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Evaluation of some *Trichoderma* isolates for biological control of potato wilt disease (*Fusarium oxysporum*) under laboratory and greenhouse conditions

Farkhondeh Ommati and Masoud Zaker*

Plant Protection Department, Shahrood Agricultural Research Center, P. O. Box: No. 36155-313, Shahrood, Iran.

Abstract: Biological efficacy of *Trichoderma* species may differ due to variations in ecosystems. This study was conducted to assess the biocontrol efficacy of some native *Trichoderma* isolates against *Fusarium oxysporum*, an important causal agent of potato wilt disease under laboratory and greenhouse conditions in Shahrood Agricultural Research Center, Shahrood, Iran during 2006-2007. Fourteen isolates were collected among which eight showed promising ability in inhibiting growth of the pathogen through dual culture and production of volatile and non-volatile metabolites but *T. asperellum* (T2) and *T. atroviride* (T3) were almost more efficient than other isolates in inhibiting the mycelial growth of the pathogen in comparison to control ($P \leq 0.01$). Eight isolates were evaluated against the disease under green house condition. Potted plants treated with *Trichoderma* isolates + *F. oxysporum* showed lower disease incidence in comparison to Fusarium infested control ($P \leq 0.05$). Best disease control was observed in potted plants treated with *F. oxysporum* + *T. asperellum* (T2) showing 2.5% disease incidence in contrast to Fusarium infested control, in which disease incidence was 73%.

Keywords: Biocontrol, *Trichoderma*, Efficacy, Potato**,** Fusarium wilt.

Introduction

Application of biological methods in plant disease control are of unique importance because of reducing environmental pollutions. The potential of *Trichoderma* species as biocontrol agents in plant disease control was first recognized in the early 1930s (Weindling, 1932) and subsequently they were applied successfully as biocontrol agents against several plant diseases in commercial agriculture (Howell, 2003). Several superior strains have been identified and formulated into commercial biopesticides (Agrios, 1997). Control may be achieved by competition, production of antibiotics or by mycoparasitism (Campbell, 1989). Otadoh *et al*., (2011) evaluated the antagonistic ability of *Trichoderma asperellum, T. atroviride, T. koningii, T. harzianum* and *T. reesi* against *Fusarium oxysporum* f. sp. *phaseoli* under lab. and green house conditions and found *T. reesei and T. koningii* as the most effective isolates against the pathogen and for stimulation of plant growth. Akrami *et al*., (2011) evaluated *T. harzianum* (T1), *T. asperellum* (T2), *T. virens* (T3) against *F. oxysporum* of lentil and found that all of them could effectively inhibit growth of the fungus in laboratory tests. They tested the three isolates alone and in combination in green house and observed that disease severity was reduced from 20 to 44% while dry weight increased from 23 to 52% when T1 and T2 were

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* **Corresponding author**, e-mail: mzakerus@yahoo.com
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combined. In another study Boureghda and Bouznad (2009) evaluated biological efficiency of several isolates of *Trichoderma atroviride, T. harzianum* and *T. longibrachiatum* against *Fusarium* wilt of chickpea under lab. and green house conditions and reported 65.64% and 48.71% reduction in colony diameter of the pathogen by *T. atroviride* isolate (Ta. 13) through dual culture and production of volatile inhibitors respectively and a complete inhibition by the same isolate through production of non-volatile inhibitors. In green house, they observed the lowest disease severity with the same isolate which led to 83.92% disease reduction compared to the control. The most effective isolates in protecting chickpea seedlings from the disease were three strains of *T. atroviride* (Ta. 3, Ta. 7 and Ta. 13) as well as *T. harzianum* (Th. 16) and reduction in disease severity was associated with an increase in vegetative growth including the stem height as well as the plant fresh and dry weights. Ozbay *et al*., (2004) tested the commercial and noncommercial strains of *T. harzianum* against *F. oxysporum* f. sp. *radicis-lycopersici* of tomato plants grown in two different hydroponic media (coir and rockwool) and found that *T. harzianum* strains, especially applied at transplanting, decreased disease incidence by 79% for coir and 73% for rockwool and increased fruit yield 37% for coir and 25% for rockwool. Tsai *et al*., (2008) studied the biological control of Fusarium stem rot of *Anoectochilus formosanus* caused by *F. oxysporum* using five antagonistic *Trichoderma* strains isolated from *Anoectochilus* rhizospheres for their growth promoting effectiveness in a gnotobiotic culture system and reported that among the five tested isolates, *T. asperellum* TA strain was the best for promoting the growth of *Anoectochilus* plants and reducting the disease in greenhouse.They suggested that *T. asperellum* TA strain has excellent potential to be used as biocontrol agent for the control of stem rot disease of Taiwan *Anoectochilus*. Hajieghrari *et al.,* (2008) evaluated the antagonistic potential of some selected Iranian isolates of *Trichoderma*, (*T. hamatum* T614, *T. hamatum* T612, *T. harzianum* T447, *T. harzianum* T969 and *T. virens* T523) against four isolates of soil borne pathogenic fungi including *Fusarium graminearum* through dual culture method and production of volatile inhibitors and found maximum inhibitions in *F. graminearum-T. hamatum* (T614) interaction and that *F. graminearum* was most susceptible to the volatile inhibitors produced by *T. hamatum* (T612). Soltani *et al*., (2005) used *T. harzianum* againt some potato root and tuber diseases including Fusarium wilt (*F. oxysporum*) under green house and field conditions and reported that better control of the disease was observed under green house condition, while an increase in yield was noticed under field condition. Ashrafizadeh *et al*., (2002) studied the potential of *Trichoderma* species in controlling Fusarium wilt of melon and found *T. virens* (DAR-7429) as most effective for its control.

Materials and methods

Pathogen isolation

Potato plants showing wilt symptoms were collected from different fields at flowering stage. Small pieces of diseased specimen were grown on Fusarium selective medium (Nash and Snyder, 1965). After purifications, isolates were identified according to morphological characteristics with the help of standard key (Nelson *et al.*, 1983). Pathogenicity tests were conducted on potted plants and after reisolation, a pathogenic isolate of *Fusarium oxysporum* was selected for further studies.

Sources of *Trichoderma* isolates

Isolation of *Trichoderma* spp. from soil was done following the technique used by Rifai (1969). For this purpose soil samples were collected from potato root rhizosphere (20 cm. deep) of different fields. Twenty g of each soil sample were gently mixed with 500 ml distilled water containing 0.2% citric acid, five ml of prepared solution were added to Petri plates containing 15 ml water agar at 50

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ºC and shaken to mix properly. After solidification, five mm plugs of these cultures were transfered into Petri plates containing Davet selective medium (Davet, 1979) and were incubated at 25 ºC. After proper growth, isolates were purified and identified according to standard keys (Rifai, 1969; Bissett, 1991a; Bissett, 1991b).

Three *Trichoderma* isolates performing good antagonistic ability against *Fusarium* wilts were also obtained from Abu – Ali – Sina University, west Iran (Table 1).

Table 1 Source of *Trichoderma* isolates.

Isolate	Source
T. atroviride (T3)	Abu-Ali-Sina University, West Iran
T. asperellum (T7)	Abu-Ali-Sina University, West Iran
T. longibrachiatum (T5)	Abu-Ali-Sina University, West Iran
T. harzianum (T13)	Potato fields, Shahrood, North Iran
T. brevicompactum (T6)	Potato fields, Shahrood, North Iran
T. asperellum (T2)	Potato fields, Shahrood, North Iran
T. brevicompactum (T10)	Potato fields, Shahrood, North Iran
T. brevicompactum (T1)	Potato fields, Shahrood, North Iran

Inhibitory mechanisms of *Trichoderma* isolates against *F. oxysporum* mycelial growth

Macroscopic and microscopic observations in dual culture method

Five mm plugs of seven-day-old cultures of *F. oxysporum* and *Trichoderma* were placed against each other on plates containing PDA. In case of control instead of *Trichoderma* plugs, PDA plugs were used. Plates were incubated at 25 ºC and checked daily for their reactions such as growth speed and sporulation (Skidmore and Dickinson, 1976). Radial growth of the pathogen was measured daily and data were obtained.

For microscopic observation of *Trichoderma* reaction such as penetration and coiling on mycelia of the pathogen, PDA was poured into plates and at about 50 ºC, sterilized microscopic slides were placed in the middle of plates in such a way that a tiny layer of the medium covered the slides. Plugs of both fungi were then placed inside plates according to above mentioned method and incubated. After proper mycelial growth of both fungi over microscopic slides, these slides were removed and observed under microscope for their mode of interactions (Burgess and Hepworth, 1996).

Effect of non-volatile inhibitors of *Trichoderma* isolates against *F. oxysporum* colony growth

Cellophane layers (9 cm dia.) were kept in between layers of filter papers and sterilized. Sterilized cellophane layers were placed on PDA plates. Five mm plugs of seven-day-old culture of *Trichoderma* were placed on the cellophane layers. For control only PDA plugs were used. After 24 and 48 hours cellophane layers and *Trichoderma* plugs were replaced with 5 mm plugs of *F. oxysporum* and were daily inspected. After 5 days radial growth of *F. oxysporum* from different treatments was measured (Dennis and Webster, 1971a).

Effect of volatile inhibitors of *Trichoderma* isolates on *Fusarium oxysporum* colony growth

Five mm plugs of seven-day-old culture of *F. oxysporum* and *Trichoderma* isolates were placed in middle of plates containing PDA separately. The plates containing *F. oxysporum* were inverted over plates containing *Trichoderma* isolates. The two plates were sealed together with parafilm under sterilized condition and were incubated at 26 ºC (Dennis and Webster, 1971b). The radial growths of *F. oxysporum* was measured after 24, 48, 72, 96 and 120 h.

Data of laboratory tests were calculated by the following formula: % of inhibition = Dia. of colony growth in control - Dia. of colony growth in treatment × 100

Dia. of colony growth in control

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Evaluation of *Trichoderma* isolates against Fusarium wilt disease in greenhouse

Preparation of inocula of *F. oxysporum* and *Trichoderma* isolates

In order to prepare *F. oxysporum* inocula, Erlenmeyer flasks containing 100 g of wheat (Var. Durum) and 100 ml of sterilized water were autoclaved at 121 ºC for 1 h on three successive days. After cooling, about 5-7 small plugs of seven- day- old culture of *F. oxysporum* were dropped into each Erlenmeyer under sterilized condition. The flasks were kept at 25 ºC for 4 weeks. Colonized wheat grains were then transferred into paper pockets, and were dried and ground. Fourteen g of prepared powder was used to infest 1 Kg of soil (Frommel *et al.,* 1991). For preparation of *Trichoderma* inocula moistened wheat bran was poured into Erlenmeyer flasks which were autoclaved at 121 ºC for 1 h on three successive days. The substrate mixture was then inoculated with a homogenized suspension of spore + mycelia of seven days old culture of *Trichoderma* isolates under aseptic condition. Erlenmeyer flasks were incubated at 27 ºC for 14 days under fluorescent lamp and 70% relative humidity. Ten g of this inoculum (10^5 – 10^7 CFU) was used to add to 1 Kg of pot soil. A mixture of 1: 1 clay soil and peat moss were autoclaved at 121 ºC for 1 h on three successive days for filling pots. Surface sterilized potato mini tubers (Var. Agria) were grown in pots. All of the *Trichoderma* isolates performing well in lab. tests, were used in this experiment. Disease incidence was measured every week according to disease assessment model used by Chandra *et al*., (1983) (Table 2) and data of green house evaluations were calculated by the following formula: percentage of plants infected =

$$\frac{\text{No. of healthy plants in healthy control} - \text{No. of healthy plants in treatment} \times 100}{\text{No. of healthy plants in healthy control}}$$

Table 2 Disease assessment model (Chandra *et al*., 1983).

Symptom	% of disease incidence	Scale
Healthy plants	0	0
Yellowing of older leaves	10	0
Yellowing and wilting of older leaves	25	2
Wilting of two or some branches	50	3
Wilting of all branches except apical shoot	75	4
Wilting and dying of whole plant	100	5

Statistical method

Completely randomized design with four replications was used in all experiments. Data were analyzed in MSTAT-C and means were compared according to Duncan's multiple range test.

Results

In the present study out of 14 collected isolates, 8 showed promising ability against *F. oxysporum* under laboratory and green house conditions (Table 1). In dual culture method *Trichoderma* isolates demonstrated different degrees of inhibition against *F. oxysporum* growth and showed significant differences in comparison to control. Microscopic studies revealed hyphal coiling, hyperparasitism, of isolates T3 and T2 around *F. oxysporum* hyphae. These two isolates showed highest inhibitory effect against *F. oxysporum* (table 3). In macroscopic study growth of *Trichoderma* isolates toward mycelia of *F. oxysporum* was observed due to mycoparasitism.

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Table 3 Inhibitory effect of *Trichoderma* isolates on growth of *Fusarium oxysporum* in comparison to control after 5 days.

Isolates	% growth inhibition		
	dual culture	non-volatile inhibitors	volatile inhibitors
T. atroviride (T3)	84.33 a	42.33 ab	35.33 ab
T. asperellum (T2)	83.33 a	59.33 a	44.33 a
T. harzianum (T13)	81.00 ab	25.33 bc	25.33 b
T. longibrachiatum (T5)	79.33 ab	40.33 ab	32.33 ab
T. brevicompactum (T6)	78.67 abc	38.33 ab	32.33 ab
T. asperellum (T7)	73.67 bc	25.97 bc	25.97 b
T. brevicompactum (T10)	70.00 cd	55.67 a	23.67 b
T. brevicompactum (T1)	62.00 d	28.66 ab	22.67 b

Numbers in a column followed by the same letter(s) are not significantly different ($P \leq 0.01$) according to Duncan's multiple rang test.

The non-volatile metabolites of our tested isolates presented various degrees of inhibition against growth of the pathogen among which T2 followed by T10 with 59.33 and 55.67% growth inhibition showed the best ability. In this test T3 stood in the third place (Table 3).

Volatile inhibitors of all isolates also inhibited mycelial growth of *F. oxysporum* in comparison to control and as it is shown in Table 3, there were significant differences between the isolates and control. In this test T2 and T3 showed highest inhibitory effects against the pathogen respectively.

Under green house condition, reduction of potato wilt disease was observed by all tested isolates in comparison to infested control two months after simultaneous inoculations with pathogen and the antagonist. Best disease control was achieved in treatment *F. oxysporum* + *T. asperellum* (T2), demonstrating only 2.5% of disease incidence (Table 4). As it is obvious from laboratory and green house evaluations *T. asperellum* (T2) and *T. atroviride* (T3) were quite competitive against the pathogen and can be focused on for field evaluations.

Table 4 Effect of different treatments on *Fusarium oxysporum* under green house condition (after two months).

Treatment	% of Disease incidence
Infested control	73.00 a
F. oxysporum + *T. brevicompactum* (T6)	68.75 a
F. oxysporum + *T. asperellum* (T7)	62.50 ab
F. oxysporum + *T. longibrachiatum* (T5)	52.00 ab
F. oxysporum + *T. brevicompactum* (T10)	52.00 ab
F. oxysporum + *T. harzianum* (T13)	50.00 ab
F. oxysporum + *T. brevicompactum* (T1)	40.00 ab
F. oxysporum + *T. atroviride* (T3)	30.00 ab
F. oxysporum + *T. asperellum* (T2)	2.50 c
Healthy control	0.00 c
T. brevicompactum (T1)	0.00 c
T. asperellum (T2)	0.00 c
T. atroviride (T3)	0.00 c
T. longibrachiatum (T5)	0.00 c
T. brevicompactum (T6)	0.00 c
T. asperellum (T7)	0.00 c
T. brevicompactum (T10)	0.00 c
T. harzianum (T13)	0.00 c

Numbers in a column followed by the same letter(s) are not significantly different ($P \leq 0.05$) according to Duncan's multiple rang test.

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Discussions

Several *Trichoderma* spp. could be effectively used in biocontrol of soil borne plant pathogens and identifying efficient species adapted to different agroecosystems seem to be useful for their further evaluation. Several reports have indicated that biocontrol efficiency of *Trichoderma* spp. against Fusarium wilts may differ in different regions of the world ie, a highly antagonistic species against a particular pathogen in a given region may react poorly against the same pathogen in another region (Otadoh *et al*., 2011; Hajieghrari *et al.,* 2008; Ashrafizadeh *et al*., 2002) which could be due to differences in various agroclimatic conditions. Our results confirm the usefulness of evaluating different isolates of this antagonistic genus for their mycoparasitic properties which could lead to preparation of biopesticides. Isolates of some species namely *T. harzianum, T. viride* and *T. virens* (Syn. *Gliocladium virens*) are well known and their formulations are used by many growers but there might be more effective species of *Trichoderma*, therefore evaluating strains of other species of this environmental friendly genus would be beneficial. The dual culture findings of Akrami *et al*., (2011); Boureghda and Bouznad (2009) and Tsai *et al*., (2008) are in agreement with our results. They found *T. asperellum* and *T. atroviride* as the best inhibitors against *F. oxysporum*, while Otadoh *et al*., (2011); Ozbay *et al.,* (2004); Hajieghrari *et al.,* (2008) and Ashrafizadeh *et al*., (2002) in their screening experiments mentioned other species of *Trichoderma* as most efficient isolates against this particular pathogen. Regarding inhibitory effect of non-volatile inhibitors of tested isolates, Boureghda and Bouznad (2009) reported complete inhibition of *Fusarium* of chickpea with culture filterate of *T. atroviride* isolate (Ta. 13) which proves high antagonistic ability of this species. In our results although performance of *T. atroviride* (T3) was acceptable yet was not so good as *T. asperellum* (T2). Akrami *et al*., (2011) claimed that the volatile inhibitors of *T. asperellum* were as effective as those of *T. harzianum* against *F. oxysporum* of lentil and Boureghda and Bouznad (2009) reported 48.71% growth inhibition of *Fusarium* wilt of chickpea by volatile inhibitors of *T. atroviride* which match with our results. In this relation Otadoh *et al*., (2011) reported that volatile inhibitors of *T. reesei and T. koningii* were most effective inhibitors against *F. oxysporum* f. sp. *Phaseoli* which is in contrast with our results. Zepa *et al*., (1991) reported that the quality and quantity of volatile antibiotic compounds produced by *Trichoderma* spp. greatly depend on its isolate.

Our results of greenhouse experiment are in agreement with some reports and in contrast with some others. Akrami *et al*., (2011) reported that 44% disease reduction was observed when a combination of *T. harzianum* and *T. asperellum* were used against the pathogen while Boureghda and Bouznad (2009) observed that *T. atroviride* isolate (Ta. 13), with 83.92% disease reduction was the most effective species under green house condition. Also Tsai *et al*., (2008) reported that among five tested isolates, *T. asperellum* TA strain was the best for promoting the growth of *Anoectochilus* plants and reduction of the disease, but others such as Otadoh *et al*., (2011); Ozbay *et al*., (2004); Soltani *et al*., (2005) and Ashrafizadeh *et al*., (2002) mentioned other *Trichoderma* species as effective antagonists against Fusarium wilt of several crops under green house condition. In our evaluations, out of eight isolates belonging to five Trichoderma species, two isolates: *T. asperellum* (T2) and *T. atroviride* (T3) showed proper performance against potato wilt pathogen under laboratory and green house conditions, although these isolates need to be evaluated under field condition they, however, seem to be promising isolates for further studies.

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ارزیابی چند جدایه تریکودرما در کنترل بیولوژیکی پژمردگی فوزاریومی سیب‌زمینی (*Fusarium oxysporum*) در شرایط آزمایشگاه و گلخانه

فرخنده امتی و مسعود ذاکر*

محققان بخش تحقیقات گیاه‌پزشکی، مرکز تحقیقات کشاورزی شاهرود، صندوق پستی ۳۱۳ – ۳۶۱۵۵، شاهرود.
* پست الکترونیکی نویسنده مسئول مکاتبه: mzakerus@yahoo.com
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چکیده: بسته به شرایط مختلف آب و هوایی موجود در اکوسیستم‌ها ممکن است در قدرت آنتاگونیستی گونه‌های تریکودرما تفاوت‌هایی وجود داشته باشد. این تحقیق به‌منظور ارزیابی توانایی آنتاگونیستی چند جدایه تریکودرما در کنترل *Fusarium oxysporum* یکی از عوامل پژمردگی فوزاریومی سیب‌زمینی در طی سال‌های ۸۶-۸۵ در مرکز تحقیقات کشاورزی شاهرود در شرایط آزمایشگاه و گلخانه انجام شد. از میان ۱۴ جدایه جمع‌آوری شده، تعداد ۸ جدایه قابلیت خوبی در جلوگیری از رشد کلنی عامل بیماری در آزمایشات کشت متقابل و تولید متابولیت‌های فرار و غیرفرار از خود نشان دادند لکن جدایه‌های *T. asperellum* (T2) و *T. atroviride* (T3) در بین جدایه‌های مؤثر بهترین کارآیی را از خود نشان دادند ($P \leq 0.01$). این ۸ جدایه جهت ارزیابی میزان کارایی آنها درکنترل بیماری پژمردگی فوزاریومی سیب‌زمینی در شرایط گلخانه مورد بررسی قرار گرفتند. نتیجه مطالعات نشانگر کاهش میزان بیماری در بوته‌های گلدان‌هایی بود که بعد از آلوده‌سازی مصنوعی بوته‌ها با عامل بیماری، توسط گونه‌های تریکودرما تیمار شده بودند ($P \leq 0.05$) ولی از بین آنها تیمار *F. oxysporum* + *T. asperellum* (T2) با میزان ۲/۵٪ وقوع بیماری در مقایسه با شاهد آلوده که شدت بیماری آن ۷۳٪ برآورد گردید بهترین عملکرد را از خود نشان داد.

واژگان کلیدی: بیوکنترل، توانایی، تریکودرما، پژمردگی فوزاریومی، سیب‌زمینی

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