

Original Research Article

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Early Screening of Fusarium Wilt and Molecular Analysis of Banana Variants

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ABSTRACT

Banana and plantains is the most important tropical fruit crop in the world, which serves as a major food and source of income in many countries. Bananas are severely infected by several pathogens one such *Fusarium oxysporum* f. sp. *Cubense* which causes significant crop losses in the fields. In this study fusarium wilt screening was carried out on chemically induced mutants and molecular analysis was done to assess the acquired resistance to *Fusarium oxysporum* f. sp. *cubense*. From the early screening we observed that Ethyl Methane Sulphonate derived mutants are more sensitive which ranged from 9.5-11.4%. Whereas, several mutants obtained from Sodium Azide were resistant and was comparable with resistant cv. Poovan. Similar correlation analysis with molecular markers also revealed comparable results with the early screening. RAPD primers (OPN-06 and OPR-07) showed resistance specific bands and were used for the testing variation among mutants, after confirming its reproducibility. Putative resistant and susceptible mutants along with Poovan banana when amplified with OPN 06 showed the presence of a major band at 1500 bp and 200 bp which evidenced the occurrence of random mutations in genome.

Keywords

Mutation, FOC, Screening, Molecular analysis

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Introduction

Bananas and plantains are large monocotyledonous herbs originated from southern parts of Asia (Simmonds, 1962). The evolution of cultivated bananas are the result of two diploid, seeded species of *Musa*, *M. acuminata* Colla and *M. balbisiana* Colla (Kress, 1990) contributed lot for the evolution

of present day cultivars. India is also one of the region for banana diversity development in terms of varieties, landraces are among the most important ecotypes grown since time immemorial for its unique features of quality and its acclimatization to local ecological conditions. One such ecotype is Nanjanagudu Rasabale once a leading cultivar of Mysore province is now under the threat of extinction

due its severe susceptibility to *Fusarium oxysporium* f. sp. *cubense* (Pooja *et al.*, 2013; Khan, 2015).

When susceptible varieties are cultivated in disease infected soils, management of Fusarium wilt becomes very difficult due to its long term surveillance of fungal spores in the soil. Control of this *FOC* under such conditions is restricted to the use of disease-free planting material and clean soils (Ploetz, 2006). Resistant genotypes exist for some applications, but resistance is still needed in other situations. Attempts at developing clones resistant to *Fusarium oxysporum* f. sp. *Cubense* using traditional breeding methods has been relatively weak or even restrained due to narrow genetic variability resulting from the low female fertility (Silva *et al.*, 2001; Pua, 2007; Bidabadi *et al.*, 2012).

Mutation breeding and biotechnological techniques may provide more possibilities for banana improvement. Mutation breeding *in vitro* is a powerful tool for the induction and selection of desirable mutants which can be utilized in banana improvement either for higher yields, good quality and resistance to biotic and abiotic factors (Purseglove, 1998). The mutants so produced facilitate the isolation, identification and cloning of genes used in designing crops with improved yield and quality traits (Ahloowalia and Maluszynski, 2001). Molecular markers have a number of perceived advantages over the morphological markers for the assessment of genetic diversity (Shao *et al.*, 2010). Initially, the DNA based markers like Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), and Amplified Fragment Length Polymorphic DNA (AFLP) were used which provide excellent tools to study the genetic diversity. Later, ISSR and SRAP markers have been shown to be effective for finger printing, genetic diversity analysis and germplasm

evaluation since they can identify many informative loci (Budak *et al.*, 2004; Ruiz and Garcia-Martinez, 2006 and Liu *et al.*, 2008).

In the light of above information, an investigation was carried out to screen the mutants against fusarium wilt and molecular analysis of these putative mutants for possible acquisition of resistance genes.

Materials and Methods

The experiments were conducted at Centre for Horticulture Biotechnology, UHS, Bagalkot, with banana cultivar Nanjanagudu Rasabale. Chemical mutations were induced by Ethyl Methane Sulphonate (EMS-0.30 %, 0.60 %, 0.90 % and 1.20 %) and Sodium Azide (NaN₃-0.01 %, 0.02 % and 0.03 %). The putative mutants obtained after mutagenesis was screened against *Fusarium oxysporum* f. sp. *cubense*, Race 1.

Isolation and maintenance of *Fusarium* pathogen

The plants showing typical symptom *viz.*, older leaves yellowing followed by drooping all along the stem, basal leaf sheath breakage (stem splitting) the vascular discoloration of inner core of the pseudostem and rhizome. These discolorations appeared as dark brownish red at centre with a dark brown margin surrounding the core. These infected vascular bits were surface sterilized with 0.10 per cent mercuric chloride solution for 30 seconds. The bits were washed thoroughly in sterile distilled water for three successive changes and were transferred aseptically to petriplates containing PDA medium.

The pathogenic fungus grown on PDA slants was allowed to grow at 25±1°C for 10 days and such slants were preserved in a refrigerator at 5°C and renewed once in 30 days.

Measurement of cells concentration of fungal conidia

The haemocytometer was used for counting the fungal spores in liquid suspension. It is a special microscope slide with a counting chamber 0.10 mm deep so that volume of liquid over a one sq. mm is 0.10 cubic mm.

Methodology of *Fusarium oxysporum* f. sp. *cubense* Race 1 inoculation

The putative mutants obtained from the experiment one were screened against *Fusarium oxysporum* f. sp. *cubense*, Race 1 using the procedure given by the Musoke, *et al.*, (1999). Screening was carried out using *Fusarium oxysporum* f. sp. *cubense* Race 1 spore population adjusted to 3×10^4 spores/ml using the immersion method. Each plantlets were dipped in the spore suspension and placed on growing medium. Control plantlets were dipped in distilled water.

DNA isolation, PCR amplification and sequencing

About 2 gram leaf samples of DNA extraction were collected from putative mutants of Banana cv. Nanjanagudu Rasabale for extraction of DNA. Leaf samples were also collected from Poovan variety for comparison with the putative mutants against FOC resistance. DNA was extracted from banana variants using young cigar leaf. Standard protocol for the isolation and extraction of DNA by CTAB method was used.

Agarose solution of 0.80 per cent was prepared in 1X TAE buffer for 30 ml [1.5 % agarose solution for RAPD]. It was heated to dissolve completely, cooled to 40°C, and then ethidium bromide solution (0.5 g/ml) was added. It was then poured into the cast and the comb inserted. Nano drop spectrophotometer was used for the quantification of DNA. The

blank was made with TE buffer, 2 µl of DNA sample was taken for the quantification.

The PCR reaction was carried out using Randomly Amplified Polymorphic DNARAPD markers in Master Cycler gradient 533 (Eppendorf, India). The amplified products (after PCR) were stored at 4°C till GEL electrophoresis in a 25 µl reaction volume containing 10X incomplete buffer, 25 mM MgCl₂, 1 mM dNTP's, 0.30 µM primers, 0.50 U of Taq DNA polymerase (Genei, Bangalore) and 20 ng template DNA in Eppendorf master cycler.

Statistical analysis

Analysis of variance for effect of chemical mutagens were analysed in a Completely Randomized Design (CRD). The levels of significance used for F-test was at 1 per cent probability. Critical difference values were given in the table was at 1 per cent level of significance, where the F-test was significant and used to compute the means.

Results and Discussion

Hardened plants obtained after mutagenesis was subjected to early screening for any acquired resistance to *Fusarium oxysporum* f. sp. *cubense* Race 1. A total of 105 plants were screened and revealed varied results which can be differentiated by various external symptoms and molecular analysis. Some of results are expressed as follows.

Per cent plants with external symptoms of FOC

The reaction study of putative mutants screened against *Fusarium oxysporum* f. sp. *cubense* inoculation exhibited several noted symptoms like older leaf yellowing, plantlet wilting and pseudo stem splitting. The combinational effects of external symptoms

were recorded in several treatments. The data pertaining to disease symptoms of the mutants are presented in the Table 1.

Among the mutagenic treatments, maximum yellowing (66.6 %) and wilting (33.33 %) was recorded in untreated control. The maximum pseudo stem splitting (20 %) was recorded in EMS 0.30 % followed by EMS 0.90 % and NaN_3 0.01 % (each 13.33 % plants). The minimum stem splitting (6.66 %) symptoms was observed in untreated control.

Number of plants devoid of external symptoms of *FOC*

The effect of *Fusarium oxysporum* f. sp. *cubense* Race 1 culture on *in vitro* derived mutants was found to be effective in inducing resistant plants among the treatments. The highest number of plants (3) with no external symptoms was obtained from NaN_3 0.01%. The treatments EMS at 0.30 %, 0.90 %, NaN_3 at 0.02% and NaN_3 at 0.03% have also recorded minimum number of plants (1) with no external symptoms of *FOC* Race 1.

External symptoms exhibited by the plants included leaf yellowing, wilting and pseudostem splitting were recorded. The effects of *Fusarium oxysporum* f. sp. *cubense* Race 1 culture on various chemical mutants showed varied symptoms. Our results are comparable with the results of Chen *et al.*, (2013) statements, Brazil banana plantlets began to show external symptoms of fusarium wilt within 10–14 days of inoculation with *FOC* race 4, but Zhongshan Dajiao plantlets were resistant to *FOC* race 4 and did not show any symptoms. In Brazil banana plantlets, the first symptom was yellowing of lower leaves, which began along the margin and advanced toward the midribs. Subsequently, brown spots appeared on the leaves, followed by browning of petioles and buckling, and these symptoms developed from older to younger

leaves. The injured roots of the *in vitro* derived plantlets dipped in *FOC* spore suspension showed symptoms of fusarium wilt within fifth day of incubation. The leaf veins showed chlorosis gradually entire lamina turned yellow and resulted in the wilting of the leaves as reported by Krishna *et al.*, (2013). The partially resistant plantlets exhibiting the symptoms were recorded. Among the mutants maximum external symptoms are exhibited by control plantlets which showed maximum susceptibility to *FOC*. This is because of a differential response of the genomic composition of the cultivar to *Fusarium oxysporum* f. sp. *cubense* Race 1 culture.

Analysis by using RAPD markers

It was difficult to distinguish these mutants using traditional morphological or phenotypic differences. Random amplified polymorphic DNA markers were used to detect the variation among the resistant and susceptible mutants. OPN, OPJ, OPS, OPT, OPA and OPR series primers were used to determine genetic variation between the various phenotypic mutants, resistant and susceptible mutants along with mother plant.

For molecular analysis 10 Operon primers were used. Among these 09 primers showed amplification and 03 primers amplified unambiguous, readable and showed polymorphic bands. A total of 631 amplification products were produced from the selected 09 primers and the number of bands varied from 1-11 with an average of 7.7 bands per primer. The bands which are more than 100 kb are selected for analysis. The details are presented in Table 2. Each and every individual could be identified using gel profiles. A polymorphism was found among the various phenotypic mutants and mother plant, resistant and susceptible plant indicating there was a high molecular variability among the mutants.

Plate.1 Response of putative banana mutants to *Fusarium oxysporum* f. sp. *cubense* Race 1

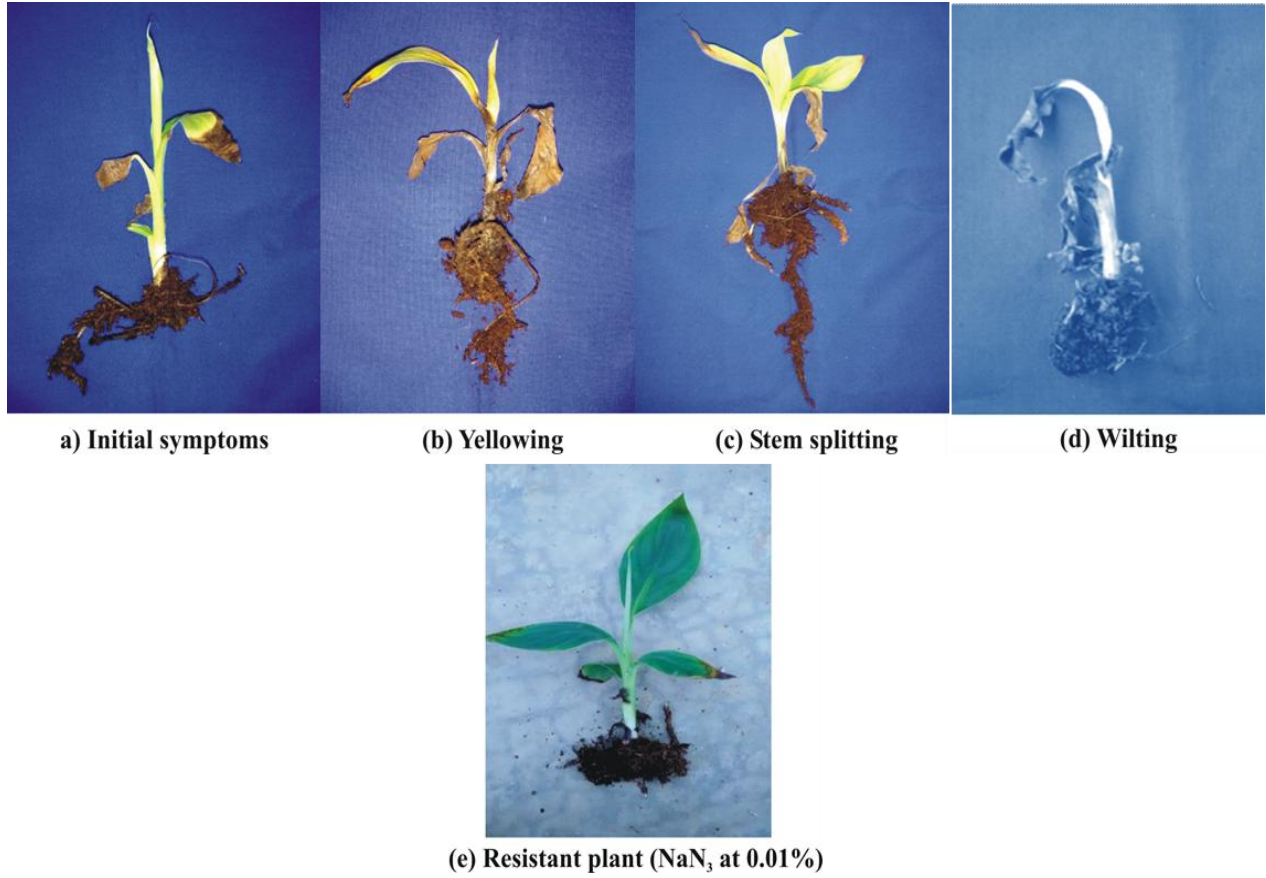


Plate.2 RAPD profile of Poovan, FOC resistant and susceptible mutants obtained with OPN 06

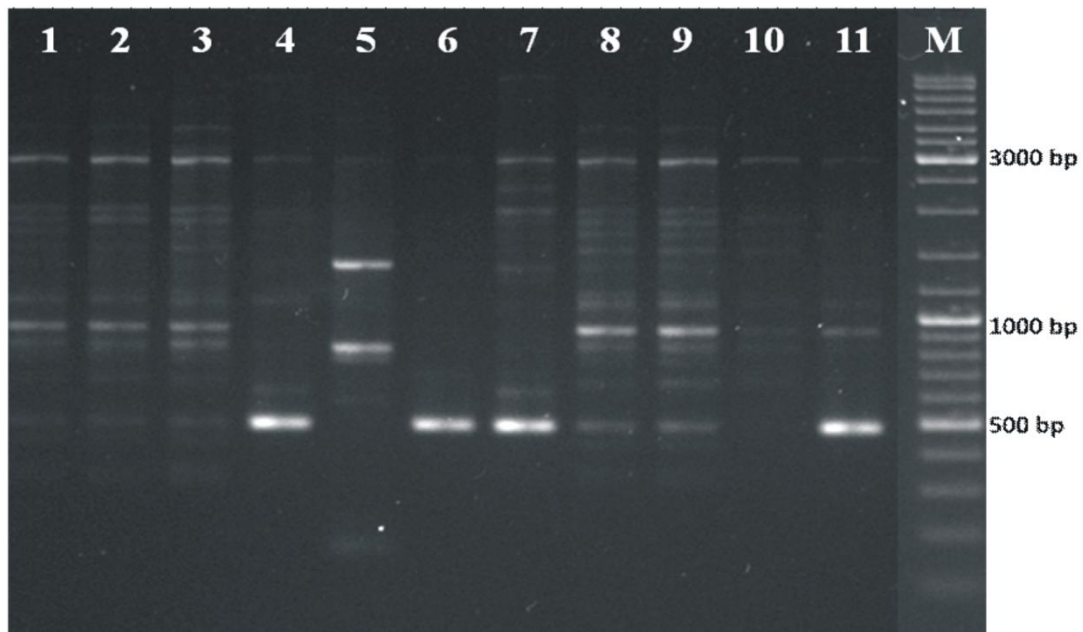
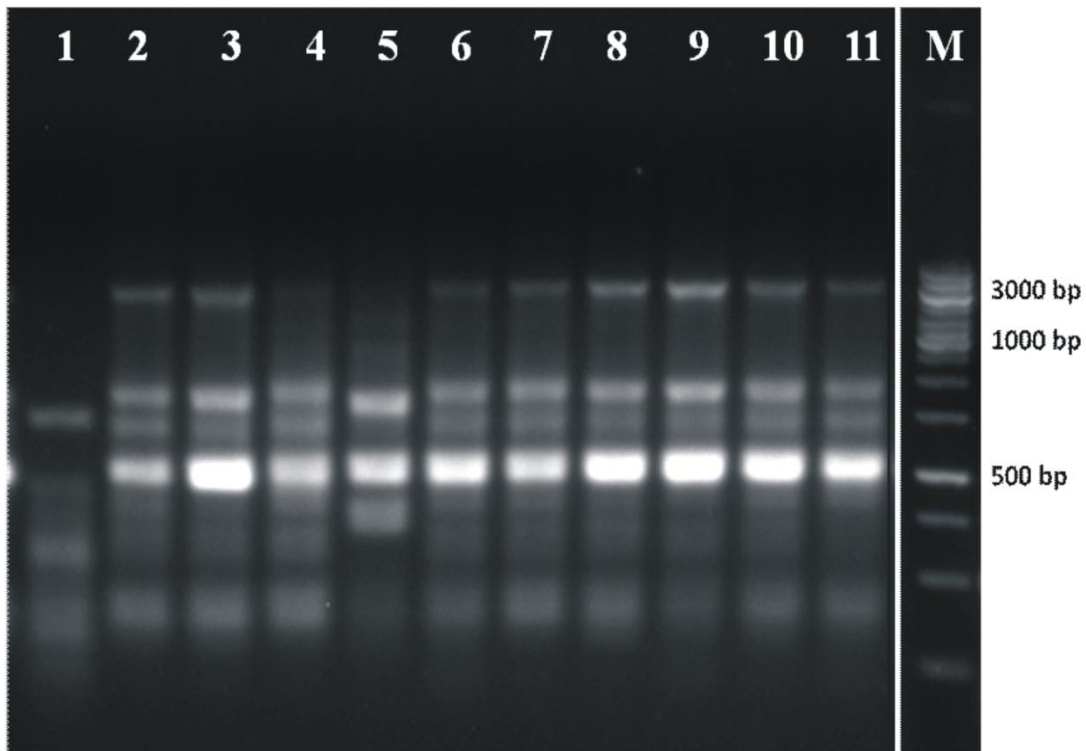


Plate.3 RAPD profile of Poovan, FOC resistant and susceptible mutants obtained with OPR 07



Legends; 1-Poovan, 2,3,4- Resistant mutants of NaN₃ at 0.01 %, 5- Resistant mutants of NaN₃ at 0.02%, 6- Resistant mutant of NaN₃ at 0.03 %, 7- Resistant mutant of EMS at 0.90 %, 8- Susceptible mutant of NaN₃ at 0.01 %, 9- Susceptible mutant of NaN₃ at 0.02%, 10-Susceptible mutant of NaN₃ at 0.03 %, 11-Susceptible mutant of EMS at 0.90 %

Table.1 The response of putative mutants of banana cv. Nanjanagudu Rasabale on artificial inoculation of *Fusarium oxysporum* f. sp. *cubeense* Race 1

Treatments	Per cent plants with external symptoms of <i>FOC</i>			Per cent plants with no external symptoms
	Older leaf yellowing	Plantlet wilting	Pseudo stem splitting	
T ₁ -EMS at 0.30 %	40	33.33	20	1
T ₂ -EMS at 0.60 %	33.33	26.66	0	0
T ₃ -EMS at 0.90 %	46.66	26.66	13.3	1
T ₄ -EMS at 1.20 %	0	0	0	0
T ₅ -NaN ₃ at 0.01%	40	20	13.33	3
T ₆ -NaN ₃ at 0.02%	60	26.66	0	1
T ₇ -NaN ₃ at 0.03%	53.33	20	0	1
T ₈ -Untreated control.	66.66	33.33	6.66	0
Total	49.52	26.66	7.61	7

Table.2 Analysis of genetic variation using random operon primers

Primers	Nucleotide sequence (3' to 5')	Total bands	Polymorphic bands	Monomorphic bands
OPJ 10	AAGCCCGAGG	100	78	22
OPJ 16	CTGCTTAGGG	67	38	29
OPN 06	GAGACGCACA	121	96	25
OPN 04	AAGCGACCTG	55	37	18
OPN 16	AAGCGACCTG	88	60	28
OPR 07	ACTGGCCTGA	88	70	18
OPR 08	CCCGTTGCCT	101	90	11
	Total	620	469	151

The seven primers used provided a large number of polymorphism among the resistant, susceptible mutants, morphological mutants and mother plant. Only few of the bands observed were specifically shared either by resistant, susceptible mutants, phenotypic mutants and mother plant. Putative resistant and susceptible mutants along with Poovan banana when amplified with OPN 06 showed the presence of a major band at 1500 bp and 200 bp in lane 5 and absence of the band in all other lanes shows the polymorphism of the mutant lines. With OPR 07 absence of a major band at 600 bp and presence of major band at 400 bp in lane 5 was clearly indicating the changes under molecular level. These bands may be associated with the resistant character of putative mutants (Plate 2&3).

Molecular analysis

Observing to the banding pattern of RAPD primer OPN-06, it was clear that 1500 bp and 200 bp band size was found to be resistance specific since it was absent in mother plant and susceptible individuals and other resistant mutants. Observing to the banding pattern of another RAPD primer OPR-07, it was clear that absence of a major band at 600 bp and presence of band at 400 bp in lane 5 is clearly indicating the changes in molecular level was found be resistance specific. Javed *et al.*,

(2004) reported that 1.0 k bp band derived from the primer-21 was observed to be present only in the resistant and absent in the susceptible seed progenies resistant of *Musa acuminata* to *Fusarium oxysporum* using RAPD markers. Damasco *et al.*, (1996) successfully demonstrated the use of RAPD markers and detected a marker linked with dwarfness in Cavendish bananas.

External symptoms exhibited by the plants included leaf yellowing, wilting and pseudostem splitting were recorded. The effect of *Fusarium oxysporum* f. sp. *cubense* culture on various chemical mutants showed varied symptoms. Our results are comparable with the results of Chen *et al.*, (2013) statements, Brazil banana plantlets began to show external symptoms of fusarium wilt within 10–14 days of inoculation with FOC race 4, but Zhongshan Dajiao plantlets were resistant to FOC race 4 and did not show any symptoms. In Brazil banana plantlets, the first symptom was yellowing of lower leaves, which began along the margin and advanced toward the midribs. Subsequently, brown spots appeared on the leaves, followed by browning of petioles and buckling, and these symptoms developed from older to younger leaves. The injured roots of the *in vitro* derived plantlets dipped in FOC spore suspension showed symptoms of fusarium wilt within fifth day of incubation. The leaf

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