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### Scanning Electron Microscopic Studies on Mycoparasitic Activity of *Trichoderma* spp. against *Rhizoctonia solani*, Incitant of Sheath Blight of Rice

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#### ABSTRACT

Cultural and morphological characteristics of sheath blight pathogen; *Rhizoctonia solani* was observed on different solid media viz., potato dextrose agar (PDA) medium, Czapeck's dox agar (CDA) medium and rose bengal agar (RBA) medium. On PDA, mycelial growth was abundant, but sclerotia production was delayed. On CDA, though the mycelial growth was moderate and slower than on PDA, sclerotia production was early. On both the media, the sclerotia were aggregated. The mycelium growth was very slow on RBA and there was no sclerotia production. Scanning Electron Microscopy (SEM) of hyphal interaction between *Trichoderma* spp. and *R. solani* indicated that biocontrol agent parasitized the mycelium of *R. solani*. Hypha of *R. solani* was highly susceptible to hyphal parasitic attack by the species of *Trichoderma*. Studies of hyphal interaction between *Trichoderma* and *R. solani* indicates penetration of parasite and finally resulting into lysis or collapse of *R. solani* hypha. Among the *Trichoderma* spp. TK3 isolate showed more mycoparasitic activity by making contact with host hyphae, running parallel to it, production of hook like structures and emptied the cells of pathogen.

**Keywords:** *Rhizoctonia solani*, variability, antibiosis, mycoparasitism, scanning electron microscopy

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## INTRODUCTION

*Trichoderma* pers. Ex. Fr., a genus under Deuteromycotina, Hyphomycetes, Hyphales, Dematiaceae has gained immense importance since last few decades due to its biological control ability against several plant pathogens. The researchers are interested in this genus because of its novel biological properties and biotechnological applications. Biocontrol mechanisms are likely to be specific for particular antagonists and plant pathogens and several mechanisms could operate independently or synergistically in any microbial interaction. *Trichoderma harzianum* is one efficient biocontrol agent that is commercially produced to prevent development of several soil pathogenic fungi. Different mechanisms have been suggested as being responsible for their bio-control activity, which include competition for space and nutrients, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds. *Trichoderma harzianum* are found to be capable of lysing mycelia of *Sclerotium rolfsii* and *Rhizoctonia solani*. *Rhizoctonia solani* Kühn is the major fungus responsible for sheath blight in Rice. In this work, the interaction between *T.harzianum* and *R. solani* was observed by using SEM techniques. The effect of culture filtrate of *Trichoderma* spp. on radial growth and on germination or viability of sclerotia of *Rhizoctonia solani* was studied.

## MATERIALS AND METHODS

The present study was conducted using facilities available in the Department of Plant Pathology, S.V. Agricultural College, Tirupati and Scanning Electronic microscopic studies were done in Department of Physics, S.V. University, Tirupati. Rice plants showing characteristic symptoms of sheath blight were collected from Agricultural Research Station (ARS), Nellore. The infected leaf sheath samples were thoroughly washed in running tap water and cut into small pieces of 3 mm size along with the lesion having half healthy and half diseased tissue. The pieces were surface sterilized with 0.1% mercuric chloride solution for 30 sec. The tissue pieces were subsequently washed in three changes of sterile distilled water to eliminate excess mercuric chloride and then the pieces were transferred onto PDA medium in Petri dishes. Plates were incubated at  $28 \pm 2$  C and observed periodically for growth of the fungus. Axenic culture of the pathogen was obtained by single hyphal tip method and maintained on PDA slants throughout the present investigation.

Sheath blight pathogen was identified on the basis of cultural and morphological characteristics. Slides were prepared in cotton blue and the variation of *R. solani* in colony diam and growth rate was determined by measuring the radial growth of the fungus at 24 h interval. The colony growth was measured along two diam at right angles and averaged and examined under compound microscope for morphological characteristics of the fungus. Potato Dextrose Agar (PDA), Czapeck's Dox Agar (CDA) and Rose Bengal Agar medium (RBA) were used to assess the differential growth of *R. solani* under the study. Twenty milliliters of autoclaved and cooled medium was poured in

each Petriplate and allowed to solidify at room temperature. Later the mycelial disc of 5 mm diam was cut using sterilized cork borer from the periphery of an actively growing 7 d old culture of the pathogen grown on PDA, transferred aseptically to the centre of the plate containing PDA and CDA and the plates were incubated at  $28 \pm 2$  C. Cultural characteristics like growth pattern, growth rate and pigmentation observed in three solid media. Growth pattern was recorded by visual observation according to growth of hyphae as abundant (aerial mycelium, obscured surface mycelium and touched the cover of the Petridish), moderate (aerial mycelium, obscured surface mycelium and without touching the cover), slight (aerial mycelium obscured surface mycelium). The colour, texture (smooth or rough), formation of sclerotia (scattered, peripheral or central) and location of sclerotia (aerial or surface) formed were studied [10].

### **Isolation of native antagonistic *Trichoderma* isolates against *Rhizoctonia solani***

Soil samples were collected from Kadapa, Chittoor and Nellore districts of Andhra Pradesh where the disease incidence is high. Serial dilution plate technique was used to isolate native antagonistic *Trichoderma* spp. on PDA. *Trichoderma* species were isolated using *Trichoderma* specific medium [5,6]

### **Effect of non-volatile compounds produced by *Trichoderma* spp.**

The effect of culture filtrates of the nine isolates of *Trichoderma* spp. on the growth of *R. solani* was studied as per method given by Dennis and Webster (1971). Fifty ml of sterilized potato dextrose broth (PDB) was poured into 250 ml conical flask and inoculated with a 5mm mycelia disc of the biocontrol agent(s) cut from the edge of 4 d old culture. Inoculated flasks were then incubated at  $28 \pm 2$  C for 5 d with constant shaking in water bath. The culture filtrate was collected after passing through Whatman no.1 filter paper. The culture filtrate of bioagent and molten double strength PDA were mixed together in equal proportion (1:1). The medium was then sterilized and poured into the Petriplate @ 20 ml/plate. After solidification the Petriplates were carefully inoculated with 5 mm discs of the test pathogen cut from the 4 d old culture. PDA plates inoculated with the test pathogen which are not amended with culture filtrate were treated as control. Plates were then incubated in an incubator at  $28 \pm 2$  C. Three replications for each treatment were maintained. Periodic observations on radial growth of mycelium were recorded [1]. Inhibition percentage of mycelial growth of test pathogen was calculated by the formula:  $I = (C - T / C) \times 100$ . Where, I = Per cent inhibition in growth of test pathogen, C = Radial growth of pathogen (mm) in control, T = Radial growth of pathogen (mm) in treatment

### **Effect of *Trichoderma* spp. on viability of sclerotia**

Sclerotia of *R. solani* were placed on the surface of PDA which was overgrown with the mycelium and spores of a 4 d old colony of *Trichoderma* spp. Ten sclerotia were placed for each treatment and three replications were maintained. The cultures were

incubated at 26 C in the darkness for upto 30 d. Untreated sclerotia were served as the control. The viability of *R. solani* sclerotia was estimated by placing them on water- agar for 24 h at 26C and the germination was detected with a stereomicroscope [8].

### **Preparation of specimens for scanning electron microscopy**

The parasitism of hyphal cells of *R. solani* by *Trichoderma spp* was studied in detail by scanning electron microscopy (SEM). To obtain interaction sites of hyphae, 10% PDA was inoculated at a constant distance from the edge of the Petri dish with a mycelial disc (5mm) cut from the leading edge of a colony of *Trichoderma* and the pathogen. *R. solani* was inoculated 24 h before *Trichoderma*. The mycoparasite and its host grew toward each other and their hyphae intermingled. After 48 h of incubation, the plate cultures were observed under a light microscope to verify the early stage of interaction. The interaction site was marked and an agar block of 1 cm<sup>2</sup> was removed for SEM preparation. Mycelia samples from the interaction region were fixed for 24 h with vapors of glutaraldehyde and Osmium tetroxide (3:1), air dried for 48 h, sputter coated with gold [8].

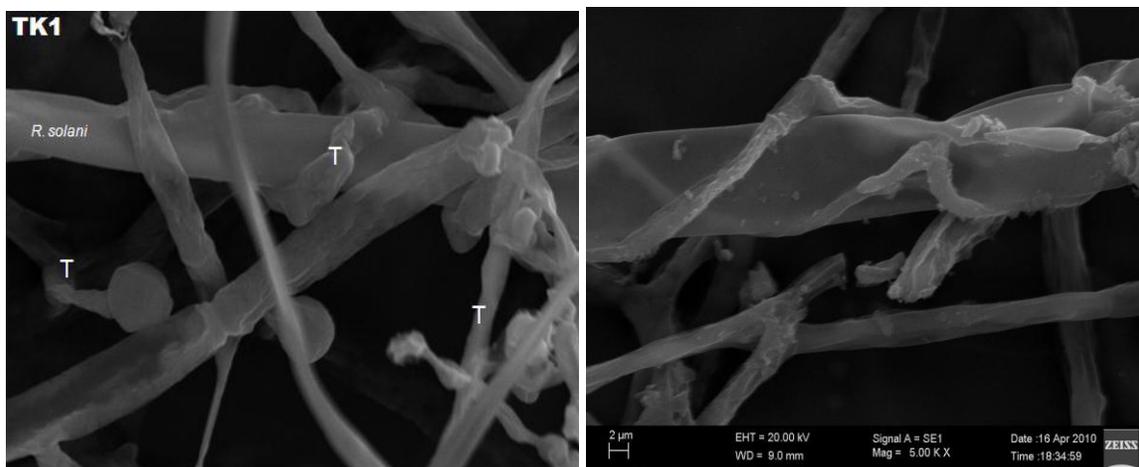
## **RESULTS AND DISCUSSION**

In the present investigation, susceptible rice cultivar NLR-34449 was used and it was collected from Agricultural Research Station (ARS), Nellore. The pathogen was isolated from the infected leaf sheath by tissue segment method using potato dextrose agar medium (Rangaswami and Mahadevan, 1999). The mycelial growth of sheath blight pathogen *R. solani* was observed on different solid media viz., potato dextrose agar (PDA) medium, czapeck's dox agar (CDA) medium and rose bengal agar (RBA) medium. The cultural and morphological differences of mycelial and sclerotial characters were studied to know the influence of different solid media on the growth of mycelium. Among these, the mycelium growth was fast on PDA and slow on RBA. The mycelium occupied the Petri plate with in 3 d on PDA, but it has taken 6 d on RBA to cover the entire Petri plate. The mycelial growth was abundant on PDA i.e. aerial mycelium obscured surface mycelium and touched the cover of the Petri dish and it was moderate (aerial mycelium obscured surface mycelium and without touching the cover) and slight (mycelium was not produced visibly) on CDA and RBA respectively. Sclerotia were produced early on CDA (i.e. on fifth day), while on PDA, sclerotia were produced after 20 d. Black mustard spore like appearance was observed on RBA with out formation of any sclerotia. The present investigation indicated that the mycelial growth was abundant on PDA, but sclerotia production was delayed. On CDA, though the mycelial growth was moderate and slower than mycelium growth on PDA, sclerotia production was early. The mycelium growth was very slow on RBA and there was no sclerotial production observed [11]. The results were in agreement with Lal and Janki Kandhai (2009) who studied the morphological variability of 25 isolates of *R. solani* viz., colony size, colony growth, colour and sclerotia formation (central, peripheral or scattered), location (aerial surface) and texture (smooth or rough). Morphological studies of the different crop

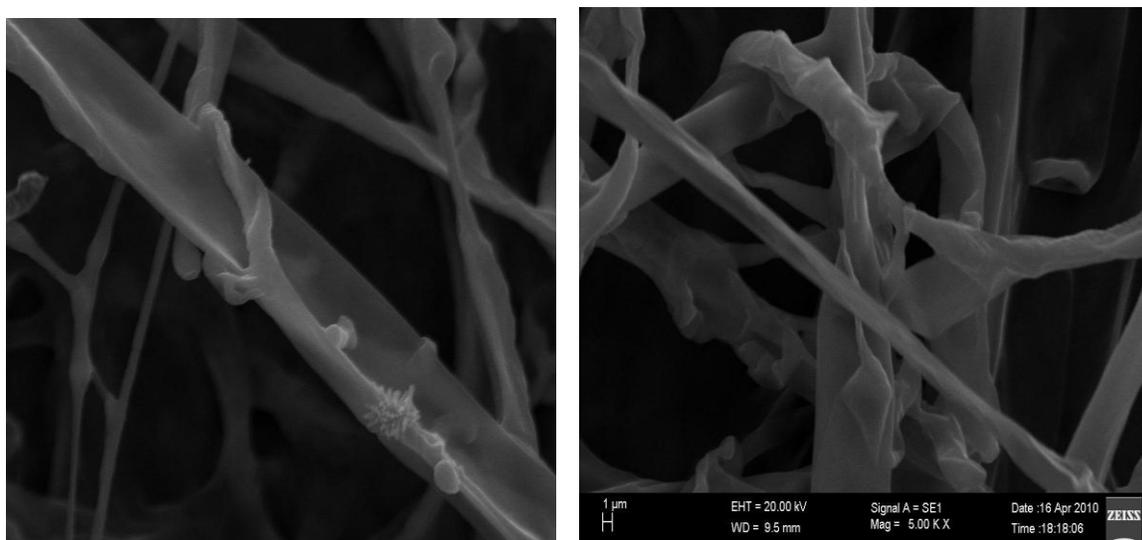
isolates of *R. solani* revealed significant differences in hyphal width, sclerotial size, colour of the mycelium and growth. The sclerotial formation of different isolates on Petri plates was also varied. Morphological studies of the different crop isolates of *R. solani* revealed significant differences, in hyphal width, sclerotial size, colour of the mycelium and growth. The sclerotial formation of different isolates on Petri plates was also varied [10]. Weindling and Emerson (1936) observed that the production of metabolites by *T. lignorum* was toxic to *R. solani*. Production of chloroform soluble antibiotics such as Trichodermin which was different from viridian has been reported by Dennis and Webster (1971). There are several reports on the antibiotic action of *Trichoderma* spp. against *R. solani* (Dubey, 1995). Previous reports and present investigation suggests that effect of non-volatile compounds (culture filtrates of antagonists) towards *R. solani* is aided by antibiotics which are released into the medium [14].

The viability of sclerotia was decreased after 30 d of incubation (Table 1 ). TN3 was able to inhibit the viability of sclerotia upto 62.04% followed by TK3 and TC3 equally (55.53%). In fact, in this investigation TN3 has shown to be an efficient mycoparasite of *R. solani*. The results were in agreement with Itamar Soares and Jane, 2000 who observed that the viability of sclerotia was decreased after 30 d of incubation. *T. harzianum*, Th-9 was able to reduce the germination of sclerotia in 72% and *T. koningii* in 43%. In fact, in this investigation *T. harzianum*, Th-9 has shown to be an efficient mycoparasite of *R. solani*. On the other hand, *T. koningii* has proved to be a good antibiotic producer. To be considered a successful biocontrol agent, a mycoparasite should be effective against resistant survival structures of plant pathogens. The results revealed that there was a reciprocal relationship between the culture filtrates of *Trichoderma* spp. and the radial growth of *R. solani*. Maximum inhibition of the mycelial growth of *R. solani* was observed with the TC3 (22.20%) followed by TK2 (18.80%) and TN2 (18.80%).

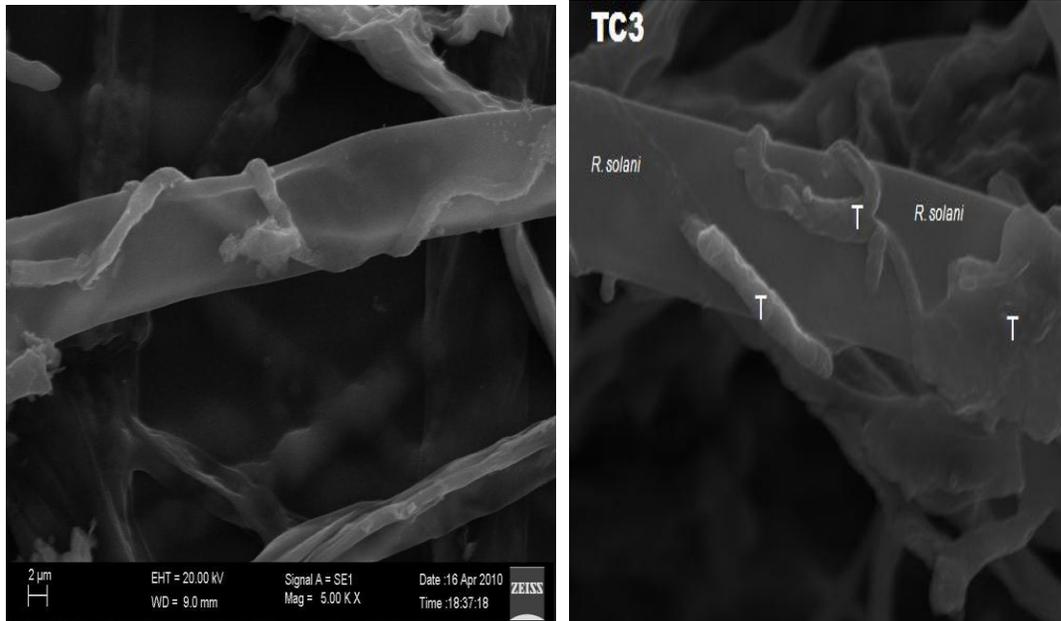
The results were in conformation with Ashraf Alikhan and Sinha (2007) who reported that the all the five isolates of *Trichoderma* spp. exhibited antibiotic potential against *R. solani* by inhibiting its mycelial growth. With the increase in concentration of culture filtrates of the bioagents, the radial growth of test pathogen was proportionally decreased in general. Maximum inhibition (76.3%) of the mycelial growth of *R. solani* was observed with the culture filtrate of *T. harzianum* used at 50 per cent concentration [1]. Krishnam Raju *et al.* (2008) found that the cultures or cell free filtrates of all the *Trichoderma* spp. viz., *T. viride*, *T. harzianum* and *T. hamatum* suppressed the radial growth of *R. solani*. The bioagent, *T. harzianum* was found very effective in inhibiting the radial growth of test pathogen to an extent of 44.50% when 100% concentration of the culture filtrate of the antagonist was used. This was followed by *T. hamatum* (38.63%) and *T. viride* (35.37%) [9].



**Plate 1 : (A) Production of haustoria and excessive coiling around the *R. solani* by *Trichoderma* spp.(TK1).  
 (B)The mycelium of *Trichoderma* (TK2) runs parallel with that on the mycelium of *R. solani***

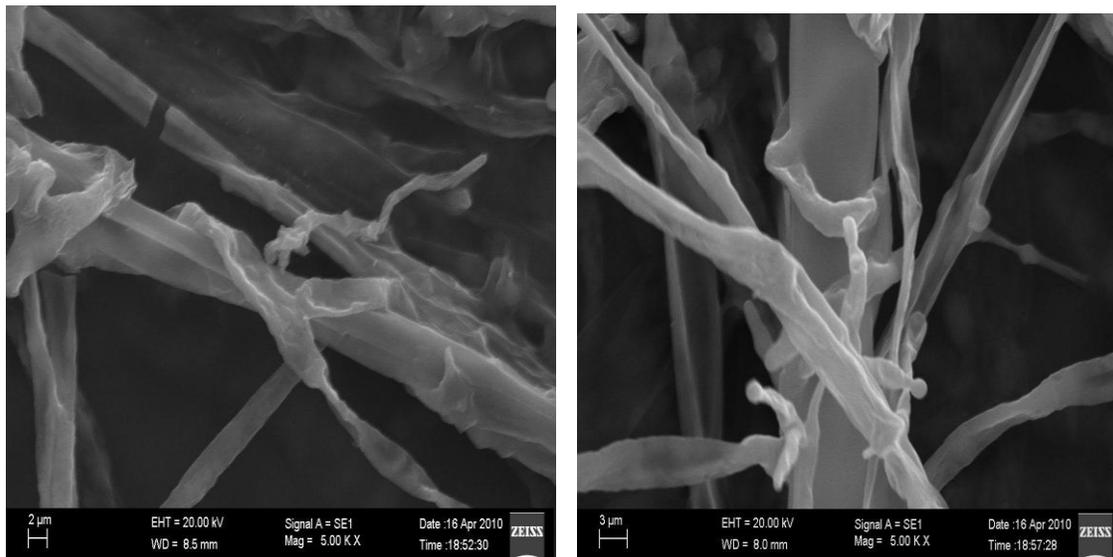


**Plate 2: (A) Hooks of *Trichoderma* (TK3) attached to hypha of *R. solani*.  
 (B) Appressorium like structures formed by *Trichoderma* spp. (TC1) attached to a hypha of *R. solani***



**Plate 3 : (A) The mycelium of *Trichoderma* (TC2) penetrates and runs parallel with that on the mycelium of *R. solani*.**

**(B) Mycelial (TC3) tips stick onto the large hypha of *R. solani***



**Plate 4 : (A) Coiling and hyphal tip penetration of *Trichoderma* (TN1) on *R. solani*.**

**(B) Excessive coiling of *Trichoderma* ( TN2) and attached itself to host mycelium by forming hooks**

**Table 1: Comparative effect of non-volatile compounds of *Trichoderma* spp. on the growth and sclerotium viability of *Rhizoctonia solani***

S.No	Isolates	Radial growth of <i>Rhizoctonia solani</i> (mm)*	Per cent inhibition over control	Number of sclerotia germinated *	% inhibition on viability of sclerotia
1	TK1	80.0	11.10(19.46)	6.33	34.85(36.18)
2	TK2	73.0	<b>18.80(25.70)</b>	5.33	45.19(42.24)
3	TK3	75.0	16.67(24.09)	4.33	<b>55.53(48.17)</b>
4	TC1	80.0	11.10(19.46)	7.33	24.50(29.67)
5	TC2	90.0	00.00(00.00)	7.67	20.68(27.05)
6	<b>TC3</b>	<b>70.0</b>	<b>22.20(28.12)</b>	4.33	<b>55.53(48.17)</b>
7	TN1	80.0	11.10(19.46)	5.67	41.36(40.02)
8	TN2	73.0	<b>18.80(25.70)</b>	4.67	51.70(45.97)
9	TN3	76.0	15.50(23.18)	<b>3.67</b>	<b>62.04(51.97)</b>
10	Control	90.00	00.00(00.00)	9.67	00.00 (00.00)
	S.Ed		0.0476		0.2505
	CD (0.05)		0.0992		0.5262
	CV (%)		0.31		0.82

\*Mean of three replications

Figures in parenthesis are angular transformed values

TK – *Trichoderma* Kadapa

TC - *Trichoderma* Chittoor

TN – *Trichoderma* Nellore

### Scanning Electron Microscopy studies

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 *R. solani* indicated that biocontrol agent parasitized the mycelium of *R. solani*. Hypha of *R. solani* was highly susceptible to hyphal parasitic attack by the species of *Trichoderma*. Studies of hyphal interaction between *Trichoderma* and *R. solani* indicates penetration of parasites and finally resulting into lysis or collapse of *R. solani* hypha. Among the *Trichoderma* spp., TK3 showed more mycoparasitic ability making contact with host hyphae, running parallel to it, production of hook like structure and emptied the cells. Itamar Soares *et al.* (2000) reported that the fungus *Trichoderma* has been shown to be particularly effective in the control of the *R. solani*. Thus, this research was carried out to screen fourteen *Trichoderma* spp. against *R. solani* under *in vitro*. Electron microscopic observations revealed that all *T. harzianum* spp. interacted with *R. solani*. Th-9 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 [8]. Shalini *et al.* (2007)

revealed that the *Trichoderma* has been shown to be particularly effective in the control of *R. solani*. About seventeen *Trichoderma* strains were screened against *R. solani* under *in vitro*. All strains including *T.harzianum*, *T. viride* and *T. aureoviride* that were tested inhibited the growth of *R. solani*. Light microscopic observation on dual culture assay showed that the hyphae of all *Trichoderma* isolates could grow parallel to the hyphae of *Rhizoctonia solani*. However *Trichoderma* isolates coiled around the hyphae of *Rhizoctonia solani* and formed appressoria and hook-like structures[13]. Elad *et al.*, (1983) demonstrated that hyphal interaction between either *T. harzianum*, or *T.hamatum* and *Sclerotium rolfsii* or *Rhizoctonia solani* were observed by Scanning Electron Microscopy (SEM). *Trichoderma* spp. attached to the host by hyphal coils, hooks or appressoria. Lysed sites and penetration holes were found in hyphae of the plant pathogenic fungi, following removal of parasitic hyphae [5].

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