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Control of sheath blight disease in rice by thermostable secondary metabolites of *Trichothecium roseum* MML003

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Abstract The aim of the study is to investigate the biocontrol mechanisms of *Trichothecium roseum* MML003 against the rice sheath blight (ShB) pathogen, *Rhizoctonia solani* as the former exhibited strong antagonistic activity against the latter. It has been found that *T. roseum* MML003 did not show any hyperparasitic interaction against *R. solani.* Further, it did not produce siderophores and hydrogen cyanide. However, the culture filtrate of *T. roseum* MML003 strongly inhibited the mycelial growth and sclerotial formation, its germination and viability, which proved that the biocontrol activity is antibiosis-mediated. The extracellular crude antifungal metabolites of *T. roseum* MML003 were thermo and photo-stable. Potted plant experiment showed that the crude metabolites of *T. roseum* MML003 effectively reduced the ShB disease in rice up to 47.7%. Thus, this study assumes significance as it provided further scope for the identification of antifungal metabolites from *T. roseum* MML003 and their possible use against sheath blight disease of rice.

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Keywords *Trichothecium roseum* · Antifungal secondary metabolites · Thermostability · *Rhizoctonia solani* · Sheath blight disease

Abbreviations

BCA	Biological control agent
GPA	Glucose peptone agar
GPYEA	Glucose peptone yeast extract agar
HCN	Hydrogen cyanide
NSA	Nutrient sucrose agar
PDA	Potato dextrose agar
PDYEA	Potato dextrose yeast extract agar
TLC	Thin layer chromatography
TPM	Trichothecene production medium
TPYEM	Trichothecene production yeast extract medium
ZOI	Zone of inhibition

Introduction

Search for environmentally and toxicologically safe, more selective and efficacious fungicides for the control of plant diseases is gaining momentum at present. Since our nature has diverse resources, regular attempts are being made to discover novel fungicidal molecules from plants and microorganisms. Antibiotic secondary metabolites of microorganisms are an attractive alternative for chemical fungicides (Schulz et al. 2002; Prabavathy et al. 2006;

2008). The successful development and usage of microbial compounds such as streptomycin, validamycin, oomycin, strobilurins and others have attracted researchers across the globe (Tanaka and Omura 1993). These compounds have also formed the basis for chemical synthesis programmes as lead molecules to derive new chemical products (Copping and Duke 2007). Fungicidally-active secondary metabolites have been found throughout the microbial system, with major discoveries especially from actinomycetes and basidiomycetes (Lange et al. 1993). Production of antimicrobial secondary metabolites has been reported in many fungal biocontrol agents (BCAs) (Gottlieb and Shaw 1970; Sivasithamparam and Ghisalberti 1998; Vyas and Mathur 2002; Mathivanan et al. 2008).

Trichothecium roseum is a common soil-inhabiting saprophytic fungus and it is also a weak pathogen to some crops especially trees in temperate regions (Hong and Michailides 1997). *Trichothecium roseum* has a synonym of *Cephalothecium roseum* (Skinner et al. 1947). *Trichothecium roseum* was reported as mycotoxicants, antiviral agents (Bawden and Freeman 1952) and producer of cell wall-degrading enzymes such as chitinase, cellulase, pectinase, amylase and protease (Balasubramanian et al. 2003; Srikalaivani 2004). *Trichothecium roseum* was also reported as a BCA against many phytopathogenic fungi both in temperate and tropical regions (Huang and Hokko 1993; Huang et al. 2000; Srikalaivani 2004; Selvakumar 2006). Besides its biocontrol potential, the relative importance of this antagonistic fungus in comparison with the most studied *Trichoderma* spp. is less. The antagonism exhibited by *T. roseum* varies with the target pathogens. It was identified as a mycoparasite and in some cases it was shown that the antimicrobial metabolites produced by this fungus make it as an effective BCA (Srikalaivani 2004; Selvakumar 2006). However, the role of *T. roseum* secondary metabolites in crop protection has not been adequately addressed. Therefore, this fungus can be an ideal choice to the researchers who are looking for newer bioactive metabolites.

Rice has been severely affected by several diseases and insect pests, of which, sheath blight (ShB) disease caused by *Rhizoctonia solani* [teleomorph: *Thanetophorus cucumeris*; anastomosis group 1 IA (AG-1 IA)] is becoming a serious concern for the successful cultivation of rice. It is one of the most destructive diseases of rice that occurs globally (Lee and Rush 1983; Slaton et al. 2003). The yield loss due to ShB was estimated between 6% and 50% in other countries, whereas in India, it was recorded up to 69% (Roy 1993; Savary et al. 2000; Tang et al. 2007). Hence, the present study has been focused on *T. roseum* for its antagonistic potential against the rice sheath blight pathogen, *R. solani* and investigates antifungal secondary metabolites.

Materials and methods

Cultures

The biocontrol strain of *T. roseum* MML003 was obtained from Prof. D. Lalithakumari, (Former Director, Centre for Advanced Studies in Botany, University of Madras) and the ShB pathogen, *R. solani* MML4001 was isolated from infected rice sheath. Both the cultures were maintained on potato dextrose yeast extract agar (PDYEA) containing (g l^{-1}) potato 200, dextrose 20, yeast extract 3, agar 20, pH 6.5–6.8 at our Biocontrol and Microbial Metabolites Laboratory.

In vitro evaluation of *T. roseum* MML003 against *R. solani* MML4001 for antagonistic activity

In vitro evaluation of *T. roseum* MML003 for antagonistic activity against *R. solani* MML4001 was carried out by the classical dual culture assay (Huang and Hoes 1976) in three different media such as glucose peptone agar (GPA) containing (g l^{-1}) glucose 10, peptone 1, agar 2% and pH 6.5; trichothecene production agar (TPA) containing (g l^{-1}) ammonium tartarate 2, dipotassium hydrogen phosphate 1, magnesium sulphate 0.5, potassium chloride 0.5, ferrous sulphate 0.01, glucose 50, corn steep liquor 10 ml, agar 2% and pH 6.5 and potato dextrose agar (PDA) containing (g l^{-1}) potato 200, dextrose 20, agar 2% and pH 6.5 with and without supplement of yeast extract (0.3%).

Mechanisms of biological control exhibited by *T. roseum* MML003 against *R. solani* MML4001

Trichothecium roseum MML003 was studied for various biocontrol mechanisms using standard techniques. A microscopic study was carried out to find out the mycoparasitism of *T. roseum* MML003 on *R.*

solani MML4001. Production of hydrogen cyanide (HCN) by *T. roseum* MML003 was tested on nutrient sucrose agar (NSA) medium supplemented with 4.4% glycine as suggested by Lorck (1948) using sodium picrate impregnated filter papers. A simple plate assay was performed to find out the effect of volatile compounds of *T. roseum* MML003 on the mycelial growth and sclerotial formation in *R. solani* MML4001. In this experiment, a mycelial disc of *T. roseum* MML003 measuring 8 mm was inoculated onto the surface of NSA medium in a basal lid of a Petri plate and the mycelial disc (8 mm) of *R. solani* MML4001 was inoculated onto the PDA medium in a basal lid of another Petri plate. Both the fungi-inoculated basal lids were combined together by parafilm such that the *T. roseum* MML003 inoculated lid was as basal and *R. solani* MML4001 inoculated lid as the upper sides. Separate *R. solani* MML4001 on PDA and *T. roseum* MML003 on NSA were also maintained for controls. Further, an uninoculated NSA medium was kept for the control with *R. solani* MML4001 in the basal lid to check the effect of NSA alone on mycelial growth, if any. All the plates were incubated at room temperature (28±2°C) and the growth of *R. solani* MML4001 was observed after 3 days. Siderophore production by *T. roseum* MML003 was tested as suggested by Meyer and Abdallah (1978) using both iron-amended and iron-free media. The role of iron competition for the inhibition of *R. solani* was assessed by dual-plate assay on PDA plates amended with 1 mM $FeCl_3$. Qualitative assay was performed to determine the lytic enzymes chitinase, amylase, lipase, caseinase, gelatinase and cellulase production by *T. roseum* MML003 using water agar supplemented with 1% of the respective substrate such as colloidal chitin, starch, Tween 20, casein, gelatin and CM cellulose.

Effect of culture filtrates of *T. roseum* MML003 on mycelial growth of *R. solani* MML4001

In order to select a suitable medium for the production of antifungal secondary metabolites, *T. roseum* MML003 was grown in three different media *viz.,* potato dextrose yeast extract broth (PDYEB), glucose peptone yeast extract broth (GPYEB) and trichothecene production yeast extract broth (TPYEB). Well diffusion assay (Prabavathy et al. 2006) was performed with filter-sterilised 7 day-old culture filtrate of *T. roseum* MML003 to determine the antifungal activity on *R. solani* MML4001.

Effect of *T. roseum* MML003 culture filtrate on sclerotial germination of *R. solani* MML4001

Plate assay

Uniform-sized sclerotia obtained from a 10 day-old *R. solani* MML4001 culture were soaked in filter-sterilised culture filtrate of 7 day-old *T. roseum* MML003 obtained from GPYEB for different time periods, blot-dried and inoculated on PDA. Sterile GPYEB-treated and also untreated sclerotia were maintained as controls. A sclerotium was considered non-germinating in the conditions where even after 3 days, no mycelial growth was observed.

Detached leaf assay

Pathogenicity of *R. solani* MML4001 sclerotia after *T. roseum* MML003 culture filtrate treatment was tested using a detached leaf assay. Healthy leaves from rice (*Oryza sativa*) cv. IR50 were collected, cut into 6 cm length segments, surface-sterilised using 2% sodium hypochlorite and washed with sterile distilled water. A small wound was created on the midrib of the abaxial side of each leaf segment. Culture filtrate-treated sclerotia for different time periods were placed on the leaf segments. Sterile medium-treated and untreated sclerotia were used as controls. The set-up was maintained in a moist chamber for 5 days, after which, the ShB lesion development in rice leaf segments was observed.

Effect of ethyl acetate extract of *T. roseum* MML003 culture filtrate on mycelial growth of *R. solani* MML4001

Trichothecium roseum MML003 was grown in 100 ml GPYEB in each 250 ml Erlenmeyer flasks. From the third day onwards, the culture was harvested up to 12 days to prepare ethyl acetate extracts. An equal amount of ethyl acetate was added to 100 ml of cell-free culture filtrate, kept under shaking conditions (150 rpm) overnight and the solvent fraction separated using a separating funnel. Filter-sterilised ethyl acetate fraction (100 µl) was placed in a well made in PDA medium using an 8 mm cork borer in the

periphery of the Petri plate. At the centre of the plate, a mycelial disc (8 mm) of *R. solani* MML4001 was placed. Ethyl acetate (100 µl) alone was placed in another well of the plate as the control. The plates were incubated at room temperature for 3 days and the zone of inhibition was measured.

Thin layer chromotographic analyses of ethyl acetate extracts

Ethyl acetate extracts of *T. roseum* MML003 were obtained as described above at different ages of culture. Ten µl from each of the ethyl acetate fractions were spotted on pre-coated silica gel TLC plates of grade F_{274} (E-Merck, Germany). The plates were developed with ethyl acetate: hexane at a 1:1 ratio and the R_f values of each fluorescent and non-fluorescent compounds were calculated.

Effect of temperature and light on crude metabolites of *T. roseum* MML003

Ethyl acetate extract was prepared as mentioned earlier using 500 ml of a 7 day-old culture filtrate of *T. roseum* MML003. It was concentrated in a rotoevoporator, dissolved in 50 ml of distilled water and filter-sterilised. The crude metabolites of *T. roseum* MML003 were incubated for 30 min at different temperatures *viz.,* 40 °C, 60 °C, 80 °C and 100 °C in a water bath and also autoclaved at 121 °C for 15 min. In addition, the crude metabolites were exposed separately to UV, fluorescent and sunlight for 30 min. Then the antifungal activity against *R. solani* MML4001 was tested using 100 µl of treated and untreated metabolites in a well diffusion assay.

Effect of crude metabolites of *T. roseum* MML003 on ShB suppression

A glasshouse experiment was conducted in potted plants using complete randomised block design (CRBD) with six replications. Nursery was prepared with rice seeds of cv. IR50, susceptible to ShB in earthenware pots contained sterile field soil. Rice seedlings were removed after 25 days and transplanted in bigger pots in different treatments. About 500 ml of 7 day-old culture filtrate of *T. roseum* MML4001 was lyophilised to powder, dissolved in 50 ml of distilled water and filter-sterilised. The obtained crude metabolite preparation was used to spray on rice plants. A prophylactic spray of 15 ml per pot was given prior to pathogen inoculation. Another spray was given after 2 days of pathogen inoculation and a final spray at 7 days after pathogen inoculation. The details of treatments are as follows:

Treatment 1 : Control (no pathogen, fungicide and crude metabolites of *T. roseum* MML003 inoculated or sprayed).
Treatment 2 : Pathogen (*R. solani* MML4001) alone inoculated without any spray.
Treatment 3 : Pathogen inoculated+crude metabolites of *T. roseum* MML003 sprayed at 15 ml pot^{-1}.
Treatment 4 : Pathogen inoculated+fungicide (carbendazim sprayed at 1 g l^{-1}).

Fifteen grams of rice hull: grain (1:3) inoculum of *R. solani* MML4001 was inoculated at the lower sheath of experimental plants at 45 days after transplanting. The ShB disease was scored after 15 days of pathogen inoculation following the scale of Standard Evaluation System developed by the International Rice Research Institute, Philippines (IRRI 2002). The disease incidence and disease suppression in the treated and control plants were calculated using the following formulae.

$$\text{ShB incidence}(\%) = \frac{\text{ShB lesion height}}{\text{Plant height}} \times 100$$

$$\text{ShB suppression}(\%) = \frac{\text{Control} - \text{Treated}}{\text{Control}} \times 100$$

Statistical analysis

All the experiments were run with six replicates with the exception of the glasshouse evaluation. The data were analysed by ANOVA using AGRES statistical software (Agres 1994).

Results

In vitro evaluation of *T. roseum* MML003 against *R. solani* MML4001 for antagonistic activity

Trichothecium roseum MML003 grew well and caused more mycelial suppression in *R. solani*

MML4001 when grown on GPYEA. In addition, the supplement of yeast extract at 0.3% greatly increased the antifungal activity of *T. roseum* MML003, irrespective of the medium used (Table 1). On GPYEA alone, a clear zone of *R. solani* MML4001 mycelial inhibition was observed. In all the yeast extract-amended media, a whitish thread-like mycelial tuft was formed at the line of mycelial interaction between *T. roseum* MML003 and *R. solani* MML4001. Moreover, no sclerotia were produced by *R. solani* MML4001 in the presence of *T. roseum* MML003 and the effect was seen more prominently in GPYEA (Fig. 1).

Fig. 1 Antagonistic activity of *T. roseum* MML003 against *R. solani* MML4001 Top row Left: Control *T. roseum* on GPA; Top Middle: *R. solani* and *T. roseum* on GPA; Top Right: Control *R. solani* on GPA; Bottom Left: Control *T. roseum* on GPYEA; Bottom Middle: *R. solani* and *T. roseum* on GPYEA; Bottom Right: Control *R. solani* on GPYEA

Mechanisms of biological control exhibited by *T. roseum* MML003 against *R. solani* MML4001

Observations under the microscope revealed that there was no hyperparasitic interaction such as coiling and penetration between *T. roseum* MML003 and *R. solani* MML4001. There was no colour change in picric acid-saturated filter paper when *T. roseum* MML003 was grown on NSA medium, which confirmed that the BCA did not produce HCN. However, an experiment was conducted to find the production of volatile metabolites other than HCN. Dual-bottom plate assay has confirmed that unknown volatile metabolites (not HCN) seem to have some effect on *R. solani* MML4001 mycelium (Fig. 2). However, the slight inhibitory effect of the unknown volatile metabolites was not consistent after 3 days of incubation. Interestingly, the sclerotial development in *R. solani* MML4001 was arrested by these unknown volatiles of *T. roseum* MML003. The spectrophotometric assay revealed that *T. roseum* MML003 did not produce any iron-binding molecules in GPYEB, even after 8 days of incubation. Since, there was no increased antifungal activity in the iron-free medium, it was concluded that *T. roseum* MML003 did not inhibit *R. solani* MML4001 by producing the iron-binding compounds, siderophores (data not shown). However, it was interesting that the addition of iron at 1 mm as $FeCl_3$ increased the antagonistic activity of *T. roseum* MML003. Qualitative enzyme production assay revealed that this antagonist did not produce chitinase,

Table 1 Antagonistic activity of *T. roseum* MML003 against *R. solani* MML4001 in different media

Medium	Growth in control plate (cm)		Growth in dual plate (cm)		Inhibition of *R. solani* mycelium (%)
	T. roseum	*R. solani*	*T. roseum*	*R. solani*	
PDA	5.5 ± 0.36^{a}	3.2 ± 0.11^{e}	5.1 ± 0.1^{b}	1.2 ± 0.05^{de}	62.2 (53.1)^{c}
PDYEA	4.3 ± 0.11^{c}	5.9 ± 0.19^{c}	4.1 ± 0.11^{e}	1.2 ± 0.1^{ae}	78.6 (62.5)^{b}
TPA	5.1 ± 0.1^{b}	3.4 ± 0.25^{e}	4.7 ± 0.1^{c}	0.8 ± 0.15^{b}	74.8 (60.0)^{b}
TPYEA	4.9 ± 0.05^{b}	4.5 ± 0.1^{d}	4.5 ± 0.2^{d}	0.96 ± 0.05^{bc}	78.6 (62.5)^{b}
GPA	4.1 ± 0.15^{c}	6.7 ± 0.2^{b}	4.7 ± 0.1^{c}	1.0 ± 0.05^{cd}	85.0 (67.3)^{a}
GPYEA	5.5 ± 0.11^{a}	7.1 ± 0.1^{a}	5.3 ± 0.32^{a}	0.66 ± 0.05^{a}	90.5 (69.5)^{a}

Values are mean of triplicates with standard deviation

Values in the parentheses are arcsine-transformed values

Values in a column with different letter(s) are significantly different at 5% level

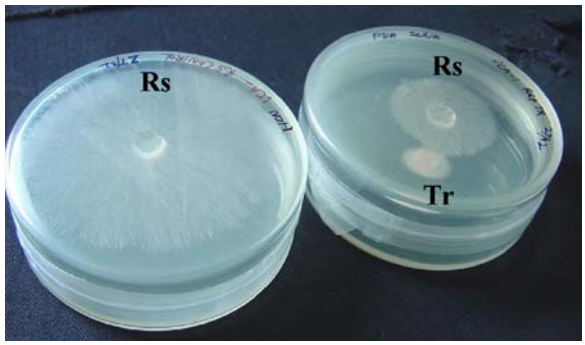

Fig. 2 Effect of volatile metabolites of *T. roseum* MML003 against *R. solani* MML4001 Left plate: *R. solani* (Rs) control plate with uninoculated NSA plate in the bottom lid; Right: *R. solani* in the top lid and *T. roseum* (Tr) in the bottom lid

amylase and lipase up to 10 days of incubation, but it produced caesinase, gelatinase and cellulase at varying degrees (data not shown). The culture filtrate of *T. roseum* MML003 obtained from GPYEB exhibited remarkable antifungal activity (17.3±0.57 mm ZOI) against *R. solani* MML4001 when compared to the culture filtrates of other media followed by PDYEB (11±0 mm) and TPYEB (13.6±0.5 mm).

Effect of *T. roseum* MML003 culture filtrate on sclerotial germination of *R. solani* MML4001

The culture filtrate treatment of *T. roseum* MML003 for 24 h significantly reduced the sclerotial germination of *R. solani* MML4001, in which only 10% germination was observed as compared to 100% and 50% germination in untreated and water-treated, respectively, controls (Table 2). Detached leaf assay also confirmed that treatment with culture filtrate of *T. roseum* MML003 for 12 h and 24 h greatly reduced the viability of sclerotia. Further, the sclerotia failed to establish ShB symptoms on detached leaves, whereas other treatments showed different levels of lesion development (Table 3).

Effect of ethyl acetate extract of *T. roseum* MML003 culture filtrate on mycelial growth of *R. solani* MML4001

Trichothecium roseum MML003 started to produce the antifungal metabolites on day three onwards and attained a maximum on day 7 and thereafter declined. However, the inhibitory effect of ethyl acetate extracts was observed until 10 days (Fig. 3). Further, lysis of

Table 2 Effect of *T. roseum* MML003 culture filtrate on sclerotial germination of *R. solani* MML4001

Treatment time	Sclerotial germination (%)		
	Untreated	Water	Culture filtrate
0 min	100 (89.09)^{h}	100 (89.09)^{h}	100 (89.09)^{h}
5 min	100 (89.09)^{h}	100 (89.09)^{h}	90 (71.56)^{g}
30 min	100 (89.09)^{h}	90 (71.56)^{g}	80 (63.43)^{f}
1 h	100 (89.09)^{h}	90 (71.56)^{g}	70 (56.79)^{e}
5 h	100 (89.09)^{h}	90 (71.56)^{g}	60 (50.76)^{d}
12 h	100 (89.09)^{h}	80 (63.43)^{f}	40 (39.23)^{b}
24 h	100 (89.09)^{h}	50 (48.84)^{c}	10 (18.33)^{a}

Values are mean of three replications

Values in the parentheses are arcsine-transformed values

Values in all the columns with different letter are significantly different at 5% level

R. solani MML4001 mycelia was observed following the treatment of ethyl acetate extracts, which suggested that *T. roseum* MML003 extracts, might have had fungicidal activity.

TLC analysis of ethyl acetate extract of *T. roseum* MML003

It was observed that the ethyl acetate extract obtained from 7 day-old culture filtrate of *T. roseum* MML003 had four major compounds as visualised after iodine reaction. In addition, two UV fluorescent minor

Table 3 Effect of *T. roseum* MML003 culture filtrate on sclerotial viability of *R. solani* MML4001 in detached leaf assay

Treatment time	ShB lesion size (cm)		
	Untreated	Water	Culture filtrate
0 min	5±0^{h}	5.0±0^{h}	5.0±0^{h}
5 min	5±0^{h}	5.0±0^{h}	4.7±0.14^{h}
30 min	5±0^{h}	5.0±0^{h}	4.3±0.04^{fg}
1 h	5±0^{h}	4.7±0.9^{g}	3.6±0.4^{c,d}
5 h	5±0^{h}	3.8±0.8^{df}	2.1±0.4^{b}
12 h	5±0^{h}	4.2±0.2^{f}	0±0 ^{a}
24 h	5±0^{h}	3.3±0.2^{c}	0±0^{a}

Values are mean of three replications with standard deviation

Values in all the columns with different letter(s) are significantly different at 5% level

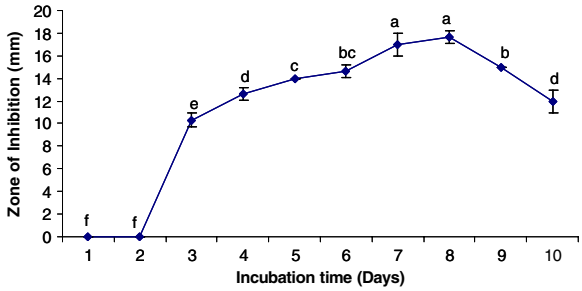

Fig. 3 Antifungal activities of ethyl acetate extracts of *T. roseum* MML003 against *R. solani* MML4001 Values are mean of three replications with standard deviation Values with same letter(s) are not significantly different at 5% level

compounds were also observed. The non-fluorescent compounds had R_f values of 0.97, 0.91, 0.75 and 0.51.

Effect of temperature and light on *T. roseum* MML003 metabolites

It was observed that *T. roseum* MML003 metabolites were relatively heat stable so that they could retain their antifungal activity even after autoclaving. However, autoclaving reduced the antifungal activity by 24.46% when compared to the untreated control (Fig. 4). Exposure to different light sources did not affect the antifungal activity of *T. roseum* MML003 metabolites as the values did not differ statistically at P=0.05.

Effect of crude metabolites of *T. roseum* MML003 on ShB suppression

In pathogen-alone treated plants, the number of both total tillers and productive tillers was reduced when

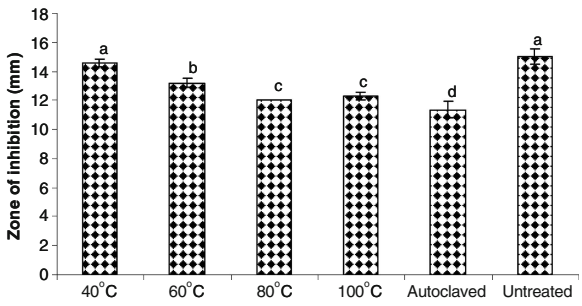

Fig. 4 Thermal stability of *T. roseum* MMl003 metabolites Values are mean of three replications with standard deviation Values with same letter are not significantly different at 5% level

compared to the uninoculated control where the total number of tillers was 24.2, and 15.2 of them were productive tillers. Spraying *T. roseum* MML003 crude metabolites significantly suppressed the ShB disease in rice compared to the untreated pathogen control. Minimum ShB incidence of 14.8% was recorded following the application of carbendazim and 16.8% incidence in crude metabolites-sprayed plants as against 32.1% in pathogen-inoculated plants. The disease reduction was 53.9% and 47.7% in carbendazim and crude metabolites-treated plants, respectively, compared to the pathogen-inoculated control (Table 4).

Discussion

The discovery of new biologically-active secondary metabolites is the goal of both pharmaceutical and agrochemical industries. Since these secondary metabolites are biologically-synthesised, they are highly selective for the target organism and hence, have little effect on beneficial organisms; besides, these metabolites are inherently biodegradable and often do not accumulate in nature (Suzni 1992; Yamaguchi 1996). From fungi, agrochemicals with new and interesting modes of action have been obtained (e.g., the unique way in which strobilurin inhibits mitochondrial respiration or trehalase inhibition by validamycin). However, a real breakthrough, resulting in a major new group of fungicides, based on naturally occurring metabolites is yet to come (Lange et al. 1993). Therefore, screening of various microorganisms for antifungal activities is always a continuing line of research, because ample possibilities exist to obtain newer molecules even from highly characterised microorganisms. In this context, *T. roseum* MML003 was studied for its biocontrol potential and role of its antifungal metabolites in controlling rice ShB pathogen, *R. solani*.

Initial screening revealed that the antagonistic effect of *T. roseum* MML003 was more prominent in GPYEA medium. It was found that yeast extract supplement enhanced the growth and antagonistic activity of *T. roseum* MML003, irrespective of the medium used. This could be due to the promotional effect of yeast extract on the synthesis of antimicrobial principles by *T. roseum* MML003, which confirms a previous report (Srikalaivani 2004). In TPYEM, a dark brown-coloured mycelial tuft was

Table 4 Evaluation of crude metabolites of *T. roseum* MML003 on ShB suppression

Treatment	Plant height (cm)	Lesion height (cm)	No. of tillers	No. of infected tillers	No. of productive tillers	Disease incidence (%)	Disease suppression (%)
Untreated	56.2±3.42[a]	0±0[a]	24.2±1.3[a]	0±0[a]	15.2±3.34[a]	0 (0)[a]	0
Pathogen alone	48.6±3.57[b]	15.6±4.50[c]	14.0±1.41[c]	7.8±2.59[c]	6.0±1.73[b]	32.1 (30.2)[c]	0
Pathogen+crude metabolites	48.2±4.71[b]	8.2±1.3[b]	20.8±2.28[b]	8.6±4.5[c]	14.2±6.68[a]	16.8 (24.0)[b]	47.7
Pathogen+carbendazim	46.4±3.91[b]	8.0±1[b]	22.4±3.28[ab]	4.4±1.67[b]	14.4±4.39[a]	14.8 (23.8)[b]	53.9

Values are mean of three replications with standard deviation

Values in the parentheses are arcsine-transformed values

Values in a column with different letter are significantly different at 5% level

observed whereas in other media it was white. This indicated that in TPYEM, *T. roseum* MML003 could have produced more trichothecene compounds since this medium was designed for the synthesis of trichothecenes (Freeman and Morrison 1948). The reduction of *R. solani* MML4001 mycelial growth and inhibition of sclerotial production by *T. roseum* MML003 was greater in GPYEA; the medium was therefore selected for further studies.

Huang and Hokko (1993), Vakkili (1985) and Urbasch (1992) have reported mycoparasitism as a biocontrol mechanism exerted by *T. roseum* against *Sclerotiana sclerotiorum, Botrytis cinerea, Fusarium oxysporum* and *Pestalotia funerea*. However, our microscopic studies did not show any involvement of mycoparasitism in the control of *R. solani* MML4001 by *T. roseum* MML003. The biocontrol mechanisms of antagonistic fungi such as *Trichoderma* depend on the biocontrol strain, the pathogen, the crop plant, and the environmental conditions, including nutrient availability, pH, temperature, and iron concentration (Benitez et al. 2004; Harman 2000; 2006; Woo et al. 2006; Vinale et al. 2008). This is also the case with *T. roseum*. Even though an antagonistic fungus may be genetically equipped with multiple biocontrol mechanisms, the operation of these depends on various factors (Benítez et al., 2004).

Mercier and Manker (2005) provided several examples of fungal BCAs suppressing plant pathogens by the production of volatile metabolites. Upadhyay (1981) demonstrated the inhibitory effects of volatile metabolites produced by *T. roseum* against *P. funerea*, a leaf spot pathogen of *Eucalyptus globules.* Although *T. roseum* MML003 did not produce the major antifungal volatile metabolite HCN, the possibility of the involvement of other volatile metabolites as seen in the dual bottom plates assay was not ruled out. The momentary inhibitory effect on *R. solani* MML4001 mycelium might be possible due to the fungistatic nature of the volatiles.

Several BCAs were reported to produce low molecular weight iron chelating substances called siderophores that suppress the pathogen (Jagadeesh et al. 2001; Thomashow and Weller 1990). Both spectrophotometric and plate assays have confirmed that there has been no involvement of siderophores in the inhibition of *R. solani* MML4001 mycelial growth by *T. roseum* MML003. Although *T. roseum* was reported to produce several lytic enzymes including chitinase (Srikalaivani 2004) and Li et al. (2004) have shown the inhibitory effect of *T. roseum* chitinase on the myecelial growth of *Fusarium moniliformae,* we could not detect the chitinase production by *T. roseum* MML003 even when the medium was supplemented with colloidal chitin, an inducer of chitinase enzyme. Interestingly, our microscopic observations revealed disintegration along with some spherical swelling in the pathogen's mycelium. The reason for these changes in host structures could be either lytic enzymes like protease, or the other secondary metabolites produced by *T. roseum* MML003, could have affected the plasma membrane integrity of *R. solani* MML4001 as suggested by Sbrana et al. (2000). Finally the well diffusion assay revealed that antifungal agents of *T. roseum* MML003 were in the culture filtrates, probably the secondary metabolites.

Sclerotial production is the main advantage of *R. solani* for survival in soil. Moreover, sclerotia are

highly resistant resting bodies and can easily be transmitted to other fields through irrigation. Hence, the successful antifungal metabolites should suppress sclerotial germination and viability. Huang and Hokko (1993) established the inhibitory effect of culture filtrate of *T. roseum* against the sclerotia of *S. sclerotiorum*. *Trichothecium roseum* MML003 culture filtrate has also been shown to inhibit sclerotial germination in *R. solani* MML4001 when soaked for >12 h. Soaking the sclerotia for a lesser duration did not show any significant reduction in germination as well as ShB lesion development in the detached leaf assay. The inhibitory effect on sclerotial germination might be due to the toxic effects of metabolites of *T. roseum* MML003 present in the culture filtrate.

Freeman and Morrison (1948) extracted antibacterial metabolites from the culture filtrate of *T. roseum* using chloroform and Machida and Nozae (1972) have reported the biosynthesis of various trichothencenes and related compounds. Subsequently, Urbasch (1985) reported the production of water-soluble heat-resistant metabolites by *T. roseum* and their toxic effects on mycelial growth and conidial germination of *P. funerea.* Similarly, in the present study, the antifungal agents from *T. roseum* MMl003 culture filtrate were successfully extracted using ethyl acetate and the antifungal activity of the obtained extracts were confirmed through a well diffusion assay. In a similar study, the crude metabolites of *Helminthosporium gramineum* were tested for their antagonistic effects towards *R. solani,* where both the culture filtrate and ethyl acetate extract significantly reduced the *in vitro* growth of *R. solani* (Duan et al. 2007). Kavitha et al. (2005) isolated a heat-stable antifungal protein from *Bacillus* sp. that suppressed the mycelial growth and sclerotial germination of ShB pathogen of rice. Similarly, the crude metabolites of *T. roseum* MML003 retained their antifungal activity even after autoclaving at 121 °C for 15 min. Further, they also exhibited photo-stability under different light regimes. These properties may be of more practical benefit, as the metabolites need to be stable in various temperatures and in sunshine in rice fields of different regions.

Management of ShB is considered important in the present day situation because the disease is most prevalent and also a serious limiting factor for the successful cultivation of rice worldwide. In spite of the use of many modern chemical fungicides, management of the ShB in the farmers' fields is still a difficult task. Interestingly, the crude metabolites of *T. roseum* MML003 effectively reduced ShB disease and this effect was comparable with the commonly used fungicide, carbendazim. Typical ShB symptoms caused by *R. solani* MML4001 were clearly visible in pathogen-inoculated plants but it was rare in crude metabolites and carbendazim-treated plants. This clearly indicated the efficacy of the antifungal metabolites produced by *T. roseum* MML003 and there is great scope for developing these metabolites as agrochemicals. Interestingly, the culture filtrate in its aqueous state itself greatly reduced ShB incidence. In addition, the antifungal metabolites of *T. roseum* MML003 exhibited astonishing thermo- and photostability which are added advantages. Besides, they also increased the number of productive tillers compared to the control plants. In a similar study, the treatments of *H. gramineum* crude metabolites effectively reduced the development of ShB with no significant adverse effects on the growth and yield attributes of rice plants (Duan et al. 2007). Ma et al. (2008) also reported the suppression of *Phytophthora* rot of chilli pepper using sterilised culture filtrates of *Penicillium striatisporum* Pst10. While a large number of reviews have clearly reported the voluminous studies on *Trichoderma* (Benitez et al. 2004; Harman 2000; 2006; Woo et al. 2006; Vinale et al. 2008), results of the present study undoubtedly prove the potency of the less-studied *T. roseum* as a fungal BCA.

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