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ORIGINAL PAPER

Isolation, characterization, and bioactivity of endophytic fungi of *Tylophora indica*

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Abstract *Dothediomycetes* sp., *Alternaria tenuissima*, *Thielavia subthermophila*, *Alternaria* sp., *Nigrospora oryzae*, *Colletotrichum truncatum,* and *Chaetomium* sp. were isolated as endophytic fungi from leaves and stems of the medicinal plant, *Tylophora indica,* based on rDNA sequencing of ITS region and microscopic examination. *Alternaria tenuissima*, *Colletotrichum truncatum,* and *Alternaria* sp. were found to be active against both *Sclerotinia sclerotiorum* and *Fusarium oxysporum*. *Chaetomium* sp. showed very mild activity against *Sclerotinia sclerotiorum* and *Fusarium oxysporum*. Whereas *Dothideomycete* sp. and *Thielavia subthermophila* showed high activity against *Sclerotinia sclerotiorum*. Methanol extract of *Dothediomycetes* sp. showed 66.5% growth inhibition (GI) at 500 μg/ml.

Keywords Endophytic fungi · *Tylophora indica* · *Sclerotinina* · Bioassay

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Introduction

Endophytes are the micro-organisms that colonize the interior of the plant parts, without having any negative effect on the host (Arnold et al. 2003), rather helping the plant by imparting resistance to host plant against biotic (Breen 1994; Schulz et al. 1999; Dingle and McGee 2003) and abiotic stresses (Siegel et al. 1990; West 1994). Every plant species examined till date harbors endophytic fungi (Strobel 2006). In addition to providing wide range of activities against plant pathogens and herbivores, endophytic fungi are known to produce bio-molecules of pharmaceutical and agricultural importance. Many of the bioactive metabolite from endophytic fungi act as plant defence activator and have been useful in novel drug discovery (Owen and Hundley 2004). *Muscador albus*, an endophytic fungus of rainforest plant, is known to produce volatile organic compound responsible for fumigant activity against stored grain pests (Strobel 2006; Strobel et al. 2001). Several antimicrobial metabolites, such as colletotric acid (Zou et al. 2000), griseofulvin (Park et al. 2005), are reported from endophytic fungi. Nematicidal activity is also reported from the culture filtrate of *Fusarium oxysporum*, an endophytic fungus of tomato (Hallmann and Sikora 1996). Metabolites of endophytic fungi responsible for pesticidal activity have been reviewed by Kumar et al. (2008).

Tylophora indica, a medicinal plant of Asian origin, is known to host several metabolites having insecticidal property (Kathuria and Kaushik 2006) and medicinal property (Reddy 2009). The plant is not reported to be attacked by many plant pathogens and pests, so endophytic micro-biota of the plant can be of great help as its presence probably protects the plant. Present paper describes the diversity of endophytic fungi isolated from *Tylophora indica* and their bioactivity against plant pathogenic fungi.

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Materials and methods

Sample collection

Leaf and stem samples of *Tylophora indica* (Family: Ascalpediaceae) were collected from pot grown plants at TERI, New Delhi, India, during October 2006–June 2007. Immediately after the collection, plant parts were washed with tap water and processed for isolation of endophytic fungi.

Media preparation

Malt extract agar medium [Malt extract (15 g/l); Agar (15 g/l), pH: 7.4–7.8] was used for isolation and purification of endophytic fungi. Antibiotic, chloramphenicol @ 0.2 g/l of the medium was added to the media to avoid bacterial contamination. Wickerham medium [Malt extract (3 g/l); Yeast extract (3 g/l); Peptone (5 g/l); Glucose (Qualigens) −10 g/l; pH −7.2 to 7.4] was used for small-scale multiplication of endophytic fungi, being used for extracting metabolite. Potato dextrose agar (PDA) was used for bioassay. All the media chemicals and antibiotics were purchased from Himedia, India.

Isolation of endophytic fungi

Endophytic fungi were isolated from the healthy plants of *T. indica* as per the procedure of described by Wang et al. (2006) with minor modification in surface sterlization. The plant parts were surface sterilized with 70% ethanol for 2 min followed by 1% sodium hypochlorite for 3 min. Surface sterilized plant parts were dried on sterile blotting sheet, chopped, and transferred to malt agar plates, after taking imprint of dried sterile plant part. These plates were incubated at 24°C for 3–15 days. Hyphal tips of the developing fungal colonies were transferred to fresh malt agar plates to get pure culture.

Identification of endophytic fungi

Slide preparation

Fungal mycelium was stained in cotton blue and mounted in polyvinyl lactic acid glycerol (PVLG) by heating at 65°C for 2–3 days and observed under microscope.

DNA Isolation and amplification

Total genomic DNA of the endophytic fungi was isolated directly from actively growing mycelium scraped from PDA plates, using the DNeasy plant minikit (Qiagen), according to manufacturers' protocol. DNA amplification was performed by PCR using Hot Star Master mix Taq polymerase (Qiagen) and the primer pair ITS 1: TCCGTAG GTGAACCTGCGG; and ITS 4: TCCTCCGCTTATTGA TATGC (Sigma) (White et al. 1990) in a thermocycler (9,800 Fast Thermal Cycler, Applied Biosystems, USA).

PCR was carried out according to the following protocol: initial denaturation 95°C for 15 min; denaturation 95°C for 1 min; annealing 56.0°C for 30s; extension 72°C for 1:00 min; final extension 72°C for 10 min and steps 2–4 were repeated 35 times. Each sample prepared for PCR consisted of 25 µl Taq polymerase mastermix, 3 µl primer mix (10 pmol/ µl each), 3 µl template DNA, and 19 µl Molecular biology grade water.

Purification of the PCR product was done by Montage® PCR centrifugal filter devices as per the suppliers' (Millipore) protocol. Sequencing of the purified PCR product was carried out at LabIndia (Gurgaon, India) on an automated multicapillary DNA sequencer, ABI Prism 3130xl genetic analyser (Applied Biosystems, the USA) using the Big Dye Terminator v.3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, the USA). To identify the isolates, sequences were subjected to the BLAST search with the NCBI database. Multiple sequence alignment of approximately 500 bp sequences was performed using CLUSTAL W version 1.8. A phylogenetic tree was constructed with the evolutionary distances using the neighbour joining method (Saitou and Nei 1987). Tree topologies were evaluated by performing bootstrap analysis of 1,000 dataset (Felsenstein 1985) with the MEGA 4 package (Tamura et al. 2007). The rDNA sequences of representative isolates from this study have been submitted to NCBI GenBank database with accession no **GQ 176264–GQ176270**.

Bioassay of endophytic fungi against plant pathogenic fungi

Bioassay of endophytic fungi against plant pathogenic fungi was done by dual culture technique. Potato dextrose agar medium (Himedia) was selected for dual culture as it favours growth of plant pathogenic fungi, *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Fusarium oxysporum*. The pathogen cultures were obtained from Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi. Plant pathogenic fungi and endophytic fungi were inoculated on PDA plate at periphery, opposite to each other. After incubation at 24°C for 3–7 days, plates were observed and antagonism was expressed by the presence of inhibition zone at the point of interaction.

Small-scale multiplication of endophytic fungi

Endophytic fungi showing antagonistic property against plant pathogenic fungi were inoculated in wickerham medium (300 ml in 1 l conical flask) and incubated at 24°C

for 3–4 weeks. Upon cooling of the media, four flasks of wickerham medium were inoculated with each endophytic fungus. One flask of medium was kept for control.

Extraction of metabolite of endophytic fungi

The metablolites from the fungal cultures were extracted as per the procedure of Wicklow et al. (1998). After attaining full growth, each fungal culture was immersed in 250 ml of ethyl acetate (Rankem, India) for 24 h. The contents were mixed thoroughly with hand blender (INALSA Tech., India), and then filtered. The filtrate was extracted thrice with ethyl acetate and filtered; the residue was re-extracted with n-butanol (Qualigens, India) in a similar manner. The ethyl acetate extract was dried on rotary evaporator (Buchi, Germany). The dried ethyl acetate extract was further partitioned between 90% methanol (Qualigens, India) and *n*-hexane (Qualigens, India). The aqueous phase was discarded by immersing it in detergent. Solvent from hexane, butanol, and methanol was removed with vacuum rotary evaporator (Heidolph Inc, Germany) and subjected to bioassays.

Bioassay of extracts of endophytic fungi against *Sclerotinia sclerotiorum*

The fungal extracts were tested for bioactivity against *Sclerotinia sclerotiorum*. The dried extract (30 mg) was dissolved in 800 µl of methanol. From this solution, 200 and 400 µl were mixed to 30 ml PDA to make 250 and 500 µg/ml concentration respectively. Intoxicated media (30 ml) was poured in three plates, and upon solidification of the media, *Sclerotinia sclerotiorum* was inoculated at the centre of the plate and radial growth was measured until the check plate attained full growth. To check if the growth inhibition is due to methanol, 400 µl of it was added to 30 ml media and poured in three plates. Percent growth inhibition of the extract was calculated with respect to growth in methanol containing plates.

Data analysis

Growth inhibition (GI) was calculated as per the following formula

$$GI = \{(A - B)/A\} \times 100$$

where A = radial diameter of plant pathogenic fungi in check plate with solvent only; B = radial diameter of plant pathogenic fungi in extract/fraction intoxicated plates.

All experiments were conducted in triplicate. GI for each replicate was calculated. Analysis of variance of the GI was done by online statistical package (Web Agri Stat Package-WASP1) of ICAR-Goa regional centre, Goa, India. Least significant difference (LSD) was calculated and means were compared.

Results

Isolation of endophytic fungi

Seven endophytic fungi EF1-EF7 were isolated from 192 tissue segments (68 from stem and 124 from leaf) of *Tylophora indica*. These endophytic fungi were identified by rDNA sequencing of ITS region, as *Dothediomycetes* sp., *Alternaria tenuissima*, *Thielavia subthermophila*, *Alternaria* sp., *Nigrospora oryzae*, *Colletotrichum truncatum,* and *Chaetomium* sp. Table 1 shows the percent similarity of these isolates with the fungi identified and their respective accession number obtained by GenBank. Endophytic fungi, *Colletotrichum truncatum* and *Alternaria* sp., were isolated from the leaf and *Dothediomycetes* sp., *Alternaria tenuissima*, *Thielavia subthermophila*, *Nigrospora oryzae,* and *Chaetomium* sp. were isolated from the stem. Phylogenetic relationship of these isolates with their related fungi is shown in Fig. 1. All the isolated endophytic fungi belong to phylum Ascomycota. Endophytic fungi were also identified microscopically by Prof. K G Mukerjii having characters as described earlier.

Bioactivity of the endophytic fungi

Dual culture test revealed that EF1 (*Dothideomycete* sp.) and EF3 (*Thielavia subthermophila*), are showing high activity against *Sclerotinia sclerotiorum.* (Figure 2), while EF2 (*Alternaria tenuissima*), EF6 (*Colletotrichum truncatum*), and EF4 (*Alternaria* sp.) were found active against both *Sclerotinia sclerotiorum* and *Fusarium oxysporum. Chaetomium* sp. showed mild activity against *Sclerotinia sclerotiorum* and *Fusarium oxysporum. Nigrospora oryzae* did not show any activity against the test fungi. None of the endophytic fungus was found effective against *Rhizoctonia solani*. Based on comparative efficacy, among the seven endophytic fungi isolated from *T. indica*, EF1 (*Dothideomycete* sp.) and EF3 (*Thielavia subthermophila*) were found most active.

Bioactivity of extract of the endophytic fungi against *Sclerotinia sclerotiorum*

Methanol and butanol extract of endophytic fungi EF1 (*Dothideomycete* sp.) and EF3 (*Thielavia subthermophila*), showing high activity against *S. sclerotiorum* in dual culture test, were tested at 250 and 500 µg/ml against *S. sclerotiorum*. Methanol extract of *Dothideomycete* sp. showed 66.5% mycelial growth inhibition (GI) higher than

Table 1 Identification of endophytic fungi, their % similarity in BLAST and activity against plant pathogenic (PP) fungi

Code	Identified as	GenBank accession no.	% similarity	Activity against PP fungi		
				R	F	S
EF1	*Dothideomycete* sp. 7,405	GQ176264	99	–	+	++++
EF2	*Alternaria tenuissima* strain L4	GQ176265	99	–	++	++
EF3	*Thielavia subthermophila*	GQ176266	99	–	–	++++
EF4	*Alternaria* sp. ZJ13-7A	GQ176267	99	–	+++	+++
EF5	*Nigrospora oryzae* strain xsd08022	GQ176268	100	–	–	–
EF6	*Colletotrichum truncatum* isolate CT0531	GQ176269	100	–	+++	+++
EF7	*Chaetomium* sp. INBI 2-26(-)	GQ176270	96	–	+	+

R Rhizoctonia solani, *S Sclerotinia sclerotiorum*, *F Fusarium oxysporum*

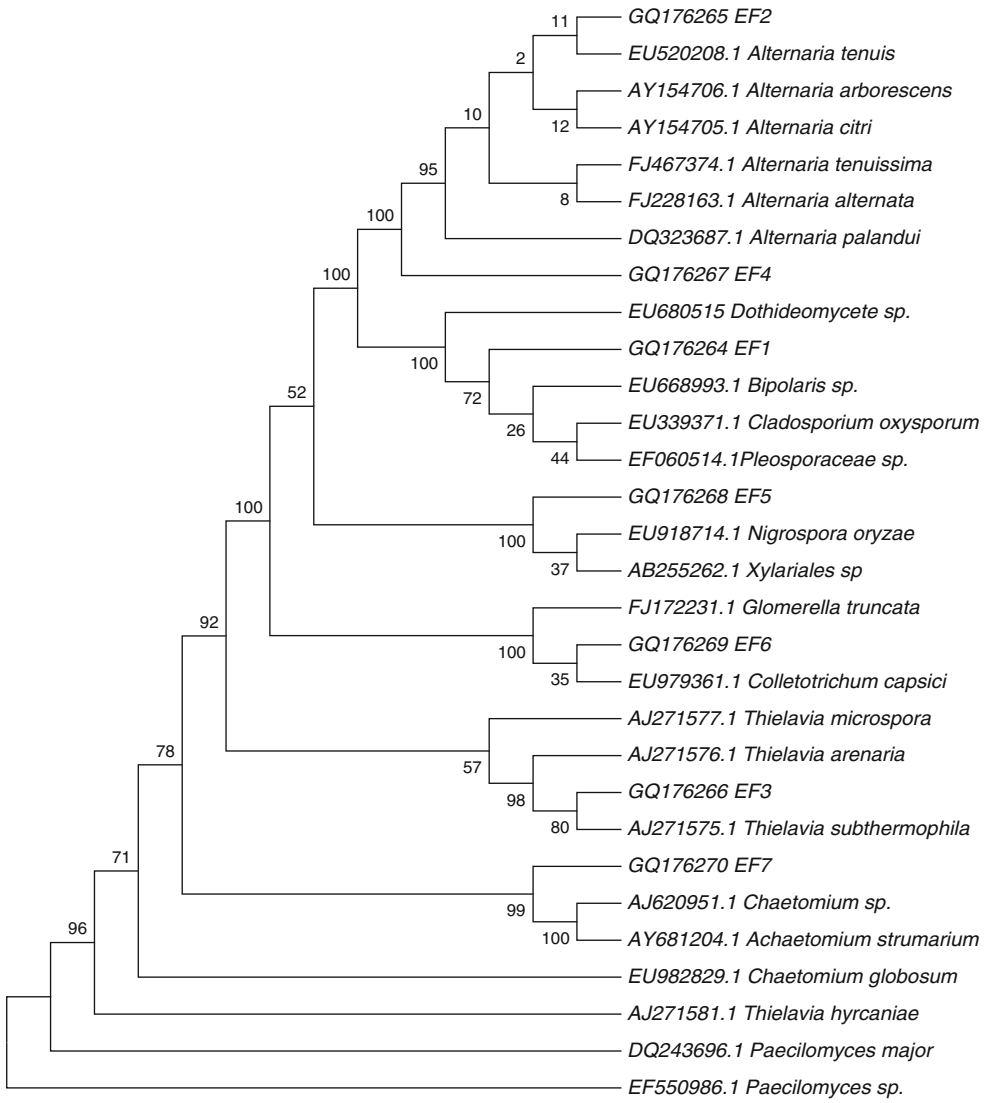

Fig. 1 Phylogenetic tree showing the relationship between endophytic fungi of *T. indica* and other related fungal species. The tree was constructed based on rDNA sequence (ITS1, 5.8S and ITS 4) by using neighbor-joining method. The bootstrap consensus tree inferred from 1,000 replicates. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 378 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4

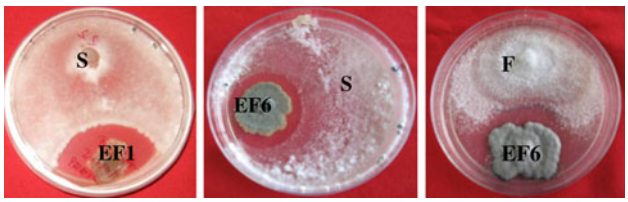

Fig. 2 Endophytic fungi from *T. indica* showing activity in dual culture antagonistic study against fungal pathogens *Scleretonia* (*S*) and *Fusarium* (*F*) of Chickpea

EF3 (*Thielavia subthermophila*) which showed 52.2% GI at 500 µg/ml. The butanol extract of EF1 exhibited 62.4% GI at 500 µg/ml which is at par with its methanol extract. While butanol extract of EF-3 was slightly less effective than butanol extract of EF1 (Fig. 3). Efficacy of the extracts on different days after the treatment is given in Table 2.

Discussions

Huang et al. has recently reported *Colletotrichum* spp., *Fusarium* spp. *Gliocladium* sp., *Phoma* spp., *and Xylariales* from *Tylophora ovata* (Huang et al. 2008). The present work is the first report of endophytic fungi *Dothediomycetes* sp., *Alternaria tenuissima*, *Thielavia subthermophila*, *Alternaria* sp., *Nigrospora oryzae*, *Colletotrichum truncatum,* and *Chaetomium* sp. from *T. indica.*

Dothideomycete taxon includes many important plant pathogens affecting all major crop plants. However, it has also been reported as endophytic fungi from the stem of Thai medicinal plant *Leea rubra* and has proved to be a good source for the production of building blocks for organic synthesis (Chomcheon et al. 2006). The present isolate shows high activity against *S. sclerotiorum* in antagonistic assay as well as assay of its extracts. Hence, this isolate can be of immense value in bio-rational pest management and need further investigation on its accurate identification and metabolite characterization.

Alternaria sp. Nees. are parasitic or saprophytic on plant material (Barnett and Hunter 1972). However, it has been isolated as endophyte from several host plant including the medicinal plants of western ghat of India (Shankar Naik et al. 2008), *Rosa damascaena* (Kaul et al. 2008), *Azadirachta indica* (Verma et al. 2008), *Catheranthus roseus* (Kharwar et al. 2008), *Polygonum senegalense* from Egypt (Aly et al. 2008), and mangrove plants in China (Liu et al. 2007). It is reported that the purified compounds of *Alternaria* sp. have antifungal activity. In the present study, species of *Alternaria* has shown antifungal activity against *S. sclerotiorum* and *F. oxysporum*. Further studies, particularly of its pathogenicity, are required for its utilization as biocontrol agent.

Thielavia subthermophila, Ascomycetes is reported from wood of *Abies webbiana, Plantaginis semen,* and *P. Herba* (Horie and Li 1997). The fungus has also been isolated from the stems of the medicinal herb, *Hypericum perforatum* (St. John's Wort), where it produced the napthodianthrone derivative hypericin and emodin (Kusari et al. 2008).This is the first report of antifungal activity of *Thielavia subthermophila* against any plant pathogenic fungus.

Endophytic association of *N. oryzae* is well documented and reported from crop plants, including maize (Saunders

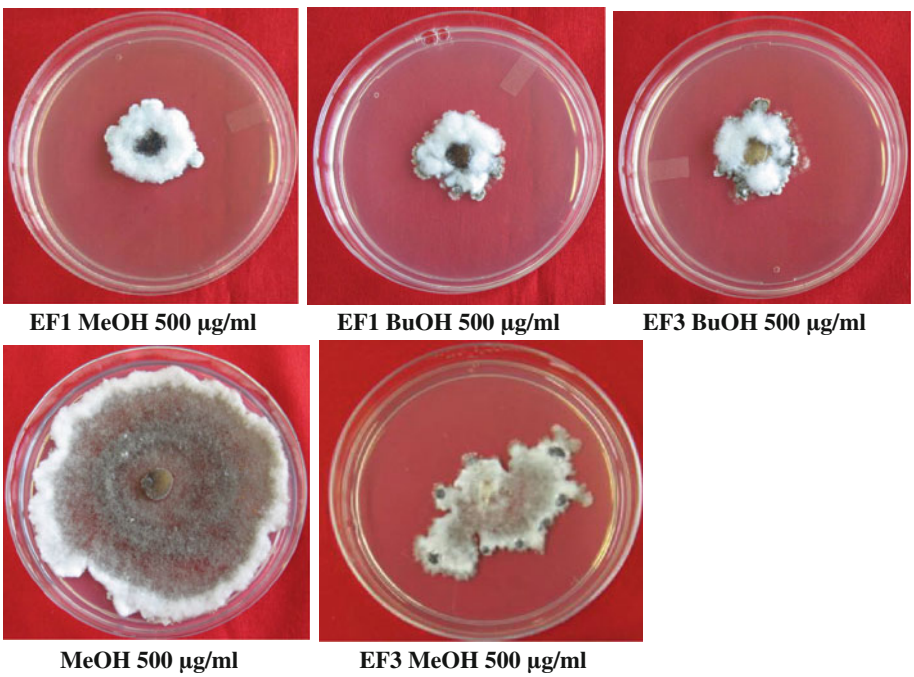

Fig. 3 Growth inhibition of *Scleretonia* by methanol (MeOH) and butanol (BuOH) extracts obtained from *T. indica* endophytic fungi

Table 2 Activity of extracts of endophytic fungi against plant pathogenic fungi tested by poisoned food technique

No. of days	Growth inhibition (%)							
	EF1 MeOH		EF1 BuOH		EF3 MeOH		EF3 BuOH	
	250 μg/ml	500 μg/ml	250 μg/ml	500 μg/ml	250 μg/ml	500 μg/ml	250 μg/ml	500 μg/ml
4th	34.41 ± 4.6	43.05 ± 5.0	34.41 ± 11.0	33.33 ± 2.4	0.0	71.91* ± 1.6	23.75 ± 3.8	45.83 ± 4.8
5th	32.96 ± 4.4	60.16* ± 4.3	25.39 ± 9.2	56.90* ± 2.2	0.0	63.78 ± 7.4	17.82 ± 2.4	56.09 ± 1.4
6th	32.44 ± 3.9	66.45 ± 4.6	19.55 ± 8.2	63.92 ± 2.9	0.0	62.8 ± 8.4	9.77 ± 2.7	63.28 ± 0.6
7th	22.67 ± 1.3	67.87 ± 4.4	7.62 ± 4.2	64.84 ± 2.2	0.0	55.55 ± 12.9	0.0	64.24 ± 0.6
10th	19.55 ± 1.2	66.47 ± 3.1	5.02 ± 4.8	62.42 ± 2.1	0.0	52.15 ± 14.4	0.0	58.95 ± 0.6

Coefficient of variation = 24.366, $CD_{0.05}$ = 14.52, $CD_{0.01}$ = 19.26

and Kohn 2008), ornamental plant, *Rosa hybrida* (Salgado and De García 2005), fruit plant, banana (Brown et al. 1998), and weed, *Parthenium hysterophorus* (Romero et al. 2001). *Crataeva magna*, a medicinal plant growing along the streams and rivers constituting riparian vegetations in Karnataka, South India is reported to host *N. oryzae* as an endophyte (Nalini et al. 2005). Phytotoxic and antibacterial metabolite, nigrosporin A and B were isolated in pure form (Tanaka et al. 1997). However, in the present study it was not found active against any of the test fungi.

Colletotrichum truncatum causes disease in most of the leguminous plants. It has worldwide occurrence as plant pathogen, causing disease in several plants including soybean (Begum et al. 2008), Broad bean, lentil (Latunde-Dada and Lucas 2007), cowpea (Bankole and Adebanjo 1996), *Pisum sativum* (O'Connell et al. 1993), and urdbean (Kaushal and Singh 1988). Despite the pathogenic nature of the fungus, it has been reported as a biocontrol agent against several noxious weeds. *Sesbania exaltata,* a noxious weed of soybean, has been successfully managed by the pathogen (Boyette et al. 2007). Its activity against plant pathogenic fungi, such as *S. sclerotiorum* and *F. oxysporum*, is reported for the first time in this work.

Chaetomium globosum has been reported as endophyte from several host plant including *Canvalia maritime* (Seena and Sridhar 2004), *Ipopmea pes-caprae*, *Launea sarmentosa, Polycarpaea corymbosa* (Beena et al. 2000), and some medicinal plants viz., *Terminalia arjuna*, *Crataeva magna*, *Azadirachta indica*, *Holarrhena antidysentrica* (Tejesvi et al. 2006). It showed mild activity against *S. sclerotiorum* and *F. oxysporum*.

Conclusion

In the present study, *Dothideomycete* sp. and *Thielavia subthermophila* have been found as potential biocontrol agent for the management of *S. sclerotiorum.* Further work on these fungi will be useful in developing new biocontrol agent against crop pests. Growth inhibition activity of the extract of endophytic fungi is quite crucial because the technological barrier in production, maintenance, and storage of living organism can be minimized by developing extract based biopesticide. Also, the use of living microbe for pest management could cause loss of crops, if these living microbes turn pathogenic in due course of acclimatization to the introduced condition. From quality and appearance point of view, adherence to biocontrol agent on the edible portion is of concern. However, extract based biopesticide will not be having such limitations.

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