DOI: 10.1515/cerce-2016-0008
Available online: www.uaiasi.ro/CERCET_AGROMOLD/
Print ISSN 0379-5837; Electronic ISSN 2067-1865

Cercetări Agronomice în Moldova
Vol. XLIX , No. 1 (165) / 2016: 91-98

BIOCONTROL OF TOMATO *FUSARIUM* WILT BY *TRICHODERMA* SPECIES UNDER *IN VITRO* AND *IN VIVO* CONDITIONS

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Received August 27, 2015. Accepted: January 31, 2016. Published online: April 08, 2016

ABSTRACT. *Trichoderma* spp. have long been used as biological control agents against plant fungal diseases, but the mechanisms by which the fungi confer protection are not well understood. Our goal in this study was to isolate species of *Trichoderma*, that exhibit high levels of biocontrol efficacy from natural environments and to investigate the mechanisms by which these strains confer plant protection. In this study, efficacy of the native isolates of *Trichoderma* species to promote the growth and yield parameters of tomato and to manage *Fusarium* wilt disease under *in vitro* and *in vivo* conditions were investigated. The dominant pathogen, which causes *Fusarium* wilt of tomato, was isolated and identified as *Fusarium oxysporum* f. sp. *lycopersici* (FOL). Twenty eight native *Trichoderma* antagonists were isolated from healthy tomato rhizosphere soil in different geographical regions of Mazandaran province, Iran. Under *in vitro* conditions, the results revealed that *Trichoderma harzianum*, isolate N-8, was found to inhibit effectively the radial mycelial growth of the pathogen (by 68.22%). Under greenhouse conditions, the application of *T. harzianum* (N-8) exhibited the least disease incidence (by 14.75%). Also, tomato plants treated with *T. harzianum* (N-8) isolate showed a significant stimulatory effect on plant height (by 70.13 cm) and the dry weight (by 265.42 g) of tomato plants, in comparison to untreated control (54.6 cm and 195.5 g). Therefore, the antagonist *T. harzianum* (N-8) is chosen to be the most promising bio-control agent for *F. oxysporum* f. sp. *lycopersici*. On the base of present study, the biocontrol agents of plant diseases might be exploited for sustainable disease management programs to save environmental risk.

Key words: Biological control; Fungi antagonist; *Fusarium* wilt; *Lycopersicon esculentum*.

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INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is an important vegetable crop grown in almost all parts of Iran. Its popularity is due to its high nutritive value. The product is quite rich in many important minerals (especially, phosphorus and potassium) and vitamins (B and C). This crop is also very important in diet against common cancers like breast and prostate cancer. Notwithstanding the importance of tomato and its economic value for farmers, soil-borne pathogens inflict a lot of diseases and economic yield loss (Babalola and Glick, 2012). Such diseases include bacterial wilt, root knot nematodes disease, early blight, late blight and *Fusarium* wilt.

Among pathogenic fungi, the *Fusarium* remain to be a challenging task in terms of management (Agrios, 2005; Srinon, 2006). Tomato wilt caused by *F. oxysporum* f. sp. *lycopersici* (Sacc.) Snyder and Hansen is one of the most economically important pathogen world-wide (Srinon *et al.,*2006; Cal *et al.,* 2004). As *Fusarium* wilt is soil-borne in nature, application of fungicides to control this disease is not practical. Besides, chemicals cause serious health hazards to an applicator as well as to a consumer of the treated material. In addition to target organism, pesticides also kill various beneficial organisms. Their toxic forms persist in soil and contaminate the whole environment (Hayes and Laws,1991). Prospects of biological control of soil-borne plant pathogens using the genus *Trichoderma*, as one of the promising bio-control agent, has been described (Morsy *et al.,*2009; Sabalpara *et al.,* 2009). Successful control of *Fusarium* wilt in many crops by application of different species of *Trichoderma* has been reported (Bell *et al.,* 1982; Elad and Kapat, 1999; Ramezani, 2009). However, all isolates of *Trichoderma* spp. are not equally effective in control of the pathogen *in vitro* (Biswas and Das,1999; Ramezani, 2008) and in *vivo* conditions. Therefore, the objective of the present study was to assess the ability of different isolates of *Trichoderma* species in suppressing the populations of FOL in tomato under *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Isolation and purification of pathogens and antagonists

The used strains were isolated from the infected vascular tissues of stem and root of tomato, which collected from different fields of Mazandaran (Iran), including Pahnab and Larim (Jouibar), Baiekola and Nozarabad (Neka), Dasht-e Naz, Farahabad (Sari), during 2011-2012 cropping season. Tissues were sterilized with 10% sodium hypochlorite, for 5-10 min, and subsequently three passages of sterile distilled water. Then, they were placed on potato dextrose agar (PDA) medium separately and incubated at the laboratory conditions at 25 ± 3^{o}C, for five days. The fungi were purified by transferring the hyphal tip into PDA slants and maintained as stock cultures for further studies.

For isolation of the *Trichoderma* strains, soil samples of the rhizophere area were dried by keeping them at room temperature, for eight days. Then, dried samples were serial diluted in sterile distilled water (Wijesundera *et al.,* 1991). After dilution, 100 µL aliquots of 10^{-4} to 10^{-6} dilutions were separately plated out on selective media of McFadden and Sutton (McFadden and Sutton, 1975).

Identification of the antagonists strains were achieved by morphological characterization of the colonies, measurement of hyphal diameter and conidiophores and conidia dimensions (Rifaee, 1969; Bisett, 1991).

Totally, seven *F. oxysporum* and 28 *Trichoderma* strains were isolated from the collected tomato plants and soil samples. Pathogenicity of the *F. oxysporum* isolates were proved on PKM1 tomato cultivar, and then the most virulent of *F. oxysporum* (L-8) was selected for further studies.

In vitro effect of *Trichoderma* antagonists against FOL pathogen

Dual culture was used to determine the effect of *Trichoderma* isolates on pathogen (Dennis and Webster, 1971). All antagonist pathogen combinations were examined on 15 mL of PDA in 90 mm Petri dish. Nine millimeter disc of fifteen old days fungal cultures were placed on PDA medium, one centimeter away from the edge of plate. *Trichoderma* spp. (9 mm disc) was placed at opposite side of the Petri plate.

The cellophane overlay technique was also used in antagonistic test. Nine cm diameter cellophane membranes (Australia Cellophane, Victoria) were boiled in distilled water, interleaved between filter papers and autoclaved before place on the agar medium. For control, a plug sterile PDA medium was used instead of antagonist. After 48 h, the cellophane membrane and adhering antagonist mycelia or agar plug were removed (Etebarian *et al.,* 2000).

For fungicide or fungistatic activity test of *Trichoderma* strains, a 5 mm diameter of not-grown plug inoculated with *F. oxysporum* mycelial, was transferred to PDA. Randomized completed design with three replications was used at this study and samples incubated at 25±3°C. Percent inhibition over control was calculated (Vincent, 1927) as the formula:

$$\% \text{ GI} = \frac{a - b}{a} \times 100 ,$$

where GI is the percent inhibition over control, a is the growth of tested pathogen in absence of antagonist (mm) and b is the growth of tested pathogen against antagonist (mm).

F. oxysporum L-6 (from Larim) with the most virulence in pathogenicity test, *T. harzianum* P-3 (from Pahnab), *T. harzianum* N-8 (from Nozarabad), *T. harzianum* D-21 (from Dasht-e Naz), with production of the most inhibition zone against the pathogen were selected for greenhouse test.

Biological control of *F. oxysporum* L-6 on tomato in greenhouse

The selected antagonists (P-3, N-8 and D-21) were tested for their ability to reduce the incidence and yield parameters of tomato under greenhouse conditions.

For this propose, *F. oxysporum* L-6 and *Trichoderma* isolates were grown on PDA for one week. Inoculum of *F. oxysporum* L-6 was multiplied by transferring the pieces of 5 cm diameter culture to Eerlenmeyer flasks containing 100 g sand, 5 g corn meal and 20 ml of sterile distilled water, and inoculums of *Trichoderma* strains were multiplied by transferring the pieces of 5 cm diameter

culture to Erlenmeyer flasks containing 100 ml of moist wheat bran. Then, the inoculated substrates were incubated at room temperature for three weeks (until all substrates were covered by *F. oxysporum* L-6 and *Trichoderma* isolates) (Frommel *et al.,* 1991).

Potting mixture (red soil: sand: decomposed FYM at 1:1:1 w/w/w) was prepared and autoclaved one hr for two consecutive days and filled in pots of 5 kg capacity. Tomato (var. PKM1) seeds were sown in autoclaved potting mixture. After 25 days, the seedlings were transplanted in pots at the rate of four seedlings per pot. Both multiplied inoculums of the pathogen and antagonists were incorporated into the pots at 5% (w/w) (*F. oxysporum* L-6 was used one day before transplanting and *Trichoderma* strains were applied just the day of seeding). The observation on the percent disease incidence was recorded at the time of harvest.

Each treatment was replicated thrice in completed randomized design (CRD). Treatments were: *F. oxysporum* L-6 + *T. harzianum* P-3, *F. oxysporum* L-6 + *T. harzianum* N-8, *F. oxysporum* L-6+ *T. harzianum* D-21, Carbendazim (0.1%), Inoculated control with *F. oxysporum* L-6 (diseased control) and un-inoculated control (healthy control).

Plants were maintained in the greenhouse of Gharakheil Crop Research Station of Mazandaran, Iran.

RESULTS

Effect of *Trichoderma* strains on mycelial growth of *F. oxysporum* L-6 *in vitro*

All tested *Trichoderma* strains inhibited mycelial growth of *F. oxysporum* L-6 in dual culture. There were significant differences among the *Trichoderma* strains. Growth inhibition of *F. oxysporum* L-6 was reduced by *T. harzianum* P-3, *T. harzianum* N-8 and *T. harzianum* D-21 by 63.33, 68.22 and 56.16%, respectively (*Fig. 1*).

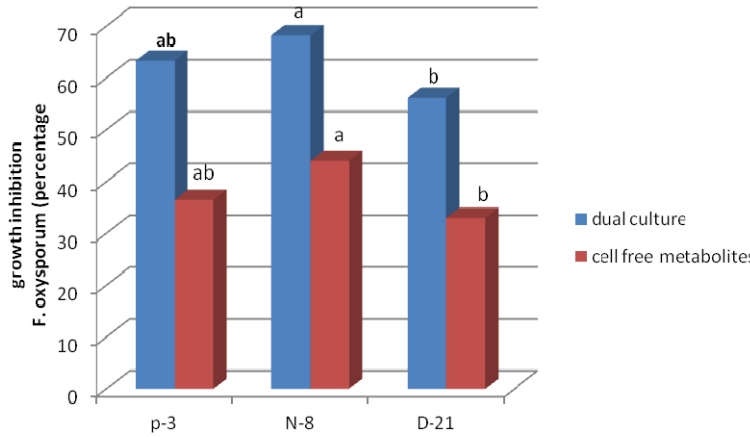

Figure 1 - Effect of *Trichoderma* strains and their cell free metabolites on growth inhibition of *F. oxysporum* L-6 (cellophane method). Treatment with the same letters do not differ significantly ($p \leq 0.5$) according to the Duncan's multiple range test. The vertical bars represent standard deviation with three replicates.

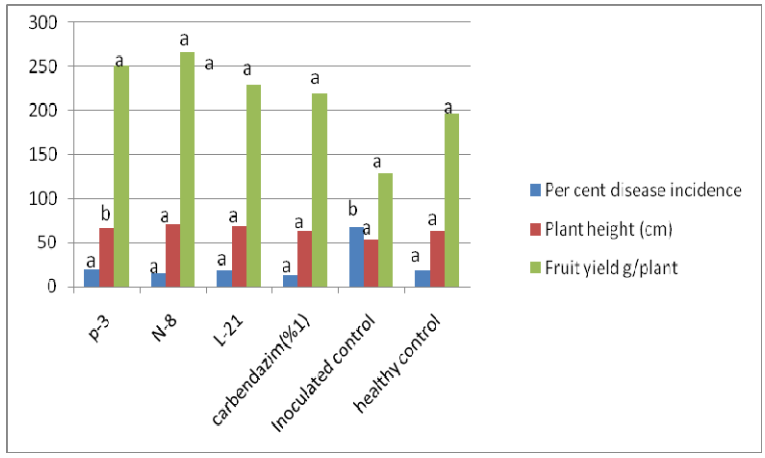

Figure 2 - Effect of *Trichoderma* strains on disease incidence, plant height and fruit yield in greenhouse. Treatment with the same letters do not differ significantly ($p \leq 0.5$) according to the Duncan's multiple range test. The vertical bars represent standard deviation with three replicates.

Cell free metabolites of *T. harzianum* P-3, *T. harzianum* N-8 and *T. harzianum* D-21 reduced the growth of *F. oxysporum* L-6 at the rates of 36, 44 and 33%, respectively (*Fig. 1*).

Effectiveness of native *Trichoderma* antagonists on wilt incidence and yield parameters under greenhouse conditions

The application of *Trichoderma* native antagonists was found effective in suppressing wilt incidence (between 14.75 and 20.15%). Conspicuously, an application of N-8 antagonistic fungal formulation was recorded as minimum wilt incidence (by 14.75%), followed by D-21 (by 18.33%) (*Fig. 2*). Among the treatments, Carbendazim (0.1%) was found to be the most effective by wilt incidence of 12.75%, compared to control (67.50%). Also, the results of this experiment revealed that the application of N-8 antagonistic fungal formulation significantly increased the plant height (by 70.13 cm) and fruit yield (by 265.42 g), when compared to untreated control (54.6 cm and 195.5 g) (*Fig. 2*).

DISCUSSION

In dual culture, all *Trichoderma* strains inhibited the growth of *F. oxysporum* L-6. Zones of inhibition were observed between the colonies of the pathogen and *Trichoderma* strains. The inhibition zone could be due to the effect of diffusible inhibitory substances produced by the *Trichoderma* strains, which suppressed the growth of *F. oxysporum* L-6. The presence and size of the zone of inhibition have

been used as evidence of the production of antibiotics by the *Trichoderma* strains (Jackson *et al.,* 1991; Crawford *et al.,* 1993).

Cell-free metabolites produced by the strains of *Trichoderma* could also reduce the colony area of *F. oxysporum* L-6. Even though the cellophane overlay technique has been used mainly for investigating non-volatile metabolites of *Trichoderma* (Jackson *et al.,* 1991; Dennis and Webster, 1971). Although antibiotic substances from *Trichoderma* strains were not extracted and determined in this study, but some antibiotics such as tubercidin, candicidin, phosphlactomycin, phenasin and 4-diacetylphloroglucinol, which have been produced by some antagonists, like *Pseudomonas flourescens*, *Streptomyces* spp. and *Trichoderma* spp., have been reported by researchers (Hwang *et al.,* 1994; Mazzolla *et al.,* 1992; Shanahan *et al.,* 1992).

In the present study, the plant height and fruit yield were also increased in *Trichoderma* (N-8)-treated plants. Similar results were reported on plant growth of cereals and legume crops due to application of *Trichoderma gamsii* (Rinu *et al.,* 2013). The increase of plant growth might be associated with secretion of auxins, gibberellins and cytokinins (Hwang *et al.,* 1994). The effectiveness of inoculation of tomato plants with antagonists in reduction of diseases and increase of yield was reported by others (Shahriari and Barari 2008a,b; Mazzolla *et al.,* 1992). Bochow and Fritzsche (1991) reported that inoculation of plant with *Streptomyces* in greenhouse reduced the severity of *Phytophthora infestans*. This reduction was due to the induction of host resistance by the *Streptomyces* strain. The effect of wheat plants inoculation with bacterial antagonists in reduction of root rot (*F. graminearum*) and increase of yield were reported (El-Abyad *et al.,* 1993; Etebarian *et al.,* 2000; Jones and Samac, 1996; Liu *et al.,* 1995; Luz, 2000; Nourozian *et al.,* 2006; Okhovat *et al.,* 1996).

CONCLUSION

The increase in biomatter production may be due to the production of plant growth promoters or through indirect stimulation of nutrient uptake and by producing siderophore or antibiotics to protect plants from deleterious rhizosphere organisms. Therefore, the antagonist *T. harzianum* (N-8) is chosen to be the most promising bio-control agent for *F. oxysporum* f. sp. *lycopersici*. On the base of present study, the biocontrol agents of plant diseases might be exploited for sustainable disease management programs to save environmental risk.

Acknowledgements. I would like to express my deep sincere of gratitude to Agricultural and Natural Resources Research Center of Mazandaran for supporting during this project and making available all the facilities.

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