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Genetic Variability of *in Vitro* Raised Plants of *Punica granatum* L. by RAPDs

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ABSTRACT

Random amplified polymorphic DNA (RAPD) markers were used to evaluate the genetic variation of *in vitro* raised plants of *Punica granatum* L. derived from cotyledonary callus. Twenty four random decamer primers were used to amplify DNA; the total number of bands amplified was 116, out of which 71 were monomorphic and 45 were polymorphic, equivalent to 38.8% polymorphism. Dice similarity coefficient ranged between 0.74-0.94 among 15 plants. All 15 plants showed 74.0% similarity in the dendrogram constructed using Dice's coefficient and UPGMA analysis. Our results suggest that the genetic variation may be related to somaclonal variation among *in vitro* raised plants of wild pomegranate.

Keywords: genetic variation, polymorphism, primer, wild pomegranate

INTRODUCTION

The wild pomegranate (*Punica granatum* L.) belongs to family Punicaceae, is a multipurpose fast growing and of economic significance in mid hill zone of Western Himalayas. Although the species is considered to be native of Iran, Afghanistan and Baluchistan but it is also well adapted to subtropical and subtemperate parts of Western Himalayas (Evreinoff 1949). In India it can be seen growing wild in hills especially between 900-1800 m above mean sea level (Anonymous 1982).

Wild pomegranate is an important cash crop tree among marginal farmers. Its fruit is good source of sugar and vitamin C and fair source of iron. Seed with arils are sun dried and commercially marketed as Condiments. The powdered flower buds are used in bronchitis. The unripe fruits and flowers are useful in inducing vomiting and the rind of the fruit is given in diarrhoea and dysentery. Ripe fruits are a tonic and laxative and can enrich the blood. All parts of this tree particularly rind, stem bark and root bark contains tannins which are utilized for the manufacturing of dye used for the production of Morocco leather (Howes 1953). Besides this, the species has a deep and spreading root system which helps in binding soil particles together and thus protects soil from erosion. Moreover, wild pomegranate being resistant to drought and disease, requires less after care. Therefore, a massive plantation of trees on barren hills can metamorphose the ecology of mid hills by rehabilitation (Anonymous 1982).

In the present laboratory work, was done on *in vitro* raised plants of wild pomegranate and after hardening these plants have been demonstrated under field conditions.

In vitro culture techniques provide an alternative means of plant propagation and a tool for crop improvement (Vasil 1988). Methods for pomegranate tissue culture have been developed (Moriguchi *et al.* 1987; Omura *et al.* 1987, Bhansali 1990, Naik *et al.* 2000; Kanwar *et al* 2004; Chaugule *et al.* 2005; Kanwar *et al.* 2010; Raj Deepika and Kanwar 2010). In pomegranate, suspension cultures were produced from calli derived from leaf explants but no somaclonal variation was detected among the regenerated plants (Omura *et al.* 1990). Tetraploids and tetraploid/diploid chimeras of pomegranate were also induced by colchicine treatment of leaf segments (Omura *et al.* 1987). Plants regenerated from *in vitro* culture might exhibit somaclonal variation as reviewed from time to time in other woody species (Kaushal and Kanwar 2003; Kaushal *et al.* 2006) and which is often heritable. Several cytological, isozyme and molecular markers have been used to detect variation and/or confirm the genetic fidelity of micropropagated plants (Kaushal and Kanwar 2003; Kanwar *et al.* 2005; Kaushal *et al.* 2006; Sreedhar *et al.* 2007). Random amplified polymorphic DNA (RAPD) is a simple, cost-effective technique to detect variation (Masoud *et al.* 2008; Hasnaoui *et al.* 2010). Such precise and refined techniques appear to have not been utilized thus far for assessing the *in vitro* plants of wild pomegranate and hence the present study has attempted to check the homogeneity/variation in plants of wild pomegranate raised from cotyledon-derived callus.

MATERIALS AND METHODS

Plant material

Fifteen *in vitro* raised plants of *Punica granatum* L. were characterized for their genetic variation using RAPD markers. Briefly, the protocol of raising plants was as follows. Seedling of wild pomegranate was germinated on solid MS (Murashige and Skoog 1962) basal medium and used as a source of cotyledon explants. Callus was induced from cotyledonary explants on MS medium supplemented with 21 µM α-naphthaleneacetic acid (NAA; Sisco Research Laboratory, Mumbai, India) and 9 µM benzylamino purine (BAP; Sisco). The well-proliferated callus was then transferred onto shoot induction medium (MS medium supplemented with 8 µM BAP, 6 µM NAA and 6 µM gibberellic acid (Sisco)) and root induction medium (MS medium supplemented with 0.02% activated charcoal) for the development of shoots and roots, respectively (Kanwar *et al.* 2010). The regenerated plantlets were successfully acclimatized to the external environment and transferred to field conditions in 2005.

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Original Research Paper

DNA extraction

Genomic DNA from fresh green leaves of *in vitro*-raised plants was isolated based on the method of Doyle and Doyle (1987). About 2.5 g of leaf material was ground to a fine powder in liquid nitrogen and dispersed in 15 ml pre warmed (65°C) extraction buffer [2.0% CTAB (Merck Ltd. Darmstadt, Germany), 1.4 M NaCl (Sisco), 20 mM EDTA (Sisco), pH 8.0, 100 mM Tris HCl (Sisco), pH 8.0 and 0.2% β-mercaptoethanol (Himedia Laboratories, Mumbai, India)]. After incubation for 1 hr at 65°C, the mixture was emulsified with an equal volume of chloroform: isoamyl alcohol (Sisco) (24: 1, v/v) to denature proteins and facilitate phase separation. Following centrifugation at 15,000 rpm for 20 min at 24°C, the supernatant was collected and mixed with 0.6 volume of isopropanol. The precipitated DNA was spooled out, washed twice in 70% ethanol at 10,000 rpm for 10 min at 4°C, dried under vacuum and dissolved in TE buffer (pH 8.0). RNA contaminants was digested with RNase (10 mg/ml) at 37°C for 1 hr, emulsified twice with phenol: chloroform: isoamyl alcohol (25: 24: 1, v/v/v), then with chloroform: isoamyl alcohol (24: 1, v/v) and precipitated by adding $1/10^{th}$ volume of 3 M sodium acetate (pH 5.2) and two volumes of absolute ethanol, washed with 70% ethanol and dissolved in TE buffer (pH 8.0). DNA was tested for its quality on 0.8% agarose gel, quantified spectrophotometrically (Nano Drop™ Spectrophotometer) and purity was determined using the 260/280 ratio.

PCR amplification

The PCR reaction was performed based on the protocol of Williams *et al.* (1990). The reaction mixture contained 25 ng of genomic DNA, 3.0 U of *Taq* DNA polymerase (Banglore GeNei Ltd., India), 10X *Taq* DNA polymerase buffer with 15 mM $MgCl_2$ (Banaglore GeNei), 100 µM of each dNTP (Promega Corp., Madison, USA), 25 pM of primer (Operon Technologies Inc., Alameda, USA) and 25 mM $MgCl_2$ (Bangalore GeNei) in a total volume of 25 µl. DNA amplification was carried out in BioRad Applied Biosystem PCR. After initial denaturation at 95°C for 5 min, PCR was run for 45 cycles consisting of a denaturation step at 95°C for 1 min, 37°C primer annealing step for 1 min and amplification step at 72°C for 2 min, at the end of run final amplification was appended for 72°C for 8 min. The amplification products were size separated by electrophoresis in 1.5% agarose (Sisco) gels in TBE buffer, containing ethidium bromide at 65 V, viewed under UV transilluminator (Alpha Innotech) and photographed using gel documentation system (BioRad). 100 bp DNA ladder (Gene Ruler™) was used as standard.

Data analysis

Each amplified product was scored across all 15 *in vitro*-raised plants for 24 primers for its presence or absence. Co-migrating bands were considered to represent the same locus and thus treated as the same band while scoring. Data matrices were prepared in which the presence of a band was coded as '1' whereas the absence as '0'. Electrophoretic DNA bands of low intensity were not scored. NTSYS-pc, version 2.02 (Numerical Taxonomy System, Exeter Software) was used to perform cluster analysis of the complete RAPD data. A pair wise similarity matrix was constructed by using the Dice similarity index SD = 2a/(2a+b+c), Dice (1945) where, a is the number of bands present in a pair of plant 'i' and 'j', b is the number of bands present in plant 'i' and absent in plant 'j', c is the number of bands present in plant 'j' and absent in plant 'i'. Similarity estimates were analyzed by the unweighted pair group method with arithmetic averages (UPGMA) and the resulting clusters were represented as dendrograms.

Table 1 Description of twenty four random decamer primers used for fingerprint analysis of *in vitro* raised plants of *Punica granatum* L.

Primer Name	Sequence of Primer (5'-3')	Scorable band	Monomorphic band	Polymorphic band	Fragment size (bp)
OPA-01	C A G G C C C T T C	3	3	0	480-1180
OPA-04	A A T C G G G C T G	4	3	1	200-1060
OPA-12	T C G G C G A T A G	2	2	0	500-1031
OPA-13	C A G C A C C C A C	4	3	1	350-920
OPA-18	A G G T G A C C G T	4	2	2	500-1200
OPA-19	C A A A C G T C G G	5	3	2	500-1200
OPE-02	G G T G C G G G A A	7	2	5	450-1100
OPE-05	T C A G G G A G G T	6	1	5	450-2850
OPE-08	T C A C C A C G G T	6	1	5	500-2000
OPE-10	C A C C A G G T G A	7	4	3	300-1230
OPE-15	A C G C A C A A C C	7	5	2	220-1200
OPE-20	A A C G G T G A C C	5	3	2	480-1150
OPF-03	C C T G A T C A C C	6	4	2	250-1230
OPF-07	C C G A T A T C C C	5	4	1	480-1180
OPS-02	C C T C T G A C T G	4	2	2	380-2000
OPS-07	T C C G A T G C T G	4	3	1	400-1180
OPS-15	C A G T T C A C G G	2	1	1	500-1050
OPS-17	T G G G G A C C A C	6	3	3	420-1200
OPS-20	T C T G G A C G G A	4	3	1	500-1200
OPV-04	C C C C T C A C G A	1	1	0	1150
OPV-05	T C C G A G A G G G	4	2	2	550-1150
OPV-08	G G A C G G C G T T	4	3	1	580-1021
OPV-10	G G A C C T G C T G	10	8	2	100-1500
OPV-14	A G A T C C C G C C	6	5	1	530-1500

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

Total number of primers used	24
Number of polymorphic primers	21
Number of monomorphic primers	3
Total number of scorable bands amplified by polymorphic primers	116
Size range of amplified products	100-2850 bp
Average number of bands per polymorphic primer	5.52
Total number of polymorphic bands identified	45
Total number of monomorphic bands identified	71
Average number of polymorphic bands per polymorphic primer	2.14
Percentage of total polymorphic bands	38.80%

RESULTS AND DISCUSSION

RAPD analysis

The amplification profiles of 15 *in vitro* plants were obtained using 24 RAPD primers. Out of these, 21 primers were found to be polymorphic between different plants. Out of 116 amplified fragments, 45 were polymorphic and 71 were monomorphic. Each primer generated a unique set of amplification products ranging in size from 100 to 2850 bp (**Table 1**). The average number of bands amplified per primer was 5.52, while the number of polymorphic fragments per primer ranged from 1 to 10 with an average of 2.14. Maximum number of polymorphic fragments (10) was obtained with the primer OPV-10 (**Fig. 1**). Percentage of polymorphic bands was 38.8% (**Table 2**).

De Laia *et al.* (2000) obtained 62 bands from 15 arbitrary decamer primers during analysis of genotypic identities in four clones of *Eucalyptus.* Kaushal and Kanwar (2003) studied RAPD analysis using 19 random decamer DNA primers and 268 RAPD bands were examined which showed 30% polymorphism in *Robinia pseudoacacia*. Kaushal *et al.* (2006) obtained 263 scorable (87 monomorphic and 176 polymorphic bands) amplification products ranging in size from 65 to 2686 bp in micropropagated plants of a single clone M_5 of *Morus alba* L. Sreedhar *et al.* (2007) used 30 random primers which showed 317 reproducible bands in micropropagated plants of *Vanilla planifolia*.

Cluster analysis was done on the basis of similarity coefficients generated from RAPD data of 116 bands. The similarity indices ranged from 0.74-0.94 among *in vitro*-raised plants. All the plants could be grouped into two major clusters by RAPD at 74.0% similarity level. Thirteen plants were placed in the same cluster and were very close to each other. Plants 1 and 2 were completely sidelined and were found to differ the most from all others. Major cluster I at 82.8% similarity consisted of 13 plants and cluster II contained only two plants with a 79.0% similarity. However, three different sub-clusters could easily be identified (**Fig. 2**).

Bogani *et al.* (1996) found the average values of similarity coefficients among different clones of tomato ranging between dendrograms constructed from 0.55 to 0.98. Kaushal and Kanwar (2003) obtained 0.86 to 0.96 similarity indices among 18 micropropagated plants of *Robinia pseudoacacia* with 86.0% similar level in the dendrogram depicting their origin from the same plant. Kaushal *et al.* (2006) observed 88.0% similarity level among 17 micropropagated plants of *Morus alba.* Major cluster I consisted of 16 plants and cluster II contained only one plant. However, 9 different clusters were obtained at a 95.0% similarly level.

From the present investigation, it is apparent that variation at the DNA level is present among *in vitro* raised plants of *Punica granatum* L. This variation that arise *de novo* during the period of explant culture and production of regenerants after successive subcultures is termed somaclonal variation (Larkin and Scowcroft 1981).

In the past, some authors reported somaclonal variation among tissue-cultured plants of *Populus deltoides* (Rani *et al.* 1995), *Robinia pseudoacacia* (Major *et al.* 1998), *Populus tremuloides* (Rahman and Rajora 2001), *Robinia pseudoacacia* (Kaushal and Kanwar 2003), *Morus alba* (Kaushal *et al.* 2006), and *Ananas comosus* (Santos *et al.* 2008).

Nondal *et al.* (2001) observed genetic instability among micropropagated tea cultivar 'T-78' through RAPD markers. Rahman and Rajora (2001) demonstrated in their study that somaclonal variation using microsatellite DNA markers occurred in micropropagated plants of *Populus tremuloides*. Kaushal *et al.* (2006) reported somaclonal variation in micropropagated plants of a single clone M_5 of *Morus alba* through RAPD markers. Santos *et al.* (2008) observed somaclonal variation in micropropagated plantlets of ornamental pineapple through RAPD markers.

From the present results we have demonstrated that *in vitro*-raised plants derived from cotyledonary callus showed

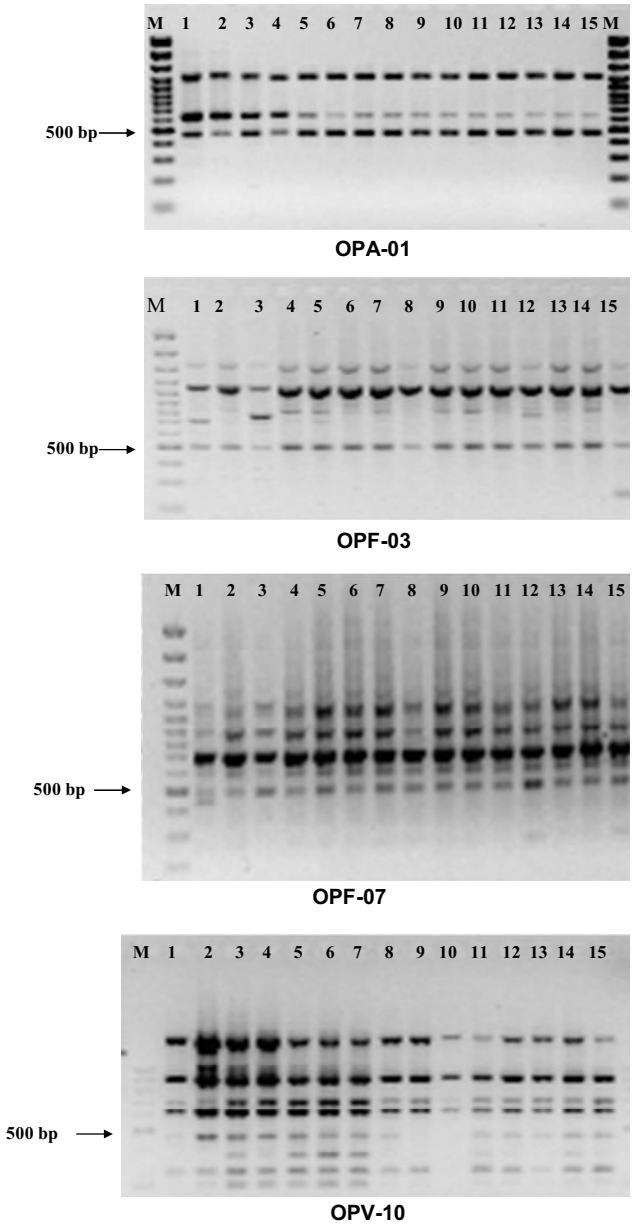

Fig. 1 RAPD pattern of 15 *in vitro*-raised plants of *Punica granatum* L. generated by primers OPA-01, OPF-03, OPF-07 and OPV-10. Lane M represents the molecular size marker used (100 bp ladder, Gene Ruler™).

74.0% similarity among 15 plants in two clusters. However, if cluster II represented by only two odd plants were to be ignored then the similarity reaches 82.8% among 13 plants. This variation may be related to somaclonal variation among *in vitro*-raised plants. It also demonstrates that genetic integrity of somaclones should be invariably confirmed before transfer of hardened plants to the field. RAPD analysis can be applied to assess the genetic variation of *in vitro*-derived plants. However, further improvement to this type of study is desired in the near future using new techniques such as AFLP, SSR and STR for molecular characterization of *in vitro* raised plants.

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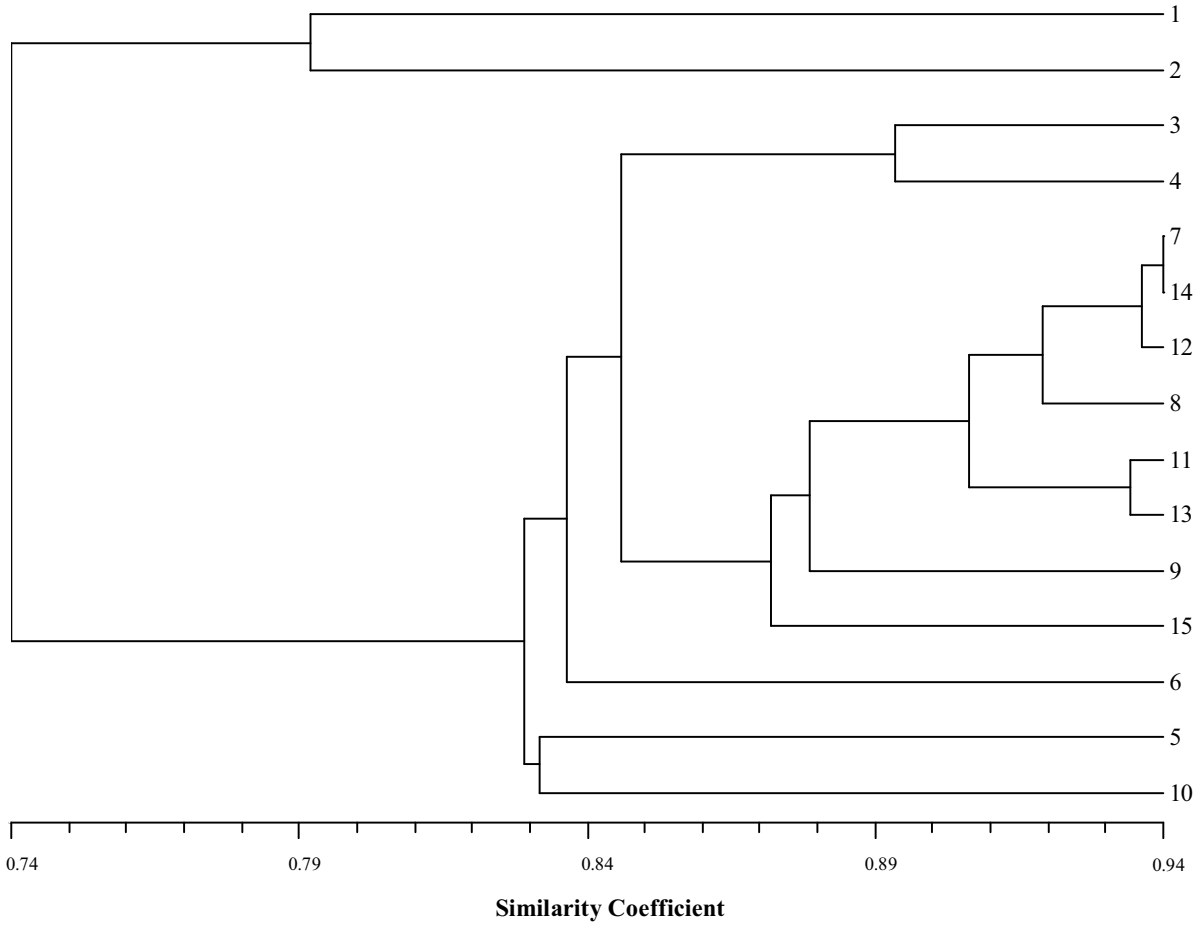

Fig. 2 UPGMA dendrogram with similarity coefficients between *in vitro*-raised plants of *Punica granatum* L. based on RAPD markers.

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