Research Paper

DNA Fingerprinting of Putative Mutants of Kolikuttu Banana Variety Agra

D.A. Shirani[1*], W.L.G. Samarasinghe[2], S.K. Wasala[2], N.M. Ubeysekara[2], S. Abeysinghe[3], P.D. Abeysinghe[3]

[1]*Grain Legume and Oil Crops Research and Development Centre, Angunakolapelessa, Sri Lanka*
[2]*Plant Genetic Resource Centre, Gannoruwa, Peradeniya, Sri Lanka*
[3]*Department of Botany, Faculty of Science, University of Ruhuna, Matara, Sri Lanka*

[*]*Corresponding author: shirani_da@yahoo.com*

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Abstract

Induced mutation coupled with *in-vitro* propagation technique has been established as a tool to generate mutations in a number of vegetatively propagated crops including banana. The need for alternative crop improvement methods for banana is owing to its sterility and polyploidy nature which make it extremely difficult in applying conventional methods. Susceptibility of Kolikuttu banana (AAB; silk) for the devastating disease; fusarium wilt (*Fusarium oxysporum* f.sp. *cubense* (*Foc*) is the major threat in commercial cultivation. Therefore, in the present study, *in-vitro* mutagenesis of shoot tips was applied aiming to develop resistant or less susceptible Kolikuttu banana against fusrium wilt. Ethyl methanesulfonate (EMS) treated shoot tips were further multiplied and subjected to screening using different techniques under *in-vitro*, protected house and sick plot conditions. Samples from the survived plants after each screening were collected and molecular characterization was performed with 21 RAPD primers to identify the variations. Gels scored with 'quantity one' software and analyzed using POPGENE illustrated that all the primers used in the analysis showed varying degrees of polymorphism ranging from 57.14 to 100%. The dendrogram generated using Nei's genetic distance and UPGMA algorithm showed that the survived plant in sick plot was different from the untreated control plant with 0.689 genetic distance. Genotypic stability of identified mutated plants was studied using selected polymorphic primers in the consecutive generation and it was observed 100% similarity which revealed that the selected mutants are stable.

Keywords: Fusarium wilt, *In-vitro* mutagenesis, RAPD, Silk banana

Introduction

Kolikuttu banana which fetches high market price is highly susceptible to fusarium wilt or panama disease caused by soil borne pathogen *Fusarium oxysporum* f.sp. cubense (*Foc*). There are no effective control measures against this devastating disease. Considering the importance of the disease for local Kolikuttu production, a research programme on *in-vitro* mutagenesis of shoot tip cultures of Kolikuttu banana was conducted aiming to develop fusarium wilt resistant or less susceptible Kolikuttu variety.

Molecular markers play an important role in screening of plants against abiotic and biotic factors and facilitate rapid and early detection of variations. Random amplified polymorphic DNA (RAPD) markers have been widely used to examine the genetic relationships in banana germplasm (Uma *et al*., 2006; Nsabimana and van Staden, 2007) identifying duplications in germplasm conservation and monitoring of genetic stability (*i.e*. somaclonal variation) of tissue culture materials (Ray *et al*., 2006; Lakshmanan *et al*., 2007; Sheidai *et al*., 2008) and identifying polymorphism between morphologically different banana mutants obtained through chemical mutagenesis (Kishor *et al*., 2017). Molecular markers to detect *Foc* resistance have not been identified for race 1 of the pathogen. However, two Sequence Characterized Amplified Region (SCAR) markers have been developed to detect the resistance against Foc race 4 (Wang *et al*., 2012).

Therefore, in the present study, RAPD analysis was selected to characterize the identified putative mutants generated through chemical mutagenesis of shoot tips of Kolikuttu banana variety Agra.

Materials and Methods

Samples were selected from the survived plants which underwent screening against *Foc* using *in-vitro*, protected house and *in-vivo* techniques for RAPD analysis.

DNA was extracted from the most immature leaf using CTAB protocol (Doyle and Doyle, 1990) with some modifications and DNA was treated with RNAse and diluted at 20 $ng/\mu l^{-1}$. Altogether there were 16 samples (14 plants derived from Ethyl methanesulfonate (EMS) treated plants used for the study + untreated variety Agra + *Foc* resistant Ambul variety Nadee).

Molecular analysis was done at Plant Genetic Resources Centre, Department of Agriculture, Gannoruwa, Peradeniya.

Out of 16 samples (shown in Table 1), 13 were selected from survived plants after different screening techniques for *Foc*. However, those plants did not show any distinct morphological variations during their vegetative growth period. Genotype 16 (Ambul variety Nadee) was taken as resistant check. Genotype 2 was the mother plant (Agra) used to establish the *in-vitro* cultures for mutagenic treatment. There was only one morphologically distinct plant (less pigmented but susceptible to Fusarium wilt) was also taken for molecular analysis. The sample number 10 was the only plant which produced a bunch during screening under sick plot conditions and it survived in the sick plot for more than 2 years.

Table 1. Samples selected for RAPD analysis

Sl. no.	Description
1	Morphological variant
2	Control (variety Agra)
3	Survived plant after *in-vitro* screening using fungal co-cultivation technique
4	Survived plant after *in-vitro* screening using fungal co-cultivation technique
5	Survived plant after *in-vitro* screening using crude culture filtrate
6	Survived plant after *in-vitro* screening using fusaric acid
7	Plant selected from *Foc* "hot spot" (Mahagama farm)
8	Survived plant after *in-vitro* screening using crude culture filtrate
9	Plant selected from sick plot screening (ARS, Telijjawila)
10	Plant selected from sick plot screening (ARS, Telijjawila)
11	Plant selected from double pot screening
12	Plant selected from double pot screening
13	Plant selected from *Foc* "hot spot" (ISTI, Angunakolapelessa)
14	Plant selected from *Foc* "hot spot" (ISTI, Angunakolapelessa)
15	Plant selected from sick plot screening (GLORDC, Angunakolapelessa)
16	Nadee (*Foc* resistant Ambul variety)

Twenty one RAPD primers used in the study are listed in Table 2. Annealing temperatures for each primer has been optimized for better performance and varied from 30 - 44 ^{0}C.

PCR amplification

Polymerase Chain Reaction (PCR) was carried out in a total volume of 15 µl containing 3 µl of 5x buffer (Promega), 2.1 µl of 25 mM $MgCl_2$ (Promega), 0.375 µl of 10 mM dNTP (Promega), 1.2 µl of 20 mM primer (IDT), 0.3 µl of 5U Taq polymerase (Promega Flexi Cat No. M829), 3 µl of genomic DNA and 5.025 µl of de-ionized water. Amplification was performed in Verity™ 96 wells Thermal Cycler with temperature gradient facility. PCR amplification consisted of an initial denaturation step at 94 ^{0}C for 7 minutes followed by a process of 40 cycles consisted of 3 steps namely, a denaturation step at 94 ^{0}C for 1 minute, annealing step for 1 minute in which the specific temperature for each primer was maintained and an extension step at 72 ^{0}C for 2 minutes. At the end of the final cycle, final extension was carried out at a temperature of 72 ^{0}C for 7 minutes, with subsequent holding temperature at 4 ^{0}C. PCR products were separated on a 1.5% agarose gel with 100bp DNA ladder. The gel pictures were taken using BIORAD Gel documentation system and used for scoring.

Identified new genotypes (2 samples; number 1 and 10) through the first RAPD analysis were confirmed for their stability in the second generation using ten selected RAPD primers (OPB-15, OPB-18, OPH-4, OPH-8, OPX-1, OPX-4, OPX-7, OPA-7, OPA-9 and UBC 292).

Table 2. RAPD primers, banding profile and percentage of polymorphism observed with the primers employed in the study

Sl. No.	Primer code	Sequence (5'- 3')	Number of RAPD products (bands)		
			Total bands	Polymorphic bands	% polymorphism
1	J 13	CCA CAC TAC C	11	08	72.73
2	J 14	CAC CCG GAT G	11	10	91.93
3	OPA 20	GTT GCG ATC C	09	08	88.89
4	OPA-7	GAA ACG GGT G	13	10	76.92
5	OPA-9	GGG TAA CGC C	12	10	83.33
6	OPB 15	GGA GGG TGT T	13	12	92.31
7	OPB 17	AGT CGT CCC C	12	10	83.33
8	OPB 18	CCA CAG CAG T	10	07	70.00
9	OPC 10	TGT CTG GGT G	08	07	87.50
10	OPC 15	GAC GGA TCA G	06	06	100.00
11	OPH 5	AGT CGT CCC C	11	11	100.00
12	OPH 3	AGA CGT CCA C	10	10	100.00
13	OPH 4	GGA AGT CGC C	10	09	90.00
14	OPH 8	GAA ACA CCC C	13	13	100.00
15	OPX 1	CTG GGC ACG A	07	04	57.14
16	OPX 4	CCG CTA CCG A	11	11	100.00
17	OPX 7	GAG CGA GGC T	12	12	100.00
18	OPY 2	CAT CGC CGC A	13	13	100.00
19	UBC 292	AAA CAG CCC G	10	09	90.00
20	UBC 465	GGT CAG GGC T	12	07	58.33
21	UBC 620	TTG CGC CCG G	11	11	100.00

Amplified fragments were scored with 'Quantity one' software. Presence of a band was scored as '1' and the absence of the corresponding band represented as '0'. POPGENE (version 1.32) software was used for genetic analysis. Nei's genetic distances and UPGMA algorithm were used to generate the dendrogram.

Results and Discussion

Altogether 225 bands were yielded and number of bands per primer varied from 6 to 13. The average number of bands per primer was 10.71. Primers OPH 8, OPA 7, OPB 15 and OPY 2 produced maximum number of bands (13) while least number of bands was produced by OPC 15. The percentage of polymorphic loci was 86.67 revealing that the primers used in the present study were able to detect the variations

of the selected plants. Polymorphism in RAPD in mutated population by chemical mutagen could be attributed to change in a nucleotide base which alters the binding site of a primer. EMS used in the present study is an alkylating agent and known to increase point mutation basically GC-AT transmission rather than choromosomal changes (Kurowska *et a*l., 2011; Jankowicz-Cieslak *et al*., 2012) when compared to physical mutagens.

The high level of polymorphism observed in this study was mainly due to the variations of sample number 1, 10 and 16 (Figure 1). The percentage of polymorphism observed in this study was higher than some other RAPD studies conducted with banana. Kundu *et al*. (2018) reported 73.91% polymorphism in different banana genotypes while Sheidai *et al*. (2008) reported 51.4% in tissue cultured banana. Comparatively higher degrees of polymorphisms have also been reported. In the RAPD analysis conducted by Poerba and Ahmad (2010) for cooking banana reported 96.82% polymorphism whereas in induced mutants of Dwarf Cavendish for salinity resistance the polymorphism observed was 92.4% (Miri *et al*., 2009).

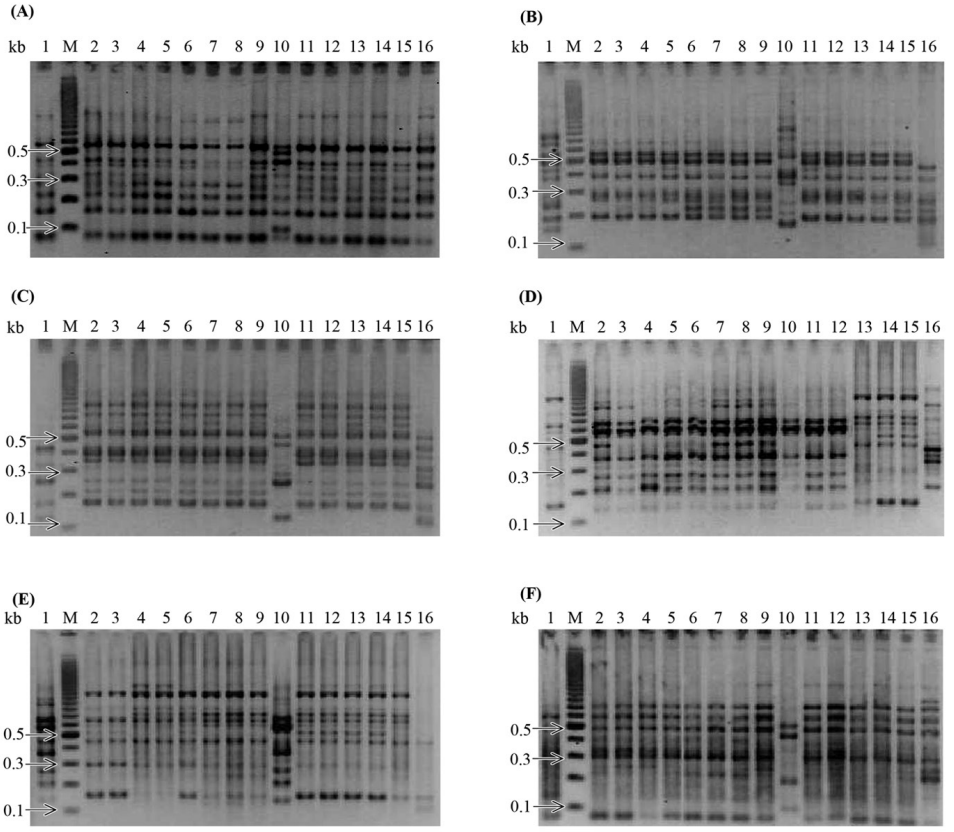

Figure 1. Random Amplified Polymorphic DNA profiles of 16 samples using primer OPA 7 (A), OPA 9 (B), OPH 4 (C), OPC 10 (D), OPH 8 (E) and OPB 17 (F). Lane: 1 (morphologically different plant), M (100bp ladder), 3,4,5,6,7,8,9,11,12,13,14,15 selected treated plants with ability to withstand to different screening techniques, (2) untreated mother plant, (10) survived plant in sick plot (16) *Foc* resistant Ambul variety *Nadee*

Out of 21 primers tested OPH 8, OPA 7, OPH 4, OPA 9, OPB 17 (Figure 1), OPX 4, OPX 7, OPB 18 and UBC 620 produced very distinct banding with sample number 10 compared to untreated Agra. OPH 8, OPA 9, OPC 10 and UBC 620 were the primers which showed significantly different banding pattern to identify the sample number 1.

This suggested that RAPD can detect intra-varietal differences. Present results are also supported by Kishor *et al*. (2017) who have effectively used RAPD markers to evaluate morphologically different putative mutants of banana. Martin *et al*. (2006) and Sheidai *et al*. (2008) have also proved that RAPD can effectively be used to identify tissue culture variants.

There were 4 major clusters in the dendrogram generated through Unweighted Pair Group Method with Arithmetic mean (UPGMA). Plant number 10, 16, and 1 separated in to 3 single clusters while the other samples were clustered into one group. Within this cluster four sub clusters were observed (Figure 2). The genetic distance values for 16 samples ranged from 0.036 to 0.743 (Table 3). The highest genetic distance (0.743) was observed between sample 6 (survived plant after *in-vitro* screening using fusaric acid) and 10 (plant selected from sick plot screening) followed by 1 (Morphological variant) and 10.

Plant number 10 was much deviated from the mother plant (Figure 2) showing a distance of 0.689 between them. Therefore, it could be assumed that many alterations at DNA level have been occurred due to mutation induction. Interestingly, plant number 10 drastically deviated from the resistant check (16) with a genetic distance of 0.689 (Table 3). According to the results it is clear that new genotype which may be resistant/less susceptible has been created by mutation induction. However, due to high genetic distance or the high dissimilarity with the control, the agronomic traits, yield and the quality of the fruits might have altered due to mutations. However, the morphological variant (sample No.1) exhibited comparatively low genetic distance (0.359) from the untreated control (sample No.2).

The genotypes which are closer to the mother plant in the dendrogram may possess favorable agronomic traits. However, except the plant no. 10 (survived plant in sick plot) all other plants which were selected from different screening methods were infected with fusarium wilt in subsequent screening in sick plot.

Confirmation of identified genotypes

In varietal improvement, the stability of the variations/mutations over the generations is an important issue. Therefore, for confirmation, plant number 1 (morphological variant, but susceptible to fusarium wilt) and plant number 10 along with untreated Agra were confirmed for their genetic stability with 10 RAPD primers.

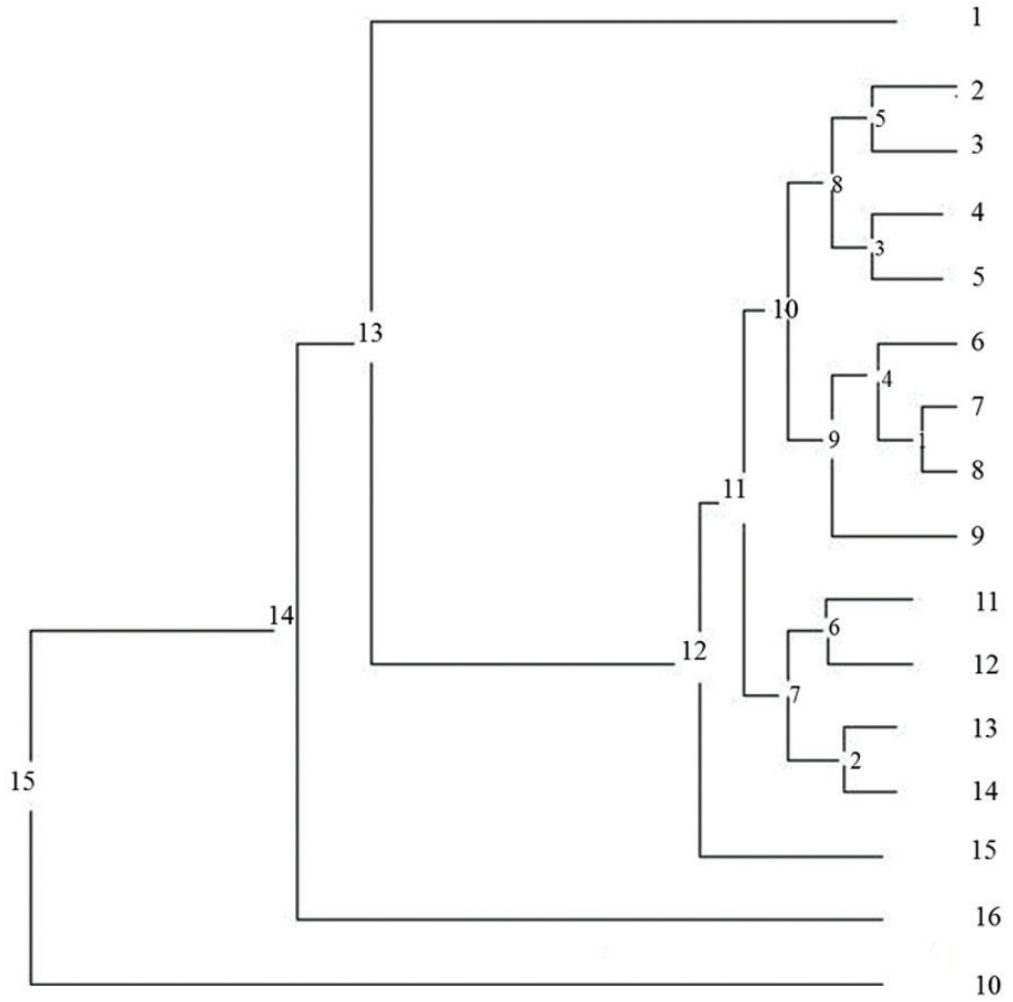

Figure 2. UPGMA derived dendrogram based on RAPD analysis by 21 primers for 16 individuals. Code numbers are as those listed in Table 1.

Table 3. Genetic distance index of 16 selected individuals based on Nei's genetic distance values

Pop ID	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1	***															
2	0.359	***														
3	0.398	0.074	***													
4	0.419	0.118	0.098	***												
5	0.379	0.069	0.088	0.055	***											
6	0.426	0.113	0.123	0.118	0.108	***										
7	0.386	0.103	0.133	0.108	0.088	0.074	***									
8	0.399	0.123	0.153	0.108	0.108	0.064	0.036	***								
9	0.405	0.128	0.128	0.123	0.143	0.118	0.088	0.098	***							
10	0.689	0.689	0.654	0.629	0.645	0.743	0.637	0.689	0.662	***						
11	0.489	0.169	0.138	0.113	0.133	0.138	0.128	0.148	0.103	0.662	***					
12	0.419	0.138	0.128	0.103	0.113	0.159	0.108	0.128	0.103	0.698	0.074	***				
13	0.432	0.128	0.138	0.103	0.103	0.158	0.128	0.138	0.133	0.662	0.103	0.093	***			
14	0.439	0.164	0.143	0.138	0.148	0.164	0.133	0.153	0.108	0.671	0.088	0.088	0.050	***		
15	0.419	0.190	0.179	0.164	0.185	0.148	0.138	0.148	0.153	0.662	0.164	0.143	0.133	0.098	***	
16	0.604	0.453	0.467	0.460	0.460	0.453	0.453	0.482	0.405	0.689	0.419	0.446	0.474	0.453	0.489	***

Sample ID from 1-16 are as listed in the table 1.

The banding pattern in each gel plates (from A to F) in Figure 3 clearly distinguished the identified two genotypes from the untreated control. First and second generation plants of each genotype showed 100% similarity (Figure 4). This suggests that the genotypes identified through the first RAPD analysis are stable over the generations. This study also confirmed that RAPD markers can be successfully used for identifying the variations induced through mutagenic treatment. Performances of the identified genotypes on yield and agronomic traits are being evaluated.

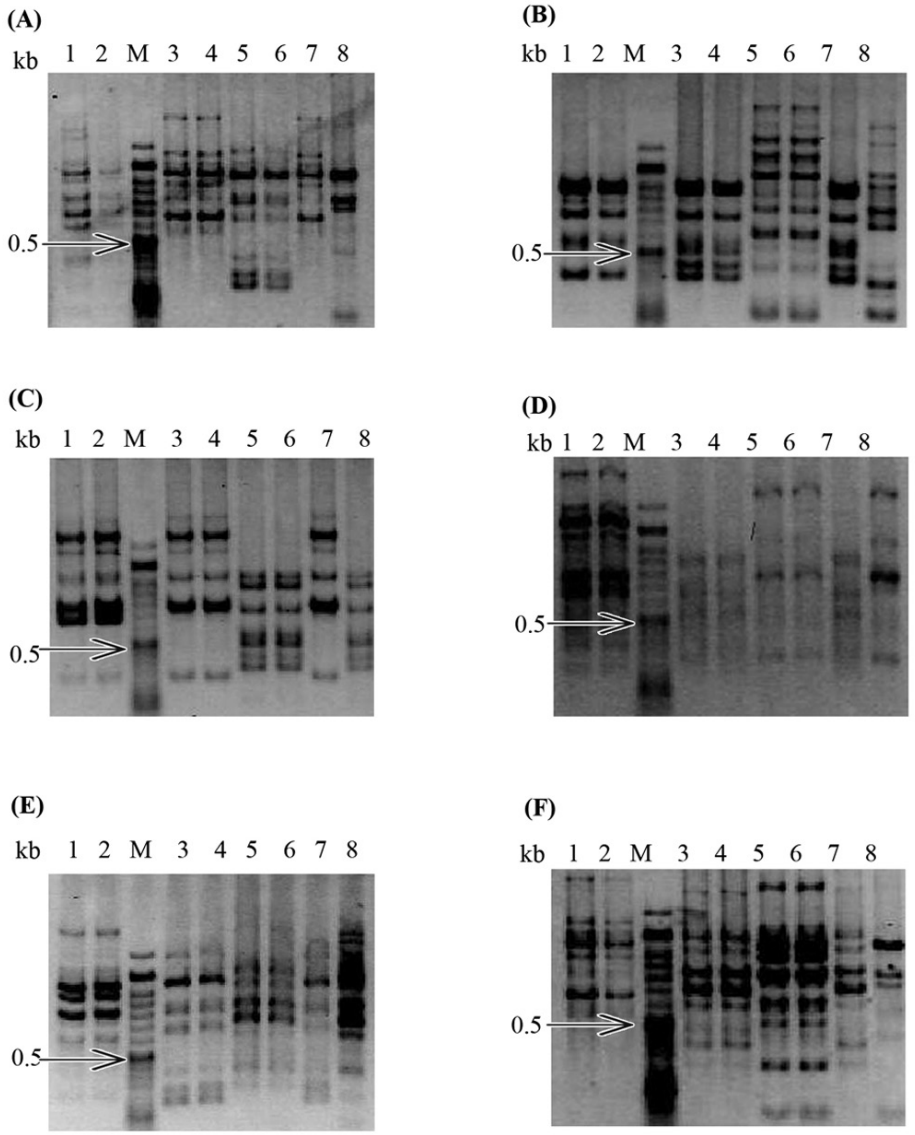

Figure 3. Random Amplified Polymorphic DNA profiles of 08 samples using primer OPA 7 (A), OPA 9 (B), OPH 4 (C), OPX 4 (D), OPB 15 (E) and OPH 8 (F). Lanes: 1 and 2 (morphological variant, 1st and 2nd generation), M. 100bp ladder, 3 and 4 (selected plant through *in-vitro* screening 1st and 2nd generation) 5 and 6 (survived plant in sick plot 1st and 2nd generation) 7 (a new plant selected from sick plot) 8 (untreated Agra: mother plant)

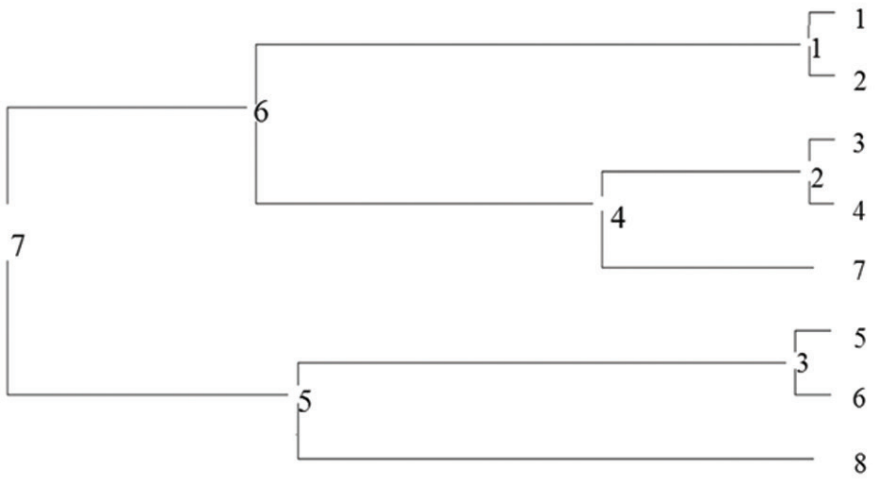

Figure 4. UPGMA derived dendrogram based on RAPD analysis by 10 primers for 8 samples. Explanation: 1st and 2nd generation of morphological variant (No.1), 3 and 4 (selected plant from *in-vitro* screening 1st and 2nd generation) 5 and 6 (survived plant in sick plot 1st and 2nd generation) 7 (a new plant selected from sick plot) 8 (untreated Agra; the susceptible plant)

Conclusion

All the primers used in this study could be used to detect DNA polymorphism in putative mutants. 86.67% of polymorphism was observed in the total of 225 numbers of amplified bands. The morphologically different putative mutant was confirmed as a new genotype. The plant survived in sick plot for 24 months was genetically different from the variety Agra and can be recognized as another genotype. In this research it was shown that RAPD can be used to characterize mutagenic populations to identify the genetic variants.

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