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Biocontrol evaluation of wheat take-all disease by *Trichoderma* screened isolates

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Wheat take-all disease, caused by *Gaeumannomyces graminis* var *tritici* (Ggt), 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 were evaluated against take-all pathogen. Five (*Trichoderma virens* T65, *Trichoderma virens* T90, *Trichoderma virens* T96, *Trichoderma virens* T122 and *Trichoderma koningii* T77) isolates were selected after screening tests for antifungal effects against take-all pathogen and three (*Trichoderma koningiopsis* T149, *Trichoderma brevicompactum* T146 and *Trichoderma viride* T150) isolates were used as random selection. Also, Trichodermin B and Subtilin as commercial bioproducts were also used to evaluate biocontrol effects against take-all in greenhouse. In dual culture tests, five (T65, T90, T96, T122 and T77) isolates covered and colonized the colony of the pathogen. Microscopic studies revealed hyphal coiling (hyperparasitism) of isolates T65, T96 and T90 around Ggt hyphae. Culture filtrates and volatile metabolites of all isolates reduced the mycelial growth of take-all pathogen. T96, T65, mixture of eight isolates and Trichodermin B were more effective than other treatments to control the disease and reduced 25 to 55% disease severity and increased 27 to 59% dry weight of shoots and 23 to 58% dry weight of roots compared with control while disease reduction of seed coating method was more than soil treatment application. Although, based on shoots and roots dry weight, all of treatments showed significant differences with healthy plants.

Key words: Biocontrol, mycoparasitism, *Trichoderma*, take-all, volatile and non volatile metabolite.

INTRODUCTION

Take-all of wheat (*Triticum aestivum*) caused by *Gaeumannomyces graminis* var. *tritici* (Ggt) fungus, is the most important root disease of wheat worldwide (Weller and Cook, 1983). In the recent years, take-all of wheat has been reported from different parts of Iran such as Tehran, Mazandaran, Gorgan, and Markazi (Frootan Naddafi et al., 1989; Amini, 1996; Ghalandar, 2001). Take-all pathogen colonize root superficially and then penetrate into the epidermal cell, cortex, and vascular bundle. Finally, root dead results from occlusion of the xylem vessels that restricts the flow of nutrients and water. Diseased plants appear as white patches in a few

fields due to the bleached, empty heads or white heads. Other symptoms such as stunting, root and crown blackening and reducing of root system are also considerable (Colbach et al., 1997; Huber and McCay-Buis, 1993). Application of Herbicides and fungicides, soil fertilization management, crop residue burning, nutrition management and crop rotation methods have been applied but so far, the most effective method to reduce take-all incidence is rotation with non-cereal crops which is not practical in some conditions (Stromberg et al., 1999a; Yarham, 1981). Therefore, searching for other control methods such as biological control is necessary.

Garrett (1938) found the phenomenon of take-all decline in monoculture systems of Australia wheat fields. Simon and Sivasithamparam (1989) related take-all decline to the population of *Trichoderma* Species and they reduced the saprophytic growth of take-all fungus

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with soil application of their inoculum. Seed coating with *Trichoderma koningii*, one of the most important observed antagonist in suppressive soils, limited saprophytic and parasitic potential and reduced take-all disease (Simon and Sivasithamparam, 1989). A *T. koningii* strain isolated from western fields of Australia reduced disease severity of take-all and increased yield in field trials in the United State and China (Duffy et al., 1996; Tang et al., 1996). Combination of *T. koningii* with fluorescent *Pseudomonads* increased yield more than application of either agents alone (Duffy et al., 1996). Kucuk and Kivance (2003, 2004) studied the effects of 10 *Trichoderma harzianum* isolates on take-all pathogen and reported that *Trichoderma* isolates inhibited 28 to 100% colony growth of take-all agent *in vitro*. Frootan Nadafi et al. (2002) reported the biocontrol effects of a few *T. harzianum* and *Trichoderma viride* isolates on take-all agent in greenhouse trials.

Trichoderma species are useful, avirulent plant symbionts that act as bicontrol 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 majorities of *Trichoderma* species is antagonist of phytopathogenic fungi and have been broadly used as the most important biocontrol agent (Tjamos et al., 1992). Nowadays, several kinds of commercial products are formulated based on *Trichoderma* strains.

In this research, biocontrol effects of *Trichoderma* native isolates and two commercial bioproducts on wheat take-all disease were evaluated.

MATERIALS AND METHODS

Take-all agent and *Trichoderma* isolates

Ggt fungus was isolated on Rifampicin diluated-PDA (R-dPDA) medium from an infected field in Markazi Province of Iran (Duffy and Weller, 1994) (Figure 1). Five isolates of *Trichoderma* including *T. virens* T65, *T. virens* T90, *T. virens* T96, *T. virens* T122 and *T. koningii* T77 were selected after laboratory tests of their antifungal activities against take-all agent out of 154 *Trichoderma* isolates. In addition, *T. koningiopsis* T149, *Trichoderma brevicompactum* T146 and *T. viride* T150 were used as random isolates. 110 *Trichoderma* isolates were isolated from rhizosphere and bulk soil samples of wheat and barley fields using Elad and Chet (1983) semiselective medium from Markazi and Hamadan Provinces in Iran and 44 *Trichoderma* isolates was prepared from Plant Protection Department of BuAli Sina University. The isolates were shortly time maintained on PDA.

Inoculum preparation of take-all agent and *Trichoderma*

To produce inoculum, 60 g sand, 60 g millet seeds, and 10 ml distilled water for take-all agent, and 80 g wheat seeds, 80 g sand and 80 ml distilled water for *Trichoderma* isolates were separately added to 500 ml Erlenmeyer flasks and sterilized twice at 121°C for 20 min, with a 3 day interval between autoclaving. Flasks containing autoclaved substrates were inoculated with four disks of Ggt

or *Trichoderma* young colonies grown on 1/4-strength PDA respectively and incubated in light at 21 to 25°C until completely colonized (10 days for *Trichoderma* isolates and 30 days for Ggt). For soil treatment application, the prepared inoculums of *Trichoderma* were aired, milled, and incubated until application time in icehouse (Duffy et al., 1997). To supply suspension of *Trichoderma* spore for seed coating method, 20 ml distilled water was added to flasks containing *Trichoderma* inoculum (Notmilled) that was completely sporulated. Stock suspension was collected into 20 ml tubes and homogenized by vibrator. Quality of inoculum (the number of propagul per gram or milliliter inoculum) was set 10⁶ propagul per gram inoculum for soil treatment and 10⁷ conidium on seed surface for seed coating method using colony forming unit and haemocytometer laboratorial process, respectively.

Dual culture test

Mycelial disks (5 mm in diameter) of Ggt were placed on one edge of petri dishes containing PDA and incubated at 25°C. 48 h later, mycelial disks (5 mm in diameter) of *Trichoderma* isolates were placed on the opposite side of Ggt in previous Petri dishes and they were incubated in the same of thermal condition. Interactions between *Trichoderma* isolates and take-all agent 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 hyperparasitism (mycelial coiling) (Dennis and Webster, 1971c; Kucuk and Kivance, 2004).

Culture filtrate (nonvolatile metabolites) and early volatile metabolites tests

Mycelial disks of each *Trichoderma* isolate grew on 1/4-strength PDA was separately inoculated into 100 ml flasks containing potato dextrose broth and incubated at 20 to 29°C and 120 RPM in rotary shaker incubator for 10 days. The cultures were then filtered through 0.22 mm millipore filters and 15 ml of these filtrates were added into sterile Erlenmeyer flasks containing 50 ml 1/4-strength PDA with 25% further agar at 45°C. After medium solidifying, mycelial disks of the take-all agent derived from actively growing colonies were placed on one edge of medium plates and were incubated at 25 ± 3°C (Dennis and Webster, 1971a; Kucuk and Kivance, 2003, 2004). For early volatile metabolites test, pathogen and *Trichoderma* actively growing colonies were subcultured on PDA and incubated in dark condition at 25°C. Then, opened Petri dishes containing 48 h old colony of take-all agent were placed on 24 h old colony of *Trichoderma* and were airtight using paraphylm. Control was Petri dishes containing PDA medium. The Petri dishes were incubated in the same temperature and dark conditions (Dennis and Webster, 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 completely design by SAS software.

Greenhouse tests

Silty loam soil with 1% organic matters, was collected from the field which were not under wheat and barley four last years. Soil samples were pasteurized with water vapor for 4 h at 95 ± 5°C. Two completely randomize designs of seed coating and soil treatment methods were accomplished in greenhouse and biocontrol effects of each eight *Trichoderma* isolates, mixture of eight isolates, Trichodermin B and Subtilin on take-all disease were studied. Pots (15 cm deep, 15 cm width) were filled with pasteurized soil contami-

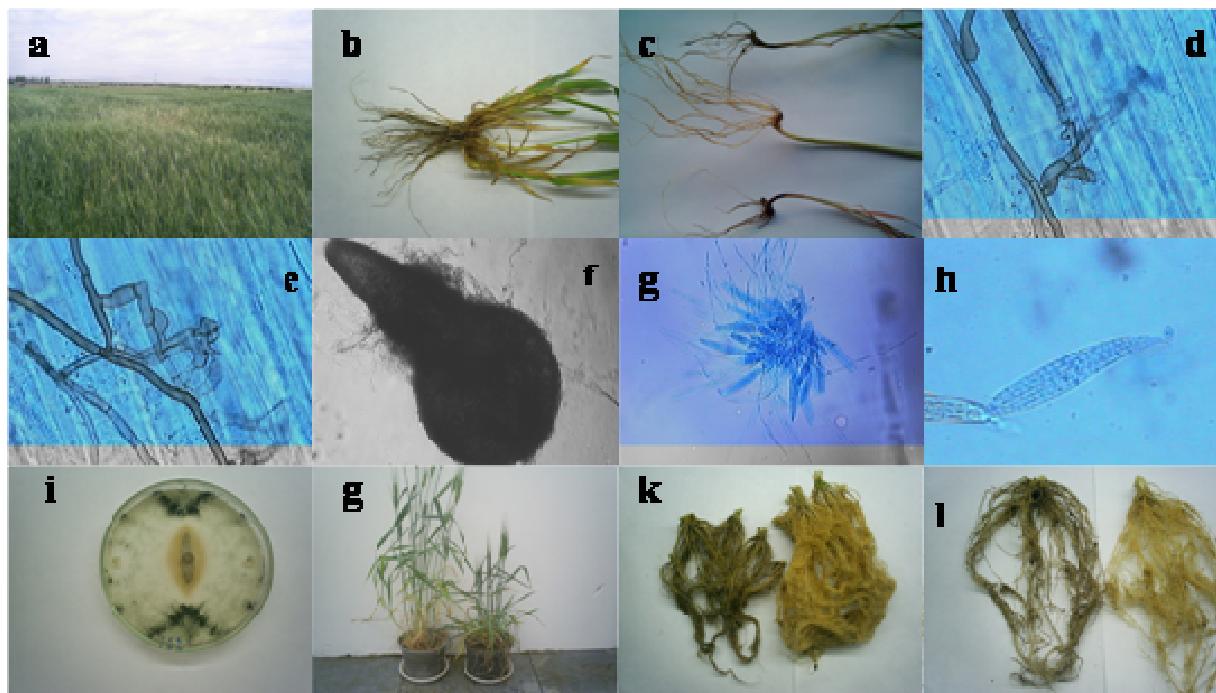


Figure 1. **a:** Take-all patches in field. **b:** Infected plant collected from field. **c:** Root necrosis in control than healthy plant in pathogenicity test. **d:** Apical simple hyphopodia of Ggt with pore. **e:** Middle simple hyphopodia of Ggt that have formed aggregated. **f:** Long neck perithecioid of Ggt. **g:** Ascii and paraphyses in hymenium of Ggt **h:** Ascus with apical operculum. **i:** Growth reduction, overgrowth, and abnormal production of yellow pigment. **g:** The height of control and healthy plant. **k, l:** Root necrosis of control and healthy plant.

nated with 1600 propagules (infested millet seeds) of take-all agent per 1 kg soil. Wheat seeds were coated with *Trichoderma* at rate of 10^7 conidium/seed averagely. In soil treatment method, contaminated soil was treated with milled inoculum of *Trichoderma* isolates averagely containing 10^6 propagule per gram at the rate of 10 g/kg. Five vernalized winter wheat seeds (cultivar of Alvand) were planted in each pot at 2 cm depth of pasteurized soil mixed with 50% sand.

Biologic commercial agents

Trichodermin B and Subtilin produced by Talhigh Daneh Company containing 10^7 and 10^{10} propagul of *T. harzianum* and *Bacillus subtilis* strains per gram biological agent respectively. These two products were used at the rate of 10 g/kg seed for seed coating and 10 g/kg soil for soil treatment.

Growing conditions of plants in greenhouse

Pots were fully randomized placed on garden seats and were needfully irrigated during Feb until April. Treatments and control plants were sufficiently and equally fertilized by NH_4NO_3 in Tilling and Jointing stages. Plants were incubated at 14 to 26°C in Feb and 20 to 28°C during Mar until April with a regime of fully natural light during the growing period.

Evaluation indicators and data analysis

Wheat plants were harvested at flowering stage. Shoots and roots

dry weight and percentages of roots and crowns necrosis were recorded. Disease severity was determined 0 to 5 based on following indicators (Rothrock, 1986). Recorded indicators were then analyzed by SAS software. 0 = no lesions, 1 = one to several lesions (roots blacking <25%), 2 = extensive lesions or several entire roots necrotic (25 to 50% roots blacking), 3 = lesions on roots and darkening of crown (50 to 75% root blacking), 4 = extensive darkening of crown (75 to 100% roots blacking) and 5 = dead plant.

RESULTS

Antifungal abilities of *Trichoderma* isolate *invitro*

In dual culture test, each of eight tested *Trichoderma* isolates differentially limited the colony growth of the pathogen while T77, T90, T96, T122, and T65 isolates overgrew the pathogen colony. Overgrow speed, unnatural production of yellow pigment and mycoparasitism are shown (Table 1 and Figure 1). In culture filtrate test, all of the isolates reduced pathogen colony growth except T149 isolate which enhanced the pathogen growth on the second 48 h after inoculation. The inhibitory effects of filtrated metabolites reduced as the incubation period increased (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 T149 and T90 isolates respectively (Table 1).

Table 1. Reaction and antifungal activities of *Trichoderma* isolates on take-all agent *in vitro*.

Isolates	Hyphal interaction in dual culture test			Inhibitory effect of Culture filtrate (percent) ⁴			Inhibitory effect of early volatile metabolites (%)
	Overgrowth ¹	Yellow pigment ²	Hyperparasitism ³	First 24 h	Second 24 h	Second 48 h	
Control	-	-	-	0a ⁵	0a	0a	0a
T65	+	Nil	Yes	69e	67e	64e	10b
T149	-	Nil	No	49d	0a	-3a ⁶	50d
T146	-	Medium	No	51d	46cd	39c	10b
T122	+	High	No	51d	40c	34c	9b
T96	+	High	Yes	66e	56ed	52d	13b
T90	+	High	Yes	54d	52d	41c	31c
T77	++	Nil	No	23b	27b	20b	9b
T150	-	Low	No	34c	21b	11b	15b

¹The growth of *Trichoderma* isolates on pathogen colony. -: Not overgrowth, +: complete with slow speed and ++: complete with rapid speed.

²Production of yellow pigment more than growth normal condition.

³Observation of hyphal coiling of *Trichoderma* isolates around pathogen hypha.

⁴Inhibitory percentages of *Trichoderma* isolates on mycelial growth of take-all agent in periods after inoculation in Culture filtrate trial.

⁵Significant differences are denoted by different letters within each column according to Duncan's Multiple Range test at P<0.01 and values are average of three replicates.

⁶Negative symbols mean that this treatment increased 3 percent mycelial growth.

Biocontrol effects of *Trichoderma* isolates in greenhouse

In the seed coating and soil treatment tests, treatments of T96, T65, mixture of isolates and Trichodermin B showed more biocontrol effectiveness than the other treatments. The decrease of disease severity was 49, 47, 34, and 25% in soil application of isolates T65, T96, Trichodermin B and mixture of isolates, respectively, while in seed treatment, the decrease of disease severity was 55, 52, 47 and 37% for isolates T96, T65, Trichodermin B and mixture of isolates, respectively. However, there was variation among isolates effect on reduction of disease severity in both soil and seed application methods. All of the soil application treatments increased the shoot dry weight of the wheat, but there was difference among their effect in this regard. The maximum and minimum shot dry weight obtained in T65 soil application and T150, respectively. All of the seed application treatments increased the shot dry weight and the maximum and minimum shot dry weight resulted from T96 and T149, respectively. The root dry weight of wheat also increased in soil and seed application tests. The maximum and minimum root weight obtained from mixture of isolates and T90, respectively (Table 2). Disease reduction in seed coating method was more than soil treatment method based on used indicators. On the whole, T96, T65, mixture of eight isolates and Trichodermin B were more effective than the other treatments to control the disease and reduced 25 to 55% disease severity and increased 27 to 59% dry weight of shoots and 23 to 58% dry weight of roots compared with control (Table 2). Although, all of treatments showed significant differences

with healthy plants and reduced 21 to 37% shoots dry weight and 27 to 44% roots dry weight in the best condition of biocontrol effects against disease resulted by treatments of T96, T65, mixture of isolates and trichodermin B.

DISCUSSION

In dual culture test, all of the eight isolates reduced the colony growth of pathogen; due to the rapid growths of *Trichoderma* isolates rapidly colonized medium surface and substrate. These observations are similar to Kucuk and Kivanc (2004) findings. Five isolates (T65, T22, T96, T90 and T77) completely overgrew on the colony of the pathogen, while this phenomenon was accompanied with production of yellow pigment (more than normal condition) and mycoparasitism for a few isolates (Table 1). Hermosa et al. (2000) also placed a few plant pathogens fungi versus a few *Trichoderma* isolates on different culture media and indicated that the sporulation and overgrowing of *Trichoderma* on pathogenic fungi are related to fungal pathogenic kind and medium compound. Mycoparasitism of pathogenic fungi and production of hydrolytic enzymes such as chitinase and gluconase during mycoparasitism stages by *Trichoderma* species have been previously demonstrated (Harman et al., 2004; Howell, 2003). Early volatile and nonvolatile metabolites of eight *Trichoderma* isolates reduced the pathogen colony growth; however this reduction effect was more with nonvolatile than early volatile metabolites (Table 1). Previous studies have showed that most of *Trichoderma* strains produce volatile and nonvolatile metabolites that

Table 2. The effects of used treatments on disease severity of take-all and shoots and roots dry weight in greenhouse.

Treatments ¹	Disease severity (0 - 5)		Shoot dry weight (g)		Root dry weight (g)	
	Soil treatment	Seed coating	Soil treatment	Seed coating	Soil treatment	Seed coating
Healthy plant	-----	-----	18.4a	18.4a	5.37a	5.37a
Control	4.1a	4.17a	9.13fe	9.23fe	2.47c	2.37d
T90	3.17edf	2.93de	10.87de	10.43ed	2.07bc	2.97dbc
T65	2.07g	1.98g	14.53b	13.5b	3.9b	3.7b
T77	3.03ef	3.07dce	11.03de	10.83cd	2.97bc	2.87dbc
T96	2.17g	1.87g	13.47bc	14.03b	3.03bc	3.37bc
T122	3.5cedb	3.67b	10.2fde	9.9fed	2.57c	2.57dc
T146	3.3ced	3.3dbc	9.67fde	9.8fed	2.43c	2.4d
T149	4.0ab	3.77ab	9.0fe	8.83f	2.48c	2.4d
T150	3.95ab	3.6b	8.17f	9.03f	2.2c	2.2d
Isolates mixture	3.07ef	2.62fe	11.67dc	11.73c	3.2bc	3.5b
Trichodermin B	2.67f	2.2fg	11.8dc	11.93c	3.2bc	3.33bc
Subtilin	3.63cadb	3.37dcdb	9.17fe	9.37fe	2.5c	2.6dc

¹Significant differences are denoted by different letters within each column according to Duncan's Multiple Range test at P>0.05 and values are average of three replicates.

inhibit the growth of pathogenic fungi (Vey et al., 2001). These metabolites are composed of harzianic acid, alamthicins, tricholin, peptaboles, antibiotics, 6-phenethyl-alpha-pyrone, massoilactone, viridian, glioviridin, glisoprenins, heptelidic acid, and other suppressive compounds (Vey et al., 2001; Simon and Sivasithamparam, 1989; Kucuk and Kivance, 2004). Percentage of growth inhibitory effect of filtrated metabolites were reduced as the incubation period increased (Table 1), which shows that a little inhibitory effects is due to late volatile compounds present in the filtrated metabolites and can be the only agent of growth inhibitory for T149 isolate, because this isolate did not show any inhibitory effects on the second 24 h after inoculation (Table 1).

Treatments of T65, T96, T77, T90, T122, T149, mixture of eight isolates and Trichodermin B significantly decreased disease severity. Duffy et al. (1997) have previously reduced disease severity of take-all in a few soils with different physicochemical characteristics of *T. koningii* isolates that were isolated from suppressive soils of Western Australia. Results of this study are also confirmed by findings of Simon and Sivasithamparam (1989), Dewan and Sivasithamparam (1989), Tang et al. (1996) and Frootan Nadafy et al. (2002) that reported the decrease of disease severity of take-all by *Trichoderma* isolates under field and greenhouse conditions. The more biocontrol effectiveness of seed coating method than soil treatment method in this study is similar to Ashrafizadeh et al. (2005) results which showed that seed coating of melon seeds with *Trichoderma* inoculum decrease fusarium wilt more than soil application of the same.

In the both of applied methods in greenhouse, the more biocontrol effective isolates were T65 and T96 treatments and these two isolates rapidly overgrew the

pathogen colony, colonized the pathogen mycelium (hyperparasitism) and inhibited the pathogen colony growth by their nonvolatile metabolites in laboratory trials. T90 and T146 isolates moderately inhibited the pathogen growth in laboratory and decreased disease in greenhouse. Therefore, laboratory and greenhouse results of these four treatments support each other. Pérez de Algaba et al. (1992) also found significant relation between antifungal effects of a few *Trichoderma* isolates on *Phoma betae* in laboratory and bicontrol effects of the same isolates in field. The results of laboratory and greenhouse trials confirm that metabolites (antibiotics and hydrolytic enzymes) of *Trichoderma* are very important in the decrease of disease severity by the four T65, T96, T90, and T146 isolates. Chet et al. (1997) reported that a few of *Trichoderma* strains have extraordinary capacity for producing antibiotics. For example, *T. virens* strains, that produced highly amount of glioviridin antibiotics, protected cotton seedlings from seedling blight caused by *P. ultimum* (chet et al., 1997). The most effective *T. harzianum* isolates against take-all agent produced pyron antibiotic (Howell, 1998; Monte, 2001). T149 treatment did not significantly show biocontrol effect on take-all disease in greenhouse while this isolates moderately inhibited the colony growth of the pathogen in nonvolatile metabolite test and greatly reduced colony growth of pathogen in early volatile metabolite test. This contradiction may be interpreted in that the gaseous metabolites do not play very important function in the biocontrol effectiveness. Kucuk and Kivance (2003) also showed that the inhibitory effects of volatile metabolites on the pathogen growth are far less than the effect of nonvolatile metabolites *in vitro*. T77 treatment moderately decreased take-all disease in green-

house while this isolate rapidly colonized (overgrew) the pathogen colony in dual culture test and very weakly inhibited pathogen growth in the produced metabolites trials. Therefore, rapid colonization and rhizosphere competence may be the main function in its biocontrol effects in greenhouse. Rhizosphere competent strains of *Trichoderma* species completely colonize roots surface for a few weeks or months and protect it from invading pathogenic fungi (Thrane et al., 1997; Harman, 2000). However, laboratory and greenhouse results of this research relatively showed similarity and scant contradiction of *in vivo* and *in vitro* results, which are confirmed by Hermosa et al. (2000) findings that we can hardly generalize antifungal activities of a *Trichoderma* strains in laboratory to natural environments.

In future, it will be possible to select the confirmed *Trichoderma* isolates with high biocontrol capacity and the preparation of more effective formulations to combat the pathogens.

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