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*Full Length Research Paper*

# An assessment of genetic fidelity of *in vitro* grown plantlets of rose (*Rosa hybrida*) through molecular markers

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**A simple and routine method for the analysis of somaclonal variation among tissue culture derived rose plants is a prerequisite for precise monitoring of quality control during rapid mass micropropagation. Random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) molecular marker techniques were employed to validate the genetic fidelity in three varieties of *Rosa hybrida* [Culture varieties (cvs) First Red, Cri Cri and Pusa Gaurav] multiplied *in vitro* by axillary multiplication for up to 26 subcultures. Twelve RAPD and seventeen ISSR primers generated a total of 119, 104 and 114 amplicons in cvs First Red, Cri Cri and Pusa Gaurav, respectively. A homogeneous amplification profile was observed between the explant source and all the micropropagated plantlets. The result indicated the clonal fidelity of the tissue culture raised *R. hybrida* plantlets and corroborated the assumption that axillary multiplication is the safest mode for multiplication of true to type plants without any somaclonal variation.**

**Key words:** *Rosa hybrida, in vitro,* genetic fidelity, molecular markers.

## INTRODUCTION

Rose is one of the most important commercial flowers. Due to its global importance as an ornamental plant, much research has focused on rose improvement through plant breeding. In the last few years, *in vitro* propagation has revolutionized commercial nursery business (Pierik, 1991; Rout et al., 2006). Important features for which *in vitro* propagation are adopted include its enormous multiplicative capacity in a relatively short span of time, production of healthy and disease free plants and its ability to generate propagules throughout the year (Dhawan and Bhojwani, 1986). The earliest references of rose micropropagation were those of Jacob et al. (1969, 1970a, b) and Elliott (1970) in *Rosa hybrida* cv. Superstar and *Rosa multiflora,* respectively. Such a method has considerable implications for the rose breeder as it allows rapid multiplication of desired varieties. Plant propagation by tissue culture usually aims at the possible highest multiplication rate without hyperhydricity and mutation directly from nodal segments, lateral and auxiliary buds. Despite the advantages of *in vitro* propagation, genetic instability has been observed frequently in micropropagated species which severely hinders the scaling up of any micropropagation protocol. The somaclonal variation are sometimes considered as noble method for developing new varieties but these variations are not desirable when the aim is to develop identical propagules of a desired variety. Hence, a stringent quality check in terms of genetic similarity of the progeny becomes mandatory. Any system which can significantly reduce or eliminate variation generated during tissue culture can be of much practical utility.

Several strategies were used to assess the genetic fidelity of the *in vitro* derived plantlets, which includes morphological description, physiological supervision, karyotype analysis, biochemical assessment and field assessment (Breiman et al., 1989; Chowdhury and Vasil,

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1993), but all methods have some limitations. Morphological and physiological methods are subject to environmental effects, karyological analysis cannot reveal alteration in specific genes or small chromosomal rearrangements (Isabel et al., 1993), biochemical assessment vary with the developmental stages of plant. Isoenzyme markers provide a convenient method for detecting genetic changes, but are subjected to ontogenic variations. They are also limited in number, and only DNA regions coding soluble protein can be sampled. Molecular markers have now come up as the most desirable tool for detecting and characterizing variation at the DNA level (Cloutier and Landry, 1994). Out of the available techniques, both random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) techniques have proven to be a reliable, reproducible, easy to generate, inexpensive and versatile set of markers that relies on repeatable amplification of DNA sequences using single primers. This technique is apparent in studies conducted to screen somaclonal variation produced tissue culture plant, as in *Populus deltoides* (Rani et al., 1995), *Plumbago zeylanica* (Rout and Das, 2002) and S*wertia chirayita* (Joshi and Dhawan, 2007). The present study was undertaken to assess the genetic stability/variability of micropropagated plantlets of *R. hybrida* varieties "Pusa Gaurav", "Cri Cri" and "First Red" derived from nodal culture using RAPD and ISSR marker techniques.

## MATERIALS AND METHODS

### Donor plant material

The material used for the present study consisted of three commercial varieties of *R. hybrida* (cvs First Red, Cri Cri and Pusa Gaurav). Micropropagation protocol by axillary methods was standardized for these three varieties of *R. hybrida* and employed for large scale commercial propagation (Senapati and Rout, 2008). Nodal segment of donor plants (cvs First Red, Cri Cri and Pusa Guarav) obtained from Rose Nursery of Regional Plant Resource Center, Bhubaneswar (India) served as explant source.

### Rapid plant multiplications

Shoot containing nodes were cut from the donor plants during the early hours in the months of January 2005. They were surface sterilized with 0.1% (w/v) mercuric chloride ($HgCl_2$) and after four subsequent washing with distilled water, explants were trimmed and transferred to agar basal culture medium containing macro and micro nutrients as recommended by Murashige and Skoog (MS) (1962), supplemented with 100 mg/L myo-inositol, 30 gm/L sucrose, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.5 mg/L thiamine-HCl, 2 mg/L glycine and also fortified with benzyl amino purine (BAP), kinetin (K)n in combination with indol acetic acid (IAA) in a range of concentration. After six weeks of initial culture, the frequency of explants eliciting response was calculated as a percentage of explants producing shoot buds per total number of explants. Axenic secondary explants with induced multiple shoot buds were further tested for their proliferation and/or regeneration efficacy on MS basal medium supplemented with cytokinin/auxin in different combinations. Afterwards, healthy regenerated shoots (>4.5 cm) on excision were transferred on half MS medium containing 2% sucrose (w/v) supplemented with 0.25 mg/L indol butyric acid (IBA) for root initiation. Operations from surface sterilization to inoculations were carried out under sterile conditions in a laminar air flow. Cultures were incubated at 25 ± 1°C under 16 h/photoperiod with light intensity of 3000 lux and were subcultured every 4 weeks to avoid callusing after an initial culture for 6 weeks. All the growth regulators were added prior to autoclaving. The media was solidified with 0.7% (w/v) agar. Media was sterilized for 15 min at 121°C (15 lb psi pressure) rooted micropropagules were thoroughly washed and planted in earthen pot containing only sterile sand. The pots were kept in green house at 25 to 30°C and >85% relative humidity for acclimatization. When the micro-propagules formed a clump of roots after about 2 weeks, they were transferred to 6 inch earthen pots containing a mixture of sand, soil and cow dung manure in the ratio of 1: 1: 1 (w/v) under field conditions and a hardening success rate of 68% was obtained.

### DNA fingerprinting: Preparation of genomic DNA and polymerase chain reaction (PCR) amplification

Genomic DNA was extracted from leaves of three months old tissue cultured raised plants maintained in field at 10 and 26th multiplication passage. DNA was also extracted from *in vivo* mother plants which were considered as explant source. DNA was isolated following the modified procedure of Doyle and Doyle (1987). An aliquot of 2 µl of the preparation was checked on 0.8% agarose gel for estimating the amount and purity of DNA isolated comparing it against a known weight of uncut lambda DNA. The yield of DNA was less per gram of leaf tissue (40 to 150 ng). In a pre-screen with 30 RAPD primers (Operon Technologies, USA) and 21 synthesized ISSR primers (M/S Genie Bangalore, INDIA) based on amplification of DNA from the mother plant and tissue cultured plants, 12 RAPD and 7 ISSR primers were selected for further analysis. These primers produced distinct amplification profiles that were easily scorable. Polymerase chain reactions (PCR) with single primers were carried out in a final volume of 25 µl containing 20 ng template DNA, 100 µM of each deoxyribonucleotide triphosphate, 20 ng of primer, 1.5 mM magnesium chloride ($MgCl_2$), 1X Taq buffer (10 mM Tris-HCl [pH-9.0], 50 mM potassium chloride (KCl), 0.01 % gelatin) and 0.5 U Taq DNA polymerase (M/S Bangalore Genie, Bangalore, India). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) programmed for a preliminary 2 min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 20 s, annealing at required temperature (depending on nucleotide sequence of RAPD and ISSR primer) for 30 s and extension at 72°C for 1 min, final extension at 72°C for 10 min. The separation of amplified DNA fragments was performed by electrophoresis in 1.5% (w/v) agarose gel along with a molecular weight marker (1.0 Kb plus ladder, M/S Bangalore Genie, Bangalore, India), pre stained with ethidiumbromide and visualized under UV light and amplification product sizes were evaluated using the software quantity one (BioRad, California, USA). All the PCR reactions were repeated at least twice to ensure the reproducibility.

## RESULTS AND DISCUSSION

### Rapid plant multiplication

A varied morphogenetic response was obtained with different combinations and concentrations of cytokinins and auxins. The percentage frequency of shoot buds arising from explants ranged from 30 to 96% after 7 to 8 days inoculation. Maximum frequency of bud break found

**Table 1.** Effect of BAP, Kn and IAA on axillary bud growth of three varieties of rose.

| MS + Growth regulators (mg/L) | | | Days of bud break (percentage of explants showing bud break) | | |
|---|---|---|---|---|---|
| BAP | Kn | IAA | Name of the variety | | |
| | | | First Red | Cri Cri | Pusa Guarav |
| 0 | 0 | 0 | 16 (30)$^{f}$ | 20 (45)$^{f}$ | 16 (45)$^{f}$ |
| 0.5 | 0 | 0 | 8 (70)$^{a}$ | 8 (65)$^{a}$ | 8 (64)$^{a}$ |
| 1.0 | 0 | 0 | 8 (75)$^{a}$ | 9 (80)$^{b}$ | 8 (75)$^{a}$ |
| 1.5 | 0 | 0 | 9 (80)b | 9 (82)$^{b}$ | 8 (80)$^{a}$ |
| 2.0 | 0 | 0 | 10 (75)*$^{c}$ | 9 (75)*$^{b}$ | 10 (62)*$^{c}$ |
| 3.0 | 0 | 0 | 10 (60)*$^{c}$ | 10 (52)*$^{c}$ | 10 (50)*$^{c}$ |
| 0 | 0.5 | 0 | 9 (54)$^{b}$ | 9 (60)$^{b}$ | 9 (54)$^{b}$ |
| 0 | 1.0 | 0 | 10 (68)$^{c}$ | 9 (72)$^{b}$ | 10 (65)$^{c}$ |
| 0 | 1.5 | 0 | 10 (70*$^{c}$ | 12 (66)*$^{e}$ | 9 (60)*$^{b}$ |
| 0.5 | 0 | 0.01 | 8 (90)$^{a}$ | 8 (92)$^{a}$ | 8 (96)$^{a}$ |
| 1.0 | 0 | 0.1 | 8 (80)$^{a}$ | 8 (80)$^{a}$ | 8 (80)$^{a}$ |
| 1.5 | 0 | 0.10 | 8 (88)$^{a}$ | 8 (86)$^{a}$ | 8 (85)$^{a}$ |
| 1.5 | 0 | 0.25 | 9 (80)$^{b}$ | 9 (80)$^{b}$ | 9 (72)$^{b}$ |
| 1.5 | 0 | 0.50 | 10 (60)*$^{c}$ | 10 (65)*$^{c}$ | 9 (60)*$^{b}$ |
| 2.0 | 0 | 0.25 | 9 (42) *$^{b}$ | 9 (34)*$^{b}$ | 9 (45)*$^{b}$ |
| 0 | 1.5 | 0.10 | 9 (75)*$^{b}$ | 10 (70)*$^{c}$ | 9 (65) *$^{b}$ |
| 0 | 2.0 | 0.10 | 12 (56)*$^{e}$ | 11 (54)*$^{d}$ | 12 (42)*$^{d}$ |
| 0 | 1.5 | 0.25 | 11 (65)*$^{d}$ | 12 (40)*$^{e}$ | 10 (60)*$^{c}$ |

20 replicates for each treatment, repeated thrice. Mean having the same letter in a column were not significantly different by Ducan's Multiple Range Test, P ≤ 0.05. *Callusing at the basal end of the axillary bud.

was 90, 92 and 96% in cvs First Red, Cri Cri and Pusa Gaurav, respectively when cultured on MS medium supplemented with 0.5 mg/L BA and 0.01 mg/L IAA after 8 days of initiation (Table 1, Figure 1a, b and c). The three cultivars exhibited best multiplication response on the MS medium supplemented with 2 mg/L BA, 50 mg/L Adenine disulphate (Ads) and 0.25 mg/L IAA. Shoot number per explant averaged 4.84 ± 0.52 (cv First Red), 5.26 ± 0.73 (cv Pusa Gaurav) and 4.34 ± 0.87 (cv Cri Cri) with mean shoot length of 5.62 ± 0.46 (cv First Red), 5.81 ± 0.64 (cv Pusa Gaurav) and 4.92 ± 0.72 (cv Cri Cri) (Table 2, Figure 1d to i). This hormonal combination also showed the desirable organogenic response with very negligible intervening callus phase.

## RAPD and ISSR fingerprinting

The present study was conducted to screen for tissue culture (TC)-induced genetic variations in three varieties of *R. hybrida* plantlets by employing RAPD and ISSR marker techniques. The samples analyzed represents *in vitro* raised plantlets at 10 and 26th multiplication stage (three months old tissue cultured derived plantlets maintained in the field) and *in vivo* plants that served as explants source. The leaf samples were collected at randomly taking 15 plants at each culture period. Initially, twenty RAPD and seventeen ISSR primers were tested, out of which 12 RAPD and 7 ISSR primers were selected finally based on their ability to detect distinct and clearly resolved amplified products for further analysis. Other primers either did not yield amplified products or showed very complex pattern of amplification that was difficult to analyze. These RAPD and ISSR primers amplified a total number of 119, 104 and 114 fragments in cvs First Red, Cri Cri and Pusa Gaurav, respectively which is comparable with the numbers of bands scored (65, 119, 150 and 86) in various other plant taxa by employing RAPD and ISSR marker assay (Leroy et al., 2000; Devarumath et al., 2002; Joshi and Dhawan, 2007; Panda et al., 2007). In our study, all primers amplified scorable bands between 0.32 to 2.9 Kbp molecular size ranges. Amplification profile of three varieties of TC-raised *R. hybrida* plantlets at different stages along with their explant donor has been shown by representive gel profile of RAPD primer OPD-08 (Figure 2a to c) and ISSR primer IG-18 (Figure 2d to f).

The banding pattern of PCR amplified product from micropropagated plantlets along with their explant donor plant was found to be monomorphic for all the primer tested except a few weak bands. These weak bands were not consistently polymorphic in replicate PCR

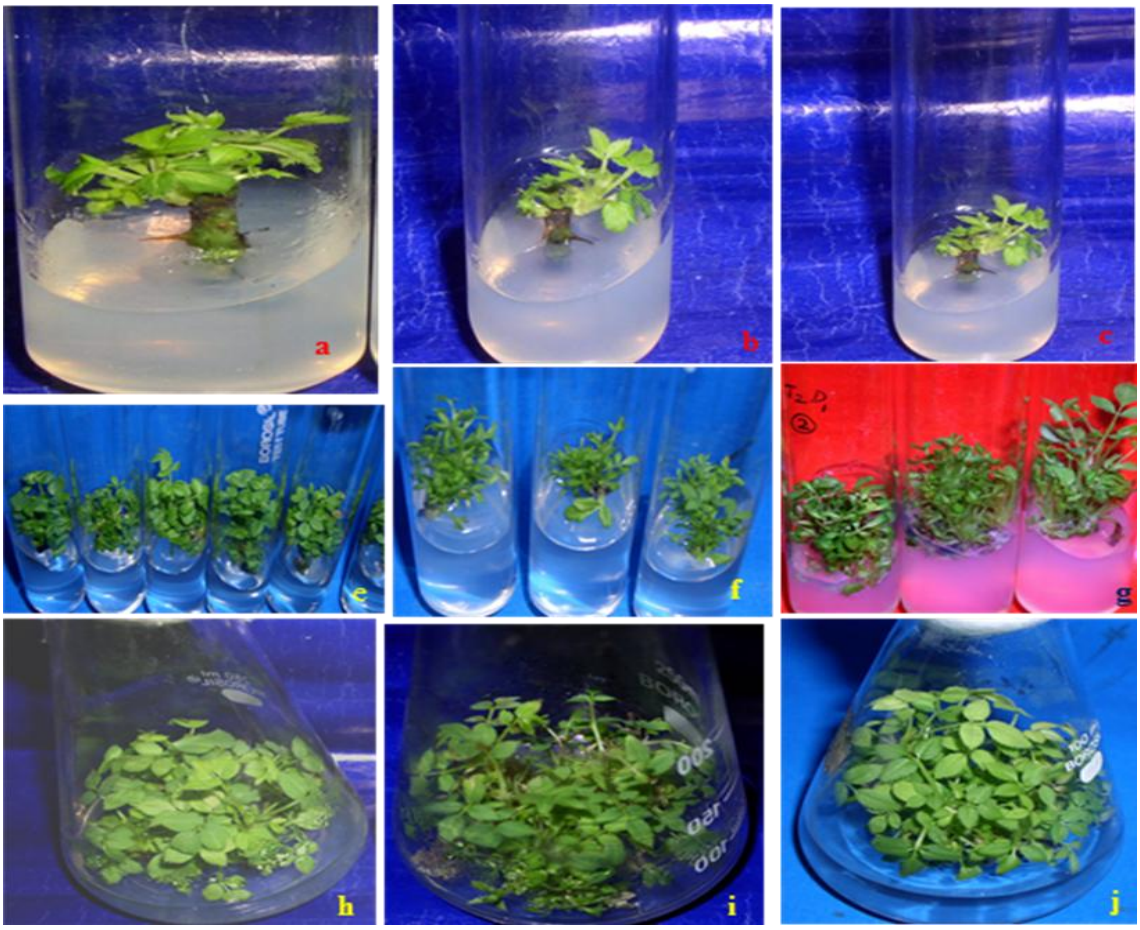

**Figure 1.** a, b and c: Initial of cvs First Red, Cri Cri and Pusa Gaurav, respectively; d, e and f: Shoot multiplication of cvs First Red, Cri Cri and Pusa Gaurav, respectively after 10th subculture; g, h and i: Shoot multiplication of cvs First Red, Cri Cri and Pusa Gaurav, respectively after 26th subculture.

**Table 2.** Effect of growth regulators on multiplication of micro shoots of *R. hybrida* (cvs First Red, Cri Cri and Pusa Gaurav).

| Name of the variety | MS + Growth regulators | | | | Average number of shoots per explant (Mean ± SEM)† | Mean shoot length (cm) (Mean ± SEM)† |
|---|---|---|---|---|---|---|
| | BA | Kn | Ads | IAA | | |
| cv First red | 1 | 0 | 50 | 0.10 | 4.30 ± 0.61$^{d}$ | 4.86 ± 0.68$^{d}$ |
| | 0 | 2 | 50 | 0.25 | 3.24 ± 0.73$^{a}$ | 4.85 ± 0.58$^{d}$ |
| | 2 | 0 | 50 | 0.25 | 4.84 ± 0.52$^{e}$ | 5.84 ± 0.63$^{h}$ |
| | 2 | 0 | 100 | 0.25 | 4.31± 0.48$^{d}$ | 5.62 ± 0.46$^{g}$ |
| cv Cri Cri | 1 | 0 | 50 | 0.10 | 3.43 ± 0.62$^{b}$ | 3.84 ± 0.76$^{a}$ |
| | 0 | 2 | 50 | 0.25 | 3.26 ± 0.85$^{a}$ | 4.68 ± 0.86$^{c}$ |
| | 2 | 0 | 50 | 0.25 | 4.34 ± 0.87$^{d}$ | 4.92 ± 0.72$^{d}$ |
| | 2 | 0 | 100 | 0.25 | 3.68 ± 0.63$^{c}$ | 4.34 ± 0.68$^{b}$ |
| cv Pusa Gaurav | 1 | 0 | 50 | 0.10 | 4.79 ± 0.81$^{e}$ | 4.63 ± 0.48$^{c}$ |
| | 0 | 2 | 50 | 0.25 | 3.40 ± 0.54$^{b}$ | 5.31 ± 0.71$^{f}$ |
| | 2 | 0 | 50 | 0.25 | 5.26 ± 0.73$^{f}$ | 5.21 ± 0.64$^{e}$ |
| | 2 | 0 | 100 | 0.25 | 4.92 ± 0.88*$^{e}$ | 5.81 ± 0.81*$^{h}$ |

†20 replicates for each treatment, repeated thrice. Mean having the same letter in a column were not significantly different by Ducan's Multiple Range Test, P ≤ 0.05. *Callusing at the basal end of the axillary bud.

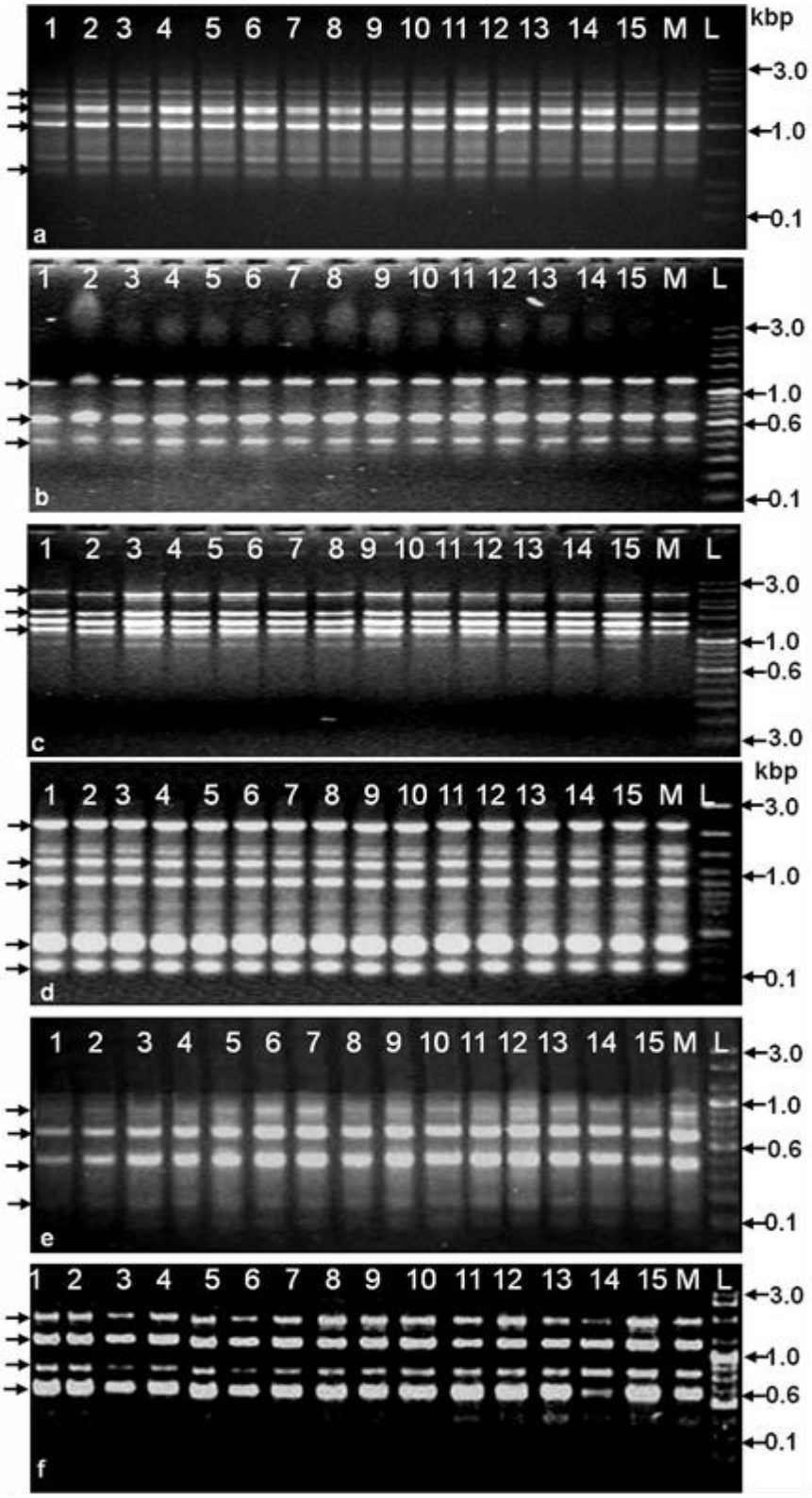


**Figure 2.** Amplification of genomic DNA from tissue cultured derived plantlets cv. (a) Cri Cri, (b) First Red and (C) Pusa Gaurav by primer OPD-08. (d) Cri Cri, (e) First Red and (f) Pusa Gaurav by primers IG-18. L corresponds to a 100 bp DNA ladder; M represents amplification of genomic DNA from mother plant serving as explants source. Arrows point to markers that were consistently both reproducible and scorable monomorphic bands.

**Table 3.** List of different RAPD and ISSR primers used for detecting genetic stability in micropropagated plants and explant donor in three varieties of *R. hybrida.*

| Primer code | Primer sequence | Monomorphic Bands In TC-raised plants + Explant donor | | | Total number of bands amplified |
|---|---|---|---|---|---|
| | | First Red | Cri Cri | Pusa Guarav | |
| **RAPD primers** | | | | | |
| OPA-02 | TGCCGAGCTG | 4 | 5 | 7 | 7 |
| OPA-04 | AATCGGGCTG | 4 | 7 | 7 | 8 |
| OPD-02 | GGACCCAACC | 5 | 6 | 9 | 10 |
| OPD-08 | GTGTGCCCCA | 4 | 6 | 8 | 11 |
| OPD-11 | AGCGCCATTG | 6 | 5 | 3 | 8 |
| OPD-18 | GAGAGCCAAC | 7 | 3 | 5 | 9 |
| OPD-20 | ACCCGGTCAC | 6 | 6 | 4 | 6 |
| OPN-02 | ACCAGGGGCA | 4 | 6 | 7 | 8 |
| OPN-07 | CAGCCCAGAG | 8 | 7 | 6 | 9 |
| OPN-08 | ACCTCAGCTC | 5 | 5 | 5 | 7 |
| OPN-15 | CAGCGACTGT | 6 | 7 | 4 | 7 |
| OPN-16 | AAGCGACCTG | 5 | 3 | 4 | 6 |
| **ISSR primers** | | | | | |
| IG-01 | AGGGCTGGAGGAGGGC | 7 | 7 | 5 | 9 |
| IG-11 | (AG)8C | 7 | 6 | 8 | 10 |
| IG-16 | $(CT)_8G$ | 10 | 4 | 5 | 11 |
| IG-18 | CAG $(CA)_7$ | 9 | 7 | 9 | 12 |
| IG-19 | TGG$(AC)_7$ | 8 | 5 | 6 | 9 |
| IG-22 | $(GACAC)_2$ | 8 | 4 | 5 | 9 |
| IG-23 | $(GA)_8C$ | 6 | 5 | 7 | 8 |
| Total | - | 119 | 104 | 114 | 164 |

reactions. The size and number of DNA fragments produced by respective primers has been noted in Table 3. No polymorphism was detected in the major bands profile, however some weak bands were absent in some of the regenerates though their frequency was quite low. The mean genetic similarity among three months old tissue cultured derived plantlets maintained in the field along with *in vivo* plants that served as explants source and was found to be 99.16, 98.08 and 98.24% for cvs First Red, Cri Cri and Pusa Gaurav, respectively. So, the mean frequency of polymorphism (when the weak bands are also considered) accounts to < 2% in all the three varieties. Absence of polymorphism in RAPD and ISSR profile of micropropagated plantlets suggests a high level of genetic fidelity and also indicates that this micro-propagation protocol is efficient enough to maintain genetic stability of three varieties of *R. hybrida.*

Shenoy and Vasil (1992) reported that micro-propagation through meristems culture are generally less subjected to genetic changes that might occur during cell differentiation under *in vitro* conditions and is the safest mode of micropropagation to produce true to type progeny. Srivastava et al. **(**2006) evaluated < 6% frequency polymorphism of *in vitro* raised sugarcane varieties produced from shoot-tip culture through RAPD markers. Joshi and Dhawan (2007) confirmed that the plants of *S. chirayita* multiplied through axillary method of micropropagation maintain genetic fidelity even after prolonged period of 168 weeks under *in vitro* condition.

Micropropagated plantlets are often subjected to *in vitro* stress that provokes changes at preferential sites, such as repetitive DNA, thereby activating transposable elements (Srivastava et al*.,* 2006) which may cause variation in genetic level due to insertion or deletion. The insertion/deletion of transposons and point mutation results in the loss or gain of primer annealing sites that shows variation in the RAPD and ISSR pattern (Peschke et al*.,* 1991). Variation induced by genetic and epigenetic mechanism is likely to be reflected in banding profiles by employing different marker systems. However, the reliability and efficiency of molecular markers are frequently questioned. A better analysis of genetic stability of plantlets can be made by using a combination of two types of markers which amplify different regions of the genome (Martins et al*.,* 2004). Palombi and Damiano (2002) suggested the use of more than one DNA amplification technique as advantageous in evaluating somaclonal variation while working on micropropagated

plants of kiwi fruit. They found that 11 ISSR primers were able to detect polymorphism (48 polymorphic bands out of 56 reproducible fragments) in comparison to RAPD primers that produced all monomorphic bands. So, in this study, two types of markers study have been employed to detect any occurrence of somaclonal variations. The somaclonal variation are sometimes considered as noble method for developing superior clones, but it could also be a very serious problem in the plant tissue culture industry resulting in the production of undesirable plant off-types (Karp, 1993; Cassells et al., 1999). Off-types have been detected from micropropagated plants, such as in tea clones (Devarumath et al*.,* 2002). Polymorphism at DNA level which is phenotipically normal was reported among date palms (Saker et al*.,* 2005). Hence, it becomes imperative to regularly check the genetic integrity of micropropagated plants in order to produce clonally uniform progeny while using different techniques of micropropagation.

## Conclusion

From the study, it was concluded that the genetic stability of rose plantlets observed in our study indicates good genetic fidelity obtained in plantlets raised *in vitro* when examined by two marker systems*.* A polymorphism rate of < 2% was detected by RAPD and ISSR assay which may be due to point mutations occurring outside the priming sites. Thus, this method can be applied efficiently to determine the genetic purity of rose plantlets derived *in vitro.*

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