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Biological control of *Verticillium* wilt and growth promotion in peach by endophytic and rhizospheric soil fungi from stone fruit trees

A. Esmaeilzadeh
D. Zafari
M. Ketabchi✉
Department of Plant Protection, College of Agriculture, Bu-Ali Sina University, Hamedan, Iran
R. Aletaha
Department of Soil Science, College of Agriculture, Bu-Ali Sina University, Hamedan, Iran
A. Nourian
Department of Pathobiology, Faculty of Veterinary Sciences, Bu-Ali Sina University, Hamedan, Iran

Abstract: *Verticillium* wilt of stone fruit trees caused by *Verticillium dahliae* occurs worldwide and causes serious economic losses. Control of *Verticillium* is difficult and costly due to its wide host range and resistant soil-borne microsclerotia. Also, increased concerns about agrochemicals have encouraged the development of biocontrol strategies. In this study, we evaluated antagonistic fungi for biocontrol of *V. dahliae in vitro* and greenhouse. A total of 85 endophytic and rhizospheric fungal isolates of peach and other stone fruit trees were isolated in the west Azarbaijan province, Iran. The identified fungi included *Alternaria*, *Aspergillus*, *Aureobasidium*, *Clonostachys*, *Cryptococcus*, *Fusarium*, *Penicillium*, and *Trichoderma*. The potential control of the isolates was initially evaluated by a dual culture assay. Furthermore, the antagonistic activity of fungi metabolites on the germination of microsclerotia both *in vitro* and in the soil was evaluated. In total, *Trichoderma asperellum* AE66 showed the highest inhibitory activity (73.85%) and was selected for greenhouse experiments. In the greenhouse assay on peach, *T. asperellum* AE66 not only reduced the progress of *Verticillium* wilt but also its severity. Moreover, the plant growth was promoted. These findings suggest that biocontrol provides a potentially effective strategy for the management of *Verticillium* wilt.

Keywords: Disease assessment, Endophytic Fungi, Rhizospheric Fungi, Stone Fruits, *Trichoderma asperellum.*

INTRODUTION

The genus *Verticillium*, with 10 major pathogenic species, is a vascular and soil-borne phytopathogen. Among these, *Verticillium dahliae* causes wilt in 74 plant families, including woody plants e.g., olive, almond, and peach trees (Inderbitzin et al. 2011). Control of the disease is difficult because of the wide host range of *V. dahliae* and the lack of effective fungicides. Moreover, melanized microsclerotia are able to survive in the soil for 13-14 years (Maldonado-González et al. 2015).

Biological control has been proposed for the management of *V. dahliae*. For example, biocontrol of *Verticillium* wilt in olive trees has been successful using *Trichoderma* spp. (Aleandri et al. 2015, Carrero-Carrón et al. 2016, Ruano-Rosa et al. 2016). The bacteria *Serratia plymuthica* (Müller et al. 2008), *Pseudomonas fluorescens*, *Pseudomonas putida* (Mercado-Blanco et al. 2004, Prieto et al. 2009, Sanei and Razavi, 2011, Aranda et al. 2011, Maldonado-González et al. 2015), and *Paenibacillus alvei* (Markakis et al. 2016) have also served as biological control agents against *Verticillium*. However, since previous studies indicated that using a biocontrol agent for *Verticillium* wilt in woody plants would be time-consuming and labor-intensive, reports to date have been limited to olive and maple trees (Chandelier et al. 2003, Deketelaere et al. 2017). Accordingly, the use of biocontrol agents for *Verticillium* wilt in peach trees has not been reported yet.Peach (*Prunus persica* (L.) Batsch, Rosaceae) originated in China and transported to Persia (Iran) (Luriea & Crisosto 2005). Annual world production of peaches has reached 25 million tons. Iran, with approximately 687.000 tons of annual production, was a major producing country after China, the European Union, and Turkey in 2022/23 (FAO 2023). Despite the prevalence of *Verticillium* wilt in peach trees in Iran, to our knowledge, no study has investigated the biocontrol of the disease.

This research was carried out both *in vitro* and in the greenhouse to determine whether endophytic and rhizospheric fungi isolated from stone fruit trees were able to protect peach trees against *V. dahliae*.

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✉ Corresponding Author: E-mail: mahru.ketabchi@gmail.com

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MATERIALS AND METHODS

Isolation of *V. dahliae*

A number of 250 samples were collected from stone fruit trees in west Azarbaijan province, located in northwestern Iran. Several locations were selected for the collection of peaches with disease symptoms. Fungi isolated from infected tissues were identified according to Inderbitzin et al. (2011). The modified method of Tjamos & Fravel (1997) was used to isolate *V. dahliae* microsclerotia larger than 70 µm for their higher fitness (Hawke & Lazarovits 1994).

Isolation of endophytic and rhizospheric soil fungi

Endophytic and rhizospheric fungi were isolated from internal plant tissues and rhizospheres. For isolation of endophytic fungi, roots, leaves, petioles, veins, and branches were cut into 0.5-1 cm, washed with sterile distilled water, and surface-sterilized according to Hamim et al. (2017). Healthy parts of each sample were surface-sterilized in 95% ethanol for 60 s, 4% sodium hypochlorite for 5 min, 30 s in 95% ethanol, rinsed three times in sterile distilled water, and plated on potato dextrose agar (PDA) for 2-8 weeks at 23 ºC in the dark.

For isolation of rhizospheric fungi, serial dilutions of the rhizospheric soils were cultured on PDA, water agar (WA), Davet (Davet 1979), ethanol-agar (Ausher et al. 1975), and TM media (Marois et al. 1982). Eight grams of rhizospheric soil was suspended in 100 mL sterile distilled water and shaken for 20-30 min. The suspension was then serially diluted. One mL dilutions of 10^{-3} - 10^{-7} were plated in triplicate on culture media and incubated at 25 ºC. The emerged colonies with different morphological appearances were purified. Fungi were maintained on plum extract agar (Talboys 1960) at 4 °C in the dark.

Morphological and molecular identifications of the fungi

Morphological identification of the isolates was performed according to the standard identification keys (Samson 1994, de Hoog & Hermanides-Nijhof 1997, Schroers 2001, Leslie & Summerell 2006, Simmons 2007, Samuels et al. 2012, Visagie et al. 2014). All isolated fungi were deposited in the Iranian Fungal Culture Collection.

The genomic DNAs were extracted according to Möller et al. (1992) with minor modifications. Four primer pairs were used for PCR amplifications according to the references (Table 1). Amplicons were sequenced using the same primers as applied in PCR (Takapouzist, Iran). The sequences were analyzed and edited using the Chromas software, then identified by BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi), with those from a group of species obtained from various substrates. The sequences were deposited in NCBI GenBank.

Table 1. PCR primers used in this study for the amplification of specific genes in the fungal isolates

Locus	Primers	Annealing Tm (°C)	Sequence (5′–3′)	References
Internal Transcribed Spacer (*ITS*) region of the rRNA	ITS1 ITS4	56	CTTGGTCATTTAGAGGAAGTAA TCCTCCGCTTATTGATATGC	White et al., 1990
Translation elongation factor 1-alpha (*tef1*)	EF1-728F EF1-986 R	58	CATCGAGAAGTTCGAGAAGG TACTTGAAGGAACCCTTACC	Carbone and Kohn, 1999
beta-tubulin (*tub2/BenA*)	Bt2a Bt2b	55	GGT AAC CAA ATC GGT GCT GCT TTC ACC CTC AGT GTA GTG ACC CTT GGC	Glass and Donaldson, 1995
Calmodulin (*CaM*)	Cmd5 Cmd6	55	CCGAGTACAAGGARGCCTTC CCGATRGAGGTCATRACGTGG	Hong et al., 2006

Antagonistic effects of fungal isolates against *V. dahliae in vitro*

Dual culture assay

The inhibitory effects of the isolated fungi on the growth of *V. dahliae* were evaluated on PDA by the modified method of Jabnoun–Khiareddine et al. (2009). Briefly, three plugs of *V. dahliae* were placed at the periphery of a sterile Petri plate. A plug for each antagonistic fungus was placed in the center of the plate. Plates without antagonistic fungi were used as controls. Treatments and control, in triplicate, were incubated for nine days at 25 ºC in the dark. The protocol was modified for *Trichoderma* species so that *V. dahliae* was grown on PDA for three days, the *Trichoderma* was inoculated, and the plates were incubated for six days. In the end, colony growth inhibition of *V. dahliae* was measured and reported using the formula: GI= [C-T/C] × 100, where GI is *V. dahliae* growth inhibition (%), C is the *V. dahliae* colony diameter of the control, and T is the *V. dahliae* colony diameter in the treatments (Royse & Ries 1978).

Effects of fungal non-volatile metabolites on the germination of *V. dahliae* microsclerotia

Four mycelia discs (3 mm) of each fungal isolate were transferred to an Erlenmeyer flask (250 mL) containing 100 mL Czapek dox broth (CDB, Sigma). Four PDA discs were added to control flasks. The flasks were incubated in the dark for 14 days at 25 ºC, 150 rpm. On the 7th day, one plug of *V. dahliae* was added to each flask for enhancement of metabolite production. Following 14-day incubation, the supernatants were filtered through a 0.22 µm membrane. An aliquot of 2.5 mL of the supernatant was transferred into a pot containing 3 g perlite, which was previously infected with *V. dahliae* microsclerotia (100 per g perlite).

After three days of incubation at 25 ºC in the dark, to isolate the microsclerotia, 100 mL sterile distilled water was added to each pot, filtered, and one mL of filtered supernatant was poured into a water agar (WA) plate. After 14 days of incubation at 20 ºC, the total number of germinated microsclerotia was recorded. The percentage of germination inhibition of microsclerotia was calculated using the following formula: I = [1-n/100] × 100, where I is the percentage of germination inhibition of microsclerotia, and n is the number of the germinated *V. dahliae* in the treated plate according to Jabnoun–Khiareddine et al. (2009) with minor modifications.

The effect of antagonistic fungi on the stability of *V. dahliae* microsclerotia

Inhibition of *V. dahliae* microsclerotia germination was evaluated in the pot using the modified method of Varo et al. (2016). Each pot was inoculated with 100 microsclerotia per g of soil. Spore suspensions (10^5 conidia per mL) of each antagonistic isolate were added to each pot, but distilled water was used for the control. All pots were covered with plastic bags and incubated for five days at 25 ºC in the dark, after which the pot contents were air dried. Sterile water (100 mL) was added to each pot, and the contents were shaken for one hour, followed by filtration (30 nm mesh). The material that remained on the filter was thoroughly suspended in 100 mL sterile water, and 1 mL aliquots of the suspension were transferred into each of 10 plates containing ethanol agar. After 14 days of incubation at 18 ºC in the dark, colonies with characteristics of *V. dahliae* were counted, and the total number of colonies was compared to that of the original sample. The percentage of inhibition (MV) was calculated by the same formula.

The most potent antagonistic isolate

To determine the most potent antagonistic isolate in all three previous experiments, the following formula was used: I_T (%) = [(GI%) + (I%) + (MV%)×2] / 4 , where I_T is the total inhibition (%) of *V. dahliae,* GI is the growth inhibition (%) of *V. dahliae* in dual culture, (I) is the inhibition (%) of *V. dahliae* as the result of antagonistic metabolites and (MV) is the inhibition (%) of microsclerotia stability by antagonistic metabolites.

Suppressive effect of *Trichoderma asperellum* AE66 on *V. dahliae* in greenhouse

Since *Trichoderma asperellum* AE66 was the most potent isolate, according to I_T, it was used for greenhouse experiments. *T. asperellum* was mass-produced, according to Naraghi et al. (2006). *T. asperellum* AE66 effect on the development of *Verticillium* wilt and the growth of peach plants under greenhouse conditions was assessed using the highly virulent isolate *V. dahliae* AE_2. A mixture of soil, dry manure, and sand (3:1:1 v/v/v) was inoculated by *V. dahliae* (20 microsclerotia per g soil) and *T. asperellum* (20 g per kg soil) (Ausher et al. 1975), then added to the bottom of a 12 L pot. Peach seedlings were transplanted into the pots and irrigated. Controls included the pots inoculated only with *V. dahliae* and the pots without inoculation. All treatments were done in three replicates.

Disease assessment

Any symptoms of disease in the plants were recorded weekly for 10 weeks. Disease severity was evaluated using a 0-4 score according to Tjamos et al. (1991), where scores ranging from 0-4 represent: 0 = no symptom, 1= moderate symptom with <33% wilt, 2 = moderate symptom with 34-66% wilt, 3 = severe foliar symptom with 67-99% wilt, and 4 = dead plant. The disease severity index (DSI) was calculated as follows: DSI (%) = [Σ (P×X) / (M×N)] × 100, where P is the score, X is the number of plants with the same score, M is the total number of plants, and N is the highest score.

Areas under the disease progress curve (AUDPC) were calculated as follows (Campbell & Madden 1990): AUDPC = $\Sigma [(Y_i + Y_{i+1}) / 2] (t_{i+1} - t_i)$, where Y is the average of disease severity, and t is the average of time (day).

The percentage of plant protection was calculated according to Li et al. (1996): PPP = [1 – (X/Y)] × 100 , where X is the amount of AUDPC in treated plants and Y is the amount of AUDPC in control plants.

The disease incidence was calculated from the percentage of infected plants.

Scanning electron microscopy (SEM)

The physical interaction of *Verticillum-Trichoderma* was monitored by scanning electron microscopy. The fungal samples were fixed in 2.5% buffered glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol, and dried in a critical point dryer. The samples were then sputter-coated with gold and examined in a JEOL JSM-840 scanning electron microscope operating at an accelerating voltage of 6 kV.

Statistical analyses

All experiments were performed in triplicates. Comparisons of means were conducted based on analysis of variance (ANOVA). Statistical analyses were done using the Duncan's test with SAS 9.4.

RESULTS

Morphological and molecular identifications of fungi

Fungi isolated from infected stems of stone fruit trees were identified as *V. dahlia,* according to Inderbitzin et al. (2011). Moreover, 46 endophytic and 39 rhizospheric fungal isolates were recovered from stone fruit trees. Eight fungal genera were identified by morphological and molecular studies (Table 1S).

Antagonistic assays

***In vitro* antagonism of fungi in dual culture assay**

The inhibitory data of fungal isolates against *V. dahliae* are shown in Table 1S, as GI, I, and MV.

The highest percentage of mycelia growth inhibition (GI) in *V. dahliae* was 63.7%, obtained by *Aspergillus carbonarius* AE82, followed by *T.harzianum* AE63 (60.3%), *Fusarium proliferatum* AE51 (58.5%) and *A. carbonarius* AE86 (58.02%). Overall, 21% of isolates inhibited the mycelia growth

of *V. dahliae* >50% (Table 1S, Figure 1). All *Trichoderma* isolates in this assay, including *Trichoderma asperellum* AE66, *Trichoderma harzianum* AE65, *T. harzianum* AE69, and *T. harzianum* AE54, inhibited the growth of *V. dahliae.*

***In vitro* effect of fungal non-volatile metabolites on the germination of *V. dahliae* microsclerotia (I)**

As seen for (I) data in Table 1S, after 14 days of incubation in perlite-containing pots, the fungal metabolites inhibited the germination of *V. dahliae* microsclerotia. Here, *Aspergillus carneus* AE88 showed the highest inhibition (86%), followed by *F. oxysporum* AE44 and *Penicillium* sp. AE84 (82%). In total, 17% of the isolates had inhibitory scores >50% in this assay (Table 1S).

Effect of fungal metabolites on the stability of *V. dahliae* microsclerotia in soil (MV)

The antagonistic activity of fungi metabolites on *V. dahliae* microsclerotia stability in soil is reported in Table 1S as (MV). Data indicated that *Trichoderma asperellum* AE66 inhibited *V. dahliae* microsclerotia germination (95%), followed by *F. oxysporum* AE44, *Fusarium* sp. AE43 and *Fusarium solani* AE52 (74%, 72% and 67 %, respectively). In total, 12% of the isolates showed an inhibitory effect >50% (Table 1S).

The most potent antagonistic isolate

The total percentages of inhibition of *V. dahliae* in all experiments are represented as I_T in Table 1S, according to which *T. asperellum* AE66 showed the highest level of inhibition (73.85%), followed by *F. oxysporum* AE44, *P. spinolosum* AE80, and *A. flavus* AE89 (67.05%, 59.04%, and 58.38%, respectively). In general, 8.2% of the isolates showed >50% inhibitory activity (Table 1S).

Suppressive effect of *Trichoderma asperellum* AE66 on the development of *Verticillium* wilt in greenhouse

Symptoms of *Verticillium* wilt, including defoliation and necrosis, were developed after 32 days in peach inoculated with *V. dahliae*. The plants co-inoculated with *T. asperellum* showed less disease severity. Disease severity was evaluated from days 32 to 70. On day 32, the disease severity index (DSI) in the control plants was 18.7%, compared to 6.3% in the plants co-inoculated by both pathogen and *T. asperellum*. On day 70, the control plants showed complete defoliation and necrosis with a DSI of 100% (score 4). However, in comparison, the DSI of the co-inoculated plants was 37.5% (score 2). *T. asperellum* significantly reduced disease incidence, disease severity, and AUDPC, which increased plant protection by 62.17% (Table 2).

Peach growth promotion by *T. asperellum*

The effect of *T. asperellum* on the growth of peach plants is shown in Table 4. *V. dahliae* inoculation ($Control^+$) reduced all growth parameters in comparison with uninfected plants ($Control^-$), especially the stem height and stem wet weight. In comparison with uninfected plants, treatment with *T. asperellum* significantly ($P\leq0.01$) increased all growth parameters except for the root length. In addition, stem height, stem wet weight, root wet weight, and root dry weight values in plants treated with *T. asperellum* were significantly ($P\leq0.01$) higher than those of the plants co-inoculated with *T. asperellum* + *V. dahliae*. On the other hand, except for the root length, all growth parameters in the co-inoculated plants were significantly higher than those of the *V. dahliae*-infected plants (Table 3).

SEM study on *V. dahliae* -*T. asperellum* interaction

As shown in Figure 2, *T. asperellum* AE66 colonized *V. dahliae* hyphae and penetrated the mycelium, which resulted in deformation of the hyphae.

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Table 2. Effects of root treatment with *T. asperellum* on development of *Verticillium* wilt in peach plants grown in soil inoculated with *V. dahliae*

Treatment	Disease parameters			
	Disease incidence (%)	Disease Severity (%)	AUDPC (Area under disease progress curve)	Plant protection (%)
V. dahliae (Control)	95.83 [a*]	100 [a]	2296.5 [a]	0
T. asperellum +*V. dahliae*	54.16 [b]	37.5 [b]	868.75 [b]	62.17

*Different letters indicate significant difference at $P\leq0.01$. Disease parameters were assessed 70 days after inoculation of plants (in soils infected - with 20 microsclerotia per g- *V. dahliae*). The data are the means of three replicates.

Table 3. Effect of *T. asperellum* on growth parameters of peach plants in the presence of *V. dahliae*

Treatment	Growth parameters					
	Stem height	Root length	Stem wet weight	Root wet weight	Stem dry weight	Root dry weight
Non-inoculated ($Control^-$)	114[c*]	24[a]	34.6[c]	16.9[c]	23.46[b]	8.5[c]
V. dahliae ($Control^+$)	105[d]	24[a]	28.55[c]	15.8[c]	22.1[b]	8.2[c]
T. asperellum	145[a]	23[a]	55.7[a]	32.56[a]	32.1[a]	16.6[a]
T. asperellum+ *V. dahliae*	124[b]	24[a]	40[b]	23.5[b]	30.66[a]	13[b]

*Different letters indicate significant difference at $P\leq0.01$. The data are the means of three replicates

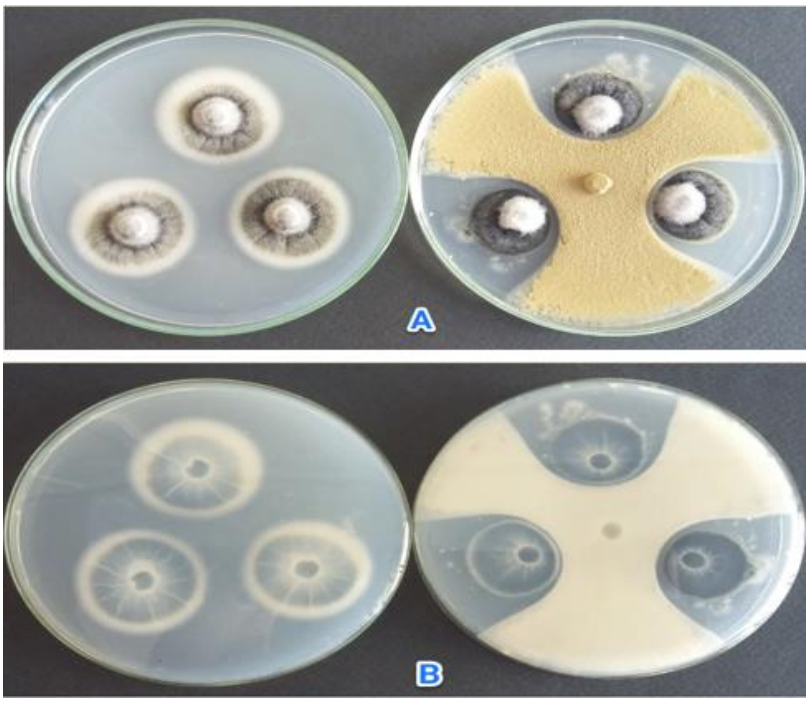

Fig 1. Dual culture of *Penicillium* sp. AE80 and *V. dahliae*. A. Top B. Reverse. The right side plate is control

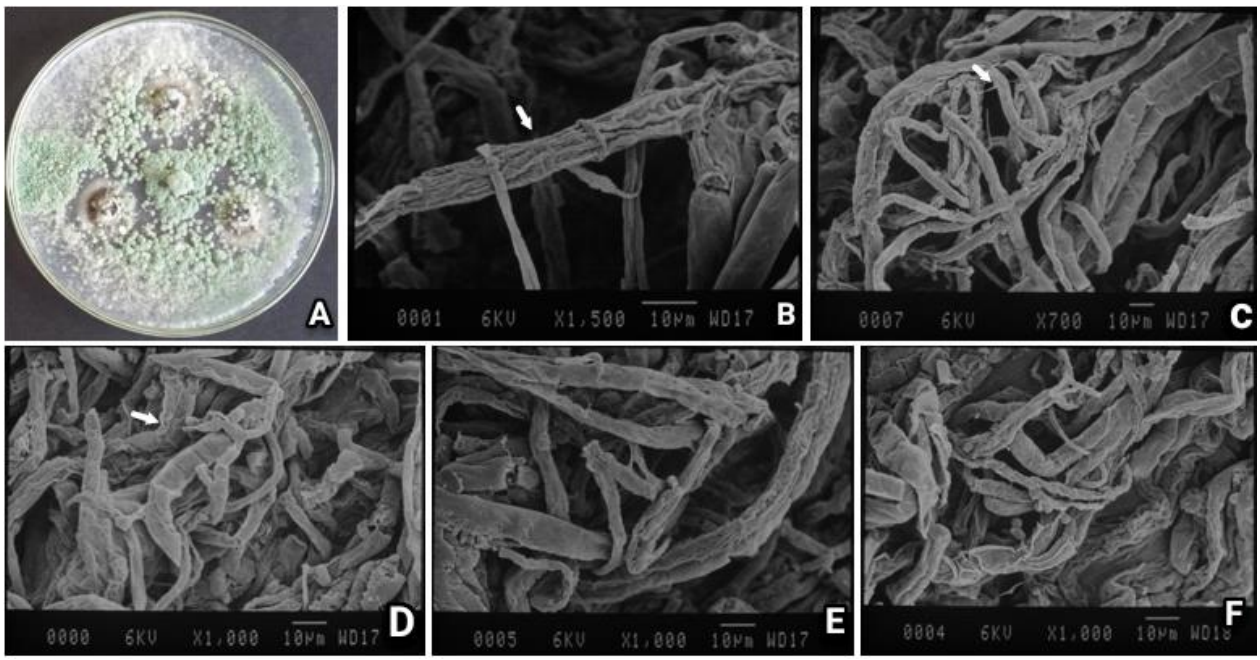

Fig 2. SEM electron micrographs of *T. asperellum* AE66 and *V. dahliae* in dual culture. A. Dual culture in Petri plate. B. *T. asperellum* AE66 (arrow) is twisted around *V. dahliae* hyphae. C. Penetration of *T. asperellum* AE66 mycelium (arrow) into *V. dahliae* hyphae. D, E and F. Deformation of *V. dahliae* hyphae (arrow).

DISCUSSION

Biological control has been recognized as a promising and sustainable disease management strategy in agriculture. It is regarded as being safe for the environment, conserving biodiversity, and lowering chemical use. *Trichoderma* species show high antagonistic activities against plant pathogens and have been successfully used as biocontrol agents to control diverse plant diseases. They also promote plant health and growth.

Here, 15 *V. dahliae* isolates were recovered. Also, 85 antagonistic fungal isolates were obtained, among which *Fusarium* with a frequency of 24.74% and *Trichoderma* with a frequency of 21.18% comprised the most prevalent fungi. The biological control assays carried out in the present study revealed that both endophytic and rhizospheric isolates recovered from stone fruit orchards inhibited *V. dahliae* by different mechanisms, including antagonism and mycoparasitism. Some fungi produced antagonistic metabolites, while others same as the *Trichoderma* isolates parasitized the *V. dahliae* colonies. *In vitro* assays also indicated the high inhibitory activity of *Trichoderma* against *V. dahliae*. The mycelia of *Trichoderma* species interfered physically with the mycelium of *V. dahliae* to inhibit its growth, whereas *T. asperellum* (isolates Bt3 and T25) inhibited the *V. dahliae* in dual cultures by metabolites without physical contact (Carrero-Carron et al. 2016). *T. harzianum* has also been reported to inhibit *V. dahliae in vitro* (Ruano-Rosa et al. 2016).

Although metabolites of *T. asperellum* are effective in antibiosis against various pathogens such as *Rhizoctonia solani*, *Botrytis cinerea*, and *F. oxysporum* (Taghdi et al. 2015), we found no evidence of such an effect against *V. dahliae*. However, metabolites of other fungi, most notably *A. corneus* AE88, *F. oxysporum* AE44, and *Penicillium* sp. AE84 highly inhibited the germination of *V. dahliae* microsclerotia. It is reported that the metabolites could activate the genes encoding hydrolytic enzymes that are destructive for the pathogenic fungus (Lorito et al. 2010). The high inhibitory effect of *T. asperellum* on the germination of *V. dahliae* microsclerotia could be due to its physical interaction with the pathogen, which leads to disruption and deformation of its mycelium (Deketelaere et al. 2017). Therefore, the inhibition of microsclerotia germination could be an effective control strategy for *Verticillium* wilt.

The high biocontrol potential of *T. asperellum* AE66 and *F. oxysporum* AE44 against *V. dahliae* was demonstrated in the total inhibition assay, which is represented as the I_T index. Although *F. oxysporum* isolates have been reported as effective biocontrol agents against *Verticillium* wilt in eggplant (Angelopoulou et al. 2014), pepper (Veloso & Díaz 2012), olive (Varo et al. 2016), cotton (Zhang et al. 2015) and tomato (García et al. 2011), *T. asperellum* AE66 was shown in the present study to be more effective than *F. oxysporum* AE44 in the inhibition of *V. dahliae.*

Other *Trichoderma* isolates, including *T. viride* T46 and T117, have been shown to reduce the disease severity of *Verticillium* wilt in eggplant by 30% (D'Ercole et al. 2000). Also, *Trichoderma* isolates reduced disease severity in tomato, eggplant, and pepper by more than 80% (Narisawa et al. 2002, Slusarski & Pietr 2009). Factors determining the effectiveness of a biocontrol isolate include the host plant variety and age, the accessibility of nutrients, and the nature of the interactions between the biocontrol agent and the pathogen (Deketelaere et al. 2017).

Microsclerotia of *V. dahlia* could penetrate and colonize the xylem of the host plant in 2-4 days (Fradin & Thomma 2006). It was reported that only 5 microsclerotia of *V. dahliae* per g soil was adequate to infect plants (Stapleton et al. 1993). However, in this study, we co-inoculated the biocontrol agent with a four-fold density of the pathogen (20 microsclerotia of *V. dahliae* per g soil). We observed 62% protection in comparison with the infected control plants, which all died.

In several studies, *T. asperellum* reduced the severity of *Verticillium* wilt but had no significant effect on disease incidence (Carrero-Carrón et al. 2016, Varo et al. 2016). However, in strawberries, a decreased incidence of disease was associated with yield enhancement (≥70%) (Berg et al. 2001). In this study, it is observed that *T. asperellum* AE66 reduced incidence and severity of *Verticillium* wilt. Moreover, it promoted the growth of peach plants. Since *T. asperellum* AE66 was isolated from the peach rhizosphere, it could be able to colonize the rhizosphere. It is suggested that for control of disease in the greenhouse, successful root colonization was the most important index for an effective biocontrol agent (Rubio et al. 2014). It is reported that *T. asperellum* T25 had a higher potential for root colonization and control of *Verticillium* wilt than other isolates tested. In contrast, it showed a lower inhibitory effect *in vitro* in comparison with the others (Carrero-Carrón et al. 2016). Therefore, the ecological niche is a significant factor in determining the biocontrol potential of a given strain (Deketelaere et al. 2017).

The present experiment was conducted in non-sterile soil to simulate the field conditions. In sterile soils, there is no competition with natural plant microflora. So, the results are often not reproducible under field conditions (Zhou et al. 2006). When plants are under disease pressure, it is expected that their growth could be stopped as a defense mechanism to conserve energy (Hermosa et al. 2012). Here, under natural conditions, *T. asperellum* AE66 suppressed *Verticillium* wilt and promoted plant growth even under disease pressure. Although there was no

evidence of *V. dahliae* inhibition by *T. asperellum* AE66 metabolites, it was highly effective in suppressing disease in the greenhouse. The inhibition of cortex or xylem colonization and the reduction of the pathogen mycelia growth in the rhizosphere are currently considered the main factors in *Verticillium* wilt suppression (Deketelaere et al. 2017).

In conclusion, this is the first assessment of the antagonistic effects of a broad range of endophytic and rhizospheric fungi from stone fruit orchards against *V. dahliae* in peach trees. We found that *T. asperellum* AE66 was the most effective isolate *in vitro* by several modes of action, including direct physical contact and suppressing microsclerotia germination. Besides, it was the superior strain in suppressing *Verticillium* wilt under natural conditions, which resulted in plant growth promotion. *T. asperellum* AE66 increased the length and biomass of stems and roots even in *V. dahliae*–infected plants. These findings indicate that *T. asperellum* AE66 could be a promising biocontrol agent for *Verticillium* wilt in peach trees. This necessitates further investigations to evaluate its efficacy in peach and other stone fruit trees grown under diverse environmental conditions.

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REFERENECES

Aleandri MP, Chilosi G, Bruni N, Tomassini A, Vettraino AM, Vannini A. 2015. Use of nursery potting mixes amended with local *Trichoderma* isolates with multiple complementary mechanisms to control soil-borne diseases. Crop Protection 67: 269-78.

Angelopoulou DJ, Naska EJ, Paplomatas EJ, Tjamos SE. 2014. Biological control agents (BCAs) of *Verticillium* wilt: influence of application rates and delivery method on plant protection, triggering of host defense mechanisms and rhizosphere populations of BCAs. Plant Pathology 63: 1062–1069.

Aranda S, Montes-Borrego M, Jiménez-Díaz RM, et al. 2011. Microbial communities associated with the root system of wild olives (*Olea europaea* L. subsp. *europaea var. sylvestris*) are good reservoirs of bacteria with antagonistic potential against *Verticillium dahliae*. Plant and Soil 343: 329–345.

Ausher R, Katan J, Ovadia S. 1975. An improved selective medium for the isolation of *Verticillium dahliae*. Phytoparasitica 3: 133-137.

Berg G, Fritze A, Roskot N, Smalla K. 2001. Evaluation of potential biocontrol rhizobacteria from different host plants of *Verticillium dahliae* Kleb. Journal of Applied Microbiology 91: 963–971.

Campbell CL, Madden LV. 1990. Introduction to Plant Disease Epidemiology. John Wiley, New York, USA. 531 p.

Carbone I, Kohn LM. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. Mycologia 91: 553-556.

Carrero-Carrón I, Trapero-Casas JL, Olivares-García C, Monte E, Hermosa R, Jiménez-Díaz RM. 2016. *Trichoderma asperellum* is effective for biocontrol of *Verticillium* wilt in olive caused by the defoliating pathotype of *Verticillium dahliae*. Crop Protection 88: 45-52.

Chandelier A, Laurent F, Dantinne D, Mariage L, Etienne M, Cavelier M. 2003. Genetic and molecular characterization of *Verticillium dahliae* isolates from woody ornamentals in Belgian nurseries. European Journal of Plant Pathology 109: 943–952.

D'Ercole N, Nipoti P, Di Pillo L, Gavina F. 2000. In vitro and in vivo tests of *Trichoderma spp*. as a biocontrol agent of *Verticillium dahliae* Kleb. in eggplants. In: Advances in *Verticillium*: Research and Disease Management; Tjamos EC, Rowe RC, Heale JB, Fravel DR, Eds.; APS Press: St. Paul, MN, USA, 2000; pp. 260–263.

Davet P. 1979. A technique for analyzing soil populations of *Trichoderma* spp. and *Gliocladium virens*. Annual Review of Phytopathology 11: 529-534.

Deketelaere S, Tyvaert L, França SC, Höfte M. 2017. Desirable traits of a good biocontrol agent against *Verticillium* wilt. Frontiers in Microbiology 8: 1186.

FAO 2023. Faostat, Production Statistics. http://faostat. fao.org.

Fradin EF, Thomma BP. 2006. Physiology and molecular aspects of *Verticillium* wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Molecular Plant Pathology 7: 71–86.

García M, Arriagada C, García-Romera I, Ocampo JA. 2011. Are plant cell wall hydrolysing enzymes of saprobe fungi implicated in the biological control of the *Verticillium dahliae* pathogenesis. Crop Protection 30: 85– 87.

Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. Applied and Environmental Microbiology 61: 1323-30.

Hamim A, Miche L, Douaik A, Mrabet R, Ouhammou A, Duponnois R, Hafidi M. 2017. Diversity of fungal assemblages in roots of *Ericaceae* in two Mediterranean contrasting ecosystems. Comptes Rendus Biologies 340: 226-37.

Hawke M, Lazarovits G. 1994. Production and manipulation of individual microsclerotia of *Verticillium dahliae* for use in studies of survival. Phytopathology 84: 883-890.

Hermosa R, Viterbo A, Chet I, Monte E. 2012. Plant-beneficial effects of *Trichoderma* and of its genes. Microbiology 158: 17e25.

Hong SB, Cho HS, Shin HD, Frisvad JC, Samson RA. 2006. Novel *Neosartorya* species isolated

from soil in Korea. International Journal of Systematic and Evolutionary Microbiology 56: 477–486.

Inderbitzin P, Bostock RM, Davis RM, Usami T, Platt HW, Subbarao KV. 2011. Phylogenetics and taxonomy of the fungal vascular wilt pathogen *Verticillium*, with the descriptions of five new species. PLoS one 6: e28341.

Jabnoun-Khiareddine H, Daami-Remadi M, Ayed F, El-Mahjoub M. 2009. Biological control of tomato *Verticillium* wilt by using indigenous *Trichoderma* spp. The African Journal of Plant Science and Biotechnology 3: 26-36.

Leslie JF, Summerell BA. 2006. The Fusarium Laboratory Manual. Ames, Iowa, USA: Blackwell Publishing; 2006. 387 p.

Li J, Zingen-Sell I, Buchenauer H. 1996. Induction of resistance of cotton plants to *Verticillium* wilt and of tomato plants to *Fusarium* wilt by 3-aminobutyric acid and methyl jasmonate. Journal of Plant Diseases and Protection 103: 288-299.

Lorito M, Woo SL, Harman GE, Monte E. 2010. Translational research on *Trichoderma*: from omics to the field. Annual Review of Phytopathology 48: 395-417.

Lurie S, Crisosto CH. 2005. Chilling injury in peach and nectarine. Postharvest biology and technology 37: 195-208.

Maldonado-González MM, Schilirò E, Prieto P, Mercado-Blanco J. 2015. Endophytic colonization and biocontrol performance of *Pseudomonas fluorescens* PICF7 in olive (*Olea europaea L.*) are determined neither by pyoverdine production nor swimming motility. Environmental Microbiology 17: 3139–3153.

Markakis EA, Tjamos SE, Antoniou PP, Paplomatas EJ, Tjamos EC. 2016. Biological control of *Verticillium* wilt of olive by *Paenibacillus alvei*, strain K165. Biocontrol 61: 93–303

Marois J, Johnston S, Dunn M, Papavizas G. 1982. Biological control of *Verticillium* wilt of eggplant in the field. Plant Disease 66: 1166-1168.

Mercado-Blanco J, Rodrıguez-Jurado D, Hervás A, Jiménez-Dıaz RM. 2004. Suppression of *Verticillium* wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. Biological Control 30: 474–486.

Möller E, Bahnweg G, Sandermann H, Geiger H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Research 20: 6115.

Müller H, Berg G. 2008. Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on *Verticillium* wilt in oilseed rape. Biocontrol 53: 905-16.

Naraghi L, Heydari A, Ershad D. 2006. Sporulation and survival of *Talaromyces flavus* on different plant material residues for biological control of cotton wilt caused by *Verticillium dahliae*. Iranian Journal of Plant Pathology 42: 382-397.

Narisawa K, Kawamata H, Currah RS, Hashiba T. 2002. Suppression of *Verticillium* wilt in eggplant by some fungal root endophytes. European Journal of Plant Pathology 108: 103–109.

Prieto P, Navarro-Raya C, Valverde-Corredor A, Amyotte SG, Dobinson KF, Mercado-Blanco J. 2009. Colonization process of olive tissues by *Verticillium dahliae* and its greenhouse interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. Microbial Biotechnology 2: 499–511.

Royse DJ, Ries SM. 1978. The influence of fungi isolated from peach twigs on the pathogenicity of *Cytosporacinata*. Phytopathology 63: 603- 607.

Ruano-Rosa D, Prieto P, Rincón AM, Gómez-Rodríguez MV, Valderrama R, Barroso JB, et al. 2016. Fate of *Trichoderma harzianum* in the olive rhizosphere: time course of the root colonization process and interaction with the fungal pathogen *Verticillium dahliae*. Biocontrol 61: 269–282.

Rubio MB, Quijada NM, Perez E, Domínguez S, Monte E, Hermosa R. 2014. Identifying *Trichoderma parareesei* beneficial qualities for plants. Applied and Environmental Microbiology 80: 1864e1873.

Samson RA. 1994. Taxonomy—Current Concepts of *Aspergillus* Systematics. In: Smith JE (eds) *Aspergillus*. Biotechnology Handbooks, vol 7. Springer, Boston, MA.

Samuels GJ, Ismaiel A, Mulaw TB, et al. 2012. The Longibrachiatum clade of *Trichoderma*: a revision with new species. Fungal Diversity 55: 77–108.

Sanei SJ, Razavi SE. 2011. Suppression of *Verticillium* wilt of olive by *Pseudomonas fluorescens*. Journal of Experimental Agriculture International 8: 294-305.

Schroers HJ. 2001. A monograph of *Bionectria* (Ascomycota, Hypocreales, Bionectriaceae) and its *Clonostachys* anamorphs, Centraalbureau voor Schimmelcultures. 214 pp.

Simmons EG. 2007. *Alternaria*: An Indentification Manual. CBS Biodiversity Series 6. 775 pp.

Slusarski C, Pietr SJ. 2009. Combined application of dazomet and *Trichoderma asperellum* as an efficient alternative to methyl bromide in controlling the soil-borne disease complex of bell pepper. Crop Protection 28: 668–674.

Stapleton J, Paplomatas E, Wakeman R, De Vay J. 1993. Establishment of apricot and almond trees using soil mulching with transparent (solarization) and black polyethylene film: effects on *Verticillium* wilt and tree health. Plant Pathology 42: 333-338.

Taghdi Y, Hermosa R, Dominguez S, Rubio MB, Essalmani H, Nicolas C, Monte E. 2015. Effectiveness of composts and *Trichoderma* isolates for control of *Fusarium* wilt of tomato. Phytopathologia Mediterranea 1: 232-40.

Talboys P. 1960. A culture-medium aiding the identification of *Verticillium albo-atrum* and *V. dahliae*. Plant Pathology 9: 57-58.

Tjamos EC, Fravel DR. 1997. Distribution and establishment of the biocontrol fungus *Talaromyces flavus* in soil and on roots of solanaceous crops. Crop Protection 16: 135–139.

Tjamos EC, Biris DA, Paplomatas EJ. 1991. Recovery of olive trees from *Verticillium* wilt after individual application of soil solarization in established olive orchards. Plant Disease 75: 557-562.

Varo A, Raya-Ortega MC, Trapero A. 2016. Selection and evaluation of micro-organisms for biocontrol of *Verticillium dahliae* in olive. Journal of Applied Microbiology 121: 767–777.

Veloso J, Díaz J. 2012. *Fusarium oxysporum* Fo47 confers protection to pepper plants against *Verticillium dahliae* and *Phytophthora capsici*, and induces the expression of defence genes. Plant Pathology 61: 281–288.

Visagie CM, Houbraken J, Frisvad JC, Hong SB, Klaassen CH, Perrone G, Seifert KA, Varga J, Yaguchi T, Samson RA. 2014. Identification and nomenclature of the genus *Penicillium*. Studies in Mycology 78: 343-71.

White TJ, Bruns T, Lee SJWT, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications 18: 315–322.

Zhang Q, Yang L, Zhang J, Wu M, Chen W, Jiang D, et al. 2015. Production of anti-fungal volatiles by non-pathogenic *Fusarium oxysporum* and its efficacy in suppression of *Verticillium* wilt of cotton. Plant and Soil 392: 101–114.

Zhou L, Hu Q, Johansson A, Dixelius C. 2006. *Verticillium longisporum* and *V. dahliae*: infection and disease in *Brassica napus*. Plant Pathology 55: 137–144.

کنترل بیولوژیکی پژمردگی ورتیسیلیومی و افزایش رشد هلو توسط قارچ‌های اندوفیت و ریزوسفری از درختان میوه هسته‌دار

ابوطالب اسماعیل زاده[۱]، دوستمراد ظفری[۱]، مهرویه کتابچی [۱]✉، راحله آل‌طه[۲]، علیرضا نوریان[۳]

۱- گروه گیاهپزشکی، دانشکده کشاورزی، دانشگاه بوعلی سینا، همدان، ایران

۲-گروه علوم خاک، دانشکده کشاورزی، دانشگاه بوعلی سینا، همدان، ایران

۳-گروه پاتوبیولوژی، دانشکده علوم دامپزشکی، دانشگاه بوعلی سینا، همدان، ایران

چکیده: پژمردگی ورتیسیلیومی درختان میوه هسته دار ناشی از *Verticillium dahliae* در سراسر جهان رخ می‌دهد و باعث خسارات اقتصادی جدی می‌شود. کنترل ورتیسیلیوم به دلیل دامنه میزبانی وسیع و میکرواسکلروت‌های خاکزاد مقاوم آن دشوار و پرهزینه است. همچنین، افزایش نگرانی‌ها در مورد سموم شیمیایی کشاورزی، باعث توسعه استراتژی‌های کنترل زیستی شده است. در این مطالعه، قارچ‌های آنتاگونیست برای کنترل بیولوژیکی *V. dahliae* در شرایط آزمایشگاهی و گلخانه‌ای ارزیابی شدند. در مجموع ۸۵ جدایه قارچی اندوفیت و ریزوسفری از هلو و سایر درختان هسته‌دار در استان آذربایجان غربی، ایران، جدا شدند. قارچ‌های شناسایی شده شامل *Alternaria*، *Aspergillus*، *Aureobasidium*، *Clonostachys*، *Cryptococcus*، *Fusarium*، *Penicillium* و *Trichoderma* بودند. توان کنترلی جدایه‌ها ابتدا با روش کشت متقابل ارزیابی شد. علاوه بر این، فعالیت آنتاگونیستی متابولیت‌های قارچی بر جوانه‌زنی میکرواسکلروت‌ها هم در شرایط آزمایشگاهی و هم در خاک مورد ارزیابی قرار گرفت. در مجموع، جدایه *Trichoderma asperellum* AE66 با ۷۳/۸۵ درصد بیشترین بازدارندگی را داشت و برای آزمایش گلخانه‌ای انتخاب شد. در آزمایش گلخانه‌ای روی هلو، *T. asperellum* AE66 نه تنها وقوع بیماری، بلکه شدت پژمردگی را نیز کاهش داد و باعث افزایش رشد گیاه شد. این یافته‌ها نشان می دهد که کنترل بیولوژیک، استراتژی بالقوه موثری برای مدیریت پژمردگی ورتیسیلیومی در درختان میوه هسته‌دار است.

کلمات کلیدی: ارزیابی بیماری، قارچ‌های اندوفیت، قارچ‌های ریزوسفری، درختان میوه هسته‌دار، *Trichoderma asperellum*

مکاتبه کننده: مهرویه کتابچی Email: mahru.ketabchi@gmail.com

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