Study of Antagonistic Effects of Trichoderma Species on Growth of Verticillium dahliae, the Causal Agent of Verticillium Wilt of Pistachio under Laboratory Condition

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Abstract
Verticillium wilt is a serious disease of pistachio caused by Verticillium dahliae. Control of the disease is difficult due to soil borne nature of the causal agent. Verticillium wilt has been biologically controlled by Trichoderma spp. In the present study, Trichoderma spp. was isolated from soils of pistachio orchards and their effect was investigated on radial growth of Verticillium dahliae by using dual culture and production of volatile and non-volatile metabolites. Five isolates of T. harzianum and one isolate of T. asperellum, T. koningii and T. crassum were used in the present study. All the isolates of Trichoderma produced volatile and non-volatile metabolites that reduced radial growth of the pathogen. T. harzianum 30 and T. koningii isolates had the highest effect on radial growth of the pathogen but T. harzianum 42 and T. crassum showed the lowest effect in non-volatile test. Six days after culture of pathogen on the medium was the best time for study of effect of the non-volatile metabolites. In volatile metabolite test, T. harzianum 85 and T. koningii reduced radial growth of V. dahliae further than others. Overall, T. harzianum 30, 85 and T. koningii isolates showed the highest inhibitory effect of radial growth of V. dahliae.

Keywords: Antagonistic Effects, Pistachio, Trichoderma, Verticillium dahliae.

Introduction
Verticillium dahliae Kleb. is one of the most important pathogens that limit planting of pistachio trees in some countries such as the United States. The use of biological control methods is one of the best ways for control of soil-borne diseases caused by fungi. One of the most important biocontrol agents against pathogenic fungi is Trichoderma (Cohen-Kupiec and Chet, 1998). Various species of Trichoderma has been reported as antagonist agents against various pathogens due to soil-borne properties and rapid establishment in soil (Mohammadi, 2003).

In an investigation, systemic resistance in cotton caused by seed treatment with strains of T. virens and decreased the severity of Verticillium wilt (Linda and Hanson, 2000). In another study, it was observed that three species of T. koningii, F. solani and A. alternata could colonize microsclerotia of V. dahliae (Grunden et al., 2001). Furthermore three species of Trichoderma including T. viride, T. virens and T. harzianum prevented the activity of microsclerotia and reduced the mycelial growth of Verticillium wilt in tomato by production of metabolites and volatile compounds (Jabnoun-Khiareddine et al., 2009).

Materials and Methods
Dual culture of Trichoderma isolates and Verticillium dahliae
In this experiment, the ability of Trichoderma species was examined on the growth inhibitory of V. dahliae in culture medium based on Jabnoun-Khiareddine et al (2009). For this purpose, mycelia disks (5 mm in diameter) of V. dahliae was cultured in the center of the 10-cm Petri dish containing PDA (Potato Dextrose Agar) medium. Trichoderma species were cultured in both sides of colonies of V. dahliae after colony diameter reached to 1.5 cm. Petri dishes were incubated at 25 °C. Then, the isolates were selected with inhibitory effect on radial growth of V. dahliae.

For study of effects of secondary metabolites (Non-volatile) produced by different species of Trichoderma on mycelial growth of Verticillium dahliae, erlenmeyer flasks (250 ml) containing 100 ml PDB (Potato Dextrose Broth) were sterilized and four mycelial disks (5 mm in diameter) of the 3-day colonies of Trichoderma species were placed in each flask. Four mycelial disks were added to PDA medium in Erlenmeyer flask of control. Erlenmeyer flasks were shaken in a rotary shaker at 90 rpm for 10 days. The contents of each Erlenmeyer flask were passed through two layers of sterile filter paper by Buchner funnel and vacuum pump for separation of solid particles in a liquid medium. Then prepared liquid was passed through the milipore filter (0.2 µ in diameter) for sterilization. This extraction was mixed into ratios of 10, 20 and 30% with PDA medium and was added in Petri dish (Haghdeh, 2009). Then, a mycelium disk (5 mm in diameter) of the V. dahliae was cultured in center of each Petri dish. After 7 days, colony diameter of Verticillium was recorded. Percentage of inhibition of Trichoderma extraction on vegetative growth of Verticillium was calculated based on the following formula:

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A: The average diameter of the control colony
B: The average diameter of the treatment colony
C: The average diameter of the control colony

For study of volatile compounds effects on the growth of V. dahliae, a mycelial disk (5 mm in diameter) of the pathogen was cultured in center of 8-cm Petri dishes containing PDA medium. After the colonies diameter reached to 1 cm, a mycelial disk (5 mm in diameter) of Trichoderma was cultured in center of another Petri dish. After removing the lids of the Petri dishes under sterile conditions, two Petri dishes were placed on each other which Trichoderma plates were placed below and the around of Petri dishes was completely blocked by Para film. The Petri dishes were incubated at 25 °C. Then, the radial growth of V. dahliae was measured after 48, 72 and 96 hours. Eventually, percentage of inhibition of volatile compounds produced by Trichoderma species on growth of V. dahliae was calculated as described above (Haghdel, 2009).

Results

The effect of 10, 20 and 30% of secondary metabolites of Trichoderma isolates has been showed in (Fig. 1). All ratios of the metabolites reduced radial growth of V. dahliae. T. harzianum 30 and T. harzianum 42 isolates showed the highest and lowest inhibition of radial growth, respectively. T. crassum and T. harzianum 42 had the lowest effect on growth of the pathogen. These results showed that the ability of secondary metabolites production is different in Trichoderma species and isolates.

Maximum inhibition of radial growth of the pathogen was observed at sixth day after inoculation of V. dahliae (Fig 2). The difference between the sixth days with other days for radial growth inhibition was significant only in T. harzianum 30, 85, 36, 82 and T. koningii isolates. The percentage of radial growth inhibition had no significant difference between Trichoderma isolates after 2-days. So, this time is not sufficient for study the effects of Trichoderma metabolites on radial growth of V. dahliae. This result was agreed well with Henni (1987) and El Rafai et al. (2003). One reason for this could be increase of secondary metabolites produced by Trichoderma isolates over the time.
Inhibition of radial growth of *Verticillium dahliae* at different times (2, 4 and 6 days) of pathogen culture on medium containing non-volatile metabolites of *Trichoderma* isolates.

The ability of volatile compounds production was different in *Trichoderma* species and isolates (Fig. 3). Volatile compounds were reduced radial growth of pathogen significantly at sixth day compare with other times. The difference was not significant in *T. crassum* and *T. harzianum* isolates. It seems that these two strains have been little ability in production of volatile compounds and inhibition of pathogen growth. Etebarian et al. (2003) reported that volatile compound produced by *T. virens* can inhibit growth of *Phytophthora drechsleri*.

Fig. 2. Effect of volatile secretions of *Trichoderma* isolates on inhibition of mycelial growth of *Verticillium dahliae* under laboratory condition.
Discussion
The present research showed Trichoderma species from soils of pistachio orchards can produce volatile and non-volatile compounds. Exactly similar to non-volatile, these compounds inhibit radial growth of pathogenic fungi (Dennis and Webster, 1971; Jahnoun-Khiareddine et al., 2009). The present study showed that antagonistic activity of T. harzianum 85 and T. koningii can reduce radial growth of V. dahliae more than other isolates and species. It seems that these species can be used for the control of Verticillium wilt disease in greenhouse and field conditions.

Several volatile compounds included Lactones, Alcohols, Terpene derivatives and α-Pierion derivatives were obtained from Trichoderma species under different culture conditions (Zeppa et al., 1991). As well as volatile compounds, lipids and amino acids of T. harzianum have been isolated and reduced radial growth of pathogenic fungi (Zeid et al., 1998).

It seems that the ability of radial growth inhibition of V. dahliae is different among isolates and species of Trichoderma. So for biological control of verticillium wilt, volatile and non-volatile compounds production were examined in collected isolates of Trichoderma.

References