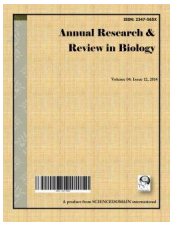

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In vitro Regeneration of Banana and Assessment of Genetic Fidelity in the Regenerated Plantlets through RAPD

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Authors' contributions

This work was carried out in collaboration between all authors. Authors PS and SS designed and conceptualized the experiment. Authors KD and MBG performed the experiment. Authors KD and PS prepared the manuscript. Authors BKS and MKM reviewed the manuscript. All the authors reviewed and approved the manuscript.

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ABSTRACT

Amrit Sagar belonging to banana (*Musa acuminata*) genome group AAA is popularly grown in North Eastern part of India for its high yield and natural disease resistance potential. The explants were established initially on supplemented Murashige and Skoog's (MS) mediums followed by subculturing for multiple shoot induction. Various concentrations of BAP were tested to improve the quality and quantity of multiple shoots induction, out of which 10 mg/l BAP gave the best result. A total of 6-8 cycles of subcultures were carried out, each with an interval of 20-25 days. Among these, well established healthy shoots of 4-5 cm were transferred onto rooting medium containing 10 mg/l sucrose. After 3 weeks, plantlets were carefully acclimatized to adapt the green house condition and subsequently transferred to field. Appearance of off-types of plantlets during the

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course of micropropagation were assessed with random marker *i.e.* RAPD for precise monitoring of quality control during rapid mass micropropagation. Out of 15 random decamer primers used, 12 generated well distinguished and reproducible pattern of amplified DNA. The RAPD profile analyzed with NTSYS-pc 2.02, revealed that the tested plantlets were similar to that of the mother plant with a very low level of polymorphism (9%). Thus, it can be asserted that the protocol used to generate the *in vitro* plantlets is safe and conforms to genetic fidelity.

Keywords: Micropropagation; BAP; Musa acuminate; somaclonal variation.

ABBREVIATIONS

BAP : 6 Benzylaminopurine
RAPD : Random Amplified Polymorphic DNA
PCR : Polymerase Chain Reaction
MS : Murashige and Skoog's Medium
FYM : Farm Yard Manure
mg/l : Milligram per Litre

1. INTRODUCTION

Banana is native to tropical South and Southeast Asia, and is likely to have been first domesticated in Papua New Guinea [1]. At present, it is being cultivated throughout the tropics and subtropics (agroforestry.net). In terms of production volume India ranks top in the world. Diverse groups of different cultivar are being grown in different agroclimatic zones of India based on environmental acceptability and soil types. Botanically, banana is the largest monocotyledonous herb and its genome groups are classified as AA, AB, AAA, AAB, ABB, AABB, and ABBB with letters A and B representing the contribution of *Musa acuminata* and *Musa balbisiana* respectively. Only the parthenocarpic and seedless genotypes of bananas and plantains are cultivated for their fruits [2].

Banana is the fourth most important food crop, with many high yielding and good quality cultivars being developed across the globe depending on the agroclimatic condition. A very high yielding and popular cultivar, Amrit Sagar, with genotype AAA, and having fruits with fairly high quantity of protein and vitamin is a very popular among the people of the North East region of India. Unlike the other popular cultivars, Amrit Sagar has natural resistance against most of the viral diseases and has good demand owing to its high yield potential. In spite of these importances, there is still no well organized systematic effort to cultivate this particular cultivar. This crop is specially grown in few pockets in an unorganized way due to lack of adequate and timely supply of healthy planting material. Till date, commercial mass propagation has not been embraced as far as this particular cultivar is concerned. Thus, mass production of its planting material through tissue culture can provide the solution to this problem and can make the cultivation more profitable and economically viable. *In vitro* regeneration of banana has been well documented [3-10] because of its ability to maintain true-to-type and also to initiate rapid multiplication of disease free cultivars. Of late, farmers have also shown interest in growing banana over a large area owing to its increasing demand and vast export potential.

One of the major important criteria of successful rapid propagation of banana is the efficient development of multiple shoots. Venkatachalam et al. [11] reported exogenous supply of a single cytokinin was sufficient to induce shoot multiplication to some extent in Nanjanagudu Rasabale (AAB). The specific concentration of cytokinin is very critical for the proper shoot proliferation. On the contrary, Sahijram et al. [8] reported that using high concentration of kinetin (cytokinin) in culture medium may cause high level of somaclonal variation. Thus, optimization of concentration of hormone in the culture medium is of utmost importance to produce high level of multiple shoot but with less somaclonal variation.

The most important part of *in vitro* micropropagation is the generation of genetically homogenous and uniform plantlets with all desirable characters of the mother plant. Somaclonal variation is usually observed during tissue culture when it is regenerated through callus/suspension culture. But even during adventitious shoot formation, production of off-types cannot be avoided completely. This may lead to generation of off-types planting material with undesirable character(s) and which may not be suitable for the industry. Factors such as genotype, explant type, number of subcultures, degree of dedifferentiation and concentration of hormone etc. contribute to somaclonal variation in micropropagated banana [12]. Since

somaclonal variation was first defined by Larkin & Scowcroft [13], 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 [14-16]. Although somaclonal variation is undesirable in the context of micropropagation, it can also be an advantageous phenomenon for genetic improvement of banana as reported by Vidal and Garcia [17], who found a somaclonal variant 'CIEN BTA-03' resistant to yellow Sigatoka during *in vitro* cultures. However, majority of the off-types recorded in this crop are agronomically inferior to the parental clone [18]. It has, therefore, been suggested that commercial application of tissue culture in banana must await adequate quality check under field conditions [19]. With the advent and application of molecular markers that include Restriction Fragment Length Polymorphisms (RFLPs), Random Amplified Polymorphic DNAs (RAPDs), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs), Inter Simple Sequence Repeats (ISSRs), DNA Amplification Fingerprinting (DAF) and Microsatellite Primed-PCR (MP-PCR), the detection of any kind of genetic variability at DNA level has become easier and more precise. RAPD analysis is extensively used for the identification of germplasm, screening of off-types, assessing genetic diversity and monitoring the genetic stability of conserved germplasm [11,20]. This marker system has been successfully applied to detect the somaclonal variations in a number of species such as *Musa spp*. [10], *Swertia chirayita* [21], *Saccharum officinarum* [22], *Dendrocala mushamiltonii* [23], and *Cymbopogon martini* [24]. The present study seeks to optimize a suitable regeneration protocol for, Amrit Sagar (AAA) and subsequent confirmation of its ability to sustain genetic fidelity with the help of RAPD markers.

2. MATERIALS AND METHODS

2.1 Plant Material and Mother Plant

Sword suckers weighing around 0.5 – 1 kg were collected from disease free banana orchard of Assam Agricultural University, Jorhat India wherein the mother plants were maintained in disease-free condition and used as propagating material. . The shoot tip uniform in size (5cm) were excised from rhizomes and surface sterilized with 0.1% mercuric chloride for 2 min followed by thorough washing with sterile distilled water for 3-4 times under aseptic condition. For each batch of tissue culture experiment 40 explants were used.

2.2 Establishment of Explant

The sterile explants were transferred to initiation medium (MS medium supplemented with 0.2 mg/lit thiamine and 10 mg/lit BAP) for establishment of cultures under *in vitro* condition and maintained at 25±2°C with 12 hr light (3000 lux). After 20-25 days of culture the explants turned green and were ready for first subculture. After two cycles of 24 days interval, explants were subsequently subcultured on fresh multiplication medium.

2.3 Growth Regulator Treatments and Multiple Shoot Proliferation

In order to improve the rate of shoot multiplication, the growth regulators BAP (cytokinins) in shoot multiplication medium (MS supplemented with 20 mg/lit glycine, 0.8mg/lit Adenine, 10 mg/lit ascorbic acid, 1 mg/lit Nicotinic acid, 1 mg/lit Pyrodoxine and 30 g/lit sucrose) were progressively increased from 5 mg/l to 15 mg/l, and their effects on both shoot proliferation and shoot growth were studied. The established explant, each with an average 4–6 multiple shoot buds of uniform size, was selected, and five such segments were transferred to multiple shoot medium with different concentration of BAP. The observations on growth and morphological changes were recorded at every 21 days, after which the cultures were transferred to fresh respective medium by vertically splitting the culture into three pieces. Three such successive subcultures were made after which the final morphological observations were recorded after 72 days. This experiment was replicated three times and data were analysed by Duncan's multiple range test and the experimental design used was randomize block design (RBD). Finally, the BAP concentration which gave best results in multiple shoot proliferation was selected for each cycle of subsequent subculture. The subculture was confined for 6 cycles in every 24 days interval for shoot proliferation.

2.4 Rooting and Acclimatization in Greenhouse

Well established shoots of 4-5 cm length were transferred onto rooting medium (MS medium

same as multiple shoot induction but, with 10 g/lit sucrose). After 3 weeks of culture, well established individual plantlets with healthy root system were shifted carefully to acclimatize in the green house conditions. Primary hardening was done by transferring the plantlets into coco peat mixture and maintained in plastic sheet covering tunnels for 15 days and later on transferred to soil mixture (25% vermi compost, 25% FYM, 50% soil) and maintained in shade net house for another 30 days for secondary hardening. Finally, the plants were transferred to field.

2.5 Preparation of Template DNA

Total DNA was extracted from tender leaves of 21 numbers of randomly selected micropropagated plants from a single batch and the mother plant. Fresh leaves (~ 500 mg) were ground to fine power with liquid nitrogen in a mortar and pestle. The powder was transferred to a 50 ml sterile polypropylene tube containing 5ml of extraction buffer (200 mM NaCl, 25 mM EDTA, 200mM Tris-HCl, pH 8.0, 0.5% SDS, 0.2 % (v/v) β-mercaptoethanol (BME), 0.2 % (v/v) Bovine serum albumin and 1% polyvinyl pyrrolidone (PVP). Banana being high polyphenol containing plant, additional measure was adopted in the extraction buffer with addition of BME and PVP. The homogenate was centrifuged at 10000 g for 10 minutes. To the supernatant, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and centrifuged at 10000 g for 10 minutes. The DNA was precipitated from the aqueous phase by mixing with an equal volume of isopropanol and kept at -20ºC overnight followed by washing with 70% ethanol. The DNA pellet was then air dried and resuspended in sterile distilled water. The RNase treatment at a concentration of 1 mg/ml was given by incubating the solution at 37°C for 20 minutes. The quality and quantity of purified total DNA was estimated by taking absorbance at 260 and 280 nm using Nanodrop Spectrophotomer (NanoDrop 1000 spectrometer, Thermo Scientific Corp., USA). The DNA was then separated on 0.8% agarose gel using ethidum bromide and photographed in a Gel Documentation System.

2.6 RAPD Analysis

A total of twenty universal decamer oligonucleotides, were procured from Operon (Operon Biotechnologies GmbH, Cologne, Germany) to assess the genetic fidelity in the micropropagated plantlets at the secondary hardening stage that were selected randomly from a single batch of plantlets as well as the mother plant. RAPD analysis was performed in a 25 µl reaction mixture containing 10x *Taq* Buffer 2.5 µl (Genei, India), 10 mM dNTP mix (Genei, India)1 µl, 1U *Taq* DNA polymerase 1µl (Genei, India), 30 pM primers (Operon Technologies, Germany) 1µl and 100 ng of genomic DNA. The total reaction volume was then subjected to PCR in a thermocycler (Gene Amp* PCR System 9700 Applied Biosystems). The PCR cycle used by Damasco et al. [18] to generate RAPD in *Musa* pp. was followed to generate RAPD profile.

2.7 Statistical Analysis

Only consistently reproducible, well-resolved fragments, in the range of 150bp to 1400bp were scored as 1 or 0 for presence or absence of the band on the gel. Bands of equal molecular weight and mobility generated by same primer were considered to be of identical locus. The distance matrix and dendrogram were constructed using the NTSYS-pc 2.02. Genetic similarities between tissue culture-derived plants and their donor mother plant were measured by the Jaccard's similarity coefficient with SIM-QUAL module. Similarity coefficients were used to construct a dendrogram using the UPGMA (unweighted pair group method with arithmetic average).

3. RESULTS

3.1 Growth Regulator Treatments

The results showed that multiple shoot formation occurred in all the combinations of growth regulators tested, but significant differences were observed in terms of number and quality of shoots formed under each treatment (Table 1). The number of shoot buds increased with an increasing concentration of BAP up to 10 mg/l, beyond which a suppression of shoot development occurred. BAP at low concentrations fostered optimum proliferation of shoots, whereas at higher concentrations it resulted in clustering of shoots and buds with a maximum number of 100 shoot buds per segment (at 10 mg/l). BAP at 10 mg/l was considered optimal for shoot multiplication as the shoot morphology appeared appropriate for routine application. With higher levels of BAP (11–15 mg/l), a reduction in number as well as length of shoot buds occurred (Fig. 1).

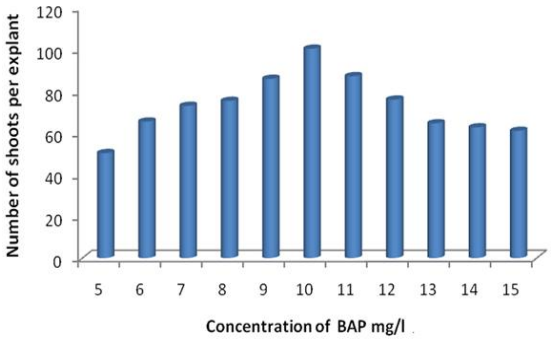

Fig. 1. Effect of BAP on multiple shoot development calculated by Duncan's Multiple Range Test (DMRT)

Table 1. Effect of different concentrations of BAP on multiple shoot proliferation of banana cv. Amrit Sagar (AAA)

BAP conc.	No. of shoot
5	50.27g
6	65.4e
7	72.93cd
8	75.33c
9	85.89b
10	100.33a
11	87.16b
12	75.93c
13	64.56ef
14	62.63f
15	60.96f

Means with common letters are not significantly different at P ≤ 0.05, according to Duncan's Multiple Range Test (DMRT)

3.2 Micropropagation Protocol

Sword suckers weighing around 0.5-1 kg were found to be the best to excise the explant for tissue culture (Fig. 2A). Within 5-6 days of culture the explants started appearing green and undergo basal swelling. After two cycles of 24 days interval, the buds started to produce multiple shoots (Fig. 2B). Each shoot was cut into two pieces and subcultured on the fresh multiple-shoot induction media. Generally, 6-8 cycles of subcultures were carried out for each explant (Fig. 2C, D). Prolonged subculture was avoided to check somaclonal variation in the clones. Appearances of roots from shoots were observed within 18 days of culture on rooting medium. After 3 weeks of cultures, 10-15 numbers of roots of 6-10 cm length were observed, (Fig. 2E) which was found to be optimum for hardening. The plantlets were irrigated regularly. During hardening, the plants underwent physiological adaptation to changing external factors like water, temperature, relative humidity and nutrient supply (Fig. 2F, G).

3.3 RAPD Profile

In order to confirm the genetic stability of micropropagated plants for maintaining genetic fidelity, RAPD fingerprinting of 21 randomly selected micropropagated plants of cv. Amrit Sagar along with the mother plants were carried out (Fig. 3). Out of 15 arbitrary RAPD primers tested initially, 12 produced clear and scorable band patterns (Table 2). The number of loci ranged from 5(OPB-01 and OPA-11) to 11(OPB-06) with an average of 7.75 loci per primer, size ranging from 150 bp (OPA-11) to 1400 bp (OPA-16). Out of all the scorable bands 90.4% were found to be monomorphic across all the micropropagated plants and their mother plant. A very low polymorphism was recorded (9.6%) as evidenced by the presence of few additional bands (Fig. 3). Maximum numbers of 5 polymorphic bands were obtained with the primer OPA-16. Clone f showed a unique band with numbers of primers *viz.,* OPA 17_{650bp}, $OPA11_{700bp}$, OPB 06_{350bp}. This particular clone also showed polymorphism in terms of absence of major band (OPA 12_{350bp}, OPB 07_{630bp} and OPB 08_{300bp}) or presence of minor band with most of the primer tested. Thus, clone 'f' can be considered as off-type.

3.4 Statistical Analysis

Cluster analysis of RAPD using UPGMA showed close relationship of the regenerated plantlets with the mother plant except very few. The similarity matrix established through the analysis showed more than 80% similarity between the clones and the mother plant (Table 3), which is also evident from the dendogram (Fig. 4). Similarity coefficient for micropropagated and the donor plants ranged from a minimum of 0.387 (38.7% similarity among clone no. f and g to a maximum of 0.968 (96.8% similarity among clone no. b and c) with an average of 76.5% similarity. As observed in Fig. 2 (A-D) the micropropagated plant 'f' showed different banding pattern as compared to all other plants and the mother plant indicating the chance of a probable somaclonal variation. All other micropropagated plants and the donor plant shared the same banding patterns, implying that they possibly were genetically identical to each other. The cluster analysis of RAPD using UPGMA showed that regenerated plants compared to their respective

donor mother plants had high affinity (86%), and the affinity was more than 90% among most of the micropropagated plants. The dendogram grouped all the 17 clones out of 21 tested into a single group with the mother plant with more than 80% similarity. There are four off-types clones recorded in this study also quite evident from their different RAPD profile.

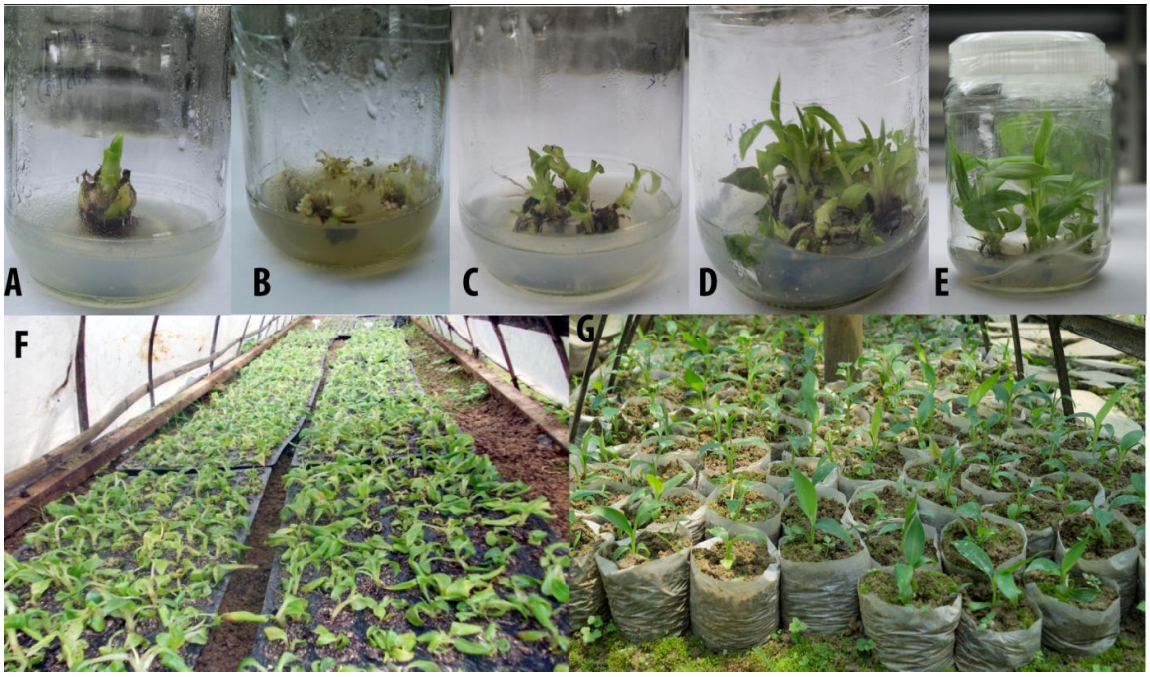

Fig. 2. Micropropagation of Banana (*Musa acuminata*) (A) Establishment of explant; MS Basal +4% sucrose 0.2 mg/l thiamine 10 mg/l BAP 0.8% agar pH5.8 (B) Initiation of multiple shoots; MS basal + 3% sucrose 10 mg/l BAP 0.5 mg /NAA+ 0.8% agar pH 5.8 (C) Sub-culture-I; MS basal + 3% 10 mg/l BAP 0.5 mg /l NAA+0.8 % agar Ph 5.8(D) Sub-culture-VI; MS basal + 10 mg/l BAP +0.5 mg /l NAA +3% sucrose 0.8% agar pH5.8 (E) Rooting of *in vitro* generated plantlets; MS basal + 0.2 mm/l BAP +1 mg /l IBA +1% sucrose 0.8% agar pH5.8+ (F) Primary hardening; (G) Secondary hardening

Table 2. List of RAPD primers, their sequences and size of amplified fragments generated along with number of bands, loci and polymorphic bands for detecting the genetic fidelity in micropropagated plants of banana cv. Amrit Sagar

Primer code	Primer sequence (5'-3')	Number of bands	Number of Loci	Number of polymorphic bands	Size range (bp)
OPB-01	GTTTCGCTCC	110	5	1	480-1000
OPB-04	GGACTGGAGT	198	9	1	200-900
OPB-05	TGCGCCCTTC	154	7	1	300-1100
OPB-06	TGCTCTGCCC	242	11	1	220-1400
OPB-07	GGTGACGCAG	132	6	0	270-750
OPB-08	GTCCACACGG	176	8	3	350-1000
OPA-11	CAATCGCCGT	110	5	2	200-900
OPA-12	TCGGCGATAG	198	9	0	150-1030
OPA-13	CAGCACCCAC	154	7	0	150-650
OPA-16	AGCCAGCGAA	154	7	5	550-1400
OPA-17	GACCGCTTGT	198	9	0	300-1100
OPA-18	AGGTGACCGT	220	10	0	290-1200
Total		1,947	93	9	

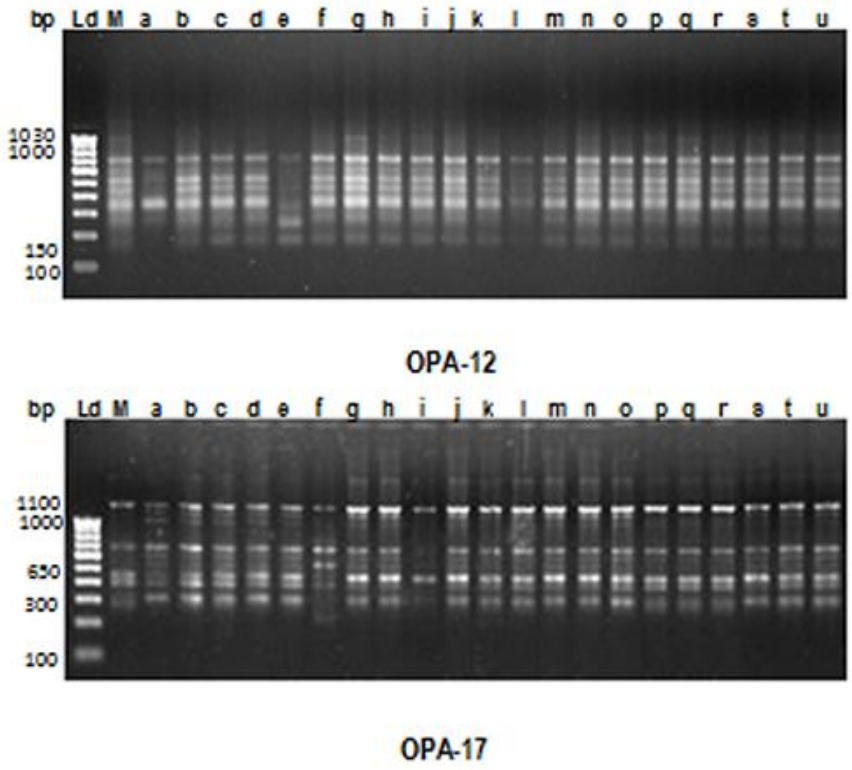

Fig. 3. DNA fingerprinting of micropropagated plants of banana cv. Amrit Sagar. Lane Ld represents DNA Ladder (Genei, India), Lane M represents mother plant, Lanes a–u represents the micropropagated plants

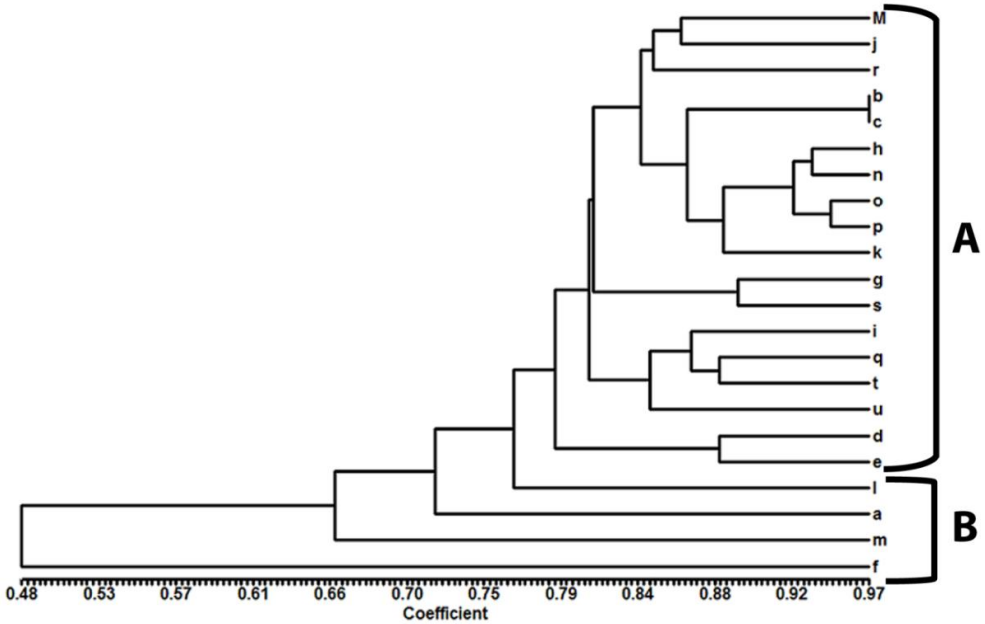

Fig. 4. RAPD Dendrogram based on Jaccard Similarity indices, showing the genetic distance among the regenerated plants of banana (a-u) and the mother plant (M)

Table 3. Similarity matrix for Jaccard's coefficient based on RAPD banding pattern for the micropropagated plants and mother plant of *Musa acuminata* cv. Amrit Sagar

	M	a	b	c	D	e	f	g	h	i	j	k	l	m	n	O	p	q	r	s	t	u
M	1.000																					
A	0.785	1.000																				
B	0.839	0.774	1.000																			
C	0.828	0.763	0.968	1.000																		
D	0.753	0.731	0.871	0.860	1.000																	
E	0.785	0.742	0.839	0.849	0.882	1.000																
F	0.5.38	0.581	0.527	0.516	0.527	0.516	1.000															
G	0.742	0.634	0.774	0.785	0.710	0.699	0.387	1.000														
H	0.785	0.699	0.882	0.871	0.817	0.785	0.452	0.892	1.000													
I	0.753	0.645	0.763	0.774	0.763	0.753	0.419	0.796	0.796	1.000												
J	0.860	0.731	0.828	0.839	0.763	0.774	0.505	0.796	0.860	0.806	1.000											
K	0.806	0.742	0.860	0.849	0.860	0.828	0.495	0.806	0.914	0.817	0.839	1.000										
L	0.710	0.667	0.785	0.774	0.763	0.753	0.462	0.753	0.796	0.742	0.742	0.839	1.000									
m	0.667	0.624	0.677	0.667	0.677	0.710	0.527	0.559	0.645	0.656	0.720	0.667	0.656	1.000								
n	0.806	0.699	0.839	0.849	0.774	0.785	0.452	0.871	0.935	0.796	0.882	0.892	0.817	0.688	1.000							
o	0.806	0.699	0.839	0.849	0.774	0.785	0.430	0.892	0.914	0.796	0.839	0.849	0.817	0.624	0.935	1.000						
p	0.839	0.753	0.892	0.903	0.828	0.817	0.462	0.860	0.925	0.828	0.892	0.882	0.806	0.656	0.925	0.946	1.000					
q	0.742	0.677	0.796	0.785	0.774	0.785	0.452	0.763	0.806	0.860	0.774	0.828	0.731	0.688	0.828	0.806	0.839	1.000				
R	0.828	0.763	0.817	0.828	0.796	0.828	0.495	0.763	0.828	0.796	0.860	0.871	0.753	0.774	0.871	0.806	0.860	0.785	1.000			
S	0.763	0.699	0.796	0.806	0.753	0.720	0.409	0.892	0.828	0.796	0.753	0.806	0.710	0.559	0.785	0.849	0.860	0.763	0.763	1.000		
T	0.817	0.753	0.849	0.860	0.806	0.817	0.484	0.860	0.882	0.871	0.849	0.882	0.763	0.677	0.860	0.839	0.892	0.882	0.860	0.860	1.000	
U	0.806	0.699	0.753	0.763	0.710	0.742	0.473	0.763	0.785	0.839	0.817	0.763	0.688	0.624	0.763	0.785	0.839	0.828	0.785	0.785	0.860	1.000

4. DISCUSSION AND CONCLUSION

A high rate of shoot multiplication needs high level as well as a precise combination of different cytokinins, because each cytokinin is known to trigger different molecular pathways [25,26]. In the present study, it was observed that multiple shoot formation occurred in all the concentration of BAP but with significant differences in the number of shoots regenerated. Although BAP at concentrations of 10 mg/l fostered an enhanced rate of shoot multiplication, but it was found to be detrimental to shoot proliferation at very high concentrations i.e > 10 mg/l. Similar suppressive effects of BAP at very high concentrations have also been previously reported in AAB genotype of banana [11,27,28]. Very high concentration of BAP in combination with adenine was reported to cause genetic variability in micropropagated banana [29].

True-to-type clonal fidelity is one of the most important prerequisites in the micropropagation of any crop species. A major problem often encountered with the *in vitro* cultures is the presence of somaclonal variation amongst subclones of one parental line, arising as a direct consequence of *in vitro* culture of plant cells, tissues, or organs. According to RAPD analysis, the group of 21 (non-preferably chosen) similarity plantlets and the mother plant of banana cv. Amrit Sagar showed high degree of monomorphism with few polymorphic bands (Table 2). Similar levels of monomorphism was also reported by Sharma et al. [30] in *Jatropha curcas* whereas, 28.8% polymorphism was reported by Damasco et al. [18] in banana. In most of the micropropagation program, 3–5% somaclonal variation is permissible [31], whereas, in banana, up to 10% variation is permitted (as practised by commercial micropropagation outfits) due to the flexible genetic make-up of the crop [5,32]. A high level of genetic similarity ranging from 80 to 96% in most of the micropropagated plants was prominently observed. Except the clone f, which showed only 53.8% similarity with the mother plant and high level of polymorphism with other clones also. This particular clone can be regarded as off-types.

The dendogram generated from similarity matrix value, grouped the clones into two distinct clusters. Cluster A consists of most of the micropropagated line i.e. 17 out of 21 studied with more than 80% similarity with the mother plant. Cluster B consist of only four clone viz., “l”, “f”, “m” and “a”. These clones showed less than 75% similarity with the mother plant. Among these four, clone “f” can be regarded as off-types or somaclonal variants as it shows only 50% similarity with the mother plant and also evidenced from banding pattern using almost all the primers. Since, even single base change at the primer annealing site can manifest as appearance or disappearance of RAPD bands, it could be suggested that tissue culture conditions have induced varied amount of genetic changes in this clone. In addition, such polymorphism in the RAPD banding profile may be due to repeated sub-culturing after a specific intervals which may lead to genetic alternations, such as deletions, substitutions, or duplication resulting in random changes within the primer binding sites [33]. Though *in vitro* transfers over a long duration are known to induce somaclonal variations [34], molecular data regarding the effect of the number of sub-cultures on the stability of micropropagated plant material are not documented [35]. In the present study the similarity percentage was high, with most of the clones showing more than 90% similarity with the mother plant, which is within the safe limit of monomorphism according to Commercial banana micropropagation outfit and it meant that all these plantlets are genetically very close to each other, as previously reported by Zoghlami et al. [36]. In the case of micropropagation of banana, though the protocol is known to yield clonal material, there are reports of somaclonal variation especially in cultivars Williams (AAA) [18,37], Robusta (AAA), and Giant Governor (AAA) [10]. Occurrence of somaclonal variation in *in vitro* regeneration of banana also depends on genome group [8]. Genome group AAA most probably is prone to more genetical variation [38]. The occurrence of off-type in tissue cultured plantlets was reported to range from 6 to 38% in Cavendish (AAA) cultivars [38]. Banana derived from shoot tip culture may vary from 0-70% according to genotype as reported by Smith [5] and Vuylsteke et al. [27]. So occurrence of 9.6% polymorphism in the present study among many other factors may be attributed to the genome group of the selected cultivar i.e. Amrit Sagar (AAA) and also may be due to use of shoot-tip as explant. Such somaclones were relatively stable without generally reverting to normal phenotypes, where the PCR-based tests were found very useful in genotyping such new clones [39]. The protocol optimized for *in vitro* regeneration of this important cultivar Amrit Sagar conform through the genetic fidelity test and thus, can be considered safe for commercial multiplication.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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