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ESTABLISHMENT OF GENETIC FIDELITY OF *IN-VITRO* RAISED BANANA PLANTLETS

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Abstract

A simple and routine method for the analysis of tissue culture-derived banana plants for somaclonal variations is a prerequisite for precise monitoring of quality control during rapid mass micropropagation. This study reports on the use of RAPD-PCR and SSR for the detection of genetic variations in micropropagated banana plants. Thirteen varieties of the cultivated banana, procured from INIBAP, Belgium, were screened using RAPD-DNA markers. Only three RAPD primers (among 20 tested) were chosen as producing polymorphic DNA bands differentiating the investigated cultivars. Based on those identity markers, the genetic fidelity between various subculture levels were determined. Although minor morphological variations were recorded in the leaves of some clones, the developed RAPD and SSR profiles of different micropropagated clones were typical to that of the donor mother plants up till eight subculture levels.

Introduction

The genus *Musa* L. (family Musaceae, order Zingiberales) contains a wide range of triploid (2n = 3x = 33) cultivars and landraces of plantains and bananas that evolved from interspecific and intraspecific hybrids of the two wild diploid species, *M. acuminata* Colla, and *M. balbisisana* Colla.,which provide the A and B genomes, respectively (Simmonds & Shepherd, 1955; Gayral, 2008a, b). Only the parthenocarpic and seedless genotypes of bananas and plantains are cultivated for their fruits (Jambhale, *et al*., 2001). Sterility and polyploidy of the edible bananas constitute an important handicap for conventional breeding programs.

Historically a tissue culture cycle was seen essentially as a method of cloning a particular genotype (Etienne *et al*., 2002; Kadota *et al*., 2003). It was often considered a sophisticated method of asexual propagation enabling a more rapid rate of propagation. Thus it became the accepted dictum that all plants arising from tissue culture should be exact copies of the parental plant. However, phenotypic variants were frequently observed amongst regenerated plants. Sometimes the variants have been viewed as consequences of the exposure to exogenous phytohormones and sometimes they have been labeled as 'epigenetic' events.

Since somaclonal variation was first defined by Larkin & Scowcroft (1981), it has been widely documented in tissue culture-raised plants at morphological, chromosomal, biochemical and molecular levels in many plant species and has been extensively reviewed (Brown, 1991; Karp, 1991, Geering, 2005). Polymorphism at DNA level among the somaclonal families which were phenotypically normal was reported in strawberry (Damiano, 1997) in *Triticum* (Brown *et al*., 1993), in rice (Godwin *et al*., 1997), in *Populus deltoids* (Vijay *et al*., 1995) and in date palm (Saker *et al*., 2000; Saker *et al*., 2005). Such modifications included gene methylation changes, DNA rearrangements and alterations in copy number.

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Commercial production of banana using micropropagation processes bears several risks. Plant off-types, and genetically not identical to the mother plant, may be among the resulting plants. *In vitro* production of plants involves the application of plant growth regulators, such as auxins and cytokinins. Changes to these phytohormone habituations are known to be associated with genetic instability in plants. The failure to observe gross changes or abnormalities in morphology of plants does not negate the possibility of genetic variations which careful and specific analyses might reveal. In order to evaluate genetic variability, reliable methods of identification are required. Unambiguous identification is especially important in clonally-propagated crops such as banana (Kestner, 1983). Commercial cultivators need to be sure that they are investing their time and money in propagating the specific cultivar that they have chosen on the basis of yield, harvest time, size and shape.

Presently, there are various methods available which can be used to detect and monitor tissue culture-derived plants and cultivar identification. The most reliable methods are the molecular marker techniques that identify the variance depending on the plant proteins, which are expressed from defined regions of DNA, or DNA polymorphisms. RAPD (random amplified polymorphic DNA's) is a powerful technique for identification of genetic variation (Welsh *et al*., 1990). It has the distinct advantage of being technically simple and quick to perform, requiring only small amounts of DNA.

In the present study, the primary objectives were to use RAPD's and microsatellites to examine the genetic integrity and uniformity of the important banana cultivars produced employing tissue culture technique.

Materials and Method

Plant material: The banana variety used in this study was Cavendish Basrai (CB), a locally grown cultivar in the Sindh province of Pakistan.

***In vitro* propagation of banana:** Meristematic tips were isolated from suckers, sterilized and cultured onto the medium as recommended by Khan *et al*., (2001) supplemented with 6-benzylaminopurine (BAP, 0.50 mg/L), Kinetin (1.00 mg/L), table sugar (20.00 g/L) using cotton as a supporting medium. The shoots were multiplied and subsequently rooted *In-vitro* medium containing Indole-3 Butyric Acid (IBA 1.00 mg/L). Rooted plants were transplanted to mixture containing farm yard manure and sand (1:1 v/v) and fertilized weekly with ½ strength MS salts.

Genomic DNA isolation: DNA of 12 different successive passages/ subculture levels was extracted from the leaves following the method described by Haq *et al*., (2009) using extraction buffer I(1Molar Tris-Cl, 5M NaCl, 2% CTAB,50 mM EDTA, 1% PVP, and 0.2% 2-mercaptoethanol) with some modifications such as elimination of washing before adding extraction buffer.

RAPD-PCR conditions: Twenty 10-mer oligonucleotide primers (Operon Technology®, USA) were randomly chosen for the study. PCR reactions were performed in total volume of 25 µL reaction mix containing 2.5 µL of 1× reaction buffer, containing 2 mM $MgCl_2$, 2.5 µL dNTP at 0.1 mM, 0.3 µL (1 U) of Taq DNA polymerase (Promega, USA), 12 ng of genomic template DNA and 16 pmol primer in a preheated thermocycler (Eppendorf, Gradient Mastercycler). PCR was initiated by a denaturation step at 92°C for

3 min., and then the reaction was subjected to 45 cycles of 92°C for 30 sec, 35°C for 1 min., 72°C for 2 min., with a final elongation step of 10 min., 72°C.

Visualization and analysis of RAPD-PCR products: The amplification products were resolved by electrophoresis on a 1.2% agarose gel with Ethidium bromide and visualized under UV. The presence and absence of bands between samples was scored and data were transcribed into binary format (1, 0, respectively).

Microsatellite-PCR analysis: A set of 6 microsatellite primers (Table 2) was procured from Invitrogen®. These primers were obtained from the *Pst* I and *Taq* I libraries, (Invitrogen®). Initially, 3 genotypes namely Petite Naine, William Hybrid and FHIA-23 were used for PCR amplification using all the 6 primers. The primers generating clear and polymorphic patterns of DNA profile were selected for further analysis with all the 13 genotypes. Ultimately, *C. Basrai* was selected for the variation studies along each of the successive 12 subcultures. Extracted samples were amplified using the selected primers. For each primer, 25µl amplification reaction contained 10mM Tris-HCl pH 9.0, 50mM KCl, 0.1% Triton X-100, 1.5mM $MgCl_2$, 1mM dNTP, 0.2mM of each primer, 15ng of genomic DNA and 0.8 units of *Taq* DNA polymerase (Fermentas, USA). PCR amplifications were performed in an Eppendorf® Gradient 9700 thermal cycler with initial denaturation at 94°C for 4 min followed by 35 cycles of 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 sec, with final extension at 72°C for 10 min.

Development of microsatellite gels by silver staining: For the resolution of DNA image, electrophoresis was employed using Polyacrylamide Gel Electrophoresis (PAGE). A 5% acrylamide gel was employed with 8M Urea for denaturation purposes. Scoring of bands were conducted and images were captured using a Kodak 33mm camera on a Gel Documentation System (UV Tech®, UK), and the software UV Band (UV Tech®, UK) was employed for digital scoring of the gel. For each subculture level, each fragment / band that was amplified using Microsatellite was treated as a unit character. Unequivocally scorable and consistently reproducible amplified DNA fragments were transformed into binary character matrices (1 for presence, 0 for absence).

Results and Discussion

RAPD and genetic stability analysis: Genetic molecular markers are considered to be reliable in monitoring variability in the DNA sequences of the plants (Piña-Escutia *et al*., 2010, Zerihun *et al*., 2009). Several authors have applied the RAPD technique to investigate the genetic variability and found it to be very efficient and reliable (Brown *et al*., 1993). The results obtained from RAPD analysis were compared with those obtained with restriction fragment length polymorphism (RFLP) and isozymes (Sabir *et al*., 1992) and found no difference in them.

On the basis of the number, intensity and reproducibility of RAPD bands, three primers (OPF-07, OPF-11 and OPF-13) were selected out of the twenty primers, which were previously tested. Bands with the same mobility were treated as identical fragments. Weak bands with negligible intensity and smear bands were both excluded from final analysis. Figure 1 demonstrates the RAPD profiles obtained with one of the best primer (OPF-13). The number of scored bands varied from eight to thirteen, with an average of 11 bands per primer and an average of 4.5 polymorphic bands per primer.

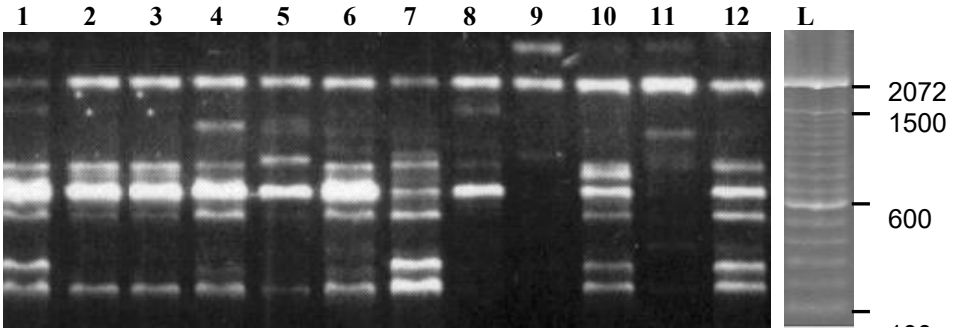

Fig. 1. RAPD Profiles obtained using OPF-13 primer M = 100 bp marker, Lane numbers = 12 successive subculture levels.

Screening of the 12 subculture levels revealed that banding profiles obtained with OPF 07, OPF 11, and OPF 13 primers were enough to distinguish all the cultivars (results not shown here). The results indicated that the RAPD technique is effective to develop genotype-specific banding patterns valuable for cultivar identification. Our results are in agreement with Oebrowska & Tyrka (2003), who used RAPD markers to identify and assess the level of genetic diversity among 9 strawberry cultivars differing in their response to photoperiod. They have confirmed since RAPD technique does not require previous DNA sequence information and uses very small quantity of DNA, it is considered as one of the most widely used techniques for genetic diversity studies. However, there is a problem with RAPD regarding its reproducibility.

The reproducibility of amplification profiles of RAPD is influenced by any variation in the method used for DNA isolation (Korbin *et al*., 2000) concentration of template DNA and primer, Taq-DNA polymerase concentration, temperature of annealing, number of thermal cycles and $MgCl_2$ concentration (Basam *et al*., 1992; Kernodle *et al*., 1994). 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 (Hedrick, 1992; Gibbs *et al*., 1994).

Similar results were observed when the various subculture levels were analyzed using microsatellites. At subculture level 1, a very high similarity index of 0.99 was obtained, which kept on declining up to level 12. At subculture level 9 a similarity index of 0.81 was observed, and at subculture level 10, a similarity index of 0.78 was obtained (Table 1), which further declined to 0.71 in sub culture 12, which depicted tremendous amounts of variations.

These results correlated with the morphology of the plants in the fields, as it was observed that when the banana plants were shifted to the greenhouses after 8 subcultures they gave normal growth and morphology as the parent/stock plants. However, if the plants were shifted after 9 or more subcultures, the leaves exhibited variegated patterns on the surface in the form of white and green streaks (Fig. 3a), and in the form of dark and green patches as observed in Fig. 3b. These variations continued with an increase in subculture levels, and with prolonged exposure of the plants to *In vitro* conditions. Morphological variations were also observed in plants that were acclimatized as clones of bananas. It has been noticed to have a high level of morphological flexibility in response to micropropagation conditions. It was also observed that leaves showed variegated patterns of different shades (Fig. 3).

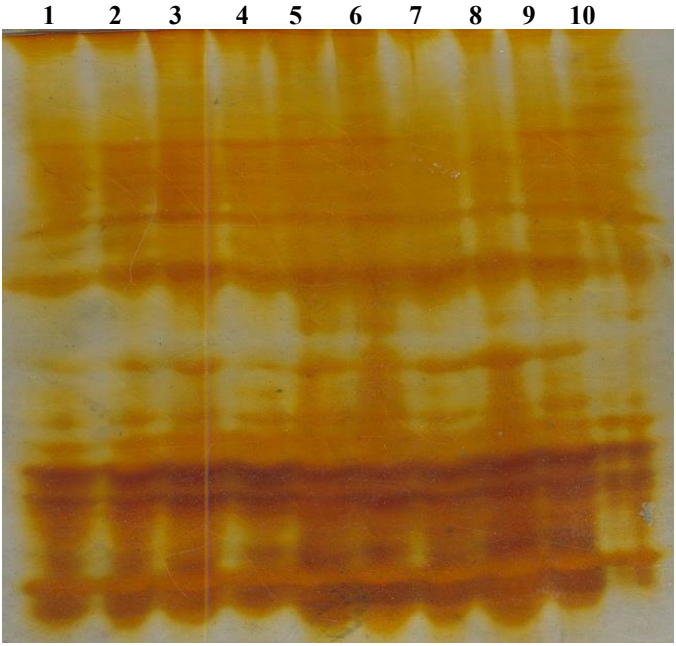

Fig. 2. Different subculture levels exhibited by Microsatellites.

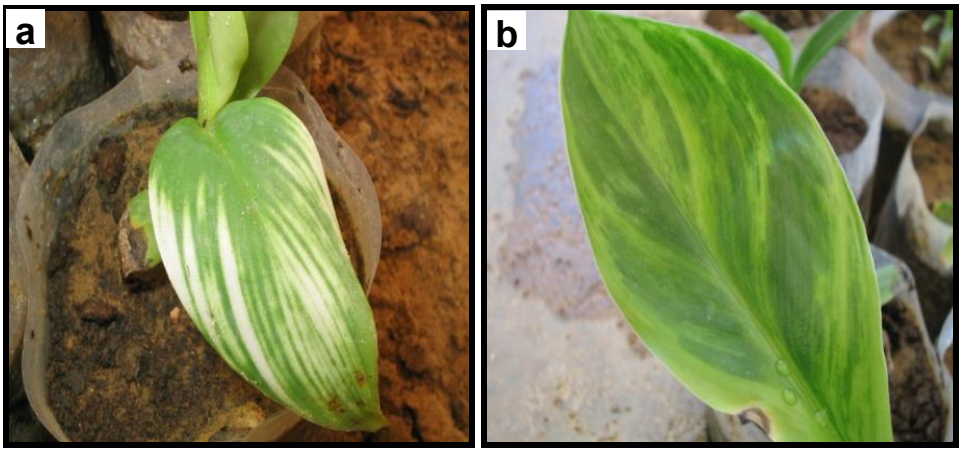

Fig. 3. Morphological variations observed in Banana plants subjected to micropropagation at 10th subculture level.

The phenotype of cells, tissues or plants can be altered either by transient environmental or by permanent changes to the genotype. Temporary variation often results from the expression of genes in some cells, tissues or organs which are not normally active. If such changes occurs during *In vitro* culture, the phenotype of the cells, tissues or organs may then be altered or be atypical. Temporary changes are often noted in cultured plant cells and tissues, and also in plants regenerated from and

propagated by tissue culture. For example, during successive subcultures it is sometimes possible to isolate strains of callus which differ in such characteristics such as colour, shape and growth rate. Changes of this kind are often reversible (Ogihara & Tsumewaki, 1979). Non persistent variation is sometimes observed amongst micropropagated plants which might display a change in growth habit, a more rapid development to flowering and the production of more axillary shoots. These temporary changes in phenotypic expression may result from unstable changes.

Variants may also arise as a result of more suitable changes due to single mutations in cultures. Recessive mutations are not detected in plants regenerated *In vitro* from any cell or tissue, but expressed in progeny, this shows that variants are the mutants. Somaclonal variants may also be due to changes caused by mitotic crossing over in regenerated plants. Such changes may also occur due to changes in organelle DNA, isozymes and protein profiles. Genomic DNA exhibits normal methylation patterns. Prolonged exposure of plant tissues to *In vitro* cultures has resulted in the alteration of normal methylation patterns.

The amount of variability released during propagation can be modulated by limiting the number of subculture and the duration of sub culture proliferation. Repeated *In vitro* sub culturing increases the chance of mutation and looses the regeneration potential, thus it should be avoided (Cassells & Plunkett, 1986).The period of time during which tissues have been maintained in culture commonly influences their morphogenetic potential. There is often a short period (one or more sub cultures) during which morphogenesis increases (Rhode, 1990; Renfroe, 1984). This is followed by a period in which capacity of the tissues to undergo morphogenesis often declines. Genetic change is often accentuated by prolonged intervals between subcultures.

Table 1. Similarity index of various subculture levels, with respect to the Parent (initial explant).

Subculture level	RAPD's	SSR's
1	0.98	0.99
2	0.94	0.94
3	0.90	0.92
4	0.91	0.90
5	0.89	0.89
6	0.88	0.90
7	0.89	0.91
8	0.86	0.89
9	0.73	0.81
10	0.75	0.78
11	0.84	0.72
12	0.60	0.71

Table 2. Characteristics of STMS clones from *Pst* I And *Taq* I libraries (*Invitrogen*®) used as microsatellites for the detection of variation in sub culture levels of banana.

Template	5`--Primer sequence—3`	Anneal temp (T_m) product length	Clone	Accession	Allele number
AGM-159	AATCGAAATCGAGT`AACAAGG	52 °C	MaCIR 503	X-9017	12
AGM-160	TTTTGTGGATGGTTGGTTCC	309 bp			
AGM-12	TTTGATGTCACAATGGTGTTCC	55 °C	MaCIR 108	X-87262	19
AGM-125	TTAAAGGTGGGTTAGCATTAGG	28 bp			
AGM-133	AGTTTCACCGATTGGTTCAT	55 °C	MaCIR 09	X-87265	08
AGM-13	TAACAAGGACTAATCATGGGT	151 bp			

Thus it is of utmost importance and the need of the time to devise an appropriate system which is economical, robust and technically simple to identify off types at an early stage among large number of populations of the micropropagated plants. In this study RAPD's and Microsatellites 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 (Rafalski *et al*., 1996).

In the series of experiments conducted for RAPD's, the Operon primers displayed very encouraging results, as all the results obtained were reproducible. Likewise SSR's also gave very much similar results to RAPD's in which it was clearly depicted that the genetic stability of CB banana is valid up to subculture level 8 after which the *In vitro* plants should not in any case be subjected to subculturing, thus a micropropagation regime should be materialized accordingly so as to strictly monitor the level a certain plant was being bulked up.

The most common types of somaclonal variant which are prevalent in the Cavendish subgroup; are stature variants (dwarfism); foliage variants (mosaic, rubbery or mottled-green thin leaves resembling virus infection); pseudostem variants (black, red or pale green stems); and bunch variants (affecting peduncle, fruit, sex ratio of flowers, bracts and male buds) as have been observed by Simmonds (1955).

Low levels of morphological variations were also observed in banana, and this variation includes variegated leaves, stalks with a shorter diameter and shortened internodes. This was most probably due to the *In vitro* stress which causes the genome to respond by DNA methylation, and this had most probably modified the marker profiles through insertion or excision of transposons (Hirochika *et al.,* 1996) The RAPD technique reveals DNA polymorphism as differences in the amplification patterns, and uses primers of random sequences that search for complementarities in the genome. It is suggested that DNA bands possibly represent repetitive sequences (Grattapaglia *et al*., 1992, Liliana *et al*., 2009). Polymorphism in repetitive DNA sequences has frequently been observed during plant propagation (Smulders *et al*., 1995; Santos *et al*., 2010). *In vitro* stress may provoke changes at preferential sites, such as repetitive DNA, thereby activating transposable elements. In order to reduce the proportion of somaclonal variation to a commercially acceptable level, it is recommended that subcultures and multiplication should be limited to only 1000 plants per explant (Yu, 2007); and micropropagated plants should be screened in the nursery for early detection of variants.

Shenoy & Vasil (1992) reported that micropropagation through meristem culture is generally associated with low risk of genetic stability because the organised meristems are generally more resistant to genetic changes that might occur during cell division or differentiation under *In vitro* conditions.

Microsatellite markers are a reliable mean of detecting high levels of polymorphism using relatively simple technique. In this study, polyacrylamide denatured gels were used followed by silver staining for facilitating the identification of greater levels of detectable polymorphisms, as compared to agarose gel for visualization.

Several mechanisms governing somaclonal variation induced during subculturing includes gene amplification, single nucleotide base change, transposon migration, altered methylation states, chromosome instability, chromosome inversion, single gene mutations, translocations, cytoplasmic genetic changes, ploidy changes, rearrangements and partial chromosome deletions (Duncan *et al*., 1986, Yu, Becker, 2006). It is believed

that high concentrations of plant growth regulators can modify the frequency of ploidy changes and point mutations. Factors such as explant source, time of culture, number of subcultures, phytohormones, genotype, media composition, the level of ploidy and genetic mosiacism are capable of inducing *In vitro* variability (Silvarolla, 2000, Yu Holland, 2008).

Thus by the utilization of different markers, it was clearly depicted that Banana plants could be subjected to mass multiplication up to 8 subcultures, which can be considered a 'safe level' of multiplication. New plants must be initiated on a regular basis so as to develop a continuous cycle of plant production, keeping very sure not to exceed subculture level 8.

In conclusion, our results demonstrate that molecular analysis using RAPD's and microsatellites can be applied to assess the genetic fidelity of plants derived *In vitro* on an industrial scale as part of crop improvement programs.

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