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A Potential Isolate of *Trichoderma viride* NRCB1 and its Mass Production for the Effective Management of Fusarium Wilt Disease in Banana

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ABSTRACT

Thirty-one isolates of *Trichoderma viride*, out of 110 isolates of *Trichoderma* spp. isolated from the rhizosphere of banana were effective in the initial screening tests conducted *in vitro* against banana wilt pathogen *Fusarium oxysporum* f.sp. *cubense* (*Foc*). These 31 isolates of *Trichoderma viride* and 6 isolates of other species of *Trichoderma* viz., *T. viride*-6, *T. harzianum*, *T. koningii*, *T. pseudokoningii* and *T. hamatum*, were subjected to intensive *in vitro* screening by dual culture and spore germination assay. Among these, *T. viride* isolate NRCB1 resulted in the maximum reduction in mycelial growth and spore germination. Different organic substrates were tested with two jaggery concentrations (1 and 5%) to mass-produce this isolate. The results showed that the fastest coverage of the entire substrate (5 days) as well as high population density (6×10^{31} CFU/g of material) occurred in rice chaffy grains added with 5% jaggery solution, compared to other substrates. Studies on shelf life indicated that population density of *T. viride* NRCB1 was significantly higher in rice chaffy grains formulation at both storage temperature (37 and 25°C), even after 5 months compared to other substrates and talc powder formulation. The pot culture and field studies indicated that the soil application of *T. viride* NRCB1 as chaffy grain formulation significantly reduced the Fusarium wilt disease of banana (up to 80%) and increased the plant growth parameters as compared to the talc powder formulation. The analyses of induction of PO and PAL and total phenolic content both in *T. viride* applied and *Foc* challenge inoculated plants revealed that these were significantly higher compared to control plants and plants inoculated with the pathogen (*Foc*) plants alone.

Keywords: *Fusarium oxysporum* f.sp. *cubense*, lytic enzymes, phenylalanine ammonia lyase rice chaffy grains, shelf-life, total phenol

INTRODUCTION

Fusarium wilt, also known as Panama disease, caused by *Foc* (*Fusarium oxysporum* f.sp. *cubense* (E.F. Smith) W.C. Snyd. & H.N.Hans. Snyder and Hansen 1940) is regarded as one of the most significant threats to banana (*Musa* spp.) production worldwide (Perslay and De Langhe 1987). It is a serious problem in many banana cultivars grown by small holders in the Philippines, Malaysia, India, Brazil, Australia, Vietnam, East Africa, and Thailand. In addition, the widely grown clones in the ABB 'Bluggoe' and AAA 'Gros Michel' and 'Cavendish' sub groups are also highly susceptible to this disease. The fungus survives in soil for up to 30 years as chlamydospores in infested planting material or in the roots of alternative hosts (Moore *et al*. 1995). Generally, infected plants produce no bunches and if produced the fruits are very small and only few fingers develop. In India, the disease is ranked as the number 1 disease of banana (Molina and Valmayor 1999) and is considered as the main impediment in increasing the production and productivity of banana. At present, Panama disease is widespread in almost all the banana growing regions of India (Thangavelu *et al*. 2001).

Since the discovery of Fusarium wilt of banana, various control strategies like soil fumigation (Herbert and Marx 1990), fungicides (Lakshmanan *et al*. 1987); crop rotation (Hwang 1985; Su *et al*. 1986), flood-fallowing (Wardlaw 1961; Stover 1962) and organic amendments (Stover 1962) have evolved and been tested. Despite these methods, the disease cannot be controlled effectively except by the deployment of resistant cultivars (Moore *et al*. 1999). Under these circumstances, the use of antagonistic microbes especially *Trichoderma* spp. which protect and promote plant growth by colonizing and multiplying in the rhizosphere may be a potential alternative approach for the management of Fusarium wilt of banana. Biological control of soil borne diseases caused especially by *Fusarium oxysporum* is well documented (Marois *et al*. 1981; Sivan and Chet 1986; Datnoff *et al*. 1995; Larkin and Fravel 1998; Thangavelu *et al*. 2004). However, a consistent, effective bio-control management system specifically against Fusarium wilt of banana, for wide use is lacking. It is evident that one of the important factors for achieving the effective control of soil borne disease is to ensure a high population level in the rhizosphere region of the plant. The formulations of several commercial products of *Trichoderma* spp., which are available in the market, are mainly based on inert carriers (Lewis and Papavizas 1983), which do not support the multiplication of the antagonists and moreover the small-scale farmers cannot afford to purchase these commercial formulations due to its high price. Hence, the development of methods for the mass production of efficient strains of *Trichoderma* spp. in the cheaply and easily available and biodegradable organic substrates by farmers themselves would be beneficial to the farming community. In this paper, the identification of an efficient strain of *Trichoderma viride*; its mass production in rice chaffy grains and their evaluation under *in vitro* and *in vivo* conditions for the effective management of Fusarium wilt of banana are discussed.

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MATERIALS AND METHODS

Fungal culture

The fungus, *Fusarium oxysporum* f. sp. *cubense* race 1 was isolated from dried vascular strands of wilt infected banana (cv. 'Rasthali', silk group) using ¼ strength potato dextrose agar (PDA) medium amended with streptomycin sulphate at 1.2 mL/240 mL PDA. 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 according to Correll *et al*. (1986). The pathogenicity of the fungus was tested under pot culture conditions using the tissue cultured plants of cv. 'Rasthali'.

Isolation of *Trichoderma* spp. from rhizosphere soils

A survey was conducted in different banana growing states of India viz. Tamil Nadu, Kerala, Andhra Pradesh, Karnataka and Pondicherry and rhizosphere soils were collected from different banana cultivars and also from coconut and coffee (**Table 1**). From these soils, *Trichoderma* spp. isolates were isolated by serial dilution technique using *Trichoderma* special medium (Elad and Chet 1983). Isolates of *Trichoderma* spp. were identified to species level by microscopic examination of the fungal structures as well as the morphological characters (Biseett 1984, 1991a, 1991b, 1991c; Barakat *et al*. 2006). Additional species of *Trichoderma*, which were maintained in the division of plant pathology, were also used in this study.

In vitro screening of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *cubense*

1. Growth inhibition assay

Screening of different isolates of *Trichoderma* spp., 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 the Petri dishes (9 cm diameter) and the plates were then incubated under room temperature (28 ± 2°C) for 3 days. Later, an 8 mm diameter mycelial disc cut from actively growing colonies of *Trichoderma* was placed on the opposite side in the Petri dish perpendicular to the *Foc* mycelial disc. After incubation at room temperature (28 ± 2°C) for 4 days, inhibition of *Foc* growth in terms of growth of *Foc* mycelium (in cm) was measured. Ten replications per *Trichoderma* isolate were maintained.

2. Spore germination assay

The individual isolates of *Trichoderma* spp. were grown separately in 250 ml conical flasks containing 150 ml of PDA broth for 7 days at room temperature (37°C) and culture filtrate was collected by filtering through two layers of muslin cloth and finally through 0.2 µm size Millipore filter to remove all the fungal cells. One ml of the cell free culture filtrate of respective *Trichoderma* spp. was placed in the cavity of the depression slide. Then one ml of conidial suspension (4×10^6 spores/mL) of *Foc* (prepared in sterile distilled water) was added and mixed thoroughly. The cavity slide was kept in Petri dishes glass bridge chamber and incubated at 25°C. The spore suspension of *Foc* in sterile distilled water alone, served as a control. The germination of spores was observed after 48 h of incubation in three different microscopic fields and percentage germination was calculated (CSFT 1947).

Based on the results obtained from the above two *in vitro* experiments, one of the *T. viride* isolates viz., NRCB1 which inhibited both the mycelial growth and spore germination of *Foc* effectively compared to all other isolates of *T. viride* isolates, was used in further studies.

Mass production of *Trichoderma viride* and its shelf life on different organic substrates

Different organic substrates viz. wheat flour, maize flour, rice chaffy grains and pearl millet flour were evaluated for the mass production of *T. viride* isolate NRCB1 under *in vitro* condition as per the method described by Thangavelu *et al*. (2004). 250 g of each organic substrate was added with 50 mL of 1 and 5% jaggery (A type of sugar obtained from sugarcane juice by an indigenous method) solution separately in polypropylene bags and the excess jaggery solution was drained. For each organic substrate, control (without jaggery solution) was also maintained by adding 50 mL of distilled water. The bags containing individual organic substrates were sterilized at 121°C for 2 h for two consecutive days. After sterilization, the organic substrates were inoculated with an 8 mm mycelial disc of *T. viride* obtained from 5-day-old culture separately under aseptic conditions. For each treatment three replications were maintained. The bags were incubated at 25 ± 2°C for 10 days. The days taken for the 100% coverage of the substrate were recorded. After 100% coverage of the substrate, the samples were drawn at monthly intervals up to 5 months and the population of *T. viride* NRCB1 isolate on different organic substrates was estimated by serial dilution technique using *Trichoderma*-selective agar medium (TSM) (Elad and Chet 1983).

Effect of temperature on shelf-life of *T. viride* mass-produced in rice chaffy grains under *in vitro* conditions

About 250 g chaffy grains added with 50 mL of 5% jaggery solution was filled in polypropylene bags and excess jaggery solution was drained. The bags containing chaffy grains were sterilized at 121°C for 2 h for two consecutive days. After sterilization, the chaffy grains were inoculated with an 8 mm mycelial disc of *T. viride* obtained from a 5-day-old culture, separately under aseptic conditions. For comparison, talcum powder formulation of the same strain of *T. viride* was prepared in polythene bags as per the method described by Vidhyasekaran *et al*. (1997). To one kg of sterilized talc powder, 500 mL of *T. viride* biomass along with the growth medium was added, mixed thoroughly and air dried. Then carboxymethyl cellulose was added at 5 g/kg of talc powder and filled in polythene bags. These bags were incubated at room temperature (35 ± 2°C) and also at 25°C. For each treatment, three replications were maintained. Samples from rice chaffy grains as well as from talcum powder formulations were drawn at monthly intervals for five months and the population of *T. viride* was estimated using *Trichoderma* special medium by serial dilution technique (Elad and Chet 1983).

Pot culture evaluation of *T. viride* NRCB1 against Fusarium wilt of banana

1. Mass production of T. viride NRCB1 in field

To suit the local farming condition, a small shed of 210 cm × 150 cm × 150 cm size was made with gunny bags under a tree and sand was spread on the ground inside the shed and moistened. At the outside of the shed, a small channel was opened in all the four sides and water was allowed to stand so as to create high humidity. The humidity and temperature inside the shed was 90% and 32°C, respectively.

Rice chaffy grains (1 kg) were soaked in a plastic bucket containing 500 mL of 5% jaggery solution and left overnight. Then the chaffy grains along with jaggery solution were steam boiled for 20 min. The excess jaggery solution was drained and the chaffy grains were transferred to a plastic tray of 45 cm × 30 cm size. Then 50 g *T. viride* NRCB1 inoculum in chaffy grains was added to the plastic tray, covered with polythene sheet at the top of the tray and kept in the shed. The full coverage of substrate by *T. viride* NRCB1 was observed in 5 days (**Fig. 1**) and the population density was 1×10^{31} CFU/g of material.

Evaluation of *T. viride* NRCB1 in rice chaffy grain formulation for suppression of Fusarium wilt disease

Disease-free, 3-month old tissue cultured 'Rasthali' plants were obtained from the tissue culture lab of NRC for Banana, Thiruchirapalli, Tamil Nadu and were planted in mud pots of 30 cm ×

30 cm × 30 cm size, filled with 7 kg sterilized pot mix (1: 1: 1 ratio of red soil, sand and decomposed farm yard manure). Another set of plants, disease-free suckers of 'Rasthali' banana, each weighing 1.5 kg was extracted from disease free banana plantations, were also used in this experiment. These suckers were planted after pralinage in the pots as detailed above. One month after planting, *T. viride* NRCB1 of rice chaffy grains formulation multiplied under shed condition, was applied to the pots in the soil around the plants at 25 g/plant. For comparison, talc powder formulation of *T. viride* NRCB1 was applied to the pots at 25 g/plant. After ten days, the sand maize meal inoculum (Ricker and Ricker 1936) of *Foc* was applied around the plants in soil at 20 g/pot. For each treatment, eight replications were maintained. All the pots were fertilized with the normal recommended dose of organic and inorganic fertilizers and watered regularly. The observation on growth parameters such as plant height, girth, length of petiole, no. of leaves, and leaf area were taken at regular intervals. The disease severity was estimated by observing both internal and external symptoms by following the INIBAP's Technical guidelines number 6 (Carlier *et al*. 2002) and the descriptions of Ploetz *et al.* (1999) respectively as follows: Internal score: 1) corm completely clean, no vascular discoloration; 2) isolated points of discoloration in vascular tissue; 3) discoloration of up to one-third of the vascular tissue; 4) discoloration of between one-third and two thirds of the vascular tissue; 5) discoloration of greater than two-thirds of the vascular tissue; and 6) complete discoloration of the vascular tissue; external score: 1) healthy; 2) slight chlorosis and wilting with no petiole buckling; 3) moderate chlorosis and wilting with some petiole buckling and or no splitting of leaf bases; 4) severe chlorosis, severe wilting, petiole buckling and dwarfing of the newly emerged leaf; and 5) dead.

Induction of plant defense mechanisms by treatment with the potential isolate of *T. viride* NRCB1

1. Greenhouse studies

Tissue cultured, 3-month old 'Rasthali' banana plants (Vuylsteke 1989) were obtained from the tissue culture laboratory of NRC for Banana and planted in earthen pots (30 cm diameter) filled up with 7 kg of sterilized soil and kept inside the net house. *T. viride* isolate NRCB1 was grown in PDB for 7 days as static culture and the mycelium was harvested by filtering through sterile cheese cloth and washed with sterile distilled water for three times to remove the medium. The harvested mycelium was ground in a pestle and mortar using sterile distilled water and the resulted suspension was adjusted with sterile distilled water to contain 10^9 spores/mL. After 20 days of planting, the individual plant was treated with *T. viride* NRCB1 by pouring 250 ml of spore suspension in each pot. Simultaneously, the plants were then inoculated with sand maize culture of pathogen *Foc* at 10 g/plant. Necessary control treatments were also maintained. At different intervals, leaf samples were collected from the 2nd leaf of each plant and analyzed for chemical changes viz., total phenolic content, phenylalanine ammonia lyase and peroxidase activities. Samples from each treatment were analyzed thrice. All the chemicals for the estimation of the abovesaid chemical changes were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai, India.

Estimation of total phenolic content

One gram of banana leaf was homogenized in 10 mL of 80% methanol and agitated for 15 min. at 70°C. One mL of the methanolic extract was added to 5 mL of distilled water and 0.25 mL of 1 M Folin-Ciocalteau reagent and the solution was kept at 25°C. After 3 min 1 mL of saturated solution of Na_2CO_3 and 1 mL of distilled water were added and the reaction mixture was incubated for 1 h at 25°C. The absorption of the developed blue colour was measured using a spectrophotometer at 725 nm. The content of total soluble phenols was calculated according to standard curve obtained from a Folin-Ciocalteau reaction with phenol (Swain and Hills 1959).

Peroxidase activity

To assess Peroxidase (E.C. 1.11.1.7) activity, 1 g of banana leaf was homogenized in 0.1 M phosphate buffer (pH 6.5) at 4°C. The homogenate was filtered through four layers of muslin cloth and the filtrates were centrifuged at 12,000 × *g* at 4°C for 20 min. The supernatant served as an enzyme source. In a spectrophotometer sample cuvette, 1.5 mL of 0.05 M pyrogallol and 0.1 mL of sample extract were taken. In the reference cuvette, inactivated enzyme (by boiling) extract (0.1 mL) was taken along with 1.5 mL of 0.05 M pyrogallol. The reading was adjusted to zero at 420 nm in a spectrophotometer. To initiate the reaction, 0.1 mL of 1 % H_2O_2 (v/v) was added to the sample cuvette and the changes in absorbance were recorded at 30 s interval (Hammerschmidt *et al*. 1982).

Estimation of Phenylalanine ammonia lyase activity

One gram of banana leaf was homogenized in 5 mL of 0.1 M sodium borate buffer (pH 7.0) containing 0.1 g insoluble polyvinyl pyrrolidone (PVP – MW 40000). The extract was filtered through muslin cloth and the filtrate was centrifuged at 12,000 × *g* at 4°C for 20 min. The supernatant served as an enzyme source. Phenylalanine ammonia lyase (PAL; E.C. 4.3.1.5) activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm as described by Dickerson *et al*. (1984). Samples containing 0.4 ml of enzyme extract was incubated with 0.5 mL of 0.1 M borate buffer, pH 8.8 and 0.5 mL of 12 mM L-phenylalanine in the same buffer for 30 min at 30°C. In the reference cell, 0.4 mL of enzyme extract was taken along with 1.0 mL of borate buffer. The amount of *trans*-cinnamic acid synthesized was calculated.

Field evaluation of *T. viride* NRCB1 for suppression of Fusarium wilt disease

A field experiment was conducted in 2005 at research farm of National Research Centre for Banana, Thiruchirapalli (TN) to assess the effect of *T. viride* NRCB1 multiplied in rice chaffy grain to manage the Fusarium wilt disease in highly susceptible cv. 'Rasthali' (Silk-AAB). Uniform sized (1.5 kg) and disease free suckers were collected from the disease free banana fields. The roots and the outer skin of the corm were removed with a knife to remove pathogens and nematodes present and planted in the field at a distance of 6 feet between rows and 6 feet between plants within a row. One month after planting, the chaffy grain formulation of *T. viride* NRCB1 was applied 2-3 cm below the soil around the plants at 25 g/plant and covered with soil. For comparison, talc powder formulation of *T. viride* NRCB1 was applied at 25 g/plant in the soil around the plants. After 10 days, 20 g of *Foc* as sand maize inoculum was applied 15-30 cm away from the plant and 2-3 cm below the soil surface around the plant and covered with soil. Plants applied with Fusarium pathogen alone and plants applied with neither fusarium wilt pathogen nor the *T. viride* served as controls. The trial was laid out in a randomized block design with 20 replicates per treatment. Watering and fertilizer application was done regularly. After 8 months of planting, the disease severity of Fusarium wilt was recorded by following the INIBAP's Technical guidelines number 6 (Carlier *et al*. 2002) for internal symptoms and the descriptions of Ploetz *et al*. (1999) for assessing the external symptoms.

Data analysis

The experiment on i*n vitro* screening of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *cubense* was conducted by completely randomized block design and all the remaining experiments such as, mass production of *Trichoderma viride* and its shelf life on different organic substrates, Effect of temperature on shelf-life of *T. viride* mass-produced in rice chaffy grains under *in vitro* condition, pot culture and field evaluation of *T. viride* NRCB1 against Fusarium wilt of banana, were conducted by randomized block design. The data for percentage of germinated spores were transformed into the arcsine square root values to normalize distributions

before analysis of variance. Data on the population of *Trichoderma* in different organic substrates (CFU/g) were log-transformed to improve the homogeneity of variances. The incidence of *Fusarium* wilt disease, the percent coverage of the substrate and the effect of bioagent on the growth parameters were subjected to analysis of variance (ANOVA). Statistical significance was assessed at the level of $P < 0.05$ using AGRES programme version 7.01 and Duncan's multiple range test was used to separate means.

RESULTS AND DISCUSSION

In vitro screening of *Trichoderma* spp. against *Fusarium oxysporum* f. sp. *cubense*

Totally, out of 110 isolates of *Trichoderma* spp. which were isolated from the rhizosphere soils collected from different varieties of banana and also from coconut and coffee grown in different parts of India, and also the Trichoderma isolates maintained at Div. of Plant Pathology, NRCB, Thiruchirapalli such as *T. viride*-6, *T. harzianum*, *T. koningii*, *T. pseudokoningii* and *T. hamatum*, 31 isolates of *T. viride* were found to be effective in inhibiting the mycelial growth of *Foc* under *in vitro* condition. The percent reduction of mycelial inhibition was ranged from 44.44 to 73.33. Among these, *T. viride* isolate NRCB1 recorded the maximum reduction in mycelial growth (2.4 cm) followed by *T. koningii* and *T. hamatum*-1 (2.7 cm) (**Table 1**). When the culture filtrate of all the *Trichoderma* spp. isolates tested for their ability to inhibit the spore germination of Fusarium wilt pathogen under *in vitro* condition, the culture filtrate of *T. viride* NRCB1 recorded 94.80% inhibition of spore germination which was followed by *T. viride*-6 and *T. hamatum*-1 (72.03%) and *T. koningii*-1 (69.35%) (**Table 1**). Several investigations have revealed that antagonistic organisms generally restrict the mycelial growth and inhibit the germination of spores of the pathogen. Jee and Kim (1987) found that *T. harzianum* isolated from the rhizosphere region of cucumber effectively inhibited the growth of *F. oxysporum* f.sp. *cucumerinum*. Padmodaya and Reddy (1996) also reported that out of ten antagonistic microorganisms, *T. viride* was highly inhibitory to *F. oxysporum* f.sp. *lycopersici*. Though *in vitro* antagonism is not a clear indicator of the potential antagonism in actual field situations (Broadbent *et al.* 1971), different bio-control agents have shown promising results in *in vitro* tests as well as reducing disease severity *in vivo* (Thangavelu *et al*. 2001, 2004; Nel *et al*. 2006). Getha *et al*. (2005) also selected the *Streptomyces* sp. strain g10 for further *in vivo* assays, based on results of *in vitro* tests, as the strain exhibited strong antagonism towards *Foc* races 1, 2 and 4 in plate assays, therefore the selection of isolates from *in vitro* testing for further mass production and *in vivo* evaluations is substantiated.

Mass production of *Trichoderma viride* and its shelf life on different organic substrates

One of the most important factors in deciding the efficiency of a bio control agent is the type of formulation which supports high populations of the agent and at a minimal cost so that even, small and medium type farmers can afford to use the bio-agents for successful control of the disease. The *T. viride* isolate NRCB1 which performed the best under *in vitro* conditions was selected for mass production studies using four different organic substrates viz., wheat flour,

Table 1 Effect of *Trichoderma* spp on mycelial growth and spore germination of Fusarium wilt pathogen.

Trichoderma viride isolates	Mycelial growth of the pathogen (cm)	% Decrease over control	% Spore germination	% Decrease over control
Ras-1	3.0 ± 0.0 cd	66.66	75.60 m	21.45
PK-1	3.8 ± 0.6 gh	57.77	90.00 uv	6.49
Nd-1	3.8 ± 0.4 gh	57.77	90.00 uv	6.49
DC-1	3.2 ± 0.0 de	64.44	75.60 m	21.45
Ras-2	3.6 ± 0.8 fg	60.00	89.74 u	6.76
Ras-3	3.2 ± 0.4 de	64.44	71.42 j	25.79
Kar-1	4.4 ± 0.0 i	51.11	77.77 no	19.20
Ras-4	3.0 ± 0.6 cd	66.66	66.10 h	31.32
Nd-2	4.6 ± 0.6 ij	48.88	90.24 uv	06.24
Mor-5	3.2 ± 0.0 de	64.44	69.23 i	28.07
Pk-2	3.2 ± 0.4 de	64.44	78.94 op	17.98
Nd-3	3.2 ± 0.7 de	64.44	75.60 m	21.45
NP-1	3.4 ± 0.0 ef	62.22	77.14 mn	19.85
Rob-1	2.6 ± 0.4 ab	71.11	76.31 mn	20.71
Re-1	3.2 ± 0.0 de	64.44	80.00 pq	16.88
Rob-2	3.6 ± 0.8 fg	60.00	83.78 rs	12.95
Nd-4	3.6 ± 0.0 fg	60.00	72.34 jk	24.84
Np-2	3.2 ± 0.4 de	64.44	34.38 d	64.28
Np-3	3.0 ± 0.6 cd	66.66	75.60 m	21.45
Np-4	2.6 ± 0.4 ab	71.11	40.81 e	57.60
Nd-5	3.2 ± 0.0 de	64.44	44.92 g	53.32
Nd-6	3.4 ± 0.6 ef	62.22	42.59 f	55.75
PK-3	3.0 ± 0.7 cd	66.66	29.50 c	69.35
KV-2	3.0 ± 0.0 cd	66.66	76.31 mn	20.71
Nd-7	2.8 ± 0.7 bc	68.88	82.26 r	14.53
Co-1	4.8 ± 0.4 jk	46.66	90.24 uv	06.24
Poo-1	2.6 ± 0.0 ab	71.11	75.60 m	21.45
Co-2	3.0± 0.7 cd	66.66	66.10 h	31.32
Co-3	5.0 ± 0.6 kl	44.44	90.24 uv	06.24
Poo-2	3.8 ± 0.7 gh	57.77	84.21 st	12.50
NRCB1	2.4 ± 0.0 a	73.33	5.00 a	94.80
T. viride-6	3.0 ± 0.8 cd	66.66	26.92 b	72.03
T. harzianum-3	3.5 ± 0.6 fg	61.11	77.77 no	19.20
T.koningii-1	3.2 ± 0.6 de	64.44	29.50 c	69.35
T. pseudokoningii-1	2.5 ± 0.4 ab	72.22	73.80 kl	23.32
T. hamatum-1	2.7 ± 0.6 bc	70.00	26.92 b	72.03
Control (*Foc*)	9.0 ± 0.0 m	00.00	96.25 w	-------

*Mean of 10 replications; In a column means followed by a common letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

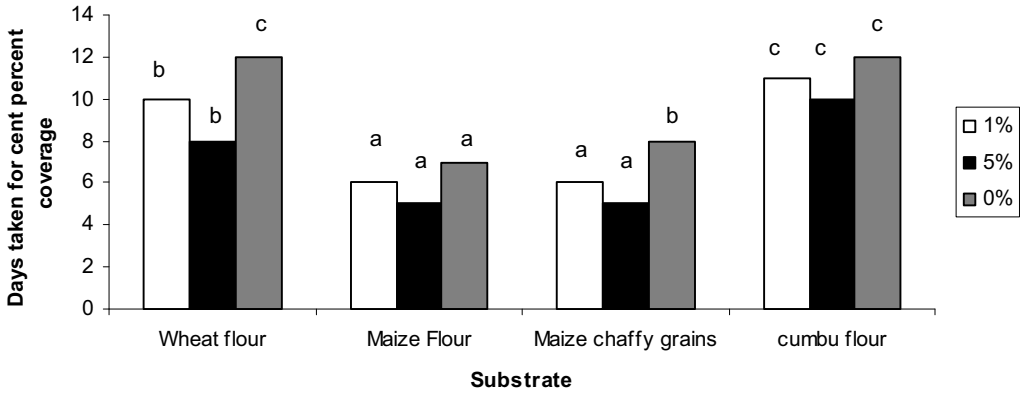

Fig. 1 Time taken for the complete coverage of different substrates by *Trichoderma viride* NRCB1. Data are means of 10 replications. Different letters indicate significant differences between treatments according to Duncan's multiple range tests ($P \leq 0.05$).

maize flour, rice chaffy grains and pearl millet flour. Among the four substrates evaluated at two concentrations of jaggery on the growth of *T. viride* NRCB1 under room temperature condition (35 ± 2°C), the rice chaffy grains and maize flour at 5% concentration recorded 100% coverage in just 5 days as against 7-10 days in substrates without jaggery. It was also observed that as the concentration of jaggery decreased, the number of days taken for the coverage of the substrate increased in all the substrates (**Fig. 1**). In the past a number of formulations of *Trichoderma* have been developed and evaluated against 32 different fungal pathogens (Ramakrishnan *et al.* 1994; Lewis and Larkin 1997; Lewis *et al*. 1998; Mathivanan *et al.* 2000). Ahmed and Baker (1988) found that when simple sugars were added along with cellulose substrate, *T. harzianum* grew more rapidly on complex carbon sources and increased its biomass on the substrate. Lewis and Papavizas (1983) also reported that bran, cornmeal and peanut hull meal were better than eight other solid substrates for production of chlamydospores and conidia. They could produce 22×10^7 CFU of UV induced mutants of *T. viride*/g of bran added with sucrose nitrate as liquid nutrients.

Effect of temperature on shelf-life of *T. viride* mass-produced in rice chaffy grains under *in vitro* condition

The viability of bio-control agents is an important factor in deciding the quality of a commercial bio-control product and the viability in turn is decided by the temperature at which the formulations are stored and also the period of storage. The formulation should have a minimum population of 2×10^8 CFU/g of product in order to be an effective agent (Ragunathan 1997). In this study, among the substrates, the population was very high in rice chaffy grains during all the 5 months of storage as compared to other substrates. The population recorded was 6×10^{31} CFU/g after one month storage and then gradually decreased to 1×10^{11} CFU/g after 5 months of storage at 5% jaggery concentration. A similar trend was observed in other substrates as well as in all the concentrations of jaggery studied. Interestingly in all the substrates, 5% jaggery supported a significantly high population of *T. viride* compared to substrate with 1% and without jaggery (**Table 2**). The population obtained (6×10^{31} CFU/g of rice chaffy grain) in the present study, was 10^{22} higher than the talc-based formulation (Ramakrishnan *et al*. 1994) of *Trichoderma* spp. and several

Table 2 Shelf life of *Trichoderma viride* in different substrates at different concentration of jaggery solutions.

Substrate	Concentration of jaggery solution added	CFU / g of material				
		Months after storage				
		1	2	3	4	5
Wheat flour	1%	$1.0 \pm 0.0 \times 10^{20}$ e (20.00)	$9.0 \pm 0.8 \times 10^{16}$ e (16.95)	$4.0 \pm 0.0 \times 10^{11}$ cd (11.60)	$1.0 \pm 0.0 \times 10^{7}$ bc (7.00)	$3.0 \pm 0.5 \times 10^{4}$ c (4.47)
	5%	$2.0 \pm 0.0 \times 10^{24}$ gh (24.30)	$3.0 \pm 0.0 \times 10^{20}$ h (20.47)	$2.0 \pm 0.0 \times 10^{16}$ ij (16.30)	$2.0 \pm 0.0 \times 10^{10}$ de (10.30)	$1.0 \pm 0.0 \times 10^{6}$ d (6.00)
	None	$3.0 \pm 0.8 \times 10^{15}$ a (15.47)	$2.0 \pm 0.0 \times 10^{12}$ a (12.30)	$3.0 \pm 0.0 \times 10^{8}$ a (8.47)	$1.0 \pm 0.0 \times 10^{5}$ a (5.00)	$5.0 \pm 0.8 \times 10^{3}$ ab (3.69)
Maize flour	1%	$3.0 \pm 0.8 \times 10^{20}$ ef (20.47)	$3.0 \pm 0.8 \times 10^{18}$ f (18.47)	$7.0 \pm 1.6 \times 10^{14}$ g (14.84)	$5.0 \pm 0.8 \times 10^{13}$ f (13.69)	$2.0 \pm 0.0 \times 10^{10}$ ef (10.30)
	5%	$4.0 \pm 0.8 \times 10^{22}$ fg (22.60)	$4.0 \pm 0.5 \times 10^{20}$ hi (20.60)	$1.6 \pm 1.0 \times 10^{16}$ i (16.20)	$1.2 \pm 0.0 \times 10^{15}$ g (15.07)	$3.0 \pm 0.0 \times 10^{10}$ ef (10.47)
	None	$1.0 \pm 0.0 \times 10^{16}$ ab (16.00)	$1.0 \pm 0.0 \times 10^{13}$ ab (13.00)	$6.0 \pm 0.8 \times 10^{10}$ bc (10.77)	$3.0 \pm 0.8 \times 10^{6}$ b (6.47)	$1.0 \pm 0.0 \times 10^{4}$ ab (4.00)
Rice chaffy grains	1%	$1.0 \pm 0.0 \times 10^{26}$ i (26.00)	$4.0 \pm 0.8 \times 10^{22}$ j (22.60)	$2.0 \pm 0.0 \times 10^{18}$ k (18.30)	$2.0 \pm 0.0 \times 10^{15}$ gh (15.30)	$4.0 \pm 0.5 \times 10^{9}$ e (9.60)
	5%	$6.0 \pm 0.5 \times 10^{3}$ lj (31.77)	$4.0 \pm 1.4 \times 10^{26}$ k (26.60)	$3.0 \pm 0.0 \times 10^{21}$ l (21.47)	$2.0 \pm 0.0 \times 10^{17}$ i (17.30)	$1.0 \pm 0.0 \times 10^{11}$ fg (11.00)
	None	$1.0 \pm 0.0 \times 10^{18}$ c (18.00)	$3.0 \pm 0.8 \times 10^{16}$ cd (16.47)	$1.0 \pm 0.0 \times 10^{12}$ dc (12.00)	$1.0 \pm 0.0 \times 10^{10}$ d (10.00)	$3.0 \pm 0.0 \times 10^{6}$ d (6.47)
Pearl millet flour	1%	$3.0 \pm 0.0 \times 10^{21}$ ef (21.47)	$3.0 \pm 0.0 \times 10^{18}$ f (18.47)	$3.0 \pm 0.0 \times 10^{13}$ f (13.47)	$1.0 \pm 0.0 \times 10^{11}$ de (11.00)	$1.0 \pm 0.0 \times 10^{7}$ d (7.00)
	5%	$7.0 \pm 1.4 \times 10^{22}$ fg (22.84)	$1.0 \pm 0.0 \times 10^{19}$ fg (19.00)	$1.0 \pm 0.0 \times 10^{15}$ gh (15.00)	$2.0 \pm 0.0 \times 10^{10}$ de (10.30)	$1.0 \pm 0.0 \times 10^{7}$ d (7.00)
	None	$2.0 \pm 0.0 \times 10^{18}$ cd (18.30)	$2.0 \pm 0.0 \times 10^{15}$ c (15.30)	$1.0 \pm 0.0 \times 10^{10}$ b (10.00)	$3.0 \pm 0.0 \times 10^{6}$ b (6.47)	$2.0 \pm 0.0 \times 10^{3}$ a (3.30)

*Mean of three replications; Figures in parentheses are $\log_{10}$ value; In a column means followed by a common letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$).

Table 3 Shelf life of *Trichoderma viride* NRCB1 in chaffy grains at 37 (room temperature, RT) and 25° C.

Substrate	CFU / g of material				
	Months after storage				
	1	2	3	4	5
Rice chaffy grain formulation at RT	$1.0 \pm 0.0 \times 10^{24}$ b (24.00)	$2.0 \pm 0.0 \times 10^{21}$ b (21.47)	$4.0 \pm 0.8 \times 10^{17}$ c (17.60)	$1.6 \pm 0.4 \times 10^{12}$ c (12.20)	$3.0 \pm 0.8 \times 10^{8}$ b (8.47)
Talcum powder formulation at RT	$1.0 \pm 0.0 \times 10^{13}$ a (13.00)	$6.0 \pm 0.8 \times 10^{10}$ a (10.77)	$1.0 \pm 0.0 \times 10^{8}$ a (8.00)	$2.0 \pm 0.8 \times 10^{6}$ a (6.30)	$9.0 \pm 1.4 \times 10^{5}$ a (5.95)
Rice chaffy grain formulation at 25°C	$2.0 \pm 0.8 \times 10^{30}$ c (30.30)	$4.0 \pm 0.8 \times 10^{26}$ c (26.47)	$7.0 \pm 0.9 \times 10^{21}$ d (21.84)	$1.3 \pm 0.4 \times 10^{16}$ d (16.11)	$9 \pm 1.4 \times 10^{11}$ c (11. 95)
Talcum powder formulation at 25°C	$2.0 \pm 0.0 \times 10^{13}$ a (13.30)	$1.0 \pm 0.0 \times 10^{11}$ a (11.00)	$5.0 \pm 0.0 \times 10^{10}$ b (10.69)	$8.0 \pm 0.0 \times 10^{8}$ b (8.70)	$1.0 \pm 0.0 \times 10^{6}$ a (6.00)

*Mean of three replications, Figures in parentheses are $\log_{10}$ value. In a column means followed by a common letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

Table 4 Effect of *Trichoderma viride* NRCB1 in rice chaffy grains formulation on the Fusarium wilt severity in banana plants (cv. 'Rasthali') in both tissue cultured and sucker derived plants.

Treatments	Internal score (1- 6 scale)*		External score (1-5 scale)*	
	Tissue cultured plants	Sucker derived plants	Tissue culture plants	Sucker derived plants
T. viride NRCB1 in rice chaffy grains formulation	1.5 ± 0.5 b	1.5 ± 0.5 ab	2.00 ± 0.0 b	1.0 ± 0.0 a
T. viride NRCB1 in talc cum powder formulation	3.2 ± 0.6 c	3.0 ± 0.7 c	2.5 ± 0.5 bc	2.5 ± 0.5 b
Control (*Foc* pathogen alone)	5.5 ± 0.5 d	5.0 ± 0.9 d	5.0 ± 0.5 d	4.5 ± 1.2 c
Control (water alone)	1.0 ± 0.0 a	1.00 ± 0.0 a	1.0 ± 0.0 a	1.0 ± 0.0 a

*Mean of 20 replications, In a column means followed by a common letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

fold higher than the populations observed when using bran and ground corn cobs as the solid substrate for the mass multiplication of *Trichoderma* spp. (Lewis and Papavizas 1983). Though the rice chaffy grain and maize flour equally supported high population of *T. viride*, the maize flour turns watery after 2 months of storage at 25°C and thus rice chaffy grain formulation was selected for shelf life and other *in vivo* studies against Fusarium wilt pathogen.

When the rice chaffy grains formulation was evaluated for its shelf life at two different temperature conditions (37 and 25°C), in comparison to talc powder formulation which is a usual formulation available in the market, the population of *T. viride* NRCB1 was significantly high at 25°C during the 5 months storage period. Furthermore, the population of *T. viride* NRCB1 was several folds higher even after 5 months (9×10^{11} CFU/g) of storage as compared to talc powder formulation of the same strain (9×10^{8} CFU/g) at 25°C (**Table 3**). Several authors have reported that storing the formulations at low temperature increases shelf life of the bio agents. Sabaratnam *et al.* (2002) found that at 4°C, the powder and granular formulations of Streptomyces were the most stable and were shown to be 100% viable after 14 and 10 weeks of storage, respectively. The nematophagous fungus *Verticillium chlamydosporium* retained its viability when mixed with carrier Kaolin and binder and stored in vacuum–sealed bags at 25°C for 12 months (Stirling 1991).

Pot culture evaluation of *T. viride* NRCB1 against Fusarium wilt of banana

The pot culture experiment conducted indicated that the application of *T. viride* NRCB1 in chaffy grain formulation significantly reduced the external and internal symptoms of Fusarium wilt disease in tissue culture as well as sucker derived plants of banana cv. 'Rasthali' as compared to talc powder formulation. The percent reduction of the internal score over the pathogen inoculated plants was 72.73 and 70 in tissue cultured and sucker plants respectively, whereas it was 40 and 42% in the case of *T. viride* applied as chaffy grain formulation. With regard to the external score, the ap-

Table 5 Effect of *Trichoderma viride* NRCB1 in rice chaffy grain formulation on growth parameters of banana plants of both tissue cultured (TC) and sucker derived plants.

Treatments	Height (cm)		Girth (cm)		Total leaves	
	TC	Sucker	TC	Sucker	TC	Sucker
T. viride NRCB1 in rice chaffy grains formulation	28.3 ± 0.2 c	118.2 ± 0.8 d	10.6 ± 0.5 cd	32.60 ± 1.0 d	04.8 ± 0.4 ab	06.0 ± 0.4 bc
T. viride NRCB1 in talc cum powder formulation	26.0 ± 1.5 b	95.2 ± 1.30 c	09.1 ± 0.5 b	28.40 ± 1.0 c	04.6 ± 0.3 ab	05.5 ± 0.3 b
Control (*Foc* pathogen alone)	18.6 ± 1.0 a	73.5 ± 1.4 a	08.2 ± 0.3 a	21.10 ± 0.8 a	04.2 ± 0.3 a	04.5 ± 0.5 a
Control (water alone)	34.3 ± 0.7 d	80.2 ± 0.8 b	10.2 ± 0.2 c	24.60 ± 1.3 b	05.0 ± 0.5 bc	06.2 ± 0.3 bc

Table 5 (Cont.)

Treatments	Petiole length (cm)		Leaf area (cm)	
	TC	Sucker	TC	Sucker
T. viride NRCB1 in rice chaffy grains formulation	05.4 ± 0.6 bc	29.7 ± 0.50 d	204.1 ± 1.7 c	2.940.4 ± 3.6 d
T. viride NRCB1 in talc cum powder formulation	05.0 ± 0.4 ab	26.3 ± 1.3 c	180.3 ± 2.3 b	2.246.2 ± 4.8 c
Control (*Foc* pathogen alone)	04.3 ± 0.3 a	20.2 ± 0.6 a	137.7 ± 1..34 a	1.210.1 ± 3.2 a
Control (water alone)	06.8 ± 0.4 d	23.8 ± 0.6 b	257.8 ± 2.3 d	1.362.4 ± 2.0 b

*Mean of 20 replications, In a column means followed by a common letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

Table 6 Field evaluation on the potentiality of *T.viride* in chaffy grain formulation for the suppression of Fusarium wilt disease in banana.

Treatments	Disease severity of Fusarium wilt disease *	
	Internal score (1- 6 scale)	External score (1-5 scale)
T. viride NRCB1 in rice chaffy grains formulation	1.0 ± 0.0 a	1.0 ± 0.0 a
T. viride NRCB1 in talc cum powder formulation	1.5 ± 0.5 b	2.0 ± 0.0 b
Control (*Foc* pathogen alone)	5.0 ± 1.0 c	4.0 ± 0.7 c
Control (water alone)	1.0 ± 0.0 a	1.0 ± 0.0 a

*Mean of 20 replications, In a column means followed by a common letter are not significantly different according to Duncan's multiple range test ($P \leq 0.05$)

plication of *T. viride* NRCB1 has resulted in the reduction of 60 to 78% over the pathogen (*Foc*) alone inoculated plants, whereas it was 44 to 50% in talc powder based formulation (**Table 4**). In addition, the application of *T. viride* NRCB1 as chaffy grain formulation also resulted in a significant increase in plant growth parameters such as height (52 to 61%), girth (29 to 55%), number of leaves (14 to 33%) petiole length (26 to 47%) and leaf area (48 to 143%) in both TC and sucker plants as compared to the pathogen alone inoculated plants. The effect was more pronounced in *T. viride* NRCB1 applied as chaffy grain formulation as compared to *T. viride* NRCB1 in talc powder formulation (**Table 5**).

Field evaluation of *T. viride* NRCB1 for suppression of Fusarium wilt disease

In a field trial conducted using cv. 'Rasthali', the application of *T. viride* NRCB1 has resulted in the reduction of 70-80% of internal symptoms and 50 to 75% external symptoms as compared to the pathogen alone-inoculated plants at 9 months after planting. Among the two formulations, the maximum reduction of wilt score was observed in chaffy grain formulation (80% in internal and 75% in external scores) (**Table 6**). The reason for increased reduction of wilt incidence might be due to high population of *T. viride* in rice chaffy grain formulation. The severity of wilt disease of cotton caused by *F. oxsyporum* f.sp. *vasinfectum* also reduced significantly due to the application of high density of propagules of *T. viride* (Cano and Catedral 1994). In a recent investigation, Thangavelu *et al*. (2004) reported that

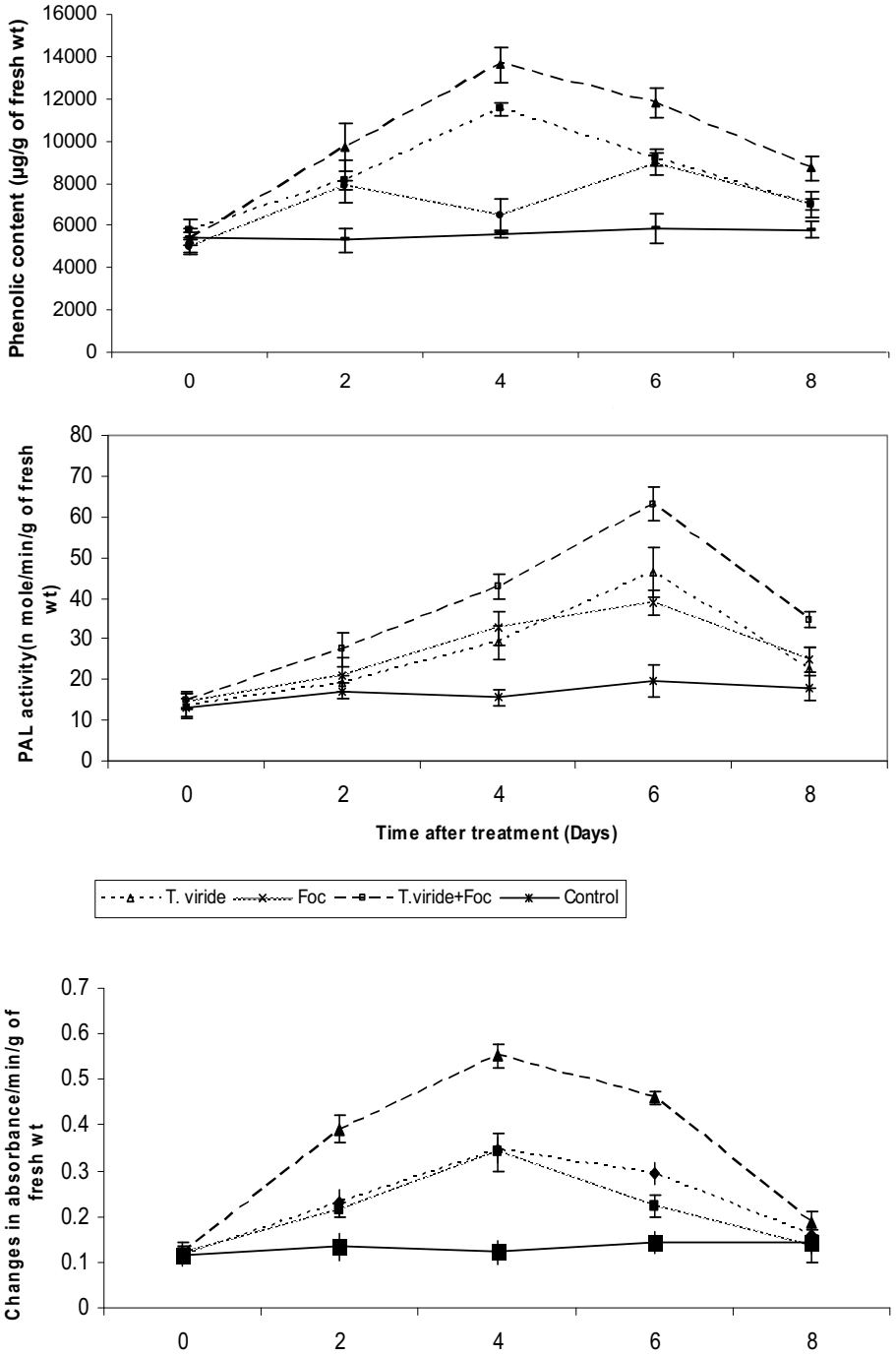

Fig. 2 Induction of defense related enzymes by treatment with the potential isolate of *T. viride* NRCB1. (**A**) Total phenolic content (µg/g of fresh tissues), (**B**) changes in Phenylalanine ammonia-lyase activity (nmole of transcinnamic acid/min/g of fresh weight) and (**C**) changes in peroxidase activity (min/g of fresh tissues) in *Trichoderma viride* applied and *Foc* challenge inoculated banana plants cv. 'Rasthali'. Error bars = ± standard error of the mean (n =5).

application of dried banana leaves formulation of *T. harzianum*-Th-10 with high population of propagules decreased the wilt incidence significantly as compared to talc powder formulation. Lewis and Lumsden (2001) reported that a biocontrol formulation of *Trichoderma* and *Gliocladium* consisted of mixing vermiculite, powdered wheat bran and dry fermentor-produced biomass of isolates of *Trichoderma* and *Gliocladium* effectively controlled damping-off diseases in several crops caused by *R. solani*.

Induction of plant defense mechanisms by treatment with the potential isolate of *T. viride* NRCB1

The induction of systemic resistance by various microorganisms may protect plants against soil and foliar pathogens (Paulitz 1990). However the speed and magnitude of activation of the defense mechanism appears to be critical for the expression of resistance (Reuveni *et al*. 1992). Morpurgo *et al*. (1994) also concluded that PO activity could be used as a parameter to discriminate between Fusarium wilt susceptible and tolerant clones of banana. Several authors have reported the increase of PO and PAL activity and also the phenolic content due to the application of bio-agents like *Trichoderma* spp., etc. in different crops (Hoffland *et al*. 1995; Podile and Laxmi 1998; Yedidia *et al*. 1999; Chen *et al*. 2000). In the present study, the analyses of PO and PAL both in *T. viride* applied and *Foc* challenge inoculated plants revealed that the induction of these defense related enzymes were significantly higher (> 50%) as compared to control plants and also the pathogen *Foc* alone inoculated plants. The total phenolics content also increased significantly in the *T. viride* NRCB1 applied and pathogen *Foc* challenge inoculated plants as compared to control. It was also observed that the increase of PO activity and the total phenolic content was maximum at the 4th day after treatment whereas the PAL activity was maximum at the 6th day after treatment (**Figs. 2A-C**). Morpurgo *et al*. (1994) reported that the activity of peroxidase was at least five times higher in the roots and corm tissues of *Foc*-resistant banana variety than in the susceptible variety. Inoculation of resistant plants with *Foc* resulted in a 10-fold increase in PO activity after seven days of inoculation, whereas, the susceptible variety exhibited only a slight increase in PO activity. Yedidia *et al*. (1999) reported that root inoculation of *T. harzianum* increased peroxidase and chitinase activities in leaves of cucumber seedlings. The application of *Trichoderma* isolate T39 also induced plant defense against *Botrytis cinerea* in tomato, lettuce, pepper, bean and tobacco (Demeyer *et al*. 1998). When cucumber roots were inoculated with *Pseudomonas corrugate* strain 13, peroxidase and polyphenol oxidase activities were increased in roots 2-5 days after bacterization in cucumber seedlings and also due to challenge inoculation with *Pythium aphanidermatum* (Chet *et al*. 1979). Podile and Laxmi (1998) also reported that bacterization of pigeonpea seeds with *Bacillus subtilis* AF1 increased the peroxidase activity from day 1 to day 7 and reduced Fusarium wilt incidence caused by *Fusarium udum*. The increased PO and PAL activities and thus increased accumulation of phenolics in the *T. viride* NRCB1 applied banana plants might have induced resistance against *Foc* by either making physical barrier stronger or chemically impervious to the hydrolytic enzymes produced by the pathogen. To conclude, the rice chaffy grain formulation of *T. viride* NRCB1 would be beneficial to the banana growers worldwide as the *T. viride* stain NRCB1 is most effective and its mass production method is easy and cheap for the management of this highly destructive disease of banana.

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