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ORIGINAL ARTICLE

Effects of Arbuscular Mycorrhizae and *Verticillium dahliae* on Activity of Antioxidant Enzymes in Two Pistachio Rootstocks

Sakineh Jamali Paghaleh[1], Nasser Radman[2], Amir Hossein Mohammadi[*3], Mahdi Pirnia[2], Abdol Hossein Taheri[4]

[1]*Ph.D student in Plant Pathology, University of Zabol, Zabol, Iran*

[2]*Associate Professor, Department of Plant Protection, University of Zabol, Zabol, Iran*

[3]*Pistachio Research Center, Horticultural Sciences Research Institute, Agricultural Research, Education and Extension Organization (AREEO), Rafsanjan, Iran*

[4]*Associate Professor, Department of Plant Protection, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran*

K E Y W O R D S

Biological control;
Mycorrhizal symbiosis;
Resistant;
Verticillium wilt

A B S T R A C T

Inoculations of plant roots with arbuscular mycorrhizae can reduce Verticillium wilt severity. In present research, the effect of inoculation of *Verticillium dahliae* (Vd) and three species of arbuscular mycorrhizae (AM), *Funneliformis mosseae*, *Rhizophagus irregularis* and *Claroideoglomus tunicate* were studied on the activity of catalase, peroxidase, superoxide dismutase and phenylalanine ammonia-lyase in the root of Ahmad Aghaei and Badami Zarand pistachio rootstocks. The roots of the pistachio seedlings were inoculated with 100 propagules per gram of three species arbuscular mycorrhizae and two months later, *Verticillium dahliae* was inoculated. Measurement of enzyme activity was done after *V. dahliae* inoculation at 11 different times. The experiment was conducted as a factorial in a completely randomized design with 5 replications. The results showed that in Vd treatment, the specific activity of enzymes in Ahmad Aghaei decreased after an increasing period in days 8 to 12 after vd inoculation, then decreased less than AM inoculations, but in Badami Zarand, the activity of enzymes increased in a (days 8-16) and after that, despite the decreasing trend, it was still higher than AM treatment. In AM+Vd treatment, enzyme activity increased faster in both pistachio rootstocks compared to other treatments. In Badami Zarand, the time of the enzymes' peak activity was longer than in Ahmad Aghaei rootstocks. In general, the results of the present research indicated that increasing antioxidant enzyme activity can reduce the severity of Verticillium wilt in pistachio seedlings.

Introduction

Pistachio (*Pistacia vera* L.) is a very important horticultural product that is mainly traded as dry nuts and being third in Iran export products (Alipour, 2018; Sharifkhah *et al*., 2020; Karamozian *et al*., 2021; Nazoori *et al*., 2022). In 2020, 387000 tons of pistachio was produced from about 534000 ha of pistachio

[*]**Corresponding author**: Email address: ah-mohammadi@pri.ir
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cultivation area in Iran (Ahmadi *et al*., 2020). Pistachio industry has been affected by abiotic and biotic stresses (Behzadi Rad *et al.*, 2021; Mohit Rabari *et al*., 2023). Verticillium wilt is a very serious disease of the pistachio trees reported in the US, Greece, Iran, and Spain (Mohammadi *et al*., 2007; Epstein *et al*., 2004; Mohammadi; Banihashemi, 2008; Moral *et al*., 2010). In Iran, Kerman, Semnan, Fars, Qazvin, and Khorasan Razavi provinces are infected with this disease (Mohammadi and Banihashemi, 2008; Mohammadi *et al*., 2007). The causal agent of the disease, *Verticillium dahliae*, is a very serious plant pathogen, with a wide host range in vegetables, legumes, forest trees, weeds, and herbaceous plants (Kowalska, 2021). The pathogen affects the plants' water-nutrient transfer process by blocking the xylems (Klosterman *et al*., 2009; Shaban *et al*., 2018), causing wilting symptoms, yellowing of the leaves and, finally, the plant death (Temple *et al*., 1973; Sanei and Razavi, 2017). Controlling the Verticillium wilt disease is quite difficult and costly, the reasons for which are the monoculture production, the inefficiency of soil fumigation methods, and production of a large number of microsclerotia which resistant to chemical fungicides (Kowalska, 2021). Since fungicides are ineffective in controlling the Verticillium wilt, the use of biological control agents has become very important (Kowalska, 2021).

Arbuscular mycorrhizae (AM) are useful soil microorganisms that play a key role in soil fertility and health because they have symbiotic relationships with the plant root (Behrooz *et al*., 2019; Giovannini *et al*., 2020). They can increase the macro/micronutrients and water absorption (Jamali Paghaleh, *et al*., 2022) and protect the plant against environmental stresses such as salinity, drought, and pathogens (Mercado-Blanco, 2012; Zahedi *et al*., 2022) and, hence, reduce the need for fungicides (Fred & Davies, 2000). Many studies have shown that arbuscular mycorrhizae can reduce Verticillium wilt damage in pistachio (Jamali Paghaleh, *et al*., 2022), strawberries (Sowik *et al*., 2016), cotton (Norouzi *et al*., 2011) and pepper (Garmendia *et al*., 2006). In mycorrhizal plants, the biological protection against soil-borne pathogens can be due to the host's defense system activation, including structural variations and accumulation of pathogenicity-related proteins (Garmendia *et al*., 2006; Sowik *et al*., 2016). Inoculation of *V. dahliae* in pepper has resulted in the production of new isoforms of acidic chitinases, superoxide dismutases (SOD), peroxidases, and phenylalanine ammonia-lyase (PAL) (Garmendia *et al.*, 2006).

Improving the efficiency of pistachio rootstocks is needed by the industry (Vahdati *et al.*, 2021). In this study, we investigated changes in specific activities of the antioxidant enzymes in the roots of two pistachio rootstocks inoculated with a mixture of three species of arbuscular mycorrhizae and *V. dahliae*.

Materials and methods

Biological material and growth condition

The isolate of *Verticillium dahliae* used in this research was previously isolated by Mohammadi *et al.* (Mohammadi *et al.*, 2007) from pistachio trees in Rafsanjan. This isolate (Accession number: **OL361776)** is registered in the National Collection of Living Fungi of Iran under the **IRAN 2432C** Code. For Inoculum production the method proposed by Sowik *et al.* (2016) was used, with minor changes. Fifty ml of 3% malt extract (Merck) was added to 250 ml flasks and sterilized at 121°C under 15 lb/inch^2 pressure for 20 minutes. Five discs (6 mm in diameter) from the edge of the 14-days-old *V dahliae* colonies were transferred to the flasks and kept at 25°C for 21 days. Fungal mycelium was mixed with the liquid medium and the contents of each flask were added to a sterilized mixture of washed sand-corn flour (2:10:1). This mixture was kept at 25°C for one month. A mixture of three species of arbuscular mycorrhizal fungi (*Funneliformis mosseae*

(Syn: *Glomus mosseae*), *Rhizophagus irregularis* (Syn: *G. intraradices*) and *Claroideoglomus etunicatum* (Syn: *G. etunicatum*) was prepared from Zistfanavar Pishtaz Varian (ZPV) Co. as a commercial product named **MycoRoot,** which contained at least 100 active propagules per gram (with equal proportions of each species).

Kernels of Ahmad Aghaei and Badami Zarand pistachio cultivars (susceptible and tolerant to verticillium wilt, respectively) (Hadizadeh and Banihashemi, 2014) were surface sterilized in 10% sodium hypochlorite solution for 10 min, washed three times and soaked in sterile distilled water for 7 hr.

Inoculation of arbuscular mycorrhizal fungi, Verticillium dahliae, and Experiment design

The planting bed contained virgin soil-sand mixture (1:1) which was pasteurized for 2 hr at 95°C. In each 3 kg pot, the planting bed was mixed with 5% (w/w) of a mixture of arbuscular mycorrhizal fungi propagules and then 7 pistachio seeds were sown and the final weight of pots was to 2 kg. Irrigation of the pots was done using tap water and up to the FC border. Pots were kept greenhouse under 25 ± 3°C temperature and 6000 lux light intensity (combined natural-artificial, 16 hr light 8 hr darkness). After germination of seeds, 4 uniform seedlings were kept in each pot for eight weeks and the rest were removed, with their roots, from the soil. Inoculation of *V. dahliae* was done according to the method proposed by Huang *et al.,* (2006) with minor changes. At the bottom of 4-kg pots, 600 gr of planting bed containing 7.5% and 5% (w/w) of arbuscular mycorrhizal fungi and *V. dahliae* were added and the pistachio seedlings, with the roots and surrounding soil, were transferred to the pots.

The experiment was conducted as factorial in a randomized complete design with 5 replications. The factors contained inoculations of roots with arbuscular mycorrhizae (AM), *V. dahliae* (Vd), and arbuscular mycorrhizae-pathogen interactions (AM + Vd). To determine the enzyme activity changes, plants were harvested at 11 different times after pathogen inoculation.

For extraction of protein from root tissues (0.5 g), three ml of extraction buffer, containing 100 mM (citric acid/Na_2HPO_4) with pH 6.8, 8% (w/v) PVP (Polyvinylpyrrolidone), 1 mM DTT (Dithiothreitol), 1 mM PMSF Phenylmethylsulfonyl fluoride) and 0.1% Triton X-100 was used. Root extract was centrifuged at 12,000 g for 40 min at 4°C and the supernatant was used to assay the enzymes.

For determination of catalase (CAT) activity 500 µl of root extract was mixed with 1 ml Phosphate buffer (pH: 7) containing 33 mM H_2O_2. The decrease in absorbance of H2O2 at 240 nm was recorded for 1 min, immediately (Aebi, 1984).

Guaiacole peroxidase (GPX) activity was assayed based on the Polle *et al* (1994) method. Three ml of phosphate buffer 100 mM (pH=7) containing 20 and 20 mM guaiacole and H_2O_2 was mixed with 50 µl root extraction. An increase in absorbance was recorded at 436 nm for 3 min in 30 s intervals.

Fifty µl enzyme extract mixed with 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 2 µM riboflavin, and 0.1 mM EDTA. After adding riboflavin, the final volume of the reaction mixture was increased to 3 ml. Tubes were shaken and placed 30 cm below two 40 W fluorescent lamps. The reaction was started by switching on the light and was allowed to run for 10 min during which time it was found earlier to be linear. The reaction was stopped by switching off the light and the tubes were covered with a black cloth. The absorbance by the reaction mixture at 560 nm was read. (Dhindsa *et al.,* 1981)

For determination of Phenylalanine ammonia-lyase (PAL) activity, 100 mmol L^{-1} Tris-HCl buffer (pH= 8.5), 1 mmol L^{-1} 2-mercaptoethanol, 15 mmol L^{-1} L-phenylalanine were added to 100 µl enzyme extract. The

reaction mixture was incubated at 30 °C for 15 min, and the reaction was terminated by the addition of 6 mmol L^{-1}HCl and then measured at 290 nm (Godwin *et al*., 1996). Data were analyzed using SAS Software to compare the mean values, and Duncan's multiple range test at the 5% probability level was used.

Results

The percentage colonization of stem by the pathogen (Vd) and pathogenicity index in both pistachio rootstocks increased with increasing time after pathogen inoculation. In all sampling times, these characteristics were significantly higher in Ahmed Aghaei than in Badami Zarand (Jamali *et al*., 2022). Inoculation of AM in both pistachio rootstocks significantly decreased the stem colonization percentage by the pathogen (Vd) and the pathogenicity index compared to AM + Vd treatment (Jamali *et al*., 2022).

Catalase (CAT) activity

In AM treatment, specific activity of the catalase enzyme (CAT) increased during the experiment in both pistachio rootstocks; in the Ahmad Aghaei rootstock, this increase was significant on days 84 and 98, and in Badami Zarand rootstock, it started after day 35 (Fig. 1). In Vd treatment (*V. dahliae* inoculation) the CAT activity in Ahmad Aghaei rootstock, showed a significant increase ($p \leq 0.05$) only on days 8 and 12 after pathogen inoculation compared to AM treatment, after which it decreased. From day 28, the decrease in the CAT activity was significant compared to AM treatment, but in the Badami Zarand rootstock, the CAT activity highly increased from day 4 compared to AM and it reached the maximum level on days 8 to 16 after the pathogen inoculation. From day 28 to the end of the experiment CAT activity decreased but it was higher than AM treatment. In the AM + Vd treatment, the CAT-specific activity in Ahmad Aghaei rootstock increased significantly from day 8 after the pathogen inoculation compared to the other two treatments, and reached its highest level on days 12 to 20, after which the CAT activity showed a decreasing trend, but still much higher than the AM treatment. In the Badami Zarand rootstock too, the CAT activity highly increased from day 4, reaching the highest on days 8 to 35 after pathogen inoculation. The CAT activity decreased from day 42 to the end of the experiment but it was higher than the other two treatments.

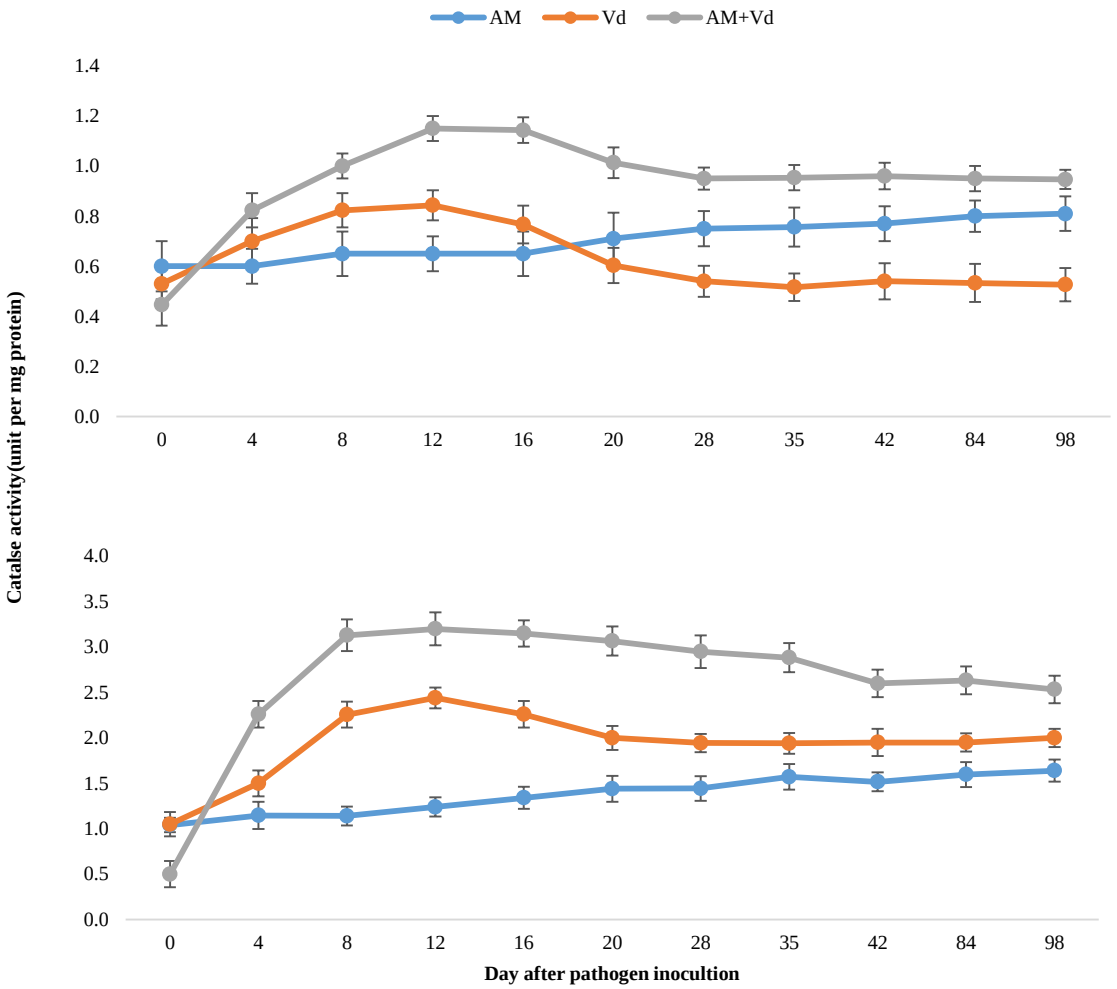

Fig. 1. Specific activity of catalase (CAT) after impregnation of root with arbuscular mycorrhizae and inoculation with *Verticillium dahliae* in Ahmad Aghaei and Badami Zarand pistachio rootstocks. LSD ($P \leq 0.05$)= 0.56

Guaiacol peroxidase (GPX) activity

The specific activity of the guaiacol peroxidase (GPX) enzyme in the AM treatment, increased significantly from day 84 in Ahmed Aghaei rootstock and from day 35 after the pathogen inoculation in Badami Zarand rootstock until the end of the experiment (Fig. 2). In the *V. dahliae* inoculation treatment, the highest GPX specific activity was on day 12 after inoculation in Ahmad Aghaei rootstock, after which it showed a decreasing trend and reached its lowest value on day 28 after inoculation compared to AM treatment. In the Badami Zarand rootstock, the GPX activity highly increased on days 8 and 12 after pathogen inoculation, after which it showed a decreasing trend, but it was still higher compared to AM treatment. In AM + Vd treatment, the GPX-specific activity increased in Ahmad Aghaei rootstock from day 8 compared to other treatments and reached its highest during days 12-20 after the pathogen inoculation; the enzyme activity decreased after day 35, but it was still higher than AM treatment. In Badami Zarand rootstock, the GPX activity increased from day 4 after pathogen inoculation compared to others and reached its highest value from days 8-20 after pathogen inoculation. It showed a relatively stable trend until the end of the experiment.

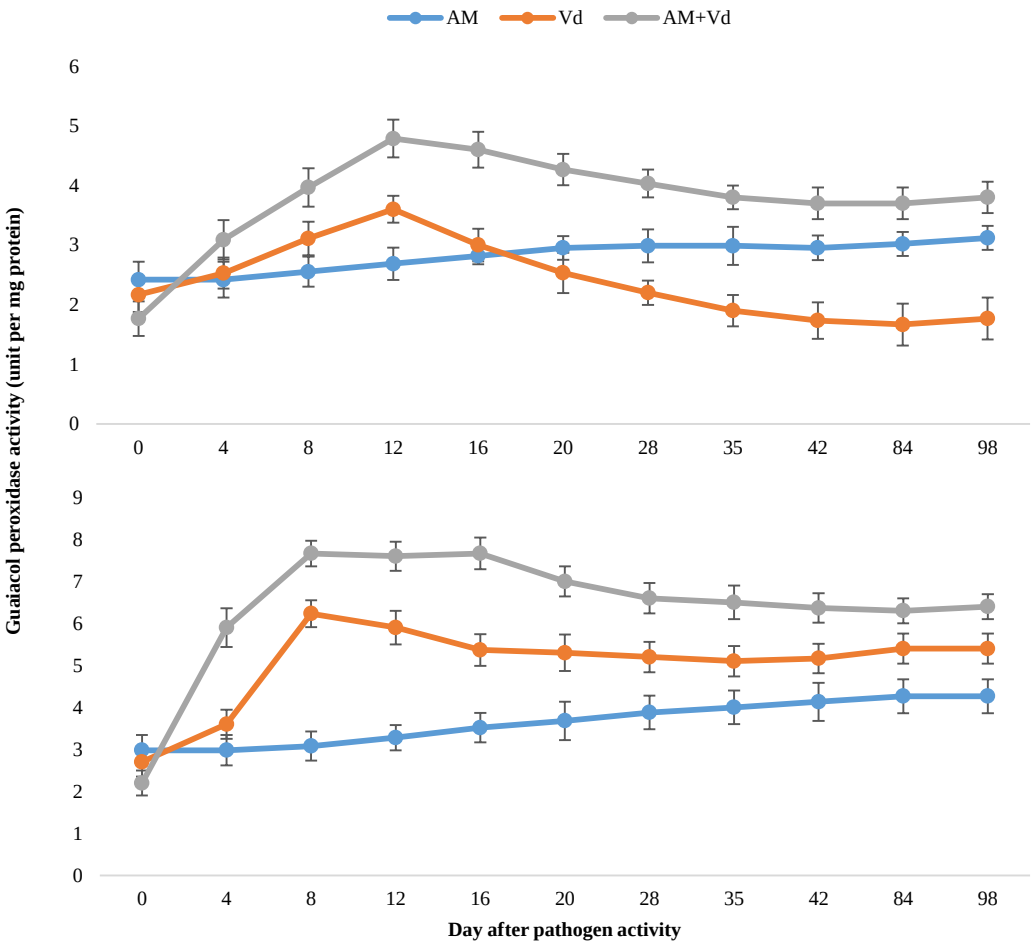

Fig. 2. Specific activity of Guaiacol peroxidase (GPX) after impregnation of root with arbuscular mycorrhizae and inoculation with *Verticillium dahliae* in Ahmad Aghaei and Badami Zarand pistachio rootstocks. LSD ($P \leq 0.05$)= 0.53

Superoxide dismutase (SOD) enzyme

In AM treatment, the specific activity of the superoxide dismutase (SOD) enzyme increased during the experiment (Fig. 3). In Ahmed Aghaei rootstock maximum activity was observed at the end of the experiment, and in the Badami Zarand rootstock it was on days 42 and 98 after pathogen inoculation compared to the starting time (day zero). In the *V. dahliae* inoculation treatment, the specific activity of SOD highly increased in Ahmad Aghaei rootstock on day 8 after pathogen inoculation compared to AM treatment and then decreased; from day 20 after pathogen inoculation until the end of the experiment, the enzyme activity was much lower than AM treatment. In Badami Zarand rootstock, the SOD activity highly increased from day 4 and reached its highest value on days 8-16 after pathogen inoculation. From day 20 after pathogen inoculation until the end of the experiment, the enzyme activity was much lower than the peak days, but was still higher than AM treatment. In AM+Vd treatment, a significant increase in the SOD activity compared to other treatments was observed on days 8 and 4 in Ahmad Aghaei and Badami Zarand, respectively. On day 4 after pathogen inoculation. In the Ahmed Aghaei rootstock, the highest SOD activity was during days 8-

20 after pathogen inoculation; the trend was then decreasing, but still higher than the other treatments. In Badami Zarand rootstock, the SOD activity peak was on days 8-35 after pathogen inoculation. A significant reduction of SOD activity was observed from day 42 until the end of the experiment, but it was much higher than in AM and *V. dahliae* treatments.

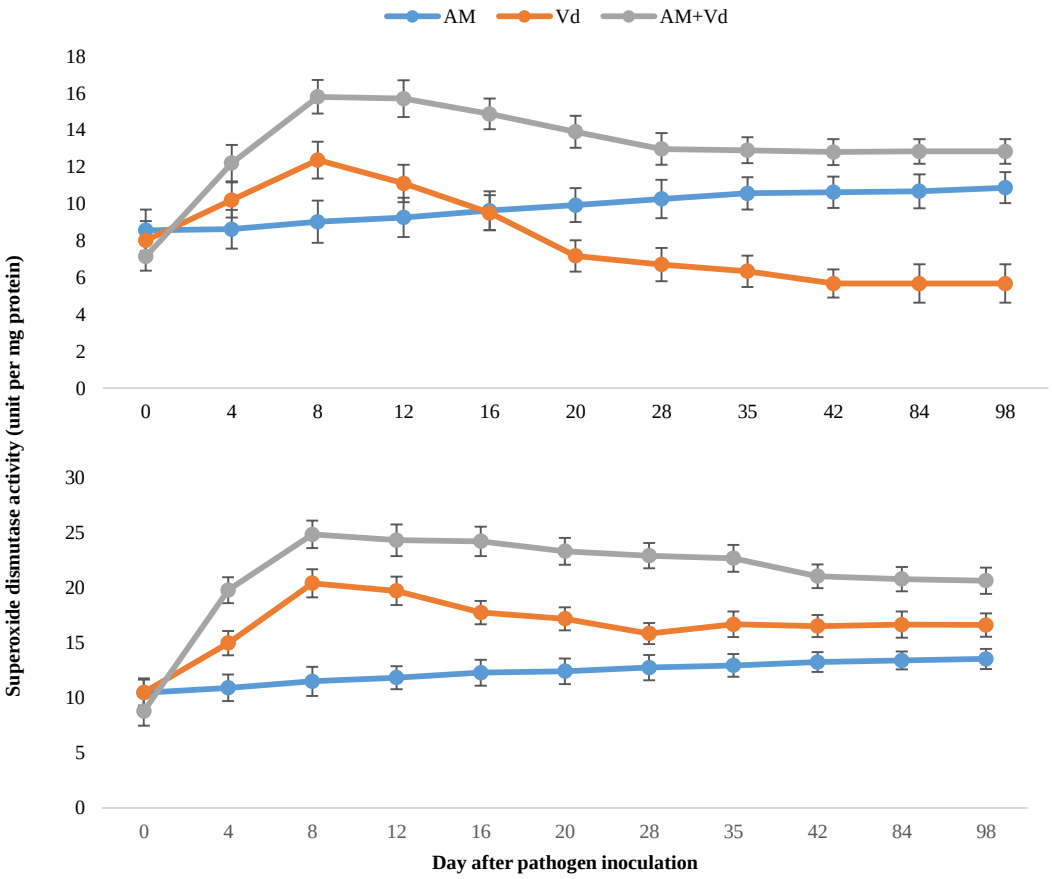

Fig. 3. Specific activity of superoxide dismutase (SOD) after impregnation of root with arbuscular mycorrhizae and inoculation with *Verticillium dahliae* in Ahmad Aghaei and Badami Zarand pistachio rootstocks. LSD ($P \leq 0.05$)= 1.69

Phenylalanine ammonia-lyase (PAL) enzyme

In AM treatment, the specific activity of the phenylalanine ammonia-lyase (PAL) enzyme increased during the experiment in both pistachio rootstocks; the increase was significant in the Ahmad Aghaei rootstock during days 84-98 and in the Badami Zarand rootstock during days 42-98 after pathogen inoculation compared to when the experiment started (Fig. 4). In the *V. dahliae* inoculation treatment, the significant increase and the maximum of the PAL activity in the Ahmad Aghaei rootstock was observed in day 12 and days 12-20 after pathogen inoculation, respectively. The enzyme activity decreased significantly compared to AM treatment on day 35. In the Badami Zarand rootstock, the significant increase in the PAL activity started from day 8 reaching its peak during days 12-20 after pathogen inoculation. The enzyme activity in the *V.*

dahliae inoculation treatment highly decreased from day 28 after pathogen inoculation, but it was still higher than the AM treatment. In the combined AM + Vd treatment, the significant increase in the PAL activity in the Ahmed Aghaei rootstock was on day 4, peaked during days 8-16, and started decreasing from day 20 afterward; although the PAL activity had a decreasing trend, it was still higher than AM treatment. In the Badami Zarand rootstock, the PAL activity increased significantly from day 4 after pathogen inoculation compared to the other treatments. The highest enzyme activity was on days 8-35 after pathogen inoculation. After PAL activity showed a significant reduction but it was significantly higher than in other treatments.

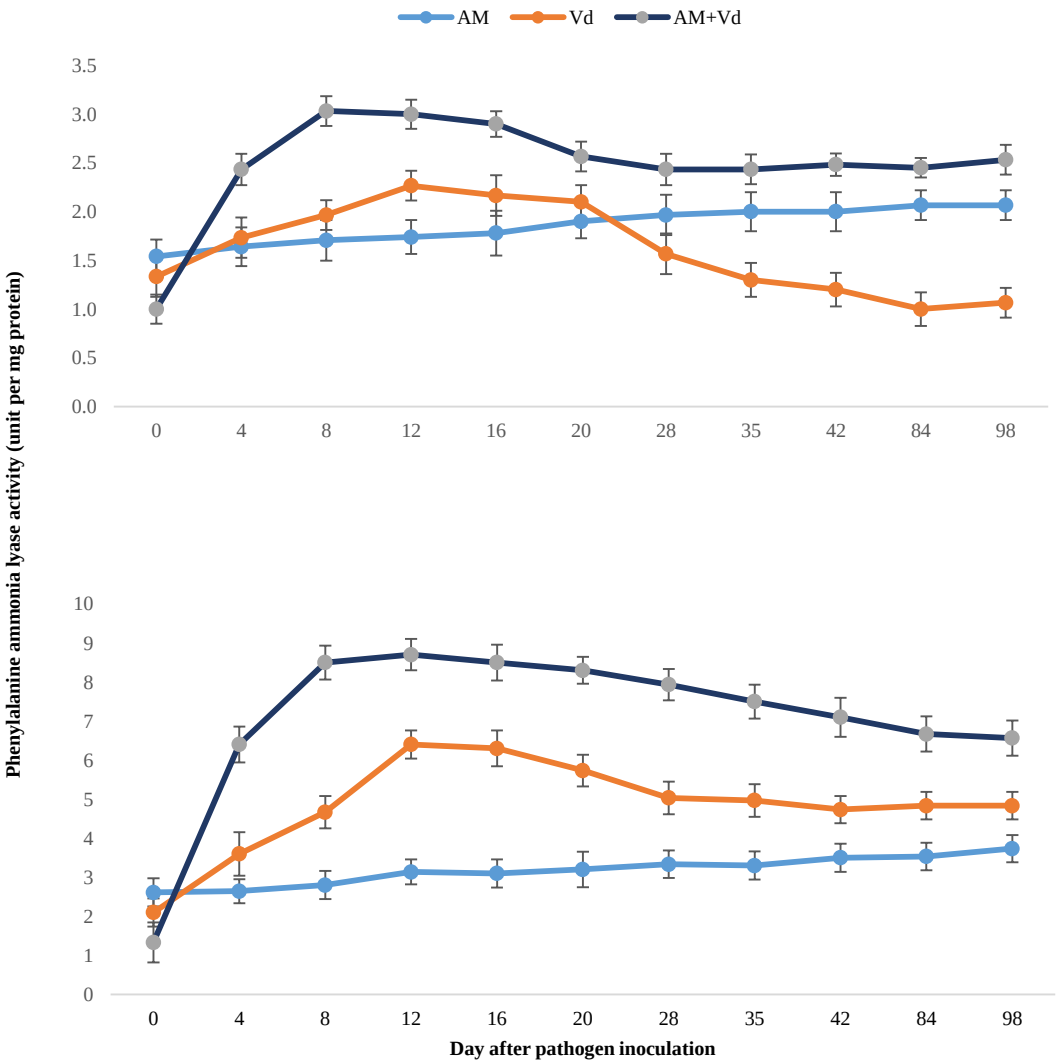

Fig. 4. Specific activity of phenylalanine ammonia lyase (PAL) after impregnation of root with arbuscular mycorrhizae and inoculation with *Verticillium dahliae* in Ahmad Aghaei and Badami Zarand pistachio rootstocks. LSD ($P \leq 0.05$)= 0.5

Discussion

AM fungi and *V. dahliae* can stimulate the production of new isoforms of antioxidant enzymes such as catalase, guaiacol peroxidase, superoxide dismutase, and phenylalanine ammonia-lyase

(Garmendia *et al.*, 2006), the increasing activity of which by the AM fungus can reduce the harmful verticillium effects in plants and increase their resistance against the pathogens (Boutaj *et al.*, 2019).

Results of this study revealed that the pathogen inoculation increased the specific activity of antioxidant enzymes in the Ahmad Aghaei rootstock (susceptible to *V. dahliae*), but the trend decreased immediately and reached lower than that of AM treatment. Contrarily, in the Badami Zarand rootstock (resistant to pathogen), *V. dahliae* inoculation increased the activity of the antioxidant enzymes with a decreasing trend, which was still higher than that of AM treatment. According to the results, antioxidant enzymes play important roles in increasing the resistance of pistachio seedlings, especially in disease-resistant rootstocks. Several studies (Garmendia *et al.*, 2004, 2006; Lambais *et al.*, 2003; Blilou *et al.*, 2000; Giocoechea *et al.*, 2010; Jung *et al.*, 2005) have reported that inoculation with various pathogens has increased the specific activity of antioxidant enzymes in different plants, which is consistent with the results of this research.

In mycorrhizal seedlings, the specific activity of antioxidant enzymes increased faster and more than the *V. dahliae* inoculation, and the time when the enzyme activity reached a maximum was also longer. In the Ahmed Aghaei rootstock, the activity of antioxidant enzymes decreased after reaching a peak, but it was still higher than in other treatments, In the Badami Zarand rootstock, the enzyme activity was more than in the other two treatments and showed a slight decrease after reaching a peak.

Increasing the activity of the inhibiting enzymes of reactive oxygen species (ROS) such as peroxidases and SOD is the most common mechanism to reduce their harmful effects and the pathogen spreading and penetration during plant-pathogen interactions (Mittler, 2002).

In mycorrhizal peppers, the increased activity of the catalase enzyme relates to the delayed occurrence of the disease symptoms and the photosynthesis process lasts longer (Garmendia *et al.*, 2004). The AM fungi catalyze the oxidative polymerization of phenylpropanols to produce lignin, cross-link the proteins in the cell wall, and strengthen it in the pathogen penetration process; in addition, they may play a role in deactivating H_2O_2 as an oxidizing agent (Mittler, 2002). Various studies (Fries *et al.*, 1996; Garmendia *et al.*, 2006; Spanu and Bonfante-Fasolo, 1988) have reported a temporary and transient induction of the peroxidase activity accompanied with the pathogen suppression in the roots of the mycorrhizal plants, which agrees with the results of the present research. Pomar *et al.*, (2004) showed that changing the concentration of the stem lignin compounds and peroxidase activity in the roots, stems and leaves of pepper-resistant cultivars can reduce the penetration of the *V. dahliae* hyphae. Garmendia *et al.*, (2006) showed that in the AM+Vd inoculation treatments, the PAL enzyme activity is higher than in the AM or *V. dahliae* treatment alone.

Conclusions

Results of this research showed that using a mixture of three AM fungi can result in a faster and longer-term increase in the activity of antioxidant enzymes, which play an important role in increasing the resistance of the pistachio seedlings and biologically controlling the Verticillium wilt by limiting the penetration and spreading of the *V. dahliae*.

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Conflict of interests

The authors declare that there is no conflict of interest.

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