

Trichoderma spp. As Safe Bio-Control Tool Against *Rhizoctonia solani* Root Rot on Basil Plants.

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ABSTRACT

This study was conducted to investigate the suppression effect of some antagonistic fungi against *Rhizoctonia solani* as the causal agent of basil root rot. Growth of *R. solani* was inhibited (*in vitro* and *in vivo*) in the presence of some antagonistic fungi (*Trichoderma harzianum*, *Trichoderma hamatum*, *Trichoderma viride*, *Gliocladium virens*, *Gliocladium roseum*). *In vitro* the results showed that *T. harzianum* was the best antagonistic fungi in inhibiting the radial growth of *R. solani* gave (96.6%) inhibition, while *G.roseum* showed the lowest effect on *R.solani* (31.75%) inhibition compared with the untreated control and the chemical control treatment that led to complete reduction in *R. solani* (100%) inhibition. *In vivo* experiments *T. harzianum* used by drench method (D) was the best antagonistic fungus had 0% disease severity followed by *T.viride* (D), *T.hamatum* (D) treatments had 7.41% disease severity. On the other hand, *T.viride* used by soaked method (s) gave less effect on the pathogen with (40.74%) disease severity. Compared with the controls (0%) disease severity in the untreated control, also 55.56% in the artificially infested control and the chemical control it led that 22.22% disease severity, respectively).

Keywords: *R. solani*, *T. harzianum*, *T. hamatum*, *T. viride*, *G. virens*, *G. roseum*, chemical control, Basil.

INTRODUCTION

Sweet basil (*Ocimum basilicum* L.) is one of the aromatic plants that have a major role in agriculture and food industry. Basil crop is the main source for drugs and raw substances used in manufacturing of pharmaceuticals. *R. solani* was the main causal of root rot of basil plants as recorded high frequency (Gamiel *et al.*, 1996 and Chiocchetti *et al.*, 2001)

In recent years, large number of synthetic fungicides has been prohibited in many countries because of their hazard properties such as high and sharp toxicity. In some developing countries, they are still being used despite their harmful effect. Many pathogenic microorganisms have developed resistance against chemical fungicides (Behzad, *et al.*, 2008). This seriously hamper the management of diseases of crops and agricultural plants and the continue, i use of chemicals due to inhibition effects on non-target organisms, the undesirable changes they act on the environment (Arcury and Quandt 2003). Considering the harmless effects of synthetic fungicides on life supporting systems, there is an urgent need for alternative agent for management of pathogenic microorganisms (Dennis *et al.*, 1971; Bell, *et al.*, 1982 and Gaigole, *et al.*, 2011)

Recently, there has been a worldwide swing to the use of eco-friendly methods for protecting the crops from pest and disease (Rao *et al.*, 1998). Biological control of plant disease especially soil borne plant pathogens and nematodes by antagonistic microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods (Robert, *et al.*, 1993; Barker and Panlitz, 1996; Eziashi *et al.*, 2007 and Saba, *et al.*, 2008).

Several strains of the fungus *Trichoderma* species have been isolated and found to be effective biocontrol against various soil-borne plant pathogenic fungi under greenhouse and field conditions (Papavizas, *et al.*, 1985 and Vincent, *et al.*, 1990).

The antagonistic activity of *Trichoderma* depends on multiple synergistic mechanisms (Howell, 2003 and Nallathambi *et al.*, 2009). The various mechanisms include antibiosis, parasitism, inducing host-plant resistance, competition, secretion of chitinolytic enzymes, mycoparasitism and production of inhibitory compounds (Harman *et al.*, 2004). *Trichoderma* produces a number of secondary metabolites with biological activity (Ghisalberti and Sivasithamparam, 1991 and Harman, 2006).

This study aims to control *R. solani* the main causal of root rot of basil plant by using selected antagonistic fungi.

MATERIALS AND METHOD

1. Isolation of the causal root rot and damping off in basil:

Infected roots and stems were washed repeatedly with tap water. Roots and stems were cutting then sterilized with 1% hypochlorite-Na solution, after that left for dried. Then placed on potato dextrose agar media in Petri's dishes and incubated at 25±2°C for 7 days. Growing colonies were purified using hyphal tip method, then transferred onto water agar plates using the methods suggested by Booth (1971) and the purified single colonies were then transferred to WA slants. Isolates obtained were identified by morphological features; microscopically according to Ellis (1971) and Barentt and Hunter (1972.) The purified fungal isolates were identified at Dept. of Plant Pathology, Faculty of Agriculture, Mansoura University. PDA slants from isolated fungus were kept on 4°C for further studies.

2-Pathogenicity test:

R .solani was tested for pathogenicity on basil seeds in Petri plates in the laboratory and on planting in soil in the greenhouse.

Preparation of inoculum:

Stock cultures of *R.solani* were obtained from PDA slants kept in 4°C as described before and then transferred to the surface of PDA; Cultures were incubated at 22°C for 7days, 250 ml flasks containing 100 ml of (PDBroth) were inoculated with 2 disks from

the edge of fresh culture and were incubated at (20±2°C) in darkness for 7 days at (22±2°C). Flask contain were blended with 200 ml of sterilized water in a low speed of elctric blender for 20 seconds.

Pathogenicity test:

Autoclaved soil Mixture (50 % sand and 50 % loam)were put on Polyethylene bags (25cm in diameter), soil then artificially infested with 25ml of a suspension from the prepared culture of the pathogen on top of the surface soil of bags (2cm depth from the top of bags) then completed with additional soil to fill the bags of 25cm diameter, (Mclean and Stewart, 2000). The infested soil was watered and left for 7 days before sowing to stimulate growth and ensure its distribution of the pathogen in the soil. The control treatment was inoculated with pathogen free medium at the same rate. Basil seeds were surface sterilized by dipping in 0.5% Nahypochlorite solution for 5 minutes , then washed Repeatedly in sterilized distilled water. Seeds were planted at five plants per plastic bag, take into consideration that each bag was a replicate one. Four replicates were used for each treatment. Determination of disease incidence was calculated as the percentage of healthy plants after 15 and 30 days from planting date, respectively and calculated as (Brix and Zinkernagel,1992);

$$dI = \left[\frac{C-I}{C} \right] \times 100$$

(dI) = Disease incidence %.

(I)=number of replicates.

(C)=mean number of survived plants in the control.

Inoculated fungus was re-isolated from infected plants and microscopically examined

3-Effect of fungal antagonistic action on radial growth of *R. solani*:

Five fungal antagonists (*T. viride*, *T. harzianum*, *T.hamatum*, *G. virens* and *G.roseum*). were isolated from the rhizosphere zones of healthy basil plants grown in Dakahlia governorate. Roots of plants were washed carefully with tap water to remove the adhering soil particles. The washed roots were cut into small pieces and divided into two groups. The first group was sterilized by root pieces dipped in 1% Na hypochlorite solution for 5 min. then washed with sterilized distilled water, while the second group was left without sterilization in order to isolate all surface organisms. The washed root pieces were air dried, then transferred onto potato dextrose agar (PDA) media mixed with 0.003 %rose Bengal and 0.01 % streptomycin sulfate in 9 cm diameter Petri's dishes and incubated at 25±2°C for 4-7 days. The grown colony were separatly transferred to PDA medium; purification was carried out using hyphal tip technique. Then identified by Dept. of Plant Pathology, Faculty of Agriculture, Mansoura University. These antagonistic fungi were studied of inhibition on radial growth of *R.solani*. The fungal antagonists under study and the fungal pathogen were grown on PDA media for 5-7 days at (25±2°C) in darkness. Antagonistic effect was

done using one disc (5 mm. in diameter) of antagonists facing one disc (5 mm. diameter) of the pathogen on the edges of PDA media . The control treatment was done as the same method in Petri's dishes but without antagonistic disc; only pathogenic disc. Three replicates were used and plates incubated at (25±2°C) in dark for 7 days; the diameter rate of growing zone of the pathogen was recorded as stated above.

4-Effect of some antagonistic fungi on controlling root rot and damping-off of basil plants caused by *R.solani* under greenhouse conditions:

All the antagonistic fungi under study were tested in pots under greenhouse conditions for controlling root rot and damping off diseases. Two application methods were used to apply these antagonists; soaking seed and soil drenching;

Seed soaking trial:

Black polyethylene bags (25 cm in diameter x 25cm height) were filled with autoclaved sandy loam soil (50 % sand + 50 % loam, about 2 Kg/ bag), then artificially infested with 25 ml suspension from the culture of the pathogen. Seeds were surface sterilized with Na-hypochlorite 0.5 % for 3 minutes, washed with sterilized water after that were air dried, then soaked in antagonistic fungal suspensions for 2.5 hr.Khaleifa *et al.*(2007) before planting. The wetted seeds were spread in a thin layer and left about 24 hours then sown in the infested potted soil with *R. solani*. Antagonistic fungal suspensions were adjusted to (1 x 10⁶ cfu/ml) that prepared by inoculating 1disc in 250 ml conical flask mixed with 100ml PDB for 7days at (22±2oC) then were blended for 20 sec at the low speed. Colony forming units (cfu) in the resulted suspension were determined.

Control treatments were done by planting surface sterilized seeds in non-infested soil (as untreated control) and by planting surface sterilized seeds soaked in distilled water in infested soil (as infested control) . Also chemical control was done by soil drenching with solution of Rizolex at the rate of 0233 ml/ bag in concentration of 0.5 g/ L. The three control treatments were used in all greenhouse tests. Three pots as replicates were used for each treatment Mazen (2004). Pre-and post- emergence and root rot incidence were recorded after 15 and 30 days of sowing. The survival of basil plants was also recorded after 60 days from sowing.

Infection severity was calculated by the following formula:

$$Disease\ Severity = \frac{Sum\ of\ all\ ratings}{Total\ number\ of\ plants\ X\ maximum\ score} \times 100$$

Soil drenching

Soil drenching application was done by drenching soil with antagonistic fungal mycelium and or spore suspension which were adjusted to (1 x 10⁶ cfu / ml) for each antagonistic fungus, added to the infested soil in polyethylene bags at the rate of 20 ml/ bag, directly at the time of planting. Five seeds were planted in each bag. Each treatment contained 3 replicates, each replicate contained 3 pots each was planted with 5 seeds.

Control treatments were done by planting surface sterilized seeds in non-infested soil (as absolute control) and by planting surface sterilized seeds in infested soil without inoculation with antagonistic fungi (as infested control). Also chemical control was used by soil drenching with solution of Rizolex at the rate of .02338 ml / bag in concentration of 0.5 g / L. The tree control treatments were used in all greenhouse tests. Three pots for each treatment were used as replicates (Mazen, 2004). Pre- and post- emergence and root rot incidence were recorded after 15 and 30 days of sowing. The survival of basil plants was also recorded after 60 days from sowing.

Statistical analysis:

Data collected from all experiments were statistically analyzed using the Statistic Analysis System Package (SAS institute, Cary, NC, USA). Differences between treatment were studied using Fisher's least significant difference (LSD) test and Duncan's Multiple Range Test (Duncan, 1955). All analysis were performed at P5% level.

RESULTS

1. Pathogenicity tests:

Data presented in Table (1) point up that there was severely artificially infested plants, showed several symptoms, the above ground parts were collapsed, root rotted achieved 86.667% disease incidence, 86.667 %disease severity on basil plants. On the other hand, no root or above ground plant part infections was recorded in the control treatment, roots were healthy and the above ground foliage was bright and green with 0% disease incidence, 0%disease severity and 100% living plants.

Table 1. Pathogenicity test of *R.solani* on basil plants after 15 days from planting

Treatment	Disease severity	Living plants	Disease incidence %
Control(non-infected)	0.0 B	15	0.0 B
Infested control	86.667 A	2	86.667 A

Values within a column followed by the same letter are not significantly different according to Duncan's multiple range test (P=0.05).

2- Effect of fungal antagonists on radial growth of the pathogen

Table (2) showed that, five fungal species of *T. viride*, *T. harzianum*, *T.hamatum*, *G.virens*, *G.roseum* were used in antagonism experiment against *R.solani* to examine their ability to suppress the fungal growth. Data presented showed significant differences among each of the five fungal species against *R.solani*. The antagonistic effect of the five fungi was determined after 3 periods of incubation (3 , 5 and 7 days. After 7 days of incubation *T.harzianum* was the best antagonistic fungus in inhibiting the radial growth of the pathogen giving 0.26 cm =(96.6%) inhibition, when compared with the untreated control. This followed by *T.viride*,*T.hamatum* and *G. virens* which gave 83cm=(89.6%), 1.36cm=(83.75%) and 2.76cm=

(65.5%) inhibition, while *G.roseum* showed the lowest effect on *R.solani* giving 5.46 =(31.75%)cm inhibition when compared with the untreated control. In comparison with the chemical control treatment which gave the highest reduction in *R.solani* giving 0.0cm=(100%) inhibition. Results obtained by these antagonistic treatments come in the second class after the chemical treatment.

Table 2. Effect of antagonistic fungi on the radial growth of *R.solani*

Incubation time	3days		5days		7days	
	*R.G	Inh. %	*R.G	Inh. %	*R.G	Inh. %
<i>T.harzianum</i>	0.26 C	96.6%	0.26 F	96.6%	0.26 F	96.6%
<i>T.viride</i>	0.33 C	95.8%	0.83 E	89.6%	0.83 E	89.6%
<i>T.hamatum</i>	1.00B	87.5%	1.36 D	83. %	1.36 D	83. %
<i>G.virens</i>	1.50 B	81.25%	2.50 C	68.75%	2.76 C	65.5%
<i>G.roseum</i>	2.33 A	70.87%	4.66 B	41.75%	5.46 B	31.75%
Chemical control	0.00 C	100%	0.00 F	100%	0.00 F	100%
Rizolex-T						
Untreated control	2.90 A	63.75%	5.23 A	35%	8.00 A	0.0%

R.G= mean average of Radial growth (cm.)
Inh. %=growth inhibition %

3. Effect of some antagonistic fungi on disease incidence and disease severity of damping off and root rot caused by *R.solani* on basil plants:

• After 15 day from planting:

Data presented in Table (3) showed that there was a slight significant difference between all treatments using was soaking seed (S), and soil drenching (D) on disease incidence%, living plants% and disease severity% of damping-off and rot root. *T.harzianum* (D) was the best antagonistic fungal giving 6.66% disease incidence. The same antagonistic fungus gave 94% living plants and 0%disease severity followed by *T.viride* (D) and *T.hamatum* (D) treatment gave 33.33% disease incidence, 86.6% living plants and 7.41%disease severity. Followed by *G.roseum* (s) were giving 22.22% disease incidence, 73.3% living plants and 11.11%disease severity. On the other hand *T.viride* (s) gave less effect on the pathogen giving 71.11% disease incidence, 28.8% living plants and 40.74% disease severity. When compared with the controls (0% disease incidence, 100% living plants and 0% disease severity in the untreated control, also 46.66%, 53.33% and 55.56% in the artificially infested control and the chemical control gave 6.66%diseases incidence, 93.33% living plants and 22.22%disease severity, respectively).

• After 45 day from planting:

The best antagonistic fungus was *T.harzianum*(D) treatment giving 93.3% living plants and 6.66% disease incidence and 7.41 %disease severity, there was no significant differences between another treatments in disease severity. *T. viride* (s) treatment gave less effect on the pathogen compared with the chemical, infested and untreated control.

DISCUSSION

Results showed that *T.harzianum*, achieved the best inhibition in radial growth of *R. solani* followed by *T. viride*, *T. hamatum* and *G. virens* respectively, while

the *G. roseum* gave moderate inhibition when compared with the untreated control. These results agree with the finding of (Hadar (1978); Shalini, et al. (2007); Montealegre, et al. (2010); Gaigole, et al.(2011) and Osman, et al. (2011).

Under greenhouse condition, evaluated the efficiency of these bio antagonists, compared with chemical controls.

Results showed slight significant difference among all treatments using soaking seed (S), and soil drenching (D) methods on disease incidence%, living plants% and disease severity% of damping off and rot root. *T. harzianum* (D) was the best antagonist, followed by *T. viride* (D), *T. hamatum* (D). On the other hand *T. viride* (s) gave less effect on the pathogen when compared with the controls.

These results agree with results obtained by Abdel-Kader, et al. (2011); Osman, et al. (2011) and Mays, et al. (2015)

On the other hand these results disagree with results obtained by Abd-Elkhair, et al. (2011) who reported that in greenhouse experiment the best protection to damping-off disease was obtained by *T. hamatum*, followed by *T. viride*, *T. album* and *T. harzianum*, respectively.

Trichoderma fungal strains exerted biocontrol activity against fungal phytopathogens. It have several mechanisms of action that allow them to control pathogens, including mycoparasitism, production of antibiotics or enzymes, competition for nutrients and space and the induction of plant host defenses (Brimmer and Boland, 2003 and Harman, 2006). *Trichoderma* spp. are the commonly found soil inhabitants, especially in organic soil. These fungi can live either saprophytically or parasitically with or on other fungi. One of the most well-known characteristics of the group is their ability to parasitize on other fungi, grows rapidly on many substrates and effects a wide range of plant pathogens. One of the mechanism which was observed to be adopted by *Trichoderma* to parasites *R. solani* was by means of competition. *Trichoderma* suppressed the growth of *R. solani* through the over growth. In second case, the *Trichoderma* was observed to be cluster around *R. solani* by the formation of small tufts thus limiting the growth of the pathogen. In both the cases, formation of sclerotial bodies of *R. solani* were suppressed (Shalini, et al., 2007)

Microscopic observation indicated that the mycoparasitism of *R. solani* hyphae by the hyphae of the biocontrol agent, including coiling around pathogen hyphae. The mycelial tip of *Trichoderma* runs parallel with that on the mycelium of *R. solani* and with its mycelial tips it sticks on the large hyphae of *R. solani*. this process is followed by a very rapid and excessive coiling on the target fungus, penetration and subsequent dissolution of the host cytoplasm (Vipul, et al., 2006), then followed by the production of enzymes (Metacalf and Wilson 2001 and Sharon, et al., 2001) such as chitinases, glucanases, which function by breaking down the complex polysaccharides, chitin and beta glucans that are responsible for the rigidity of the fungal

cell wall, thereby destroying the cell wall integrity (Sivan et al., 1984)

The lytic enzymes produced by *T. harzianum*, β -(1-3)glucanase activity was much higher than that of chitinase, the lytic extracellular enzymes were capable of degrading *Rhizoctonia* cell walls. *R. solani* belongs to the basidiomycetes the cell wall of which are composed mostly of glucans with only about 6-8% chitin for this reason β -(1-3)glucanase is more important in the degradation of cell wall (Hadar, 1978).

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استخدام بعض انواع من فطر التريكوودرما كوسيله أمنة لمقاومة مرض عفن الجذور والمتسبب عن فطر الريزكتونيا سولاني في نبات الريحان

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¹ جامعة المنصورة كلية الزراعة قسم امراض النبات.

² جامعة المنصورة كلية الزراعة قسم النبات الزراعي.

يعتبر نبات الريحان من النباتات العطرية التي لها استخدامات متعددة في المجالات الطبية وفي مجال الصناعة وهو من النباتات التي تصدر الى عديد من دول العالم مثل ألمانيا وإيطاليا وهولندا والولايات المتحدة. وقد تم في هذا البحث عزل بعض الفطريات المحمولة في التربة والمسببة لمرض عفن الجذور في الريحان وقد وجد ان فطر الريزكتونيا سولاني هو اكثر الفطريات تكرر. كما تم في هذا البحث استخدام بعض اجناس من الفطريات المضادة في مقاومه فطر الريزكتونيا المسبب لمرض عفن الجذور في الريحان وهي (تريكوودرما هرزيانم و تريكوودرما هماتم و تريكوودرما فردي وجلاجلديوم فيرينس وجلاجلديوم روزيم) وقد تم اخبار هذه الاجناس الفطرية معمليا وتحت ظرف الصوبة. واطهرت النتائج المعملية ان فطر التريكوودرما هرزيانم كان له افضل تأثير مثبت لفطر الريزكتونيز. في حين ان نتائج الصوبه اظهرت ان فطر التريكوودرما هرزيانم كان لها افضل تأثير مثبت للفطر.