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Molecular analysis of variants identified from orchards growing with micro propagated Robusta banana (*Musa* spp. 'AAA') using RAPD markers

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Abstract: *The molecular fingerprinting using RAPD markers of thirteen morphological variants (off-types) and a normal from farmers' field planted with micropropagated Robusta banana (Musa spp.) and Robusta and Grand Naine clones from authenticated source was carried out. Out of fourteen arbitrary (10-mers) primers showing intense unambiguous and reproducible amplification using PCR, two primers viz., OPA-19 and OPC-03 showed only monomorphic bands while OPA-02, OPA-09, OPA-13, OPA-14, OPB-6, OPB-15, OPC-01, OPD-10, OPF-04 and OPF-12 primers showed polymorphic amplification pattern. Out of 123 amplified fragments scored, 53 fragments were polymorphic in nature. In addition, a dendrogram generated based on Ward's method of cluster analysis revealed that all morphological variants grouped into a cluster that was separate from the other cluster which consisted normal plant from field, Robusta and Grand Naine. In the genetic dissimilarity matrix generated based on Squared Euclidean Distance, 20-35 percent variation between morphological variants and normal plant was noticed in 'Extra dwarf plant with pseudo stem' and 'All foliage plant' (35%). The field plant showed genetic variation of 5% from somaclonal variants of 'Robusta' clone, while that variation has not affected its morphology as well as fertility. Results showed that morphological variations in field planted micropropagated Robusta plants were due to somaclonal variations, which suggests that the need for use of molecular means in commercial tissue culture units for testing genetic fidelity of plants as a safety measure to avoid passing of off-type planting material to farmer.*

Keywords: *Grand Naine, Polymerase Chain Reaction (PCR), Random primers, Robusta, Somaclonal variations*

I. Introduction

Banana and plantains (*Musa* sp.) are important crops in the global fruit industry. In the world it is grown on an area of 9 million tones with total production of 92 million tones. In India, banana occupies 20% area under fruit crops and it supports livelihood of millions of people. The cultivar Robusta of Cavendish subgroup (*Musa* sp. 'AAA') of banana is very popular because of high yield, wide market acceptability and high economic returns per unit area. Of late, this is overtaking other fruit crops in production and productivity. Consequently, there is huge demand for planting material and major part of it is being met through micropropagation. Apart from the higher and faster multiplication rates, micropropagated banana plants are advantageous due to regular availability of planting material, earliness, synchronized blooming and comparatively higher yields.

However, increasing reports on array of morphological variations and high yield loss in micropropagated banana plants after several months of planting are the cause of concern for both growers and biofactories. Few possibilities for such morphological irregularities are viz., nutrient deficiencies in soils [1], environmental conditions during hardening [2] and occurrence of somaclonal variations due to features of the *in vitro* technique such as growth regulators and their concentrations, number of subcultures etc. [3, 4 and 5]. In this regard there is need to identify field variations in plants and characterize them to ascertain whether it is somaclonal or mere morphological variation to help farmer as well as tissue culture industry.

Several strategies can be used to characterize the variability and genetic fidelity in *in-vitro* derived clones, with their own limitations. Karyotyping analysis, for example, cannot reveal alterations in specific genes or small chromosomal rearrangements [6]. Biochemical markers like isozyme markers provide a convenient method for detecting genetic changes, but are subject to ontogenic variations. They are also limited in number and only DNA regions coding for soluble proteins can be sampled. Presently, DNA based molecular markers are highly suitable, particularly RAPD markers [7], because of its based on PCR with random primers, fast and less cumbersome as it involves simple technology and analyzes variation at many loci in minute quantities of DNA, even in closely related organisms such as near isogenic lines (NILs). RAPD markers have been successfully

employed in fingerprinting somaclonal variants [8, 9, 10, 11, 12, 13, 14, 15 and 16] as well as assessment of genetic stability in long-term micropropagated bananas [17]. In the present study, a survey was carried out to identify the morphological variants in farmers fields planted with micropropagated Robusta banana plants and an effort has been made to characterize those using RAPD markers.

II. Material and Methods

Plant material

In a survey on orchards growing micropropagated Robusta banana plants, 13 plants showing morphological variations (off-types) were identified along with a normal yielding plant (Table 1; Fig. 1). Cigar or newly opened leaf samples that were free from any probable pest and pathogenic damages were collected from all variant plants and normal Robusta plant in field. The leaf samples of Robusta (as authentic source of Robusta clone) and Grand Naine (as additional clone from Cavendish subgroup) clones from National Research Centre for Banana (NRCB), Trichy, India were also collected. The DNA was extracted using a protocol based on CTAB that was standardized for leaves rich in carbohydrates, phenols and proteins [18] with a few modifications.

DNA isolation

One gram of fresh leaf sample was ground in liquid nitrogen and suspended in 20 ml of 2X CTAB extraction buffer [3% CTAB, 100 mM Tris (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 3% PVP and 0.1% β-Mercaptaethanol], the suspension was incubated at 60^{o}C for 45 minutes, then brought down to room temperature. An equal volume of chloroform: isoamylalcohol (24:1) was added, centrifuged at 10,000 rpm (revolution per minute) for 15 minutes at 4^{o}C, supernatant was collected into fresh tube and step was repeated twice. Finally, supernatant was transferred to fresh tube, 0.2 volume of NaCl was added and mixed gently, then one volume of ice-cold isopropanol was added, mixed gently and incubated overnight at 4^{o}C. Next day, contents were centrifuged at 10,000 rpm for 20 minutes at 4^{o}C to pellet DNA, supernatant was discarded, the DNA pellet was washed twice with 70% ethanol, air dried for 30 minutes and dissolved in 500 µl of TE buffer (10 mM Tris HCl, pH 8.0 and 1 mM EDTA, pH 8.0). Further, it was treated with 3 µl RNase (10 mg/l) at 37^{o}C for 1 hour. Then it was extracted with equal volume phenol, phenol: chloroform: isoamylalcohol (25:24:1) and chloroform at 10,000 rpm for 10 minutes at room temperature. The DNA was precipitated with 95% chilled ethanol in the presence of 0.3 M sodium acetate (pH 5.2) at –20^{o}C for 2 hours, pelleted at 10,000 rpm for 20 minutes at 4^{o}C, washed twice with 70% ethanol, air-dried for 30 minutes and resuspended in 250 µl TE buffer. The quality of DNA was assessed using both agarose gel (0.8%) electrophoresis and spectrophotometer (Shimadzu, UV visible; 260/280 ratio) and quantity was calculated by using standard formula (OD 260 nm *50 µg* Dilution factor/1000).

Polymerase Chain Reaction (PCR)

PCR amplification of genomic DNA was carried out using random primers (10-mers; M/S Operon Tech. USA). Amplification reaction was performed in a final volume of 25 µl reaction mixture containing template DNA (25-30 ng), primer (5 picomoles), $MgCl_2$ (2.00 mM), dNTP's (200 µM; Bangalore Genei), Taq DNA Polymerase (1 unit; Bangalore Genei) and 1X buffer [10 mM Tris HCl (pH 8.8); 500 mM KCl; 15 mM; 0.1% gelatin; 0.05 per cent Tween-20 and 0.05% NP 40]. A drop of mineral oil (Sigma Chemical Co.) was overlaid on the reaction mixture to prevent the evaporation at high temperature. PCR was carried out in a thermal cycler (MJ Research, PTC 100) using program profile consisting of an initial denaturation at 95^{o}C for 5 minutes, followed by 45 amplification cycles each consisting denaturing for 1 minute at 94^{o}C, annealing at 35^{o}C for 2 minutes and extension for 2 minutes at 72^{o}C and final extension at 72^{o} C for 10 minutes was also included in the program. Amplified product was mixed with 6X loading buffer (30% sucrose, 0.05% xylene cynol and 0.05% bromophenol blue) and loaded along with 500 bp (base pair)/0.5 kb (kilo base pair) ladder (Genei, Bangalore) on 1.2% agarose gel containing ethidium bromide (0.001%). Electrophoresis was conducted at 50 volts for five hours and gel was photographed under UV light by using Alpha Digi Doc system (Herolab, Germany).

Primer screening

Primer screening was taken up using DNA isolated from a variant 'very dwarf', normal Robusta from field and Robusta and Grand Naine clones from NRCB. Totally, 200 random primers (-10 mers; Operon Technologies, USA) were screened (OPA, OPB, OPC, OPD, OPE, OPF, OPH, OPJ, OPK and OPI series) including 10 random primers from previous research studies on fingerprinting of somaclonal variation in micropropagated bananas. There was a negative control (with no template) and a positive control (using DNA of normal Robusta plant from field) reaction in all the amplifications carried out in the study to assess amplification conditions.

Gel scoring and analysis

Gels were observed closely for differences in amplification pattern between morphological variants and normal Robusta from field. Later, gels were scored for presence (1) or absence (0) of amplified fragments. Data was analyzed by using 'STATISTICA' software. Dendrogram based cluster analysis was carried out by using Wards method [19] and genetic dissimilarity matrix was generated using Squared Euclidean Distance.

III. Results and Discussions

In the survey, on an average 2.3 percent off type plants were observed in the fields planted with micropropagated Robusta banana plants, based on morphological irregularities. Israeli et al., [20] recorded variations up to 50 percent among micropropagated Cavendish bananas and linked the dwarf phenotypes to morphological anomalies viz., split fingers, mosaic leaves, deformed lamina, black pseudo stem, choking etc. Smith et al., [21] identified an off type micropropagated ladyfinger bananas that is characterized by its slow growth and poor bunch size. However, in this study using literature from those studies, plants showing morphological irregularities along with less or no yield compared to normal plants have been identified as morphological variants.

The genomic DNA isolated from leaves using the present protocol was of good quality (no shearing and free from protein and RNA contamination) and quantity (250-350 ng/µl) (data not shown). In preliminary round 50 random primers were selected that showed amplification out of 200 primers screened and in second round 14 primers have been selected that showing intense, unambiguous and reproducible banding pattern were selected RAPD analysis (Table 2). There was no amplification in negative control and intense, consistent and reproducible amplification patterns in replications in positive control and other samples, showed that the amplification conditions were optimum. In gel scoring a total of 123 amplified fragments were scored from 14 primers of which, 53 fragments were polymorphic in nature. The number of amplification fragments produced ranged from 5-19 with an average of 8.3 per primer and size of amplification fragments ranged from 250-2500 bp (Table 2). The primers OPA-19 and OPC-03 produced monomorphic fragments (Table 2; Fig. not shown) and rest of the selected primers showed polymorphic amplification pattern indicating the existence of genetic difference in morphological variants compared to normal plant (Table 2; Fig. 2). Kaemmer et al., [22] used random amplified polymorphic DNA (RAPD) and micro-satellite fingerprinting to construct phylogenetic dendograms of *Musa* spp., and characterized a somaclonal variant of Grand Naine (AAA) named Novaria. Ten micropropagated variants were isolated and described morphologically and analyzed by random amplified polymorphic DNA (RAPD) using three arbitrary 10-mer oligonucleotide sequences as compared to normal plant [23]. There were only two primers from previous studies [8, 9, 10, 11, 12, 13 and 14] viz., OPA-2 and OPC-1 were useful in differentiating morphological variants from and normal plant from field, while rest of them was generated monomorphic amplification pattern. Rout et al., [24] reported the use of ISSR marker for identification of different varieties of banana and detection of genetic uniformity of micropropagated plantlets. In previous studies, primers have been selected on variants of cultivar Grand Naine, Red and Williams and hence, it might be possible that all of them could not differentiate present variations in Robusta plants. However, primers from present studies can be used in future molecular characterization of morphological variants in micropropagated Robusta banana plants.

Additionally, the score for present or absent of bands obtained from RAPD amplification pattern was used in developing dendrogram using cluster analysis (Fig. 3) based on Ward's method and genetic dissimilarity matrix based on Squared Euclidean distances. Tomato and Chilli F1 hybrids and their parents were studied for identification and genetic purity testing using RAPD primers. Molecular marker tools can be effectively used to find out contaminations in DNA polymorphism of respective hybrids [25].

The cluster analysis allowed a clear separation among variants and the healthy clones. In the dendrogram, there were two major clusters separated at a distance of 140 units. Cluster one contained all 13 field identified morphological variants which were further sub grouped, whereas the second major cluster contained normal Robusta plant from field, Robusta and Grand Naine clones of Cavendish group owing to the close genetic relationship within the Cavendish subgroup. Anitha [9] noticed similar results in Cavendish subgroup of *Musa* spp. Rout et al., [24] carried out Genetic characterization of cultivated banana based on ISSR bands scoring and dendrogram based on the similarity matrix using UPGMA. The four varieties were divided into two clusters at 54% similarity. 'Grand Naine' and 'Robusta' has been grouped together sharing a similarity of 66%. In this study, genetic Dissimilarity analysis showed 20-35% dissimilarity between morphological variants and normal plant and it was highest in 'extra dwarf plant with pseudo stem' and 'all foliage plant' (35%) (Table 1). There was 8% dissimilarity between the cultivars Grand Naine and Robusta clones. The morphologically normal plant from field was genetically dissimilar (5%) to 'Robusta' clone from NRCB. However, that genetic variation in normal plant neither appeared morphologically nor affected fertility, which could be novel source for crop improvement.

The results showed that morphological variations in field planted tissue culture Robusta banana plants were due to somaclonal variation. Extensive use of *in vitro* techniques, somaclonal variation is commonly

observed in *Musa* propagation [26]. Grillo et al., [2] reported that mass propagation of bananas through *in vitro* techniques can lead to a high percentage of somaclonal variants. Masoud et al., [27] has opinioned that the genetic variations induced in the regenerated plants increase with the time-period of the sub-culture.

The genetic changes detected here may be due to culture conditions including excision of explants through subcultures and growth regulators. The molecular mechanisms underlying somaclonal variations have been attributed to chromosome breakage, single base changes, and changes in copy number of repeated sequences and alteration in DNA methylation patterns [28]. The polymorphisms in the amplification products may be either from changes in the sequences of the primers binding sites (e.g. point mutations) or changes which alter the size or prevent the successful amplification of the a target DNA (e.g. insertion, deletions, inversions). Rani et al., [29] have attributed similar opinion in genetic assessment of micropropagated *Populus* sp. Finally, this study suggests for testing genetic fidelity of micropropagated banana plants at early stages using modern and reckonable detection tools such as molecular markers to prevent loss of inputs, time and money to farmers. In addition, biofactories are advised to train personnel for the purpose of quality control during nursery stage, who can apply the detection tools whenever applicable in a comprehensive manner. This can ensure the supply of quality planting materials, which will build the confidence in growers about the suppliers and themselves. After all these resolutions, obviously one can expect a spurt in productivity which can play a major role in the betterment of growing community and ultimately the national economy.

IV. Figures and Tables

Figure 1: A representation of normal and morphological variants in field planted micropropagated Robusta banana plants used for RAPD characterization A) Normal B) Dwarf variant; C) Rosette

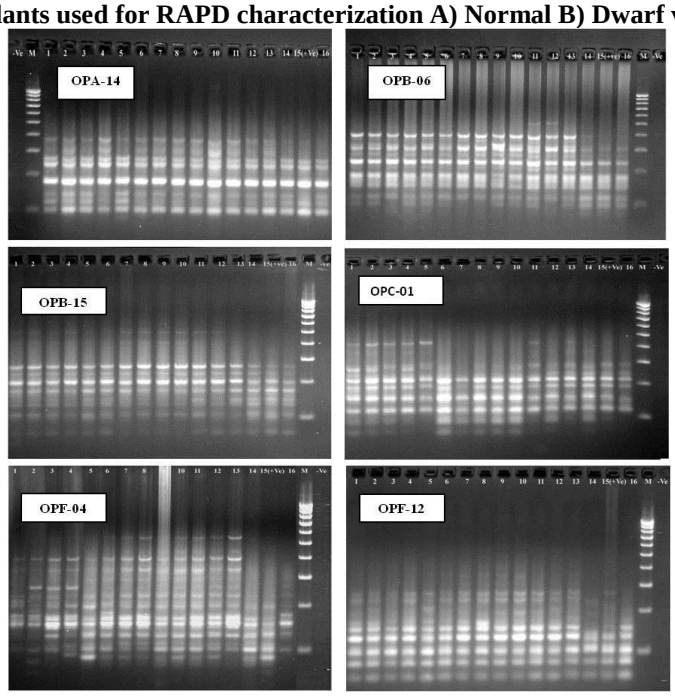

Figure 2: RAPD gel profiles of thirteen morphological variants and a normal Robusta plants from field, Robusta and Grand Naine clones developed using various random primers [Legend: Lane1-16 remains same as in Table I; -ve-negative control: +-positive control; M-500 bp ladder (Bands from lower to higher-0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5 kb)]

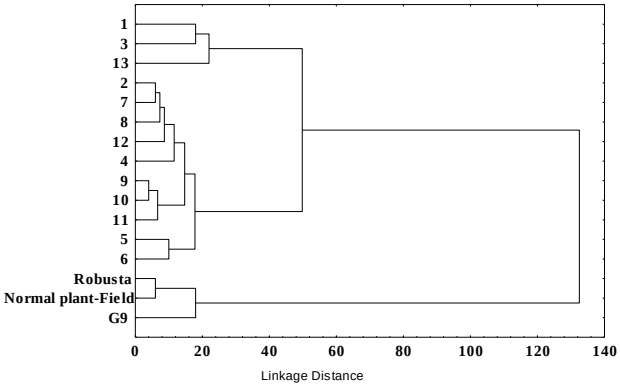

Figure 3: Genetic association among thirteen morphological variants and a normal Robusta banana plant from field and Robusta and Grand Naine clones

Table 1: List of plant materials used in RAPD characterization

Sl. No.	Plant material	Genetic Dissimilarity (%)	
		Normal Robusta-Field	Robusta (Trichy)
1.	Choking	29.00	26.00
2.	Dwarf	34.00	31.00
3.	Streaking with bunchy appearance	20.00	19.00
4.	Rosette	35.00	34.00
5.	Tip Burn	31.00	28.00
6.	Foliage Mosaic	32.00	29.00
7.	Extra Dwarf	34.00	31.00
8.	Medium Dwarf with brown spots	32.00	31.00
9.	Extra dwarf plant with split pseudo stem	35.00	32.00
10.	All foliage plant	35.00	32.00
11.	Small fruited	33.00	30.00
12.	Chlorophyll mutant	31.00	28.00
13.	Yellowing with burnt appearance	24.00	25.00
14.	Robusta (NRCB, Trichy, India)	05.00	00.00
15.	Normal Robusta-Field	00.00	05.00
16.	Grand Naine (NRCB, Trichy, India)	11.00	08.00

Table 2: Synthetic random (10 mer) primers used in present study for RAPD characterization

Primer No.	Nucleotide sequence (5'-3')	No. of bands amplified	Monomorphic bands	Polymorphic bands	Range of band size (≈bp)
OPA-02	TGCCGAGCTG	10	08	02	500-1750
OPA-09	GGGTAACGCC	12	04	08	500-2000
OPA-13	CAGCACCCAC	05	04	01	700-1600
OPA-14	TCTGTGCTGG	08	06	02	500-2000
OPA-19	CAAACGTCGG	07	07	0	500-1500
OPB-06	TGCTCTGCCC	10	06	04	450-2500
OPB-10	CTGCTGGGAC	05	02	03	600-1000
OPB-15	CCAGGGTGTT	10	08	02	300-2500
OPC-01	TTCGAGCCAG	10	06	04	300-2300
OPC-03	GGGGGTCTTT	05	05	00	600-1600
OPD-10	GGTCTACACC	06	02	04	600-2300
OPD-11	AGCGCCATTG	06	03	03	300-1500
OPF-04	GGTGATCAGG	19	03	16	300-2500
OPF-12	ACGGTACCCC	10	06	04	250-2000
	Total	123	70	53	

V. Conclusion

In conclusion, the molecular analysis of morphological variants of Robusta group of bananas from the farmers' orchards showed that an optimal detection of variants could be achieved using RAPD markers. These primers can be used by tissue culture industry in early identification of such variations.

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