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Induction of Systemic Resistance by *Trichoderma harzianum* Isolates in Pistachio Plants in-Fected with *Verticillium dahliae*

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Abstract

Twenty isolates of *Trichoderma harzianum* wereisolated from the rhizosphere of healthy pistachio plants from different localities of Kerman Province, Iran.Five isolates with high antagonistic activity in *in vitro* assays against *Verticillium dahliae* (the causal agent of pistachio wilt), were investigated for their effect on the defense enzymes, peroxidase (PO), phenyl alanine-ammonia lyase (PAL) as well as the total phenol and protein contents in pistachio seedlingsexposed to *V. dahliae*-under greenhouse conditionsfor one month after inoculation. The results indicated that all of five isolateshad the ability to induce defense enzymes in treated pistachio seedlings;the Tr8 isolate had the maximum PAL activity and a corresponding increase in the total phenol content.The maximum PO activity and increase in total of protein contentwere seen with the Tr5 and Tr19 isolates, respectively. The increase in the activity of these enzymes when pistachio seedlings treated with antagonist alone or in combination with pathogen was greater than for plants inoculated with pathogen alone. In addition, Tr8 induced a significantly higher level of resistance in pistachio seedlings; therefore it showed the highest inhibition about 45.4% of verticillium wilt disease. This study suggests that the increased induction of defense related enzymes results in increased total phenol and protein contentsdue to enhanced resistance to invasion of pistachio seedlings by verticillium wilt. Outcomes of the study will be useful in formulating *T.harzianum* isolates for control of verticillium wilt in pistachio plants.

Keywords: Induced resistance, Peroxidase, Phenyl alanine-ammonia lyase, *Trichoderma harzianum*, *Verticillium dahliae*.

Introduction

Verticillium dahliae, isone of the important soilborne plant pathogens. It causes vascular wilts in more than 300 plant species including pistachio (Agrios, 2005; Williamson *et al*., 2007). In some countries, including Iran, verticilliumwilt is a serious pistachioproblem (Aminaee and Ershad, 1999). Because of the lack of specificity of the host and the extreme variability of *V. dahliae* pathogenicity, control of *V. dahliae* is difficult (Pegg, 2002). The use of chemical compounds, resistant rootstocks and soil disinfestation methods areparticularly important elements in current management strategies. However, the effectiveness of these management prac

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tices is loweredbecause *Verticillium's* mode of protection in soil as microsclerotia and the occurrence of new physiological races and chemical control is expensive and may be subject to future governmental restrictions due to environmental and health concerns (Rowe and Powelson, 2002). Recently, there has been a worldwide tendency to use eco-friendly methods in plant protection management that complement current strategies (Hajieghrari *et al*., 2008; Mbarga *et al*., 2012). Hence the interest in applying biological controls, for example, by using beneficial microorganisms that occur naturally in the soil and are antagonists of the pathogen (Karkachi *et al.*, 2010, Abano and Sam-Amoah, 2012).

Trichoderma spp. areamong the most important biocontrol agents used for management of different diseases (Papavizas, 1985; Harman, 2004). They are free living fungi that are common in soil and root ecosystems and currentlyare being successfully used and commercialized to combat a broad range of soil phytopathogenic fungi (Spiegel and Chet, 1998; Yedidia and Chet, 2001; Jabnoun-Khiareddine *et al*., 2009; Kakvan *et al*., 2013). *Trichoderma* spp. are well known to antagonise other fungi by a variety of active and passive mechanisms. One of the mechanisms of biocontrol is the defense response that occurs during early stages of root colonization by *Trichoderma* (Howell *et al*., 2000; Howell, 2003).As a result of interaction of *Trichoderma* with the plant, variousenzymes, avirulence-like gene products and lowmolecularweight compounds are released from fungal or plant cell walls by the activity of *Trichoderma* enzymes (Djonovic *et al*., 2006; Woo *et al*., 2006; Woo and Lorito, 2007). These compounds elicite a further reaction in the plant, by activating the mycoparasitic gene expression cascade thus enhancing the biocontrol ability of *Trichoderma* (Rasmussen, 1991; Ramanathan *et al*., 2000; Mandal, 2010). In addition, the defense reaction is enhanced due to the accumulation of PR-proteins, phytoalexins, chalcone synthase, phenylalanine-ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), phenolics, lipoxygenase, superoxide dismutase and β-1,3-glucanase in plants (Shivakumar *et al*., 2002; Babitha *et al*., 2004; Girish *et al*., 2005). PAL, one of the most extensively studied enzymes in plants because it is the first enzyme in the phenyl propanoid pathway and, catalyses the conversion of L-phenylalanine to trans-cinnamic acid which in turn enters different biosynthetic pathways leading to lignin synthesis. Thus, changes in PAL activity are the key events in controlling the synthesis of phenyl propanoids and this defense mechanism is used for protection against pathogen invasion. Induction of PAL as a response to pathogen infection is well documented in various host pathogen interactions (Geetha *et al*., 2005, Kavitha *et al*., 2012). Also, peroxidase is a component of an early response in plants to pathogen infection and plays the most important role in cell wall lignifications, substrate oxidation, photosynthesis, respiration and growth regulation and the plants biochemical defense against pathogens (Srivastava, 1987; Bruce and West, 1989). The products of the enzyme in the presence of hydrogen donor and hydrogen peroxide have antimicrobial activity (VanLoon and Callow, 1983). PO is one of the key enzymes involved in phenyl propanoid pathway and it is associated with disease resistance in plants (Hammerschmidt *et al*., 1982). Several studies reported that *Trichoderma* spp. could induce resistance in different plant species against a variety of fungal pathogens (De Meyer *et al*., 1998; Han *et al*., 2000; Yedidia *et al*., 2003; Shoresh *et al*., 2005; Moreno *et al*., 2009). The induction of plant defense responses and an increased resistance to pathogensby *Trichoderma* spp., also was observed when pre-treated with biocontrol agents in the field (Yedidia *et al*., 1999; Hanson and Howell, 2004; Shoresh *et al*., 2010). Induced resistance may provide an alternative approach to plant protection especially for problems not satisfactorily controlled by various fungicides (Schoenbeck, 1996).

Recent reports suggest that *Trichoderma* isolates can stimulate production of biochemical compounds of a phenolic nature associated with host defense. However,

more knowledge about these biochemical responses is needed to improve efficient formulations and potential biocontrol agents with suitable antagonistic characteristics must be screened carefully for other traits relevant to their use in a given application. There is little information on the use of *T. harzianum* as biocontrol agent against pistachiowilt caused by *V. dahliae.*Therefore in the present study we have screened local isolates of *T.harzianum* isolated from rhizosphere soil samples of healthy pistachio plants in different locations of Kerman Provincefor their ability to induce protection against verticilliumwilt in pistachioplants by production of biochemical compounds and theirpotentialas biocontrol agents.

Materials and Methods

Isolation of microorganisms

During 2012 – 2013, *Verticillium dahliae* isolates were obtained on selective media (Christen, 1981) from pistachio shoots with wilt symptoms. In 2013 – 2014, *Trichoderma harzianum* isolates were obtained from the rhizosphere of plants in healthy pistachio orchardsin different areas of Kerman Province, on DAVET selective medium (Davet, 1979) using the technique of Rifai (1969). After proper growth, isolates were purified and identified by standard keys according to their morphology and microscopic characteristics (Goud *et al*., 2003; Rifai, 1969; Bissett, 1991; Samuels *et al*., 2015). From 20 isolates of *T. harzianum*, five isolates that exhibited high antagonistic activity in *in vitro* assays against *Verticillium dahlia* (in dual culture tests (Morton and Stroube, 1955) and production of volatile and non-volatile metabolites (Dennis and Webster, 1971)) wereinvestigated for their ability to induce defense enzymes. Thepathogenicity of *V. dahliae* isolateswastested by the root-dipping method on pistachio seedlings (Badami zarand cultivar) at the 3rd-4th true leaf stage (Singleton *et al*., 1992). The collected isolates were preserved on potato dextrose agar (PDA) and stored at 4°C.

Greenhouse evaluations

Preparation of plants

Pistachios seeds (Badami zarand cultivar) were washed thoroughly with sterile distilled water and then the surface was sterilized by placing the seeds in 1% sodium hypochlorite for 1 minute, after which they were rinsed three times in sterilized distilled water and placed in sterilled perlite for germination. After 3-4 days and appearance of the plumule and radicle, three germinated seeds were transferred to pots and in a mixture of soil, sand, and perlite (1/1/1, v/v/v), that had been autoclaved at 121ºC for 30 minon two successive days,and were allowed to grow in a greenhouse at 25°C for two months (Mohammadi and Banihashemi, 2002).

Preparation of inoculum of pathogen

Microsclerotia of *V. dahliae* were produced on a liquid medium as described by Hall and Ly, 1972. The cultures were inoculated in darkness at 25°C, incubated for 3 weeks under continuous shaking at 120 rpm in sterile conditionsand checked regularly for the formation of microsclerotia. The inocula were then separated from the media by vacuum filtration, rinsed with sterile distilled water, dried aseptically in the shade for 72 h, weighed and passed through a 200 mesh screen to obtain smaller sizes of microsclerotia, from which 0.5 g of preparedmicroslerotia used to infect 1 kg of soil.

Preparation of inoculum of Trichoderma

Erlenmeyer flasks containing 100 g of wheat seed and 100 mL of sterilized water were autoclaved at 121ºC for one hour on three successive days. After cooling, about 5-7 small plugs of seven day old culture of *T. harzianum* isolates were dropped into each Erlenmeyer under sterilized conditions. The flasks were kept at 27ºC for 4 weeks. Colonized wheat grains were then transferred into paper pockets, and were dried and ground to a powder. Ten g of prepared powder was used to infect 1 kg of soil (Frommel *et al*., 1991).

Greenhouse biocontrol tests

Five isolates *viz*. Tr8, Tr19, Tr4, Tr5 and Tr18, of *T. harzianum*, all having high antagonistic activitesin *in vitro* assays against *V. dahliae*,were evaluated for bio-control experiments in the greenhouse.The seedlings with 4-5 true leaves were inoculated with pathogen and antagonist in four treatments: 1) Control (neither pathogen nor *T. harzianum*); 2) *T. harzianum*; 3) Pathogen + *T. harzianum* and 4) Pathogen. In treatments containing the antagonist, soils were inoculated with *T. harzianum* isolates seven days before infection with microslerotia of *V. dahliae*. All pots were watered as needed. Pots were kept under greenhouse conditions at 25±2°C for one month. Pot culture experiments were conducted in the greenhouse using a completely randomized design with 4 replicates. Wilt symptoms were recorded at 10 day intervals for one month after inoculation. Disease severity was evaluated from 0 – 5 using the following scale (Huang *et al.*, 2006) 0, Healty plants, 1, <25% of the plants wilted with scarcely any browning of the crown; 2, 25% of plants wilted and showed slight browning; 3, 50% of the plants wilted and showed progressive browning; 4, ≥75% of plants wilted and showed complete browning; 5, dead plants.

Trichoderma as inductor of plant defence responses

Preparation of sample

Freshly leaf samples were collected at 5, 10, 20 and30 days after inoculation with pathogen and antagonist to assay the changes in activities of defense related enzymes *viz*., peroxidase, phenylalanine-ammonia lyase and total of phenol and protein contents. Samples (0.5 gram) werehomogenized with 1 mL of 0.1 M sodium phosphate buffer (pH 7.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatant was used for estimating plant defense enzymes activity. Crude enzyme extract in 0.1 M sodium phosphate buffer (pH 7.0) was used for the estimation of peroxidase andphenylalanine-ammonia lyase activity.The enzyme extractswere stored in deep freezer (-70°C) and utilized for later biochemical analysis.

Estimation of peroxidase (PO)

Peroxidase activity was assayed by measuring the oxidation of guaiacol in the presence of hydrogen peroxide as described by HammerSchmidt*et al*. (1982). A 1.5-mL aliquot of 0.05 M pyrogallol and 0.1 mL of enzyme extract were added to a cuvette. To initiate the reaction 0.5 mL of 1% H_2O_2 was added. Crude enzyme preparations were diluted to give changes in absorbance at 470 nm with differences of 0.1 to 0.2 absorbance units/min. The change in absorbance was recorded at 470nm at 30-sec intervalsfor three min from zero second of incubation at room temperature. The peroxidases enzyme activity was determined for all the treated as well as control plants.The specific activity of PO was expressed as Unit/mgprotein.

Estimation of Phenylalanine Ammonia Lyase (PAL)

PAL activity was determined as the rate of conversion of L-phenylalanine to *trans*-cinnamic acid at 290 nm (Dickerson *et al*., 1984). The reaction mixture contained 1 mL enzyme extract, 0.5 mL substrate (50 mM L-phenylalanine) and 0.4 mL 25 mM Tris-HC1 buffer (pH 8.8). After incubation for1 h at 30°C, the reaction was stopped by the addition 0.5 mL of 2 N HC1 and the absorbance was read at 290 nm against a blank consisting of the same volume of reaction mixture without L-phenylalanine. The specific activity of PAL was expressed as Unit/mgprotein.

Estimation of the total phenols

One gram of plant sample was homogenized in 10 mL of methanol/water, 8/2 (v/v) and agitated for 15 min at 70°C (Zieslin and Ben-Zaken, 1993). One mL of the methanolic extract was added to 5 mL of distilled water and 250 mL of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25°C for 3 min. The absorbance of the developed blue color was measured at 725 nm using a spectrophotometer. Catechol was used as the standard. The total phenol content was expressed in mg/g of fresh tissue.

Estimation of the total proteins

Total protein concentration in the filtrates was assayed by theBradford method (1976) using Coomassie blue reagent (Coomassie Protein Assay Reagent, Piere) and bovine serum albumin (BSA) as the standard protein. The specific activity of the enzymes in the total filtrate was calculated using protein concentrations determined by this method.

Statistical analysis

Data were analysed on SAS system version 9.1 (SAS Institute Inc., 1996). Mean separation was tested using Duncan's multiple range test at p=0.05. The test for induction of defense related enzymes and the total phenolic and protein content of pistachio seedlings by *T. harzianum*isolates against *V. dahliae*was established under a factorial in completely randomized design with a control and four replications for each test pathogen.

Results

Isolation of microorganisms

One isolateof *Verticillium dahliae* with high pathogenecity was isolated and used for further biocontrol investigations. Twenty islolates of *T. harzianum* collected from pistachio orchards in different areas of Kerman Province (Fig. 1), were selected and designated as Tr1, Tr2, Tr3, … Tr20. These 20 isolates showed the highest in*in vitro* activity.

Fig. 1.Sites in Kerman Province where samples were collected and isolates of *Trichoderma harzianum* were obtainedare shown as white diamonds.

Greenhouse evaluations

The results of the greenhouse experiments revealed that all five isolatesof *T. harzianum* had the ability to reducewilt disease in treated pistachio seedlings (Fig. 2).The maximum wilt disease reduction observed in pots treated with Tr8 (45.4%) and Tr5 (32.9%) isolates respectively. Statistical analysis of the greenhouse experiments revealed that wilt disease reduction of theplants with the antagonist in combination with pathogen was enhanced in comparison with treatment in which the plants were inoculated with pathogen alone. The Tr8 isolate showedthe highest degree of inhibition about 45.4% of verticillium wiltdisease under greenhouse conditions after one month. (Fig. 3)

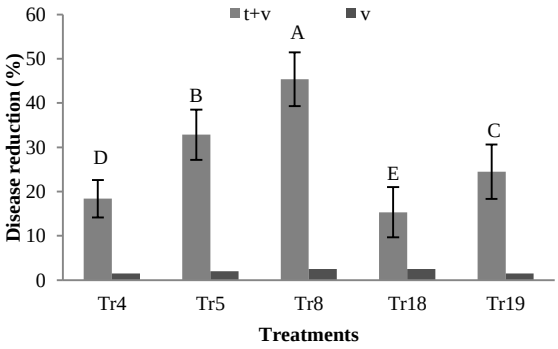

Fig.2. Effect of the treatments of *Trichoderma harzianum* isolates and *Verticillium dahliae* on wilt disease reductionin pistachio seedlings under greenhouse conditionsone month after inoculation.V= *V. dahliae* andTr= *T.harzianum*

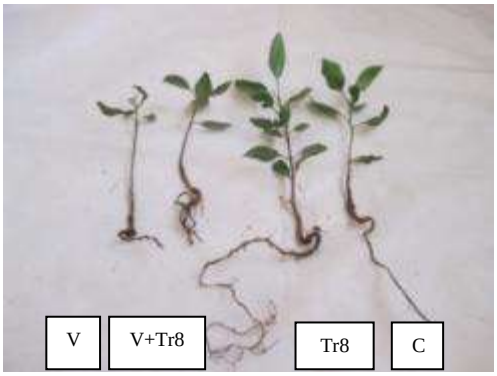

Fig.3. Effect of the treatments ofTr8 isolate and *Verticillium dahliae* in pistachio seedlings under greenhouse conditions one month after inoculation. C= Control, V= *V. dahliae* andTr= *T.harzianum*

Greenhouse assay for biological control

The results indicated that all five isolateshad the ability to induced defensive enzymes in treated pistachio seedlings,and that Tr8 isolate had maximum PAL activity and led to the maximum increase in total of phenol content.The maximum PO activity and increase in total protein contentwas seen withthe Tr5 and Tr19 isolates, respectively. The increase in the activity of these enzymes was greater for pistachio seedlings treated with antagonist alone or in combination with pathogen than for plants inoculated with pathogen alone. In addition, Tr8 induced a significantly higher level of resistance in the pistachio seedlings, and therefore showedthe highest level of inhibition about 82.5% of *Verticillium* wilt disease.

Estimation of peroxidase (PO)

The specific activity of peroxidasewas found to be increased in plants treated with the all five *T. harzianum* isolatesand *V. dahliae*. There was significant difference in the activity of peroxidase between *T. harzianum* isolates (Fig. 4).The maximum PO activity was detectedin Tr5 (4.7 U/mgprotein) whereas minimum activity from Tr19 (3.7 U/mgprotein).Also, the results indicated that pistachio seedlings treated with antagonist in combina

tion with pathogen showed a significant increase in PO activityand greater than plants inoculated with antagonist or pathogen alone. At 20th day after inoculation, enhanced PO activity was observed in almost all treatments and then slowly decreased (Fig. 4).

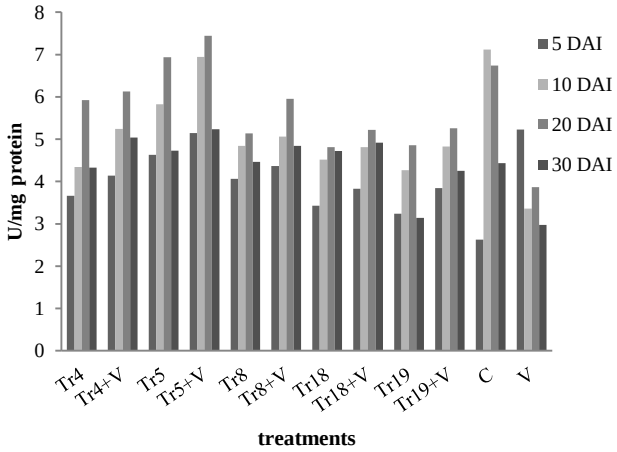

Fig.4. Effect of *Trichoderma harzianum* isolates and *Verticillium dahliae* on the PO activity in pistachio seedlings under greenhouse conditionsone month after inoculation.C= Control, V= *V. dahliae* andTr= *T.harzianum*, DAI=Days after inoculation

Estimation of Phenylalanine Ammonia Lyase (PAL)

The results indicated that the specific activity of phenylalanine-ammonia lyase of the strains varied from 2.1 to 2.8 (U/mgprotein) (Fig. 5). The highest specific activity was recorded for Tr8 (2.8 U/mgprotein) whereas Tr19 produced the lowest specific activity of PAL (2.1 U/mgprotein). Also, the specific activity of PAL was found to be higher in plants pretreated with *T. harzianum*and pathogen than in plants inoculated with antagonist or pathogen alone. The control seedlings without pathogen infection displayed the lowest PAL activity. The PALspecific activity increased significantly after the challenge inoculation and reached the highest level at the 10th day after inoculationafter which it slowly decreased (Fig. 5).

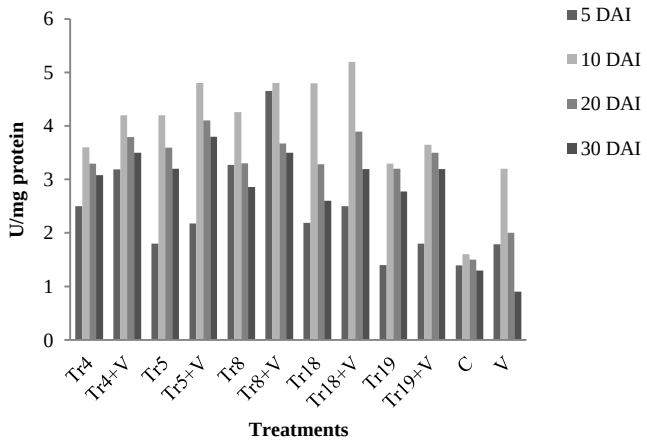

Fig.5. Effect of *Trichoderma harzianum* isolates and *Verticillium dahliae* on the PAL activity in pistachio seedlings under greenhouse conditionsone month after inoculation.C= Control, V= *V. dahliae* and Tr= *T.harzianum*, DAI=Days after inoculation

Estimation of the total phenols

From Fig. 6, it is clear that the total phenol content increased with treatment by the all five isolatesof *T. harzianum* and *V. dahliae*. All plants pretreated with *T. harzianum* isolatesand *V. dahliae* showed more accumulation of total phenol than the control and for plants pretreated with Trichoderma alone. The maximum total phenol content (0.68 mg/g) shownfor the Tr8 isolate and the minimum (0.39 Mg/g) for Tr18.The accumulation of phenol increased from the 5th day after challenge inoculation with *V. dahliae* and reached the maximum level on the 30th day (Fig. 6).

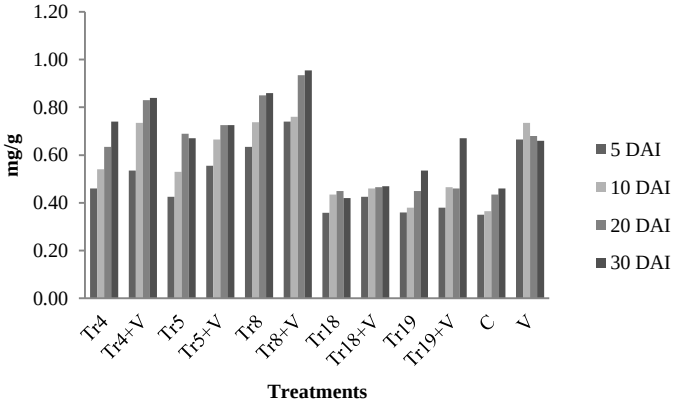

Fig.6. Effect of *Trichoderma harzianum* isolates and *Verticillium dahliae* on the total phenolcontent in pistachio seedlings under greenhouse conditionsfor one month after inoculation.C= Control, V= *V. dahliae* andTr= *T. harzianum*, DAI=Days after inoculation

Estimation of the total proteins

The total protein content was significantly higher in all specimens treated with *Trichoderma* isolates as compared with the untreated control (Fig.7). The pistachioseedlings treated with *T. harzianum* isolatesand challenged with the pathogen showed the maximum total protein content, which was higher than the corresponding pathogen challenged control. The total protein content reached its maximum on the 30thday after challenge inoculation (Fig. 7). However, the pistachioseedlings treated by Tr19 isolate exhibited significantly highertotal protein content (0.68mg/g)than other isolates.Also, pistachio seedlings treated by the Tr5 isolate led to decrease total protein content (0.35mg/g).

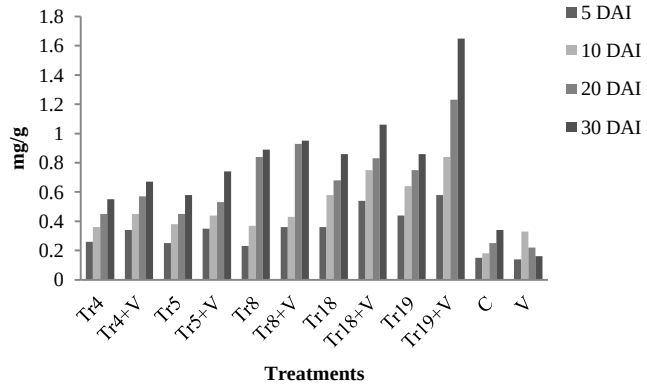

Fig.7. Effect of *Trichoderma harzianum* isolates and *Verticillium dahliae* on total protein content in pistachio seedlings under greenhouse conditions for one month after inoculation.C= Control, V= *V. dahliae* andTr= *T.harzianum*, DAI=Days after inoculation

Discussion

Application of biological control agents assists in reducing use of chemical pesticides and controlling release of their residues into the environment (Baker and Paulitz, 1996).*Trichoderma* spp. are widespread in almost any soil and rhizosphere, and have been investigated as effective biocontrol agents because of their ability to reduce of diseases caused by number of pathogenic plant fungi particularly many common soil borne pathogens (Elad, 2000; Freeman *et al*., 2004; Dubey *et al*., 2007; Vinale *et al*., 2008). The beneficial action of *Trichoderma* spp. is not limited tofighting pathogens; they have also been shown to be opportunistic plant symbionts, enhancing the systemic resistance of plants (Yedidia *et al*. 1999; Shoresh *et al*., 2010). Some isolates are also known for their ability to induce systemic resistance by production of biochemical compounds of phenolic nature in plants that are active against different pathogens (Kavino *et al*., 2008; Radjacommare *et al*., 2010). They can induce localized or systemic resistance to diseases and their causative pathogens through the release of metabolites (Wei *et al*., 1996; Zhou and Paulitz, 1994; Liu *et al*., 1995).Resistance results in an increase in the concentration of metabolites and enzymes related to defense mechanisms, such as the enzymes phenylalanine-ammonia lyase (PAL), peroxidase (PO), chalcone synthase (CHS) that are involved in the biosynthesis of phytoalexins, chitinases and glucanases and high levels of phenols.Induced PO and PAL activity in plants enhances the antimicrobial properties and plant phenolics and their oxidation products provide resistance to a wide range of pathogens (Vidhyasekaran, 1988). *Trichoderma* spp. have recently led to the proposal that besides their recognized antifungal properties, such organisms could also act as elicitors of plant defense reactions, thereby promoting the expression of plant defense related metabolomes (Yedidia *et al*., 2003, Segarra *et al*., 2007).

In this study, weevaluated theability of five strainsof *T. harzianum* as biological agents, isolated fromthe rhizosphere soil of healthy pistachio plants from Iran,to induce systemic resistance to verticillium wilt by way of the defense enzymes, PO and PAL and total phenol and protein contents in pistachio seedlings grownunder greenhouse conditions. PO is a useful marker of plant resistance to infection and stress (Welinder, 1992). POs are used primarily for the synthesis of secondary metabolites and are known to be induced by pathogen infection (Delannoy *et al*., 2003; Sasaki *et al*., 2005). The increased PO activity contributes to disease resistance in infected plants (Vidhyasekaran, 1997). Therefore, the increase of PO activity by *T. harzianum* isolatesin the all pistachio seedlings infected with *V. dahliae*, even in the absence of *V. dahliae* infection, can be considered as a marker of disease resistance during fungal phytopathogenesis in plants possibly through its utilization in cell wall lignification. Our results corroborate studies of Bradley *et al*. (1992), who reported that increased PO activity has been correlated with resistance in many species of plants and that these enzymes are involved in the polymerization of proteins and lignin or suberin precursors into plant cell walls, thus constructing a physical barrier that can prevent pathogen penetration of cell walls or movement through vessels. Other studies, (Nawar and Kuti, 2003; Hassan *et al.*, 2007; Van Wees *et al*., 2008) have delineated the induction of PO in plants infected by pathogens or insects, resulting in faster and stronger resistance to them. Also, *T. harzianum* isolatesincreased PAL enzyme activity in pistachio seedlings. PAL induced phenyl propanoid metabolism starts with the conversion of L-phenylalanine into *trans*-cinnamic acid thus supplying precursors for flavanoid pigments, lignin and phytoalexins (Massala *et al*., 1980; Hahlbrock and Scheel, 1989). An increase in PAL activity subse

quently might have led to increased levels of the signaling molecule salicylic acid and the phenolic compounds in the host thereby contributing to disease resistance (Klessig and Malamy, 1994; Charitha Devi *et al*., 2012). Induction of PAL by *Pseudomonads fluorescens*against *C. gloeosporioides* in mango and noni has been reported (Vivekananthan *et al*., 2004; Manjunath, 2009). In our study, a significant increase in the level of PAL in the treated pistachio seedlings is in agreement with earlier reports (Yedidia *et al*. 2003; Verma *et al*. 2007).

This finding is well supported by the study of Shoresh *et al*., (2005), who reported high induction of PAL in cucumber plants treated with *T. asperellum*. as well as that of Kavitha *et al.,* (2008), who reported the accumulation of PAL and PO mediated by the *T. asperellum* in tomato. Similarly, systemic resistance was enhanced due to a high accumulation of defense enzymes in response to *R. solanacearum* challenge in tomato (Vanitha *et al*., 2009). Further, the interaction between tomato and *V. dahliae* elicited enhanced activities of PO and PAL, phenylpropanoid metabolism, and synthesis of lignins (Gayoso *et al*., 2010). Also, it was observed that resistant plants contain more phenols and proteins. A multifold increase in phenol and protein content was observed in the all the pistachio plants treated-with *T. harzianum* isolates alone or with the pathogen, compared with the control plants. Some phenolics may act as signal molecules or antioxidants and thus induce resistance (Malamy *et al*., 1990). The accumulation of phenols may be due to excess production of H_2O_2 in infected plants through increased respiration (Farkas and Kiraly, 1962) or to the activation of hexose-monophosphate shunt pathway, acetate pathway and to the release of bound phenols (Goodman *et al*., 1967; De Ascensao and dubery, 2000; Mandal and Mitra, 2007). These observations closely resemblefindings ofresistance in maize roots elicited with *T. harzianum* strain T-22 (Bigirimana *et al*., 1997). Similarly, induction of synthesis of high amounts of phenols by *T. harzianum* isolates, compared with controls, suggests their role in inducing resistance against wilt in pistachio plants. The level of defense-related enzymes determines the degree of host resistance. Increase in activity and accumulation of these enzymes also depends on the plant genotype, physiological conditions and the pathogen.

In this study, it has been concluded that the pistachio plants treated with native bioagents of *T.harzianum* followed by challenge inoculation of *V. dahliae* enhances induction of defense related enzymesand that these were very effective in the control of verticilliumwilt of pistachio plants. In conclusion, plants treated with *Trichoderma* in the root zone can produce higher levels of PO, PAL, phenols, and pathogenesis related proteins. In this regard, other researchers have also shown in the greenhouse similar results; e.g. *Trichoderma* isolates have been shown to be successful in controlling soil borne diseases (Basin *et al*., 1999).*Trichoderma* spp. decreased wilt incidence in chickpea plants (Dubey *et al*., 2007).The selection of biocontrol agents and the understanding the mechanisms involved in the antagonistic effect of *Trichoderma* spp. on plant pathogens are important in designing effective and safe biocontrol strategies. The different isolates of *Trichoderma* have different combative abilities for pathogen; their indirect effects may also vary.

From the current study, it can be inferred that there is maximum disease reduction in the application of indigenous *Trichoderma* isolate Tr8 against pistachiowiltas compared to the other tested isolates. This may be to the case for a number of reasons, including pathogenecity of isolates, climatic adaptability, influence of the pathogen origin and even the influence of local pistachio cultivars used in this region (Harman, 2006; Sharon *et al*., 2007). Therefore, the Tr8 isolate native to Kerman Province could be an excellent candidate for providing long term biocontrol agent against *V. dahliae* in pistachio plants with the aim of reducing the use of chemical pesticides in this region. The mechanisms involved in induced

plant resistance are still poorly understood and to exploit such applications of *Trichoderma*, more research is needed.

Conclusions

The present investigation was aimed at understanding theinduced systemic resistance of pistachio plantstreated with native, highly potent *T. harzianum* isolates possessing rhizosphere competence and antifungal properties against *V. dahliae* at different times after challenge inoculation. Pre and post inoculation studies demonstrated that the pistachio plants responded well to select *T. harzianum* isolates, which induced some systemic resistance to *V. dahliae* infection and caused significant changes in the host plant in terms of total protein, phenol content and levels of PO and PAL. Due to the increase in plant defense enzymatic activity, and the decrease in disease incidence, the *Trichoderma* inoculum can also applied in the field in order to enhance the yield compared to control plants. Hence, this study shows clear evidence that the native isolates of *Trichoderma* inoculum can not only enrich soil fertility and crop yield but also induce disease resistance and help to increase production. Different enzymes, PAL and PO which are induced of all defense mechanisms. The mechanisms are similar for *Trichoderma* spp. against different pathogens (Bolton, 2009). Therefore, this beneficial impact of new strains of antagonist in experimental systems with different plants and pathogens aimed to determine the ability of *Trichoderma* strain to induce defense response and resistance in a variety of biotic and abiotic conditions.

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