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ORIGINAL PAPER

Fungal endophytes of turmeric (*Curcuma longa* L.) and their biocontrol potential against pathogens *Pythium aphanidermatum* and *Rhizoctonia solani*

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Abstract
Endophytic fungi have been isolated from the healthy turmeric (*Curcuma longa* L.) rhizomes from South India. Thirty-one endophytes were identified based on morphological and ITS–rDNA sequence analysis. The isolated endophytes were screened for antagonistic activity against *Pythium aphanidermatum* (Edson) Fitzp., and *Rhizoctonia solani* Kuhn., causing rhizome rot and leaf blight diseases in turmeric respectively. Results revealed that only six endophytes showed > 70% suppression of test pathogens in antagonistic dual culture assays. The endophyte *T. harzianum* TharDOB-31 showed significant in vitro mycelial growth inhibition of *P. aphanidermatum* (76.0%) and *R. solani* (76.9%) when tested by dual culture method. The SEM studies of interaction zone showed morphological abnormalities like parasitism, shriveling, breakage and lysis of hyphae of the pathogens by endophyte TharDOB-31. Selected endophytic isolates recorded multiple plant growth promoting traits in in vitro studies. The rhizome bacterization followed by soil application of endophyte TharDOB-31 showed lowest Percent Disease Incidence of rhizome rot and leaf blight, 13.8 and 11.6% respectively. The treatment of TharDOB-31 exhibited significant increase in plant height (85 cm) and fresh rhizome yield/plant (425 g) in comparison with untreated control under greenhouse condition. The confocal microscopy validates the colonization of the TharDOB-31 in turmeric rhizomes. The secondary metabolites in ethyl acetate extract of TharDOB-31 were found to contain higher number of antifungal compounds by high resolution liquid chromatograph mass spectrometer analysis. Thereby, endophyte *T. harzianum* isolate can be exploited as a potential biocontrol agent for suppressing rhizome rot and leaf blight diseases in turmeric.

Keywords Antagonism · Biocontrol · Fungal endophytes · Growth promotion · HR-LCMS · Leaf blight · Rhizome rot · SEM

Introduction

Turmeric (*Curcuma longa* L.), a member of the ginger family (*Zingiberaceae*) is one of the major rhizomatous spice used worldwide in alternative systems of medicine. The whole plant is aromatic, but the underground rhizomes, fresh or processed are the most valuable commodities. The active compound 'curcumin' is believed to have a wide range of biological effects including anti-inflammatory, antioxidant, antitumour, antibacterial and antiviral activities (Gupta et al. 2012a). India is the major producer and exporter of turmeric in the world contributing for 80% of the world production (Thiripurasundari and Selvarani 2014).

The fungus *P. aphanidermatum* and *Rhizoctonia solani* cause rhizome rot and leaf blight diseases in turmeric plants and reduce commercial value (Park 1934; Roy 1992). It has been noted that chemical fungicides like ridomil, metalaxyl, carbendazim (0.1%) and mancozeb (0.25%) were commonly used to manage rhizome rot and leaf blight diseases (Rathaiah 1982; Muthukumar et al. 2011). Use of chemical fungicides is of public concern as it causes various human health problems and also pathogens build resistance against fungicides.

The increasing awareness of fungicide related hazards has emphasized the need for adopting biological methods as an alternative ecofriendly disease control strategy (Wahab 2009) Biological control measures rely on the use of antagonistic organisms against target pathogens. Endophytes are

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microorganisms that live within the plant tissue as endosymbionts, without causing any disease symptoms. Some of them are mutualistic symbionts with beneficial effect on their host such as plant growth promotion, resistance against disease or environmental stress (Colla et al. 2015; Lledo et al. 2016). A large number of endophytic fungi have been isolated from plant species (Nisa et al. 2015). A more precise assessment of diversity and identification of fungi can be achieved using the polymerase chain reaction (PCR) of the internal transcribed spacer (ITS) of the fungal nuclear ribosomal DNA (Borneman and Hartin 2000; Rodriguez et al. 2009). ITS is recommended as universal barcode to identify fungal species (Schoch et al. 2012).

Endophytic fungi are promising biocontrol agents (BCA's) as they occupy internal living tissues of plants and due to close proximity to plant pathogens. Endophytic fungi were used as BCA's like *Trichoderma viride, Aspergillus fumigatus* and *Penicillium atrovenetum* isolated from medicinal plant *Artemisia nilagirica* against *Phytophthora infestans* infecting potato (Myrchiang et al. 2014). Similarly, fungal endophytes isolated from healthy *Theobroma cacao* tissues viz., *Colletotrichum gloeosporioides, Clonostachys rosea* and *Botryosphaeria ribis* were screened in vitro for antagonism against major pathogens of *cacao*. Endophyte *C. gloeosporioides* and *C. rosea* significantly decreased pod loss due to black pod rot and incidence of cacao pods with sporulating lesions of *M. roreri* was reported earlier (Mejia et al. 2008). Further, endophytic *C. rosea* has been reported to control *Botrytis cinerea* in roses (Morandi et al. 2000). Endophytes and mycoparasites associated with *Theobroma gileri* used as BCA's agents of *cocoa* diseases (Evans et al. 2003). Endophyte *Gliocladium catenulatum* isolated from (*Theobroma cacao* L.) used as biological control of Witches Broom Disease caused by *Crinipellis perniciosa* (Rubini et al. 2005). Endophytic fungi harbored in *Cannabis sativa* L. reported as potential BCA's against two host phytopathogens namely *Botrytis cinerea* and *Trichothecium roseum* (Kusari et al. 2013). The endophytic fungi were isolated from healthy tissues of vegetable plants viz., cucumber, red pepper, tomato, pumpkin and Chinese cabbage. Among the isolates the endophyte *F. oxysporum* isolated from roots of red pepper showed potential disease control efficacy against tomato late blight caused by *Phytophthora infestans* (Kim et al. 2007).

Trichoderma spp. are known to be the most commonly used antagonists against *P. aphanidermatum* and *R. solani* (Chet et al. 1981). Apart from their antagonistic activity, *Trichoderma* strains have also demonstrated growth promoting properties and is used as rhizome treatment in turmeric (Ushamalini et al. 2008). Promotion of plant growth is the major contribution of fungal symbiosis. The growth promotion of fungal endophytes is attributed to the production of ammonia and phytohormones like indole acetic acid (IAA), HCN and by production of cell-wall degrading enzymes such as cellulase, pectinase and protease (Fouda et al. 2015).

The production of antibiotic secondary metabolites is often correlated to the biocontrol activity of *Trichoderma* isolates (Vinale et al. 2006). *Trichoderma* secondary metabolites are chemically diverse and their production varies greatly between species and between isolates of the same species (Vinale et al. 2009). The *Trichoderma* spp. known to produce secondary metabolites with a key role in antagonistic activities and was used as BCA's (Vinale et al. 2014).

To the best of our knowledge there is no information available on the potential fungal endophytes on growth promotion and biocontrol of rhizome and leaf blight diseases of turmeric. This study was taken up to profile the endophytic fungi associated with turmeric and to evaluate their antagonistic activities, biocontrol potential and plant growth promotion both in vitro and in vivo conditions against *P. aphanidermatum* and *R. solani* pathogens which cause rhizome rot and leaf blight diseases of turmeric respectively.

Materials and methods

Collection of samples

A total of 250 healthy and 50 diseased turmeric samples were collected for endophytes and pathogens (*P. aphanidermatum* and *R. solani*) isolation respectively from different agroclimatic regions of Karnataka, Andhra Pradesh, Kerala, Tamilnadu states of India during July–December 2014 (turmeric plant growth stage was 4–6 months). The samples were collected in polythene bags, labeled and stored in refrigerator at 4 °C and processed within 48 h of collection.

Isolation of endophytic fungi

Healthy turmeric rhizomes were washed in running tap water, then surface sterilized by soaking in 70% ethanol for 1 min, followed by 3.5% sodium hypochloride for 3 min and finally rinsed in sterile distilled water three times at 1 min intervals. After surface sterilization, the epidermis was peeled off and the rhizomes cut into pieces of 5 mm^2 using sterile blades. Two hundred segments of each sample were used to isolate fungal endophytes and colonization frequency percentage (CF %) was calculated. Each petridish (90 mm diameter) containing water agar medium supplemented with streptomycin sulfate (100 mg/l to suppress bacterial growth) were placed with 10–15 segments (Nalini et al. 2014). To verify the efficacy of surface sterilization of the rhizomes, 100 µl of the last rinse was added on potato dextrose agar (PDA) medium.

The petridishes were incubated at 23 °C with 12 h light and dark cycles up to 3–4 weeks and the segments were

examined periodically. The outgrowing fungal colonies were transferred to new petridishes with PDA medium for further identification. All the endophytic fungal isolates were numbered as DOB# series, maintained in cryovials on PDA layered with 15% glycerol (v/v) at − 80 °C in a deep freezer (Cryoscientific Pvt. Ltd., Chennai, India) at the Department of Studies in Biotechnology, University of Mysore, Mysuru, India. The Colonization frequency of endophytes was calculated according to the method of Fisher and Petrini (1987) as follows,

$$\text{Colonization Frequency (CF\%)} = \frac{\text{Number of segments colonized by fungus}}{\text{Total number of segments}} \times 100$$

Isolation and pathogenicity of fungal pathogens

The surface sterilized diseased rhizome pieces (1.5 cm) were blotted dry on sterile filter paper, and placed on P5ARP medium (*Pythium* selective medium) containing 0.005 g of pimaricin, 0.25 mg of ampicillin, 0.01 g of rifampicin, and 0.10 g of pentachloronitrobenzene per liter in corn meal agar (Jeffers and Martin 1986) for *P. aphanidermatum* and on PDA medium for *R. solani* isolation. The plates were incubated at room temperature (28–30 °C), the colony morphology was studied (Van der Plaats-Niterink 1981). Hyphae were successively transferred to the new PDA medium and the cultures were maintained on slants. Pathogenicity of the isolated fungi was established by inoculating to susceptible cultivar 'Erode local' (Johnston and Booth 1983; Sriraj et al. 2014).

Morphological identification of endophytes and pathogens

Morphological studies were carried out to identify endophytes and pathogens under the stereomicroscope (Leica DM-LS 2 Trinocular Research Microscope). The slides were prepared for light microscopy by staining with lactophenol blue and identified comparing with the description given in standard manuals (Barnett and Hunter 1972; Domsch et al. 1980; Gilma and Joseph 1998).

Molecular characterization of fungal endophytes and pathogens

DNA was extracted using HipurA Fungal DNA Purification kit of Himedia. The quality and quantity of DNA was determined using NanoDrop spectrophotometer (2000 C, Thermo Scientific, Japan). The integrity of the DNA was assayed by 0.7% agarose gel electrophoresis.

The DNA was amplified using universal primers for ITS 1 and ITS 4 region [forward 5′-TCC GTA GGT GAA CCT GCG G-3′ and reverse 5′-TCC TCC GCT TAT TGA TAT GC-3′] (White et al. 1990). The PCR reaction was performed in 50 µl final reaction volume containing 5 µl of 10× PCR buffer, 8 µl of 25 mM $MgCl_2$, 2.5 µl of 1.25 mM dNTP, 0.2 µl of each primer (20 µM), 100 ng of DNA and 0.2 µl Taq DNA polymerase (5 U/µl) (Sigma-Aldrich, Bangalore) in a BioRad thermal cycler programmed for initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 56 °C for 1 min, extension at 72 °C for 2 min. At the end of the amplification reaction, a final extension step was achieved at 72 °C for 10 min. Fifteen microliters of the PCR products from each PCR reaction were electrophoresed on 1% (w/v) agarose gel containing 5 mg/ml of ethidium bromide in a 1×TBE (pH 8.4). To estimate the size of the PCR products, a 100 bp molecular ladder (Sigma-Aldrich, Bangalore) was used. The electrophoresis was carried out using 100 V. The gel was visualized and photographed using Gel Documentation system (Gel Doc 2000, BioRad, California, USA).

Sequencing and phylogenetic analysis

The amplified products were sequenced at Chromous Biotech. Pvt. Ltd, Bangalore. The sequences obtained were further analyzed by BLAST program of National Center for Biotechnology Information (NCBI, website: http://www.ncbi.nlm.nih.gov). The ITS sequence was compared using nucleotide BLAST with default settings and megablast (highly similar sequences) as the selected program. Species identification was determined from the lowest expect value (E-value) of the BLAST output and the similarity percentage. The analysed sequences were submitted to Genbank (NCBI) and accession numbers were obtained.

The ITS sequence analysis was done for molecular authentication of the endophytic species. All the nucleotide sequences of isolated endophytes and the sequences of some nearest neighbours of the endophytic fungal isolates retrieved from Genbank (NCBI) were used to construct the phylogenetic tree. All sequences were aligned using CLUSTAL W algorithm (Thompson et al. 1994) and phylogenetic analysis was conducted using Mega 6 software. Phylogenetic inference was performed by the Maximum Parsimony (MP) method, with bootstrap values calculated from 1000 replicate runs (Tamura et al. 2013).

Assessment of in vitro antagonism by dual culture method

The antagonistic effect of fungal endophytes on *P. aphanidermatum* and *R. solani* pathogens was evaluated using dual culture technique. In this method 5 mm^2 size of endophyte culture and same size of test pathogen were placed 1 cm away from the periphery of the petriplate containing PDA media in opposite direction. The petriplate inoculated with pathogen alone in the absence of antagonist (endophytes) served as control and the experiment was done in triplicates. The inoculated plates were incubated under alternating periods of 12 h darkness and 12 h of daylight at 25 ± 2 °C until the control plates were completely covered by pathogen mycelia (Lahlali et al. 2007). The radial mycelial growth of pathogen towards the antagonistic fungus (T) and that on a control plate (C) were measured and the mycelial growth inhibition (I) was calculated using the following formula:

$$I = C - T \times 100/C$$

where C is the radial growth measurement of the pathogen in control (main axis), T is the radial growth of the pathogen towards the antagonistic fungus, I is the percent inhibition.

Scanning electron microscope (SEM) studies of the interaction zone

For SEM analysis, the zone of interaction between pathogen and endophyte from the dual culture was taken and the samples were exposed to 2% osmium tetroxide for 24 h at 20 °C, later it was transferred to copper stubs over double adhesive carbon tape. They were then coated with gold in a POLARON, AU/PD sputter and scanned in SEM, S-3400N model (Japan) at 5.00 kV and the abnormalities in the fungal hyphae were recorded.

In vitro evaluation of plant growth promoting traits of endophytes

In in vitro study of antagonism the six endophytic fungal isolates viz., Thar DOB-2, Thar DOB-4, Tasp DOB-12, Tatro DOB-17, Tasp DOB-19 and Thar DOB-31 that showed > 70% inhibition against pathogens (Table 1). These isolates were tested for their plant growth promoting (PGP) traits.

Indole-3-acetic acid production

For detection of IAA production by endophytic fungal isolates, fungal disc of 1 cm diameter were inoculated to 20 ml of Czapex Dox (CD) broth supplemented with 0.5 g^{-1} of tryptophan and incubated for 7 days at 28 °C, performed in triplicates. Culture suspension (5 ml) was transferred to test tubes and centrifuged at 6000 rpm for 10 min. One milliliters of the supernatant was mixed with 4 ml of Salkowski reagent (Fouda et al. 2015) and the tubes were incubated for 30 min. IAA production was observed as the development of a pink colour. The low IAA production (light pink colour '+'); medium IAA production (Dark pink colour '++'); high IAA production (Dense pink colour, '+++') was recorded.

Hydrogen cyanide (HCN) production

HCN production by the fungal agents was tested following the method of Triveni et al. (2013) with slight modifications. The fungal strain was inoculated on PDA slants amended with glycine at 4.4 g/l. Whatman No.1 filter paper strips were dipped in 0.5% picric acid in 2% sodium carbonate solution. The strips were inserted in the top of each test tubes and sealed with parafilm and incubated at 28 °C for 7 days. A change of colour of the filter paper from yellow to light brown, brown or reddish-brown was recorded as weak (+), moderate (++) or strong (+++) reaction respectively.

Screening for cellulolytic activity

Isolated endophytic fungal strains were screened for cellulolytic activity by inoculating into petriplates containing agar medium containing carboxymethylcellulose (Pereira and Castro 2014). After 5 days of incubation at 28 °C, the plates were flooded with congo red solution for 15 min (1 mg/l). The plates were destained with 1 M Nacl for 15 min by decanting excess congo red solution. A clear zone was considered as cellulase positive (Gupta et al. 2012b).

Phosphate solubilizing ability

All endophytic fungal isolates were screened for their phosphate solubilizing activity on Pikovskaya's medium (Himedia) by spot inoculation method and incubated at 28 °C for 7 days. The production of a halo around a growing colony indicated phosphate solubilization (Singh and Reddy 2011).

Efficacy of endophytes on rhizome rot and leaf blight pathogens under green house conditions

Four promising endophytic isolates viz., *T. harzianum* (Thar DOB-2), *T. atroviride* (Tatro DOB-17), *T. asperellum* (Tasp DOB-19), *T. harzianum* (Thar DOB-31) were selected for green house studies based on in vitro antagonism studies and PGP traits. Two sets of experiments were performed to analyze the efficacy of the endophytes in controlling the rhizome rot and leaf blight diseases of turmeric under green

Table 1 Molecular identification of antagonistic fungal endophytes using rRNA gene (ITS1 and ITS4) and their effect on the in vitro mycelial growth of pathogenic fungus *P. aphanidermatum* and *R. solani*

Sl. no.	Geographic locations	Endophytic fungal isolates	CF%	Closest related species	Identity in percent (%)	Amplicon size (BP)	GenBank accession numbers	% Growth inhibition *P. aphanidermatum*	% Growth inhibition of *R. solani*
1	Dharwad, Karnataka	TharDOB-1	10	*T. harzianum*	99	541	KU365129	63.0 ± 0.93	65.6 ± 0.86
2	Chamarajanagar, Karnataka	TharDOB-2	14	*T. harzianum*	99	544	KU365130	73.6 ± 0.74	70.2 ± 0.98
3	Dharwad, Karnataka	TharDOB-3	11	*T. harzianum*	100	545	KU365131	71.0 ± 0.68	69.0 ± 0.43
4	Dharwad, Karnataka	TharBOD-4	16	*T. harzianum*	100	549	KU365132	70.8 ± 0.47	72.2 ± 0.58
5	Dakshina Kannada, Karnataka	PmicDOB-5	10	*P. microspora*	99	475	KU365133	57.0 ± 0.37	57.4 ± 0.86
6	Chamarajanagar, Karnataka	ThamDOB-6	16	*T. hamatum*	99	542	KU365134	69.6 ± 0.86	68.9 ± 0.72
7	Bhavanisagar, Tamilnadu	TharDOB-7	15	*T. harzianum*	99	531	KU365135	68.3 ± 0.77	69.5 ± 0.76
8	Kollegal, Karnataka	TharDOB-8	18	*T. harzianum*	100	531	KU365136	58.9 ± 0.39	60.2 ± 0.71
9	Dandeli, Karnataka	FsolDOB-9	9	*F. solani*	99	519	KU517655	52.0 ± 0.41	53.6 ± 0.34
10	Dakshina Kannada, Karnataka	FsolDOB-10	13	*F. solani*	99	506	KU517656	55.2 ± 0.86	52.6 ± 0.91
11	Erode, Tamilnadu	ThamDOB-11	20	*T. hamatum*	99	542	KU517657	66.8 ± 0.70	63.8 ± 0.84
12	Dharwad, Karnataka	TaspDOB-12	12	*T. asperellum*	100	541	KU662964	72.7 ± 0.93	70.4 ± 0.76
13	Dharwad, Karnataka	TaspDOB-13	10.5	*T. asperellum*	99	542	KU662965	58.0 ± 0.92	60.0 ± 0.45
14	Dharwad, Karnataka	TaspDOB-14	14	*T. asperellum*	99	546	KU662966	73.2 ± 0.56	69.6 ± 0.68
15	Coimbatore, Tamilnadu	TaspDOB-15	22	*T. asperellum*	99	538	KU662967	62.9 ± 0.72	63.6 ± 0.37
16	Coimbatore, Tamilnadu	TaspDOB-16	26	*T. asperellum*	99	549	KU662968	59.3 ± 0.64	63.0 ± 0.53
17	Kollegal, Karnataka	TatroDOB-17	18.5	*T. atroviride*	100	547	KU662969	73.8 ± 0.48	72.6 ± 0.90
18	Calicut, Kerala	TaspDOB-18	16	*T. asperellum*	99	541	KU662970	66.0 ± 0.85	68.2 ± 0.43
19	Salem, Tamilnadu	TaspDOB-19	20	*T. asperellum*	100	540	KU662971	75.0 ± 0.53	76.5 ± 0.73
20	Calicut, Kerala	TaspDOB-20	14	*T. asperellum*	99	543	KU662972	60.2 ± 0.72	62.0 ± 0.71
21	Kadapa, Andhra Pradesh	FproDOB-21	8	*F. proliferatum*	100	500	KU865563	57.4 ± 0.98	59.0 ± 0.73
22	H.D.Kote, Karnataka	FproDOB-22	18	*F. proliferatum*	100	424	KU865563	60.1 ± 0.73	61.4 ± 0.63
23	Guntur, Andhra Pradesh	TaspDOB-23	10	*T. asperellum*	98	450	KU865564	57.8 ± 0.59	60.0 ± 1.00
24	Hassan, Karnataka	TaspDOB-24	15	*T. asperellum*	100	546	KU865565	61.8 ± 0.72	62.2 ± 0.96
25	H.D.Kote, Karnataka	TaspDOB-25	20	*T. asperellum*	99	483	KU865566	62.4 ± 0.80	62.0 ± 0.73
26	Mysore, Karnataka	FsolDOB-26	24	*F. solani*	100	439	KU865567	56.0 ± 0.75	58.2 ± 0.92

Table 1 (continued)

Sl. no.	Geographic locations	Endophytic fungal isolates	CF%	Closest related species	Identity in percent (%)	Amplicon size (BP)	GenBank accession numbers	% Growth inhibition *P. aphanidermatum*	% Growth inhibition of *R. solani*
27	Wayanad, Kerala	TharDOB-27	16	*T. hamatum*	100	554	KU865568	66.7 ± 0.81	68.9 ± 0.79
28	Chamarajanagar, Karnataka	PmicDOB-28	9	*P. microspora*	100	491	KU865569	56.0 ± 0.88	54.6 ± 0.93
29	Calicut, Kerala	FfujDOB-29	6	*F. fujikuroi*	100	488	KU865570	50.1 ± 1.03	53.0 ± 0.86
30	Salem, Tamilnadu	TatrDOB-30	17	*T. atroviride*	100	496	KU865571	63.1 ± 0.73	61.8 ± 0.67
31	Erode, Tamilnadu	TharDOB-31	20	*T. harzianum*	100	550	KU865572	76.0 ± 0.59	76.9 ± 1.03

Values are mean ± SE of three replicates

house condition by using turmeric cultivar 'Erode local' (susceptible variety). Four replications were maintained for each treatment and each replication consisted of five earthen pots (20 cm diameter) in a completely randomized design (CRD) in a green house, the experiment was repeated twice.

The talc-based formulation of the fungal endophytes was prepared containing population densities of 3×10^6 spores/ml (Shanmugam and Kanoujia 2011). The talc-based formulations of endophytic fungal isolates *T. harzianum* (Thar DOB-2), *T. atroviride* (Tatro DOB-17), *T. asperellum* (Tasp DOB-19), *T. harzianum* (Thar DOB-31) were applied as rhizome treatment and soil application. The rhizomes were surface sterilized with 2% sodium hypochloride for 1 min and soaked in sterile distilled water containing 20 g/l formulation. The suspension was drained off after 12 h and the rhizomes were air dried overnight under a sterile air stream. Rhizomes each with three nodes were planted in earthen pots containing sterilized sandy loam soil of 5 kg. For first set of experiment, the pathogen *P. aphanidermatum* was multiplied on sand-corn meal medium at a ratio of 1:19 (sand–maize inoculum:soil) and were challenge inoculated (Shanmugam et al. 2013). And for second set, the 30 day old BCA treated turmeric plants were challenge inoculated with *R. solani* by inserting young immature sclerotia (2 sclerotia per sheath) (Sriraj et al. 2014).

Inoculation of pathogens was immediately followed by soil applications (8 g) with talc formulations (individual endophytes) and three times upto 90 days at intervals of 15 days. For *R. solani* inoculated pots soil application was followed by foliar spray of endophytes (individual endophytes) at 10^8 spores/ml suspended in water. Carbendazim (0.1%) with mancozeb (0.25%) combination was applied as seed treatment and soil drenching served as fungicide control. The rhizomes without treatment and pathogens treated alone served as controls. The intensity of leaf blight disease was recorded after 7 days of inoculation, with 0–9 scale of the Standard Evaluation System of rice, IIRI (2002) and expressed as Percent Disease Index (Sriraj et al. 2014).

A separate set with four treatments along with untreated control and pathogenic control was maintained for rhizome colonization assay and growth promotion studies. The Percent Disease Incidence (PDI) of rhizome rot, plant length and fresh rhizome yield were recorded at the time of harvest (October–February). PDI was calculated using the following formula:

$$\text{PDI} = \frac{\text{Number of infected plants}}{\text{Total number of inoculated plants}} \times 100$$

Rhizome colonization assay by confocal microscopy

BCA (*T. harzianum* TharDOB-31) treated turmeric rhizomes (as explained earlier) of 60 days old, were removed intact from the soil. The rhizomes were thoroughly washed in running tap water followed by distilled water. The rhizomes were surface sterilized with 2% (W/V) sodium hypochloride solution for 30 s. Experiments were performed twice, and rhizomes from three plants were analyzed for each data. The rhizome material (1 cm) was transferred to trichloroacetic acid fixation solution [0.15% (wt/vol) trichloroacetic acid in 4:1 (vol/vol) ethanol/chloroform]. Sections from rhizome were hand cut about 1 cm from the surface and approximately 50 µm thick segments were mounted on a microscope slide. Hyphae in rhizome segments were stained by 0.01% acid fuchsin-lactic acid. Subsequently, segments were incubated at room temperature for 10 min in 1× PBS (pH 7.4) containing dye at 10 µg/ml. After incubation the segments were mounted on clean glass slides and examined immediately. Confocal fluorescence images were recorded on Advanced Spectral Confocal Microscope System-LSM 710 (Carl Zeiss, Germany). It was excited with a 568-nm laser line and detected at 557–698 nm, Channels A568 and

T PMT, laser lines 488 and 543 nm were used (Deshmukh et al. 2006).

Metabolite extraction of endophyte *T. harzianum* TharDOB-31

Erlenmeyer flasks (5 l) containing 2 l of potato dextrose broth (PDB) was sterilized and inoculated with two discs (10 mm) of endophyte *T. harzianum* TharDOB-31 obtained from actively growing margins of PDA cultures. The stationary cultures were incubated for 31 days at 25 °C devoid of antibiotic, the culture broth was filtered using Whatman No. 1 filter paper and the filtrates was centrifuged at 5000 rpm for 10 min. The obtained supernatant was extracted with ethyl acetate (EtOAc), by liquid–liquid extraction of secondary metabolites using separating funnel. The extract was evaporated to dryness using flash evaporator (Vinale et al. 2006; Buatong et al. 2011) and used for further analysis.

Minimum inhibitory concentration (MIC)

Antifungal properties of crude ethyl acetate extract of endophyte *T. harzianum* TharDOB-31 was tested against the fungal pathogens (*P. aphanidermatum* and *R. solani*) using MIC. The MIC of ethyl acetate extract was determined by broth macro dilution method (Pujol et al. 1996). PDB was added with required concentration of DMSO containing endophyte *T. harzianum* TharDOB-31 ethyl acetate extracts to get a concentration of 20, 40, 60, 80, 100 µg/ml. Conical flask (250 ml) containing 100 ml of PDB was sterilized and inoculated with 5 × 5 mm disc of pathogenic fungal mycelia in each flask and incubated for 7 days at 24 ± 2 °C. The control flasks containing PDB were inoculated only with the fungal mycelial disc and DMSO without extract served as negative control, carbendazim (0.1%) and mancozeb (0.25%) combined 1 mg/ml was considered as positive control. The least concentration at which no visible growth observed was considered as MIC. Three replicates were used per concentration treatment and the experiment was repeated twice (Elsherbiny et al. 2016).

HR-LCMS Analysis

High resolution liquid chromatograph mass spectrometer (HR-LCMS) analysis of ethyl acetate extract of endophyte *T. harzianum* TharDOB-31 was carried out on a Agilent TOF/Q-TOF M S G6550A System, Model- 1290 Infinity UHPLC System, 1260 infinity Nano HPLC with Chipcube, 6550 iFunnel Q-TOFs, (mass range: 50–3200 amu, resolution: 40,000 FWHM, high mass accuracy: typically < 1 ppm, ionization method: API; ESI—positive and negative, APCI—positive and negative, direct infusion for mass analysis: MS, MS/MS; binary nano HP-LC system with mass as detector, UHPLC PDA mass spectrometer). The analysis was carried out at Sophisticated Analytical Instrument Facility (SAIF), Indian Institute of Technology, Bombay, Powai, Mumbai-400 076, India. The fragmentation patterns of mass spectra were compared with those stored in the spectrometer database using National Institute of Standards and Technology Mass Spectral database (NIST-MS).

Statistical analysis

Statistical analyses were performed using MS-Exel version 2007 and SPSS (Version 17). A CRD was used for all the experiments, with three replications for each treatment. Differences between experimental outcomes were assessed using Ducan's multiple range tests and $P \leq 0.05$ was considered statistically significant.

Results

Enumeration of endophytes

From healthy turmeric rhizomes, 31 endophytic fungi were isolated and identified morphologically and molecularly by using ITS–rDNA sequences. The rate of colonization ranged from 6 to 26% in different samples. *Trichoderma* was the dominant endophyte (Table 1).

Selection of fungal pathogens

Ten isolates of each fungal pathogen were subjected to green house pathogenicity test according to Koch's postulates. Potent virulent strain of *P. aphanidermatum Py*DOB-4 and *R. solani* RhsDOB-3 exhibiting systemic infection within 3 weeks after challenge inoculation were selected for further studies.

Morphological characterization of endophytic fungi and fungal pathogens

The 31 endophytic fungal isolates and fungal pathogens *P. aphanidermatum* and *R. solani* were identified based on micro and macroscopic features like colour of the colony, hyphae, spore, sporangia, asexual and sexual structures and oospores. The isolated endophytic fungi were assigned to three genera namely *Trichoderma, Fusarium* and *Pestalopsis* (Table 1).

Molecular characterization of endophytic fungi and fungal pathogen

The quality of endophytic and pathogenic genomic DNA was good as evident from the ratio of 260/280, which was

1.72. The integrity of isolated DNA was determined by electrophoresis using 0.7% agarose gel. The integrity was well established by DNA isolated using kit method, further used for downstream applications.

All the endophytic fungi and fungal pathogens were subjected to PCR amplification. ITS specific universal primers ITS1F and ITS4R were used for amplification. The amplified PCR products were resolved well in 1% agarose gel as compared to 100 bp ladder. The amplified product ranged between 500 and 554 bp for endophytic fungal isolates (Table 1), and 730 and 625 bp for fungal pathogens *P. aphanidermatum* and *R. solani* respectively.

Phylogenetic analysis

The sequences of all the 31 endophytic fungal isolates and the pathogens showed 98% similarity with the species in Genebank during Blast analysis. All the 31 endophytic sequences and two test pathogens sequences (*P. aphanidermatum*-KT315583 and *R. solani*- KT366922) were submitted to Genebank (NCBI) and their accession numbers were obtained (Table 1). Phylogenetic studies confirmed the affiliation of the selected endophytes into *Trichoderma, Fusarium and Pestalopsis* clades as revealed by clustering of each genus to its corresponding group formed by the sequences retrieved from Genbank. The phylogenetic tree (Fig. 1) formed consists of three major Clades, each subdivided into two clades. In Clade 1–subclade 1a, 12 isolates of *T. asperellum* along with two *T. hamatum* (Tham DOB-6 and ThamDOB-11) and one *T. atroviride* (TatrDOB-30) isolate were grouped together showing more similarity. *T. harzianum* formed Clade 1b which included one isolate of *T. atroviride* (TatrDOB-17) as well. In the Clade 2a, four isolates of *F. solani* were grouped along with other existing *F. solani* isolates with high bootstrap confidence of 99%. Clade 2b contained *F. proliferatum* and *F. fujikuroi* isolates grouped along with bootstrap value of 97 and 99% showing the divergence. In Clade 3, two isolates of *P. microspora* formed an out group.

In vitro test for antagonism by dual culture method

Most of the isolated fungal endophytes showed antagonism against *P. aphanidermatum* and *R. solani*. Among the isolates of *Trichoderma, Fusarium* and *Pestalopsis* species, *Trichoderma harzianum* TharDOB-31 and *Trichoderma asperellum* TaspDOB-19 exhibited the highest potential antagonistic activity. In dual culture method, TharDOB-31 showed 76.0 ± 0.59 and 76.9 ± 1.03 of growth inhibition against *P. aphanidermatum* and *R. solani* respectively. TaspDOB-19 showed 75.0 ± 0.53 and 76.5 ± 0.73% of growth inhibition against pathogen *P. aphanidermatum* and *R. solani* respectively (Table 1).

Scanning electron microscopic studies of the interaction zone

SEM studies showed that the endophytes cause deformities in the mycelia of both *P. aphanidermatum* and *R. solani*. The deformities included hyphal fragmentation, perforation, lysis and mycelia degeneration (Figs. 2, 3).

In vitro evaluation of plant growth promoting potentials

All the six endophytic fungal isolates viz., Thar DOB-2, Thar DOB-4, Tasp DOB-12, Tatro DOB-17, Tasp DOB-19 and Thar DOB-31 were able to produce IAA with l-tryptophan as a precursor. Except *T. atroviride* (Tatro DOB17), the other isolates exhibited positive results for HCN production, while three isolates solubilized inorganic phosphate $Ca_3(PO_4)$ and four isolates showed positive to cellulase activity. Isolate *T. harzianum* (Thar DOB-31) was positive for IAA, HCN production, inorganic phosphate solubilization and production of cellulase (Table 2). The isolates viz., Thar DOB-2, Tatro DOB-17, Tasp DOB-19 and Thar DOB-31 which produce multiple PGP traits were further considered for green house studies.

Green house experiments

The reduced severity in leaf blight and rhizome rot disease was recorded in four different treatments of endophytes viz., *T. harzianum* (Thar DOB-2), *T. atroviride* (Tatro DOB-17), *T. asperellum* (Tasp DOB-19), *T. harzianum* (Thar DOB-31). Among the tested endophytes, *T. harzianum* 'Thar DOB-31' showed the lowest PDI of rhizome rot to 13.8% and PDI of leaf blight to 11.6%. The isolate also enhanced the plant length and fresh rhizome weight (Table 3).

Rhizome colonization assay by confocal microscopy

The endophytic colonization in turmeric rhizomes was analysed by confocal microscopy. The results revealed the hyphae of endophyte *T. harzianum* TharDOB-31 formed an unrestricted net-like intra and intercellular colonization pattern, intracellularly growing hyphae of endophyte showed necks at site where the fungus transverses a cell wall (Fig. 4).

Minimum inhibitory concentration (MIC)

The MIC determined by broth macro-dilution method showed the inhibition of the tested pathogens (*P.*

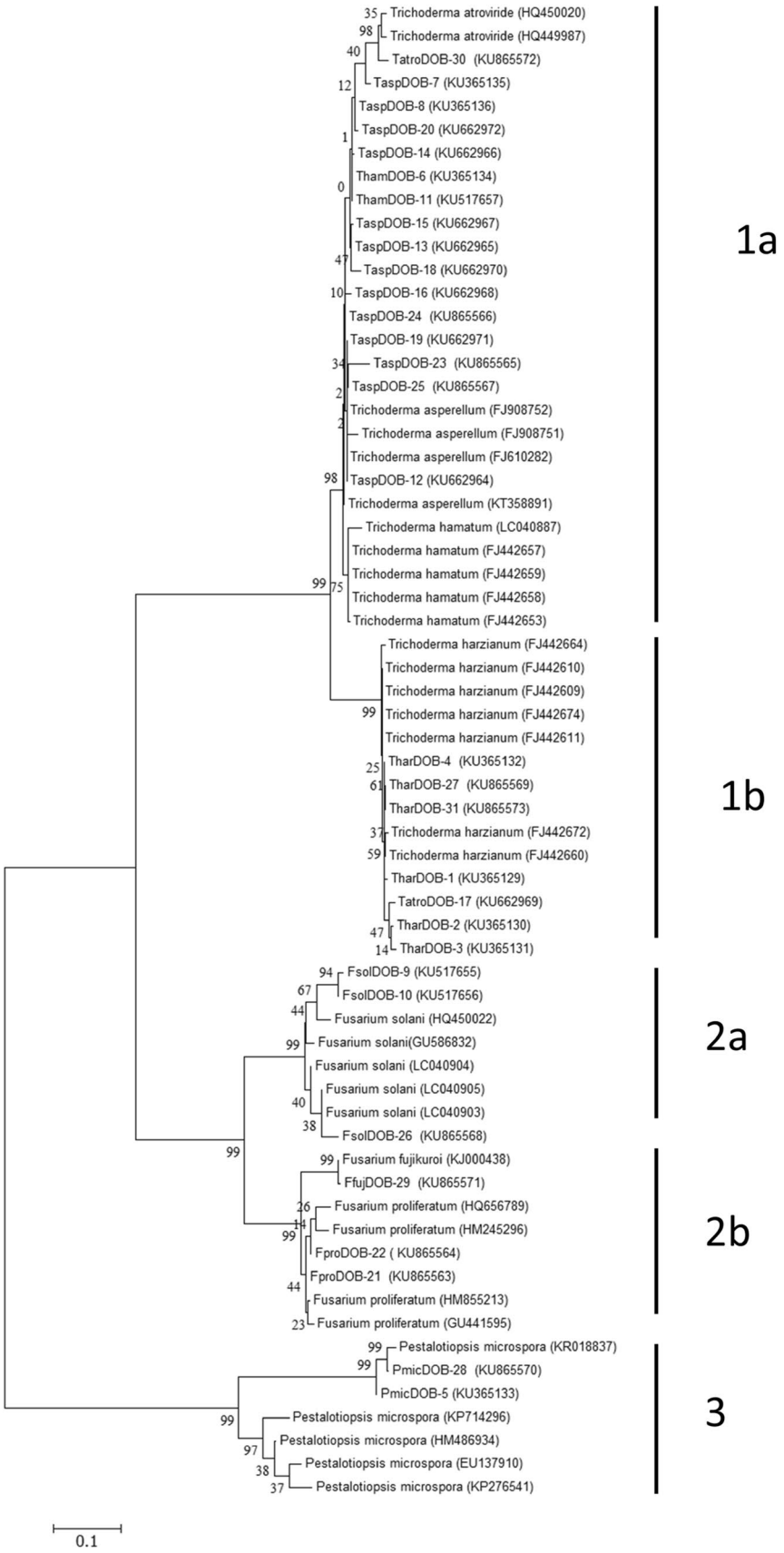

Fig. 1 Position of endophytic fungal isolates of the present study on the phylogenetic tree as inferred based on ITS- rDNA sequence from the nearest neighbour of fungal endophytes retrieved from Genbank. MP bootstrap values are indicated above branch nodes. Number of bootstrap replicates = 1000

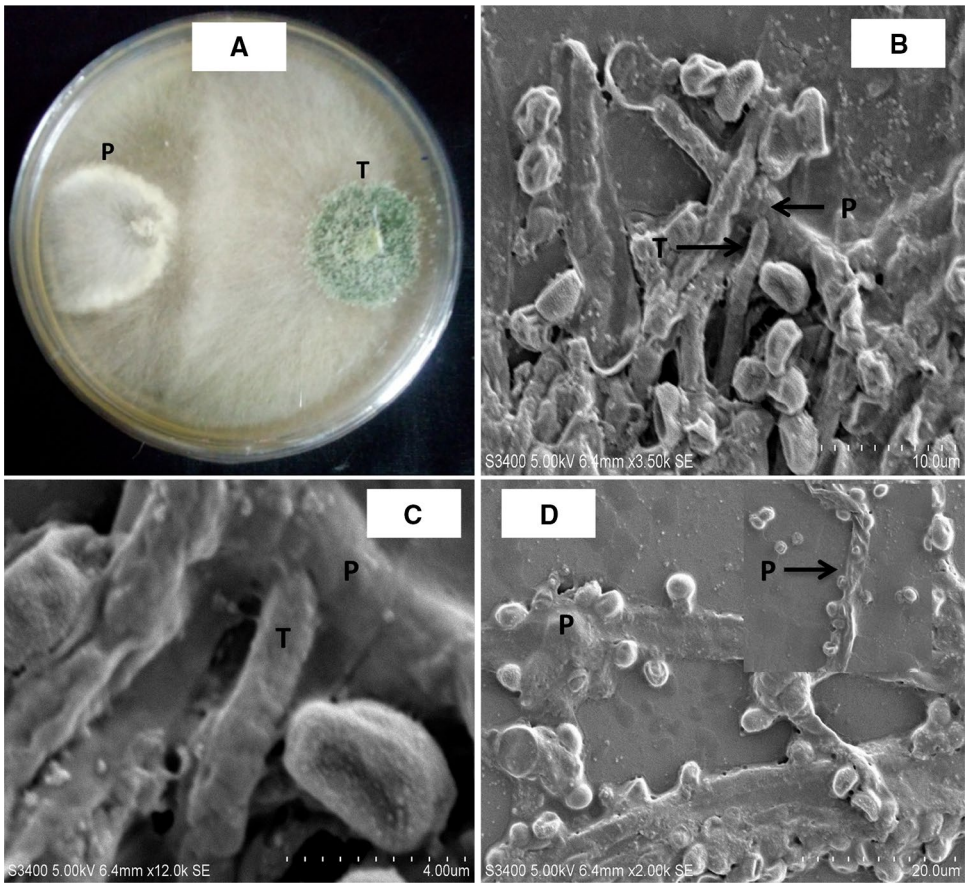

Fig. 2 Photographs of dual culture tests and scanning electron micrograph from the inhibition zone between *P. aphanidermatum* and endophyte *T. harzianum* TharDOB-31 showing abnormal features: **a** dual culture test in petri dish; **b** mycoparasitism; **c** *T. harzianum* TharDOB-31 hyphae invading *P. aphanidermatum* hyphae, and **d** arrow shows shrivelling and lysis of the mycelium along with *Trichoderma* spores are visible. *T* = *T. harzianum* TharDOB-31; P = *P. aphanidermatum*

aphanidermatum and *R. solani*) at 80 µg/ml (MIC) for ethyl acetate extract of *T. harzianum* TharDOB-31.

HR-LCMS Analysis

The crude ethyl acetate extract of *T. harzianum* TharDOB-31 was analyzed using HR-LCMS and the chromatogram of active fractions were given (Fig. 5). From the HR-LCMS analysis TharDOB-31 was found to contain different bioactive metabolites, majority of the compounds possess antimicrobial properties (Table 4).

Discussion

In this study, 31 endophytes were isolated from healthy turmeric rhizomes and the virulent pathogenic strains (*P. aphanidermatum* PyDOB-4 and *R. solani* RhsDOB-3) from the diseased rhizome and leaf material. Their identity was confirmed by morphological and ITS–rDNA sequences. The ITS–rDNA sequences were submitted to Genbank (NCBI) (Table 1). The phylogeny showed endophytic isolates grouped with the identical isolates retrieved from Genbank (Fig. 1). A majority of the endophytic isolates belonged to genera namely *Trichoderma, Fusarium*, and *Pestalopsis*. The *Trichoderma* sp. is one of the major fungi found as an endophyte in the healthy turmeric rhizomes (Table 1). All the isolates were screened for antagonism in order to select the isolates that showed the most promising results with regard to growth promotion and biocontrol of rhizome rot and leaf blight diseases in turmeric plants.

The in vitro screening of endophytic isolates for antagonism against *P. aphanidermatum* an *R. solani* reported that endophyte *T. harzianum* TharDOB-31 followed by *T. asperellum* TaspDOB-19 exhibited > 70% inhibition (Table 1) of both the pathogens in dual culture assays. *Trichoderma* spp. showed antagonism against *Sclerotium rolfsii, Ceratobasidium cornigerum, Phytophthora parasitica, P. aphanidermatum, P. myriotylum*, and *R. solani* pathogens, the isolate overgrew the pathogen, covering completely or at least of the surface was reported earlier (Bell et al. 1982). The radial growth of pathogenic fungus was retarded in the dual culture test, and the mycelia of endophytic *Trichoderma* overgrew the pathogen in petridish (Figs. 2, 3). Similarly, hyphal parasitism of *P. aphanidermatum* by *Trichoderma* spp. isolated from soil was reported earlier (Chet et al. 1981).

Endophytes can inhibit pathogen infection and proliferation within the host directly via antibiosis, competition and mycoparasitism or indirectly via inducing resistance

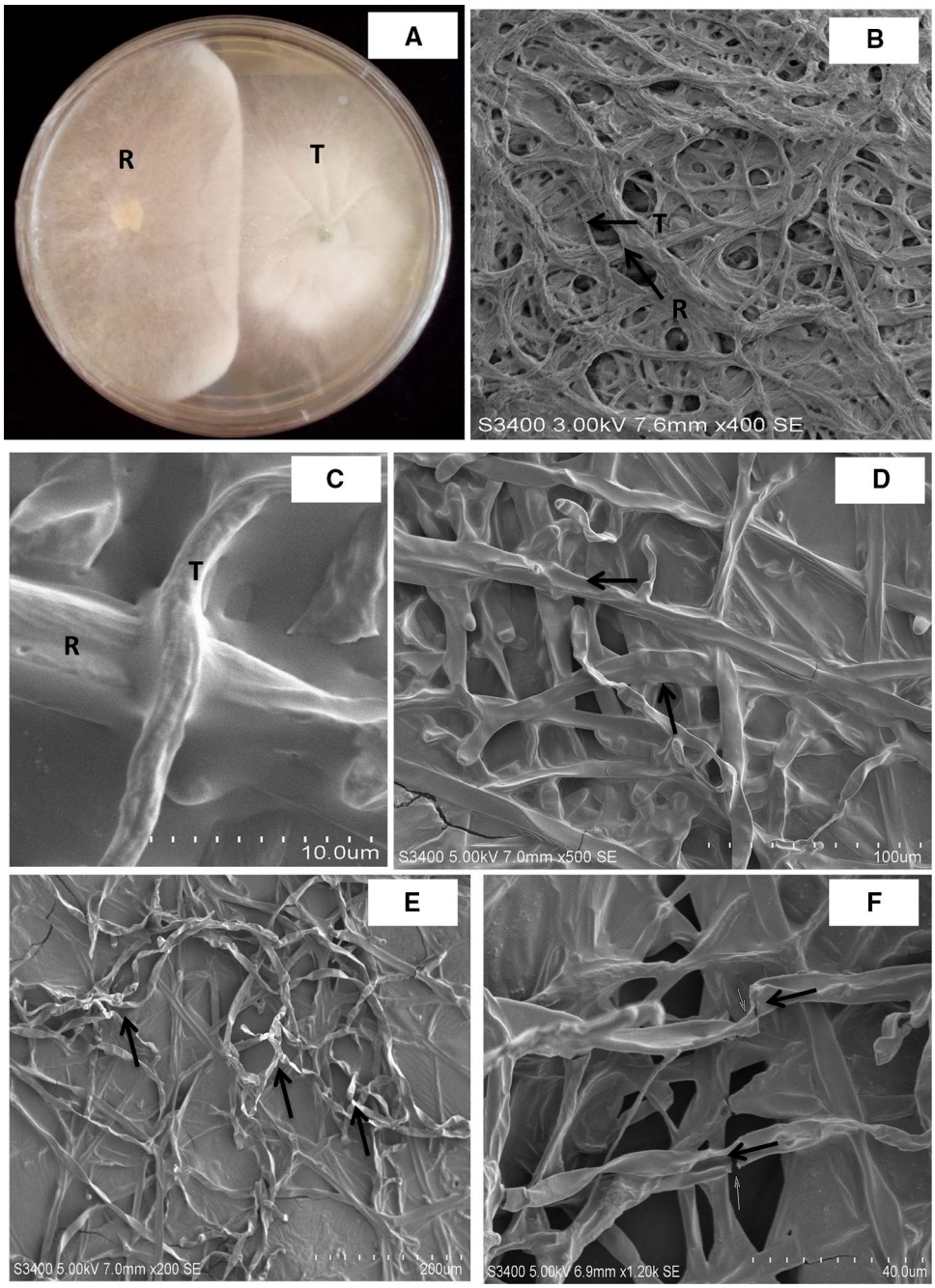

Fig. 3 Photographs of dual culture tests and scanning electron micrograph from the inhibition zone between *R. solani* and endophyte *T. harzianum* ThartDOB-31 showing abnormal features: **a** dual culture test in petri dish; **b** and **c** mycoparasitism; **d** empty mycelium; **e** shriveling of mycelium and **f** arrow shows the breakage of the mycelium of *R. solani*. *T* = *T. harzianum* TharDOB-31; R = *R. solani*

responses intrinsic to host (Benhamou and Chet 1996; Lahlali and Hijri 2010). The fungal interaction zone between pathogen and BCA (Thar DOB-31) was observed, our SEM results revealed the morphological deformities of mycelia of the pathogens. The antibiosis towards pathogenic fungi could be due to the production antagonistic compounds produced by *Trichoderma* and are also known to enhance competiveness and thereby control the disease (Yedidia et al. 1999; Howell 2003). Similar observations of hyphal deformities were made in antagonism of *Trichoderma* spp. against *P. aphanidermatum* (Chet et al. 1981). Thereby, *Trichoderma* based biocontrol could be due mainly to the production of hydrolytic enzymes and metabolites (Harman et al. 2004).

The endophyte *T. harzianum* TharDOB-31 exhibited significant PGP traits like production of cell-wall degrading enzymes, phosphate solubilization, production of IAA and HCN (Table 2). The *Trichoderma* spp. are reported to have high antibiosis, rhizosphere competency and PGP ability and activates the solubilization of phosphates (Harman et al. 2004). Cell wall degrading enzymes, such as

Table 2 Characterization of selected endophytic fungal isolates for PGP potentials

Sl. no.	Isolate no.	Species identified	IAA production	HCN production	Phosphate solubilization	Cellulase activity
1	Thar DOB-2	*T. harzianum*	++	++	++	−
2	Thar DOB-4	*T. harzianum*	+	+++	−	+
3	Tasp DOB-12	*T. asperellum*	++	++	+	++
4	Tatro DOB-17	*T. atroviride*	+	−	++	+
5	Tasp DOB-19	*T. asperellum*	++	++	−	−
6	Thar DOB-31	*T. harzianum*	+++	++	+++	++

IAA production: '+' represent low Indole acetic acid production (light pink colour); '++' represents medium IAA production (dark pink colour); '+++' represents high IAA production (dense pink colour)

HCN production: degree of activity (+++>++>+); colour, ranged from yellow ('+') to dark brown ('+++')

Phosphate solubilization: '+' represents clear halo zone < 5 mm on medium; '++' represents halo zone = 5 mm on medium; '+++' represents halo zone > 5 mm on medium; '−' represents no halo zone on medium

Cellulase activity: '+' represents positive; '++' represents intermediate; '+++' represents strong activity; '−' represents negative

Table 3 Management of rhizome rot and leaf blight diseases of turmeric caused by *P. aphanidermatum* and *R. solani* by fungal endophytes in green house

Treatment	Rhizome rot			Leaf blight		
	Fresh rhizome weight (g)	Plant length (cm)	PDI	Fresh rhizome weight (g)	Plant length (cm)	PDI
T. harzianum (Thar DOB-2)	360 ± 2.37^{d}	79.89 ± 1.27^{c}	18.6 ± 0.97^{c}	340 ± 1.27^{d}	76.97 ± 0.83^{c}	15.0 ± 0.37^{b}
T. atroviride (Tatro DOB-17)	410 ± 2.71^{b}	83.17 ± 0.97^{b}	16.4 ± 0.57^{d}	380 ± 1.27^{b}	81.27 ± 0.57^{b}	14.1 ± 0.57^{c}
T. asperellum (Tasp DOB-19)	380 ± 1.97^{c}	80.27 ± 1.21^{c}	15.2 ± 0.37^{e}	365 ± 1.27^{c}	80.50 ± 0.77^{b}	13.4 ± 0.42^{d}
T. harzianum (Thar DOB-31)	425 ± 3.12^{a}	85.71 ± 0.77^{a}	13.8 ± 0.43^{f}	410 ± 1.27^{a}	84.60 ± 1.21^{a}	11.6 ± 0.97^{e}
Carbendazim (0.1%) + mancozeb (0.25%)	300 ± 2.74^{f}	70.88 ± 0.75^{d}	19.4 ± 0.71^{b}	280 ± 1.27^{e}	69.75 ± 0.57^{d}	15.8 ± 0.33^{b}
Untreated control	320 ± 2.57^{e}	57.75 ± 0.57^{e}	0.0	300 ± 1.27^{e}	53.45 ± 0.73^{e}	0.0
Pathogenic control	220 ± 2.17^{g}	42.65 ± 0.79^{f}	79.0 ± 0.54^{a}	210 ± 1.27^{f}	40.45 ± 0.43^{f}	75.8 ± 0.37^{a}

The values are mean of three replications ± SE. Means in a column followed by same superscript letter are not significantly different according to Duncan's Multiple range test at $P \leq 0.05$

cellulases are important in breakdown of cell walls of oomycete pathogens such as *Pythium* spp. (Mishra 2010).

The four promising BCA's viz., *T. harzianum* (Thar DOB-2), *T. atroviride* (Tatro DOB-17), *T. asperellum* (Tasp DOB-19) and *T. harzianum* (Thar DOB-31) were then tested in the green house for their disease suppression and plant growth promotion abilities compared to untreated and pathogenic controls. Green house results revealed, the endophyte *T. harzianum* (Thar DOB-31) recorded less PDI (high disease reduction) also enhanced the yield of turmeric when compared to untreated control (Table 3). Similar to our reports on turmeric, there are several studies on growth promotion and disease suppression by endophytes in other crops like potato the endophyte *T. atroviride* and *E. nigrum* improved yield significantly and decreased the stem disease of potato plants in greenhouse experiments (Lahlali and Hijri 2010), tomato and pepper the endophyte *T. harzianum* and *T. atroviride* strains promoted the growth and increased the crop productivity as compared to untreated controls (Vinale et al. 2004).

Root colonization by *Trichoderma* strains frequently enhances root growth and development, crop productivity and resistance to abiotic stress. In the present study endophyte *T. harzianum* Thar DOB-31 showed positive results for colonization of rhizome by confocal microscopic studies (Fig. 4). Our results were supported by earlier reports on some Trichoderma species that colonized root surfaces and caused substantial changes in plant metabolism. Also *Trichoderma* strains promote plant growth and enhance disease resistance (Harman et al. 2004). The endophyte

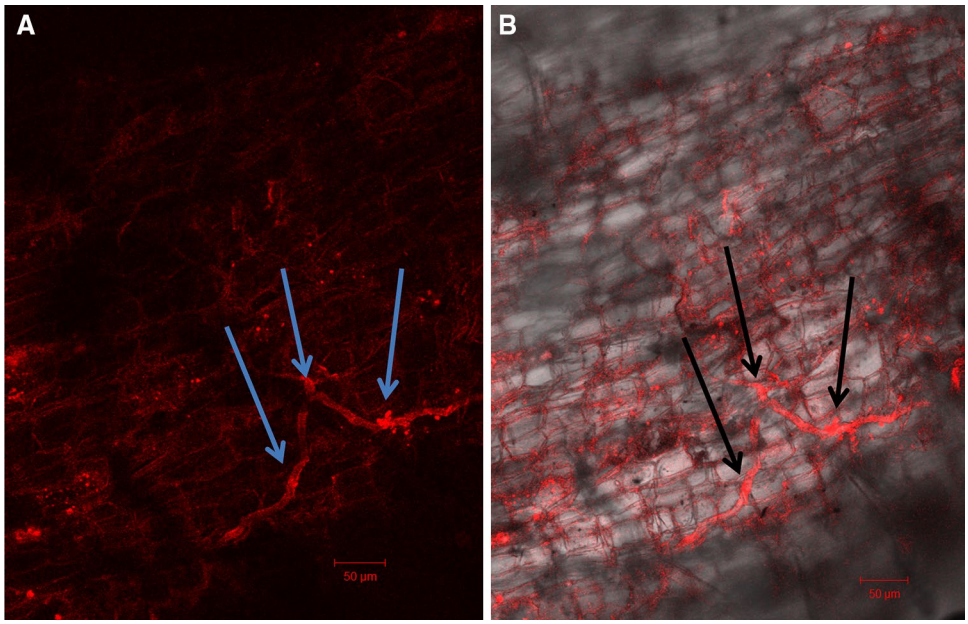

Fig. 4 Confocal microscopy observations of BCA (*T. harzianum* TharDOB-31) treated 60 day old turmeric rhizome segments for colonization. **a** The hyphae penetration site is indicated by an arrow in fluorescence image. **b** Showing intracellular mycelium in rhizomes the overlay of T PMT field image and fluorescence image-intracellular hyphae form necks (arrowheads) (scale bars 50 µm)

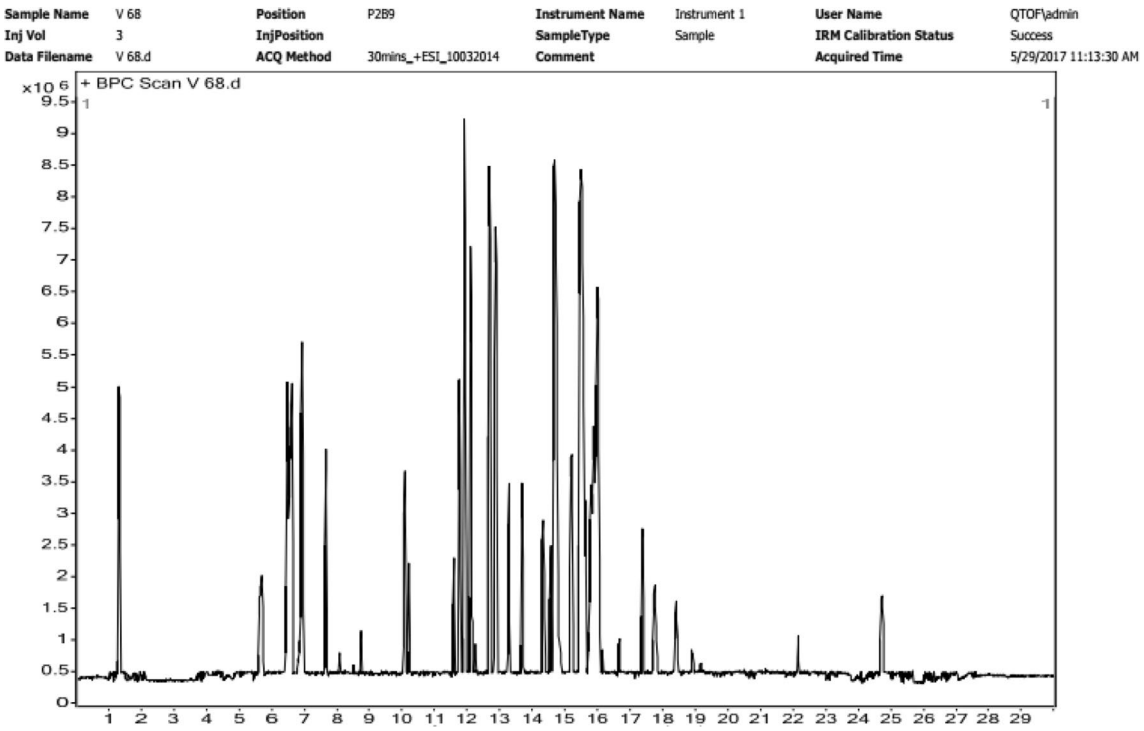

Fig. 5 HR-LCMS chromatogram of crude ethyl acetate extract of *T. harzianum* TharDOB-31 showing the bioactive metabolites

Trichoderma gamsii exhibited plant growth promotion and antagonism against phytopathogens has been reported (Rinu et al. 2014).

Different formulations using a variety of *Trichoderma* strains are available commercially for crop production worldwide (Harman 2000) its secondary metabolites affect plant metabolism and enhance growth (Vinale et al. 2012). The antibiotic fungal metabolites isolated from the biocontrol agent *T. harzianum* (strains T22 and T39) against phytopathogens *Rhizoctonia solani, Pythium ultimum* and *Gaeumannomyces graminis* var. *tritici* was reported (Vinale et al. 2006). In the present study, crude

Table 4 Major bioactive compounds present in the ethyl acetate extract of *Trichoderma harzianum* TharDOB-31

Sl. no.	Compound name	Mass	Formula	Retention time	Biological activity	References
1	Diethanolamine	105.0758	$C_4H_{11}NO_2$	1.327	Antimicrobial, antifungal, antibacterial	Medic-Saric et al. (1980)
2	2-Oxo-3-methylvaleric acid	130.0629	$C_6H_{10}O_3$	5.679	Antifungal	Guo et al. (2008)
3	2-Amino-3-methoxy-benzoic acid	167.0575	$C_8H_9NO_3$	6.597	Antifungal	Park et al. (2001)
4	Harmalol	200.094	$C_{12}H_{12}N_2O$	6.942	Antifungal	Olmedo et al. (2017)
5	Topiramate	339.1009	$C_{12}H_{21}NO_8S$	9.047	Antimicrobial	Kruszewska et al. (2012)
6	3-Hydroxy-tridecanoic acid	230.186	$C_{13}H_{26}O_3$	9.078	Antifungal	Guo et al. (2008)
7	Vanillic acid	168.0414	$C_8H_8O_4$	10.094	Antifungal	Kuete et al. (2010)
8	3-Methyl-tetradecanedioic acid	272.1966	$C_{15}H_{28}O_4$	10.213	Antimicrobial	AbdSharad et al. (2016)
9	Dirithromycin	834.5562	$C_{42}H_{78}N_2O_{14}$	11.71	Antimicrobial	Roblin and Hammerschlag (1998)
10	Punctaporin B	252.1708	$C_{15}H_{24}O_3$	12.28	Antimicrobial	Gupta (2016)
11	Acarbose	552.2404	$C_{24}H_{40}O_{14}$	12.292	Antifungal	Kruszewska et al. (2008)
12	2-Methyl-2E-hexenoic acid	128.0849	$C_7H_{12}O_2$	12.431	Antigungal	Zhang et al. (2010)
13	10-Methyl-1-dodecanol	200.2134	$C_{13}H_{28}O$	13.283	Antimicrobial	Nagoshi et al. (2007)
14	9R-hydroxy-10E-octadecenoic acid	298.2504	$C_{18}H_{34}O_3$	14.15	Antimicrobial	Abubakar and Majinda (2016)
15	Benzenemethanol, 2-(2-aminopropoxy)-3-methyl-	196.1108	$C_{11}H_{16}O_3$	14.316	Antifungal	Ubaid et al. (2016)
16	Sapindoside A	750.4582	$C_{41}H_{66}O_{12}$	14.693	Antifungal	Khan et al. (2017)
17	12-Hydroxy-10-octadecynoic acid	296.2361	$C_{18}H_{32}O_3$	15.64	Antimicrobial	Sanabria-Ríos et al. (2014)
18	3-Nonaprenyl-4-hydroxybenzoic acid	750.5946	$C_{52}H_{78}O_3$	15.748	Antimicrobial	Cho et al. (1998)
19	2-ISOPROPYL-3-Methoxycinnamic acid	220.1106	$C_{13}H_{16}O_3$	17.549	Antimicrobial	Naz et al. (2006)
20	Ophiobolin A	400.2635	$C_{25}H_{36}O_4$	17.803	Antimicrobial	Li et al. (1995)
21	2,3-Dihydroxy stearic acid	316.2606	$C_{18}H_{36}O_4$	19.445	Antimicrobial	Choi et al. (2013)
22	Calicoferol D	410.3187	$C_{28}H_{42}O_2$	22.47	Antimicrobial	Youssef et al. (2011)

ethyl acetate extract of endophyte *T. harzianum* TharDOB-31 showed antifungal activity against both the pathogens and the MIC was observed at 80 µg/m. Also partial characterization of secondary metabolites of endophyte *T. harzianum* TharDOB-31 by HR-LCMS analysis was reported (Table 4).

The success of *Trichoderma* strains as BCA's is due to their high reproductive capacity, ability to survive under unfavorable conditions by utilizing nutrients available, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defence mechanism (Benhamou and Chet 1996). *Trichoderma* as BCA controls the soil-borne pathogens namely ascomycetous, deuteromycetous, basidiomycetous and oomycetous fungi (Omero et al. 1999). Hence, *T. harzianum* TharDOB-31 can be exploited as potential BCA's in order to control the rhizome rot and leaf blight diseases in turmeric which helps to reform the chemical fungicide based disease management approaches.

Conclusion

The present study revealed the importance of isolating, screening of endophytes from turmeric rhizome for multiple PGP and biocontrol traits through greenhouse experiments. In this study based on in vitro experiments, endophyte *T. harzianum* TharDOB-31 exhibited multiple PGP traits. Also, the results of greenhouse evidenced endophyte *T. harzianum* TharDOB-31 suppressed the disease incidence of rhizome rot and leaf blight significantly and markedly enhanced the yield in turmeric compared to untreated control and chemical treatments like carbendazim–mancozeb. The colonization of *T. harzianum* TharDOB-31 treated rhizomes was confirmed by confocal microscopic studies. The secondary metabolites of *T. harzianum* TharDOB-31 reported bioactive compounds with antimicrobial properties. The study confirms the potential of endophyte *T. harzianum* TharDOB-31 as biocontrol

agent (BCA's) for sustainable turmeric cultivation. For the best of our knowledge, this is the first report on the endophyte *T. harzianum* TharDOB-31 as BCA's against *P. aphanidermatum* and *R. solani* pathogens of turmeric. Further studies concerning field applications and stable bioformulations are in progress.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests regarding the publication of this paper.

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