Indian Journal of Biotechnology
Vol 14, July 2015, pp 420-424

Molecular assessment of genetic stability using ISSR and RAPD markers in *in vitro* multiplied copies of commercial banana cv. Robusta

D Choudhary[1,2], S Kajla[1], A K Poonia[1], B Brar[3], Surekha[4] and J S Duhan[2]*

[1]Centre for Plant Biotechnology, CCS Haryana Agricultural University Campus, Hisar-125 004, India

[2]Department of Biotechnology, Chaudhary Devi Lal University, Sirsa-125 055, India

[3]Department of Molecular Biology and Biotechnology, CCS Haryana Agricultural University, Hisar-125 004, India

[4]Deprtment of Botany, Government National (PG) College, Sirsa-125 055, India

Received 3 September 2013; revised 13 July 2014; accepted 18 September 2014

The present study was carried out to monitor somaclonal variations of banana cv. Robusta during micropropagation using random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers. Shoot tips were used as explants and regenerated on MS+BAP 4.0 mg/L medium. The regenerated shoots were cultured on MS medium fortified with 4.0 mg/L BAP+0.25 mg/L NAA+30 mg/L $AdSO_4$ for multiplication. Rooting was obtained on ½ MS medium supplemented with 2.0 mg/L NAA. Rooted shootlets were separated individually and hardened in greenhouse. The hardened plants of banana were screened for genetic stability using 46 (26 RAPD and 20 ISSR) primers. The number of scorable bands for each RAPD primer varied from 1 to 9. Twenty-six RAPD primers produced 108 distinct and scorable bands, with an average of 4.15 bands per primer and the amplification products range was from 100-1150 bps. No polymorphism was detected during the RAPD analysis of *in vitro* raised clones. Twenty ISSR primers produced 81 distinct and scorable bands in the range of 100-1000 bps and the number of scorable bands for each primer varied from 2 to 8 with an average of 4.05 bands per primer. A homogenous amplification profile was observed for all the micropropagated plants when compared to mother plant in both types of markers used. The results corroborate the fact that *in vitro* multiplication is the safest mode for production of true to type plants.

Keywords: Banana cv. Robusta, genetic fingerprinting, genetic stability, RAPD, ISSR

Banana (*Musa* spp.; Family: Musaceae) is one of the oldest fruits of the world[1]. It is a basic food source for millions of people in developing countries in the tropics and subtropics[2]. During conventional propagation, generally 4-5 suckers are obtained from single plant but through plant tissue culture technology, from a single shoot tip or an auxiliary bud, a large quantity of uniform and disease free plants with good genetic potential can be produced within a short spell of time[3-5]. The *in vitro* multiplication of plants involves the application of plant growth regulators and changes to these phytohormone habituations are known to be associated with genetic instability in plants. Somaclonal variation in tissue culture is a common phenomenon, which makes it mandatory to check the genetic stability of *in vitro* raised plants. The precise monitoring of quality control during *in vitro* multiplication and analysis of tissue culture raised plants by using simple and routine method is prerequisite step in micropropagation.

During large-scale production of banana plants using micropropagation processes, the plants may not be genetically identical to the mother plant. The failure to observe morphological abnormalities in plants does not negate the possibility of genetic variations and it needs careful and specific analyses. Commercial cultivators need to be sure that they are investing their time and money in propagating the specific cultivar, which are true to type in nature. Various methods can be used to detect and monitor *in vitro* raised plants. The most reliable method is the use of molecular marker techniques. Identification of possible somaclonal variants at an early stage of development is considered to be very useful for quality control in plant tissue culture, transgenic plant production and in the introduction of variants[6]. Sheidai *et al*[7] used random amplified polymorphic DNA (RAPD) markers to detect the somaclonal variations in the tissue culture raised plants of banana cultivar Cavendish Dwarf.

In the present study, an improved and cost effective protocol was developed which can be used to produce the genetically stable and uniform plants of banana cultivar Robusta. The genetic stability and uniformity of tissue culture raised plants was examined and compared with mother plant by using random amplified polymorphic DNA (RAPD) and inter simple sequence repeats (ISSR) markers.

*Author for correspondence:
Tel: +91-1666-243147; Fax: +91-1666-248123
duhanjs68@gmail.com; duhanjs@rediffmail.com

The banana plants used in the present study was *Musa paradisica* cv. Robusta. The experiments were conducted at the Centre for Plant Biotechnology, Hisar. All experiments were performed on Murashige and Skoog's[8] basal medium having 30 g/L sucrose. Sterilized shoot tip explants of cv. Robusta were cultured on MS basal medium supplemented BAP 4.0 mg/L for shoot induction under aseptic conditions. *In vitro* multiplication was done on MS media fortified with 4.0 mg/L BAP+0.25 mg/L NAA +30 mg/L $AdSO_4$. Subculture was carried out at 28 d intervals. Separated elongated shoots were inoculated on ½ MS medium supplemented with 2.0 mg/L NAA for rooting. The rooted plantlets were transferred in greenhouse for hardening[4]. The hardened plants were screened and compared with the mother plant for genetic stability using 26 RAPD (Table 1) and 20 ISSR (Table 2) primers (IDT make).

Table 1—DNA amplification profile generated for clonal fidelity testing of *in vitro* propagated banana cv. Robusta using RAPD primers

No.	Primer	Sequence (5'-3')	Mol wt range (bps)	No. of scorable bands per primer
1	MA1	TGCCGAGCTG	200-550	5
2	MA2	AATCGGGCTG	150-350	5
3	MA3	AGGGGTCTTG	150-220	4
4	MA4	GGTCCCTGAC	190	1
5	MA5	GAAACGGGTG	120-450	4
6	MA6	GTGACGTAGG	200-450	3
7	MA7	GGGTAACGCC	180-420	7
8	MA8	GTGATCGCAG	200-400	5
9	MA9	AGCCAGCGAA	200-350	3
10	MA10	GACCGCTTGT	400-1050	5
11	MA12	AGGTGACCGT	250-350	4
12	MA13	CCGAACACGG	100-450	2
13	MA14	TCGTTCCGCA	600-700	2
14	MA15	CCTCTCGACA	190-450	2
15	MA16	TGAGCCTCAC	300-1000	3
16	MA17	CCCAAGGTCC	200-1000	9
17	MA18	GGTGCGGGAA	300-1000	5
18	MA19	CCAGATGCAC	300-1000	8
19	MA20	GTGACATGCC	500-1000	4
20	MA21	TCAGGGAGGT	400-1000	5
21	MA22	AAGACCCCTC	700-900	4
22	MA23	AGATGCAGCC	400-1000	3
23	MA25	GAGTCTCAGG	300-800	4
24	MA26	GGTGACTGTG	300-500	3
25	MA27	GGCACGTAAG	200-900	4
26	MA28	AAGTCCGCTC	200-800	4
Total				108
Mean				4.15

Total genomic DNA of the mother plant and nine hardened *in vitro* raised clones was extracted from young leaf tissue by using the modified cetyl trimethyl ammonium bromide (CTAB) method as described by Murray and Thompson[9]. PCR amplification was optimized and amplification of RAPD primer was carried out in 20 µL of reaction mix containing 1× PCR buffer, 250 µM dNTPs mix, 0.6 µM primers, 1.5 mM $MgC1_2$, 0.5 U *Taq* DNA polymerase and 50 ng of template DNA. ISSR-PCR reaction was conducted in 10 µL of reaction mix containing l× PCR buffer, 500 µM dNTPs mix, 0.5 µM primers, 1.5 mM $MgCl_2$, 1 U *Taq* DNA polymerase and 25 ng of template DNA. For RAPD primers, PCR was initiated by a denaturation step at 94°C for 3 min and then the reaction was subjected to 40 cycles at 94°C for 1 min, 36°C for 1 min, 72°C for 1 min, with a final elongation step of 5 min at 72°C for RAPD primers. However, PCR for ISSR primers was initiated by a denaturation step at 94°C for 4 min and then the reaction was subjected to 40 cycles at

Table 2—DNA amplification profile generated for clonal fidelity testing of *in vitro* propagated banana cv. Robusta using ISSR primers

No.	Primer	Sequence (5'-3')	Mol wt range (bps)	No. of scorable bands per primer
1	MM1	AGAGAGAGAGAGAGAGYT	400-1000	5
2	MM2	GAGAGAGAGAGAGAGAA	200-1000	6
3	MM3	GAGAGAGAGAGAGAGAC	140-400	4
4	MM4	GAGAGAGAGAGAGAGAT	300-1000	6
5	MM5	CTCTCTCTCTCTCTCTA	200-800	5
6	MM6	CTCTCTCTCTCTCTCTRC	800-900	2
7	MM7	ATGATGATGATGATGATG	650-900	3
8	MM8	GACAGACAGACAGACA	400-600	2
9	MM9	TCTCTCTCTCTCTCTCG	100-600	6
10	MM10	GAGAGAGAGAGAGAGAYT	200-400	2
11	MM11	GAGAGAGAGAGAGAGAYG	900-1000	2
12	MM13	CACACACACACACACARG	600-820	3
13	MM14	ACACACACACACACACYT	400-500	2
14	MM15	CTCCTCCTCCTCCTCCTC	100-800	8
15	MM16	VDVCTCTCTCTCTCTCT	100-590	6
16	MM17	DBDACACACACACACAC	100-400	2
17	MM19	VHVGTGTGTGTGTGTGT	200-900	5
18	MM20	HVHTGTGTGTGTGTGTG	500-800	3
19	MM21	TGGATGGATGGATGGATGGA	500-700	3
20	MM22	AGGGAGAGGAGGAGGAGG	200-800	6
Total				81
Mean				4.05

94°C for 1 min, 50-65°C for 1 min, 72°C for 2 min, with a final elongation step of 8 min at 72°C.

The amplified products were stored at –20°C till further use. The amplification products were resolved by electrophoresis on 2% agarose gel with ethidium bromide (5 µg/mL). PCR amplification products were viewed under long wavelength UV light (302 nm) and photographed using Alpha Digi Doc Pro™ documentation system. Consistent, well-resolved fragments in the size range of 100 bps 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. For both RAPD and ISSR profiles, the well-resolved and consistently reproducible fragments of 100 bps were scored as present or absent. For detecting any genetic change, all the RAPD and ISSR results were compared with each other for all the DNA samples.

The experiments in the present investigation were conducted for *in vitro* propagation and detection of genetic stability amongst i*n vitro* raised plants as compared to mother plant of banana cv. Robusta. The shoot tips were established on MS+ BAP 4.0 mg/L medium producing 1.8 buds /explant. The regenerated shootlets were subcultured on MS medium fortified with 4.0 mg/L BAP+0.25 mg/L NAA+30 mg/L $AdSO_4$ for multiplication. The elongated shoots were transferred to ½ MS liquid media containing 50 g/L sucrose fortified with 2.0 mg/L NAA. Profuse rooting was observed after 3 wk. *In vitro* rooted shootlets were successfully transferred in greenhouse with 100% survival[4].

Clonal fidelity of *in vitro* raised nine hardened randomly chosen plants was carried out and compared with the mother plant and screened for genetic stability using RAPD and ISSR primers. The number of scorable bands for each RAPD primer varied from 1 (MA4) to 9 (MA17) (Table 1). The 26 RAPD primers produced 108 distinct and scorable bands, with an average of 4.15 bands per primer. Each primer generated a unique set of amplification products varied in size from 100 bps (MA13) to 1150 bps (MA10). Further, 20 ISSR primers produced 81 distinct and scorable bands in the range of 100 (MM9, MM15, MM16 and MM17) to 1000 bps (MM1, MM2, MM4 and MM11). The number of scorable bands for each primer varied from 2 (MM6, MM8, MM10, MM11, MM14, MM17) to 8 (MM15) with an average of 4.05 bands per primer (Table 2). All banding profiles from micropropagated plants were monomorphic and similar to those of the mother plant. No polymorphism was detected during the RAPD (Fig. 1) and ISSR (Fig. 2) analysis of *in vitro* raised clones when compared with the mother plant. Genetic molecular markers are considered to be reliable in monitoring variability in the DNA sequences of the plants[10,11]. Similar results were also observed by Kajla *et al*[12] in banana cv. G-9 using RAPD and ISSR marker. Several authors have applied the RAPD technique to investigate the genetic variability and found it to be very efficient and reliable[13]. The results obtained from RAPD analysis were compared with those obtained with restriction fragment length polymorphism (RFLP) and isozymes[14] and no difference were found in them. The results indicated that the RAPD technique is effective to develop genotype-specific banding patterns valuable for cultivar identification. Several researchers have reported that the majority of RAPD bands are reproducible if one takes care in developing a standardized protocol, which is strictly followed in each reaction[15,16].

Clonal fidelity testing of banana cv. Robusta using RAPD and ISSR primers reveals that all the plants raised through *in vitro* propagation of banana cv. Robusta were true to type. The use of two types of

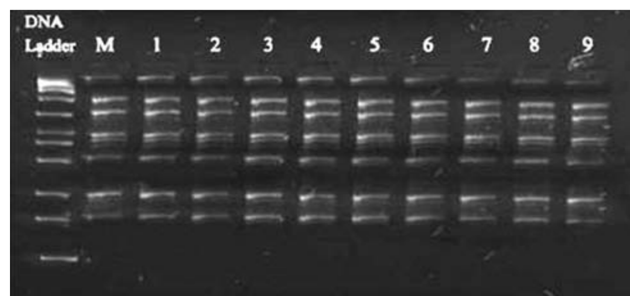

Fig. 1—RAPD profiles of mother plant (M) and tissue culture raised (1-9 lanes) plants using primer MA-17.

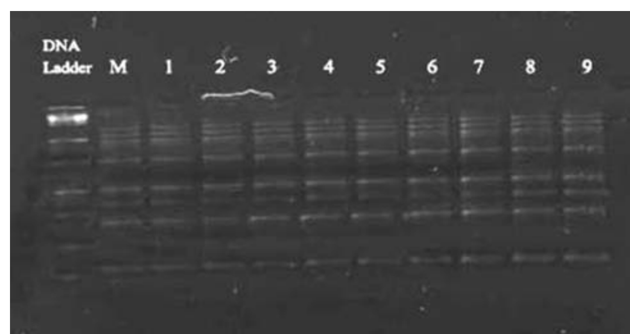

Fig. 2—ISSR profiles of mother plant (M) and nine tissue culture raised (1-9 lanes) plants using the primer MM-15.

markers, which amplify different regions of the genome, allows better chances for identification of genetic variations in the plantlets. In the present study RAPD and ISSR have been used for analysis of variants as they offer several advantages over other conventional methods. This technology is technically simple, inexpensive, quick to perform, requires very little plant material, yields true genetic markers and quick DNA extraction protocols are suitable for the study[17]. Ray *et al*[18] highlighted the genetic stability of the micropropagated plants of 3 banana cv. Robusta (AAA), Giant Govenor (AAA) and Martaman (AAB) by using 21 RAPD and 12 ISSR primers. They found 3 somaclonal variants from 'Robusta' and 3 from 'Giant Govenor". Harirah and Khalid[19] used 18 arbitrary decamer primers to study the clonal fedility of *Musa acuminate* cv. Berangan. They found that all the regenerated plants were monomorphic and no somaclonal variation was detected. In some cases, regeneration process is prone to somatic variation resulting in the off-types as in case of *Populus termuloides*[20] and tea clones[21]. Variation is induced by different genetic and epigenetic mechanisms that are likely to be reflected in the banding pattern developed by employing different marker system. However, the reliability and efficiency of molecular markers in detecting large-scale genome arrangements have been frequently questioned. Since simple sequence repeat based primers target the fast evolving and hyper variable DNA sequence, ISSR markers are considered suitable to detect variation among tissue cultured produced plants [22].

Molecular marker can be employed in monitoring somaclonal variations during rapid mass micropropgation. ISSR and RAPD have their specific advantages over other markers as they do not require prior sequence information, simple in operation, high stability and low cost. Therefore, ISSR and RAPD have been proposed as a more economical and reliable DNA marker systems. The use of ISSR and RAPD to discriminate between somatic mutants and the clones from which the mutants originated has been studied in *Musa*[23]. In the present investigation, the genetic fidelity of banana cv. Robusta during micropropgation was studied using RAPD and ISSR markers. A homogenous amplification profile was observed for all the micropropagated plants when compared to mother plant in both types of markers used. The results corroborate the fact that *in vitro* multiplication is the safest mode for production of true to type plants.

References

1 Singh R, *Fruits* (National Book Trust, New Delhi, India) 1969, p 213.

2 Daniels D, Kosky R G & Vega M R, Plant regeneration system *via* somatic embryogenesis in the hybrid cultivar FHIA-21 (*Musa* sp. AAAB group), *In Vitro Cell Dev Biol—Plant*, 38 (2002) 330-333.

3 Kajla S, Poonia A K, Kharb P & Duhan J S, Role of biotechnology for commercial production of fruit crops, in *Biotechnology: Prospects and applications*, edited by R K Salar, S K Gahlawat, P Siwach & J S Duhan (Springer, New Delh,) 2014, 127-142.

4 Choudhary D, Kajla S, Poonia A K, Duhan J S, Kumar A *et al*, An efficient micropropagation protocol for *Musa paradisiaca* cv. Robusta: A commercial cultivar, *Ann Biol*, 30 (2014) 25-31.

5 Choudhary D, Kajla S, Duhan J S, Poonia A K, Surekha *et al*, Comparative study of various growth regulators on *in vitro* multiplication of commercial cultivar of banana cv. Grand naine (G-9), *Ann Biol*, 29 (2013) 288-293.

6 Soniya E V, Banerjee N S & Das M R, Genetic analysis of somaclonal variation among callus-derived plants of tomato, *Curr Sci*, 80 (2001) 1213-1215.

7 Sheidai M, Aminpoor H, Noormohammadi Z & Farahani F, Genetic variation induced by tissue culture in banana (*Musa acuminate* L.) cultivar Cavandish Dwarf, *Gene Conserv*, 9 (2010) 1-18.

8 Murashige T & Skoog F, A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol Plant*, 15 (1962) 473-497.

9 Murray H G & Thompson W F, Rapid isolation of high molecular weight DNA, *Nucleic Acids Res*, 8 (1980) 4321-4325.

10 Pina-Escuta J L, Vazquez-Garcia G & Arzate-Fernandez A M, *In vitro* regeneration and genetic fidelity of *Tigridia pavonia* (L.f.) DC, *Electronic J Biotechnol*, 13 (2010) 15 January 2010. [Doi: 10.2225/vol13-issue1-fulltext-1]

11 Zerihun D, Vashist U & Boora K S, Molecular characterization of citrus cultivars using DNA markers, *Int J Biotechnol Biochem*, 5 (2009) 271-280.

12 Kajla S, Choudhary D, Poonia A K, Duhan J S, Rapid plant regeneration and molecular assessment of genetic stability using ISSR and RAPD markers in commercial banana cv. Grand naine (G-9), *J Adv Biotechnol*, 4 (2014) 393-403.

13 Brown P T, Lang F D, Kranz E & Lorz H, Analysis of single protoplasts and regenerated plants by PCR and RAPD technology, *Mol Gen Genet*, 237 (1993) 311-317.

14 Sabir A, Newbury H J, Todd G, Catty J & Ford-Lloud B V, Determination of genetic stability using isozymes and RFLPs in beet plants regenerated *in vitro*, *Theor Appl Genet*, 84 (1992) 113-117.

15 Hedrick P, Shooting the RAPDs, *Nature (Lond)*, 355 (1992) 679-680.

16 Gibbs H L, Prior K A & Weatherhead P J, Genetic analysis of populations of threatened snake species using RAPD markers, *Mol Ecol*, 3 (1994) 329-337.

17 Rafalski J A, Vogel J M, Morgante M, Powell W, Andre C *et al*, Generating and using DNA markers in plants, in *Analysis of non-mammalian genomes—A practical guide*, edited by B Birren & E Lai (Academic Press, New York) 1996, 75-134.

18 Ray T, Dutta I, Saha P, Das S & Roy S C, Genetic stability of three economically important micropropagated banana (*Musa* spp.) cultivars of lower Indo-Gangetic plains, as assessed by RAPD and ISSR markers, *Plant Cell Tissue Organ Cult*, 85 (2006) 11-21.

19 Harirah A A, & Khalid N, Direct regeneration and RAPD assessment of male inflorescence derived plants of *Musa acuminata* cv. Berangan, *Asia Pac J Mol Biol Biotechnol*, 14 (2006) 11-17.

20 Rahman M H & Rajora O P, Microsatellite DNA somaclonal variation in micropropagated trembling aspen (*Populus tremuloides*), *Plant Cell Rep*, 20 (2001) 531-536.

21 Devarumath R M, Nandy S, Rani V, Marimuthu S, Muraleedharan N *et al*, RAPD, ISSR and RFLP fingerprints as useful markers to evaluate genetic integrity of micropropagated plants of three diploid and triploid elite tea clones representing *Camellia sinensis* (China type) and *C. assamica* ssp. *assamica* (Assam-India type), *Plant Cell Rep*, 21 (2002) 166-173.

22 Joshi P & Dhawan V, Assessment of genetic fidelity of micropropagated *Swertia chirayita* plantlets by ISSR marker assay, *Biol Plant*, 51(2007) 22-26.

23 Venkatachalam L, Sreedhar V & Bhagyalakshmi N, Genetic analyses of micropropagated and regenerated of banana as assessed by RAPD and ISSR markers, *In Vitro Cellular Dev Biol—Plant*, 43 (2007) 267-274.