JOURNAL OF PURE AND APPLIED MICROBIOLOGY, June 2016. Vol. **10**(2), p. 1591-1598

# Antagonism and Hyphal Relationship between *Trichoderma* spp. and *Fusarium oxysporum-Rhizoctonia bataticola* causing Wilt Complex in Chickpea

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(Received: 13 January 2016; accepted: 19 April 2016)

***Trichoderma* spp. are bio control agents extensively used in management of fungal diseases of crop plants exhibiting antagonism against a wide range of plant pathogens. It has been known from many years that *Trichoderma* spp. produces wide range of antibiotic substances and they paralyze other fungi. They can also compete for key exudates from seeds that stimulates the germination of propagules of plant pathogenic fungi in soil (Howell, 2002) and more generally compete with the soil microorganisms for nutrient and space. Keeping this in view nine *Trichoderma* spp. viz; *T.asperellum, T. atroviride, T. harzianum, T. koningii, T. longibrachiatum, T. minutisporum, T. ressei, T. viride,* and *T. virens* isolated from different locations of India were characterized for their antagonistic activity against wilt and dry root rot pathogens of chickpea. The various spp. showed differential reaction against the test pathogens *Fusarium oxysporum* f.sp. *ciceri* (*Foc*) and *Rhizoctonia bataticola* (*Rb*). However, *T. harzianum* was most effective causing 55.0 and 31.8 percent inhibition of mycelial growth in *Foc* and *Rb* respectively. Scanning Electron microscopic investigations about hyphal interactions between antagonists and test fungi revealed that the mycoparasitic hyphae were usually attached longitudinally to the hyphae of the pathogen. Coiling of hyphae, short contact branches, hyphal depression and pincer shaped structures were seen during observations through SEM indicated the mode of action in biological control of the test pathogens.**

**Keywords** *Fusarium oxysporum* f.sp. *ciceri. Rhizoctonia bataticola. Trichoderma* spp., Antagonism, Hyphal relationship, Wilt Complex, Chickpea, Biological management.

Chickpea (*Cicer arietinum* L.) is one of the major grain legume widely grown in India as well as other parts of the world. Among various factors attributing to low productivity of chickpea, diseases are very important. Van Emden *et al*. (1988) estimated that yield losses due to insects and diseases ranges from 5 to 10% in temperate and 50 to 100% in tropical regions. Vishwa Dhar and Chaudhary (2001), stated that chickpea is prone to many diseases and among them wilt and dry root rot caused by *Fusarium oxysporum* f.sp. *ciceri* and *Rhizoctonia bataticola* are the major constraints in chickpea production causing 10-20% annual loss. Wilt complex caused by *Fusarium oxysporum* f.sp.*ciceri* (*Foc*) and *Rhizoctonia bataticola* (*Rb*) causes more severe damage to the crop. In chickpea, infected seed, chlamydospores and sclerotia surviving in the soil are the major sources of primary inoculums. Since 75% cultivation of chickpea in India is under rainfed, the crop faces severe moisture stress which predisposes the crop to wilt and dry root rot development. The saprophytic survival ability of the pathogens in soil makes chemical control and

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crop rotation ineffective. Cultivation of resistant varieties is an economical approach for the management of these diseases but up to now effective resistant cultivars are not available to combat with the pathogens. The preference for biological control methods is justified also by the undesirable side effects of pesticides. The technology that seems promising to manage the diseases without disturbing the equilibrium of harmful and useful composition of environment and ecosystem is the use of more and more biological control agents. The use of microorganisms to control plant pathogens, known as biological control is now in practice. It is accepted as a suitable and environmentally friendly alternative or a supplemental way of reducing the use of chemicals in agriculture against plant disease management. Generally biological formulations are considered to be ideal for pest management because of their specificity to pests and because of their lack of toxicity to humans or natural enemies of many crop pests. Natural pest enemies include pathogenic fungi, viruses, bacteria and entomopathogenic nematodes. The controlling of insect pests are chiefly carried out by the imported expensive chemicals, which are the main cause of environmental pollution, have detrimental effect on human health, cause death of not only pests, but of many useful insects. It was stated by various workers that use of *Trichoderma* spp. as biological agents has been very much successful against soil borne diseases for which no resistant sources have been identified (Mukhopadhyay,1994; Claudia *et al*,. 1997; Mukhrejee *et al*., 2012; Thiago *et al*., 2013; Xuping *et al*., 2014).

There is still considerable interest in finding more efficient mycoparasitic fungi especially within *Trichoderma* spp., which differ considerably with respect to their biocontrol effectiveness. It is important to isolate *Trichoderma* spp. having potentially higher antagonistic efficiency. The aim of this study was to screen *Trichoderma* spp. for their antagonistic ability by dual cultures and to investigate their capability of interaction and hyphal depression to the test pathogens. These observations will be helpful to identify potential *Trichoderma* spp. with strong mycoparasitic activity against wilt and dry root rot pathogens which can be used for the formation of novel bioformulations.

## MATERIALS AND METHODS

### Soil samples and Isolation

Soil samples were collected from the rhizosphere soil of different crop niches in Uttar Pradesh (India). Five- fold serial dilutions as described by Singh and Singh, (1970) for each soil sample was prepared in sterilized distilled water and 0.5 ml diluted sample was poured on the surface of Trichoderma Specific Medium (TSM) (Elad *et al*., 1981). Plates were incubated at 28 ± 2°C for 96 h. Morphologically different colonies appearing on the plates were purified on Potato Dextrose Agar Medium (PDA) (HiMedia, India) and send to ITCC, New Delhi for identification. Test pathogens were isolated from the roots of infected chickpea plants and maintained in PDA.

### Antagonistic Activity of *Trichoderma* Isolates

The dual culture technique described by Morton and Stroube (1955), was used to test the antagonistic ability of 9 *Trichoderma* spp. viz; *T. asperellum* (ITCC 8940), *T. atroviride* (ITCC 7445), *T. harzianum* (ITCC 6796) *T. koningii* (ITCC 5201), *T. longibrachiatum* (ITCC 7437), *T. minutisporum* (ITCC 7280), *T. ressei* ( ITCC 7284), *T. viride* (ITCC 8315), and *T. virens* ( ITCC 4177) against test pathogens. The purified fungal cultures of pathogens and *Trichoderma* spp. were grown on PDA for a week at room temperature (28 ± 2°C). Five mm disc of the target fungi (*Foc* and *Rb*) cut from the periphery was transferred to the Petri dish previously poured with PDA. *Trichoderma* spp. was transferred aseptically in the same plate of opposite end and were incubated at room temperature with alternate light and darkness for 7 days and observed periodically. Control plates were maintained without *Trichoderma*. The experiment was replicated thrice and percent growth inhibition was calculated by the formula of I = (C”T)/C × 100, where C is mycelial growth in control plate, T is mycelial growth in test organisms inoculated plate and I is inhibition of mycelial growth and the data was analysed using Completely Randomized Design (CRD) method (Vincent *et al*., 1999).

**Scanning electron microscopy (SEM)**

Small pieces of agar (approx. 2 mm2) were taken from the dual culture plates at the point of interaction between *Trichoderma* spp. and test fungi. The samples were fixed in 2.5% glutaraldehyde dissolved in 0.5M phosphate buffer at pH 7.2 and stored overnight at 4°C, then rinse with the same buffer. After dehydration using a graded ethanol series, samples were critical-point dried in carbon dioxide after a graded transition from ethanol to acetone. Sections (5×5mm) were mounted on stubs, coated with gold- palladium, and examined with a JEOL-JSM-T300 SEM operating at 15 kV.

## RESULTS

Macroscopic examination of the fungal dual cultures revealed that most of the strains made hyphal contact with test pathogens within three days after inoculation. *T. harzianum* was the most inhibiting antagonist that grew over the pathogen. Other *Trichoderma* spp. acted only as a barrier against *Fusarium oxysporum* f.sp. *ciceri* and *R. bataticola.*

Antagonistic potential of *Trichoderma* spp. through dual culture indicated that colony growth after 72 h was 35.0- 42.5 mm in *Foc* and 37.5- 48.7 mm in *Rb* as compared to control. Colony growth of test pathogens was appressed and after coming in contact, the antagonists grew and sporulated over the pathogen colony due to their prolific growth habit and mycoparasitic character. Inhibition percent of growth by different *Trichoderma* spp. ranged between 21.6 – 55.0 percent and 11.4 – 31.8 in *Foc* and *Rb* respectively (Table 1, Fig 1 & 2). These findings corroborate the findings by earlier workers. (Sumeet and Mukherjee, 2000; Golve and Kurundkar, 2002; Jagathambigai *et al*., 2009; Shahid et.al., 2012; Srivastava et.al., 2014.)

A similar behavior for each antagonist-pathogen combination was observed by SEM. There were similarities and differences in the antagonistic ability of all species of *Trichoderma* to invadethe pathogen in dual culture. Direct contact with the pathogen was always followed by various types of hyphal aggression. SEM investigations revealed that mycoparasitic hyphae were usually attached longitudinally to the hyphae of the pathogen. Hyphal coiling, hooks, pincer shaped structures, short contact branches and hyphal depression were also observed. (Fig 3 & 4)

In case of conidial and sclerotial inoculum of *Foc* & *Rb* it was observed that percent conidia and sclerotia killed ranged from zero to 100 percent depending upon the antagonistic potential of the *Trichoderma* species. *T. harzianum* kill all the conidia and sclerotia while other species killed some of the inoculum (Fig. 4a-h). All *Trichoderma* sp. were effective in reducing conidial and sclerotial viability. These observations revealed that penetration and multiplication of antagonist inside the conidia and sclerotia is dependent on the ability of the biocontrol agent to attack and establish on the wall of conidia and sclerotia. As the studies done so far on biological control of *F. oxysporum ciceri* and *R. bataticola* included only a few isolates of a particular species so it is difficult to draw a conclusion on the species specificity. In the present investigations antagonistic effects of nine *Trichoderma* species revealed that there is a significant variability in their ability to parasitize, macerate and kill the myceial, conidial and sclerotial inoculum of the test pathogens. Conidia and Sclerotia are first colonized by the antagonists followed by penetration and finally killing. *Trichoderma harzianum* is found best in mycoparasitism of *Fusarium oxysporum*, f.sp. *ciceri* and *R. bataticola* as compared to other antagonists studied. These findings supported by findings of earlier workers. (Rajput et.al.,2010; Amrutha *et al*., 2014; Magar et.al., 2014; Nagamani et.al., 2015).

## DISCUSSION

*Trichoderma* is a well known biocontrol agent with multiple modes of action such as competition (Howell, 2003), induced resistance (Harman, 2006), solubilization of inorganic plant nutrients (Altomare *et al*., 1999), inactivation of the pathogen's enzymes involved in the infection process (de Meyer *et al*., 1998) and mycoparasitism (Barnett and Binder, 1973). Various workers stated that *Trichoderma* spp. produces cell wall degrading enzymes (CWDEs) including chitinases, â-1,3-glucanases, proteases and â- 1,4-glucanases, antibiotics and antibiotic peptides, such as peptaibols to combat with the pathogen (Flores *et*

*al*., 1997; Elad and Kapat, 1999; Pandey et.al., 2014; Srivastava et. al., 2014).

In case of antibiosis in dual culture it was observed that *Trichoderma harzianum* was found best in controlling growth of the test pathogens among all *Trichoderma* spp. Varying modes of hyphal interactions and degree of inhibition in growth and development of *Foc* and *Rb* were studied to investigate mechanism of control. Understanding the mechanism of action involved in the biocontrol process is of primary importance in establishing these characteristics. This can provide much insight about where and when the interaction occurs and how the pathogen will be affected. In order to survive and mycoparasitize *Trichoderma* spp. produces a wide variety of toxic and antibiotic metabolites such as trichodermol, trichodermin, harzianolide, terpines, polypeptides (Vinale *et al.*, 2006; Vinale *et al*., 2008; Andrabi *et al*., 2011) and extracellular hydrolytic enzymes (Thrane *et al*., 2000; Eziashi *et al*., 2006) which were involved in the inhibition, competition, and mycoparasitism of phytopathogenic fungi. In this regard our results support these findings by showing that *Trichoderma harzianum* produces strong antibiosis and competitive growth against pathogens in agar plates (Fig. 1 & 2).

**Table 1.** In vitro antagonistic potential of *Trichoderma* isolates against *Fusarium oxysporum* and *R. bataticola* through dual culture

| *Trichoderma* spp. | Growth of *Foc* at 72h(mm) | | Growth of *R.b.* at 72h(mm) | |
|---|---|---|---|---|
| | Mycelial growth | % inhibition in mycelial growth | Mycelial growth | % inhibition in mycelial growth |
| *T.asperellum* | 18.7 | 37.6 | 45.0 | 18.1 |
| *T. atroviride* | 19.0 | 36.6 | 42.5 | 22.7 |
| *T. harzianum* | 13.5 | 55.0 | 37.5 | 31.8 |
| *T. koningii* | 23.5 | 21.6 | 47.5 | 13.6 |
| *T. longibrachiatum* | 19.0 | 36.6 | 48.7 | 11.4 |
| *T. minutisporum* | 23.0 | 23.3 | 45.0 | 18.1 |
| *T. ressei* | 20.5 | 31.6 | 46.5 | 15.4 |
| *T. viride* | 21.5 | 28.3 | 45.0 | 18.1 |
| *T. virens* | 18.5 | 38.3 | 46.2 | 16.0 |
| Control | 30.0 | - | 55.0 | - |
| CD@5% | 4.2 | | 3.3 | |

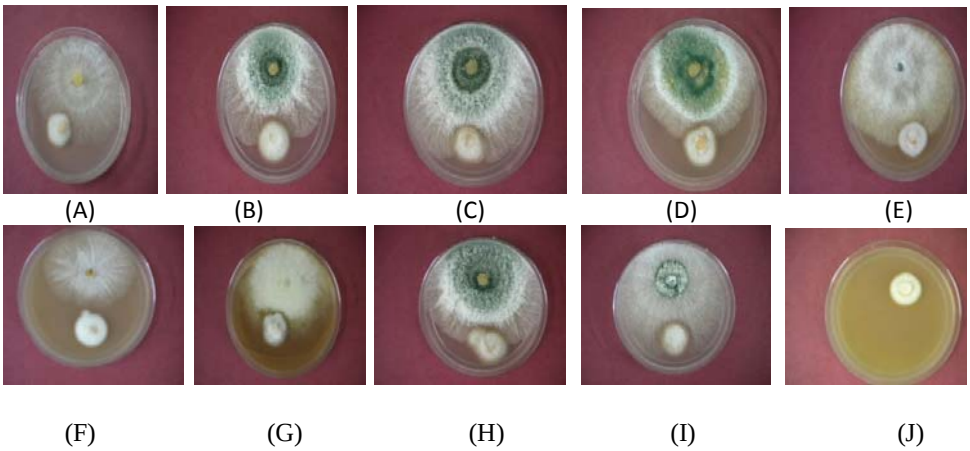


**Fig. 1.** Antagonism of *Trichoderma* spp. against *Fusarium oxysporum* f.sp. *ciceri* (A) *T.asperellum* (B) *T. atroviride* (C) *T. harzianum* (D) *T. koningii* (E) *T. longibrachiatum* (F) *T. minutisporum* (G) *T. ressei* (H) *T. viride* (I) *T. virens* (J) Control (*Foc*)

The interactions observed in the SEM analysis can be considered as typical of hyperparasitism (Agrios, 2005). Similarly, Louzada *et al*. (2009) observed hyphae of *F. solani* encoiled by *Trichoderma* in SEM images. As reported by Zeilinger & Omann (2007), during mycoparasitism, lectines from the cell wall of the pathogen can induce the association of antagonist hyphae with the pathogen and, consequently, hyphae of the pathogen may be colonized.

Knowledge on the mechanism of antagonism is must and would prove very useful for the effective disease control. Scanning Electron Microscopy (SEM) of hyphal interaction between *Trichoderma* spp. and *Fusarium oxysporum-Rhizoctonia bataticola* (wilt complex pathogens) indicated that biocontrol agents parasitized the mycelium first. They penetrate and finally resulting into lysis or collapse of hyphae of the pathogens. Among the *Trichoderma* spp. *Trichoderma harzianum* showed more mycoprasitic ability making contact with host hyphae, running parallel to it, production of hook like structure and emptied the cells. This research was carried out to screen nine *Trichoderma* spp. against wilt-dry root rot pathogens of chickpea under in vitro. Electron microscopic observations revealed that all *T. harzianum* spp. interacted with the pathogens. *T. harzianum* grew toward the pathogen and coiled around the host cells, penetrating and destroying the hyphae. Penetration into host cells was apparently accomplished by mechanical activity. Elad *et al*., (1983) demonstrated hyphal interaction between *T. harzianum* and *T. hamatum* with *Sclerotium rolfsii* and *Rhizoctonia solani* by Scanning Electron Microscopy (SEM). *Trichoderma* spp. adhere the host surface by coiling, hooks or appressoria. Lysed sites and penetration holes were found in hyphae of the plant pathogenic fungi, following removal of parasitic hyphae. Zexun et. al., (2004) described that in mycoparasitic interactions of *Trichoderma atroviride* with *Pythium ultimum* and *Rhizoctonia solani*, the mycoparasitic hyphae grew alongside the pathogen mycelia, and this was followed by coiling and formation of specialized structures similar to hooks, appressoria, and papillae. In mycoparasitic interactions of *T. atroviride* with *P.*

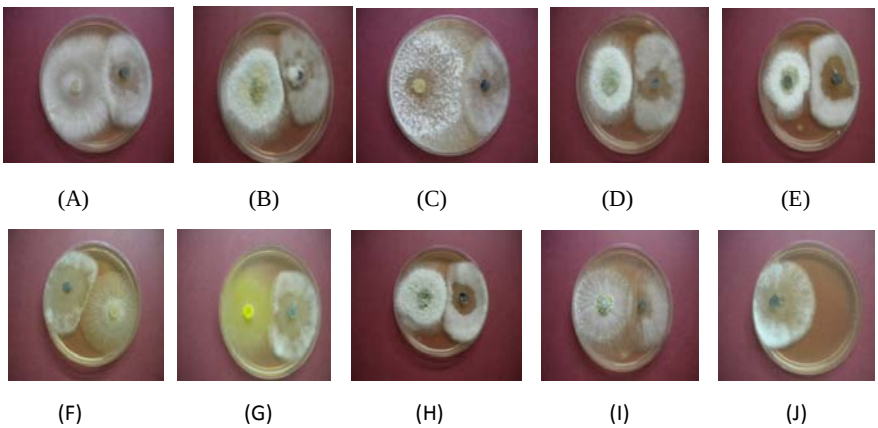


**Fig. 2.** Antagonism of *Trichoderma* spp. against *Rhizoctonia. bataticola* (A) *T.asperellum* (B) *T. atroviride* (C) *T. harzianum* (D) *T. koningii* (E) *T. longibrachiatum* (F) *T. minutisporum* (G) *T. ressei* (H) *T. viride* (I) *T. virens* (J) Control (*Rb*)

**Foc = Fusarium oxysporum* f.sp. *ciceri;* *Rb = *Rhizoctonia bataticola*

**Fig. 3.**

**Fig. 4.**

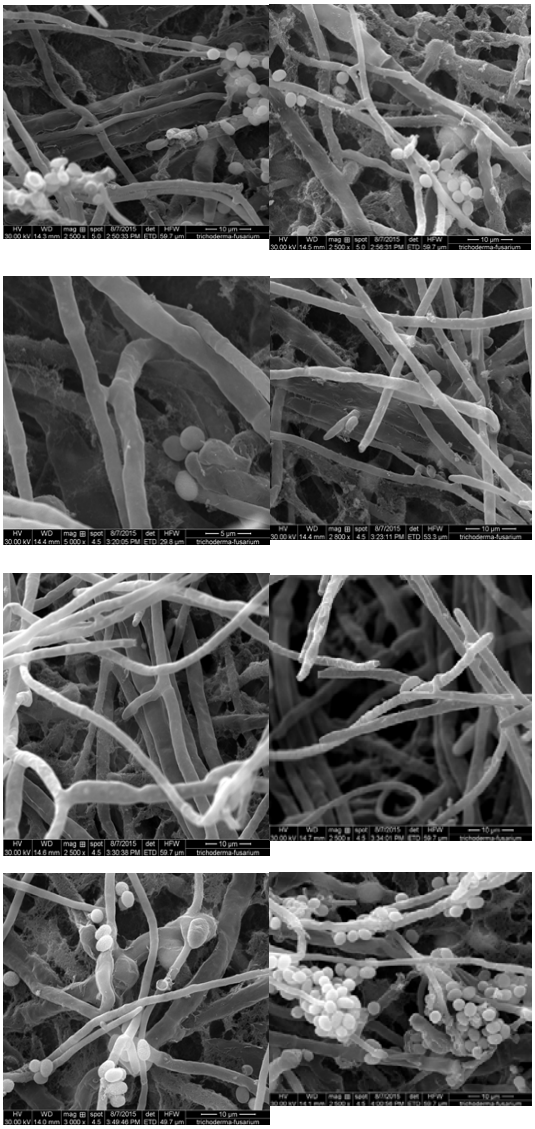

**Fig. 3.** (3a-h) Zone of interaction between *T. harzianum* and *Fusarium oxysporum* f.sp. *ciceri* (Dual culture) (a & b) Scanning electron micrograph on mycoparasitism of the *F. oxysporum ciceri* hyphae by the hyphae of *T. harzianum* with pincer shaped structure moving longitudinally and parallel to the hyphae of the pathogen (c & d) Coiling of hyphae and hyphal tip of *T. harzianum* attached to and penetrating the hyphae of *F. oxysporum ciceri* (e & f) *T. harzianum* hyphal tip, hooks and pincer shape formed against *F. oxysporum ciceri* causing hyphal depression (g & h) *T. harzianum* mycelial growth and spores adhere to the hyphae of *F. oxysporum ciceri*
**Th = Trichoderma harzianum* = T
**Foc= Fusarium oxysporum* f.sp. *ciceri* = F

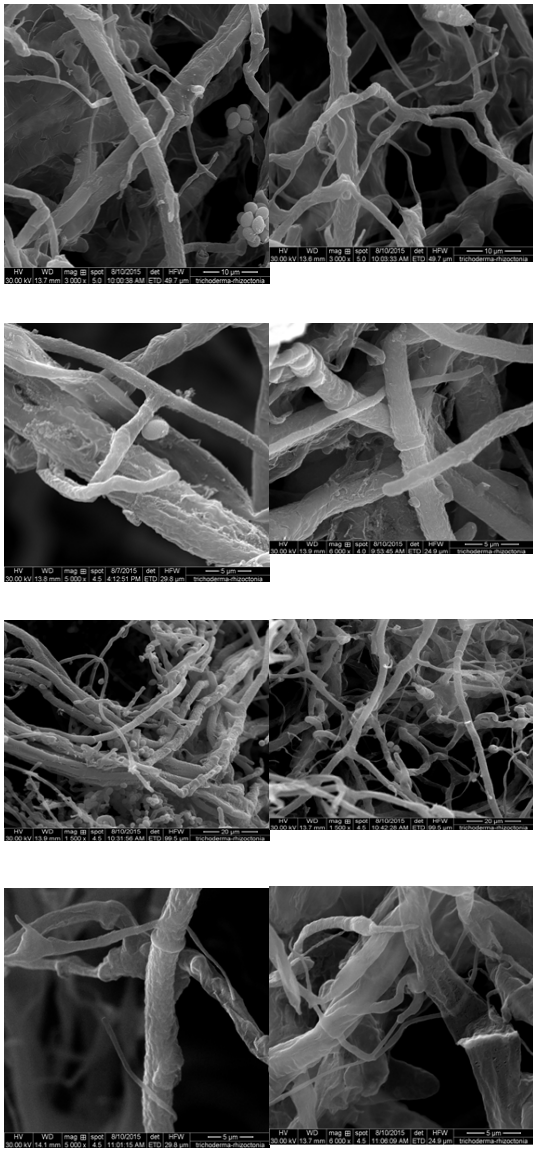

**Fig. 4.** (4a-h) Zone of interaction between *T. harzianum* and *Rhizoctonia bataticola* (Dual culture) (a & b) Scanning electron micrograph on parasitic action of *T. harzianum* against *R. bataticola*, moving longitudinally and parallel to the hyphae of the pathogen (c & d) Coiling of hyphae and hyphal tip of *T. harzianum* attached and penetrating the hyphae of *R. bataticola* (e & f) *T. harzianum* mycelium and conidia adhere to the hyphae of *R. batatocola* causing hyphal swelling (g & h) Coiling of hyphae and hyphal growth depression by *T. harzianum*
**Th = Trichoderma harzianum* = T
**Rb = Rhizoctonia bataticola* = R

*ultimum* and *Rhizoctonia solani*, the mycoparasitic hyphae grew alongside the pathogen mycelia, and this was followed by coiling and formation of specialized structures similar to hooks, appressoria, and papillae. Daniel et. al., (2014) studied intrahyphal colonization of *Fusarium oxysporum f.sp. phaseoli by T. harzianum* through SEM indicated that this type of interaction is not commonly reported for antagonistic interactions of *Trichoderma* and other fungi.

Based on the antagonistic potential and hyphal morphologies observed at SEM we would suggest *T. harzianum* as a strong antagonist. These findings are new as SEM investigations on *Trichoderma* spp. with wilt complex creating fungi in chickpea are not reported earlier from India. It may play an important role in the biological control of soil borne diseases of chickpea in U.P. (India).

## ACKNOWLEDGMENTS

My special thanks to Department of Science & Technology (DST), New Delhi, for financial support under Women Scientist Scheme (WOS-A) (SR/WOS-A/LS-1182/2014) and Central Drug Research Institute (CSIR), Lucknow, India for electron micrograph. I am also thankfull to I. C. A. R. (Niche Area of Excellence program) and CSAUA&T, Kanpur for providing facilities.

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