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ANTAGONISTIC ACTIVITY OF TRICHODERMA HARZIANUM AGAINST FUSARIUM SOLANI ISOLATES SEPARATED FROM POTATO TUBERS UNDER IN VITRO CONDITIONS

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ABSTRACT
Fusarium dry rot of potato tubers in warehouse is one of the most important fungal diseases of potato worldwide. Application of fungicides to control this disease in the fields causes loss of beneficial microorganisms and ecological imbalance, while biological control has attracted significant attention in recent decades as an environmentally friendly approach. The present study was undertaken to investigate the biocontrol potential of a *Trichoderma harzianum* isolate against Fusarium solani isolates that cause dry rot of potato. Tubers with symptoms were collected from warehouses in North Khorasan province (Iran) from January 2013 to April 2014 and transported to the laboratory. The infected tissues were cultured after disinfection and pathogen purification was performed by the single spore method. Pathogenicity confirmation test was done following inoculating Agria tubers with a mycelial plug taken from the edge of actively growing *F. solani* cultures on PDA. The antagonistic capacity of the *T. harzianum* isolate was tested using dual culture and volatile metabolites assays. In the dual culture assay, *T. harzianum* significantly reduced the growth of *F. solani* isolates at different time points compared to the control treatments. There was a significant difference in the diameter of fungal colony between the *F. solani* cultures grown with or without *T. harzianum.* Furthermore, *F. solani* cultures exposed to the volatile metabolites released by *T. harzianum* had significantly lower growth compared to controls. The results demonstrated the dramatic effects of a indigenous *T. harzianum* isolate and its volatile derivatives on *F. solani* isolates causing dry rot of potato, confirming this fungus as a natural and healthy alternative to commonly used fungicides.

KEY WORDS: potato, dry rot, biocontrol, *Fusarium solani*, *Trichoderma harzianum*

INTRODUCTION

Food supply, as the main need throughout the human history, has always been faced with difficulties, so that a large part of the world's population currently suffers from food shortages (Lelgany Dezky, 2006; Vazin Afzal and Azamy Sarduee.2013). Potato is the most important dicot source of human nutrition. In terms of production, potato is in the second and in terms of the nutritional value in the third place after wheat and rice and is one of the strategic products (Safavi and Dehdar masjedlu, 2007; Arshady, 2009). Fusarium dry rot is one of the most damaging diseases of potato in all potato growing regions in Iran and is also one of the most important diseases in warehouses (Fig 1) (Baqhaee Ravari et al., 2008a). According to the reports, 46.9% of potato tubers are wounded at harvest, of which 19.9% have been infected by Fusarium dry rot causal agents (Rich, 1992).

Habiby et al. (2005) reported *F. solani, F. roseum, and F. sulphureum* as the most common causes of potato dry rot in Iran. Some researches attributed the decay of potato seed components in to *F. solani and F. roseum f. sp. sambucinum. F. solani* is also the decay agent of the stem base rot in potato plants (Rich, 1992). On the infected tubers, small brown spots are appeared near the wound after about a month. Gradually, the infection spreads and the infected surfaces wrinkle and twist and the infected areas can sometimes be seen in the form of concentric pages. The internal tissues of the infected tubers become brownish or chocolate brown (Habiby et al., 2005; Etebarian, 2009). Symptoms in aerial parts of the infected plants include dehydration, wrapping and browning of leaves, poor growth, wilting, and in some cases rosette. Also, the brown spots, shrink scars, and mummy tumors are of obvious infection symptoms (Baqhaee Ravari et al., 2008b).

Trichoderma has the capacity to control diseases caused by Fusarium and also stimulates the defense mechanisms in plants and enhances their growth by secretion of enzymes and hormones. Finally, limiting the disease incidence and severity, it helps to maintain the ecological balance (Akrami and Ebrahimof, 2011). Trichoderma species are among the most abundant fungi in many soils due to their metabolic diversity and high competitive power (Mehrabi Kooshki et

al., 2009; Kari Dolatabadi and Mohammadi Goltapeh, 2011). The success of Trichoderma isolates as potential biocontrol agents is due to their rapid growth, high sporulation ability, ability to survive under unfavorable conditions, effective utilization of nutrients, ability to modify the rhizosphere, and their high mycoparasitic activity against fungal pathogens (Chet, 1987; Howell, 2003, Iraqi et al., 2012). Moreover, role of the Trichoderma in promoting plant growth, induction of plant defense system, and the destruction and degradation of pesticides has been already proven (Akrami and Ebrahimof, 2011). In a field study, Trichoderma isolates increased the yield of potato crop up to 45 tons per hectare as compared to the treatments without Trichoderma (32 tons per ha), and caused a reduction of 7-8% in rot diseases of crown, root, and tubers (Soltani et al., 2006). The biocontrol capacity of Trichoderma species against many soil-borne pathogens is well documented (Chet and Baker, 1980).

It has been reported that *Trichoderma virens*, *T. harzianum*, and *T. viride* can control Fusarium wilt of chickpea caused by Fusarium oxysporum f.sp. ciceris (Dubey et al., 2006). T. harzianum isolates and B. subtilis demonstrated a good potential to control the growth of *F. solani* f.sp. phaseoli and *Rhizoctonia solani* under in vitro conditions, compared with fungicides (El-Meleigi et al., 2007). The potential mechanisms of different Trichoderma isolates against various pathogens have been investigated by Iraqi and Rahnama (2011), Mohammadi et al. (2008), Nowruzy et al. (2012) and Lurito et al. (1996). Considering the huge loss caused by the Fusarium dry rot of potato in commercial warehouses, the present study was conducted to investigate the antagonistic capability of a indigenous *T. harzianum* isolate and its volatile compounds on F. solani isolates separated from infected potato tubers in North Khorasan province, Iran.

Figure 1.Symptom of Fusarium dry rot on potato tubers

MATERIALS AND METHODS

Sampling was performed from January 2013 to the end of March 2014. The infected tubers were randomly collected based on the symptoms of the disease (Nowruzi, et al., 2012) from potato warehouses especially in important areas such as Faruj, Shirvan, Bojnord, Maneh and Samalghan.

Preparation of pathogen isolates:

After transferring the samples to the lab, tubers were washed with tap water for 10 minutes. Then, slices were taken from the boundary between healthy and infected tissues. After surface disinfection using 1% sodium hypochlorite for 60 seconds, slices were washed three times with sterile distilled water. Thereafter, slices were dried on sterile filter papers and placed on PDA medium. The PDA plates were incubated at 25±1 °C for one week. After growth of pathogen colonies, microscopic slides were prepared for identifying the F. solani species (Ahmadi Mousavy, 2012; Setayesh Mehr, 2008). In the present study, the isolates were identified using the key in Dr. Saremi book.

Preparation of the *Trichoderma harzianum* isolate:

In this study, an indigenous *T. harzianum* isolate was obtained from the fungal collection at the Sciences Department, Ferdowsi University of Mashhad, Iran. Two methods were used to grow *T. harzianum:*

First method: the fungal isolate was cultured on PDA medium and incubated at 24 °C for 3-4 days, and then stored in a refrigerator at 4 °C (Iraqi et al, 2012).

Second method: the propagation of T. harzianum isolate was done on wheat grain substrate (100 g grain + 40 mL distilled water), which was autoclaved for 2 hours at 121 °C. For this purpose, five 10 mm discs of four-day-old cultures were inserted into glass bottles containing 100 g of the heat-sterilized grain. The glass bottles were then stored in an incubator at 23-26 °C until the fungus completely colonized the whole substrate (Kari Dolatabadi and Mohammadi Goltapeh, 2011).

Investigating the antagonistic activity of *T. harzianum* against *F. solani* isolates by the dual culture assay:
This test was performed to investigate the pathogen growth in the presence or absence of *T. harzianum.* For this purpose, the dual cultures were used, in which one 5 mm disk from the active margin of *F. solani* colonies and one 5 mm disk from active margin of *T. harzianum* colonies were cultured on either side of the Petri dishes containing PDA medium.

In the control treatment, the fungal pathogen was grown along with a 5 mm disc of antagonist free medium. All cultures were incubated at 25±1 °C. Measurements of radial growth of fungal colonies were made 1day, 2 days, 3 days, 4 days, 5 days, and 6 days after the cultivation. The daily growth of the pathogen was compared between the T. harzianum and control treatments. (Nasrollahnezhad et al. 2010; Iraqi et al. 2011).

Investigating the effects of T. harzianum volatile metabolites against F. solani isolates:
In this test, the Petri dishes with pathogenic fungus were placed reversely on top of plates containing T. harzianum. The Petri dishes were subsequently sealed off using Teflon to prevent the removal of volatile compounds from plates. In the control treatment, instead of the *T. harzianum*, a 5 mm disc of PDA medium was used. All cultures were stored in an incubator at 25±1 °C, and the colonies diameter was measured with intervals of 1 day, 2 days, 3days and 4 days. At the end, the growth inhibition percentage of *T. harzianum* isolates were calculated using the equation $X = (AB \times 100)/A$. In this equation, X indicates percent inhibition, A diameter of colony growth in control treatment, and B diameter of colony in each of the non-control treatments (Nasrollahnezhad et al. 2010; Iraqi et al. 2011).

STATISTICAL ANALYSIS
The experiments were conducted as a completely randomized design with factorial arrangement and were analyzed using the average of two independent populations at 1% (dual culture) 5% (volatile metabolites) probability level by SPSS software.

RESULTS
A total of 135 Fusarium isolates were initially separated from storage tubers of which 84 isolates were identified as F. solani.
The effects of *T. harzianum* on *F. solani* isolates in the dual culture assay:
In this experiment, the *T. harzianum* isolate significantly ($p<0.01$) reduced the mycelial growth of *F. solani* isolates at all the time points tested (Fig 2).

The Trichoderma fungus colonies after growth and dealing with pathogenic fungus colonies have hindered its growth and development (Fig 3). The notes were taken of the results daily during the period of six days after planting was done. In this method, Trichoderma limits the pathogen growth on the medium due to different mechanisms of inhibition. The analysis indicated that there is a significant difference between the experimental group and the control group with the probability of 1%. In all periods, there was a significant difference between control and Trichoderma in terms of inhibition of mycelial growth as pathogenic factor. The trend line has a positive slope in the absence of antagonist, while it trends toward zero in the presence of T. harzianum.

The volatile metabolites assay:
The volatile compounds released by the *T. harzianum* isolate significantly reduced the pathogen growth at all time points compared to controls (Fig. 4). The pathogen growth remained constant after 72 hours of exposure to volatile metabolites. The present study after 4 days as compared to the control group have shown that T. harzianum has largely inhibited *F. solani* growth, and its growth speed and colony size has changed a lot (Fig 5). Similar to the figure 2, the above results for the change in the mean diameter of the pathogenic factor in the presence of volatile metabolites and the control group was clearly plotted also in figure 4.

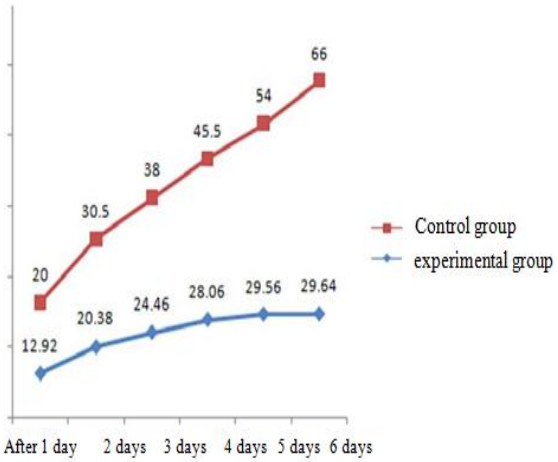

Figure 2. Colony diameter of *F. solani* isolates in the dual culture assay

Figure 3.The dual culture assay

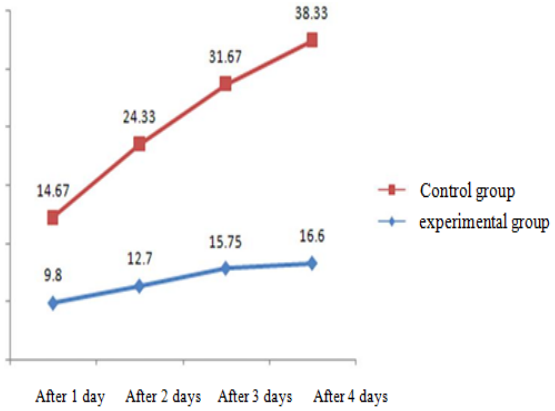

Figure 4. Colony diameter of F. solani isolates in the volatile metabolites assay

Figure 5. The volatile metabolites assay

DISCUSSION

In this study, the *T. harzianum* isolate showed a great biocontrol capability against indigenous *F. solani* isoltes in both dual culture (Fig 2) and volatile metabolites (Fig 4) assays. In the dual culture assay, T. harzianum showed a fast growth and colonized the whole PDA media after 6 days. Pathogenic fungi are among the most important factors that cause huge damages in potato in the fields and warehouses. *F. solani* and *F. oxysporum* are considered as the most important pathogens infecting potato tubers. The chemical control of soil fungi is difficult in many cases, whereas use of biological control agents in agricultural studies have shown practical and satisfactory results. The antagonistic activity of fungi can be attributed to hyphae parasitizing, inhibiting the mycelial growth by secreting volatile and nonvolatile compounds and competition over space and food (Soltani et al. 2006).

According to the existing literature, Trichoderma is the most common soil-borne fungus with a great biocontrol potential (Dubey et al., 2006). Here, the biocontrol capability of the T. harzianum isolate could be due to mechanisms antibiotics (Howell, 2003; Kucuk and Kivanc, 2003). T. harzianum and T. viride have been reported from a variety of soil types in Iran including potato growing areas. However, it seems that the abundance of Trichoderma populations is not high enough to actively control the pathogens. Intense application of fungicides might be one of the main factors that negatively affects the growth and development of Trichoderma populations in many agricultural soils. Therefore, introducing more Trichoderma into soil, can lead to a better control of soil inhabiting pathogens and the damages occurred. *T. harzianum* have been practically used to control F. solani and R. solani (Soltani et al., 2006).
In the natural environment, a complex set of factors influence the relationship among pathogen, antagonist, and host, so the more success of antagonists under laboratory conditions does not guarantee the positive outcome under field conditions. Rapid growth and high sporulation ability of Trichoderma are among the features that enable it to survive and function even in unfavorable conditions. Such characteristics have made this fungus in particular *T. harizanum* a suitable biological control agent against various soil-borne pathogens such as Fusarium, Sclerotinia and Rhizoctonia. Trichoderma has been shown to successfully colonize the rhizosphere soil due to rapid growth and high sporulation leading to effective control of pathogens (Iraqi et al., 2011).

Zafari et al. (2002) showed that there are at least 11 species of Trichoderma in Iran including seven newly identified species (Nasrollahnezhad et al., 2010). Trichoderma species can produce many non-volatile compounds such as cellulase, chitinase, lamynarynaz, B-1, 3-glucanase and antibiotics such as trichodermin, trichotoxin, alamethicin, dermadin and parasilicin.(Cuevas et al.,1991; Ghisalberti et al.,1993) The release rate and variation of these substances in different species of Trichoderma and even strains within a species may vary, so that different types of isonitril antibiotics play an important role in differentiating Trichoderma species such as T. harzianum, T. viride, T. koningii, and T. hamatum (Iraqi et al., 2012).

Abusaeedi et al. (1994) separated isolates of *T. harzianum, T. koningii* and *T. longibrachiatum* from potato fields in Kerman province in Iran and tested their biocontrol activity against F. solani that causes dry rot of potato in warehouses. The results revealed that these indigenous Trichoderma isolates can be effective in reducing the growth of *F. solani (*Etebarian, 2009). The great anatgonistic potential of T. harzianum observed in this study could be associated with its origin as it was isolated from the same area as the pathogen isolates.

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