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Full Length Research Paper

Biological potantial of some Iranian *Trichoderma* isolates in the control of soil borne plant pathogenic fungi

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In this study the *in vitro* potential of six selected Iranian isolates of three species of *Trichoderma* (*Trichoderma hamatum* T614, *T. hamatum* T612, *Trichoderma harzianum* T447, *T. harzianum* T969, *Trichoderma virens* T523 and *Trichoderma* sp. T) were evaluated against five isolates of soil borne phytopathogenic fungi (*Fusarium graminearum*, *Rhizoctonia solani* (AG4 and AG5), *Macrophomina phaseoli* and *Phytophtora cacturum*) in dual culture techniques and through production of volatile and non-volatile inhibitors, and the pH and temperature effects on *Trichoderma* mycelial growth were also evaluated. All *Trichoderma* isolates had a marked statistical inhibitory effect on mycelial growth of the pathogens in dual culture compared with controls. Maximum inhibitions occurred in *F. graminearum*-*T. hamatum* T614 interaction. Significant pathogen colony growth inhibitions were observed when exposed to the trapped atmosphere from culture of the *Trichoderma*. *F. graminearum* was most susceptible to the volatile inhibitors produced by *T. hamatum* T612 (%inhibition = 48.65). Medium filtrate obtained the *Trichoderma* isolate culture also were effected on the pathogen species significantly. Maximum growth inhibition was observed in radial growth of *F. graminearum* by *T. hamatum* T612 non volatile metabolites (%inhibition = 38.3). Evaluation of pH and temperature effects on *Trichoderma* isolates mycelial growth showed that *Trichoderma* strains were found to be able to display activities under a wider range of pH values. Also, *Trichoderma* strains are mesophilic.

Key words: *Trichoderma* spp., biological potantial, soil borne phytopathogenic fungi, Iran.

INTRODUCTION

Soil borne plant pathogens such as bacteria, fungi and nematodes annually create a major economically losses in many important crops. Some chemical compounds have been successfully used to control soil borne plant pathogens. Although in many cases, these pesticides appear to be the most economical and efficient means of controlling plant pathogens. Toxicological environmental and sociological concerns have led to drastic reduction in the availability of efficient commercial compounds, and also the use of fungicides may lead to the appearance of new resistant strains of pathogens.

In the recent years, there has been a world wide 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 microorganisms has been considered a more natural and environmentally acceptable alternative to the existing chemical treatment methods (Barker and Panlitz, 1996; Eziashi et al., 2007). Weindling (1932) over 75 years ago, demonstrated the antagonistic nature of fungal species from the genus, *Trichoderma*. The genus, *Trichoderma* is common filamentous imperfect fungi (Deutromycetes, Dematiaceae), the most common saprophytic fungi in the rhizosphere and found in almost any soil. The mycoparasite ability

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of *Trichoderma* species against some economically important aerial and soil borne plant pathogens (Papavizas, 1985; Elad et al., 1993; Elad, 2000; Freeman et al., 2004, Dubey et al., 2007) and nematodes (Windham et al., 1989; Sharon et al., 2001) allows for the development of biocontrol strategies. Several *Trichoderma* species reduces the incidence of soil borne plant pathogenic fungi under natural conditions (Sivan and Chet, 1986; Calvet et al., 1990); however the efficacy of this depends largely on the physical, chemical and biological condition of soil. There have been numerous recent attempts to use *Trichoderma* spp. on soil borne pathogens such as sclerotinia, fusarium, pythium, and rhizoctonia species in word (Elad et al., 1980; Jager et al., 1991; Ashrafizadeh et al., 2005; Dubey et al., 2007). Among these, several species of *Trichoderma* are well documented mycoparasite and have been used successfully against certain pathogenic fungi (Papavizas and Lumsden, 1980). *T. harizanum*, *T. viridae*, *T. virens*, *T. hamatum, T. roseum* and *T. koningii* are the species that most often used biological control of pathogens. *Trichoderma* produced in late years, have been developed into several commercial biological control products to prevent development of several soil pathogenic fungi. The first biocontrol agent to be commercialized registered and used in green house crops and vineyards was isolate T39 of *T. harzianum* (Trichodex® by Machteshim, Israel) (Elad, 2000; Freeman et al., 2004). ECOFIT® (*T. viridae*) and TRI 002® (*T. harzianum*) are marketed in India and Europe, respectively, for control of various plant soil borne pathogens on field and green house crops and vegetables (Koch, 1999). SOILGARD® (*T. virens* GL21) registered as biopesticide in the USA (Lumsden et al., 1996). Also SUPERESIVIT® (*T. harzianum*) has been reported to control *Pythium ultimum* (Duscova, 1995). In this regard, the first requirement of biological control is the identification and deployment of highly effective strains control of several soil borne plant pathogenic fungi in field crops and green house system.

In this study, the *in vitro* biological potential of some Iranian *Trichoderma* isolates (belonging to three species; *T. harzianum*, *T. hamatum*, *T. virens*) were evaluated against some common soil borne plant pathogens belonging to different group of fungi (*F. graminearum*, *Rhizoctonia solani* AG4 and AG5, *Macrophomina phaseoli* and *Phytophtora cactorum*). Also, powerful and highly effective isolates were selected for further field biocontrol studies.

MATERIALS AND METHODS

Isolates

The *Trichoderma* isolates that were selected for this study were obtained from collection of *Trichoderma* spp., in the Plant Pest and Disease Institute, Tehran, Iran. These include *T. harzianum* T447, *T. harzianum* T969, *T. hamatum* T612, *T. hamatum* T614 and *T. virens* T523. Also, one isolate of *Trichoderma* isolated from Moghan area soil was evaluated. *F. graminearum*, *R. solani* AG4 and AG5, *M. phaseoli* and *P. cactorum* were isolated from infested wheat, sugar beet, potato, soyabean and apple rootstock, respectively. The isolates were maintained on potato dextrose agar (PDA) medium and stored at 4°C for further use.

Dual culture technique

The *Trichoderma* isolates were evaluated against last mentioned soil borne fungi by dual culture technique as described by Morton and Strouble (1955). A 5 mm diameter mycelial disc from the margin of the *Trichoderma* 7 days-old culture of isolates and the soil borne pathogens were placed on the opposite of the plate at equal distance from the periphery. The experimental design used was a completely randomized with four Petri dishes for each isolates. In control plates (without *Trichoderma*), a sterile agar disc was placed at opposite side of the soil borne inoculated isolates plates. Inoculated plates were incubated at 25 ± 1°C until the end of the incubation period (7 days after inoculation). Two, 4, 6 days after the incubation period, radial growth of pathogen isolates was measured and percent inhibition of average radial growth was calculated in relation to growth of the controls as follows:

$$L = [(C - T)/C] \times 100$$

Where L is inhibition of radial mycelial growth; C is radial growth measurement of the pathogen in control; T is radial growth of the pathogen in the presence of *Trichoderma* isolates (Edington et al., 1971).

Slide culture method

For each pathogen-*Trichoderma* interaction, a clean slide was placed in 9 cm diameter plates and sterilized. Then a small amount of autoclaved melted potato dextrose agar was spread over the slide to make a thin PDA film on the slide. The 5 mm discs of one week old growing colonies cut from the margin of each pathogen and *Trichoderma* isolates were placed on the opposite sides of the slide 3 cm apart on the PDA surface. Then a few ml of double distilled water was added to the plate to prevent drying and then incubated at 25 ± 1°C for a week. At the end of incubation period, meeting area of *Trichoderma*–Pathogen hyphae was observed under a light microscope for the presence of coiling structures for wall disintegration.

Effects of volatile metabolites

The effect of the *Trichoderma* isolates of released volatile metabolites was evaluated on the mycelial growth of the pathogen isolates by following the methods of Dennis and Webster (1971b). The 5 mm diameter mycelial disc of 7 days-old culture obtained from the margin of each the *Trichoderma* isolates was centrally placed on the PDA plates and incubated in 25 ± 1°C for 48 h. In control plates, a 5 mm diameter of sterile PDA medium was placed in the dish as done above. At the end of incubation period, the top of each plate replaced with bottom of the PDA plate inoculated centrally with 5 mm diameter mycelial plug of the pathogen isolates and held together with adhesive tape. The experimental design used was completely randomized with four replicates. Radial growths of the pathogens were recorded each day and percent inhibitions of average mycelial growth in relation to growth of their controls were calculated by the last mentioned formula.

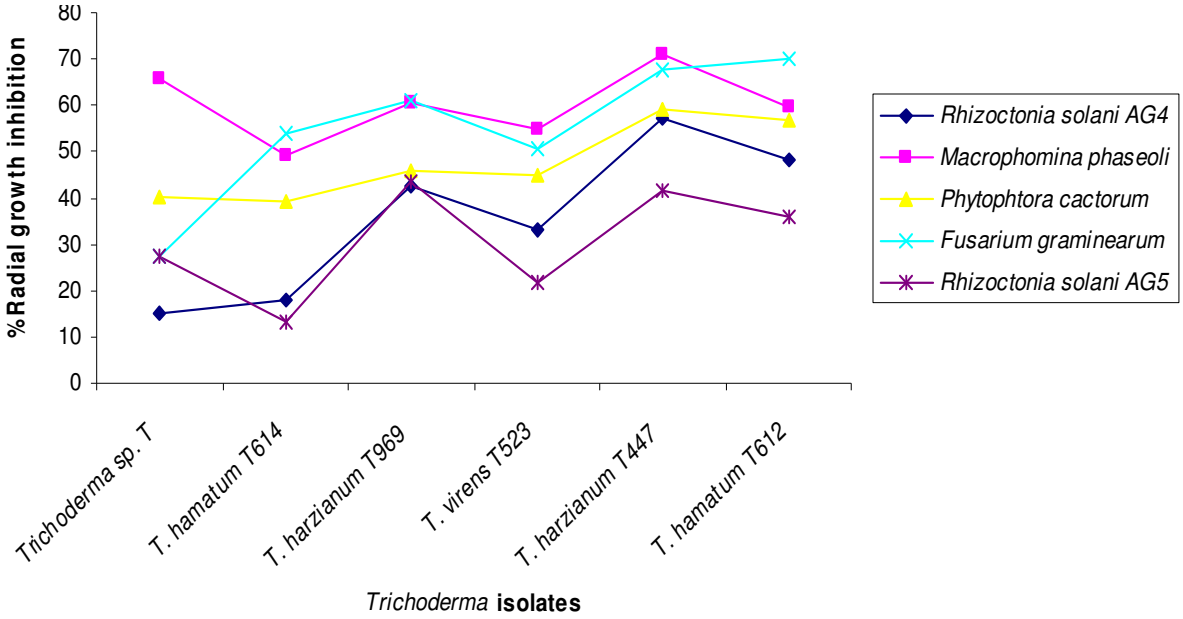

Figure 1. Pathogen growth inhibition by *Trichoderma* isolates after 6 days of inoculation in dual culture.

Effects of non volatile metabolites

To determine the effects of the Trichoderma isolate of non volatile metabolites on mycelia growth of the pathogens, three discs of mycelial agar plugs (5 mm diameter) were removed with a No. 3 cork borer from the edge of the young culture. *Trichoderma* isolates were inoculated in 100 ml sterilized potato dextrose broth (PDB) in 250 ml conical flasks and incubated at 25 ± 1°C on a rotary shaker set at 100 rpm for 14 days. The control conical flasks were inoculated with three 5 mm diameter of sterile PDA medium. The culture was filtered through Millipore filter for removing mycelial mats and then sterilized through 0.2 µm pore biological membrane filter (FP30/0.2 CA-S, Schleeicher and Schuell MicroScience GmbH).

The filtrate was added to molten PDA medium (at 40 ± 3°C) to obtain a final concentration of 10% (v/v). The medium was placed in Petri dishes at 20 ml per plate and inoculated with 5 mm mycelial plugs of the pathogens in the centre of the plates and incubated at 25 ± 1°C for 7 days or until the colony reached the plate edge (Dennis and Webster 1971c). There were 4 replicates for each treatment. Radial growths of the pathogens were recorded each day. Percent inhibition of average growth mycelial in relation to growth of the controls was calculated.

pH and temperature

To evaluate the influence of pH and temperature on the *Trichoderma* mycelial growth, a 5 mm diameter mycelial block cut from the margin of 7-day old of each *Trichoderma* isolates colonies by No. 3 cork borer was placed in PDA plates that adjusted pH to 5, 7, 8 with 0.1 N HCl and NaOH before autoclaving, and incubated at 20 ± 1°C, 25 ± 1°C and 30 ± 1°C The experiment was designed by completely randomized design (Factorial). The colony diameters of the *Trichoderma* were measured in four replicates each day after inoculation. The means were analyzed by analysis of variance (ANOVA) and Least Significant Difference (LSD) test at 5% significant level with SAS software (SAS (1985) Institute Inc., Cary, NC, USA).

RESULTS

In studying *Trichoderma* isolates and the pathogen species in dual culture, all of the *Trichoderma* isolates had a marked significant inhibitory effect on the growth of the pathogens compared with their control. Maximum pathogens growth inhibitions occurred in interacting with *T. harzianum* T447. 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. In dual cultures of *T. virens* T523 and *T. harzianum* T969 with all of the pathogens, an inhibition zone without physical contact between the colonies around the pathogen colony was observed. And also, this inhibition zone without hyphae contact was observed in *R. solani* AG4 and AG5-*T. harzianum* T447 and *P. cactorum*-*Trichoderma* sp. T interaction. No apparent inhibition zone in other pathogen-*Trichoderma* interaction was observed macroscopically. Microscopically observation of the interaction zone showed vacuolization and mycelial tip denaturizing of the *F. graminearum*-*Trichoderma virens* T523 hyphae followed by disintegration. No hyphae coiling was observed in Pathogen-*Trichoderma* interactions. *F. graminearum* was most inhibited by *T. hamatum* T612 and *R. solani* AG4 was least inhibited by *T. hamatum* T614 (Figure 1).

The significantly colony growth inhibition of the pathogens was observed when exposed to the trapped atmosphere from cultures of the *Trichoderma* isolates in comparism with their control. *F. graminearum* was found to be most susceptible to the volatile inhibitors produced by *Trichoderma hamatum* T612. The minimum inhibition percentage was recorded in *M. phaseoli*-*Trichoderma*

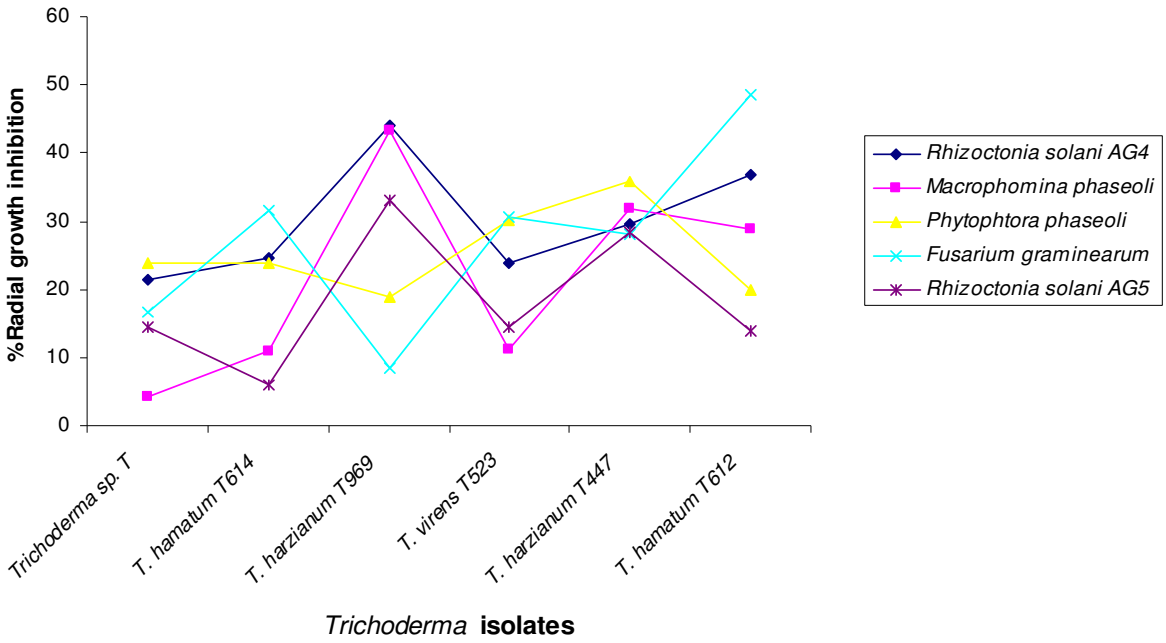

Figure 2. Pathogen growth inhibition by *Trichoderma* volatile compounds.

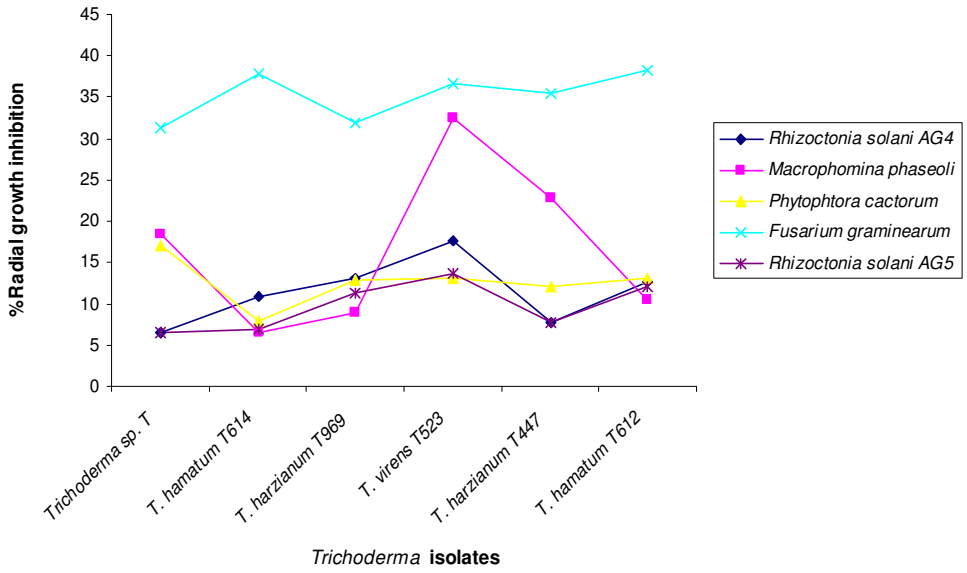

Figure 3. Pathogen growth inhibition by *Trichoderma* non-volatile compounds.

hamatum T614 interaction (Figure 2).

Maximum growth inhibitions of *F. graminearum* radial growth were observed by *T. hamatum* T612 non-volatile metabolites. Among *M. phaseoli* and *R. solani*, AG4 showed minimum growth inhibition by non-volatile inhibitors of *T. hamatum* T614 and *Trichoderma* sp. T, respectively. In addition, *F. graminearum* was more susceptible to the inhibitory medium filtrate of all the tested *Trichoderma* isolates (Figure 3).

Trichoderma isolates mycelial growth was statistically differed in respect of the tested temperatures and pH. 25°C supported the highest mycelial growth of *T. hamatum* T612, *T. harzianum* T447, *T. harzianum* T969 and *T. hamatum* T614. The mycelial growth of *T. virens* T523 and *Trichoderma* sp. T was highest at 30°C. Mycelial growth of *T. hamatum* T612, *T. harzianum* T447 and *T. virens* T523 was highest at pH 5 and mycelial growth of *T. harzianum* T969 and *Trichoderma* sp. T was

Table 1. Effects of pH and temperature on the mycelial growth of *Trichoderma* isolates.

Treatment	*T. hamatum* T612	*T. harzianum* T447	*T. virens* T523	*T. harzianum* T969	*Trichoderma* sp. *T*	*T. hamatum* T614
pH 8	33.26*	23.89	41.15	31.07	32.19	33.78
pH 7	29.3	24.3	33.96	39.7	39.45	30.85
pH 5	33.78	34.8	45.52	34.7	32.22	27.68
30°C	29.15	17.2	43.67	35.41	36.89	31.37
25°C	36.04	38.89	37.56	36.44	35.3	30.96
20°C	31.14	26.89	39.41	33.63	31.67	29.96

*Values are means of four replicates.

highest at pH 7. While *T. hamatum* T614 has the highest mycelial growth at pH 8 (Table 1).

DISSCUSION

Plant pathogenic fungi and nematodes is a widespread problem and the use of chemicals is hardly successful. However, the high cost associated with the use of fungicides to control disease caused by soil borne fungi is a limiting factor in the profitability of crop production. According to this study, biological control could be the best alternative and may be helpful, especially against soil borne pathogens and nematodes. This is an integral part of the integrated pest management philosophy, which entails the judicious use of biocontrol agents and reduced amounts of biocides/fungicides or other physical aspects (such as soil solarization).

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 soil borne plant pathogens. Evaluation of produced volatile and non-volatile components also showed the acceptable performance on inhibiting mycelial growth of pathogens. The results reported here suggest that from the six isolates of *Trichoderma* used in this study, *T. harzianum* T447, *T. hamatum* T612 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. However, *T. hamatum* T612 is also a potential bioagent for *F. graminearum* and a good candidate for further study on *F. graminearum* biocontrol in field condition.

pH and temperature are two keys parameter to manipulate for 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 present study also support these hypotheses.

The presence of an inhibition zone in dual culture without the hyphae contact in treatments *T. virens* T523 and *T. harzianum* T969 suggests the secretion of diffusible non-volatile inhibitory substance by the *Trichoderma* isolates. The results of non-volatile substance against the pathogens that were more effective support this hypothesis. However, it seems that 1:10 dilutions of cultural filtrate used in this study were not in sufficiently high concentrations to effect significant inhibitory response. 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 β-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 than 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 various strategies for fungal antagonism and indirect effects on plant health also vary. Therefore, one of the most interesting aspects

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, water potential and such like) and biotic (plant species and variety, 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.

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