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Biocontrol Characteristics of *Trichoderma* Spp. Against *Fusarium* in Iran

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Abstract: Applying chemical fungicides to control soil-born fungi may lead to environmental pollution. In recent years, biological control, using living cells of different types of microorganisms especially fungi, to control plant pathogens, holds a great promise to improve yield and quality of crop. Fungal species belonging to the genus *Trichoderma* are worldwide in occurrence and have emerged as an important component in biological control of plant diseases caused by *Fusarium*. In the previous study, 15 strains of *Trichoderma* were isolated from soil, decaying wood and other forms of plant organic matter and 5 of which selected for antagonistic assay towards *Fusarium* at lab scale. From collected data, *T. harziuanum* T3 showed different mechanisms such as mycoparasitism, production of volatile compounds and secreted materials that prevent growth of plant pathogens partly. Therefore, it selected for pot experiments. Experiment done in a way that *Fusarium* at different times, (before, at the same and after inoculation of *Trichoderma)*, added to the soil. Results revealed that it is better to add *Trichoderma* to the soil before inoculation of *Fusarium*. This finding may reflect the competition role that antagonistic fungus played.

Key words: *Trichoderma* · *Fusarium* · Plant pathogens · Antagonistic effects · Pot experiments

INTRODUCTION

Trichoderma spp. as saprophytic filamentous fungi, are worldwide in occurrence and characterized by their rapid growth, mostly bright green numerous conidia and a repetitively branched conidiophore structure [1]. The genus *Trichoderma* is well known for its biological control of important plant pathogens such as *Fusarium* spp. The mechanisms employed by the fungus to fight pathogenic fungi contain mycoparasitism, antibiosis, competitive saprophytic ability, the metabolites secretions, induction of resistance against tension and promotion of growth in plants [2]. Diversity of antagonistic mechanisms in the genus *Trichoderma* made it suitable for biological control of soil-borne phytopathogens.

In preliminary study, a total of 15 isolates belonged to genus *Trichoderma* were obtained from soil and decaying wood of different regions of Iran. In this study biological control of *Fusarium* using 5 promising isolates of those *Trichoderma* spp. (T_2, T_3, T_6, T_{12} and T_{13}) that morphologically [3] and by molecular test using ITS1 and ITS4 primers identified as *T. harziuanum* was investigated.

METERIALS AND METHODS

Colony Growth Inhibition Assay: In vitro antifungal activity of *Trichoderma* spp. against *Fusarium proliferatum* was tested using dual culture method as described by *Doley* [4]. A 3 mm diameter mycelia block from margin of 1-day-old culture of *Trichoderma* isolates and of pathogen placed near the edge on the surface of PDA medium. A similar culture of pathogenic fungus without antagonistic fungus was carried out for control. Three replicates of each treatment were incubated at 28±1°C and observed daily for one week for mycoparatisim. After contact of two fungal colonies in the plate the area of interaction of fungi was inspected by naked eyes and microscopically, as well [5].

Volatile Metabolites Test: In order to investigate the effect of released volatile metabolites of *Trichoderma* spp. on mycelial growth of the pathogen, volatile metabolites test was done based on the methods of Dennis and Webster [6].

A disc of agar plug of 7 days-old culture from the margin of each *Trichoderma* isolate and the *Fusarium* was centrally placed on seprated PDA dishes and the

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antagonistic fungal Petri plates were placed bottom to bottom with the *Fusarium* Petri plates on the top and held together with parafilm to prevent leakage and incubated in 28±1°C for 5 week. A 5 mm diameter of sterile PDA medium was placed in the dish instead of *Trichoderma* spp. as the control plate. Radial growths of the pathogens were recorded daily and percent inhibition of average pathogen mycelial growth in relation to the control was calculated.

Effect of Non-Volatile Antibiotics: *Trichoderma* spp. were cultured in PDB and incubated in 28±1°C for 1 week. Culture filtrate of *Trichoderma* spp. was obtained by passing a 7 days old PDB culture of the fungi through cellulose filter papers which enable filtration of bulk mycelia particles and then a membrane filter (Sartorius, Germany) of pore size, 0.25 µm. The amount of 0.5, 1, 1.5, 2 and 2.5 ml of each fungal extract were added to the respective amount of 9.5, 9, 8.5, 8 and 7.5 ml of concentrated PDA to produce the 5, 10, 15, 20 and 25% dilutions of the *Trichoderma* spp. filtrates. Then a 5 mm diameter mycelial plug of the pathogen positioned centrally in the prepared PDA dishes after the agar hardens. Radial growth of pathogen observed daily and inhibitory effect of non volatile metabolites of *Trichoderma* spp. on *Fusarium* growth calculated as follows:

$$I = [(C - P)/C] \times 511$$

where I is inhibition of radial mycelial growth; C is radial growth measurement of the pathogen in control; P is radial growth of the pathogen in the presence of *Trichoderma* isolates [7].

Pot Experiments: Biological control of *Fusarium proliferatum* by the antagonistic fungus in pot test was surveyed as described previously by Karampour in completely randomize designs of seed coating and soil treatment methods [8]. In this regard, inoculation of pathogen and antagonist prepared separately and added in ratio of 1:10 (w/w) to the soil of each pot containing clay: humus: sand in combination of 1:1:2. For inoculation of fungi into the pot, the suspension of antagonist and pathogen spores in sterile distilled water were adjusted to 2×106 $_{spores/ml\ with\ a}$ hemacytometer from the one-week-old culture of each fungus in PDB and added to Flasks containing equal amounts of sterile, wet wheat and clay as the carrier of each fungus. The flasks incubated in 28±1°C until the fungi grew and covered the bed completely. *Cantaloupe* seeds (sterile via washing in 70% ethanol for 30 sec, dipping in a 0.1% sodium hypochlorite solution and rinsing with sterile distilled water for 3 times) were planted in each pot at 2 cm depth of pasteurized soil and the soil treatment arranged as following order: addition of *F. proliferatum*(F) alone (control negative); *F. proliferatum* plus *T. harzianum* T3; without any fungi (control positive). The treatments were done in a way that *Fusarium* in three different onsets e.g. 5 day before, 5 day after and contemporary of antagonistic fungus inoculation added to the pots. Vegetative growth of *Cantaloupe* plants, such as plant height, wet matter weight and percentage of total weight of plant were recorded after 50 days as the index of effectiveness of the biocontrol of pathogen in the plant.

RESULTS

In studying *Trichoderma* isolates and *Fusarium proliferatum* in dual culture, all *Trichoderma* isolates grew faster than the pathogen fungus, in that, after contact of two fungi, *Trichoderma* spp. inhibited growth and development of the pathogen and then started to grow and march on the mycelia of *Fusarium* and trapped it, finally. By observing the interaction site of antagonistic and pathogenic fungi, a clear zone between the mycelia around the pathogen colony was formed (Figure 1a). Microscopically inspection of the interaction zone revealed the coiling of the *Trichoderma* spp. around hyphae of the pathogenic fungus and finally segmentation of its mycelial tips (Figure 1b).

In studying the susceptibility of *Fusarium* to the volatile inhibitors produced by *Trichoderma* spp. the growth inhibition of the pathogen compared with the control observed when the antagonistic fungi cultured 24 hours earlier. Among *Trichoderma* spp. *T. harzianum* T_3, T_{12} and T_{13} and T_3 in particular, had a marked significant inhibitory effect ($p \leq 0.05$) on the growth of the pathogen compared with the control. Maximum pathogen inhibition percentage recorded by *T. harzianum* T_3, (33.4%).

The observation of radial growth inhibition of *F. proliferatum* by non volatile metabolites of *Trichoderma* spp. showed the filtrate of 25% antagonistic fungi had the different effects on pathogen, when T_3, T_{13} and T_6 by 60%, 57.2% and 51% caused the maximum inhibition of *Fusarium* radial growth, respectively and the minimum growth inhibition by non-volatile inhibitors o T_9 and T_2 with 36.7% and 33.3% respectively, were obtained.

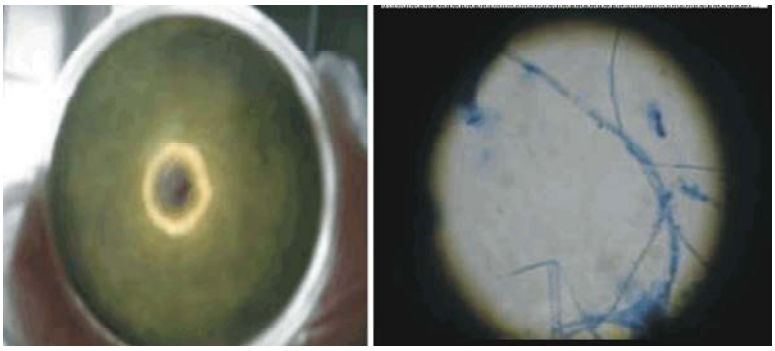

Fig. 1: A- a clear zone between the mycelia around the pathogen colony. B- the coiling of the *Trichoderma* spp. around hyphae of the pathogenic fungus

Table 1: Vegetative growth indexes of *Cantaloupe* plants in different treatments

Treatments	Plant height (cm)	Wet weight (g)	Percentage of total weight (%)
Positive control	29	10	-
Negative control	25	9	-
Treatment (T_3+F): contemporary inoculation	37.7	12.5	25
Treatment (T_3+F): inoculation of F before	30.5	10.9	-2
Treatment (T_3+F): inoculation of F after	42	14	27

Since *Trichoderma harzianum* T_3 was differed in respect of diverse antagonistic mechanisms and its fast growth rate towards other isolates of *Trichoderma* spp. this isolate selected for further investigation of pot experiments.

Treatment of *Cantaloupe* seeds by T_3 offered significantly ($P \leq 0.05$) higher plant height and more wet matter weight and percentage of total weight in comparison with control (Table 1). The remark point was the positive antifungal influence of T_3 when pathogen fungus added 5 days after antagonistic fungus inoculation.

DISCUSSION

Biocontrol of soil-borne plant pathogens such as *Fusarium* sp. by antagonistic fungus *Trichoderma* is the best alternative, due to the advantages like environmental friendly, cost- effective and expanded protection [9]. *Trichoderma* spp. are known as potential biocontrol agents because they can reduce the incidence of disease caused by plant pathogenic fungi through mechanisms such as mycoparasitism, antibiosis, competitive saprophytic ability and the metabolites secretions [10]. The remark point many authors mentioned is the diversity of mechanisms employed by various strains of *Trichoderma* [11].

Previously, 15 biocontrol agents from *Trichoderma* spp. had been isolated by the researches in the ACECR organization. In this study, the ability of the 5 *Trichoderma* spp. to control plant pathogen *Fusarium* were tested in vitro and in vivo examinations.

The antifungal activity of the tested *Trichoderma* spp. indicated that in competition for nutrient and space in PDA plates, this is the *Trichoderma* spp. to win because of their faster growth towards *Fusarium* sp., in that, all 5 *Trichoderma* spp. showed rapid colonization of the medium and effective control of colony growth of the pathogen. The microscopic investigation of interface of the pathogen and antagonist fungi revealed that coiling the mycelium of *Fusarium* by the *Trichoderma* spp. made some changes in the structure of pathogen cell wall, so that the cell wall disintegrity following to penetration of antagonistic fungi hyphea occurred. It is well documented degradation of cell wall of fungi by extra cellular enzymes of *Trichoderma* spp. which allows break down of polysaccharides, chitins and glucanase, thereby destroying cell wall integrity and making clearance zone in the plate [12, 13]. The resulted obtained from dual culture of antagonist-pathogen in which mycoparasitism and clear zone in the PDA plates was seen, is in accord with the documents.

It has been shown that different isolates of *Trichoderma* spp. can produce various volatile metabolites such as alcohol and lactones, depend on the kind of the strains and culture condition [14]. The results obtained from present study which showed different ability of the *Trichoderma* spp. to inhibit radial growth of pathogen reflected these ideas.

However, their antifungal activities are dependent on the concentration of volatile compounds produced by antagonists [14].

The results reported here suggest that from the isolates of *Trichoderma* used in this study, *T. harzianum* T3 was more capable to influence the growth of tested pathogen in dualculture and through production of volatile and non-volatile inhibitors under controlled condition. Therefore, *T. harzianum* T3 used for pot conditions.

As the pot experiments showed the selected *Trichoderma* (T_3) caused more wet weight and height in the presence of pathogen fungus which this elongation in the height of the plant was more than that seen in the control plant without any fungi. These finding may indicate the promoter effect of the T3 on growth of aerial organs of *cantaloupe*. This finding is consistent with Sajadi observations which indicated treatment of tobacco plants with *Trichoderma* isolates resulted in more height of the plant over that of the controls [15].

Several authors such as Windham in 1986 showed that amendments of soil with *Trichoderma* isolates enhanced plant growth of tomato or tobacco directly by the metabolites which contain a growth regulating factor that increase the rate of seed germination or as a secondary effect due to control of plant pathogens [16].

The evaluation of the effective time of the antagonist inoculation showed development of resistance in biocontrol of *Fusarium* sp. was time dependent. It means that application of *Trichoderma harzianum* T3 before outbreak of the pathogen effectively inhibited decay development and increased the plant growth. The results obtained from this study support the idea indicated suppression of the colonization of roots by *Fusarium* because *Trichoderma* spp. is predominant in niche and nutrient competition.

Baghani and coworkers showed some strains of *Trichoderma* could prevent germination of *Fusarium* spp. by occupying the plant surfaces [17]. Study of Aragi [18] and Inch [19] also confirm that using *Trichoderma* suspensions before development of *Fusarium* spp. are the most effective time for controlling the pathogens.

Finally, the judicious use of biocontrol agents and reduced amounts of fungicides or other physical aspects as the integrated pest management philosophy are achieved by applying natural antagonistic agents such as *Trichoderma* spp.

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