Molecular assessment of clonal fidelity in micropropagated grape (*Vitis* spp.) rootstock genotypes using RAPD and ISSR markers

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Abstract

Micropropagated plantlets derived from three different grape rootstock genotypes namely, Dogridge (*Vitis champini*), SO4 (*V. berlandieri*×*V. rupestris*) and ARI-H-144 (*V. vinifera*×*V. labrusca*) were subjected to randomly amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) analyses in order to evaluate their genetic stability and/or detect likely existing variations among *in vitro* derived plantlets. A dozen RAPD (10-mer) and ten ISSR (dinucleotide contained repeats) primers were used for PCR and reproducible band profiles were obtained. The 84 and 81 distinct and scorable band classes (a total of 1,914 and 1,980 scorable bands) with an average of 7.0 and 8.1 bands per primer were obtained by RAPD and ISSR, respectively. Although higher numbers of bands were obtained by ISSR rather than RAPD, but none of the primers showed polymorphism among micropropagated plantlets and their respective mother plants. The profiles generated based on the two marker systems were found to be highly uniform and monomorphic. Cluster analysis further confirmed genetic stability of micropropagated plantlets. Jaccard's similarity coefficients obtained for both markers in mother plants and their *in vitro* regenerants were estimated to be 1.00 but three sets of genotypes were grouped into two major clusters with similarity coefficients of 0.53 (RAPD) and 0.63 (ISSR). The molecular analyses precisely proved the production of genetically stable grape plantlets and certified the application of micropropagation protocol to be developed on a commercial scale.

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INTRODUCTION

Rootstocks have been used for propagating fruit trees for more than 2000 years. To be of any value to the early fruit tree propagator, the selected clonal rootstocks must themselves have been easy to propagate (Webster, 1995). For large-scale production, efficiency of propagation methods is of prime importance, but perhaps even more important is the genetic stability of *in vitro* regenerated plantlets (Haisel *et al*., 2001). When plant tissue is passaged through *in vitro* culture; many of the regenerated plantlets appear to be no longer clonal copies of their donor genotype, probably due to epigenetic changes. The broader utility of any micropropagation system may be limited due to occurrence of cryptic genetic changes and development of somaclones (Rani and Raina, 2000). In a rootstock micropropagation programme, it is of paramount importance to produce true-to-type planting materials, hence somaclonal variations of any kind, if induced may multiply very fast and lead to loss of the chief characteristics of the parent rootstocks. Furthermore, this genetic instability may be a risk associated with the application of *in vitro* culture techniques for handling and storage of germplasm (Ray *et al*., 2006). Variation is understood to be generated via combination of genetic and/or epigenetic changes. A lack of any phenotypic variation among regenerants does not necessarily imply a concomitant lack of genetic (or epigenetic) change (Larkin and

Scowcroft, 1981) and it is therefore of interest to assay the outcomes of *in vitro* raised plantlets at the genotypic level. The polymerase chain reaction (PCR) has previously been used in conjunction with short randomly amplified polymorphic DNA (RAPD) primers (decamer or ten-mer) to assess the genetic stability of micropropagated grape plantlets (Khawale *et al*., 2006; Singh *et al.*, 2005), MM 106 apple rootstock (Modgil *et al*., 2005), peach (Hashmi *et al*., 1997) and strawberry (Boxus *et al*., 2000). However, some reports have suggested the use of more than one DNA amplification technique as being advantageous for evaluating genetic stability of micropropagated plantlets in several crops like kiwifruit (Palombi and Damiano, 2001), almond (Martins *et al.,* 2004) and banana (Lakshmanan *et al*., 2007; Ray *et al*., 2006). The genetic stability of micropropagated grape rootstock plantlets using ISSR markers has recently been reported (Alizadeh *et al*., 2008). In the present study, a combination of two PCR-based molecular marker techniques, RAPD and inter simple sequence repeats (ISSR), was used to confirm genetic stability of micropropagated grape rootstocks. There is currently no report on evaluation of genetic stability in micropropagated grape rootstock plantlets which can be strengthened with these simple, fast, cost effective, highly discriminative and reliable DNA marker systems.

MATERIALS AND METHODS

Plant material and micropropagation: Three grape rootstocks were procured from main experimental orchard, IARI (Indian Agricultural Research Institute), New Delhi, India and micropropagation protocol was standardized for them (Alizadeh, 2007), Single-node segments (1.5 to 2 cm long) from 2 months old canes were used to initiate *in vitro* cultures following agitation in a solution of carbendazim (AGRO CARE,UP,India, Bavistin at 2 g/l) along with 8-hydroxyquinnoline citrate (8-HQC (Fi-tech chemechtron, Private Limited, Bangalore, India) (200 mg/l) for 1h, which was then followed by surface sterilization with mercuric chloride (0.1%) for 7 min. The MS medium (Murashige and Skoog, 1962) supplemented with 2.0 mg/1 of 6-benzyl adenine (BAP) and 0.2 mg/1 α-naphthalene acetic acid (NAA) was used for culture initiation, while shoot proliferation-cum-rooting was performed on MS medium supplemented with 2.0 mg/l of indole-3-butyric acid (IBA) and 200 mg/l of activated charcoal (AC). Rooted plantlets (one month old) were transferred to glass jars with polypropylene (PP) caps filled with coco peat (which consists of perlite: vermiculite (2:1:1)) for acclimatization (Fig. 1). The potting mixture was moistened with 1/4 MS macro- and micro-nutrients devoid of organics and subjected to autoclaving (30 min at 15 psi, 121ºC) prior to plantlet transfer. The glass jars were then incubated in a growth chamber with the following conditions; cool white fluorescent lights (227 µmol/m^2/sec), a controlled photoperiod (16/8h) and a temperature of 26±1ºC. Three weeks following inoculation, the PP caps were loosened for a few days prior to complete removal. Hardened plantlets were then transferred to glasshouse conditions during the 6 to 8th week after inoculation depending up on the genotype. Ninety days after *ex vitro* transfer, ten plantlets were randomly selected from each genotype for molecular studies.

Genomic DNA isolation: A modified etyltrimethylammonium bromide (CTAB) procedure described by Murray and Thompson (1980) was used to isolate DNA from young, unexpanded grape leaves (5 g). Frozen tissues in liquid nitrogen were grounded using an autoclaved, pre-chilled mortar and pestle to yield a fine powder. The content was transferred to 50 ml Oak-Ridge centrifuge tubes (Fi-tech chemechtron, Private Limited, Bangalore, India). Fifteen ml of pre-warmed (65ºC) extraction buffer (10 ml of 1M Tris-HCl, 4 ml of 0.5 M EDTA, 35 ml of 4 M NaCl, 20 ml of 10% (w/v) CTAB, 200 µl of β-mercaptoethanol, 30.8 ml of autoclaved Milli-Q (MQ) water in a total volume of 100 ml) and 100 mg/g of leaf (for each gram of leaf sample 100 mg PVP was added) polyvinylpyrrolidone (PVP) were added to the grounded sample. The mixture was then incubated at 65ºC in a water bath for 30 min, and gently rotated at 10 min intervals. An equal volume of chloroform: isoamyl alcohol (24:1 v/v) was added to each tube followed by inverting them 20 to 25 times. After centrifugation (8,000 rpm, 20 min.), the transparent supernatant was collected carefully with the help of a wide-bore pipette tip and transferred to fresh tubes. The DNA threads appeared immediately after adding 0.6 the volume of chilled iso-propanol to each tube. These tubes were kept at -20°C overnight followed by centrifugation (8,000 rpm for 20 min at 4ºC). The supernatant from each tube was poured off and the pellet was washed twice

with 70% (v/v) ethanol and finally with absolute ethanol. The pellet was then dried by leaving the tube uncovered in a laminar air-flow (approximately 1 to 1.5h). The dried pellet was dissolved in 150 to 200 µl of fresh TE buffer and the content was transferred to an Eppendorf tube. To estimate DNA concentration, samples were compared with dilutions of a known concentration of lambda uncut DNA as a standard following 0.8% (w/v) agarose gel electrophoresis. The extract was incubated with 1 µl of RNaseA (per 100 µl DNA) solution at 37ºC for 1h, in order to remove contaminating RNA. DNA samples were finally diluted with sterile MQ water to a working concentration of between 25 to 30 ng/µl.

Primers and their screening: A set of 26, 10-mer RAPD primers (Sigma-Aldrich Chemicals, St. Louis, USA) and 35 dinucleotide repeat ISSR primers (CLONI TECH, Genuine Chem. Corp., New Delhi, India) were screened initially for polymorphism with the template DNA of three mother plants. Twelve RAPD and 10 ISSR primers were finally selected for analysis on the basis of their amplification products for clear, bright and scorable banding patterns.

Amplification conditions: Amplification with both RAPD and ISSR primers was carried out in a total volume of 25 µl containing 1 µl (25 ng) of template DNA, 2.5 µl of 10X PCR buffer containing 15 mM $MgCl_2$, 0.5 µl of dNTPs (10 mM each of dATP, dGTP, dTTP, dCTP), 1 µl of primer, 0.25 µl of *Taq* DNA polymerase and 19.75 µl of sterile MQ water. Amplification conditions were performed as initial DNA denaturation at 95ºC for 4 min. followed by 40 cycles of 1 min denaturation at 95ºC, 1 min annealing at 37ºC and 2 min of extension at 72ºC with a final extension time at 72ºC for 10 min. In case of ISSR primers, optimal annealing temperature was adjusted according to the base concentrations of the primers (Table 1). Hence, annealing temperature was optimized for each primer using a gradient PCR. The temperature profile for ISSR was carried out in accordance with the procedure described above for RAPD. Amplification of DNA in both systems was performed in a BioRad thermocycler machine (BioRad, USA).

Electrophoresis of amplified DNA: Amplified DNA fragments were separated and visualized on a 1.4% (w/v) agarose gel stained with ethidium bromide.

Table 1. List of ISSR primers, their sequences and standardized annealing temperatures for polymerase chain reaction (PCR).

Primer code	Sequence (5'-3')	Standardized primer's annealing temperature (ºC)
04	$(GA)_8YG$	44.6
09	$(AC)_8YG$	54.3
S-13	$(AC)_8YC$	52.6
S-14	$(AG)_8T$	44.6
S-16	$(AG)_8G$	52.0
S-17	$(GA)_8T$	44.6
S-19	$(GA)_8A$	51.6
S-27	BDB $(CA)_7$	55.0
S-30	HVH $(TG)_7$	51.6
S-31	$(AG)_8VC$	49.6

*Y stands for pyrimidine, B for non-A, D for non-C, H for non-G, V for non-T residue.

Three µl of 6X loading dye was added to 25 µl of amplified products and following homogenization, 5 µl of the resulting mixture was loaded onto a gel prepared in 0.5X TAE buffer. DNA ladder (100 bp marker, New England Biolabs Inc., UK) was also loaded flanking the samples. The gel was visualized on a UV transilluminator and photographed by an Alphaimager Gel Documentation System (Alpha Innotech Corporation, USA).

Data analysis: In both marker systems, scorable bands were recorded as present (1) or absent (0) and based on band data, the similarity matrix was calculated using Jaccard's coefficient. Cluster analysis was carried out using the SHAN module in NTSYS pc 2.2 software (Rohlf, 2005). An unweighted pair group method of arithmetic mean (UPGMA) dendrogram was generated from Jaccard's similarity values individually for RAPD, ISSR and the pooled data.

RESULTS

In vitro regenerants (Fig. 1) developed in our research were highly uniform with regard to their phenotype and growth rate, i.e. vine length, number of leaves, leaf area, internodal length and *ex vitro* performance (data not shown). In addition, molecular analyses confirmed uniformity at the genotypic (DNA) level. In the present study, 33 samples were analyzed as three distinct sets, each set including one grape mother plant along with its ten *in vitro* clonal plantlets.

Random amplified polymorphic DNA (RAPD) analysis: Of 26 RAPD primers initially screened with DNA of mother plants of grape rootstocks, only 12

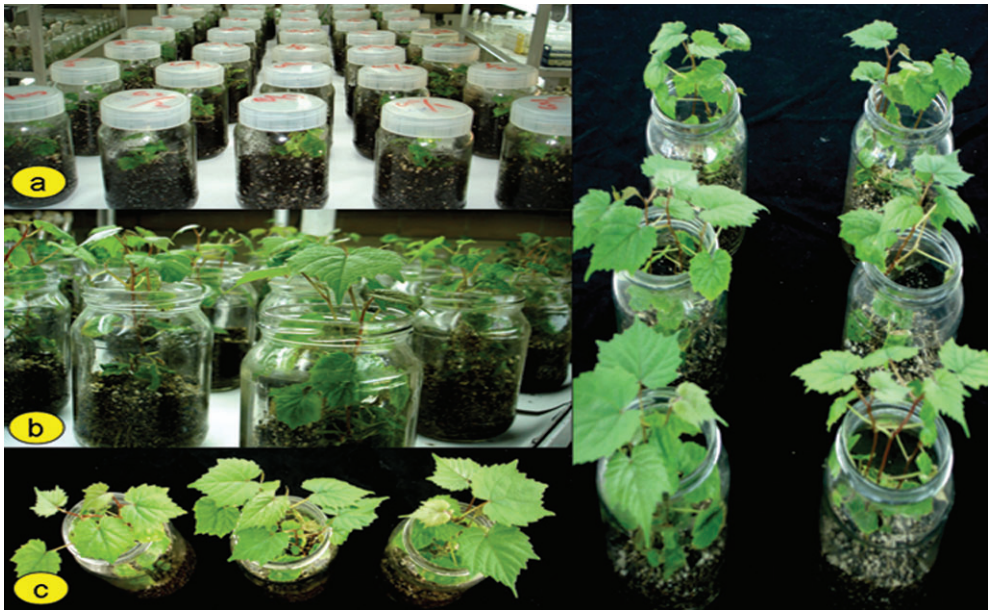

Figure 1. Micropropagated SO4 plantlets during acclimatization in glass jars filled with peat: perlite: vermiculite (2:1:1) (a), 30 DAI (b) and hardened plants ready for *ex vitro* transfer (c).

(Table 2) were used for final characterization based on screening findings. The number of scorable bands for each primer varied from 4 (P-10) to 10 (P-18). These primers produced 84 distinct and scorable band classes with an average of 7 bands per primer. Each primer generated a unique set of amplification products ranging in size from 150 (P-9) to 2000 bp (P-24). The maximum number of 37 bands was present within the size range of 500 to 1000 bp, followed by 20 bands of less than 500 bp in size, 16 bands between 1500 to 2000 bp and 11 bands between 1000 to 1500 bp. A total of 1,914 bands (number of samples analyzed×number of scorable bands with all 12 primers) were generated during RAPD analysis and all bands were found to be monomorphic. Primer P-15, amplified the highest number of bands (264), while only 99 bands were obtained by primer P-17. None of the primers revealed polymorphism for plants situated inside a particular set. Analysis of individual primers revealed that RAPD patterns were similar for both the *in vitro* raised plantlets and their respective mother plants, which indicated that there was no genetic variation in the regenerated plantlet population. The RAPD profile generated with primer P-16 is shown in Figure 2.

Inter simple sequence repeat (ISSR) analysis: Prior to ISSR analysis, primer annealing temperature was standardized for individual primers using gradi-

Table 2. Scorable and total number of bands and their sizes obtained from twelve RAPD primers.

Primer code	Sequence (5'-3')	Scorable band classes (No.)	Total No. of bands	Band size range (bp)
P-1	CAGGCCCTTC	5	143	300-1000
P-3	GAAACGGGTG	5	121	300-1517
P-5	TGCCGAGCTG	7	110	400-1517
P-9	AGGTGACCGT	9	176	150-1200
P-10	GGGTAAAGCC	4	66	400-700
P-15	CTGTCTGTGG	8	264	350-1800
P-16	ACTGGGTCGG	9	242	125-1800
P-17	GGTGTTTGCC	5	99	250-1000
P-18	CTGCCACGAG	10	220	350-1800
P-21	CCAGTCCCAA	8	143	400-1800
P-24	TGTCCTAGCC	7	198	300-2000
P-25	GTGCCTAACC	7	132	400-1600
Total	-	84	1914	-

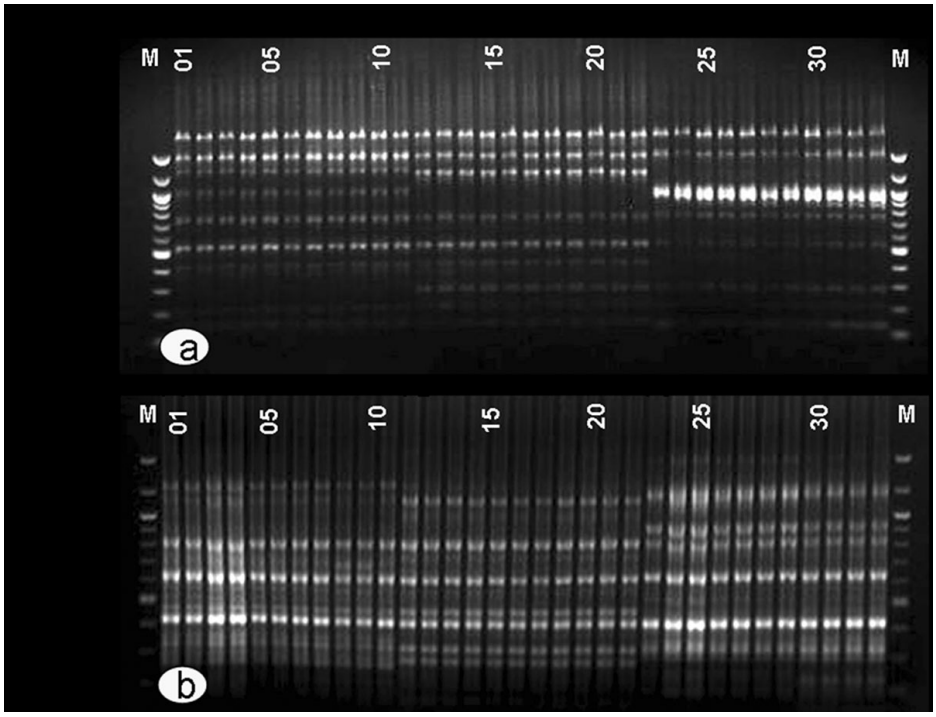

Figure 2. RAPD profile of micropropagated plantlets with primer P-16 (a) and ISSR profile generated with primer S-16 (b). (M=100 bp DNA ladder, P1=Dogridge, P2=SO_4, P3=H-144, Lanes 2-11=TC Dogridge plants; 12-22=TC SO4 plants and 23-33=TC H-144 plants).

ent PCR, which varied from 44.6 to 55°C (Table 1). The number of scorable bands for each primer varied from 5 (S-19) to 11 (S-27). The ten ISSR primers produced 81 distinct and scorable band classes with an average of 8.1 bands per primer. Each primer generated a unique set of amplification products ranging from 100 (S-27) to 1800 bp (09 and S-27). A maximum number of 38 bands were confined within the ladder size of 500 to1000 bp, which was followed by 19 bands of less than 500 bp in size, 14 bands between 1500 to 1800 bp and 11 bands between 1000 to 1500 bp. The data recorded for all three sets of genotypes revealed a total of 1,980 bands generated as per ISSR analysis. Primer S-14, amplified the highest number of bands (297), while only 110 bands were obtained by primer S-19. Furthermore, no difference was observed in banding patterns of any of the sample population for a particular primer with their respective mother plants, indicating absence of variation among the micropropagated plants (Fig. 2).

Pooled analysis: The comparative results obtained in both DNA marker systems are presented in Table 3. According to pooled data analysis using 10 ISSR and 12 RAPD primers, 165 distinct and scorable bands were generated ranging from 100 (S-27) to 2000 bp (P-24). The number of band classes amplified ranged from 4 (P-10) to 11 (S-27) with an average of 7.5 bands per primer. A maximum of 75 bands were present within the ladder size of 500 to 1000 bp, which was followed by 47 bands between 100 to 500 bp and 23 bands between 1000 to1500 bp. Only 20 bands were scored in the range of 1,500 to 2,000 bp. A total of 3,894 bands were generated and all were found to be

Table 3. Comparative data obtained by RAPD, ISSR and pooled analyses of *in vitro* grape plantlets for evaluation of clonal fidelity.

Particulars	RAPD	ISSR	Pooled analysis
Number of primers used	12	10	22
Scorable band classes per primer	4-10	5-11	4-11
Total no. of bands obtained	1914	1980	3894
Average no. of bands per primer	7.0	8.1	7.5
Band size range (bp)	150-2000	100-1800	100-2000
In vitro induced variations	Nil	Nil	Nil

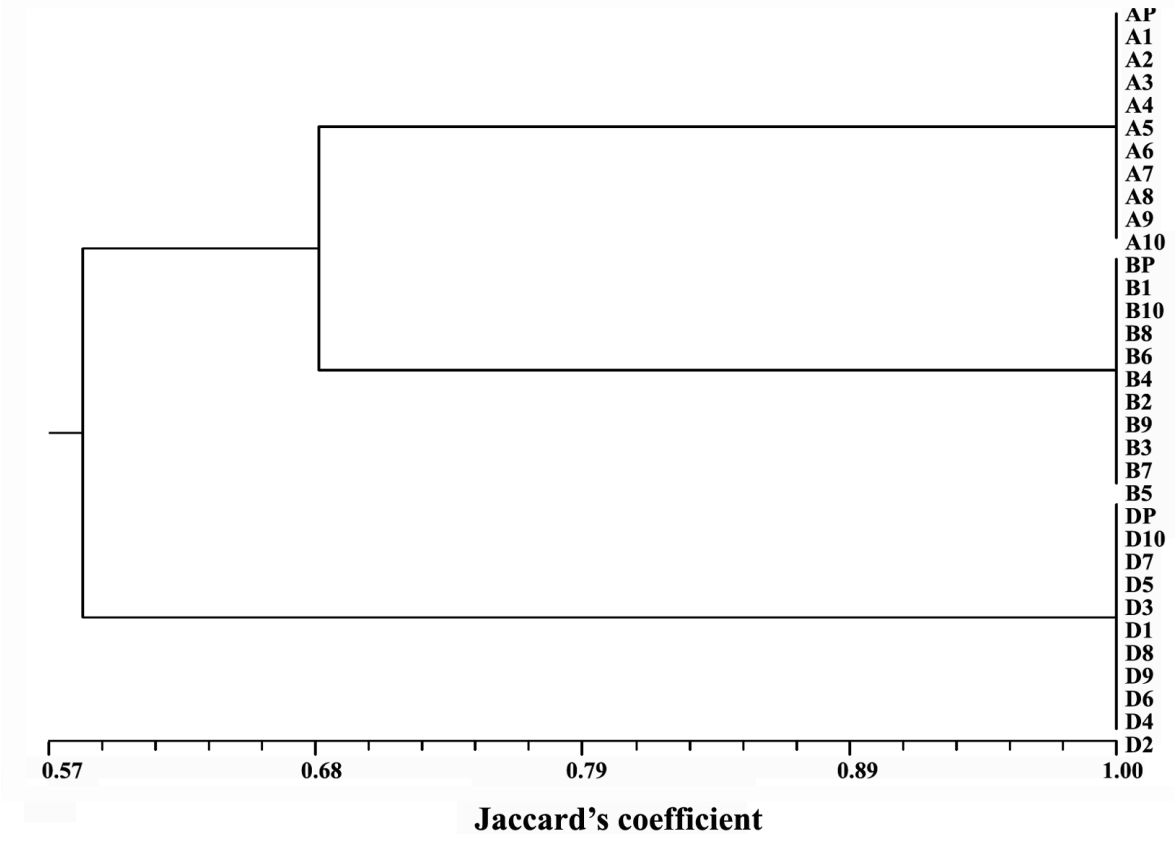

Figure 3. Dendrogram obtained based on pooled data of RAPD and ISSR corroborating high levels of similarity among *in vitro* clonal plantlets and their respective mother plants. (A1 to A10, B1 to B10 and D1 to D10=micropropagated plants of Dogridge (AP), SO4 (BP) and ARI-H-144(DP), respectively.

monomorphic, corroborating the high degree of clonal fidelity of micropropagated grape plantlets.

Dendrogram analyses: The UPGMA dendrogram generated for RAPD and ISSR (Figures not shown) and the pooled data (Fig. 3) further confirmed the true-to-type nature of *in vitro* derived clones with their respective mother plants. Furthermore, it also demonstrated the genetic relationships among three grape rootstocks. In pooled analysis, the similarity coefficient ranged from 0.58 to 1.00 for all three tested sets of genotypes. Within each set, the mother plants and their *in vitro* progenies shared the maximum similarity coefficient of 1.00. Dogridge and SO4 along with their clones were separated into two sub-clusters sharing a similarity coefficient of approximately 0.68 which was almost close to the value obtained by RAPD analysis (0.64). In ISSR analysis this coefficient was estimated to be higher (0.71) confirming its superiority in analysis of clonal fidelity.

DISCUSSION

In commercial micropropagation, it is compulsory to check regularly the clonal fidelity or genetic uniformity of micropropagated plantlets (Khawale *et al*., 2006).

In the present investigation, two PCR-based techniques namely, RAPD and ISSR were used to test clonal fidelity because of their simplicity and cost effectiveness. Furthermore, they require only a small quantity of DNA sample and do not need any prior sequence information and are simple to perform as well as fast (Lakshmanan *et al*., 2007). Besides, the uses of the two markers, which amplify different regions of the genome, allow better chances for identification of genetic variations within the clones (Martins *et al*., 2004).

All RAPD profiles from micropropagated plants were found to be monomorphic and analogous to those of their respective mother plants. Similarity matrix based on Jaccard's coefficient revealed that the pairwise value between the mother plant and the plantlets

was 1, indicating 100 per cent similarity. The true-to-the-type nature of clones was further confirmed with the phenetic dendrogram based on UPGMA analysis. Singh *et al*. (2005) have also studied genetic uniformity of micropropagated Pusa Urvashi grape plantlets, a newly released grape cultivar employing RAPD analysis. They reported no variation among micropropagated plantlets owing to direct regeneration. Working with grapevine, Khawale *et al*. (2006) have also reported the application of RAPD analysis using 30 decamer primers for adjudging clonal fidelity. They confirmed genetic stability of *in vitro* propagated grape cv. Perlette.

Absence of genetic variation using the RAPD marker system has been reported in several cases such as micropropagated shoots of *Pinus thunbergii* (Goto *et al*., 1998), somatic embryogenesis-derived regenerants of oil palm (Rival *et al*., 1998), micropropagated teak (Gangopadhyay *et al*., 2003), somatic embryo derived sweet potato plants (Sharma *et al*., 2004) and axillary bud proliferation in chestnut rootstock hybrids (Carvalho *et al*., 2004). Earlier, Raimondi *et al*. (2001) observed no intraclonal variation using RAPD analysis in asparagus plantlets. In contrast, Martin *et al*. (2002) detected small variation in *in vitro* raised chrysanthemum plantlets, which were subjected to different proliferation conditions, following the callus mediated regeneration pathway.

Wang *et al*. (1998) have also found that the dinucleotide microsatellites are prevalent in plants while mono-, tri- and tetra-nucleotide repeats are less common. All the ISSR primers used in the present study were dinucleotide repeats. Of the 10 primers tested, 3 contained $(GA)_n$, $3(AC)_n$ and $2(AG)_n$ and two 5'-tri-anchored primers. During ISSR analysis, all 10 ISSR primers showed monomorphic banding patterns within *in vitro* raised clones and with their respective mother plant. The ISSR analysis of tissue cultured plantlets of *Swertia chirayita* by Joshi and Dhawan (2007) support the results obtained in the present study. They have detected no variability among the micropropagated plantlets with their mother plants using 16 ISSR primers.

The technique which is capable of higher resolution and elimination of faint bands (while scoring), reduces the percentage of false negatives but does not affect the number of false positives and is thus ideal for determining clonal fidelity. The reliability and efficiency of markers in detecting large genomic rearrangements greatly vary with the kind of marker used. The variations due to genetic or epigenetic factors are very likely to be reflected in the banding profiles developed by employing different marker systems (Joshi and Dhawan, 2007; Leroy *et al*., 2001; Choudhari *et al*., 1998; Moreno *et al*., 1998). These differences could possibly be due to the high melting temperature for the ISSR primers which permits much more stringent annealing conditions and consequently more specific and reproducible amplification. Devarumath *et al*. (2002) have also revealed that ISSR fingerprints detect more polymorphic loci than RAPD fingerprints.

In this study, although a higher number of bands per primer was generated by ISSR rather than RAPD, but banding patterns of any sample for a particular primer was not different in either of the RAPD or ISSR systems. Corroboratory results were have been reported by Sreedhar *et al*. (2007) while testing the clonal fidelity of long term micropropagated shoot cultures of vanilla (*Vanilla planifolia* Andrews) using RAPD and ISSR markers.

In conclusion, the use of RAPD and ISSR to validate true-to-the-typeness of *in vitro* raised clones can be encouraged. The molecular data of this study clearly revealed that *in vitro* culture initiation from nodal segments may be performed to create true-to-the-type plantlets. Hence, protocols standardized for multiplication of these grape rootstock genotypes can be used commercially with minimum possibility of any *in vitro* induced variability.

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