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# *In vitro* evaluation of arbuscular mycorrhizal-like fungi and *Trichoderma* species against soil borne pathogens

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Two arbuscular mycorrhizal like-fungi (*Piriformospora indica* and *Sebacina vermifera*), and two species of *Trichoderma* (*Trichoderma viride* and *Trichoderma harzianum* (T-100)) were evaluated against two isolates of *Sclerotinia sclerotiorum*, two isolates of *Fusarium oxysporum* f. sp. *lentis*, and two species of *Rhizoctonia* (*Rhizoctonia solani* and *Rhizoctonia zeae*). Antagonistic fungi against the pathogens in dual culture, volatile metabolite and colonization were evaluated. In dual culture revealed that antagonistic fungi could produce a good zone of inhibition, and *T. harzianum* (T-100) that was observed maximum growth inhibition on mycelium of two isolates of *S. sclerotiorum*. The volatile metabolite studies revealed that *R. solani* was most susceptible to the volatile metabolite produced by *T. harzianum* (T-100), and colonization revealed that antagonistic fungi were able to overgrow the colony of pathogens and could lyse mycelia.

**Key words:** biological control, *Piriformospora indica*, *Sebacina vermifera*, *Trichoderma viride*, *Trichoderma harzianum*, soil borne fungi

## Introduction

The increased concern about the environmental and ground water pollution and lack effective chemical controls for many soils borne disease has led to considerable changes in people's attitudes towards the use of pesticides in agriculture. The control of plant pathogens by applying biocontrol techniques has potential to reduce chemical inputs to agriculture and significantly enhance global sustainability (Akhtar and Siddiqui, 2008). The biological control is the best alternative especially against soil borne pathogens. Biological control of pathogens, i.e., the total or partial destruction of pathogen populations by other

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organisms, occurs routinely in nature (Agrios, 1977). Among the various antagonists used for the management of plant diseases,

*Trichoderma* spp. plays a vital role. Among the various isolates of *Trichoderma*, *T. viride, T. harzianum, T. virens* and *T. hamatum* are used against the management of various diseases of crop plants especially with soil-borne pathogens. These filamentous fungi are very common in nature, with high population densities in soil and plant litters (Samuels, 1996). Teleomorphs of *Trichoderma* are species of the ascomycete genus *Hypocrea*. Many studies have proved the potential of *Trichoderma* spp. as biological agents antagonistic to several plant pathogens (Sivan and Chet, 1993; Inbar *et al*., 1996; Naseby *et al*., 2000; Kulling *et al.,* 2000; Tondje *et al.,* 2007; Woo *et al.,* 2006). In addition, one group of micro-organisms that shows control of plant pathogens is the arbuscular mycorrhizal fungi (AMF). They form symbiotic relationships with roots of about 90% land plants in natural and agricultural ecosystems (Brundrett, 2002), this fungi demonstrate the usefulness organisms if we could apply them as biological agents. Mycorrhizal interactions also enhance plant resistance to various toxins and pathogens (Marx, 1969; Smith and Read, 1997; Harrier and Watson, 2004). In contrast to most mycorrhizal fungi, *Piriformospora indica and Sebacina vermifera* are cultivatable fungus and can grow on synthetic or complex media without hosts (Varma *et al.*, 2001; Peskan-Berghofer *et al*., 2004). *P. indica* and *S. vermifera* belong to the Sebacinaceae, an ancient Basidiomycete family. Beyond the stimulating effect on biomass production, *P. indica* apparently supports its host by protecting it from pathogenic fungi (Waller *et al*., 2005). It was suggested that *P. indica* may target an as yet unidentified signaling pathways to induce systemic resistance (Serfling *et al*., 2007).

The purpose of this study was to evaluate the biological potential of *P. indica*, *S. vermifera*, *T. harzianum* (T-100) and *T. viride* against some soil borne plant pathogens (two isolate of *Sclerotinia sclerotiorum*; two isolate of *Fusarium oxysporum* f. sp. *lentis*; *Rhizoctonia solani* and *Rhizoctonia zeae*).

## Materials and methods

### *Fungal cultures*

Two species of *Rhizoctonia* (*R. solani* and *R. zeae*), two isolate of *Fusarium oxysporum* f. sp. *lentis* (Mashhad (F1) and Ilam (F2)), and two isolate of *Sclerotinia sclerotiorum* (Golestan (S1) and Mazandaran (S2)) were isolated from soil, *lens culinaris* and *Brassica napus*, respectively. The pathogens were maintained on Potato dextrose Agar (PDA) medium and stored at 4°C for further use.

*Trichoderma* species were used: *T. harzianum* (T-100) and *T. viride*. Cultures were maintained on Potato Dextrose Agar (PDA) medium and stored at 4°C for further use.

*Piriformospora indica* and *S. vermifera* were maintained on Kaefer's medium (Kaefer, 1977). *P. indica* was cultured as described previously (Verma *et al*., 1998; Peskan-Berghofer *et al*., 2004) in Petri dishes on a modified Kaefer's medium (KM: NaNO3, 7.0mM; KCl, 7.0mM; MgSO4, 2.1mM; KH2PO4, 9.2mM; ZnSO4, 0.77mM; H3BO4, 0.18mM; MnSO4, 0.02mM; CoCl2, 0.007mM; CuSO4, 0.0065mM; FeSO4, 0.02mM; EDTA, 0.02mM; ammonium molybdate, 0.001mM; thiamine, 0.003mM; gylcine, 0.005mM; nicotinic acid, 0.002mM; pyridoxine, 0.0004mM; glucose, 110mM; peptone, 2g/l; yeast extract, 1g/l; casein hydrolysate, 1g/l, pH 6.5) with1% (w/v) agar. The plates were inoculated with the fungi and kept in temperature 25°C for one week.

### *In vitro experiment*

*Piriformospora indica*, *S. vermifera*, *T .harzianum* (T-100) and *T. viride* were evaluated against soil borne pathogens by dual culture technique as described by Morton and Strouble (1995) and Kucuk and Kivanc (2003). Petri dishes (90 mm) containing 20 ml of sterile PDA were inoculated with a 5mm plug of 7 days old pure culture of antagonistic fungi and pathogens. One mycelial disc of each fungus, was placed on opposite poles of PDA plates and incubated at 25±1°C in incubator and the radial growth of pathogens was measured 2, 4 and 6 days after incubation. Control Petri dishes were inoculated with pathogens and a sterile agar plug. Three replications were maintained for each treatment**.** Percent inhibition of pathogen radial growth was calculated. For each interaction, a clean and sterile glass microscope slide placed in the middle of plates and sterilized. Then a thin layer of autoclaved melted potato dextrose agar spread over the slide. The 5mm discs of seven days old culture cut from the edge of each pathogen and antagonistic fungi were placed at opposite poles on PDA plates and incubated at 25±1°C in an incubator. After one week, the slides were observed microscopically for hyphal interaction. Periodic observations on interaction were made under a stereo-microscope.

The effect of volatile metabolites produced by the antagonistic microorganisms on pathogens, mycelial growth was determined by the method described by Dennis and Webster (1971) and Goyal *et al*. (1994). The antagonistic fungi were centrally inoculated by placing 5 mm diameter mycelia disc taken from 3 days old culture on the PDA plate and incubated at 25±1°C for 2 days. The top of each Petri dish was replaced with bottom of the PDA plate inoculated centrally with the pathogen. Two plates were sealed together with paraffin tape and further incubated at 25ºC. For the control, instead of *Trichoderma* spp. a 5 mm diameter of sterile PDA medium was used in the plate. Three replications were maintained for each treatment. Colony diameter of the pathogen was measured at 4 and 6 days after incubation and the inhibition of mycelial growth was calculated. The percent growth inhibition in all above experiment was calculated by using the following equation (Vincent, 1947): $\mathbf{I = [(C–T)/C] \times 100}$, where I = percent growth inhibition, C = colony growth rate in checked plates, T= colony growth rate in each treatment. Effect of colonization of antagonistic fungi on pathogens mycelium determined by the modified method described by Mohammadi Goltapeh and Danesh (2006). This study was carried out in two phased: in the first phase, 5mm discs of pathogens were placed on PDA plates and incubated at 25±1°C for 4 days before placing 5 mm discs of *P. indica*, *S. vermifera*, *T. harzianum* (T-100) and *T. viride* mycelium on center of the Petri dish.

In the second phase, 5 mm discs of pathogens mycelium were placed on PDA plates and incubated at 25±1ºC for 12 days before placing 5 mm discs of *P. indica*, *S. vermifera*, *T. harzianum* (T-100) and *T. viride* mycelium on center of the Petri dish. Three replications were maintained for each treatment.

### *Statistical analysis*

The collected data were statistically computed using SAS software. Data were subjected to analyses of variance and treatment means were compared by an approximate Duncan's multiple tests and main effectors interaction was found significant at $P < 0.05$.

## Results

Studies on the antagonistic fungi against pathogens in dual culture indicated that at 2 days after incubation, *T. harzianum* (T-100) *and T. viride* differentially limited the colony growth of the pathogens *T. harzianum* (T-100) caused maximum growth inhibition on isolate S1 of *S. sclerotiorum* (16.6 %). It was followed by *T. viride* against *R. solani* (14.2 %) and *R. zeae* (13.6 %). Isolate F1 of *F. oxysporum* f. sp. *lentis* was least inhibited by the *Trichoderma* spp. (Fig. 1a). The growth inhibition of soil borne pathogens by antagonistic fungi after 4 days of incubation revealed that *T. harzianum* (T-100) and *T. viride* resulted in maximum growth inhibition against isolate S2 of *S. sclerotiorum* (57.3 and 54.7 %, respectively). Growth inhibition recorded in all pathogens differed significantly. Isolate F1 of *F. oxysporum* f. sp. *lentis* proved to be less susceptible to *Trichoderma* spp. (Fig. 1b). Six days of incubation different degrees of mycelial growth inhibition were observed. *T. harzianum*

(T-100) caused maximum growth inhibition against mycelium isolate S1 of *S. sclerotiorum* (63.07 %). It was followed isolate S2 of *S. sclerotiorum* (61.42 %). *R. solani* was least inhibition by the *T. harzianum* (T-100) (34.54 %) (Fig. 1c). *P. indica* and *S. vermifera* at two, 4 days of incubation were ineffective in reducing radial growth of pathogens, but after 6 days of incubation, P. *indica* and *S. vermifera* caused maximum radial growth against isolate F2 of *F. oxysporum* f. sp. *lentis* (38.4 and 32.5 %, respectively). Among the pathogens, *R. solani* was least inhibition by the *S. vermifera* (6.4%). (Fig. 1d). Antagonistic fungi differentially limited the colony growth of the pathogens and *Trichoderma* species overgrew the pathogens colony and produce yellow pigment in the interaction (Fig. 4). Microscopically observation of hyphal interaction indicated that antagonistic hyphae coiled around the hyphae of pathogen, mycelial denatured and killed them. *P. indica*, *S. vermifera* and *Trichoderma* species either formed hook or bunch like structure around the hyphae of pathogens before penetration or entered directly (Fig. 2).The results of volatile metabolite revealed that at 4 days of incubation, *T. harzianum* (T-100) caused maximum growth inhibition against *R. solani* (16.2%). It was followed by T. *viride* against isolate F2 of *F. oxysporum* f. sp. *lentis* (16%) (Fig. 3a), and after 6 days of incubation, *Rhizoctonia* species showed highly susceptibility to *Trichoderma* spp. Isolate S2 of *S. sclerotiorum* showed least inhibition the *T. harzianum* (T-100) (11.2 %) (Fig. 3b). Colonization studies against *F. oxysporum* f. sp. *lentis* revealed that in the first phase *T. harzianum* (T-100) had the highest colonization of *F. oxysporum* f. sp. *lentis* (isolate F1 and F2 ) mycelium within 4 days, *T. viride*, *S. vermifera* and *P. indica* had a colonization rate of 5, 9, 10 days respectively. In the second phase *T. harzianum* (T-100), *T. viride*, *S. vermifera* and *P. indica* had a colonization rate of 5, 5, 9 10 days respectively. Similarly, in two phases antagonistic fungi colonized the surface of *Rhizoctonia* spp. completely within 5–10 days. Studies on ability of antagonistic fungi on 4 days old culture of two isolate of *S. sclerotiorum* revealed that antagonistic fungi could overgrow the mycelium of both isolates of *S. sclerotiorum* on PDA and could prevent sclerotia formation. Studies on ability of antagonistic fungi on 12 days old culture revealed that antagonistic fungi could easily overgrew the mycelium of two isolate of *S. sclerotiorum* on PDA and colonized, sporulated on sclerotia and finally lysed them (Fig. 4).

## Discussion

The diseases caused by fungal pathogens persist in the soil matrix and in residue on the soil surface defined as soil borne diseases.

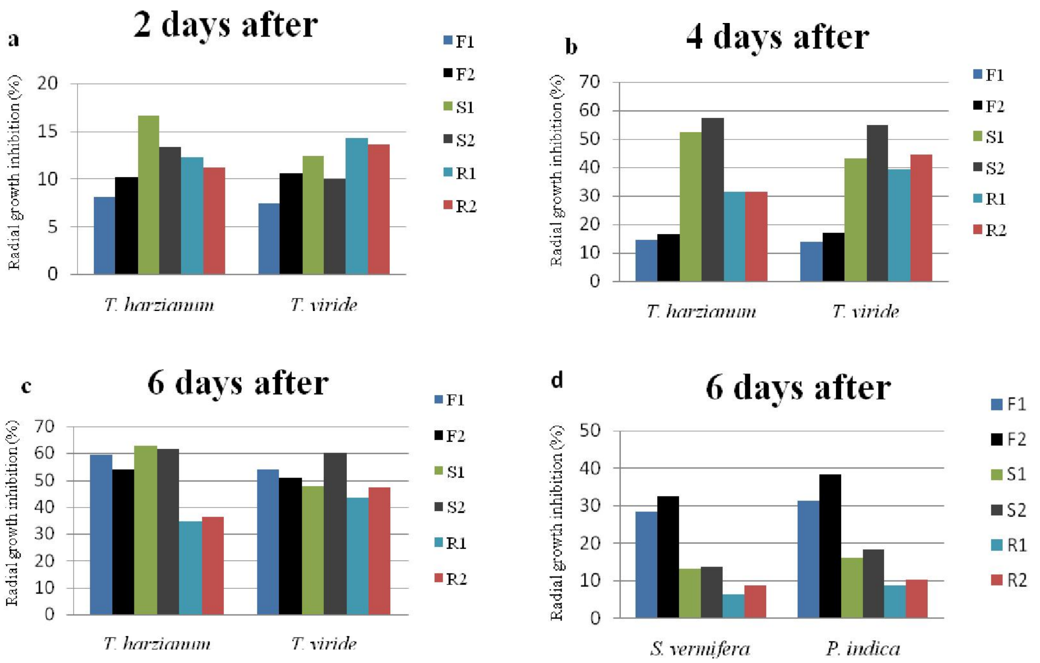


**Fig. 1.** Radial growth inhibition by *Trichoderma* spp. after 2 days of incubation in dual culture **(a)**. Radial growth inhibition by *Trichoderma* spp. after 4 days of incubation in dual culture **(b)**. Radial growth inhibition by *Trichoderma* spp. after 6 days of incubation in dual culture **(c).** Radial growth inhibition by *S. vermifera* and *P. indica* after 6 days of incubation in dual culture **(d)**. F1 = Mashhad isolate of *F. oxysporum* f. sp. *lentis*, F2 = Ilam isolate of *F. oxysporum* f. sp. *lentis*, S1 = Golestan isolate of *S. sclerotiorum*, S2 = Mazandaran isolate of *S. sclerotiorum*, R1 = *R. solani*, and R2 = *R. zeae*.

Fungal diseases are difficult to control because they are caused by pathogens that can survive for long periods in the absence of the normal crop host, and often have a wide host range including weed species The increasing cost of inorganic fertilizers and the environmental and public concern associated with pesticides and pathogens resistant to chemical pesticides, biological control is good alternative for sustainable agriculture (Akhtar and Siddiqui, 2008).

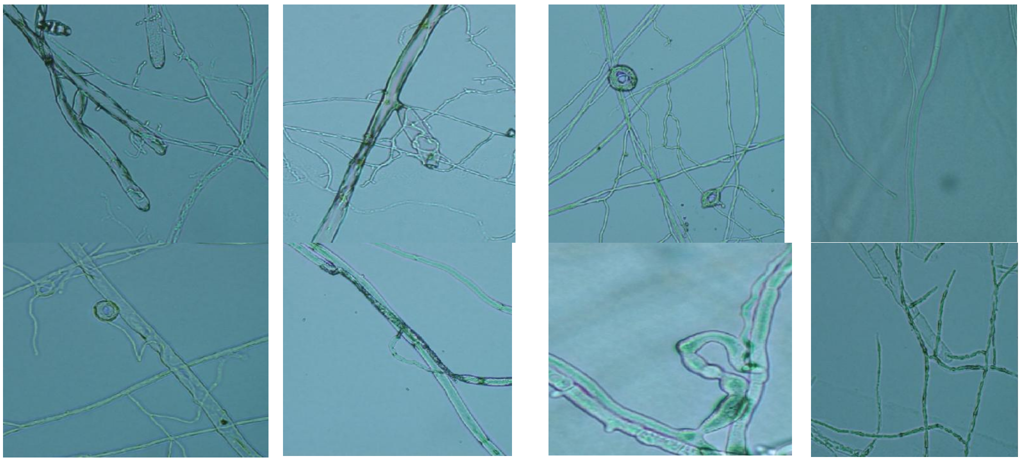

**Fig. 2.** Interaction between antagonistic fungi and soil borne pathogens. Hyphal contact between *P. indica* and *R. solani* **(a, b)**. Coiling hyphae *P. indica* around hyphae *F. oxysporum* f. sp. *lentis* **(c)**. Coiling and contact hyphae *S. vermifera* around hyphae *F. oxysporum* f. sp. *lentis* **(d, e)**. Hyphal contact between *S. vermifera* and *R. solani* **(f)**. Hyphal contact and lysis hyphae *F. oxysporum* f. sp. *lentis* by *T. viride* **(g, h)**. Hyphal contact and penetration *T. harzianum* inter hyphae *R. solani* **(i)**.

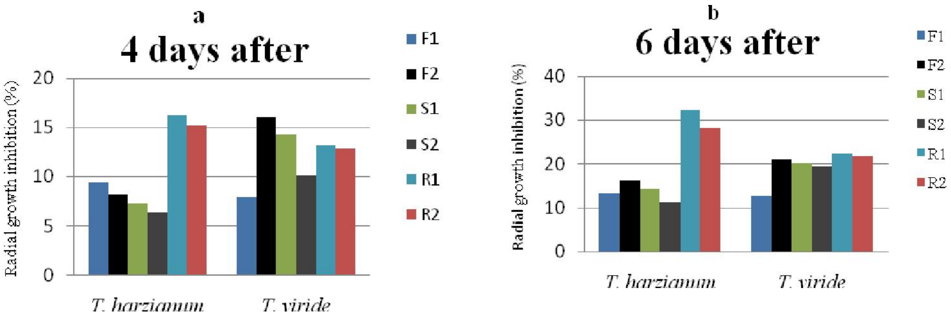


**Fig. 3. (a)** Radial growth inhibition by *Trichoderma* volatile metabolite after 4 days of incubation. **(b)** Radial growth inhibition by *Trichoderma* volatile metabolite after 6 days of incubation. F1 = Mashhad isolate of *F. oxysporum* f. sp. *lentis*, F2 = Ilam isolate of *F. oxysporum* f. sp. *lentis*, S1 = Golestan isolate of *S. sclerotiorum*, S2 = mazandaran isolate of *S. sclerotiorum*, R1 = *R. solani*, and R2 = *R. zeae*.

The ability of arbuscular mycorrhizal-like fungi and *Trichoderma* species for biocontrol of some soil borne plant pathogens were investigated. A large number of researchers had worked with AM fungi and plant pathogenic fungi (Akhtar and Siddiqui, 2006; Akhtar and Siddiqui, 2007a, b; Akhtar and Siddiqui, 2008 a, b; Berta *et al.*, 2005; Boby and Bagyaraj, 2003; Abdel-Fattah and Shabana, 2002). They form symbiotic relationships with roots of about

90% land plants in natural and agricultural ecosystems (Brundrett, 2002). Various mechanisms have been proposed to explain biocontrol ability of AMF including changes in root growth and morphology, physiological and biochemical changes in plant tissue, competition of colonization sites, changes in host nutrition, changes in microbial populations, and activation of defense mechanism (Dehne *et al.*, 1978; Cordier *et al.*, 1998; Azcon-Aguilar and Barea, 1996).

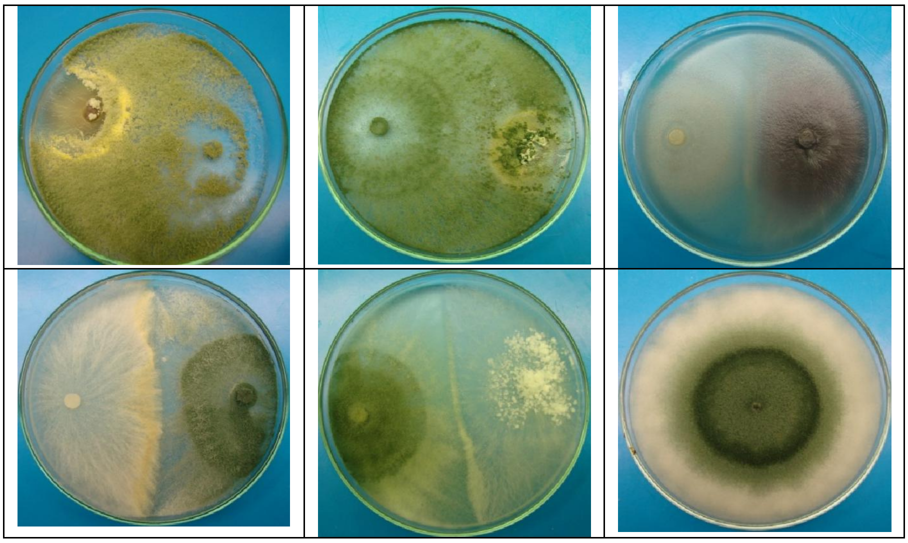

**Fig. 4.** Antagonistic biculture test. **(a)** interaction between *T. viride* and F1 of *F. oxysporum* f. sp. *lentis* in dual culture. **(b)** interaction between *T. harzianum* and F2 of *F. oxysporum* f. sp. *lentis* in dual culture. **(c)** interaction between *P. indica* and F1 of *F. oxysporum* f. sp. *lentis* in dual culture. **(d)** interaction between *T. harzianum* and *R. zeae* in dual culture. **(e)** interaction between *T. viride* and S1 of *S. sclerotiorum.* **(f)** Colonization of mycelium of S2 of *S. sclerotiorum* by *T. harzianum* and preventing sclerotia formation.

In addition, *T. viride* and *T. harzianum*, common filamentous fungi in almost any soil reported by several workers as the best antagonists for growth inhibition of several soil and seed plant pathogens (Elad, 2000; Freeman *et al.*, 2004, Poddar *et al.*, 2004; Dubey *et al.*, 2006). *Trichoderma* spp. have various mechanisms of biocontrol include antibiosis, parasitism, inducing host-plant resistance, and competition confrontation with fungal (Howell, 2003; Sivasithamparam and Ghisalberti, 1998). Our studies in dual culture revealed that all antagonistic fungi inhibited mycelial growth of the soil borne pathogens. At two, 4 and 6 days after incubation results revealed that *T. harzianum* (T-100) caused maximum growth inhibition on mycelium of two isolate of *S.*

*sclerotiorum*. Dubey *et al*. (2006) reported that *T. viride* isolated from Ranchi by *T. harzianum* (Ranchi) and *T. viride* isolated from Delhi inhibited maximum mycelial growth of the pathogen in chickpea plants. Only at 6 days after incubation *P. indica* and *S. vermifera* were effective in reducing radial growth of pathogens.

However, they were able to reduce mycelia growth of the pathogens in dual culture, suggesting that it does not act by producing volatile metabolites but by other mechanisms of competition or parasitism instead. Serfling et al. (2007) reported that in the field experiment, *Pseudocercosporella herpotrichoides* disease severity was significantly reduced in plats colonized by the *P. indica*. *Trichoderma* spp. inhibited the growth of pathogens through the production of volatile metabolites. At 4 and 6 days after incubation, percent reduction in mycelial growth of *R. solani* by *T. viride* was greater than that of the other antagonists fungi tested. Kumar and Dubey (2001) reported that *T. virens* has been found effective and inhibited maximum growth of *F. solani* f. sp. *Pisi* by the production of volatile compounds. Dubey and Pated (2001) observed that the volatile compounds produced by *T. viride* proved inhibitory against R. solani. Our studies in colonization revealed that antagonistic fungi could be able to overgrow the mycelium of pathogens.

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