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RFLP analysis of rDNA-ITS regions of native non-pathogenic *Fusarium oxysporum* isolates and their field evaluation for the suppression of Fusarium wilt disease of banana

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Abstract. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* is the most devastating disease of banana affecting commercial cultivars grown worldwide. An attempt has been made to identify antagonistic, non-pathogenic *Fusarium oxysporum* (np*Fo*) isolates from banana soil. A total of 200 rhizosphere soil samples were collected from different commercial cultivars, as well as wild bananas. Forty Fusarium isolates were recovered, of which 33 were identified as *Fo* based on mycelial and spore characters. The identity of *Fo* isolates was confirmed by specific primers FOF1 and FOR1 in PCR reactions. The 33 isolates were confirmed as non-pathogenic following inoculation studies. Molecular characterisation of the np*Fo* isolates was determined by ribosomal internally transcribed spacer (ITS) restriction fragment length polymorphism. Pathogenic isolates were also included for comparison. Among the np*Fo* isolates, 14 ITS haplotypes were observed of which groups 6 and 10 were major groups consisting of 6 and 5 np*Fo* isolates, respectively.

The *in vitro* evaluation of these np*Fo* isolates by dual culture plate and spore germination technique against the Fusarium wilt pathogen revealed the most inhibiting isolates were Ro-3 and Ra-1. Under pot culture studies, these np*Fo* isolates were evaluated by application: (i) at planting; (ii) at planting + 2 months after planting; and (iii) at planting + 2 months after planting + 4 months after planting, in both tissue-cultured as well as sucker-derived plants of the cultivar Rasthali. The Fusarium wilt severity score observed after 6 months demonstrated that the application of Ro-3 three times resulted in the reduction of Fusarium wilt severity by up to 89% as well as increasing the plant growth parameters significantly when compared with the pathogen-inoculated control plants. Field application of these np*Fo* isolates also reduced Fusarium wilt disease by 80%. The results of this study suggest that np*Fo* strain Ro-3 could be used for the management of Fusarium wilt disease of banana to sustain banana production.

Introduction

Fusarium wilt of banana, caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*), is recognised as one of the most widespread and destructive plant diseases in the recorded history of agriculture (Simmonds 1966) and remains a major constraint to banana production worldwide (Ploetz *et al*. 1992). In India, the disease affects almost all the commercial varieties except Robusta, Poovan and Nendran. The preferred Indian varieties such as Rasthali and Virupakshi are threatened with extinction because of this disease (Thangavelu *et al*. 2001). Infected plants generally produce an unsaleable bunch and the disease ultimately destroys the entire plant. Extensive surveys show that the incidence of the disease is as high as 30% in the main crop and up to 80% in the second crop (Thangavelu *et al*. 2001). Although the disease can be managed by using disease-free planting materials (suckers and tissue-cultured plants) in pathogen-free soil, the continuous cultivation of susceptible varieties in the same field has led to the non-availability of healthy suckers. Planting of resistant varieties cannot be implemented because of consumer preference (Viljoen 2002). Other management practices such as soil fumigation (Herbert and Marx 1990), corm injection with carbendazim (Lakshmanan *et al*. 1987), inclusion of paddy or sugarcane in the banana cropping system (Hwang 1985; Su *et al*. 1986), flood-fallowing or applying organic amendments (Stover 1962) are considered to be ineffective or uneconomical (Tu *et al*. 1980; Moore *et al*. 1995). Hence, the use of antagonistic microbes could be an alternative approach for managing Fusarium wilt of banana. Non-pathogenic Fusarium isolates, specifically native and endophytic isolates, are gaining importance among researchers involved in designing management strategies for Fusarium wilt (Larkin *et al*. 1996; Gerlach *et al*. 1999). The use of saprophytic or non-pathogenic isolates of Fusarium for biological control of pathogenic *Fusarium* spp. in various crops has been extensively studied and applied (Schneider 1984; Mandeel and Baker 1991; Yamaguchi *et al*. 1992; Larkin *et al*. 1996). Alabouvette *et al*. (1993) isolated an effective non-pathogenic Fusarium isolate from a soil naturally suppressive to Fusarium wilt of tomato and melon, and the same strain also reduces the incidence of Fusarium wilt of carnation (Lemanceau *et al*. 1992). In the case of banana, Nel *et al*. (2006*b*) reported that applying non-pathogenic Fusarium isolated from a suppressive soil in South Africa reduced the incidence of Fusarium wilt by 87% after 7 weeks of treatment under glasshouse conditions. Similarly, Forsyth *et al*. (2006) found that soil treatment with an endophytic non-pathogenic Fusarium isolate resulted in a significant reduction in

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expression of wilt symptoms in glasshouse trials. However, no other reports document the identification of an effective isolate of native non-pathogenic Fusarium against Fusarium wilt disease of banana. In this study, Indian non-pathogenic Fusarium isolates were tested for optimal time of application, and field evaluated. It would be useful to find a technique to discriminate the effective biological control isolates of non-pathogenic Fusarium isolates from other isolates, as there is a wide discrepancy in effectiveness among individual non-pathogenic isolates of *F. oxysporum* (np*Fo*). Recently, molecular techniques, specifically, PCR-based restriction fragment length polymorphisms (RFLP) of internally transcribed spacer (ITS) or intergenic spacer (IGS) regions have been shown to be suitable for this purpose (Nel *et al.* 2006*a*). The objectives of the present study were: (i) to isolate, identify and characterise non-pathogenic Fusarium isolates from rhizosphere soils of different banana genotypes; (ii) to conduct *in vitro* screening of non-pathogenic Fusarium isolates against Fusarium wilt pathogen; and (iii) to evaluate the ability of non-pathogenic Fusarium isolates to suppress Fusarium wilt disease under glasshouse and field conditions.

Methods

Isolation of fungus

Pathogenic Foc

Foc was isolated from wilt-infected samples (rhizomes/pseudostem) of Rasthali (race 1) and Monthan (race 2) varieties using half-strength potato dextrose agar (PDA) amended with streptomycin (Ainsworth 1971). Cultures derived from single spores were maintained on dried filter paper at 4°C.

Isolation of Fo from banana rhizosphere soil

In a survey conducted in different banana-growing regions of India in 2006, rhizosphere soil of different commercial cultivars was collected and serially diluted to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} after vigorous shaking. One mL from each dilution was plated onto Komada's medium (Komada 1975) and incubated at room temperature (28 ± 2°C) for 3–4 days. Single-spore cultures were then prepared from fungal colonies. Each single-spore isolate was grown on filter paper overlaid on half-strength PDA. The colonised filter paper was lifted from the agar plate, dried and stored under refrigerated conditions until further use.

Identification and confirmation of Fo isolated from rhizosphere soil

The single-spore culture maintained on filter paper was grown on PDA medium and the cultures were incubated at 25°C for 7 days. The fungal cultures were identified morphologically (Nelson *et al.* 1983). Cultures were further subjected to molecular methods for confirmation of the identification.

Pathogenicity testing

To confirm that the *Fo* isolates were not pathogenic to banana plants, a pot culture experiment was performed using tissue-cultured material from cultivars Rasthali and Monthan. Disease-free, 3-month-old tissue-cultured plants were obtained from the tissue culture laboratory of the National Research Centre for Banana, Thiruchirapalli, Tamil Nadu and were planted in mud pots (30 × 30 × 30 cm), filled with 7 kg sterilised potting mix (1 : 1 : 1 ratio of red soil, sand and decomposed farmyard manure). One month after planting, sand maize meal inoculum of putative *Fo* isolates, prepared according to Ricker and Ricker (1936) was applied individually in separate pots around the plants in soil at the rate of 20 g/pot. For comparison, pathogenic *Foc* of races 1 and 2 for Rasthali and Monthan plants, respectively, were included. For each isolate of *Fo*, eight replications were maintained. Pots were fertilised with the recommended dose of organic and inorganic fertilisers and watered regularly. Disease severity was estimated using the method described by Carlier *et al*. (2002).

DNA extraction from fungal isolates

All isolates of *Fo*, as well as an additional six isolates of *Foc* were grown in half-strength potato dextrose (PD) broth in sterile 250-mL conical flasks for 7 days at 25°C. Mycelia were collected on two layers of cheese cloth, washed in sterile distilled water three times and dried using sterile filter paper. DNA extraction and purification was performed as described by Raeder and Broda (1985) and by Nel *et al*. (2006*a*). DNA concentration and quality was determined spectrophotometrically using a DU640 spectrophotometer (Lamda 25, Perkin Elmer, USA) and visualised on agarose gels.

Confirmation of identity of isolates by PCR

For confirmation of the identity of *Fo* isolated from banana rhizosphere soil, PCR was performed using *Fo*-specific primers specific to the ITS region of the rDNA operon of *Fo* (Mishra *et al*. 2003). The primer pair of FOF1 (5′-ACA TAC CAC TTG TTG CCT CG-3′) and FOR1 (5′-CGC CAA TCA ATT TGA GGA ACG-3′) was synthesised by M/S Bangalore Geni, India. PCR reactions were carried out in a 20-µL volume containing PCR buffer (10 mM TRIS-HCl, 1.5 mM $MgCl_2$, 50 mM KCl pH 8.3), 0.2 mM each dNTP, 0.3 µm of each primer FOF1 and FOR1, 1U Taq DNA polymerase and 45 ng of test DNA. Sterile distilled water was added to give a final volume of 20 µL (Mishra *et al*. 2003).

DNA amplifications were performed in a Mastercycler gradient PCR machine (Eppendorf Scientific Inc., Westbury, NY) using an initial denaturation temperature of 94°C for 60 s, followed by 25 cycles of denaturation for 60 s at 94°C, annealing for 30 s at 58°C and elongation for 60 s at 72°C, with a final extension of 7 min at 72°C. The amplified products were separated by electrophoresis on a 1.5% agarose gel in 1 × Tris acetic acid EDTA (pH 8.0) buffer, pre-stained with ethidium bromide and visualised under UV light. A 100-bp ladder was used to determine the size of the PCR products.

Genotypic characterisation of npFo isolates

The ITS1–5.8S–ITS2 region of the rDNA of np*Fo* and pathogenic Fusarium isolates of banana (*Foc*) was amplified using the primers ITS1 and ITS4 (White *et al*. 1990). Each PCR reaction contained 40 ng of template DNA in 3 µL of Tris-EDTA buffer, 0.2 mM each of the four dNTPs, 0.4 µM of the primers, 2 U of Taq polymerase with PCR buffer (10 mM TRIS-HCl, 1.5 mM $MgCl_2$, 50 mM KCl pH 8.3), and autoclaved double-distilled water up to a volume of 50 µL. The reactions

were performed in a Mastercycler gradient PCR machine (Eppendorf, Hamburg, Germany) using an initial denaturation temperature of 95°C for 60 s, followed by 34 cycles of denaturation for 95 s at 60°C, annealing for 30 s at 50°C, and elongation for 1 min 20 s at 72°C, with a final elongation step of 72°C for 10 min (Singh *et al*. 2004). Negative controls (no DNA template) were also included.

Restriction digests of ITS region (RFLP) and electrophoresis

ITS products of each of the Fusarium isolates were digested individually using the restriction endonucleases *Eco*RI, *Hha*I, *Hinf*I, *Taq*I, *Msp*I, and *Hae*III (Bangalore Geni). The restriction enzyme (2 U) was added directly to 10 µL unpurified PCR amplification product, 1× restriction buffer and Millipore water to achieve a reaction volume of 20 µL, and incubated at 37°C for ~12 h. Digested fragments were separated on a pre-stained ethidium bromide gel consisting of 3.5% agarose at 70V for 5 h. Fragments were run against a 100-bp DNA ladder for size estimation. Gels were visualised under UV light.

In vitro *screening of np*Fo *isolates against* Foc

Dual culture plate technique

Screening of different isolates of np*Fo* against *Foc* race 1 was performed by dual culture plate technique using PDA medium. An 8-mm-diameter mycelial disc cut from a 7-day-old culture of *Foc* was placed on one side (1 cm from the edge) of Petri dishes (9-cm-diameter) and the np*Fo* isolates taken from the peripheries of expanding colonies growing on PDA on the opposite side in the Petri dish perpendicular to the *Foc* mycelial disc. Ten replicates of np*Fo* isolate were maintained. The plates were then incubated at room temperature (28 ± 2°C) for 7 days. Percent inhibition of *Foc* growth rate by each isolate of np*Fo* was then determined.

Spore germination assay

Individual isolates of np*Fo* were grown separately in 250-mL conical flasks containing 150 mL of PDA broth for 7 days at 25°C and culture filtrates were collected after filtration through two layers of sterile muslin cloth and a 0.2-µm-size Millipore filter. One mL of spore suspension (4×10^6 spores/mL) of *Foc* was placed into the cavity of a depression slide and 1 mL of culture filtrate of respective np*Fo* isolate was added. The spore suspension of *Foc* in sterile distilled water acted as a control. Spore germination was observed after 48 h of incubation at 25°C (CSFT 1947).

In vivo *evaluation*

Greenhouse evaluation

*Preliminary evaluation of np*Fo *isolates for the suppression of Fusarium wilt disease*

This study was performed in pots using both tissue-cultured as well as sucker-derived banana plants of cv. Rasthali. Three-month-old, disease- and nematode-free tissue-cultured Rasthali plants, obtained from M/S Growmore Biotech, Hosur (Tamil Nadu, India) were uprooted carefully and planted individually in mud pots of 30 × 30 × 30 cm, as described previously. Sand maize meal inoculum (Ricker and Ricker 1936) of the individual np*Fo* isolate, was applied separately to pots, around the plants in the soil at the rate of 20 g/pot. These plants were challenge-inoculated after 10 days by applying sand maize meal inoculum of *Foc* race 1 at a rate of 20 g/pot. Both positive (*Foc*) and negative (water) control plants were also maintained. There were 10 replicates for each isolate. Six months after planting, the tissue-cultured plants were destructively harvested and scored for internal symptoms of Fusarium wilt disease in the corm following the method of Carlier *et al*. (2002). Two np*Fo* isolates *viz*., Ro-3 and Ra-1 that significantly suppressed Fusarium wilt disease were included in further evaluations.

*Further evaluation of np*Fo *isolates for the suppression of Fusarium wilt disease*

Further evaluation was conducted in pots using both tissue-cultured as well as sucker-derived banana plants of cv. Rasthali. Three-month-old, disease- and nematode-free, tissue-cultured Rasthali plants, obtained from M/S Growmore Biotech, were uprooted carefully and planted individually in mud pots as before. Similarly, disease-free suckers of banana cv. Rasthali each weighing 1.5 kg were extracted from disease-free banana plantations and planted in pots as detailed before. Sand maize meal inoculum (Ricker and Ricker 1936) of individual non-pathogenic Fusarium isolates Ro-3 and Ra-1 was applied separately to pots as before at three different time intervals: (i) at planting; (ii) at planting + 2 months after planting; and (iii) at planting + 2 months after planting + 4 months after planting. All plants were challenge-inoculated once, 10 days after application of np*Fo* isolates by applying sand maize meal inoculum of *Foc* race 1 at a rate of 20 g/pot as previously described. Both positive (*Foc*) and negative (water) control plants were also maintained. There were 10 replicates for each np*Fo* isolate. Six months after planting, tissue-cultured and sucker-derived plants were destructively harvested and scored for internal symptoms of Fusarium wilt disease in the corm following the method of Carlier *et al*. (2002). Growth parameters such as height, girth, number of leaves, petiole length and leaf area were also measured at the time of harvest for tissue-cultured plants.

Field evaluation

A field experiment was conducted in 2006, at a research farm of the National Research Centre for Banana, Thiruchirapalli, to evaluate the ability of np*Fo* isolates to manage Fusarium wilt disease in the highly susceptible cv. Rasthali. Uniform-sized (1.5 kg) suckers were collected from disease-free banana fields. The roots and the outer skin of the corm were detached with a knife to remove pathogens and nematodes if present, and planted in the field with a distance of 1.8 m between rows and 1.8 m between plants within a row. One month after planting, the sand maize meal inoculum of np*Fo* isolate *viz.* Ro-3 and Ra-1 were applied 2–3 cm below the soil around the plants at a rate of 20 g/plant and covered with soil. After 10 days, 20 g of *Foc* in sand maize inoculums was applied 15–30 cm away from the plant and 2–3 cm below the soil surface and covered with soil. The application of np*Fo* isolates was repeated 2 and 4 months after planting. Plants treated with only *Foc* and plants not treated with either *Foc* or the np*Fo* served as the controls. A randomised block design was used with 20 replications per treatment. Eight months after planting, the disease severity of Fusarium wilt was recorded using the method of Carlier *et al*. (2002).

Data analysis

The *in vitro* experiments were conducted in a completely randomised design and the *in vivo* experiments were conducted in a randomised block design and all experiments were repeated at least twice. All statistical analysis was performed using GENSTAT 7th edition (Lawes Agricultural Trust, Rothamsted Experimental Station, UK). Data were analysed using ANOVA and if there was a significant difference, means were separated by Fisher's protected least significant difference test at $P < 0.05$.

Results

Isolation, identification and confirmation of Indian Fo

Thirty-three isolates of *Fo* were isolated from 200 different rhizosphere soil samples collected from different diploid and triploid cultivars grown in different parts of India. These isolates were identified based on the cultural and morphological characters as described by Nel *et al*. (2006*a*). The identity of isolates as being *Fo* was confirmed by PCR analysis using *Fo*-specific primers FOF1 and FOR1. The PCR revealed that all isolates of *Fo* were amplified by this primer set with a single amplicon of 340 bp.

Greenhouse studies for confirmation of the non-pathogenic nature of Fo

Greenhouse testing indicated that inoculation with np*Fo* isolates produced neither external nor internal symptoms of wilt disease even after 6 months of planting. Inoculation using *Foc* race 1 and race 2 isolates resulted in the highest rating of internal symptoms and death of plants at 38 days after inoculation (data not shown).

*Genotypic characterisation of np*Fo *isolates*

The universal primer set specific for the amplification of ITS region amplified the ITS region of the np*Fo* isolates and *Foc*. The size of the amplicon of the ITS region was ~580 bp. Restriction fragments smaller than 40 bp were not taken into consideration as they were not clearly resolved by electrophoresis in 3.5% (w/v) agarose gels. Each restriction enzyme produced unique banding patterns. Patterns that displayed fragments with similar sizes were grouped together and a letter was awarded to each specific pattern (Fig. 1). Table 1 shows the different ITS genotypes identified for the endonucleases used in this work. A total of 16 ITS genotypes were observed and these genotypes did not correlate to hosts or site of collection.

All the np*Fo* isolates were grouped into 14 different ITS genotypes and group 6 and group 10 contained 6 and 5 isolates, respectively. Two ITS genotypes were observed for pathogenic *Foc*, and each of race 1 and race 2 isolates were grouped separately (Table 1).

In vitro *evaluation*

The *in vitro* evaluation of 33 np*Fo* isolates indicated that all the isolates significantly inhibited mycelial growth (87.1–49%) of *Foc* race 1. Culture filtrate of np*Fo* also inhibited spore germination of the pathogen (99.5–11.5%) compared with controls. Isolates Ro-3 and Ra-1 recorded the maximum inhibition of mycelial growth (87.1 and 86.9%, respectively) and spore germination of the pathogen (99.5 and 98.4%, respectively) when compared with the control (data not shown).

In vivo *evaluation*

Greenhouse study

Fusarium wilt severity

The preliminary screening test resulted in the selection of two effective isolates of np*Fo*, Ro-3 and Ra-1, which reduced the wilt severity significantly, compared with other np*Fo* isolates (Table 2). Treatment of tissue-cultured and sucker-derived plants with these isolates significantly reduced Fusarium wilt severity (Table 3). The reduction of wilt severity over the pathogen-inoculated control was up to 89.4% in tissue-cultured plants and up to 77.8% in the case of sucker-derived

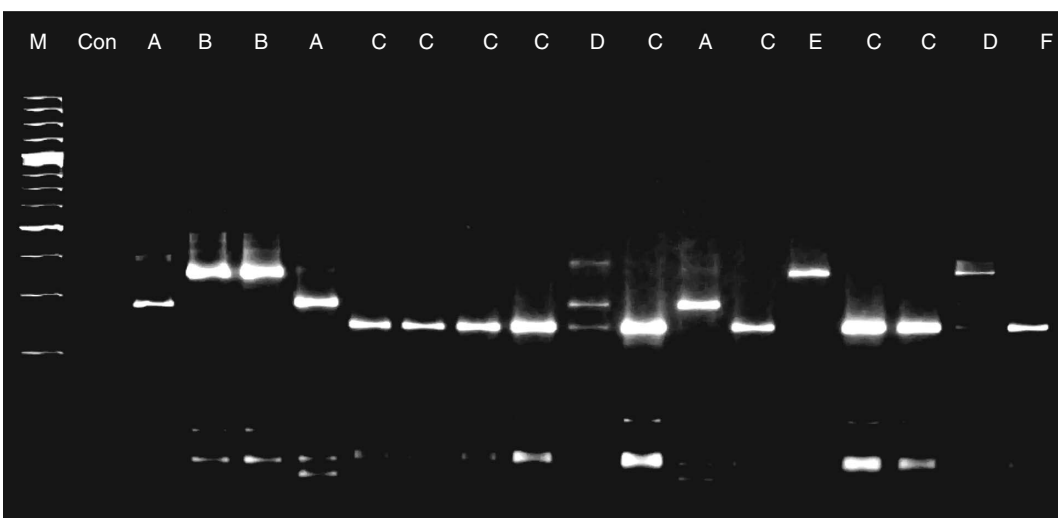

Fig. 1. Restriction fragments of amplified internally transcribed spacer products of various *Fusarium oxysporum* isolates digested with *Hae*III. Isolates of *F. oxysporum* with similar restriction patterns were grouped together and assigned the same letter. Lane M, 100 bp DNA ladder. Restriction fragments were visualised on a 3.5% agarose gel pre-stained with ethidium bromide.

Table 1. rDNA genotype groups obtained by restriction analysis (RFLP) of the PCR-amplified internally transcribed spacer (ITS) rDNA region among the *Fusarium oxysporum* isolates with their source, site of collection in India and pathogenicity status
NRCB, National Research Centre for Banana; TN, Tamil Nadu; AP, Andhra Pradesh

ITS-RFLP group	Isolate	Source	Collection site	Pathogenicity	ITS genotype					
					*Taq*I	*Hae*III	*Eco*RI	*Msp*I	*Hha*I	*Hinf*I
1	Ka-208b	Karpuravalli	Manachanallur, Trichy, TN	Non-pathogenic	A	A	A	A	A	A
2	Ka-208a	Karpuravalli	Manachanallur, Trichy, TN	Non-pathogenic	B	A	A	B	A	A
	Mon-178a	Monthan	Pudupatti, TN	Non-pathogenic	B	A	A	B	A	A
3	Ra-177	Rasthali	Kalvelipatti, Madurai, TN	Non-pathogenic	A	A	A	C	A	A
	Poo-1	Poovan	Trichy, TN	Non-pathogenic	A	A	A	C	A	A
	Poo-2	Poovan	Trichy, TN	Non-pathogenic	A	A	A	C	A	A
4	F-23	FHIA 23	NRCB, Trichy	Non-pathogenic	C	B	B	D	B	B
	Pn-1	Pisang Nanga	NRCB, Trichy	Non-pathogenic	C	B	B	D	B	B
5	F23B	FHIA 23	NRCB, Trichy	Non-pathogenic	C	C	B	E	B	B
	Mo-133c	Mortaman	East Godavari, AP	Non-pathogenic	C	C	B	E	B	B
6	Ka-140	Karpuravalli	Nellore, AP	Non-pathogenic	D	D	A	F	C	C
	Mo-138a	Mortaman	Angalaguthuru, AP	Non-pathogenic	D	D	A	F	C	C
	Mo-138b	Mortaman	Angalaguthuru, AP	Non-pathogenic	D	D	A	F	C	C
	Kb-2	Kanaibansi	NRCB, Trichy, TN	Non-pathogenic	D	D	A	F	C	C
	Mo-2	Mortaman	NRCB, Trichy, TN	Non-pathogenic	D	D	A	F	C	C
	Mon-184c	Monthan	Nilayur, Madurai, TN	Non-pathogenic	D	D	A	F	C	C
7	T-1	Tongat	NRCB, Trichy, TN	Non-pathogenic	E	E	B	G	B	B
	Mor-1	Mortaman	NRCB, Trichy, TN	Non-pathogenic	E	E	B	G	B	B
	Blu-1	Bluggoe	NRCB, Trichy, TN	Non-pathogenic	E	E	B	G	B	B
8	Mat-1	Matti	Valliyur, TN	Non-pathogenic	F	B	B	G	B	B
	T-2	Tongat	NRCB, Trichy	Non-pathogenic	F	B	B	G	B	B
9	Mon-139a	Monthan	Angalaguthuru, AP	Non-pathogenic	D	D	A	G	B	B
10	Ra-2	Rasthali	NRCB, Trichy, TN	Non-pathogenic	C	C	A	C	C	C
	Ra-3	Rasthali	NRCB, Trichy, TN	Non-pathogenic	C	C	A	C	C	C
	Nd-2	Nendran	NRCB, Trichy, TN	Non-pathogenic	C	C	A	C	C	C
	Ka-2	Karpuravalli	NRCB, Trichy, TN	Non-pathogenic	C	C	A	C	C	C
	T-1	Tongat	NRCB, Trichy, TN	Non-pathogenic	C	C	A	C	C	C
11	Pn-2	Pisang Nanga	NRCB, Trichy, TN	Non-pathogenic	B	F	B	E	D	D
12	Ak-1	Anaikomban	NRCB, Trichy, TN	Non-pathogenic	C	D	A	D	C	A
13	Mor-137a	Mortaman	Angalaguthuru, AP	Non-pathogenic	B	F	A	A	E	B
	Kvrc	Karpuravalli	NRCB, Trichy, TN	Non-pathogenic	B	F	A	A	E	B
	Kvs	Karpuravalli	NRCB, Trichy, TN	Non-pathogenic	B	F	A	A	E	B
14	Ro-3	Robusta	NRCB, Trichy, TN	Non-pathogenic	E	E	B	G	B	D
	Ra-1	Rasthali	NRCB, Trichy, TN	Non-pathogenic	E	E	B	G	B	D
15	Ra-180	Rasthali	TN	Pathogenic	A	C	B	B	B	B
	Mon-178	Monthan	TN	Pathogenic	A	C	B	B	B	B
	Ka-51	Karpooravalli	TN	Pathogenic	A	C	B	B	B	B
	Ra-131	Rasthali	AP	Pathogenic	A	C	B	B	B	B
	Ra-132	Rasthali	AP	Pathogenic	A	C	B	B	B	B
16	Mon-184	Monthan	TN	Pathogenic	G	C	B	B	B	B

plants. Among the two np*Fo* isolates tested, Ro-3 recorded the maximum reduction in disease score in both tissue-cultured as well as in sucker-derived plants. Generally, when np*Fo* isolates were applied three times (at planting + 2 months after planting + 4 months after planting) they registered the maximum suppression of Fusarium wilt severity when compared with other treatments. Disease was not recorded in the non-inoculated control plants (Table 3).

Growth promotion

The application of np*Fo* in tissue-cultured plants significantly increased the plant growth parameters (Table 4). However, the application of Ro-3 three times (at planting + 2 months after planting + 4 months after planting) registered the maximum increase in growth parameters when compared with the Ra-1 isolate and also the other treatments (Table 4).

Field evaluation

The evaluation of np*Fo* isolates (Ra-1 and Ro-3) at the field level using cv. Rasthali resulted in a significant reduction of Fusarium wilt severity (up to 84%) when relative to inoculated control plants. These np*Fo* isolates produced a similar reduction in wilt severity (Fig. 2).

Discussion

Fusarium wilt is one of the most serious and destructive diseases of banana (Ploetz and Pegg 2000) and so far no effective management practices have been found to control the disease

Table 2. Effect of non-pathogenic *Fusarium oxysporum* (np*Fo*) isolates application on Fusarium wilt disease severity in cv. Rasthali (6 months after application) under greenhouse conditions

Column means followed by the same letter are not significantly different according to Fisher's unprotected least significant difference test ($P \leq 0.05$)

np*Fo* isolate	Fusarium wilt severity (0–5 scale)[A]	ITS-RFLP group
Control (*Foc* pathogen alone)	5.0h	–
Kb-2	4.7gh	6
Mo-138b	4.6fgh	6
Kvrc	4.6fgh	13
Mat-1	4.5efgh	8
Ka-208a	4.5efgh	2
Pn-2	4.5efgh	11
Mon-178a	4.4defgh	3
Mo-138a	4.4defgh	6
F23b	4.4defgh	5
Ra-2	4.4defgh	10
Ra-3	4.3cdefg	10
Mon184c	4.3cdefg	6
T-1	4.3cdefg	7
Mor-137a	4.3cdefg	13
Ak-1	4.2cdefg	12
Mon-139a	4.2cdefg	9
Nd-2	4.2cdefg	10
Ka-208b	4.1cdefg	1
Pn-1	4.1cdefg	4
Mo-2	4.1cdefg	6
Ka-2	4.1cdefg	10
Kvs	4.1cdefg	13
F-23	4.0cdef	3
T-1	4.0cdef	7
Ra-177	3.9cde	3
Poo-1	3.9cde	3
Poo-2	3.9cde	3
Mo-133c	3.9cde	5
Ka-140	3.9cde	6
Mor-1	3.9cde	7
Blu-1	3.9cde	7
T-2	3.8cd	8
Ra-1	3.7bc	14
Ro-3	3.1b	14
Control (water)	0.0a	–

[A]Mean of 10 replicates.

in the field. Biological control is becoming a popular means of disease control, as there have been growing concerns about the environment, human health and development of resistance among plant pathogens due to the continuous use of fungicides. The use of saprophytic or non-pathogenic Fusarium isolates for biological control of pathogenic *Fusarium* spp. in various crops has been extensively studied and applied (Mandeel and Baker 1991; Yamaguchi *et al*. 1992; Larkin *et al*. 1996). However, reports on the use of non-pathogenic Fusaria at the field level against Fusarium wilt disease of banana as well as the molecular characterisation of Fusarium biocontrol agents are limited. Hence, the present study was undertaken to bridge these gaps. In the present investigation, 33 isolates of *Fo* were obtained from the rhizosphere soil of different diploid and triploid banana cultivars grown in different parts of India. These isolates were identified as *Fo* based on cultural and morphological characters and confirmed as being *Fo* by using a *Fo*-specific PCR assay. The non-pathogenic nature of these isolates was confirmed by inoculating banana plants in the greenhouse. The work presented in this paper is supported by another study (Nel *et al*. 2006*a*) in which several np*Fo* isolates were obtained from suppressive soils of banana.

Molecular analysis of the ITS region of the nuclear DNA is commonly used for grouping closely related strains of fungi; estimating the genetic relationships between fungal groups or for rapid inference of likely taxonomic affinities of isolates in the absence of sexual stages. This is because the ITS region generally evolves much faster between isolates in the same species. Therefore, in the present study, the genetic diversity analysis was carried out for all the 33 np*Fo* isolates by PCR-RFLP of the rDNA-ITS region using six different restriction enzymes. The results of the analysis indicated the presence of 14 different ITS genotypes and no correlation was found based on the geographical origin or genomic constitution of banana varieties from which these np*Fo* isolates were obtained. However, this method has clearly separated the non-pathogenic isolates of Fusarium from pathogenic isolates by placing them into separate ITS genotypes. Interestingly, the potential np*Fo* isolates Ra-1 and Ro-3 displayed identical RFLP haplotypes and were placed in a separate ITS genotype. This finding might be useful for the isolation and identification of effective np*Fo* isolates against Fusarium wilt pathogen of banana, as the differentiation of non-pathogenic isolates from pathogenic isolates is impossible by morphological characters. Similarly, Nel *et al*. (2006*a*) also reported that the most effective non-pathogenic Fusarium isolates identified against the Fusarium wilt pathogen of banana have produced identical IGS-RFLP patterns. Bao *et al*. (2002) also assessed over 300 isolates of *Fo* for biocontrol of Fusarium wilt of tomato, and found, using random amplified polymorphic DNA analyses that 15 of the 20 isolates, which provided disease protection, clustered closely. The np*Fo* strains were in two groups, while the pathogenic strains were placed in two different groups. Therefore, the results of the molecular characterisation indicate that the RFLPs generated by restriction enzyme digestion of PCR-amplified ITS region can be used as markers for the differentiation among the np*Fo* as well as between the non-pathogenic and pathogenic Fusarium isolates. However, further work will be necessary to support this research, as only 33 isolates were tested in total. Future work could focus on screening a larger range of pathogenic and non-pathogenic Fusarium isolates.

Several studies have shown that np*Fo* applied to roots can protect various hosts from Fusarium wilt when challenged by a virulent strain (Gessler and Kuc 1982; Ogawa and Komada 1986; Matta 1989). Similarly, in the present study, the soil application of two potential np*Fo* isolates Ro-3 and Ra-1 in both tissue-cultured and sucker-derived plants of banana registered up to 89% reduction of Fusarium wilt severity as well as increasing plant growth parameters significantly when compared with inoculated control plants. The Ro-3 isolate performed well in reducing the wilt disease when compared with the Ra-1 isolate. Field evaluation of these isolates using the cv. Rasthali also recorded up to 84% reduction in Fusarium wilt severity. Larkin *et al*. (1996) identified several isolates of non-

Table 3. Intensive evaluation of potential non-pathogenic *Fusarium oxysporum* (np*Fo*) isolates for the suppression of Fusarium wilt disease of banana in cv. Rasthali (6 months after application) under greenhouse conditions

Column means followed by the same letter are not significantly different according to Fisher's protected least significant difference test $P \leq 0.05$

Treatment	Fusarium wilt severity[A] (0–5 scale)			
	Tissue-cultured plants	% decrease over pathogen-inoculated control	Sucker-derived plants	% decrease over pathogen-inoculated control
np*Fo* Ra-1 (at planting) + *Foc*	3.7e	21.3	1.6d	55.6
np*Fo* Ra-1 (at planting + 2 months after planting) + *Foc*	1.7c	63.8	1.6d	55.6
np*Fo* Ra-1 (at planting + 2 months + 4 months after planting) + *Foc*	0.7b	85.1	1.2bc	66.7
np*Fo* Ro-3 (at planting) + *Foc*	3.0d	36.2	1.0bc	72.2
np*Fo* Ro-3 (at planting + 2 months after planting) + *Foc*	1.7c	63.8	1.2bc	66.7
np*Fo* Ro-3 (at planting + 2 months + 4 months after planting) + *Foc*	0.5ab	89.4	0.8b	77.8
Pathogen (*Foc*) alone	4.7f	–	3.6e	–
Control (water alone)	0.00a	–	0.00a	–

[A]Mean of 10 replicates.

Table 4. Effect of application of non-pathogenic *Fusarium oxysporum* (np*Fo*) isolates on the growth parameters of banana plants inoculated with Fusarium wilt pathogen *Fusarium oxysporum* f. sp. *cubense* (*Foc*) in cv. Rasthali

Column means followed by the same letter are not significantly different according to Fisher's unprotected least significant difference test ($P \leq 0.05$)

Treatments[A]	Height (cm)	Girth (cm)	No. of leaves	Petiole length (cm)	Leaf area
np*Fo* Ra-1 (at planting)	81.6bc	24.0b	4.5a	23.5b	1690.9d
np*Fo* Ra-1 (at planting + 2 months after planting)	84.3cd	26.2b	5.0a	23.5b	1831.4e
np*Fo* Ra-1 (at planting + 2 months + 4 months after planting)	86.1d	26.5b	6.0b	24.3bc	1854.7f
np*Fo* Ro-3 (at planting)	79.3b	24.1b	5.0a	24.7bc	1554.8c
np*Fo* Ro-3 (at planting + 2 months after planting)	102.1e	30.5c	5.1ab	25.8c	2240.4g
np*Fo* Ro-3 (at planting + 2 months + 4 months after planting)	104.2e	32.8c	7.0c	29.0d	2383.9h
Pathogen (*Foc*) alone	73.5a	21.1a	4.5a	20.2a	1210.1a
Control (without anything)	80.2b	24.6b	4.5a	23.8b	1362.4b

[A]Mean of 10 replicates.

pathogenic *Fusarium* spp. (*Fo* and *F. solani*) that effectively controlled *Fusarium* wilt of tomato, watermelon and muskmelon in greenhouse tests. More recently Nel *et al*. (2006*b*) reported that out of 24 np*Fo* isolates evaluated, 14 significantly reduced the incidence of Fusarium wilt of banana and among these potential isolates, CAV 255 and CAV 241 reduced disease incidence by 87.4 and 75.0%, respectively, under glasshouse conditions. It has been established that the efficacy of non-pathogenic Fusarium isolates depends not only on the characteristics of the strains, but also on inoculum density, that is a high non-pathogen/pathogen ratio (Couteaudier 1989). In the present study, it was observed that three applications of np*Fo* isolates (at planting + 2 months after planting + 4 months after planting) showed the maximum suppression of Fusarium wilt severity compared with one or two applications. This might be due to the high inoculum density ratio of non-pathogenic Fusarium isolates compared with pathogenic Fusarium. Larkin and Fravel (1999) also indicated that non-pathogenic isolates could differ in their efficacy as well as mechanism and dose requirements to suppress a disease. Although the mechanism of controlling the Fusarium wilt disease by non-pathogenic *Fusarium* has been proposed as saprophytic competition for nutrients (Couteaudier and Alabouvette 1990; Lemanceau and Alabouvette 1991), parasitic competition for infection sites (Schneider 1984), and induction of systemic resistance (Ogawa and Komada 1986; Larkin *et al*. 1996) the exact mode of action operating in the banana crop due to the application of these np*Fo* isolates is unknown. To conclude, the present study resulted in identification of two isolates of Indian np*Fo* for the effective suppression of Fusarium wilt disease of banana in the field. The present study also demonstrated that PCR-RFLP patterns of the ITS region were able to differentiate potential biocontrol non-pathogenic isolates from other non-pathogenic isolates and separated non-pathogenic isolates from pathogenic

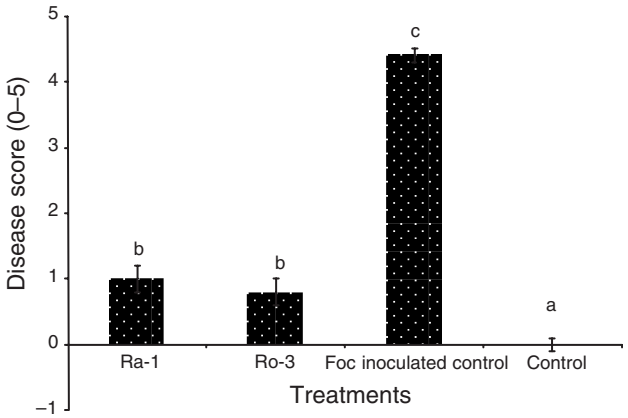

Fig. 2. Field evaluation of non-pathogenic *Fusarium oxysporum* isolates for the suppression of Fusarium wilt disease (*F. oxysporum* f. sp. *cubense* race 1) in cv. Rasthali. Column means followed by the same letter are not significantly different according to Fisher's unprotected least significant difference test ($P \leq 0.05$). Data are means of 20 replicates. Vertical bars represent the standard deviation from the mean.

isolates. However, before recommendations can be made for sustainable management of this lethal disease, further testing in different soil types in combination with fungicides is required.

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