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Study of resistance of *Musa acuminata* to *Fusarium oxysporum* using RAPD markers

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Abstract

Suckers collected from different populations of *Musa acuminata* ssp. *malaccensis* were found to be highly resistant to race 4 of *Fusarium oxysporum* f. sp. *cubense* (FOC) suggesting that local wild banana populations co-evolved with the pathogen. Seedlings from these wild banana plants segregated for resistance to the pathogen. The infected seedlings were characterized based on external and internal symptoms and the variable response to FOC was mainly due to the genetic factors. Using the technique of random amplified polymorphic DNA (RAPD), 96 major amplification products from 15 primers were identified. Only 10 out of 96 markers were monomorphic and shared among the seed progenies, whereas the remaining 86 were highly polymorphic. Three primers showed banding patterns specific to resistant or susceptible seedlings. These results showed the great potential of the wild *Musa acuminata* ssp. *malaccensis* as a source for banana improvement and also for the synthesis of segregating populations for linkage mapping, gene cloning and DNA markers related to FOC resistance.

Additional key words: DNA markers, *Fusarium* wilt, molecular breeding, zygotic embryo culture.

Introduction

Cultivated bananas have narrow genetic origin and the variability present is mainly due to somatic mutations. Banana monoculture cultivation proved a powerful driving force in disease epidemics. This has been observed in case of cv. Gros Michel resulted in the collapse of banana industry due to epidemic caused by *Fusarium oxysporum* f. sp. *cubense* race 1. Gros Michel was replaced with resistant cv. Cavendish clones for large-scale commercial production. However recent outbreak of a new FOC race 4, attacking Cavendish bananas mainly in subtropics and tropics in general caused a serious threat to banana industry. There is no chemical control available and the only way is to plant cultivars resistant/tolerant to the pathogen (Buddenhagen 1990).

As most of the cultivars are susceptible to race 4 (Pegg *et al.* 1996), it makes imperative to look for resistance in the wild banana populations. Furthermore, the breeding of most commercially acceptable banana is made difficult due to genetic sterility and triploidy (Simmonds 1962), so producing hardly any seeds. In addition, it is further complicated by low seed germination of hybrid banana plants (Shepherd 1987). The paucity of the knowledge of the genetic mechanism of the *Fusarium* wilt resistance in bananas further hampered the banana improvement.

Wild bananas could offer the possible sources of resistance to *Fusarium* wilt for the understanding of resistance mechanism. However no information is available about the resistance of wild bananas sources to FOC race 4.

Restriction fragment length polymorphism (RFLP) has been used to study the taxonomy and phylogeny of *Musa* species and cultivars (Gawel and Jarret 1991, Bhat and Jarret 1996). However, it appears that RFLP analysis detects relatively low level of polymorphism between closely related materials. Therefore efforts have been concentrated on the application of the polymerase chain reaction (PCR) for *Musa* genome analysis (Kaemmer *et al.* 1997). RAPD markers are PCR based provide a

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Abbreviations: CTAB - cetyltrimethylammonium bromide; FOC - *Fusarium oxysporum* f. sp. *cubense*; LSI - leaf symptom index; PDA - potato dextrose agar; RAPD - random amplified polymorphic DNA; RDI - rhizome discoloration index.
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convenient and rapid assessment of the differences in the genetic composition of the related individuals (Welsh and McClelland 1990, Williams *et al.* 1990).

These studies were started to characterize the Malaysian wild *Musa acuminata* ssp. *malaccensis* populations for *Fusarium* wilt resistance to race 4. RAPD markers were further used to assess genetic variability among the susceptible and resistant seedlings.

Materials and methods

Plants and fruit bunches were collected from different diploid wild *Musa acuminata* ssp. *malaccensis* (Asif *et al.* 2001a). Ten to fifteen suckers were collected for each of the samples RI, IPTJ, BD1, BD2, BC1 and Kra. These suckers were planted in the field heavily infested with FOC race 4 and infested corm tissues were also placed in each planting hole to assure no disease escape. *Musa acuminata* ssp. *malaccensis* and Novaria (AAA) suckers were 4 - 5 months old at planting. Novaria, a Cavendish banana (AAA), highly susceptible to FOC race 4 was used as susceptible check. All suckers were planted in rows with a distance of 3 × 4 m. Novaria plants were randomly planted among the tested wild banana suckers. Plants were regularly monitored for disease symptoms for a period of two years from 1996 to 1998.

Due to poor seed germination (2 - 3 %) seedlings were raised through *in vitro* zygotic embryo culture (Asif *et al.* 2001b). FOC race 4 was freshly isolated from the infected vascular tissues of Novaria (AAA) and cultured on potato dextrose agar (PDA). Cultures were maintained at 28 ± 2 °C and 16-h photoperiod (with irradiance of 37.5 μmol m^{-2} s^{-1}).

Fusarium oxysporum f. sp. *cubense* race 4 inoculum was prepared in Armstrongs' liquid medium containing, 20 g dm^{-3} sucrose, 0.4 g dm^{-3} $MgSO_4.7\,H_2O$, 1.6 g dm^{-3} KCl, 1.1 g dm^{-3} KH_2PO_4, 5.9 g dm^{-3} Ca $(NO_3)_2$, 0.0002 g dm^{-3} $FeCl_3$, 0.0002 g dm^{-3} $MnSO_4$, and 0.0002 g dm^{-3} $ZnSO_4$ (Singleton *et al.* 1992). Small pieces of PDA with a few FOC mycelia were inoculated into Armstrongs' liquid medium. Cultures were incubated at room temperature and shaken twice a day for 7 d.

M. acuminata ssp. *malaccensis* seedlings were screened following a 'double tray' method (Mohamed *et al.* 1999). Each container was planted with 20 clonal seedlings. Five Novaria (AAA) tissue cultured plantlets were randomly planted in each tray as susceptible control. Seedlings were acclimatized under shade and high humidity for 4 - 5 weeks. Plantlets were uprooted and their roots were washed with sterile water. Roots were soaked in FOC spore suspension with a concentration of 10^6 spore cm^{-3} for 3 h. The controls were immersed in sterile distilled water for the same time duration. Inoculated plants were monitored for up to 40 to 50 d. Plants were watered daily and a commercial fertilizer preparation (*GROFAS*, *ICI*, UK) was used once a week.

The disease symptom expression was based on the leaf symptom index (LSI) and rhizome discoloration index (RDI) following (Brake *et al.* 1995). Symptoms were recorded after first two weeks and again after four weeks. Final evaluation for internal symptoms was made on 5th week.

DNA extraction: Leaf samples were collected from five resistant and susceptible seed progenies. For susceptible seedlings died during the screening process, leaf samples were collected from the clonal seed progenies maintained *in vitro*. DNA was extracted using a modified CTAB method based on Doyle and Doyle (1987). Fresh leaf tissues (2 - 3 g) were ground to powder and transferred to the extraction buffer [2 g dm^{-3} CTAB (cetyltrimethyl-ammonium bromide), 1.4 M NaCl, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0)] and 0.2 % (v/v) β-mercapto-

Table 1. Nucleotide sequences of 28 arbitrary decamer primers used for the initial screening of *Musa acuminata* ssp. *malaccensis* seedlings.

Primer code	5′ Sequence 3′	G + C [%]
OPA-01	CAGGCCCTTC	70
OPA-02	TGCCGAGCTG	70
OPA-03	AGTCAGCCAC	60
OPA-04	AATCGGGCTG	60
OPA-05	AGGGGTCTTG	60
OPA-06	GGTCCCTGAC	70
OPA-07	GAACCGGGTG	70
OPA-08	GTGACGTAGG	70
OPA-09	GGGTAACGCC	70
OPA-10	GTGATCGCAG	60
OPA-11	CAATCGCCGT	60
OPA-12	TCGGCGATAG	60
OPA-13	CAGCACCCAC	70
OPA-14	TCTGTGCTGG	60
OPA-15	TTCCGAACCC	60
OPA-16	AGCCAGCGAA	60
OPA-17	GACCGCTTGT	60
OPA-18	CGGTGACCGT	70
OPA-19	AAAACGTCGG	50
OPA-20	GTTGCGATCC	60
PRI -21	CGCTGTCCTT	60
PRI -22	GGGAGAGTCA	60
PRI -23	GACGAGTACG	60
PRI -24	GTGCGTATGG	60
PRI -25	GACAGACAGA	50
PRI -26	TCACGTCCAC	60
PRI -27	CTCTCCGCCA	60
PRI -28	GAACGGACTC	60

ethanol. The suspension was extracted twice with equal volume of chloroform : isoamyl alcohol (24:1). Dried pellet was dissolved in an appropriate volume of double distilled sterile water. DNA concentration was determined through spectrophotometer (*Model DU 7500I*, *Beckman*, Coulter, USA).

Polymerase chain reaction (PCR) using RAPD: Thin walled PCR tubes were used with a total reaction mixture of 0.025 cm^3. As a negative control a complete reaction without DNA was used. Primers were bought from *Operon Technologies*, Alameda, USA (RAPD primers Kit A), and from *Life Technologies*, *Gibco BRL*, Gaithersburg, USA, (Primers 21 - 28) were derived from (Howell *et al.* 1994) (Table 1). Each PCR reaction contained 100 ng of template DNA, 10 × PCR buffer with a final concentration of 10 mM, 100 μM dNTP's, 1.5 mM $MgCl_2$, 1 unit of Taq DNA polymerase (*Promega Corporation*, Mabison, USA) and 50 pmol of primer were used. Reaction mixture was overlaid with PCR mineral oil to avoid evaporation. $MgCl_2$ was used at 0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mM for optimization. Reproducibility of RAPD profile was studied where different PCR reactions were run using same DNA sample and primer.

PCR amplifications were performed as described by Williams *et al.* (1990) with modifications by Weising *et al.* (1995) and run in a *Hybaid Omnigene Thermocycler* (*Hybaid*, Middlesex, UK); Hot start; 1 cycle at 94 °C for 4 min (initial denaturing step) followed by 45 cycles consisting of 15 s at 94 °C denaturing, 45 s at 36 °C annealing, 90 s at 72 °C elongation, and final elongation at 72 °C for 4 min. After amplification, the reaction products were stored at 4 °C. PCR products with 0.005 cm^3 of loading buffer were fractionated by electrophoresis using a 0.14 g dm^{-3} agarose gel in TBE (Tris-borate; EDTA, pH 8.0) buffer. Gel was run at 80 V for 2 h and stained with ethidium bromide (68 μg cm^3). Sizes of the amplified products were estimated using 100 bp DNA ladder (*Life Technologies*) and photographed using Polaroid film.

Results and discussions

All Novaria plants succumbed to *Fusarium* wilt in infected field within 4 - 5 months of planting whereas all *Musa acuminata* ssp. *malaccensis* suckers collected from six locations survived for more than 2 years without any disease symptoms. Dissection of the rhizomes also revealed no disease symptoms. These results confirmed the general perception that *M. acuminata* ssp. *malaccensis* is resistant to FOC race 4 and that the local wild banana populations have co-evolved with FOC (Vakili 1965). Natural selection, expressed through prolonged association between pathogen and seed propagated host, probably resulted in the survival of the more resistant progenies.

Different seedling populations showed differential response to FOC race 4 inoculations. The percentage of resistant seedlings varied from 13.3 % to (BD2) to 82.4 % (BD1). Similarly within each sample population the proportion of resistant and susceptible (R:S) plants were also varied. A total of sixty-five RI seedlings were screened and only nineteen survived after two months

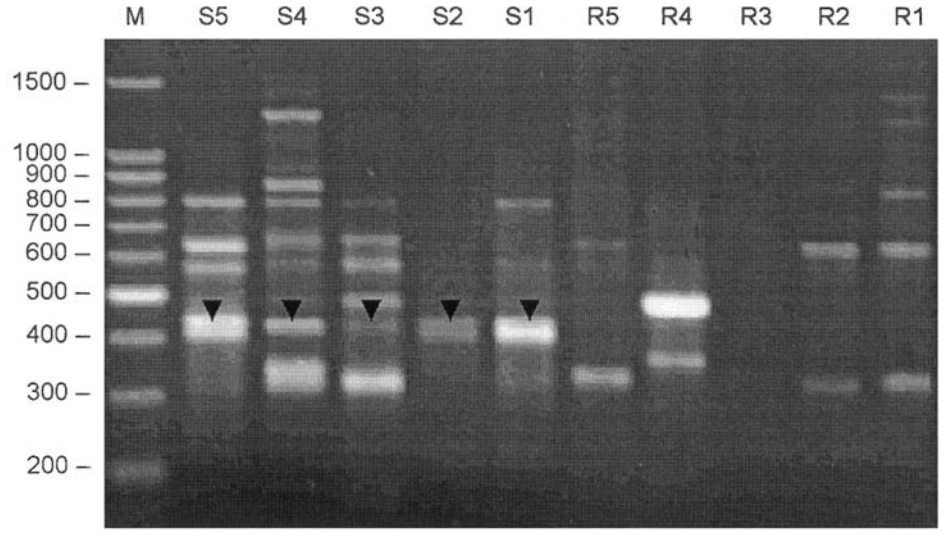

Fig. 1. Result of RAPD with primer OPA-03 (5-AGTCAGCCAC-3). Eight major bands ranging from 200 to 1 500 bp were observed. A major band of 400 bp was observed only in susceptible but absent in resistant seed progenies. M - 100 bp DNA ladder.

while forty-six eventually died, similarly IPTJ, BC1, and BD1 populations were observed with 23, 20 and 56 seedlings respectively survived. A χ^2 - test showed goodness of fit to monohybrid ratios of 3:1 for RI, IPTJ, BC1 and BD1 seed populations. Of these three populations namely RI, IPTJ and BC1 resistance seemed to be controlled by a single recessive factor whereas a single dominant factor conditioning resistance in BD1. However, for BD2 population a dihybrid ratio of 13:3 for genetic inheritance of FOC was observed.

Different genetic ratios observed among five populations suggested that the material used was very diverse genetically. Five populations were originated from different stocks of mother populations obtained from different areas of Selangor and Pahang. Therefore they may be different in their genomic compositions and thus response to FOC. *In vitro* cultured seedlings derived from IPTJ, also found segregating for albinism, a trait controlled by recessive factors (Ortiz and Vuylsteke 1993). These seed progenies seemed to be produced as a result of inbreeding.

Wild bananas are thought to have coevolved with the FOC pathogen, however there is no information available about the variation of the pathogen among the wild banana populations. Seven VCG's or VCG's complexes 0120 - 01215, 0123, 01216, 01217 and 01218 had been isolated from different cultivars of Malaysia (Pegg *et al.* 1996, Ploetz *et al.* 1997) suggesting a wide variation in the pathogen locally. Different forms of *M. acuminata* ssp. *malaccensis* may have coevolved with different VCG's groups. Response of different *M. acuminata* populations with different genetic origin may vary when screen against a particular VCG's group. Discrepancies observed in different ratios among the populations could also be related to the size of the population inoculated as Rowe and Rosales (1993) suggested that resistance to race 4 seemed to be under polygenic control. However the number of loci controlling the resistance is not known thus it is difficult to predict an affective population size for inoculations.

Inoculated plantlets expressed variable degrees of disease symptoms 2 - 3 weeks after inoculations. Seedlings showing clean or less than 5 % yellowing of the older leaves were grouped as resistant compared to susceptible seedlings where more than 5 % or complete yellowing of the older leaves was observed. For internal symptoms seedlings with clean rhizome were considered as resistant while the seedlings with slight to more than 5 % stellar region discolored or with complete blackening observed were characterized as susceptible.

Highly susceptible plants died within 2 weeks. First disease symptoms were observed on the older leaves, progressed to the younger ones. Infected plants showed a characteristic yellowing of the older leaves beginning along the margin of the leaves, subsequently advancing towards the lamina midrib. This is in sharp contrast to the normal dark-green leaf colour of the control plants. A complete blackening of the internal vascular tissues was observed in plants showing severe corm blackening. Frequently, pseudostem split longitudinally just above the soil level. Finally most of the lamina turned brown, buckled, and died. External surface of the corms showed a significant blackening compared to control plants. Corms of all the plants showing different degrees of foliage yellowing were cut longitudinally. Internal symptoms of corm were associated with very pronounced discoloration where stele joins the cortex. Plants having extensive foliage yellowing also showed extensive

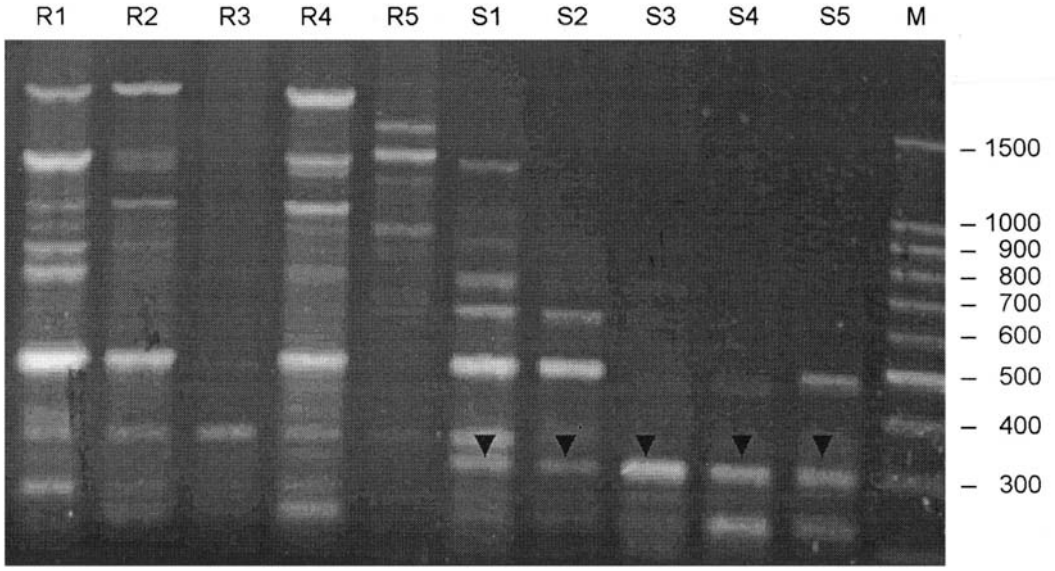

Fig. 2. RAPD profile produced using primer 24 (5-GTGCGTATGG-3). This primer produced 9 major bands with very high polymorphism. A major band of 300 bp was observed in susceptible seed progenies. M - 100 bp DNA ladder.

blackening of the corms and those showed slight or no foliar symptoms showed slight or no blackening of the corm. In addition roots of the susceptible plantlets showed marked blackening compared to the control plants, which appeared white and healthy. Some of the plants did not show external symptoms but they were observed for different degrees of internal symptoms. This was also observed in case of Pisang mas (Tang and Hwang 1999). Hence external morphological symptoms expression might not be a reliable selection criterion to estimate the degree of resistance compared to internal corm symptoms, which offered the most reliable disease symptom expression (Orjeda *et al.* 1999).

The pathogen was successfully isolated from both susceptible and resistant plants. The isolation of the pathogen from resistant plants suggested that the pathogen may have been taken up into the vascular system but further development was restricted due to the induction of resistance mechanisms. Beckman *et al.* (1961) observed no evidence of growth inhibition of FOC in resistant and susceptible bananas. They defined the resistance mechanism depends on the initial trapping of the spores at perforation plates or vessel endings and on extensive host responses permanently seal off the vascular system, providing a physical defense in depth. Finally, the resistance results from the physical occlusion of the plant vascular system by induced gels and tyloses, which prevent distribution of FOC spores within the host. The difference in response between the resistant and susceptible type interactions may be governed by some factors that become immediately operational when cells of the parasite come in contact with the cells of the host, presumably involving a difference in recognition. The response of the resistant host was extremely rapid (less than 1 h) suggesting that some mechanism *e.g*. a proton or cation pump was activated that altered the physical state of the cytoplasmic proteins (Gabriel *et al*. 1988, Beckman 1990).

Stover and Buddenhagen (1986) questioned the durability of resistance shown by small plants inoculated in flats when transferred to an infected field. Therefore *M. acuminata* ssp. *malaccensis* seedlings found resistant were screened in the infected field for further evaluations. The resistance was still durable after one year in the field.

Screening of banana cultivars in *Fusarium* infested soil has been found to be useful in selecting tolerant plants (Hwang and Ko 1987, Ho 1999). However the disease expression was generally observed in plants 4- to 5-month-old. Similarly there were problems related to quarantine to avoid disease spread, field escape due to uneven pathogen distribution and the influence of environmental and soil variables. Identification of molecular markers linked to disease resistance is an excellent alternative to allow *Musa* breeders to make marker assisted selection for disease resistance without challenging the host with the pathogen.

Initially 28 RAPD primers (Table 1) were evaluated. Only 15 (57.69 %) out of these 28 arbitrary 10-mers' primers screened produced polymorphic DNA amplifications products. Hence 15 primers were selected for the screening of the resistant and susceptible seed progenies. As PCR products were reported, to be affected by $MgCl_2$ concentrations (Damasco *et al.* 1996), the effects of different $MgCl_2$ concentrations ranging from 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mM were studied to ascertain the appropriate concentration. Amplification products were only observed at 1.5, 2.0 and 2.5 mM of $MgCl_2$. Good amplification was obtained with 1.5 mM of $MgCl_2$ hence it was used for all reactions. The different RAPD products produced were highly reproducible under the standard conditions. A total of 96 major scorable amplicons (bands) were produced from the fifteen

Table 2. Nucleotide sequences of fifteen selected arbitrary decamer primers, total number of fragments produced, their size and polymorphic markers observed among the seedlings.

Primer code	5´ Sequence 3´	Fragment [bp]	Total	Monomorphic	Polymorphic
OPA-01	CAGGCCCTTC	500-1000	8	2	6
OPA-03	AGTCAGCCAC	200-1450	8	0	8
OPA-07	GAACCGGGTG	200-1500	8	0	8
OPA-09	GGGTAACGCC	250-1500	4	0	4
OPA-10	GTGATCGCAG	200-1500	7	0	7
OPA-11	CAATCGCCGT	400-1500	5	0	5
OPA-16	AGCCAGCGAA	200- 900	6	1	5
PRI -21	CGCTGTCCTT	250-1000	8	1	7
PRI -22	GGGAGAGTCA	350-1000	7	0	7
PRI -23	GACGAGTACG	400-1000	6	2	4
PRI -24	GTGCGTATGG	200-1000	9	0	9
PRI -25	GACAGACAGA	250-1500	4	0	4
PRI -26	TCACGTCCAC	200- 800	5	1	3
PRI -27	CTCTCCGCCA	250-1500	6	0	6
PRI -28	GAACGGACTC	250-1000	5	2	3

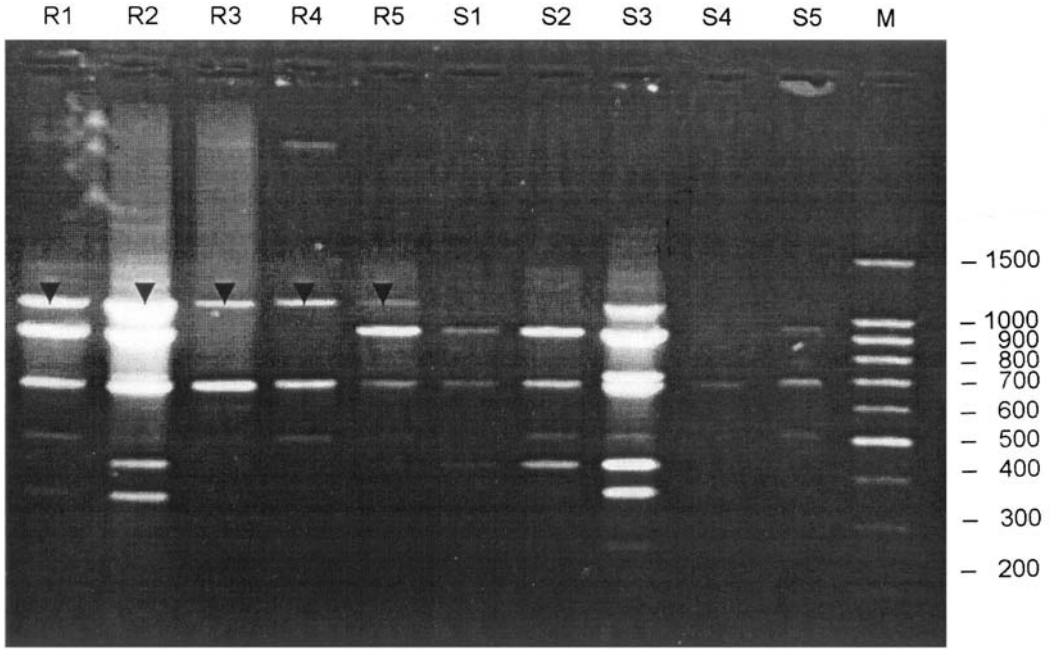

Fig. 3. RAPD profile generated with primer 21 (5-CGCTGTCCTT-3) produced a total of eight major RAPD bands with one monomorphic (700 bp). A mojor band of 1 000 bp was present in all resistant seed progenies. M - 100 bp DNA ladder.

primers used. Only 10 (9.4 %) out of the 96 (90.6 %) markers were monomorphic and shared among the seed progenies whereas the remaining 86 markers were polymorphic (Table 2). The number of scorable RAPD bands generated per primer varied from 4 - 9 with an average of 6 markers per primer. In general, the amplified DNA fragments ranged from 200 to 1500 bp (Table 2).

The fifteen primers used provided a large amount of polymorphism among the resistant and susceptible seed progenies. Only a few of the bands observed to be specifically shared by either of the resistant or susceptible seed progenies. Seed progenies amplified with the primer-03 showed a major band of 400 bp shared by all susceptible seed progenies and was absent in resistant progenies (Fig. 1). A 300 bp band was also consistently observed in most of the susceptible seed progenies and not shared by resistant progenies when primer-24 was used (Fig. 2). While a 1.0 kbp band derived from the primer-21 was observed to be present only in the resistant and absent in the susceptible seed progenies (Fig. 3). Damasco *et al.* (1996) successfully demonstrated the use of RAPD markers and detected a marker linked with dwarfness in Cavendish bananas.

These results demonstrated that RAPD methodology is robust to characterize the seed progenies, however, they did not provide any information about the degree of co-dominance. This could be due to the dominant nature of RAPD markers. Hence, they may be of low utility for the study of segregating populations where information about the heterozygous individuals is important to select parents for hybridization. Kaemmer *et al.* (1997) reported the possible use of sequence tagged microsatellites sites (STMS) markers in *Musa* breeding. They demonstrated STMS are readily applicable to the study of *Musa* genetics and being co-dominant markers can be used for segregating populations.

It was thus concluded that knowledge of the genetics of resistance in bananas to FOC wilt is of great importance for the improvement of the cultivated bananas. The wild seeded bananas could be of great importance in understanding the genetics of resistance (Buddenhagen 1990). *Musa acuminata* ssp. *malaccensis* and their seed progenies found to be resistant and could be useful for breeding segregation populations to understand resistance mechanism. Immunity to Panama wilt was reported to be polygenic (Vakili 1965, Rowe and Rosales 1993) therefore segregating populations could be useful for the development of DNA markers linked to FOC race 4 resistance and gene cloning.

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