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Bioprospecting the fungicides compatible *Trichoderma asperellum* isolate effective against multiple plant pathogens *in vitro*

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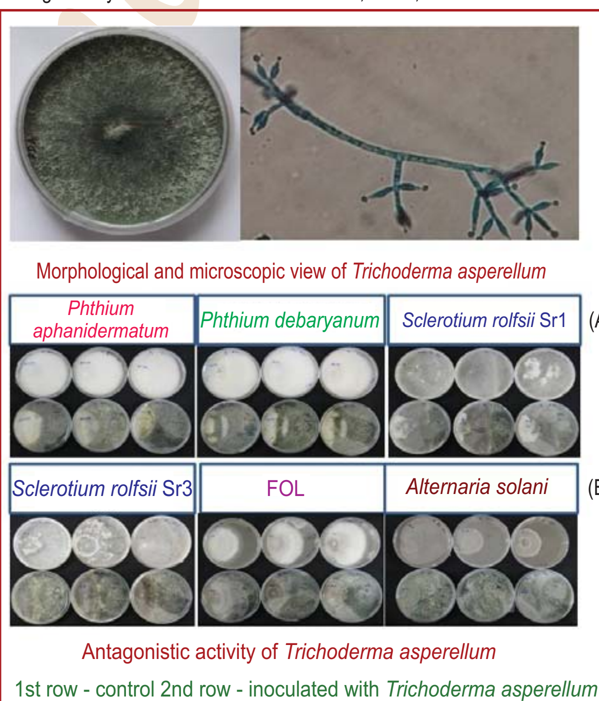
Abstract

Aim: It is essential to develop an efficient ecofriendly disease management programme which helps in gradual reduction in use of harmful chemicals. The objective of the study was to isolate, purify and identify *Trichoderma* isolate from diseased plant tissues and to identify its compatibility with commonly used fungicides for the eco-friendly management of vegetable diseases.

Methodology: *Trichoderma asperellum* was isolated from *Sclerotinia sclerotiorum* infected stem tissues of pea on potato dextrose agar. The morphological, cultural characteristics and molecular identification of *Trichoderma asperellum* was carried out using ITS primers. *In vitro* antagonistic efficiency of *Trichoderma asperellum* was studied against phytopathogenic fungi viz., *Pythium aphanidermatum, Pythium debaryanum, Sclerotium rolfsii Sr1, Sclerotium rolfsii Sr3, Fusarium oxysporum f.sp. lycopersici* and *Alternaria solani.*

Results: The nucleotide sequence of amplified ITS region was deposited at Genbank (NCBI) with accession no. KT824429. The highest mycelial inhibition values of 43.57, 38.16, 42.56 and 54.87% were obtained for *Pythium aphanidermatum*, *P. debaryanum*, *Sclerotium rolfsii* Sr1 and *Sclerotium rolfsii* Sr3, respectively, after 6 days of *T. asperellum* inoculation. *T. asperellum* exhibited 100% compatibility with Mancozeb, Azoxystrobin, Cymoxinil+Mancozeb, Metalxyl+Mancozeb at 100, 200 and 300 ppm. The per cent compatibility of 98.15, 74.82 and 50.38 with Carbendazim at 100, 200 and 300 ppm was also recorded.

Interpretation : These studies established the aggressive nature of the isolate and its suitability in integrated disease management practices.



Antagonistic activity of *Trichoderma asperellum*
1st row - control 2nd row - inoculated with *Trichoderma asperellum*

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Introduction

The increased environmental pollution due to application of agricultural chemicals for enhancing crop productivity emphasized the need for alternative methods to manage soil borne diseases and to sustain agricultural production systems (Kamal *et al.,* 2009; Holajjer *et al*., 2013; Pokhare *et al*., 2015). Biological control is an effective option to minimize the use of expensive chemical fungicides in modern agriculture, as they are the main cause of resistance development in pathogens besides polluting the environment (Harman *et al.,* 2004). In recent years, utilization of *Trichoderma* has been encouraged as an ecofriendly measure to protect the plants from diseases and increase the yields (Mukherjee *et al*., 2012). Genus *Trichoderma* has helped to sustain the agricultural yields naturally, as a multifunctional agent (Hamed *et al*., 2015; Triveni *et al*., 2015). This ubiquitous and free-living filamentous fungus, with high survival rate, reproductive ability, nutrient use and capacity to promote plant growth through diverse mechanisms, has several species that are effective biological control agents against plant pathogens (Brito *et al*., 2014, Abo-Elyousr *et al*., 2014; Dubey *et al*., 2001; Zhang *et al*., 2013). It is being used in several crops like vegetables, pulses, rice, wheat, groundnut, soybean, sugarcane, sunflower, ginger, cardamom, black pepper, coconut, cotton, castor, banana and tobacco against different plant pathogens (Sriram *et al*., 2013; Rao *et al*., 2015).

To develop an efficient disease management programme, compatibility of potential bioagents with fungicides is essential (Thoudam and Dutta, 2014). Diseases can be effectively managed by using potential bioagent which is tolerant to fungicides and keeps pathogens under sufficient pressure (Wedajo, 2015). The combination of biocontrol agents with fungicides is expected to provide the same level of disease suppression, as that of with higher fungicide use and diminish the possibility of resistance development in pathogens (Thoudam and Dutta 2014; Bhai *et al*., 2010). In addition to this, it also extends the duration of active disease control (Hjeljord and Tronosmo, 1998). Integration of bioagents which are compatible with fungicides in disease management is very important. Exploration of more effective *Trichoderma* strains with high antagonistic potential required for successful biological control programmes under different ecological niche (El Komy *et al*., 2015). The objective of the present investigation included isolation and characterization of *Trichoderma asperellum* and its *in vitro* compatabilty with commonly used fungicides in disease control.

Materials and Methods

Study area : The study was conducted at ICAR-Indian Institute of Vegetable Research, Varanasi, India having average rainfall of 1000 mm. In general, the temperature ranged from 5 ºC to 42 ºC, January being coldest and May-June hot.

Collection, isolation, purification of *Trichoderma* isolate : *Sclerotinia sclerotiorum* infected stem tissues of pea were collected during January 2015, from the research farm of ICAR-Indian Institute of Vegetable Research, Varanasi. The diseased tissues (approx. 30-40 g) were surface sterilized with 1% sodium hypochlorite solution for one minute. Then, the samples were rinsed with sterile distilled water for 6 times, air dried and placed on potato dextrose agar (PDA) medium, supplemented with 100 mg/l streptomycin sulphate. The plates were kept in a BOD for 6 days at 28 ˚C. Along with *Sclerotinia* growth green coloured mycelia were observed, which was selected and reinoculated on PDA plate. The fungus was purified by mycelial tip method and maintained on PDA slants at 4 ºC for further use.

Cultural and morphological characterization : The morphological and cultural characteristics of *Trichoderma asperellum* isolate was studied in four different media viz., PDA, Corn Meal Agar(CMA), Oat Meal Agar (OMA) and Czapek dox agar medium (Shaiesta *et al*., 2012). Mycelial discs (5 mm) of young growing culture of *Trichoderma* isolate was inoculated in the periphery of Petri plates containing previously mentioned media and incubated at 28±2 ºC for 72 hrs. The growth of mycelium was recorded at 24, 48 and 72 hrs. The experiment was repeated thrice with three replications.

Molecular identification and phylogenetic analysis: *Trichoderma asperellum* was grown on potato dextrose broth for seven days at 28±1 ºC in a BOD incubator. The fungal genomic DNA was extracted (Doyle and Doyle, 1987) and the internal transcribed spacer (ITS) regions were amplified by PCR using universal primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) (White *et al*., 1990). The PCRs were performed in a 25µl reaction volume containing 16µl PCR grade water (Sigma), 2.5 µl PCR buffer (10×), 2.5 µl of 10mM dNTPs mix (Sigma-Aldrich), 1µl of each primer (20 pmolμl^{-1}), 1 µl (5 Uμl^{-1}) of Taq polymerase (Sigma-Aldrich) along with 20–50 ng of template DNA. PCR was performed in an Eppendorf Master Cycler (Eppendorf, Hamburg). The amplification program consisted of an initial denaturation step at 95 ºC for 5 min followed by 30 cycles of denaturation at 95 ºC for 1 min, annealing for 30 seconds at 55 ºC and extension for 1 min at 72 ºC. A final extension step at 72 ºC for 7 min was included at the end of the amplification. All PCR products were electrophoresed, imaged and analyzed in a Gel Documentation System (Syngene Inc. Cambridge).

The PCR products prior to sequencing were purified using Axygen Spin PCR clean-up kit. Sequencing reaction was performed using the Big Dye Terminator cycle sequencing kit (ABI) on a thermal cycler with respective primers. The amplification products were cleaned by ammonium acetate precipitation. The cleaned product was finally run (after addition of HiDi formamide) on an automated ABI (3100Avante) DNA sequencer to get sequences in ABI format at National Fungal

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Culture collection of India (NFCCI), Pune. The PCR fragment was sequenced using ABI 3100 automated DNA sequencer. The sequence was compared using NCBI (www.ncbi.nlm.nih.gov) blast search, to determine percentage sequence identity/similarity with those species that showed maximum identity in blast search. The sequences showing highest scores with the present isolate were obtained from database and aligned using the Clustal W program (Thompson *et al*., 1994). The phylogenetic tree was generated by MEGA 5 software using the neighbour joining method with 1000 bootstrapped replications to estimate evolutionary distances between all pairs of sequences simultaneously (Tamura *et al*., 2011).

***In vitro* antagonistic studies :** *In vitro* confrontations were studied between the *Trichoderma asperellum* and the phytopathogenic fungi: *Pythium aphanidermatum a*nd *Pythium debaryanum* collected from the Indian Type Culture Collection, New Delhi; *Sclerotium rolfsii Sr1, Sclerotium rolfsii Sr3, Fusarium oxysporum f.sp. lycopersici* and *Alternaria solani* were taken from the germplasm of ICAR-Indian Institute of Vegetable Research, Varanasi. All these fungi are important pathogens in vegetables. The isolate of *T. asperellum* was tested against the phytopathogens using dual culture method (Morton and Stroube, 1955). Each Petri-dish (9 cm) containing PDA was inoculated with two 5 mm diameter mycelial discs *i.e*., one of the pathogen and one of *T*. *asperellum* were placed at 7 cm distant from each other. The discs containing pathogenic fungi were placed in the Petri-dishes, 2 days before the *Trichoderma* discs. For control treatments, a disc of individual pathogen was placed on separate PDA medium. The inoculum discs were obtained from the margin of actively growing 6 day old fungal cultures. Three replicates were maintained for each treatment. Plates were incubated at 28±2°C for 10 days. The experiment was conducted by following completely randomized design. Percent inhibition was calculated by the following formula *i.e*., I=(C-T/C) ×100, where, I is percent inhibition, C is radial growth of pathogen (cm) alone (control), T is radial growth of pathogen (cm) in the presence of *Trichoderma* isolate (Edington *et al*., 1971). The microscopic observations were recorded at 40X for possible mycoparasitism at interaction zone between two fungal cultures.

Compatibility with fungicides : The effect of different fungicides on mycelial growth of *Trichoderma asperellum* was evaluated *in vitro* (Nene and Thapliyal, 1993). Each and every fungicide was separately added to molten and cooled sterilized PDA media (Himedia, India) to get the concentration of 100, 200 and 300 ppm and poured into 90 mm petri dishes. A 5 mm mycelial plug of 3 day old culture was taken using a cork borer and placed at the centre of petri plate. The plates were kept in incubator at 28ºC and three replications were maintained. The radial growth of *Trichoderma asperellum* was measured after 72 hrs of incubation and percent growth inhibition was calculated by the following formula C-T/ C x100 where C is the growth in control and T is the growth in treatment.

Results and Discussion

The isolate was green coloured on petriplate, the conidiophores were highly branched and terminated with sub globose phialides. The phialides were flask shaped and constricted at the tip. Based on these morphological characteristics and microscopic observations, it was identified as belonging to the genus *Trichoderma* (Fig.1). Fungal members of *Trichoderma* are known to be cosmopolitan and grow in different substrates, including infected or degrading plant debris, decaying wood etc. (Garcia-Gaza *et al*., 1997). They are known as biocontrol agents, and are very much interactive in soil and roots (Zghair *et al*., 2014). They show rapid multiplication and reproduction, besides hyperparasitism. *Trichoderma* is known to be involved in biotrophic mycoparasitic associations, and the

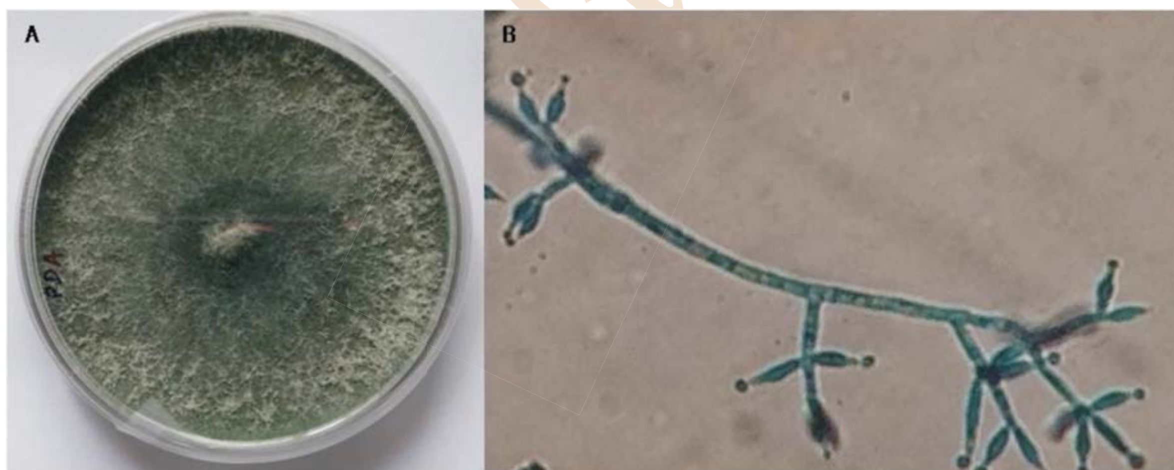

Fig. 1 : (A) Colony morphology; (B) Microphotograph of *Trichoderma* isolate

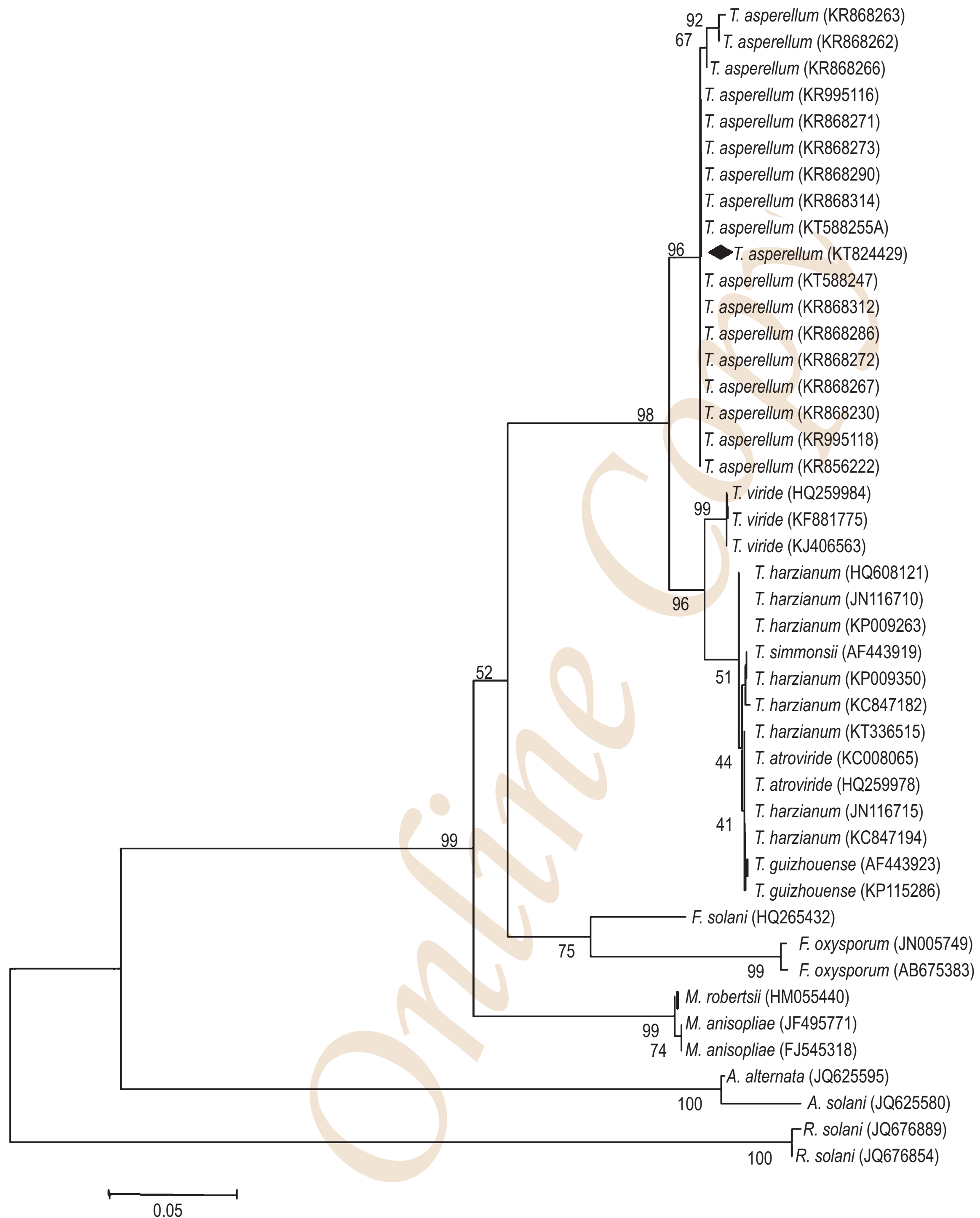

Fig. 2 : Phylogenetic tree based on the comparison of ITS sequences of *T. asperellum* and other *Trichoderma* spp. and fungi registered in GenBank. The numbers in parentheses represent the accession numbers in GenBank. The numbers in each branch points denote the percentages supported by bootstrap. The scale bar represents 0.05 substitutions per nucleotide position

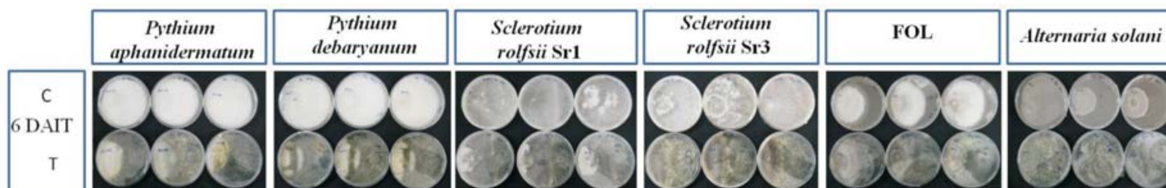

Fig. 3 : Antagonism of *Trichoderma asperellum* against different plant pathogens. FOL-*Fusarium oxysporum* f.sp. *lycopersici*; DAIT-Days after inoculation of *T. asperellum*; C-Control; T-Treatment

Fig. 4 : Compatibility of *Trichoderma asperellum* with different fungicides. A: Curzate M8; B: Ridomil Gold; C: Sixer ; D: Bavistin ; E: Amistar; F: Indofil M 45. Right to Left: Control, 100, 200, 300 ppm concentrations

isolate in the present study is such an example. Different species of *Trichoderma* (Hypocreales, Ascomycota) have been widely used as biocontrol agents against plant pathogens (Samuels 1996; Kumar and Gupta, 1999; Harman, 2000).

Out of four media tested, *Trichoderma* isolate grew rapidly on PDA and OMA with a radial growth of 4.5cm after 72 hrs of incubation. On CMA and Czapekdox agar, the radial growth was 2.82 and 4.33 cm, respectively. The *Trichoderma* isolate was purified on PDA and used for extraction of DNA. PCR based amplification of internally transcribed spacer (ITS) region of the isolate and sequencing was undertaken. *In silico* analysis revealed 100% similarity to *Trichoderma asperellum*. The phylogenetic analysis revealed the close clustering of the isolate with other *T. asperellum* isolates (Fig. 2). Based on cultural, morphological and molecular characteristics, the isolate was identified as *T. asperellum*. The sequence was deposited at GenBank (NCBI) with an accession no. KT824429. Several *T. asperellum* isolates have been reported as multifaceted bioagents (de los Santos- Villalobos *et al*., 2013; Marcello *et al*., 2010; Tondje *et al*., 2007).

T. asperellum isolates have been reported as multifaceted bioagents, and as elicitors of plant immune responses (de los Santos- Villalobos *et al*., 2013; Marcello *et al*., 2010; Tondje *et al*., 2007). The antagonistic capability of *T. asperellum* was assessed through *in vitro* confrontation studies**.** *T. asperellum* caused significant reduction in the mycelial growth of the pathogens tested (Fig. 3). The highest inhibition values of 43.57, 38.16, 42.56 and 54.87% were obtained for *Pythium aphanidermatum*, *P. debaryanum*, *Sclerotium rolfsii* Sr1 and *Sclerotium rolfsii* Sr3, respectively, after 6 days of *T. asperellum* inoculation. This isolate also inhibited mycelial growth of *Fusarium oxysporum* f.sp. *lycopersici* and *Alternaria solani* to the extent of 26.27 and 24.17% after 5 days and parasitisation after 6 days of incubation (Table 1). The isolate used in the present study

Table 1 : Inhibitory activity of the *Trichoderma* isolate against selected phytopathogenic fungi, as measured in dual culture assays after 5 and 6 days of incubation

Pathogen	Mycelial inhibition (%)	
	5 day	6 day
Pythium aphanidermatum	38.71 ± 2.24	43.57 ± 4.68
Pythium debaryanum	37.43 ± 2.78	38.16 ± 2.60
Sclerotium rolfsii Sr-1	47.43 ± 0.67	42.56 ± 0.92
Sclerotium rolfsii Sr-3	57.69 ± 0.44	54.87 ± 0.67
Fusarium oxysporum f.sp. *lycopersici*	26.27 ± 10.06	Parasitisation
Alternaria solani	24.17 ± 13.15	Parasitisation
CD (p<0.05)	25.15	12.32

Table 2 : Fungicides used for compatibility studies with *Trichoderma asperellum*

Product name	Formulation type	Active constituent	Company
Curzate M8	WP	Cymoxanil (8%) + mancozeb 64%	Dupont
Ridomil Gold	WP	Metalaxyl 4% + mancozeb 64%	Syngenta
Sixer	WP	Carbendazim 12%+ Mancozeb 63%	Dhanuka Agritech Ltd.
Bavistin	WP	Carbendazim 50%	BASF
Amistar	SC	Azoxystrobin (23.1% w/w)	Syngenta
Indofil M 45	WP	Mancozeb 75%	Indofil

was effective against all the six tested plant pathogens causing severe loss to vegetable crops (Table 1). Yedidia *et al*. (2003) reported that *T. asperellum gives protection* against soil borne, as well as foliar pathogens. It effectively managed the *Pseudomonas syringae* pv. *lachrymans* infection in cucumber by inducing systemic resistance towards a foliar pathogen. *It also* drastically controlled the *sclerotium rolfsii* infecting bean and chickpea plants (Pacheco *et al*., 2016; Prajapati *et al*., 2015). Such fast growing bioagents have additional advantage in competing with the pathogens for space and nutrients (Simon and Sivasithaparam, 1988; Benítez *et al*., 2004). *T. asperellum* isolate under study was fast growing in comparison to all the pathogens. Even though was inoculated two days after the pathogens were inoculated, it overgrew and completely parasitized them. Kumar *et al*. (2012) reported that *T. viride* inhibited mycelial growth of *S. rolfsii* more than 50%. In the present study, 54.87% mycelial inhibition of *S. rolfsii* Sr3 was recorded (Table 1).

Compatibility of bioagent is very important to reduce the use of chemical fungicides and effective management of fungal pathogens (Elshahawy *et al*., 2016). The details of the fungicides used in the study are given in Table 2. *T. asperellum* exhibited 100% compatibility with Mancozeb, Azoxystrobin, Cymoxinil+Mancozeb, Metalxyl+Mancozeb at 100, 200 and 300 ppm, (Fig. 4). The percent compatibility of 98.15, 74.82 and 50.38 % with Carbendazim at 100, 200 and 300 ppm concentrations was also recorded. The compatibility percentage for Carbendazim and Mancozeb was 90.38, 70.38 and 47.41% at 100, 200 and 300 ppm, respectively. Hjeljord and Tronsmo (1998) reported that *Trichoderma* species can use fungicides as a source of nutrient upto certain concentration levels. Fungicides at low concentrations induce stress and weaken the pathogen by making its propagules susceptible to attack by bioagent (Hjeljord and Tronsmo, 1998); increase in the concentration of fungicide inhibits the fungal growth. Carbendazim and combination of carbendazim and mancozeb reduced the growth of *T. asperellum* at higher concentration. Efficacy of bioagent is augmented when they are used in combination with fungicides at lower concentration (Silimela and Korsten, 2001). Integration of bioagents with chemical fungicides reduces seed infection more effectively than they are used alone (Srinivas and Ramakrishnan, 2002).

In the present study, *Trichoderma asperellum* isolate was effective against six phytopathogenic fungi-*Pythium aphanidermatum*, *P. debaryanum*, *Sclerotium rolfsii* Sr1, *S. rolfsii* Sr3, *Fusarium oxysporum* f.sp. *lycopersici* and *Alternaria solani. T. asperellum* exhibited 100% compatibility with Mancozeb, Azoxystrobin, Cymoxinil+Mancozeb, Metalxyl+Mancozeb at 100, 200 and 300 ppm. The presence of hyperparasitism and competitive ability in the isolate makes it an ideal candidate for inclusion in integrated disease management practices.

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