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ORIGINAL ARTICLE

Biocontrol potential of *Trichoderma harzianum* in controlling wilt disease of pistachio caused by *Verticillium dahliae*

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Abstract

Verticillium wilt caused by *Verticillium dahliae*, is one of the most devastating diseases in pistachio orchards in the world including Iran. In search for an effective non-chemical strategy for the management of this disease, we evaluated the biocontrol potential of *Trichoderma harzianum* isolates obtained from the rhizosphere of healthy pistachio trees in different locations of the Kerman province of Iran against *V. dahliae* under laboratory and greenhouse conditions. Dual culture tests in the laboratory were conducted in a completely randomized design using 72 *T. harzianum* isolates. Twenty isolates showed the highest *in vitro* antagonistic activity. The results indicated that all 20 isolates were capable of inhibiting the mycelial growth of *V. dahliae* significantly. Among them, isolates Tr8 and Tr19 were the most effective by 88.89% and 85.12% inhibition, respectively. Extracted cell free metabolites of all effective isolates also inhibited the growth of *V. dahliae* in the culture medium significantly. According to the results, isolates Tr4 and Tr6 inhibited fungal pathogen growth by 94.94% and 88.15% respectively, through production of non-volatile metabolites. In the evaluation of volatile metabolites, isolates Tr5 and Tr4 were the most effective by 26.27% and 24.49% growth inhibition, respectively. Based on the results of the *in vitro* experiments, the five most effective isolates were selected for evaluation under greenhouse conditions for their biocontrol potential in controlling *Verticillium* wilt of pistachio. Results of the greenhouse, (*in vivo*) experiments were positive and indicated that the occurrence of wilt disease in plants treated with the antagonists alone or in combination with pathogenic fungus was lower than in plants inoculated with pathogen alone. The overall results of this study suggest that *Trichoderma* fungal antagonist may be an effective biocontrol agent for the control of *Verticillium* wilt of pistachio.

Key words: antagonism, pistachio, *Trichoderma harzianum*, *Verticillium dahliae,* wilt disease

Introduction

Verticillium dahliae is a soil-borne plant pathogen worldwide which causes vascular wilts in more than 300 plant species including pistachio (Agrios 2005; Williamson *et al*. 2007; Fotoohiyan *et al.* 2015). In some countries, including Iran, *Verticillium* wilt is a serious problem of pistachio (*Pistacia vera*) production (Aminaee and Ershad 1999; Fotoohiyan *et al.* 2015). *Verticillium* wilt of pistachio is a common disease of this plant and often severe in pistachio orchards where trees are planted from susceptible rootstocks, in soil with high population densities of the fungal pathogen, or in fields where susceptible crops have previously been grown (Aminaee and Ershad 1999; Tsror and Levin 2003). Symptoms of *Verticillium* wilt vary and appear as

wilt, chlorosis, necrosis, vascular discoloration, apical leaf curling, stunting, dieback and premature plant senescence (Fradin and Thomma 2006). *Verticillium* pathogenic fungi survive in the soil as microsclerotia in the absence of host plants for many years (Fradin and Thomma 2006).

Control of *V. dahliae* is difficult because of the lack of specificity of the host and the extreme variability of fungus pathogenicity (Pegg 2002). The use of chemical fungicides, resistant or tolerant rootstocks and soil disinfestation methods are particularly important elements of current management strategies. Effective control of the pathogen has also been achieved by soil solarization (Ashworth *et al.* 1982). However, the effectiveness of these management practices is curtailed by the *Verticillium* mode of conservation in the soil as microsclerotia and the occurrence of new physiological strains. Chemical control of the disease is costly and may be subject to future governmental restrictions due to environmental and health concerns (Rowe and Powelson 2002).

Recently, a worldwide tendency is to use eco-friendly methods in plant protection strategies (Hajieghrari *et al.* 2008). Interest in biological control with beneficial microorganisms that naturally occur in the soil and plant rhizosphere, and are antagonists of the pathogens has increased (Naraghi *et al*. 2010; Jorjani *et al*. 2012; Samavat *et al.* 2014; Mahdizadehnaraghi *et al.* 2015). *Trichoderma* spp. are among the most important biocontrol agents used for the control of different diseases (Harman 2006; Mahdizadehnaraghi *et al.* 2015; Papavizas 1985). These microorganisms are free living fungi that are common in the soil and root ecosystems. Different isolates of *Trichoderma* spp. are being successfully used and commercialized to combat a wide range of soil phytopathogenic fungi such as *Fusarium oxysporum*, *Rhizoctonia solani*, *Sclerotium rolfsii*, *S. cepivorum* and *V. dahliae* (Jabnoun-Khiareddine *et al.* 2009; Kakvan *et al.* 2013; Mahdizadehnaraghi *et al.* 2015).

According to the results of previous studies, the major mechanisms and modes of action of *Trichoderma* spp. include mycoparasitism, competition for nutrients and site, and production of antibiotics and enzymes (Howell 2003; Mahdizadehnaraghi *et al.* 2015). *Trichoderma* spp. can directly have an impact on other fungi after sensing a suitable fungal host, with the production of an antibiotic, formation of specialized structures and degradation of the host cell wall, followed by the assimilation of its cellular content (Howell 2003; Mahdizadehnaraghi *et al.* 2015). Antagonistic activities of *Trichoderma* spp. have been observed both under *in vitro* conditions (Mishra *et al.* 2011) and in greenhouse and field trials (Kexiang *et al*. 2002; Mahdizadehnaraghi *et al.* 2015).

Among *Trichoderma* spp., *T. harzianum* is the most potent biocontrol agent which inhibits the growth of *V. dahliae*, the causal organism of wilt disease (Naraghi *et al.* 2010). Recently, several attempts have been undertaken to enhance plant growth and elicit plant defense reactions in some crops such as: garlic (Mahdizadehnaraghi *et al.* 2015), sugar beet (Kakvan *et al.* 2013) and tomato (Naraghi *et al.* 2010). Biocontrol of soil-borne plant pathogens by *T. harzianum* has been reported by some researchers (Ziedan *et al.* 2005; Mahdizadehnaraghi *et al.* 2015). However, there is very little information about the use of *T. harzianum* as a biocontrol agent against wilt of pistachio caused by *V. dahliae* (Jamdar *et al*. 2013).

Due to the importance of *Verticillium* wilt as a major disease and yield limiting factor in Iranian pistachio orchards and considering the environmental and health issues, the present study was conducted and executed to introduce a non-chemical and ecologically friendly strategy for the management of this devastating disease.

Materials and Methods

Isolation of microorganisms

From 2012 to 2014, *V. dahliae* isolates were obtained from pistachio shoots showing wilt symptoms using ethanol agar medium (EAM) as a selective media as well as from the orchard soil by wet sieving on soil extract agar (Christen 1981). Isolation of *Trichoderma* spp. was done from the rhizospheric soil of healthy pistachio orchards in different areas of the Kerman province, during the years 2013–2014, according to the Rifai (1969) technique on the DAVET selective medium (Davet 1979). Deveoped isolates were purified and identified according to their morphological and microscopic characteristics using standard keys (Samuels 2006; Samuels *et al*. 2015).

Pathogenicity test

The pathogenicity test was performed on susceptible pistachio cultivar seedlings using root-dipping at the 3rd–4th true leaf stage as follows: roots were washed under running tap water, placed in a conidia suspension (10^7 conidia · ml^{-1}) for 60 min and then examined for the appearance of wilt symptoms after 7 days. The obtained pathogenic fungal isolates were then identified and cultured on potato dextrose agar (PDA) medium. The most pathogenic isolate of *V. dahliae* was selected through pathogenicity trial testing for further experiments.

Evaluation of the antagonistic activity of *Trichoderma harzianum*

Dual culture procedure

The antagonistic activities of *T. harzianum* isolates against *V. dahliae* were evaluated using the dual culture

procedure (Morton and Stroube 1955). Small plugs (5 mm diameter) of the *V. dahliae* were placed near the edge of a Petri dish containing PDA culture medium for 4 days. *Trichoderma harzianum* isolates were then transferred aseptically to the opposite side of the same plate and incubated at 27°C for 7 days. Colony diameters of *V. dahliae* were recorded at 24 h intervals for 3–7 days. Each experiment consisted of two treatments and there were four replicates in each treatment. The percentage of growth inhibition was calculated using the following formula:

$$I = (C–T)/C \times 100,$$

where: C – *V. dahliae* growth on the control plate, T – *V. dahliae* growth on *Trichoderma* plates, I – the percentage of growth inhibition of *V. dahliae* (El-Naggar *et al*. 2008).

Mycoparasitism activities of *Trichoderma harzianum* isolates

Mycoparasitism activities were investigated microscopically for any morphological changes in the mycelial growth of *V. dahliae* induced by *T. harzianum*. First, both fungi were grown next to each other on the PDA culture medium. At early stages of fungal contact, mycelium agar strips (10 × 20 mm) were removed from the interaction zone, placed on sterile microscope slides and examined at 100× magnification using a compound microscope. Mycoparasitism manifestations at the different stages of the development were recorded and compared with those of the control plates containing *V. dahliae* alone (Dennis and Webster 1971c).

The effects of non-volatile metabolites of *Trichoderma harzianum* isolates on *Verticillium dahliae* growth

The effects of culture filtrates of fungal antagonists on the growth of *V. dahliae* were studied according to Dennis and Webster (1971a) as follows: *Trichoderma* isolates were grown separately in potato dextrose broth at 27°C on a rotary shaker at 150 rpm for 10 days. The cultures were filtered through Whatman No. 1 filter paper after 10 days and centrifuged at 12,000 rpm for 10 min at 4°C. The pellets were discarded and the supernatants filtered through Sartorius Millipore (0.22 µ) filters. The PDA culture medium was amended with 50, 100; 250; 500 and 1,000 ppm concentrations of cell free metabolites obtained from different isolates of *T. harzianum*. Six mm plugs of *V. dahliae* were then cultured in the center of the plates and incubated at 27°C for five days. The control plate was maintained without metabolites. There were two treatments in the experiment, each with 4 replicates. The growth inhibition of *V. dahliae* was calculated in a different treatment using the formula described in the dual culture test section.

The effects of volatile metabolites of *Trichoderma harzianum* isolates on *Verticillium dahliae* growth

The effects of volatile metabolites produced by the effective *Trichoderma* isolates on mycelial growth of *V. dahliae* were determined according to Dennis and Webster (1971b) as follows: *Trichoderma* isolates were grown on Petri dishes containing PDA culture medium for 48 h. The lid of each Petri dish was replaced with a different Petri dish covered with PDA which was inoculated with *V. dahliae* at the center. The bottom of the Petri dish containing a centrally inoculated mycelial disc of *V. dahliae* was kept inverted on the Petri dish containing only PDA media culture which served as the control. The pairs of dishes were attached together and sealed with parafilm. Observations on the radial growth of the test fungal pathogen were recorded after 24, 48 and 96 h after incubation at 27°C. The colony diameter of *V. dahliae* in different treatments was determined and was compared to that of the control. There were two treatments in this experiment each with 4 replicates. The percent of growth inhibition of *V. dahliae* caused by different isolates of *T. harzianum* was determined using the formula described in the dual culture test section.

Greenhouse experiment

Preparation of pathogenic inoculum

Microsclerotia of *V. dahliae* were produced on a liquid culture medium according to Hall and Ly (1972). Cultures were incubated in the dark at 25°C with continuous shaking at 120 rpm under sterile conditions for three weeks and checked regularly for the formation of microsclerotia. The inoculum was then separated from the culture media by vacuum filtration, rinsed with sterile distilled water, dried aseptically for 72 h, weighed and passed through a 200 mesh screen to obtain smaller sizes from which 0.5 g was prepared and used to infest 1 kg of soil.

Preparation of *Trichoderma* inoculum

Erlenmeyer flasks containing 100 g of wheat seed and 100 ml of sterilized water were autoclaved at 121°C for 1 h on 3 successive days. After cooling, about 5–7 small plugs of 7-day-old culture of *T. harzianum* isolates were transferred into each flask under sterilie conditions. The flasks were kept at 27°C for 4 weeks. Colonized wheat grains were then transferred into paper bags, dried out and comminuted. Ten g of prepared powdery inoculum was used for the treatment of 1 kg of soil (Frommel *et al*. 1991).

Biocontrol experiment

The 5 most effective antagonistic isolates: Tr8, Tr19, Tr4, Tr5 and Tr18, were evaluated in the disease biocontrol experiment in the greenhouse. The mixture of soil, sand and perlite (1/1/1, v/v/v) was autoclaved at 121°C for 30 min for 2 successive days and used as a growth substrate for plants. Each pot received 1 kg of pasteurized soil. Pistachio seedlings (Badami Zarand cultivar) with 4–5 true leaves were transplanted into the pots containing the soil mix. Pots were inoculated with pathogen and antagonist in 4 treatments as follows: 1. Control (without pathogen and antagonist); 2. *T. harzianum* alone; 3. Pathogen + *T. harzianum*, 4. Pathogen alone. In treatments containing the antagonist, the soil was treated with *T. harzianum* isolates 7 days before infestation with microslerotia of *V. dahliae*. Pots were kept under greenhouse conditions at 25°C (±2°C) for 2 months and were watered as needed. The design of the experiment was completely randomized with four treatments, each with four replicates. Wilt symptoms were recorded at 10 day intervals for two months after inoculation. Disease severity was evaluated using the following 0–5 scale (Huang *et al.* 2006) where: 0 – healthy plants, 1 – <25% of the plants was wilted with scarce browning of crown, 2 – 25% of plants was wilted and showed slight browning, 3 – 50% of the plants was wilted and showed progressive browning 4 – ≥75% of plants was wilted and showed complete browning, 5 – dead plants.

Statistical analysis

Data obtained from the different experiments were first subjected to analysis of variance (ANOVA) and means were then compared using Duncan's multiple range test by the Statistical Analysis System (SAS) software version 9.1 (SAS institute Inc., 1996). The level of significance was determined at p = 0.05.

Results

Isolation of microorganisms

Based on the pathogenicity test conducted and performed on 12 isolates of *V. dahliae* obtained from infected pistachio plants, the most virulent isolate was selected for the further experiments in this study.

For the isolation of *Trichoderma* spp., 150 soil samples were collected from rhizospheres of healthy pistachio plants in different locations of Kerman province (Fig. 1). Soils revealed pH 6.53–8.16 and electric conductivity (EC) of 1.05–9.55. Seventy-two isolates of *T. harzianum* were obtained and maintained on the PDA as pure cultures. Based on the *in vitro* dual culture test, 20 *Trichoderma* isolates with the highest antagonistic activities were selected and were designated as Tr1, Tr2, Tr3, … and Tr20. These isolates were used in further *in vitro* experiments.

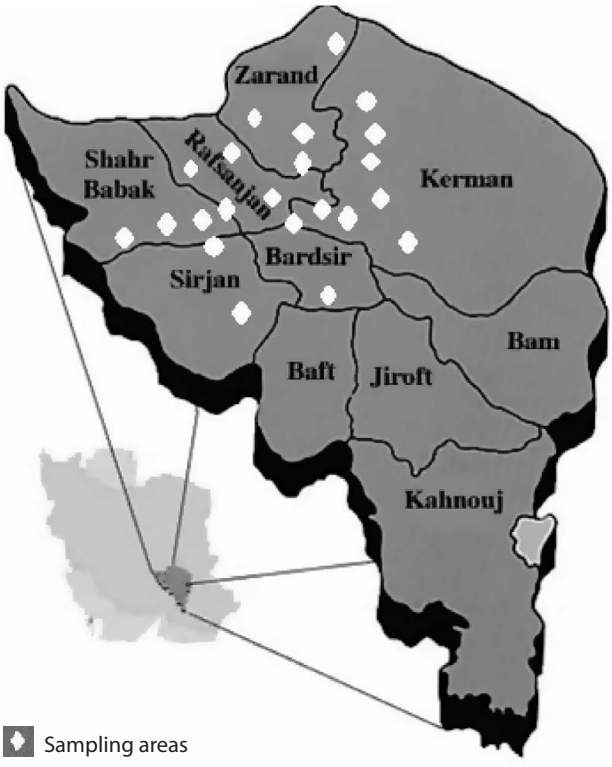

Fig. 1. Sites in Kerman province where samples were collected and isolates of *Trichoderma harzianum* were obtained

Evaluation of *in vitro* antagonistic activities

Dual culture test

The dual culture tests showed that all 20 isolates of *T. harzianum* inhibited mycelial growth of *V. dahliae* (Fig. 2). The average growth inhibition varied between 52.71% and 88.89%. Over 80% of the isolates showed a high level of antagonistic activity, ranging from 61% to 88.9%. Among the test *T. harzianum* isolates, Tr8 and Tr19 showed the maximum antifungal activity against *V. dahliae*, respectively (Fig. 3). In comparison with the other *Trichoderma* isolates, the differences were statistically significant (Fig. 3).

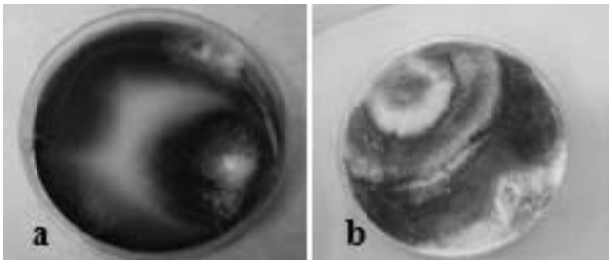

Fig. 2. Antagonistic activity of *Trichoderma* islolates against *Verticillium dahliae* in the dual culture test; a – *V. dahliae* alone, b – *Trichoderma* isolate and *V. dahliae*

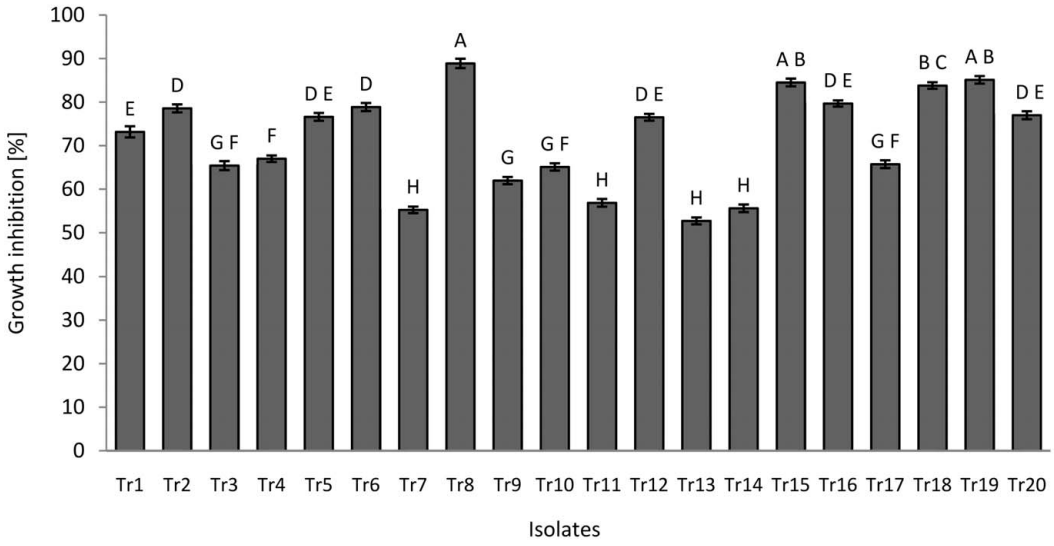

Fig. 3. Antagonistic activity of 20 active isolates of *Trichoderma harzianum* on the growth of *Verticillium dahliae* in the dual culture test. Values in the columns marked with the same letter(s) are not statistically different ($p > 0.05$) according to the Duncan's Multiple Range Test

Mycoparasitism activity of *Trichoderma harzianum*

Microscopic observation of hyphal interaction found that *T. harzianum* hyphae coiled around the hyphae of *V. dahliae*, denaturing and inhibiting their mycelia. *Trichoderma harzianum* either formed hooks or bunch-like structures around the hyphae of the pathogen before penetration or direct entry. Subsequent overlap of both the *T. harzianum* and the pathogen hyphae began to form 2–3 days after incubation at 27°C (Fig. 4).

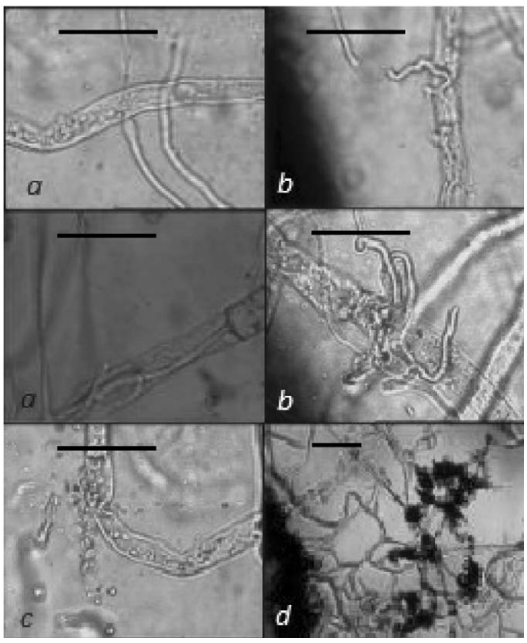

Fig. 4. Interaction between antagonistic fungi and *Verticillium dahliae*: a, b – hyphal contact and coiling of hyphae *V. dahliae* by *Trichoderma* isolates on PDA, c – lysis of hyphae *V. dahliae*, d – coiling of hyphae *Trichoderma harzianum* around hyphae *V. dahliae* (bar = 10 µm)

The effects of non-volatile metabolites of *Trichoderma harzianum* isolates on mycelial growth of *Verticillium dahliae*

The results of the antagonistic effects of non-volatile metabolites of *T. harzianum* isolates on the mycelial growth of *V. dahliae* are shown in Figure 5**.** According to these results, Tr4 and Tr12 isolates were the most effective in the growth inhibition of *V. dahliae* by 85.34% and 84.19%, respectively. Results of the inhibitory effects of culture filtrates of *T. harzianum* isolates on mycelial growth of *V. dahliae* revealed that 1,000 ppm concentrations of metabolites caused the highest mycelia growth inhibition of *V. dahliae* (Fig. 5).

The effects of volatile metabolites of *Trichoderma harzianum* isolates on mycelial growth of *Verticillium dahliae*

From 20 isolates of *T. harzianum* tested for their ability to produce toxic volatile metabolites against *V. dahliae*, Tr5 and Tr4 produced the most effective volatile metabolites for inhibition of mycelial growth of *V. dahliae* by 26.27% and 24.49%, inhibitory effects, respectively (Fig. 6).

Biocontrol activity of *Trichoderma harzianum* isolates against *Verticillium dahliae* in the greenhouse experiment

It was found that in the *in vivo* (greenhouse) experiments all 5 isolates of *T. harzianum* showed biocontrol

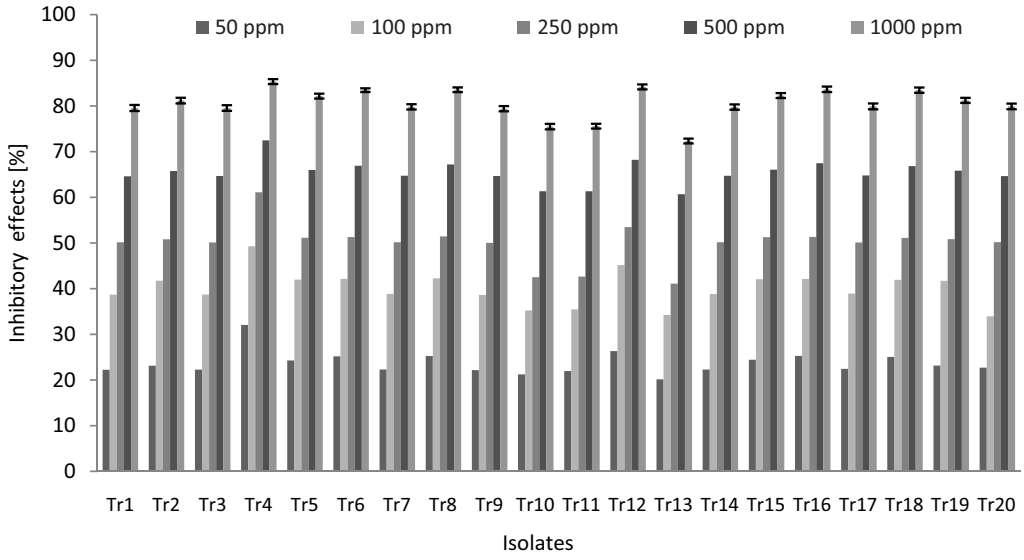

Fig. 5. Effect of different concentrations (50, 100, 250, 500 and 1,000 ppm) of metabolites obtained from different isolates of *Trichoderma harzianum*. Metabolites mixed with PDA medium and growth of *Verticillium dahliae* at 27°C measured on day 5

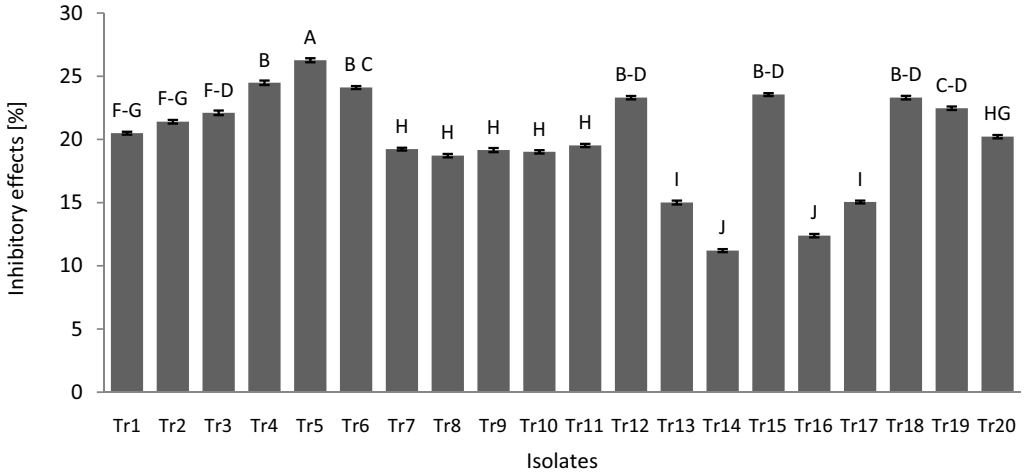

Fig. 6. Effect of volatile compounds of different isolates of *Trichoderma harzianum* on the growth of *Verticillium dahliae* at 27°C measured on day 5

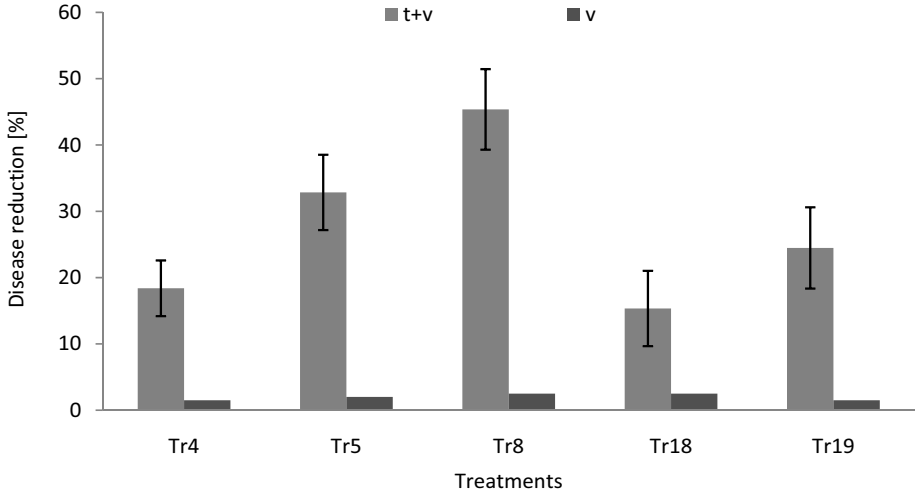

Fig. 7. Effect of *Trichoderma harzianum* isolates on the reduction of wilt disease caused by *Verticillium dahliae* in pistachio seedlings under greenhouse conditions; t – *T. harzianum*, v – *V. dahliae*, t + v – *T. harzianum* and *V. dahliae*

activity under greenhouse conditions. The statistical analysis of the greenhouse results indicated that the occurence of wilt disease in plants treated with antagonists alone or in combination with the pathogen were lower in comparison with the plants inoculated with the pathogen alone. The highest biocontrol activities were observed in those plants treated with Tr8 and Tr5 isolates, respectively (Fig. 7).

Discussion

Biological control of plant pathogens by beneficial microorganisms has been considered as a natural and environmentally safe alternative to harmful chemical methods (Mahdizadehnaraghi *et al.* 2015). *Trichoderma* spp. which are present in nearly all soils and other diverse habitats, have been introduced as potential biocontrol agents against plant pathogenic fungi, especially common soil-borne pathogens (Kataoka *et al.* 2010).

In this study we evaluated the antagonistic ability of *T. harzianum* isolates obtained from different locations of Kerman province of Iran against *V. dahliae* associated with wilt disease in pistachio plants. Isolation and identification of indigenous isolates of antagonists such as *Trichoderma* is essential for a successful selection of potential biocontrol agents (Williams and Asher 1996). In a previous study, Tong-Kwee and Boon Keng (1990) isolated 3 species of *Trichoderma* and in another study Zakaria (1989) identified 5 species of *Trichoderma* isolated from the rhizosphere of rubber in Malaysia. *Trichoderma harzianum* which was one of these species, inhibited the growth of the target pathogens through its ability to grow much faster than the pathogenic fungi (Zakaria 1989).

Trichoderma spp. are known to act against pathogenic fungi through several mechanisms such as hyperparasitism, competition and antibiosis (Hadar *et al.* 1979). The selection of biocontrol agents as well as understanding the mechanisms involved in the antagonistic effects of *Trichoderma* spp. on plant pathogens are important in selecting effective and safe biocontrol strategies. The various isolates of *Trichoderma* have different combative abilities against pathogens and their indirect effects may also vary. The variation in fungicidal activity among the *T. harzianum* isolates could be attributed to the presence of different types of chemical constituents in various isolates (Zhou *et al.* 2008).

Inhibition of mycelial growth of *V. dahliae* in the dual culture test in the present study suggests the secretion of diffusible, non-volatile, inhibitory substances by *T. harzianum* isolates. Previous studies have demonstrated that *Trichoderma* grows topically toward hyphae of other fungi, coil around them in a lectin-mediated reaction, and degrades cell walls of the target fungi by the secretion of different lytic enzymes (Ayoubi *et al.* 2014). All the chitinolytic enzymes were induced and excreted during the growth of *Trichoderma* on chitin as the sole carbon source. Before mycelia of fungi interact, *Trichoderma* spp. produces low quantities of extracellular exochitinases to dissolve the cell fragments of the host (Radheshyam *et al.* 2012). These cell fragments in turn induce the production of further enzymes (Ayoubi *et al.* 2014).

Defense mechanisms of *Trichoderma* are comprised of both enzymatic and chemical weapons, which make them efficient mycoparasites, antagonists and biocontrol agents, possessing characteristics that can be exploited by using *Trichoderma* or the metabolites secreted by this fungus as biological fungicides to fight against pathogenic fungi (Vinale *et al.* 2009). The volatile and non-volatile metabolites produced by *T. harzianum* have been shown to be responsible for the inhibitory activity against root pathogens (El-Katatny *et al.* 2006; Rahel Ratnakumari *et al.* 2011). Thrane *et al.* (2000) studied two antagonistic *Trichoderma* spp. that produced different kinds of lytic enzymes in liquid culture medium.

The effects of filterate concentrations of *T. harzianum* revealed that the aqueous extracts of *T. harzianum* reduced the mycelia growth of *V. dahliae*. The results of the present study suggest that metabolites produced by *Trichoderma* isolates were toxic and fungistatic against *V. dahliae*. Our results are in agreement with those of Anita *et al.* (2012) who observed a rapid decrease in the growth of the pathogen with a linear increase in concentrations of the metabolites.

In addition to the above-mentioned activities, *Trichoderma* isolates have been shown to be effective in controlling soil-borne diseases. In a previous study (Basin *et al.* 1999), the application of 5 isolates of *T. harzianum* against *V. dahliae* in pistachio plants showed positive results and enhanced seedlings health and growth significantly. Similar to our study, some other recent studies have reported both antagonistic activities and growth promotion potential of *T. harzianum* in various plant-pathogens interactions including tomato *Verticillium* wilt (Naraghi *et al.* 2012) and garlic white rot caused by *Sclerotium cepivorum* (Mahdizadehnaraghi *et al.* 2015).

These interesting properties and potential of our *Trichoderma* isolates may be due to a number of reasons, including a reduction of the effects on the pathogenecity of *V. dahliae* isolates, climatic adaptability, the influence of the pathogen origin and even the influence of local pistachio cultivars used in this region (Mahdizadehnaraghi *et al.* 2015).

Conclusions

The overall results of this study show that it may be possible to control and manage *V. dahliae,* the causal agent of pistachio wilt disease in the laboratory under greenhouse conditions using antagonistic isolates of *T. harzianum*. The results of our study, in particular in the greenhouse experiment which indicated the biocontrol potential of *T. harzianum*, may have practical application in the formulation of non-chemical and ecologically friendly control strategies against wilt disease of pistachio. Obtaining positive results in the pistachio orchards can help pistachio growers to increase the yield and production and will result in the protection of the agricultural environment and natural resources.

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