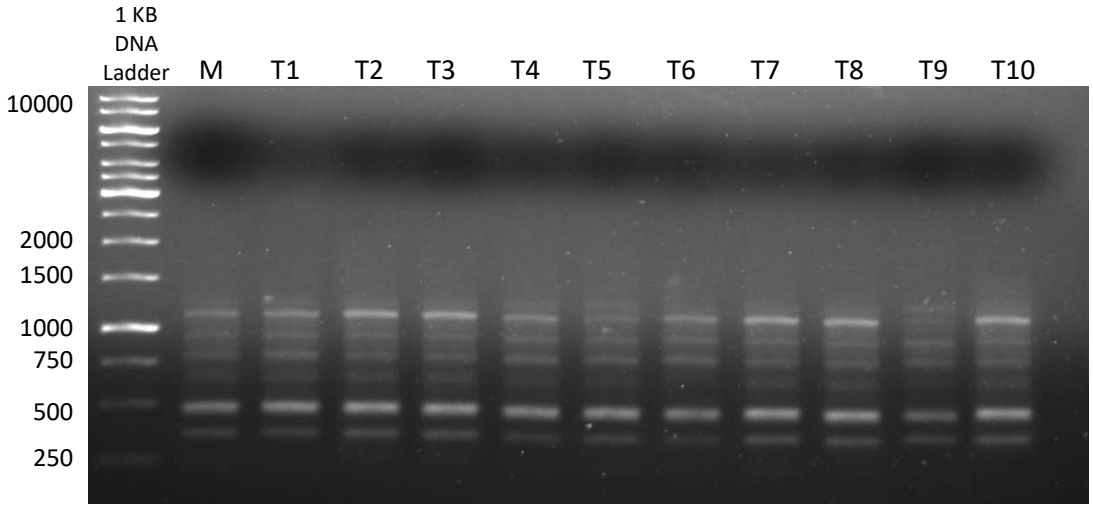
L= Ladder, M= Mother Plant, T1-T10= Tissue cultured raised plant

Fig.2 RAPD profile generated by Primer OPJ-04



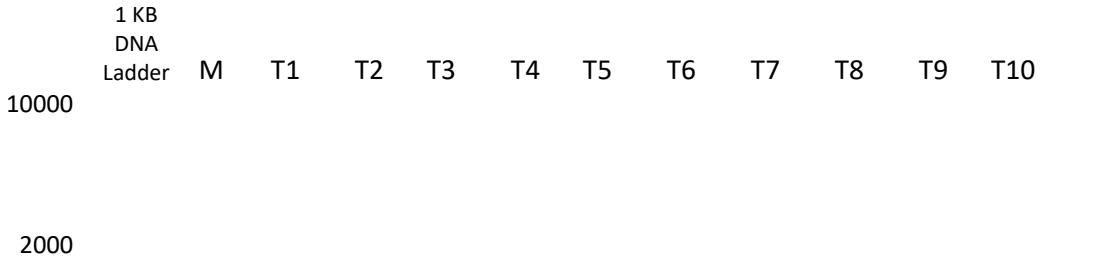
L= Ladder, M= Mother Plant, T1-T10= Tissue cultured raised plant

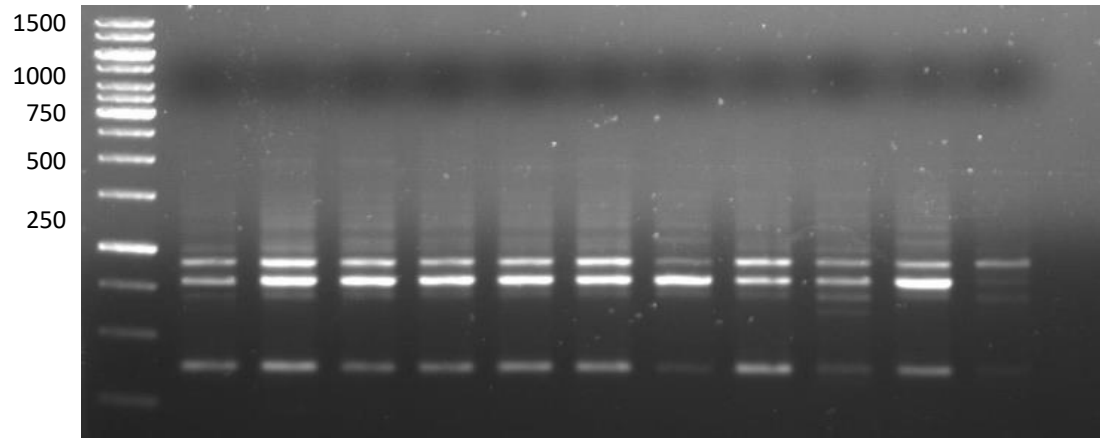
Fig. 3. RAPD profile generated by Primer OPJ-05



L= Ladder, M= Mother Plant, T1-T10= Tissue cultured raised plant

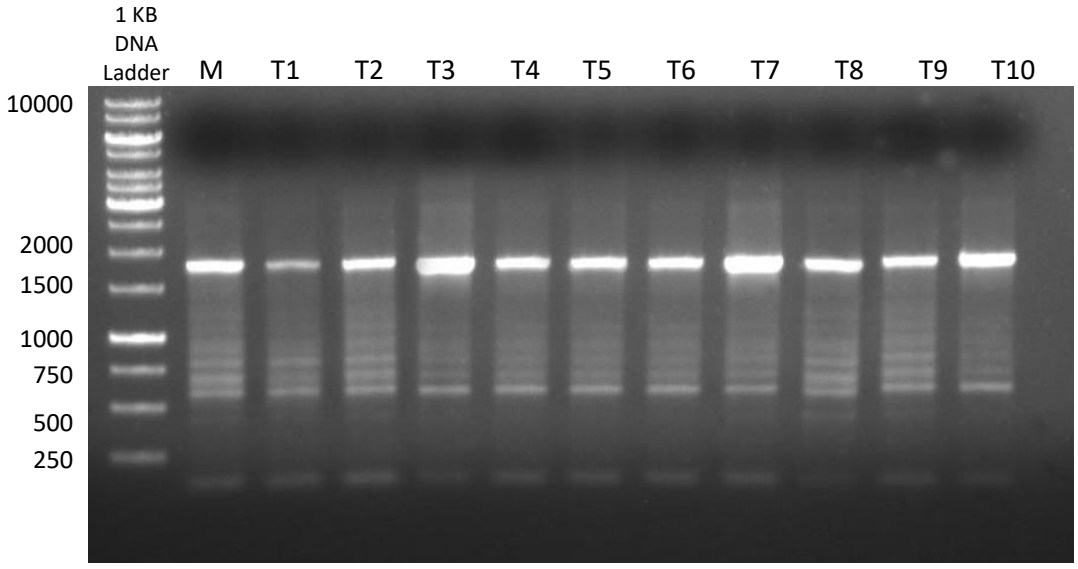
Fig.–4. RAPD profile generated by Primer OPJ-06





L= Ladder, M= Mother Plant, T1-T10= Tissue cultured raised plant

Fig. –5. RAPD profile generated by Primer OPJ – 07



L= Ladder, M= Mother Plant, T1-T10= Tissue cultured raised plant

Fig.–6. RAPD profile generated by Primer OPC – 01

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