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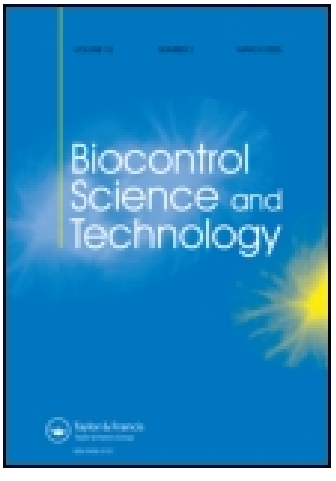

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Combined application of native Trichoderma isolates possessing multiple functions for the control of Fusarium wilt disease in banana cv. Grand Naine

R. Thangavelu[a] & M. Gopi[a]
[a] National Research Centre for Banana, Tiruchirapalli, TN, India
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RESEARCH ARTICLE

Combined application of native *Trichoderma* isolates possessing multiple functions for the control of Fusarium wilt disease in banana cv. Grand Naine

R. Thangavelu* and M. Gopi

National Research Centre for Banana, Tiruchirapalli, TN, India

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Fusarium wilt caused by *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is considered as a lethal disease of bananas worldwide. To manage the disease effectively, 20 rhizospheric and 43 endophytic *Trichoderma* isolates obtained from 12 different *Foc* resistant banana accessions were evaluated against *Foc in vitro* and *in vivo*. *In vitro* screening among *Trichoderma* isolates for their multiple functions (mycelial and spore germination inhibition, hydrogen cyanide, chitinolytic enzymes, non-volatile and volatile metabolites production) in suppressing *Foc* and promoting plant growth (IAA production and phosphate solubilisation) indicated that the multiple biocontrol actions were significantly higher in 6 isolates of rhizospheric *Trichoderma* and 10 isolates of endophytic *Trichoderma* compared to other isolates. The greenhouse evaluation of individual application of these rhizospheric and endophytic *Trichoderma* isolates against Fusarium wilt pathogen in cv. Grand Naine (AAA) indicated significant suppression of Fusarium wilt disease and increased plant growth characters as compared to *Foc* pathogen inoculated plants. However, none of these individual *Trichoderma* isolates recorded complete suppression of Fusarium wilt disease. Therefore, the greenhouse evaluation involving combination of rhizospheric *Trichoderma* sp. NRCB3 + endophytic *Trichoderma asperellum* Prr2 recorded 100% reduction of Fusarium wilt disease and increased plant growth parameters up to 250% when compared to individual isolates application and *Foc* alone-inoculated plants. Further, the field evaluation of this combination of *Trichoderma* isolates applied for three times: (1) at 15 days before planting, (2) second month after planting and (3) fourth month after planting resulting in significant reduction of Fusarium wilt disease and also increase in bunch weight as compared to untreated control plants. Therefore, these *Trichoderma* isolates may be used in combination for the effective suppression of Fusarium wilt disease in banana.

Keywords: banana; endophytes; *Fusarium oxysporum* f. sp. *cubense*; multiple functions; *Trichoderma* isolates

Introduction

Fusarium wilt of banana is one of the most devastating diseases of bananas in the world (Ploetz & Pegg, 1997). After the emergence of a virulent form of *Foc* (VCG 01213/16), the disease is posing a serious threat to the multibillion-dollar banana export industry and also to the livelihoods of small-scale banana growers (Ploetz,

*Corresponding author. Email: rtbanana@gmail.com

2005). The fungus survives as chlamydospores in soil and on plant debris and infects the host through the root hairs, root tips and natural wounds along the lateral root base, colonising the vascular system of the rhizome and pseudostem. This induces characteristic wilting symptoms (Blomme et al., 2011) both externally (yellowing and wilting of leaves, longitudinal splitting in the pseudostem, etc.) and internally (reddish-brown discoloration of vascular tissue) (Stover, 1962). Infected plants generally do not produce a bunch and under severely diseased conditions the entire plant dies.

In India, Fusarium wilt is considered as the major constraint to banana production (Thangavelu et al., 2001) and is ranked as No.1 banana disease (Molina & Valmayor, 1999). At present, the disease has become widespread and destructive in almost all the banana growing states of India and up to 95.5% of disease incidence was noticed particularly in Tamil Nadu where a greater number of susceptible varieties are grown. The important groups of banana affected by this disease are cvs. Silk (AAB), Neypoovan (AB), Pisang Awak (ABB), Virupakshi (AAB), Bluggoe (ABB) and Monthan (ABB). The cultivars such as Rasthali (Silk AAB) and Virupakshi (Pome-AAB-Hill banana) are threatened with extinction (Thangavelu et al., 2001), and the total cultivation area of Rasthali (AAB) (Syn. Amritapani, Malbhog) has been drastically reduced due to this wilt disease. For example, in Andhra Pradesh, the farmers abandoned cultivation of the most susceptible cv. Amritapani (Silk-AAB) for more than 20 years due to this disease.

In recent years, tissue-cultured bananas have become more popular among the farmers as they are free from pests and diseases and provide good yields. However, in the endemic areas of Fusarium wilt disease, particularly in Theni district of Tamil Nadu, India, the Cavendish group of bananas is severely infected by the disease. In certain fields, 100% disease incidence was recorded, and in such fields unusually the disease symptoms were observed even in 1–3 months old plants. The characterisation of Fusarium wilt pathogen infecting Cavendish revealed that the *Foc* belongs to race-1 and VCG of 0124 (Thangavelu & Mustaffa, 2010a). Fortunately, Fusarium wilt in Cavendish was so far not reported in other parts of the country. Several previous studies have revealed that tissue-cultured banana plants are more susceptible to wilt disease attack in the field than the plants derived from suckers (Blomme et al., 2004; Dewaele et al., 1997; Viaene et al., 2003). This could be attributed due to the loss of beneficial microorganisms such as endophytes through the axenic production of tissue-cultured plants, which most likely makes them more vulnerable to wilt attack in the field (Pegg et al., 1996).

Moreover, several management practices developed in the past are not very effective (Ploetz, 1990; Thangavelu et al., 2004) against Fusarium wilt disease. Hence, use of biological control agents that control Fusarium wilt disease and enhance plant growth characteristics could be a potential alternative strategy for Fusarium wilt management. Several soil antagonists, such as *Trichoderma* spp. (Nel et al., 2006; Thangavelu et al., 2004) *Burkholderia cepacia* (Pan et al., 1997), *Pseudomonas fluorescens* (Saravanan et al., 2003) and *Streptomyces* sp. (Getha et al., 2005), have been used to control Fusarium wilt disease. The endophytic nature of microbes such as non-pathogenic *Fusarium oxysporum* (np*Fo*) (Forsyth et al., 2006) and Actinomycetes (Cao et al., 2005) has also been employed to control Fusarium wilt disease. However, these efforts did not result in effective control of the disease. A possible reason may be failure in the selection of biocontrol agents with multiple functions (biological control

and plant growth promotion activities) and actions (Ploetz & Pegg, 2000). Of the many actions of pathogen inhibition, production of antimicrobial compounds, which may be volatile or non-volatile compounds, antibiotics, chitinase and hydrogen cyanide (HCN), are the most important traits to assess the biocontrol potential of antagonistic microbes, due to the fact that these traits are involved in the destruction of the cell wall integrity, leading to effective control of the pathogen (Bokhari & Perveen, 2012). Several reports suggest that *Trichoderma* isolates that produce non-volatile and volatile substances, chitinase, etc. were more inhibitory to Fusarium pathogen compared to others (Dubey & Patel, 2001; Dubey et al., 2007; Kumar & Dubey, 2001). Generally, biocontrol agents with the above said actions in addition to the plant growth promoting traits such as IAA production and phosphate solubilisation interfere with growth of various pathogens either directly or indirectly and thus contribute to disease suppression (Dwivedi & Johri, 2003). Additionally, antagonistic microbes possessing the multiple traits can also be used as broad-spectrum biological control agents under field conditions.

The search for effective microbes and investigation into their modes of action is increasing at a rapid pace, as efforts are made to exploit them commercially as bio-fertilisers and bio-agents. *Trichoderma* fungi have been widely used as biocontrol agents against several soil borne plant pathogenic fungi, invertebrates, and bacteria (Verma et al., 2007). Mohammad Akrami et al. (2011) reported that *Trichoderma harzianum* and *Trichoderma asperellum* isolates and their combination reduced Fusarium rot disease severity from 20% to 44% and increased the dry weight from 23% to 52% in lentil under glasshouse conditions. Inoculation of potted abaca plants with *Trichoderma* and yeast also provided 81.76% and 82.52% control of the disease compared to control treatment (Bastasa & Balia, 2005). Hence, the present study was designed to isolate *Trichoderma* from rhizosphere and plant tissues (endophytes) and evaluate them against a virulent strain of *Foc* race-1 (VCG O124).

Materials and methods

Isolation of fungal pathogens

Fusarium oxysporum f. sp. *cubense* race 1 (VCG 0124) was isolated from dried vascular strands of wilt-infected banana (cv. Grand Naine-AAA) using 25% strength potato dextrose agar (PDA) medium (Thangavelu & Mustaffa, 2010a). The single spore culture obtained was maintained on carnation leaf agar medium (Burgess et al., 1988) for immediate use, and for long-term use the culture was stored as dried filter paper cultures at 4°C (Correll et al., 1986). The pathogenicity of the fungus was tested under pot culture conditions using the tissue-cultured plants of cv. Grand Naine.

Isolation of Trichoderma *isolates from rhizosphere soils*

From the rhizosphere soil of different cultivars of banana, *Trichoderma* isolates were isolated by a serial dilution technique using *Trichoderma* Special Medium (TSM) (Elad et al., 1983). Isolates of *Trichoderma* were identified to species level by microscopic examination of the fungal structures as well as the morphological characters (Bissett, 1984, 1991a, 1991b, 1991c; Radwan Barakat et al., 2006). Additional species of rhizospheric *Trichoderma*, viz., *Trichoderma koningii* 140C,

Trichoderma pseudokoningii, *T. asperellum* K2T5, *T. asperellum* RT1, *T. asperellum* poo and *Trichoderma* sp. NRCB3 obtained from the Department of Pathology, National Research Centre for Banana (NRCB), were also used in this study. The effective cultures identified for the suppression of Fusarium wilt disease of banana based on *in vivo* studies such as rhizospheric *Trichoderma* sp. NRCB3 (ITCC No. 7448) and endophytic *T. asperellum* Prr2 (ITCC No 7447) were deposited at Indian Type Culture collection, Indian Agricultural Research Institute (IARI), New Delhi – 110012.

Isolation of endophytic Trichoderma *isolates*

Root (1 cm long) and corm tissues (1 cm^3) from *Foc* resistant diploid bananas of various genomic status were collected, washed in running water followed by immersion in a series of surface disinfectants, beginning with 4% sodium hypochlorite for 5 min and subsequently 70% ethanol for 3 min. These were finally rinsed three times in sterile distilled water. The surface-sterilised samples were allowed to air dry on a sterile paper towel. The sterilised roots and corm tissues were placed onto TSM and incubated at 25°C for 5–7 days. Hyphal tips of the developing fungal colonies were transferred onto PDA, and after purification the final pure cultures were transferred to PDA slants. The *Trichoderma* isolates obtained were identified to species level by microscopic examination of the fungal structures as well as the morphological characters (Bissett, 1984, 1991a, 1991b, 1991c; Radwan Barakat et al., 2006).

Screening of endophytic and rhizospheric bacterial isolates for their multiple functions against Foc *(VCG-0124) by different* in vitro *evaluation methods*

The biocontrol activities of endophytic and rhizospheric *Trichoderma* isolates were determined by inhibition of spore germination (CSFT, 1943), mycelial growth inhibition (Dennis & Webster, 1971b), volatile, non-volatile production (Dennis & Webster, 1971a, 1971b), Chitinase (Hsu & Lockwood, 1975) and HCN production (Lorck, 1948). Besides, the plant growth promoting traits such as production of indole acetic acid (IAA) (Brick et al., 1991) and phosphate solubilisation (Gaur, 1990) of all endophytic and rhizospheric bacterial isolates were assessed.

In vivo *screening of individual isolate of endophytic and rhizospheric* Trichoderma *isolates for the suppression of Fusarium wilt disease and plant growth promotion*

Six out of 19 rhizospheric *Trichoderma* isolates and 12 out of 43 endophytic *Trichoderma* isolates, which showed multiple functions of biocontrol and plant growth promoting activities, including phosphate solubilisation under *in vitro* conditions, were further evaluated for the suppression of Fusarium wilt disease in cv. Grand Naine under pot culture condition.

The individual *Trichoderma* isolates were mass-produced in rice chaffy grains separately as described by Thangavelu and Mustaffa (2010b). The individual rhizospheric *Trichoderma* isolates were applied @ 30 g pot^{-1} and planted with disease-free tissue-cultured plants cv. Grand Naine.

Similarly, for testing the endophytic *Trichoderma* isolates, the suspension of individual endophytic *Trichoderma* isolates was prepared from the rice chaffy grains formulation (@ 200 g litre of $water^{-1}$) using sterile distilled water. The spore

suspension of individual endophytic *Trichoderma* isolate was adjusted to 10^6 spores ml^{-1} in sterile distilled water. Roots of banana plantlets cv. Grand Naine were immersed in the suspension of individual endophytic *Trichoderma* isolates for 90 min and planted in pots (1′ × 1′ × 1/2′) containing sterilised potting mix prepared with red soil and sand (1:1 w/w). Ten days after the treatment with either endophytic or rhizospheric *Trichoderma* isolate, the *Foc* pathogen multiplied in sand maize meal medium was inoculated @ 30 g pot^{-1} around the plants in the soil. For each *Trichoderma* isolate, five replications were maintained and each replication contained four plants**.** Plants applied with either rice chaffy grain without *Trichoderma* isolates or the roots dipped in sterile distilled water for 90 min served as control.

In vivo *evaluation of combined application of* Trichoderma *isolate for the suppression of Fusarium wilt disease*

All the rhizospheric and endophytic *Trichoderma* isolates, which recorded effective control against *Foc* pathogen and compatible with each other under *in vitro* condition, were multiplied in rice chaffy grains (Thangavelu & Mustaffa, 2010b).

The pots (1′ × 1′ × 1/2′) were filled with a mixture of sand and clay (4:1 w/w). The tissue-cultured banana plants were removed from polybags without much disturbance to the roots. The roots of the plants were washed thoroughly to remove the adhering soil particles and immersed in a suspension of effective endophytic *Trichoderma* isolate (10^6 cfu mL^{-1}) prepared from the rice chaffy grain formulation separately for 90 min and then replanted in the pots. After 3 days, 30 g of chaffy grain formulation of rhizospheric *Trichoderma* isolates were applied. After 10 days, the sand maize meal inoculum of *Foc* (VCG 0124) was applied in the rhizosphere region of the plants @ 30 g pot^{-1}. The pots were distributed at random in a greenhouse under natural lighting and day/night temperature of 30/20°C, and five replicates were maintained for each treatment and each replicate contained four plants. The plants were watered daily. After 6 months of planting, the observations on growth parameters such as height, girth, number of leaves and leaf area (length × breadth × constant 0.83) were recorded. The plants were uprooted and observation on the number of roots was also recorded. The disease evaluation was carried out based on the extent of rhizome discoloration on 1–6 scale: where 1 – corm completely clean, no vascular discoloration; 2 – isolated points of discoloration in vascular tissue; 3 – discoloration of up to one-third of vascular tissue; 4 – discoloration of below one-third and two-thirds of vascular tissue; 5 – discoloration of greater than two-thirds of vascular tissue; and 6 – total discoloration of vascular tissue (Thangavelu & Mustaffa, 2010b). Generally, all the experiments were repeated at least once for the confirmation.

Endophytic tissue colonisation and quantification of rhizosphere populations

The tissue-cultured plants cv. Grand Naine planted in pots filled with sterile soil were treated with rice chaffy grain formulation of *Trichoderma* sp. NRCB3 (rhizospheric) and *T. asperellum* Prr2 (endophytic) separately @ 30 g $plant^{-1}$. A total of three treatments (*T. asperellum* Prr2, *Trichoderma* sp. NRCB3 and one control) each with 32 plants were maintained. Plant tissue colonisation by *Trichoderma* isolates was assessed at weekly intervals for up to eight weeks. At each sampling date, four plants from each treatment were uprooted and thoroughly washed to remove the soil with tap water. Three roots of each plant were removed and, together with the whole corm and

pseudostem, sterilised by dipping in 5% sodium hypochlorite for 1 min and then in 75% ethanol for 1 min. The samples were then washed thrice in sterile distilled water. Four root bits each of 0.25 cm long and four corm and stem pieces of size 0.25 cm^3 were then incubated on Petri dishes containing PDA. The plates were incubated at room temperature (28 ± 2°C) for 5–7 days. The emergence of *Trichoderma* isolates was identified by the characteristic conidia, conidiophores and phialids (Rifai, 1969), and percentage colonisation was assessed. For the quantification of *Trichoderma* population in the rhizosphere soil, 1 g of soil sample from the rhizosphere was collected at weekly intervals for eight weeks from each treatment and serially diluted, plated on to TSM and incubated at 28°C for 7 days. Colonies appearing on the plates were purified by single spore method and identified on the basis of morphological characters (Rifai, 1969).

Field evaluation of Trichoderma *isolates for the suppression of Fusarium wilt disease*

A field experiment was conducted in a wilt infected in Theni district of Tamil Nadu, India, with cv. Grand Naine to evaluate the *Trichoderma* isolates (which were found effective under pot culture condition) for the suppression of Fusarium wilt disease. [The soil was red loamy (amorphous) with 6.2 pH. The average annual rainfall was 1000 mm with average temperature of 20.3–38.6°C and RH of 37–80%.] The field, which was abandoned for commercial production due to severe wilt incidence, was selected for this purpose. The tissue-cultured plants obtained from Jain Irrigation Pvt. Ltd. were planted (19 August 2012) in the field with 6 × 6 feet spacing. The rice chaffy grain formulation of effective *Trichoderma* isolates viz. endophytic *T. asperellum* Prr2 + rhizospheric *Trichoderma* sp. NRCB3 was applied around the plants in the soil for three times, i.e.: (1) at 15 days before planting (in the portray itself), (2) second month after planting and (3) fourth month after planting @ 50 g/plant. Control plants were treated with only rice chaffy grains without *Trichoderma* isolates. Ten replications per treatment with four plants per replication were maintained. Timely applications of fertilisers, manures, water and other intercultural operations were followed according to standard production practices. The observations on percentage of infected plants, the yield parameters such as total number of hands, number of fingers per hand, bunch weight, percentage plants yielded saleable bunches and internal wilt disease score (as described earlier) were taken at the time of harvest.

Statistical analysis

All the experiments were repeated at least once for confirmation purposes. All the data on the effect of *Trichoderma* isolates against *Foc in vitro* were analysed by multivariate analysis of variance, and the data on the plant growth parameters and also the field studies were analysed by analysis of variance , and treatment means were compared by Duncan's multiple range test (DMRT) at $p = 0.05$. The data on the disease severity were analysed by chi-square test, and treatments means were compared by DMRT at $p = 0.05$. The data on inhibition of spore germination and tissue colonisation by *Trichoderma* isolates in root and corm were arcsine transformed, and the data on quantification of *Trichoderma* isolates were log transformed before undergoing statistical analysis (Gomez & Gomez, 1984). The package used for analysis was IBM SPSS Statistics Version 21 developed by the International Business Machines Corporation.

Results

Isolation of Trichoderma *isolates from rhizosphere and plant tissues*

A total of 13 *Trichoderma* isolates were isolated from the rhizosphere soils collected from different banana growing regions, and of which eight isolates were identified as *T. asperellum* and five as *T. harzianum*. With regard to endophytic *Trichoderma* isolates, totally 43 isolates (16 from root and 27 from corm) were obtained from 12 different *Foc* resistant banana accessions and of which 13 were identified as *T. harzianum*, 27 as *T. asperellum*, 2 as *T. koningii* and 1 as *T. pseudokoningii*.

In vitro *screening of* Trichoderma *isolates for their multiple functions against* Fusarium oxysporum *f. sp.* cubense *(race 1 VCG 0124)*

Among 19 rhizospheric *Trichoderma* isolates screened, the multiple functions were significantly ($p \leq 0.05$) higher in 6 isolates compared to the rest of the isolates (data shown only for 6 effective isolates of *Trichoderma*). These six isolates, viz., *T. koningii* 140c, *T. pseudokoningii* NRCB1, *T. asperellum* K2T5, *T. asperellum* NRCB2, *T. asperellum* Poo and *Trichoderma* sp. NRCB3 recorded 27.1–100% inhibition of spore germination and 33.4–82.5% inhibition of mycelial growth of the pathogen compared to control. Additionally, three isolates (*T. koningii* 140c, *T. pseudokoningii* NRCB1 and *T. asperellum* K2T5) were positive for HCN production and five isolates (*T. koningii* 140C, *T. pseudokoningii* NRCB1, *T. asperellum* K2T5, *T. asperellum* NRCB2 and *T. asperellum* poo) for phosphate solubilisation. However, none of these isolates except *Trichoderma* sp. NRCB3 (5 mm of lytic zone) were positive for the production of chitinase enzyme. The IAA production was observed only with *T. asperellum* poo isolate (3 μg/ml) (Table 1).

Similarly, evaluation of endophytic *Trichoderma* isolates for their multiple actions against *Foc* (race 1 VCG-0124) showed that among 43 isolates, 10 isolates of *Trichoderma* recorded significantly ($p \leq 0.05$) higher activity of multiple functions compared to other isolates (data shown only for 10 effective isolates of *Trichoderma*). These 10 isolates had completely inhibited the spore germination of the pathogen (100%) besides inhibiting their mycelial growth significantly ($p \leq 0.05$) (19.2–85%). Among 10 endophytic *Trichoderma* isolates, 7 isolates (except *T. harzianum* C4r1, *T. asperellum* Brl and *T. harzianum* Bc1) produced IAA, ranging from 3.5 to 9 μg/ml of the culture filtrate and 9 isolates were positive for HCN production (except *T. harzianum* C4rl). With regard to phosphate solubilisation, all the isolates except *T. harzianum* Pcc4 produced lytic zone of 5–7 mm in the specific medium. The *T. harzianum* Bc1 isolated from banana cv. Bluggoe produced chitinase with a 5 mm lytic zone (Table 2).

In vivo *evaluation of individual rhizospheric and endophytic* Trichoderma *isolates against Fusarium wilt pathogen (*Foc *race 1 VCG 0124) of banana*

The pot culture evaluation carried out for six isolates of rhizospheric *Trichoderma* against *Foc* (VCG 0124) in cv. Grand Naine showed that all the *Trichoderma* isolates significantly ($p \leq 0.05$) reduced the Fusarium wilt disease severity. The disease score in the *Trichoderma* isolates treated plants ranged from 2.0 to 3.2 compared to 4 in the *Foc* alone-inoculated control plants. Among the rhizospheric isolates, *Trichoderma* sp. NRCB3 recorded the lowest Fusarium wilt disease score of 2.0 compared to other isolates. In addition, the soil application of these *Trichoderma* isolates

Table 1. *In vitro* evaluation of rhizospheric *Trichoderma* isolates against Fusarium wilt pathogen (*Foc* – VCG 0124) of banana.

		Mycelial growth inhibition (cm)						
Rhizospheric *Trichoderma* isolates	Inhibition of spore germination (%)	Volatile production	Non-volatile production	Antibiosis by dual plate assay	Chitinase production (mm)	Lytic zone in Po_4 solubilisation assay (mm)	HCN production	IAA production µg/ml
T. koningii NRCB 140c	27.1f	0.9e (70.9)	0.7d (82.5)	0.8c–d (46.7)	0.0b	12a	+ve	0.0b
T. pseudokoningii NRCB1	95.1c	1.3d (58.0)	1.4b (63.7)	0.9b–c (40.0)	0.0b	12a	+ve	0.0b
T. asperellum NRCB K2T5	28.3e	1.2d (61.2)	0.8d (80.0)	1.0b (33.4)	0.0b	10a–b	+ve	0.0b
T. asperellum NRCB2	93.4d	1.6c (48.3)	0.8d (80.0)	0.7d–e (53.4)	0.0b	8b	−ve	0.0b
T. asperellum NRCB Poo	97.9b	2.0b (35.4)	1.1c (71.7)	0.6e–f (60.0)	0.0b	8b	−ve	3.0a
Trichoderma sp. NRCB3	100.0a	1.8bc (41.9)	0.7d (82.5)	0.5f (66.7)	5.0a	0c	−ve	0.0b
Control (*Foc* pathogen alone)	00.0g	3.1a (0.0)	4.0a (0.0)	1.5a (0.0)	0.0b	0c	−ve	0.0b
SEd ±	0.0	0.1	0.1	0.0	0.3	0.7	–	0.0
CD (p = 0.05)	0.1	0.3	0.2	0.1	0.6	1.6	–	0.0

Values are mean three replications; figures in parentheses are per cent inhibition over untreated control; means followed by the same letter differ non-significantly at $p \leq 0.05$ according to DMRT.

Table 2. *In vitro* evaluation of endophytic *Trichoderma* isolates against Fusarium wilt pathogen (*Foc* – VCG 0124) of banana.

Endophytic *Trichoderma* isolates	Inhibition of spore germination (%)	Mycelial growth inhibition (cm)				Lytic zone in Po_4 solubilisation assay (mm)	HCN production	IAA production µg/ml
		Volatile production	Non-volatile production	Antibiosis by dual plate assay	Chitinase production (mm)			
T. harzianum C4r1	100a	2.0b (23.0)	1.1d (72.5)	1.8b (41.9)	0a	5b	−ve	0.0f
T. asperellum Prr1	100a	2.1b (19.2)	0.6e (85.0)	0.8c–d (74.1)	0a	5b	+ve	5.0d
T. harzianum Pcc4	100a	1.8bc (30.7)	4.0a (00.0)	0.8c–d (74.1)	0a	0c	+ve	9.0a
T. asperellum Br1	100a	1.8bc (30.7)	0.6e (85.0)	1.0c (67.7)	0a	6a–b	+ve	0.0f
T. asperellum Plr2	100a	1.5c (42.3)	2.8b (30.0)	0.6d (80.6)	0a	5b	+ve	6.0c
T. harzianum Bc1	100a	2.0b (23.0)	0.6e (85.0)	1.1c (64.5)	0a	5b	+ve	0.0f
T. asperellum Kr3	100a	2.0b (23.0)	2.0c (50.0)	0.9c–d (70.9)	0a	7a	+ve	6.0c
T. asperellum Pjr1	100a	2.0b (23.0)	0.9d (77.5)	0.9c–d (70.9)	0a	6a–b	+ve	3.5e
T. harzianum Gctcr1	100a	1.9b (26.9)	0.9d (77.5)	0.6d (80.6)	0a	5b	+ve	8.0b
T. harzianum Prr2	100a	2.1b (19.2)	1.1d (72.5)	0.9c–d (70.9)	0a	5b	+ve	5.0d
Control (*Foc* alone)	0.0b	2.6a (0.0)	4.0a (0.0)	3.1a2 (0.0)	0a	0c	−ve	0.0f
SEd ±	–	0.1	0.0	0.1	0.2	0.5	–	0.1
CD ($p = 0.05$)	–	0.2	0.1	0.2	0.5	1.1	–	0.2

Values are mean three replications; figures in parentheses are per cent inhibition over untreated control; means followed by the same letter differ non-significantly at $p \leq 0.05$ according to DMRT.

increased the plant growth parameters namely plant height (0.56–25.56%), girth (32–44%), total number of leaves (6.7–26.7%), leaf area (52.8–223.6%) and number of roots (23–61.54%) significantly ($p \leq 0.05$) compared to *Foc* alone-inoculated control plants (data not shown). Similarly, the evaluation of individual application of endophytic *Trichoderma* isolates for the suppression of Fusarium wilt disease showed that all the *Trichoderma* isolates tested recorded significant ($p \leq 0.05$) reduction of internal vascular discoloration of Fusarium wilt disease and also increased the plant growth parameters such as plant height (0–66.4%), girth (0–114.3%), total number of leaves (0–86.67%), leaf area (7.5–134.5%) and total number of roots (14.3–105%) significantly ($p \leq 0.05$) compared to *Foc* alone-inoculated control plants. However, among the isolates, *T. asperellum* strain Prr2, which was isolated from the roots of cv. Pisang Rajah (AAB), recorded the maximum reduction in internal vascular discoloration of Fusarium wilt disease (disease score 1.6) compared to other endophytic *Trichoderma* isolates (disease score of 2.0–5.0) and *Foc* alone-inoculated plants (disease score of 5) (data not shown).

Evaluation of combined application of rhizospheric and endophytic Trichoderma *isolates for the suppression of Fusarium wilt disease of banana*

Generally, significant reduction of Fusarium wilt disease was observed in all the treatments having both rhizospheric and endophytic *Trichoderma* isolates. However, among all the combinations, the application of endophytic *T. asperellum* Prr2 isolated from the roots of banana cv. Pisang Rajah + rhizospheric *Trichoderma* sp. NRCB3 recorded 100% reduction of Fusarium wilt disease (disease score 1) when compared to *Foc* alone-inoculated control plants (disease score of 4.6) (Figure 1). The combined application of both rhizospheric and endophytic *Trichoderma* isolates increased the plant growth parameters such as height (31.2–169.6%), girth (42.9–205.7%), total number of leaves (15.4–130.8%), leaf area (55.8–126.4%) and total number of roots (60–230%) significantly ($p \leq 0.05$) when compared to *Foc* alone-inoculated plants. This increase in growth parameters, including total number of roots, was 15–230% higher than the effect of individual application of either rhizospheric or endophytic *Trichoderma* isolates (data not shown).

Endophytic tissue colonisation and quantification of rhizospheric Trichoderma *isolates population*

The tissue colonisation study clearly revealed that both *T. asperellum* Prr2 of endophytic origin and *Trichoderma* sp. NRCB3 of rhizospheric origin endophytically colonised the root and corm tissues and not the stem tissues. The colonisation of root tissues occurred during the first week whereas in the corm it occurred only during the second week inoculation. Both these *Trichoderma* isolates colonised 100% of root and corm tissues at four and five weeks post inoculation, respectively. Eight weeks post-inoculation, the percentage of colonisation by both the isolates of *Trichoderma* started decreasing. In general, there was not much difference in the level of colonisation between *T. asperellum* Prr2 of endophytic origin and *Trichoderma* sp. NRCB3 of rhizospheric origin in both root and corm tissues. This study revealed that irrespective of the origin of *Trichoderma* spp. both the isolates of *Trichoderma* colonised the root (Figure 2) and corm (Figure 3) tissues endophytically.

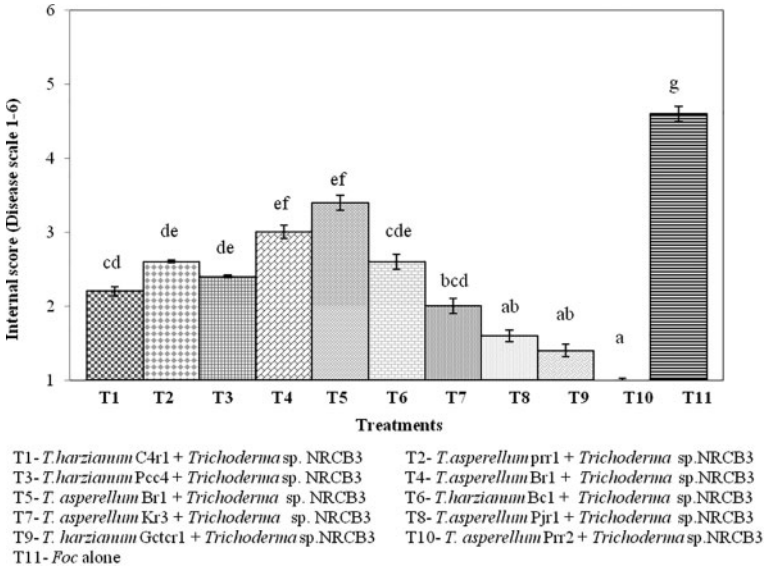

Figure 1. Effect of combined application of endophytic *Trichoderma* isolates and rhizospheric *Trichoderma* sp. NRCB3 on the severity of *Fusarium* wilt disease of banana (1–6 scale) in cv. Grand Naine under greenhouse condition.
Note: Mean ratings followed by the same letters are not significantly different according to DMRT at $p \leq 0.05$. Vertical bars indicate the standard deviation of five replications.

With regard to quantification of *Trichoderma* population, both the endophytic *T. asperellum* Prr2 and rhizospheric *Trichoderma* sp. NRCB3 were present in the rhizosphere soil and sustained the same population level (10^6 cfu/g of soil) until the seventh week of sampling and started decreasing at eighth week of sampling (data not shown). However, the population of *Trichoderma* sp. NRCB3 isolated from the rhizosphere was significantly higher in the rhizosphere soil compared to *T. asperellum* Prr2 isolated from the plant tissues (data not shown).

Field evaluation of Trichoderma *isolates for the suppression of Fusarium wilt disease*

In general, the combined application of both rhizospheric *Trichoderma* sp. NRCB3 + endophytic *T. asperellum* Prr2 at different time intervals recorded a significant reduction of Fusarium wilt disease as well as an increase in the yield parameters such as number of banana hands and bunch weight. However, maximum reduction of disease score (2.29) of Fusarium wilt disease in banana plants was recorded when the combination of *Trichoderma* isolates was applied for three times (at 15 days before planting +2nd MAP +4th MAP) as compared to untreated control plants (5.10). Moreover, the same treatment also resulted in a significant decrease in infected plants (50%) and increase in plants yielded with good bunches (90%) and also the maximum increase in the number of hands (12.5) and bunch weight (24.7 kg) as compared to untreated control plants (Table 3).

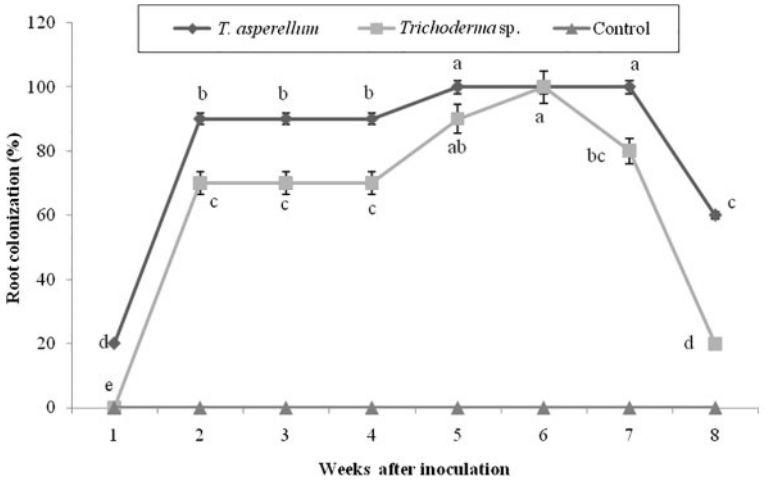

Figure 2. Colonising persistence of endophytic *T. asperellum* (Prr2) and rhizospheric *Trichoderma* sp. NRCB3 on roots of banana cv. Grand Naine.
Note: Mean ratings followed by the same letters are not significantly different according to DMRT at $p \leq 0.05$. Vertical bars indicate the standard deviation of three samples.

Discussion

Generally, use of *Trichoderma* isolates that can colonise the rhizosphere as well as the plant system would be an effective method of combating Fusarium wilt disease. Therefore, we have isolated and screened both rhizospheric and endophytic

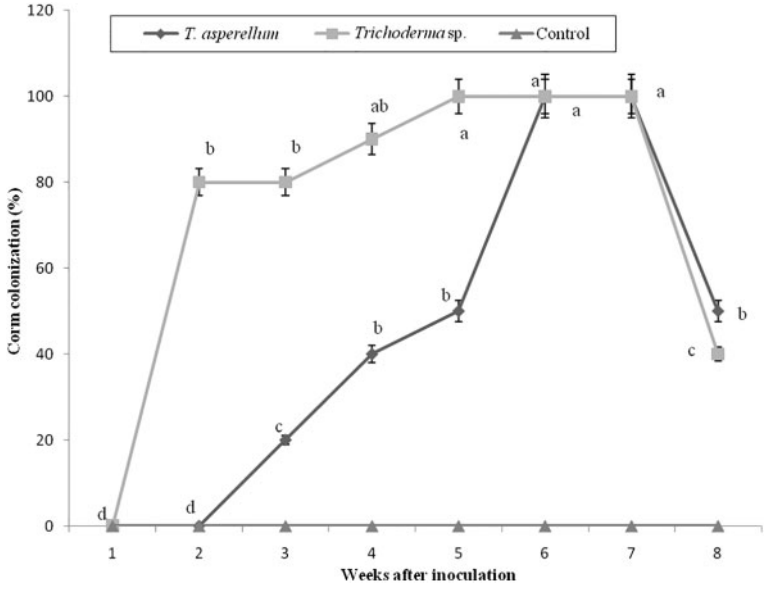

Figure 3. Colonising persistence of endophytic *T. asperellum* (Prr2) and rhizospheric *Trichoderma* sp. NRCB3 on corm of banana cv. Grand Naine.
Note: Mean ratings followed by the same letter are not significantly different according to DMRT at $p \leq 0.05$. Vertical bars indicate the standard deviation of three samples.

Table 3. Field evaluation of the combined application of endophytic *T. asperellum* (Prr2) + rhizospheric *Trichoderma* sp. (NRCB3) for the suppression of Fusarium wilts disease in cv. Grand Naine (AAA).

Treatments	Internal wilt disease score (1–6 scale)	No. of hands/ bunch*	Bunch weight (in kg)*	% of infected plants	% of plants yielded saleable bunches
A. *T. asperellum* (Prr2) + *Trichoderma* sp. (NRCB3)					
1. At 15 days before planting	3.1b	10.6c (9.3)	21.3b (25.3)	85bc	80a
2. 2nd month after planting	3.3b	11.1bc (14.4)	22.8b (34.1)	80b	80a
3. 4th month after planting	4.0c	11.3b (16.5)	22.6b (32.9)	90c	80a
4. At 15 days before planting + 2nd month after planting + 4th month after planting	2.3a	12.5a (28.9)	24.7a (45.3)	50a	90a
B. Control (without *Trichoderma* spp. application)	5.1d	9.7d (0.0)	17.0c (0.0)	95d	50b
CD (.05)	0.3	0.6	1.7	5.1	12.2
CV%	17.0	9.52	12.51	3.38	8.58

*Figures in parentheses are per cent increase over control plants.
Means followed by the same letter differ non-significantly at $p \leq 0.05$ according to DMRT; values are mean of 10 replications.

Trichoderma isolates for the control *Foc* pathogen under *in vitro* and *in vivo* conditions. From the *in vitro* evaluation, 10 isolates of endophytic *Trichoderma* and 6 isolates of rhizospheric *Trichoderma* exhibiting higher antifungal (inhibition of mycelial growth due to antibiosis, HCN, volatile and non-volatile production, inhibition of spore germination) as well as plant growth promoting characters (IAA and phosphate solubilisation) were identified. Interestingly, all the 10 isolates of endophytic *Trichoderma* and 1 isolate of rhizospheric *Trichoderma* sp. NRCB 3 effectively inhibited (100%) the spore germination of *Foc* pathogen. This could be due to the direct action of non-volatile metabolites secreted by the *Trichoderma* isolates (Akila et al., 2011). Jee and Kim (1987) have also reported that *T. harzianum* isolated from the rhizosphere region of cucumber effectively contained the growth of *Fusarium* pathogen.

Although the *in vitro* antagonistic effect may not work with similar effect under *in vivo* conditions (Broadbent et al., 1971), different biocontrol agents have shown promising results and have reduced disease severity in the field (Getha et al., 2005; Nel et al., 2006; Thangavelu et al., 2001, 2004). In the present study, pot culture experiment conducted showed that only rhizopheric *Trichoderma* sp. NRCB3 and endophytic *T. asperellum* Prr2 recorded lowest disease score of 2.0 and 1.6, respectively, as compared to other rhizospheric and endophytic *Trichoderma* isolates exhibiting multiple functions against *Foc* pathogen. Besides, these isolates enhanced all the plant growth traits significantly compared to *Foc* alone-inoculated control plants.

In general, combined application of biocontrol agents would be more effective over a single biocontrol agent in the management of several plant diseases (Crump, 1998; Pierson & Weller, 1994). In the present study, as we could not achieve complete control of the disease with individual *Trichoderma* isolates, combined application of rhizospheric and endophytic *Trichoderma* isolates was attempted and resulted in significant reduction of Fusarium wilt disease. However, among all the combinations, bio priming of banana plants with endophytic *T. asperellum* Prr2 + rhizospheric *Trichoderma* sp. NRCB3 recorded complete control (100%) of Fusarium wilt disease (disease score 1.0) when compared to *Foc* alone-inoculated plants under glasshouse condition (disease score of 4.6). Further, the field studies conducted with this combination of biocontrol agents in different time of applications in hot spot area also resulted in effective suppression of the disease (disease score of 2.29 as against 5.10 in untreated control plants) as well as increased yield parameters particularly when the biocontrol agents rhizospheric *Trichoderma* sp. NRCB3 + endophytic *T. asperellum* Prr2 applied for three times, i.e. at the time of planting +2nd month after planting +4th month after planting. Hence, it is very clear from these findings that combined application of rhizospheric and endophytic *Trichoderma* isolates possessing multiple functions is more effective in combating the Fusarium wilt disease as compared to individual application. This might be due to the fact that these two *Trichoderma* isolates acted against Fusarium wilt pathogen at the rhizosphere and also in the plant system. Moreover, unlike other combinations, this rhizospheric *Trichoderma* sp. NRCB3 + endophytic *T. asperellum* Prr2 combination showed higher biocontrol activity particularly inhibition of spore germination and mycelial growth by antibiosis, volatile and non-volatile metabolites, chitinase production and also the plant growth promoting traits such as phosphate solubilisation and IAA productions compared to other isolates under *in vitro* condition. Similarly, Fishal et al. (2010) reported that the combined application of two endophytes viz., *Pseudomonas* sp. UPMP3 and *Burkholderia* sp. UPMB3 showed significant reduction of Fusarium wilt disease in susceptible banana cv. Berangan. In another study, application of mixture of native uncultivated endophytic bacteria (mostly γ-proteobacteria) into tissue-cultured banana recorded 67% control of Fusarium wilt disease under greenhouse conditions (Jie et al., 2009). Lian et al. (2009) reported that re-introduction of naturally occurring endophytes to tissue-cultured banana plantlets resulted in a substantial reduction in the infection and severity of Fusarium wilt disease (67%) as well as increase in plant growth parameters (height, girth, leaf area).

In addition, it was also observed in the pot culture experiment that irrespective of site of isolation, both the rhizospheric and endophytic *Trichoderma* isolates were present in both the rhizosphere and the plant system endophytically. These *Trichoderma* isolates were observed only in the root and corm tissues and not in the pseudostem. Besides, it was also observed that the root and corm colonisation occurred one week after inoculation and reached the maximum of 100% colonisation at four weeks after inoculation. This study gives an idea of time interval required between bio priming and planting of banana plants in the soil for effective management of Fusarium wilt disease. In the present study, the pathogen *Foc* was inoculated 15 days after the bio priming of banana plants with *Trichoderma* isolates which enabled the biocontrol agents to colonise the tissues and also multiply in the rhizosphere soil so as to combat the invading pathogen effectively. Results from the

colonisation and quantification studies inferred that bio priming of banana plants with the combination of two *Trichoderma* isolates of rhizospheric and endophytic origin exhibiting multiple functions can effectively control the disease compared to bio priming with single *Trichoderma* isolate.

Therefore, it can be concluded that bio priming of banana cv. Grand Naine with *Trichoderma* sp. NRCB3 and *T. asperellum* Prr2 possessing multiple functions of disease suppression and plant growth promotion traits could result in effective suppression of Fusarium wilt disease besides enhancing the plant growth and yield parameters substantially. It was also found that the rhizospheric and endophytic *Trichoderma* isolates were present both in the rhizosphere and in the plant system. The present study further suggests that there should be a minimum time gap of 15 days between bio priming and planting of banana for the effective management of invading Fusarium wilt pathogen. This is the first report of achieving effective control of Fusarium wilt disease in Cavendish banana by combined application of *Trichoderma* isolates and therefore will be used for the effective Fusarium wilt disease management in endemic areas.

Highlights

1. Fusarium wilt disease is considered as a destructive disease in banana throughout the banana growing regions of the world.
2. Bio priming of banana cv. Grand Naine with *Trichoderma* sp. NRCB3 and *T. asperellum* Prr2 possessing multiple functions could result in the effective suppression of Fusarium wilt disease besides increasing the plant growth parameters significantly.
3. Identified that a minimum time gap of 15 days are required between bio priming and planting of banana for the effective management of invading Fusarium wilt pathogen.
4. This is the first report of achieving effective suppression of Fusarium wilt disease through bio priming of banana (cv. Grand Naine) with *Trichoderma* isolates.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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