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Full Length Research Paper

Evaluation of different combinations of *Trichoderma* species for controlling *Fusarium* rot of lentil

Mohammad Akrami[1], Hadi Golzary*[2] and Masoud Ahmadzadeh[3]

[1]Azerbaijan National Academy of Science (AMEA), Baku, Azerbaijan.
[2]Department of Plant Pathology, University of Tehran, Karaj, Iran.
[3]Department of Plant Pathology, University of Tehran, Karaj, Iran.

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The Fusarium disease, caused by *Fusarium oxysporum* has been observed in different areas of Iran in recent years. Current biocontrol studies have confirmed the effectiveness of the *Trichoderma* species against many fungal phytopathogens. In this study, biocontrol effects of *Trichoderma* isolates alone and in combination were evaluated against *F. oxysporum* pathogen. This study shows the ability of *Trichoderma harzianum* isolates which had been isolated from soil and as such, lentil roots were compared to the combination of the three fungal lentil *Fusarium* root. Three isolates (*T. harzianum*) T1, (*Trichoderma asperellum*) T2, (*Trichoderma virens*) T3, were selected base on good antagonist effect after screening tests for antifungal combination effects against *Fusarium* disease pathogen in greenhouse. In dual culture tests, three (T1, T2 and T3) isolates covered and colonized the colony of the pathogen. In other experiment, three (T1, T2 and T3) isolates covered and colonized the colony of the other *Trichoderma* isolates. Microscopic studies revealed hyphal coiling (hyperparasitism) of isolates T1 and T2 around *F. oxysporum* hyphae. Volatile metabolites of all isolates reduced the mycelial growth of fusarium pathogen. T1 and T2 isolates and their combination were more effective than other treatments in controlling the disease, such that it reduced disease severity from 20 to 44% and increased the dry weight from 23 to 52%. All treatments showed significant differences with control plants.

Key words: *Fusarium* rot, lentil, combination, *Trichoderma*, biocontrol.

INTRODUCTION

Lentil (*Lens culinaris* Medic.) is one of the oldest known protein-rich food legumes. Lentil is also called “poor man’s meat” (Bhatty, 1988). Lentil seeds are reach in protein, their mean value is at about 28.5% (Stoilova and Pereira, 1999). Owing to biotic and abiotic stresses, the crop yields are below attainable levels. Among the biotic factors, diseases are serious threat to lentil production in many parts of the world. Lentil (*L. culinaris* Medic.) suffers from a number of diseases which are caused by fungi, bacteria, viruses, nematodes and plant parasites (Khare et al., 1979). Diseases such as Ascochyta blight and lentil wilt play a major role in reducing lentil yield (Hamdi and Hassanein 1996). Lentil wilt, caused by *Fusarium oxysporum* f.sp *lentis* is one of the main limiting factors to successful cultivation. The effects of various environmental factors on pathogen growth and disease expression have been studied (Saxena, 1988; Stoilova and Pereira, 1999).

In recent times, there has been a worldwide swing to the use of eco-friendly methods for protecting the crops from pests and diseases. The use of potential harmful chemical sprays is viewed with dissatisfaction in many countries. As such in the present context, biological control of wilt with bioagents offers a great promise. A biological control agent colonizes the rhizosphere, the site requiring protection and leaves no toxic residues as opposed to chemicals (Dubey et al., 2007).

Significant role of *Trichoderma viride*, a well-known soil antagonist, was known from Windling and Fawcett (1936), when *Rhizoctonia* 'damping off' of citrus seedlings was found to be reduced by increasing the frequency of the antagonist in the root region. Later on, Bliss (1951)

*Corresponding author. E-mail: Hadigolzary@gmail.com. Tel: 00989132797204.

also pointed out that control of *Armillaria mellea* of citrus seedling was solely not due to carbon disulphide fumigation but because of the destruction of the pathogen by *T. viride* which was also found to be tolerant to fumigation. *Trichoderma* has also been found to be the dominant early recolonizer of soil after fumigation by various chemicals (Evans, 1955; Saksena, 1960; Moubasher, 1963; Warcup, 1952) which are generally used to control root-diseases. Other antagonist recovered from *Fusarium* wilt-suppressive soils, especially nonpathogenic *F. oxysporum*, have been used to reduce *Fusarium* wilt diseases of several different crops (Minuto et al., 1995).

The *Trichoderma* species are useful avirulent plant symbionts that act as biocontrol agents against phytopathogenic fungi via mechanisms of competition, rhizosphere competence, mycoparasitism, antibiotic and enzyme production, induced resistance, and promoting plant growth (Harman et al., 2004; Howell, 2003; Chet and Inbar, 1994). The majority of *Trichoderma* species are antagonist of phytopathogenic fungi and have been broadly used as the most important biocontrol agent (Tjamos et al., 1992). Considering these points, the present study was conducted to find out the most effective species/isolates of *Trichoderma* against the isolates of *F. oxysporum*.

MATERIALS AND METHODS

Pathogens and inoculum preparation

This study was done in Azerbaijan country and Azarbaijan city (north west of Iran) from 2008 to 2010. Root samples of 10 lentil plants infected with wilting were collected and transported to the laboratory. The root samples were cut into small pieces of up to 1.5 cm length and surface sterilized by 15% H_2O_2 for 30 to 45 s and then rinsed with distilled water for three times. These surface sterilized roots were placed onto 2% malt extract agar (MEA) medium in Petri plates and incubated at 25°C. After 6 days, the fungal isolates appearing on the root pieces were identified and transferred to 2% MEA medium Petri plates for purification. Pathogenecity of the isolates were confirmed under greenhouse conditions. Millet seed (*Panicum miliaceum* L.) inoculum was prepared as follows: 150 g millet seed, together with 200 ml distilled water was deposited in an autoclavable polyethylene bag and autoclaved at 121°C for 15 min. Each bag was subsequently inoculated with five 4 mm agar discs cut from a fresh PDA culture of *F. oxysporum*. The inoculum was incubated at 27 ± 1°C for 7 days.

Preparation of antagonistic isolates

To isolate *Trichoderma* from soil, a *Trichoderma*-selective medium was modified from Askew and Laing (1993). The basal medium consisted of 0.2 g $MgSO_4$ ($7H_2O$), 0.9 g K_2HPO_4, 0.15 g KCl, 1.0 g NH_4NO_3, 3.0 g D+ glucose anhydrous, 0.15 g rose bengal and 20 g agar. These constituents were added to 950 ml of distilled water and autoclaved at 121°C for 30 min. The biocidal ingredients, 0.25 g chloramphenicol, 0.2 ml fludioxonil, 0.02 g captan and 1.6 ml metalaxyl were mixed in 50 ml of sterilized distilled water and added to the autoclaved basal medium where it cooled to 40 to 50°C. *Trichoderma* strains were isolated from soil samples from various areas of Azarbaijan (north west of Iran) region using a soil dilution method. Ten grams of soil were suspended in 50 ml of sterile distilled water and agitated for 30 min at 200 rpm in a rotary shaker. Serial dilutions were made and 0.1 ml of each was spread on the *Trichoderma* selective medium plates with a glass rod. Three plates of each sample were prepared and incubated for 5 days at 30°C. *Trichoderma* isolates were collected and transferred onto PDA for further study.

Dual culture test

Mycelial disks (5 mm in diameter) of *F. oxyporum* were placed on one edge of petri dishes containing PDA and incubated at 25°C. Forty eight hours later, mycelial disks (5 mm in diameter) of *Trichoderma* isolates were placed on the opposite side of *F. oxysporum* in previous Petri dishes and they were incubated in the same thermal condition. Interactions between *Trichoderma* isolates and *Fusarium* were evaluated based on radial growth of pathogen, overgrowth speed of *Trichoderma* on pathogen colony, production of yellow pigment in overlapped area of two colonies and hyper parasitism (mycelial coiling) (Dennis and Webester, 1971c; Kucuk and Kivance, 2004).

Volatile metabolites tests

For volatile metabolites test, pathogen and *Trichoderma* actively growing colonies were subcultured on PDA and incubated in dark condition at 25°C. Then, open Petri dishes containing 48 h old colony of *F. oxysporum* were placed on 24 h old colony of *Trichoderma* and were airtight using paraphylm. Control check was Petri dishes containing PDA medium. The Petri dishes were incubated in the same temperature and dark conditions (Dennis and Webester, 1971b; Fiddman and Rossall, 1993). Radial growth on pathogen was measured daily in both tests. Inhibitory percentages were calculated by Abbott formula and were evaluated using randomized complete block design by SAS software.

Greenhouse tests

Sandy soil with 1% organic matters, was collected from the field Soil. Samples were pasteurized with water vapor for 4 h at 95 ± 5°C. Completely randomize designs of seed coating and soil treatment methods were accomplished in greenhouse and biocontrol effects of each eight *Trichoderma* isolates mixture of *Fusarium* isolates: *F. oxysporum* (F) alone; *Trichoderma harzianum* (T1) alone; *Trichoderma asperellum* (Tr2) alone; *Trichoderma virense* (T3) alone; *F. oxysporum* plus *T. harzianum* T1; *F. oxysporum* plus *T. asperellum* T2; *F. oxysporum* plus *T. virense* (T3); *F. oxysporum* plus *T. harzianum* + *T. asperellum* and *T. virense* per 1 kg soil. Lentil seeds were coated with *Trichoderma* at rate of 107 conidium/seed, averagely. In soil treatment method, contaminated soil was treated with milled inoculum of *Trichoderma* isolates averagely containing 10^6 propagul per gram at the rate of 10 g/kg which were planted in each pot at 2 cm depth of pasteurized soil mixed with 50% sand.

RESULTS

Antifungal abilities of *Trichoderma* isolate *in vitro*

In dual culture test, each of all tested *Trichoderma* isolates differentially limited the colony growth of the pathogen and overgrew the pathogen colony when

Table 1. Hyphal intraction in dual culture test.

Isolate	Overgrowth	Inhibitory effect of early volatile metabolites (%)	Yellow pigment	Hyperparasitism
T_1-T_2	Little	Nil	No	6b
T_1-T_3	Little	Nil	No	4b*
T_2-T_3	Little	Nil	No	5 b
T_1-T_2-T_3 +F_1	Little	Nil	No	38c
T_1-F_1	+	High	Yes	49c
T_2-F_1	+	High	Yes	47c
T_3-F_1	+	Medium	Yes	35c
Control	-	-	-	0a

Trichoderma colonies does not pitch on *Fusarium* colonies and does not produce spore but little hypha growths on *Fusarium* colonies (little). *Trichoderma* colonies pitches on *Fusarium* colonies and produces spore (+). *Trichoderma* colonies does not pitch on *Fusarium* colonies and does not produce spore (-). Significant differences are denoted by different letters within each column according to Duncan's multiple range test at $P < 0.01$ and values are averages of three replicates (*).

Table 2. Development of *Fusarium* rot in lentil plants as affected by treatment with various combinations of biocontrol organism.

Treatment	Rot (%)* (seed coting)	Reduction (%) (seed coting)	Rot (%)* (soil treatment)	Reduction(%) (soil treatment)
F1	78.0a*	0f	80.1a*	0d
T1+F1+T3	51.2bc	26.8cde	52.6b	27.5c
T2+T1+F1	46.3cd	31.7bc	48.2b	31.9bc
T3+F1+T2	55.0b	23e	48.6b	31.5bc
T1+F1	42.3d	35.7b*	44.5b	35.6b*
T2+F1	49.2bcd	28.8cd	51.7b	28.4c
T3-F1	48.1bcd	29.9cd	49.4b	30.7bc
T1-T2-T3 +F1	52.9bc	25.1de	50.2b	29.9bc
Control	0e	100a	0c	100a

Significant differences are denoted by different letters within each column according to Duncan's multiple range test at $P < 0.05$ and values are averages of four replicates (*).

compared with their control. Overgrow speed, unnatural production of yellow pigment and mycoparasitism are in Table 1. In early volatile metabolites test, all of the isolates significantly reduced the pathogen colony growth, while the maximum growth reduction was observed in *T. harzianum* T149 and *T. asperellum* T90 isolates, respectively ($P < 0.01$). By 48 h after interaction between mycelia of *Trichoderma* isolates and the pathogens mycelia, a clear zone of interaction was formed in all *Trichoderma*-pathogen combination. The yellow pigments were roducted by *Trichoderma*-pathogen combinations. The maximum hyperparasitism was observed in *T. harzianum* T149 and *T. asperellum* T90 isolates, respectively, which was significant compared with other treatments ($P < 0.01$) (Table 1).

Biocontrol effects of *Trichoderma* isolates in greenhouse

In the seed coating and soil treatment tests, treatments of T1, T2, mixture of isolates and *Trichoderm* combinations showed more biocontrol effectiveness than the other treatments (Table 2). In the seed coating, the minimum and maximum *Fusarium* rot was 42.3 and 55% for isolates of T1, T2 and T3, respectively, while in soil treatment, the minimum and maximum *Fusarium* rot was 44.5 and 52.6% for isolates of T1, T1 and T3, respec-

tively. The isolate of *T. harzianum* T149 was the best treatments for biocontrol of *Fusarium* rot.

DISCUSSION

Biological control is the best alternative, especially against soil borne pathogens such as *Fusarium* sp. The limitations to biocontrol use are scarce knowledge on the ecology of rhizosphere and use of *in vitro* antagonism for selection of biocontrol agents. However, the advantages of using biocontrol include environmental friendly, cost and extent of protection (Gohel et al., 2006b). *Trichoderma* spp. that are common saprophytic fungi found in almost any soil and rhizosphere micro flora, have been investigated as potential biocontrol agents because of their ability to reduce the incidence of disease caused by plant pathogenic fungi, particularly many common soil borne pathogens (Papavizas, 1985; Sivan and Chet, 1986; Calvet et al., 1990; Elad et al., 1993; Elad et al., 1993; Spiegel and Chet, 1998; Elad, 2000; Freeman et al., 2004; Ashrafizadeh et al., 2005; Dubey et al., 2007), although some have been occasionally recorded as plant pathogens (Menzies, 1993).

In this work, the results of dual culture revealed the rapid colonization of the medium by *Trichoderma* isolates. All *Trichoderma* isolates evaluated were effective in controlling colony growth of the *Fusarium* sp. isolates. Evaluation of produced volatile and nonvolatile components also showed acceptable performance on inhibiting mycelial growth of pathogens. The results reported here suggest that from the isolates of *Trichoderma* used in this study, *T. harzianum* T149 and *T. asperellum* T90 strains were more capable of influencing the growth of all tested pathogens in dual culture and through production of volatile and non-volatile inhibitors under controlled condition, and may be used as a broad spectrum biological control agents under field condition (Table 1). Temperature and pH are two key parameters to manipulate growth, sporulation and saprophytic ability as well as production of volatile and non-volatile metabolites, involved in nutrition, competition, mycoparasitism and extra cellular enzymes that disintegrate cell wall of fungi. Therefore, it is important to collect information about the effects of pH and temperature on the mycelial growth. It has been demonstrated that *Trichoderma* strains are active under a wider range of pH (Kredics et al., 2003). The optimum temperature for growth differs among the *Trichoderma* isolates; although most *Trichoderma* strains are mesophilic (Kredics et al., 2003). The results obtained from the present study also support these hypotheses.

It is important to mention that *Trichoderma* spp. are known to produce a number of antibiotics such as trichodernin, trichodermol, harzianum A and harzianolide (Dennis and Webster, 1971c; Kucuk and Kivanc, 2004) as well as some cell walls degrading enzymes such as chitinases, glucanases that break down polysaccharides, chitins and glucanase, thereby destroying cell wall integrity (Elad, 2000). These may also play a major role in mycoparasitism because of changes in cell wall integrity prior to penetration. Selection of biocontrol agents as well as understanding the mechanisms involved in the antagonistic effect of *Trichoderma* spp. on plant pathogens are important in designing effective and safe biocontrol strategies.

Different isolates of *Trichoderma* have different ability for fungal; their indirect effects may also vary. Therefore, one of the most interesting aspect of biology is the study of the mechanisms employed by biocontrol agents to affect disease control. Possible mechanisms of antagonism employed by *Trichoderma* spp. includes nutrient and niche competitions, antibiosis by producing volatile components and non-volatile antibiotics (Harman and Hadar, 1983; Dennis and Webster, 1971b, c) that are inhibitory against a range of soil borne fungi, as well as parasitism (Dennis and Webster, 1971a).

Also, synergism between different forms of action modes occurs as the natural condition for the biocontrol of fungal pathogens. It is widely known that environmental parameters such as abiotic (soil type, soil temperature, soil pH and water potential) and biotic (plant species and variety, and microbial activity of the soil) factors as well as other factors such as method and timing of applications may have influence on the biological control efficacy of *Trichoderma* isolates. Therefore, it is important that *Trichoderma* biocontrol potential in field condition should be further evaluated.

If the mechanisms of mycoparasitism and antibiosis are not vital to biological control by *T. virens*, so which mechanism could be present? By culturing fungi from the roots of treated plants taken at intervals from field plantings throughout the growing season, we discovered that *T. virens* could be recovered from surface-sterilized roots at any time. The fungus colonized the tap and secondary roots in the upper reaches of the soil. However, late in the season, it was found mostly on the tap and primary roots (Zhang, 1995). Because the roots were rigorously surface-sterilized, we concluded that the fungus had penetrated the roots. This hypothesis was later supported by Yedidia et al. (1999) with *T. harzianum*. They demonstrated microscopically that the fungus penetrated the epidermis and grew into the outer layers of the root cortex where it induced defense responses in plant.

Mechanism that *T. virens* employs in the biological control of the preemergence phase of seedling disease that is incited by *Pythium ultimum*, *Pythium aphanidermatum* or *Rhizopus oryzae* has recently been discovered. During the germination process, disease-susceptible plant seeds are released to the spermosphere compounds; this stimulate resting structures of the pathogens in the surrounding soil to germinate and infect the seeds of resistant cultivars which do not release these compounds. Seed treatment of susceptible cultivars

with preparations of *T. virens* prevents infection of the seed by disrupting the process. The biocontrol agent coated onto the seed metabolizes the germination stimulatory compounds before they reach the pathogen propagules in the soil (Howell, 2002). Dead cells of *T. virens* are ineffective as biocontrol agents (Howell, 2003). The enzymes responsible for metabolism of the compounds that stimulate pathogen propagule germination are contained within the hyphal cells of *T. virens*. Exposure of the stimulatory compounds to cultures of *T.* virens destroys their stimulatory activity, while exposure to cell-free filtrates of *T. virens* cultures did not result in a loss of activity when the compounds were recovered and bioassayed.

When seeds of a highly susceptible plant are treated with a strain preparation of *T. virens* and planted in soil infested with preemergence damping-off pathogens, seedlings emerge normally and appear to be healthy (Howell and Puckhaber, 2005). The biocontrol fungus was more effective in reducing the growth of the pathogen in moist than in wet soil but was more effective at 35°C. 35°C temperature into humidity is a suitable condition for the activity of fungi isolates and humidity improves the activation of the alternative bacterial. Greater amount of the antagonist was obtained from the sticks than the pathogen during the incubation in the moist soil, as the moist soil conditions appeared to be more supportive for the antagonistic activity of *T. harzianum* against the pathogen. The reasons for lower population of *T. harzianum* in the wet soil compared to the moist soil could be due to greater antagonism by bacteria that flourished under the wet conditions (Inam and Javed, 2009). In this study, because several useful isolates have been used simultaneously, a minimum of one isolate was activated and was compatible in different conditions. Thus, in this study, biocontrol is possible in wide scale in different conditions.

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