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Deciphering the genetic identity and fidelity of banana through inter simple sequence repeats fingerprinting

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ABSTRACT

Bananas and plantains are a major staple food and export product in many countries. The success and pace of progress of crop-improvement program depend to a large extent on the availability of diverse germplasm and information on their characteristics. Somaclonal variation in the tissue culture is a common phenomenon which makes it mandatory to monitor for genetic stability of plants. Present study aimed to assess the genetic identity of 16 different banana varieties and true-to-type conformity of micro-propagated banana. In the present investigation, genetic relationships of 16 bananas, including some exotic varieties, were assessed based on inter simple sequence repeats (ISSR). Out of 26 ISSR primers screened, 18 ISSR primers were produced totally 2168 clear, reproducible and scorable band classes resulting in a total of 1608 polymorphic bands. The number of scorable bands for ISSR primers varied from 1 [$(AC)_8$ YT] to 13 [$(GA)_8$ YC and $(AG)_8$ YT], with an average of 7 bands per primer. Cluster analysis classified varieties into clusters, showing similarities for evaluated banana. Further, micro-propagated banana plantlets of *Musa acuminata* cv. Bantala and cv. Grand Naine that were developed from suckers were screened for genetic variation, if any, using ISSR markers. Similarly, the mother maintained in the field was also subjected for genetic analysis, where the banding patterns for each primer was highly uniform and monomorphic to the field grown mother clone from which the culture had been established. The preset study demonstrated the utility of ISSR markers in assessing both genetic variability among 16 different banana varieties and genetic uniformity of micro-propagated banana. Further, our study confirmed the true-to-type nature of micro-propagated clones.

KEY WORDS: Banana, genetic fidelity, genetic identity, inter simple sequence repeats marker, micro-propagation

INTRODUCTION

Banana (*Musa* spp.) is grown in more than 120 tropical and sub-tropical countries, mainly by small farmers and is considered a major staple food as well as an export commodity. World banana production is around 103 million tons annually, of which banana cultivated for the export trade account for only 10% (Aurore *et al.*, 2009). India stands as the largest producer of banana with an annual production of 28.4 million tones on 796,500 ha, which contribute to 27% of the world production and about 38% of the total fruit crop production in the nation (FAOSTAT, 2011). Such numbers indicate the importance of banana as a strong commodity, playing key economic, and social roles in many countries producing bananas worldwide. Diverse pests and diseases have significantly reduced banana production. Worldwide, the most serious constraint of the banana production is considered to be yellow and black SIGATOKA diseases caused by the fungus resulting in yield lose at 30~76% (Johanson *et al.*, 2000; Rout *et al.*, 2009). The presence of more than 90 distinct clones of banana in India denotes its rich genetic diversity.

There are between 1500 and 3000 accessions within collections of *Musa* germplasm worldwide, representing a wide range of morphological variation and genome constitutions. Many of the varieties of banana have been maintained locally, although in recent years the distribution of selections with improved characteristics, as well as consumer choice for particular varieties, has led to a narrow down in the number of varieties grown. This is apparent in South India where three or four clones are most widely available (Heslop-Harrison and Schwarzacher, 2007).

In the past, morphological and time-consuming physiological assays were applied to assess genetic variability in fruit crops. Recently, several studies have been utilized DNA-based markers, including ISSRs for conducting genetic fidelity and identity of *in vitro* propagated plants such as in Gerbera (Bhatia *et al.*, 2011), Eggplant (Mallaya and Ravishankar, 2013), and Araucaria (Sarmast *et al.*, 2012), including banana (Rout *et al.*, 2009; and Lu *et al.*, 2011). Inter simple sequence repeats (ISSR) marker have gained considerable importance due to efficient, cost effective, simple, and versatile set of markers that relies on repeatable amplification of DNA sequences using single primers. ISSR fingerprinting requires a minute quantity of DNA sample, and they do not need any prior sequence information to design the primer. Further, unlike restriction fragment length polymorphism ISSR do not use radioactive probes (Lakshmanan *et al.*, 2007); thus, they are suitable for the assessment of the genetic variability and fidelity of *in vitro*-raised clones.

Production of tissue culture raised plants involves the application of plant hormones, such as auxin, for initiation. Auxins are known to be associated with genetic instability in plants, a phenomenon called somaclonal variation (Karp, 1989; and Cullis, 1992). Even at optimal levels, more number of sub-cultures often may lead to epigenetic changes or somaclonal variations in the tissue culture raised plants questioning the very fidelity of their clonal nature. Mostly somaclonal variation also occurs as responses to the stress imposed on the plant in culture conditions and are manifested in the form of point mutations, chromosome rearrangements, and DNA methylations (Phillips *et al.*, 1994). In some instance somaclonal variations may be desired as a source for variation to obtain superior clones, but, it could be a serious problem in large scale commercial production of tissue culture raised plants, where the aim is to develop identical propagules of the desired variety resulting in the production of undesirable traits or plant off-types (Cassells *et al.*, 1999). True-to-type clonal fidelity is one of the most important pre-requisites in the tissue culture industry of crop species. The occurrence of cryptic genetic changes via somaclonal variation in the regenerates can drastically limit the broader utility of the tissue culture multiplication system (Salvi *et al.*, 2001). Therefore, it is necessary to monitor the genetic uniformity of micro-propagated plantlets by recent molecular techniques.

Micro-propagation of banana has gained attention due to its potential to provide genetically identical, pest, and disease-free planting materials. Accurate verification of cultivar identity, checking propagation material and patent protection is important because very few cultivars satisfy standards for fruit quality and clonal fidelity. Micro-propagation of different cultivars of banana has been reported by several researchers using different explants sources as well as varied hormonal combinations (Bhyagyalakshmi and Singh, 1995; and Venkatachalam *et al.*, 2006).

In the present study, the objectives were to utilize ISSR markers to examine (i) genetic integrity and uniformity of different banana varieties, (ii) to develop useful DNA fingerprints to facilitate cultivar identification, and (iii) genetic fidelity testing for micro-propagated plantlets.

MATERIALS AND METHODS

Plant Material

In this study, 16 commercially grown banana cultivars were involved, namely, Cheni Champa, Red Green Banana, Hybrid H.531, Ney Poovam, Patkapura (Satasankha), Robusta, Karpuravalli, Amrutapani, Champa (Patia), Martman, Ganga tulasi, Red Banana, Champa, Grand Naine, Patkapura, and Bantala. Different batches of micro-propagated banana plantlets of cv. Bantala and cv. Grand Naine were considered for the genetic fidelity studies.

Culture Conditions

Briefly, sucker explants (cv. Grand Naine and cv. Bantala) were surface sterilized with 0.5% (w/v) mercuric chloride for 30 min and kept overnight in 1% (w/v) polyvinylpolypyrrolidone (MW 6000). The effect of cytokinins (BAP), Kinetin (KIN) combined with auxin (IAA, NAA, and 2,4-D) were evaluated on basal Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The cultures were maintained at 25°C under 16 h photoperiod with light intensity of 3000 Lux and 50-60% relative humidity. The sub-culturing was carried out at 30 days intervals. Multiple shoots proliferated from each segment were separated and sub-cultured with an interval of 30 days in MS medium with BA, Ads, and IAA, for further shoot multiplication and growth. Individual shoots with 2-3 cm in length were separated and transferred to half strength MS basal medium with IAA or IBA to induce further shoot elongation and root formation. Rooted plantlets were thoroughly washed and planted in an earthen pot containing a mixture of sand:soil:cow dung manure in the ratio of 1:1:1 (w/v) and were kept in green house with 25-28°C and >85% relative humidity for hardening and acclimatization.

Genomic DNA Isolation

Genomic DNA was isolated (Doyle and Doyle, 1990) from the leaves of 16 banana cultivars for variety identification. In the separate experiment, DNA was extracted from the leaves of the parental genotype and different batches of micro-propagated plantlets of cv. Grand Naine and cv. Bantala for genetic fidelity study. Briefly, genomic DNA was isolated by an improved CTAB (cetyl-trimethyl ammonium bromide) method. Approximately, 100 mg of fresh leaves tissue was ground with pinch of PvP to fine powder using liquid nitrogen in pre-chilled mortar and pestle. 2 ml of pre-warmed extraction buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, and 0.2% β-mercaptoethanol, pH 8) and incubated at 65°C on water bath for 30 min. The aqueous solution was extracted with equal volume of chloroform and isoamyl alcohol (24:1) and mixed by gentle inversion for 30-40 times. The sample was centrifuged at 12,000 rpm for 20 min at room temperature. The clear aqueous phase was pipette out into another centrifuge tube, and an equal volume of isopropanol was added and mixed gently by inversion for 10-20 s/20 min to precipitate the nucleic acid. It was centrifuged at 9000 rpm for 10 min at room temperature. Supernatant was discarded, and the precipitated nucleic acid was air dried for few min. The obtained nucleic acid pellet was air dried until the entire ethanol was removed (tube was spun for few seconds and was pipetted out the entire ethanol present in the tube). Dried nucleic acid pellet was dissolved in 100 µl of TE buffer. RNase (20 µg, GeNei) was added and incubated at 37°C for 35 min and stored at −20°C until use. Then, it was purified again, 200 µl of chilled ethanol was added to each tube. It was centrifuge at 9000 g for 10 min at room temperature. Supernatant was discarded. An equal volume of chloroform and isoamyl alcohol (24:1) was added and mixed by gentle inversion for 30-40 times. The sample was centrifuged at 14,000 rpm for 20 min at room temperature. Clear aqueous phase pipetted into another centrifuge tube. An equal volume of isopropanol was added and mixed gently by inversion for 10-20 s to precipitate the DNA. It was centrifuge at 9000 rpm for 10 min at room temperature. Supernatant was discarded. The obtained DNA pellet was air dried until the entire ethanol was removed (tube was spin for few seconds and was Pipette out the entire ethanol present in the tube). Dried DNA pellet was dissolved in 100 µl of TE buffer. The quality of genomic DNA was examined by agarose (1%) gel electrophoresis and quantified spectrophotometrically at A260 and A280 nm. Each sample was diluted to 50 ng/µl in TE buffer and stored at −20°C.

Polymerase Chain Reaction (PCR) Amplification

18 ISSR primers chosen for amplification of 16 commercially grown banana cultivars and micro-propagated banana plantlets from cv. Bantala and Grand Naine DNA samples. PCRs was carried out in a final volume of 25 µl containing 20 ng template DNA. 100 µM each dNTP, 20 µM of primer, 25 mM $MgCl_2$, 10× Taq buffer A (10 mM Tris-HCl [pH 9.0]), and 5U/µl Taq DNA polymerase (M/S Bangalore Genei, Bangalore, Karnataka, India). Amplification was performed in a PTC-100 thermal cycler (MJ Research, Watertown, MA, USA) programed for a preliminary 2 min denaturation step at 94°C, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at required temperature (depending on Tm of the ISSR primer) for 1 min, extension at 72°C for 2 min, and finally extension at 72°C for 7 min. The details of primers used are presented in Table 1.

Data Scoring and Statistical Analysis

The experiments were repeated twice for each cultivar, and the clear, reproducible, and scorable ISSR bands were scored using Quantity One software (BioRad) and were converted into binary data matrices on the basis of their presence (1) or absence (0) in the gel. Genetic similarity between pairs was calculated according to Jaccard's similarity coefficient (UPGMA), and a dendrogram was generated by using in NTSYS-pc version 2.1software.

RESULTS

Visualization and Analysis of ISSR-PCR Products

Amplification products were separated alongside a molecular weight marker (500 bp plus ladder, M/S Bangalore GeNei, Bangalore, India) by 1.8% agarose gel electrophoresis in 1×TAE (Tris acetate EDTA) buffer

Table 1: List of *Musa*-cultivars considered in the present study

Serial number	Cultivar	Genome
1	Cheni Champa	AAB
2	Red Green Banana	AAA
3	Hybrid H.531 ('Poovan'×'Pisang Lilin')	
4	Ney Poovan	AAB
5	Patkapura (Satasankha)	AAB
6	Robusta	AAA
7	Karpuravalli	AAB
8	Amrutapani	AAB
9	Champa Patia	AAB
10	Martman (Rasthali)	AAB
11	Ganga tulasi	AAB
12	Red Banana	AAA
13	Champa	AAB
14	Grand Naine	AAA
15	Patkapura	AAA
16	Bantala	BBB

stained with Ethidium bromide and visualized under UV light. Gel photographs were scanned through a ChemiDoc system (BioRad, California, USA), and the amplification product sizes were evaluated using the Quantity one software (BioRad, California, USA). The presence and absence of bands between samples was scored, and data were transcribed into binary format (1, 0, respectively).

Genetic Characterization of Banana Varieties

Production of ISSR amplification, a total of 2168 strong, clear, and reproducible bands, were amplified from 16 cultivars using the 18 selected ISSR primes, of which 1608 were polymorphic 74.16% (Table 1). The number of DNA amplified bands varied between one and 13 with a range of 100~2500 bp (Figure 1). The maximum number of fragments was found to be 13 and was obtained using a primer (GA)8YC and (AG) 8YT, whereas the least number of fragments was 1 that was obtained with (AC)8YT. The highest level of polymorphism (100%) was obtained with primer (AC)8 YT, whereas the lowest was 77.8% and was obtained with primer AGAGGTGGGCAGGTGG (Table 2).

Cluster Analysis

The dendrogram was constructed based on the similarity coefficient matrix based on ISSR bands scored and used UPGMA method (Figure 2), indicates that the 16 cultivars divided them into three clusters. One cluster having Cheni Champa, Ney Poovan, Karpuravalli, Patkapura (Santasankha), and Hybrid H.531. The second cluster includes Champa (Patia), Champa, Grand Naine, Patkapura, Red Banana, Ganga Tulasi, and Bantala. The third cluster includes variety Red Green Banana, Robusta, Amrutapani, and Martman. The present study clearly demonstrated that the clustering patterns based on ISSR markers data could discriminate 16 banana cultivars into groups (sub-clusters) based on DNA polymorphism content and genetic diversity. Interestingly, four cultivars, namely Martman, Amrutapani, Robusta, and Red Green Banana are genetically distant from rest of the 12 cultivars studied.

Analysis of Genetic Fidelity of *in vitro* Raised Plantlets

The quality of *in vitro* derived regenerated was screened with 16 ISSR primers that have showed monomorphic among the plantlets. The banding pattern of PCR-amplified product from micro-propagated plantlets was found to be monomorphic for analysis of genetic fidelity of *in vitro* raised plantlets. The identical ISSR banding pattern of twenty *in vitro* raised cv. Bantala plantlets and theirs mother plant have showed in Figure 3. ISSR primer P-16, UBC-841, UBC-818 showed identical DNA profiles as compared with mother plants. The identical ISSR banding pattern of twenty *in vitro* raised cv. Grand Naine plantlets and theirs mother plant have showed in Figure 4. ISSR primer P-17, UBC-834, IG-19 showed identical DNA profiles as compared with mother plants.

DISCUSSION

Genetic Characterization of Different Banana

PCR-based molecular marker techniques are efficient because of the high levels of polymorphism that can

Table 2: List of primers, their sequences, number and size of the amplified fragments generated by 18 ISSR primers

Primer code	Primer sequence (5′-3′)	Annealing temperature (°C)	Amplified bands		
			Total	Polymorph bands	% polymorphism
P-16	$(AC)_8$YT	60	66	66	100
P-17	$(CA)_8$RG	51	152	88	57.89
UBC 840	$(GA)_8$YT	53	85	85	100
UBC 818	$(CA)_8$G	52	126	110	87.3
IG-11	$(AC)_8$T	49	136	88	64.7
IG-02	AGAGGTGGG CAGGTGG	52	135	55	40.74
P-18	$(CT)_8$T	44	108	76	70.37
IG-19	TGG $(AC)_7$	59	154	122	79.22
UBC 834	$(AG)_8$YT	50	121	57	47.1
UBC 868	$(GAA)_6$	48	126	110	87.3
UBC 812	$(GA)_8$A	44	149	133	89.26
IG-03	GAGGGTGGAG GATCT	63	133	101	75.93
UBC 808	$(AG)_8$C	47	139	107	76.97
UBC 842	$(GA)_8$YG	50	118	54	45.76
UBC 841	$(GA)_8$YC	46	144	128	88.88
UBC-811	$(GA)_8$C	43.3	120	104	86.66
IG-01	AGGGCTGGAGGGC	65.7	82	66	80.48
UBC 836	$(AG)_8$YA	50	74	58	78.36

ISSR: Inter simple sequence repeat

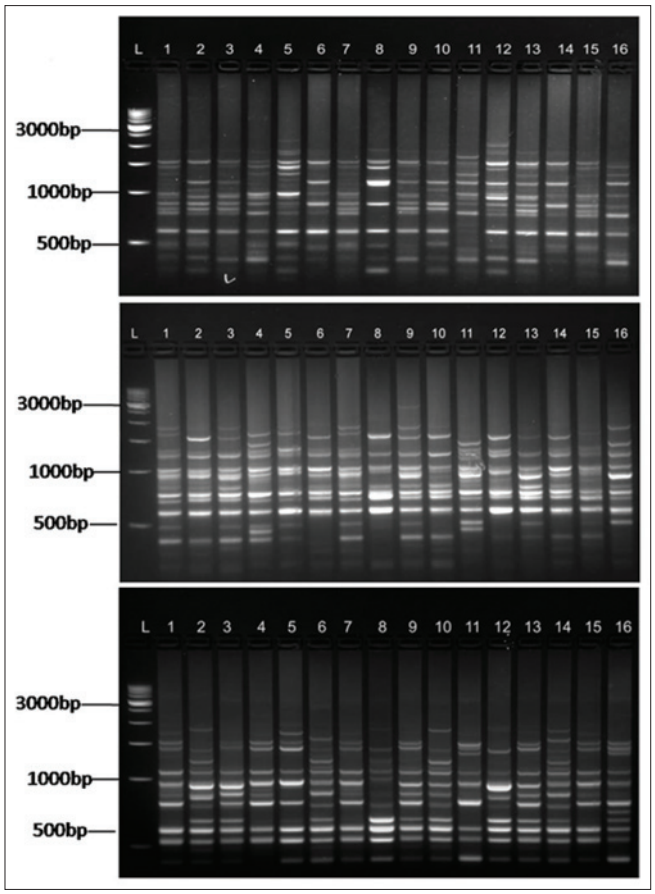

Figure 1: Polymerase chain reaction amplification pattern in 16 varieties of *Musa accuminata* produced by using inter simple sequence repeats primers. L - represents 500 bp DNA ladder and 1-16 represents "Cheni Champa," "Red Green Banana," "Hybrid H.531," "Ney Poovam," "Patkapura (Satasankha)," "Robusta," "Karpuravalli," "Amrutapani," "Champa (Patia)," "Martman," "Ganga tulasi," "Red Banana," "Champa," "Grand Naine," "Patkapura," and "Bantala," respectively

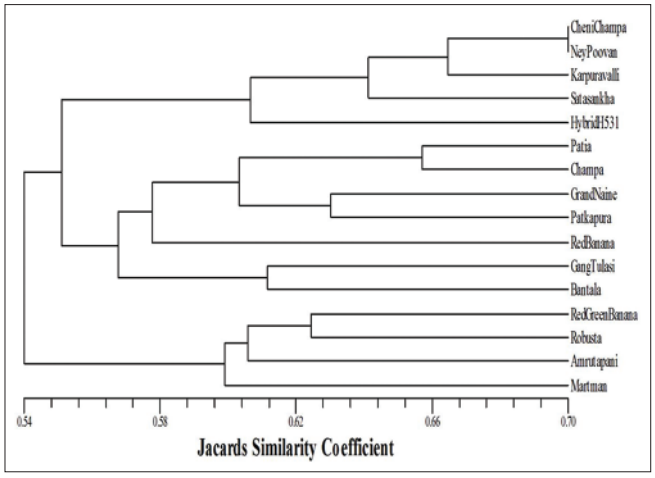

Figure 2: Dendrogram generated using UPGMA analysis showing relationship between 16 *Musa acuminata* varieties using 18 inter simple sequence repeats primers

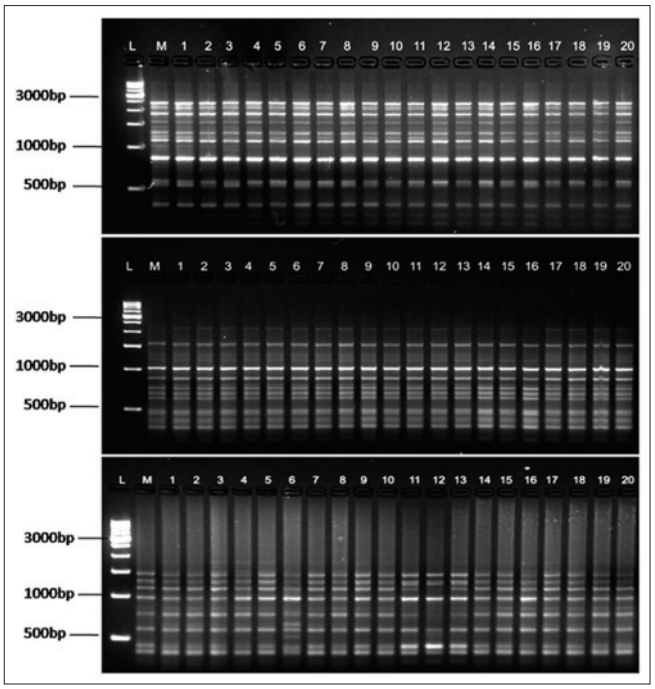

Figure 3: Gel showing the homogenous amplification pattern in 20 randomly chosen tissue culture raised plantlets of "cv. Bantala" by inter simple sequence repeats. L - represents 500 bp DNA ladder, M - represents the mother explants source, and Lane 1-10 represents 1st batch, Lane 11-20 represents 2nd batch

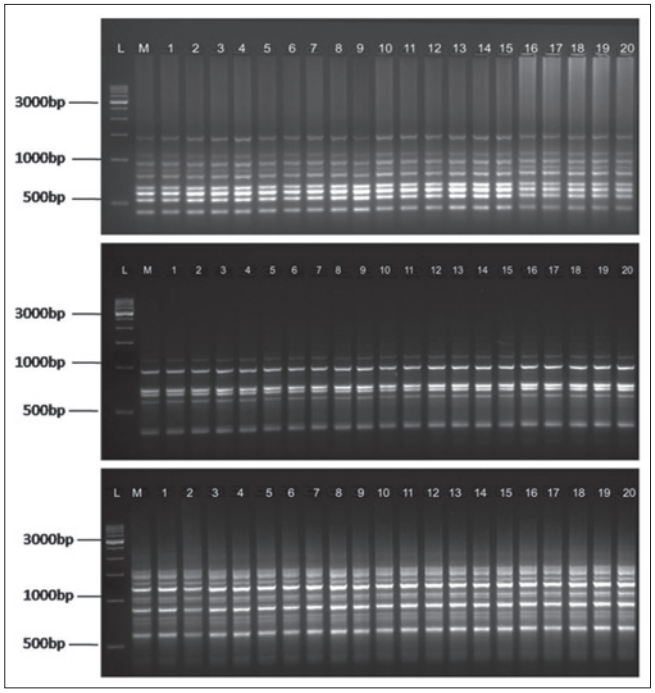

Figure 4: Gel showing the homogenous amplification pattern in 20 randomly chosen tissue culture raised plantlets of "cv. Grand Naine" by inter simple sequence repeats. L - represents 500bp DNA ladder, M - represents the mother explants source, and Lane 1-10 represents 1st batch, Lane 11-20 represents 2nd batch

be detected effectively. Molecular markers are widely utilized in breeding programs and fundamental studies of biodiversity while it is also important that genetic diversity is evaluated within the gene banks and natural populations.

Recently, different molecular markers have been applied to characterize genetic diversity in *Musa* cultivars (Rout *et al*., 2009; Opara *et al*., 2010; and Lu *et al*., 2011). Rout *et al*. (2009) used 15 ISSR primers to distinguish 4 varieties of banana and in another study by Onguso *et al*. (2004) utilized 19 RAPD primers to distinguish 20 selected banana cultivars. Furthermore, Lu *et al*. (2011) reported that 8 ISSR primers were able to distinguish 30 banana cultivars, whereas in the present study 18 ISSR primers were sufficient to distinguish 16 cultivars/varieties of banana. The ability to resolve genetic variation among different cultivars of banana may be more directly related to the number of polymorphisms detected with ISSRs in the present study.

Genetic Fidelity Analysis of *In Vitro* Raised Plantlets

All banding profile from micropropagated plants were monomorphic and similar to those of the mother plant (Figures 3 and 4). Similar results have been reported using ISSR and RAPD marker systems in almond (Martin *et al*., 2004), in banana (Venkatachalam *et al*., 2007; Lu *et al*., 2011), in *Swertia chirayita* (Joshi and Dhawan, 2007), in Gerbera (Bhatia *et al*., 2011), and in eggplant (Mallaya and Ravishankar, 2013). In contrast, 8% variation in *Araucaria excelsa* between micropropagated plants and corresponding mother plant was observed (Sarmast *et al*., 2012). The presence or absence of variations during micro-propagation depends on the method of regeneration followed and source of explants (Goto *et al*., 1998). The sub-and supra-optimal levels of synthetic plant growth hormones have been associated with somaclonal variation (Martin *et al*., 2004). Furthermore, a number of sub-cultures in tissue culture program may lead to epigenetic variations; thus, questioning the very fidelity of their clonal nature. In this study, true-to-the type nature of the *in vitro* raised clones was confirmed using ISSR markers. No variability was confirmed among the micro-propagated plantlets; hence, suckers can be successfully employed for the commercial multiplication of banana cv. Bantala and Grand Naine, without much risk of genetic instability.

CONCLUSIONS

Our study also demonstrates to identify genetic variation that exists among 16 banana varieties as revealed by ISSR marker technique. This would also aid in developing and planning breeding strategies for banana by helping breeders to identify diverse genotypes (germplasm) and makes it possible to carry out early selections and thus reduce the time between recurrent selections and increase genetic gains per year. Furthermore, true-to-type nature of the *in vitro* raised clones was confirmed using ISSR markers.

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