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Tolerance of Mycorrhiza infected Pistachio (*Pistacia vera* L.) seedling to drought stress under glasshouse conditions

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ABSTRACT

The influence of *Glomus etunicatum* colonization on plant growth and drought tolerance of 3-month-old *Pistacia vera* seedlings in potted culture was studied in two different water treatments. The arbuscular mycorrhiza (AM) inoculation and plant growth (including plant shoot and root weight, leaf area, and total chlorophyll) were higher for well-watered than for water-stressed plants. The growth of AM-treated seedlings was higher than non-AM-treatment regardless of water status. P, K, Zn and Cu contents in AM-treated shoots were greater than those in non-AM shoots under well-watered conditions and drought stress. N and Ca content were higher under drought stress, while AM symbiosis did not affect the Mg content. The contents of soluble sugars, proteins, flavonoid and proline were higher in mycorrhizal than non-mycorrhizal-treated plants under the whole water regime. AM colonization increased the activities of peroxidase enzyme in treatments, but did not affect the catalase activity in shoots and roots under well-watered conditions and drought stress. We conclude that AM colonization improved the drought tolerance of *P. vera* seedlings by increasing the accumulation of osmotic adjustment compounds, nutritional and antioxidant enzyme activity. It appears that AM formation enhanced the drought tolerance of pistachio plants, which increased host biomass and plant growth.

Introduction

In nature, plants are frequently exposed to adverse environmental conditions that have a negative effect on plant survival, development and productivity. Drought stress is considered to be one of the most important abiotic factors limiting plant growth and yield in many areas (Kramer and Boyer, 1997). The symptoms of drought are apparent by wilting of the plants, reductions in the net photosynthesis rate, stomatal conductance, water use efficiency, relative water content and gradually diminution in total chlorophyll content. Drought stress also impairs the electron transport system, leading to the formation of activated oxygen (Saraswathi and Paliwal, 2011; Smirnoff, 1993). Reactive oxygen species (ROS) such as H_2O_2, O_2^- and OH^- may accumulate during water deficit stress and damage the photosynthetic apparatus. These cytotoxic ROS can destroy normal metabolism through oxidative damage of lipids, proteins and nucleic acids. Plants under stress are well stocked with an array of protective and repair systems that minimize the occurrence of oxidative damage (Khalvati et al., 2010). According to Smirnoff (1993), these can be divided into two categories: systems that react with active forms of oxygen and keep them at a low level, i.e., superoxide dismutases, catalase (CAT), or peroxidases, and systems that regenerate oxidized antioxidants (glutathione, glutathione reductase, ascorbate and mono and dehydroascorbate reductases). CAT is considered to be the most important enzyme that eliminates H_2O_2 from cells (Abdel Latef, 2011). Peroxidase (POD) constitutes a class of heme-containing enzymes ubiquitously present in prokaryotic and eukaryotic organisms. This enzyme catalyzes the dehydrogenation of structurally diverse phenolic and endolic substances by H_2O_2 and is thus often regarded as an antioxidant enzyme, protecting cells from the destructive influence of H_2O_2 and derived oxygen species (Pandey et al., 2010; Shigeoka et al., 2002). The efficiency of the antioxidant defense system is correlated with tolerance to drought stress.

Plants can respond to drought stress at morphological, anatomical and cellular levels with modifications that allow the plant to avoid the stress or to increase its tolerance (Ruiz-Lozano, 2003). These morphological and anatomical adaptations can be of vital importance for some plant species, but they are not a general response of all plant species. In contrast, the cellular responses to drought stress seem to be conserved in the plant kingdom.

In addition to intrinsic protective systems of plants against stress, plants grow in association with a number of soil

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microorganisms that can alleviate stress symptoms. Arbuscular mycorrhizal (AM) fungi that associate with the roots of most plants not only stimulate the growth of plants, but also contribute in enhancing plant tolerance to abiotic stress such as salinity (Abbaspour, 2010), drought (Navarro et al., 2011) and temperature stress (Xiancan et al., 2010).

Previous studies have indicated that inoculation with AM fungi appeared to improve drought tolerance of host plants (Cuenca et al., 1997). Based on the research of Nelsen and Safir (1982) on onion, improved phosphorus nutrition is a crucial factor for increased drought tolerance of mycorrhizal plants. Exposure of plants to drought conditions led to increase in free proline, soluble sugar, peroxidate (POX) activities and malondialdehyde (MDA) concentration, and inhibitions of protein synthesis have been proved in many literatures (Dhindsa, 1991; Zhang and Kirkham, 1994; Arji and Arzani, 2008). However, there is very little research on improving drought tolerance of pistachio using AM associations.

Therefore, the purpose of this work was to test the effects of *Glomus etunicatum* on growth, nutrition, solute accumulation and antioxidant responses in leaves and roots of pistachio under drought stress, in order to further understand drought tolerance mechanisms in AM plants.

Materials and methods

Plant materials

Pistachio (*Pistacia vera* L. cv. Akbari) seeds were surface sterilized with 20% solution of sodium hypochlorite in distilled water and aseptically germinated on a moist mix of peat and sand in polystyrene trays. Twenty-three-day-old seedlings, uniform in size, were chosen for transplanting in order to homogenize the plant material used in the experiment. AM fungal inoculum, *Glomus etunicatum* Backer and Gerdemann (Gec) were used as the AM fungal inoculum. Pure starter cultures of *G. etunicatum* were provided by International Culture Collection of Arbuscular and Vesicular, Arbuscular mycorrhizal fungi (INVAM). *G. etunicatum* was multiplied in pot cultures with sterilized fine sand as a substrate. Maize (*Zea mays* L.) was used as a host and was cultured for 3 months in a greenhouse ($24 \pm 5\,^{\circ}C$) under natural light. Maize plants were harvested just prior to inoculation by excising and discarding shoots. Mycorrhizal inoculum consisted of soil, spores (the spore density was 10–12/g dry soil), mycelium of *G. etunicatum*, and infected root fragments with an infection level of 65% (Abbaspour, 2010).

Plant growth condition

Seedlings were transplanted in 20 cm × 15 cm plastic pots containing a mixture of salinity clay:sand (1:5, v/v) (four seedlings/pot). The characteristics of the soil after mixture with sand were: pH 6.7, EC 1.4 ds/m, 3.4% silt, 14.5% clay, 82.1% sand, 1.2% organic matter, 11.2 mg/kg P, 139 mg/kg K and 31 mg/kg N. The soil was collected from Damghan city, Iran, and P was not added to the soil in order to stimulate mycorrhiza formation. Potted plants were maintained in the greenhouse under an average maximum photosynthetic photon flux of 846 μmol m^{-2}/s. The Max/Min average temperatures were 29/18 °C and mean relative humidity was 48%. Twenty-three-day-old pistachio seedlings were inoculated with 100 g inoculum as the mycorrhizal treatment. All seedlings were irrigated twice a week until differential water treatments were initiated 90 days after transplanting. Well-watered irrigation treatment plants were watered to 100% water holding capacity, while deficit irrigation plants received 50% of the well-watered irrigation total, and for the drought treatment, irrigation was withheld for one month following transplanting (90–120 days after transplanting).

Experimental design

The experiment consisted of a randomized complete block design with two inoculation treatments: AMF and non-AMF. Six replicates of each treatment were performed for a total of 24 pots, so that half of them were cultivated under well-watered conditions throughout the entire experiment while the other half were drought stressed for one month before harvest.

Parameters measured

After 4 months, pistachio plants were harvested, and then shoots and roots were separated. Leaf area was determined using an AM-200 leaf area meter. Shoot and root dry weights were determined after over-drying at 70 °C for 48 h, and saved for mineral analysis.

The percentage of mycorrhizal root infection was estimated by the following procedures: roots from each plant were collected by gently washing out the sand under running tap water and rinsed three times in deionized water. A subsample of 0.5-g root segments was collected and cut into l-cm-long pieces. One hundred l-cm root segments per treatment were examined for the presence of arbuscules, vesicles, or hyphae. The root segments were cleared and stained for analysis of colonization by AMF using a modified Phillips and Hayman (1970) procedures. The roots were cleared for 50 min in a 10% KOH solution at 90 °C, rinsed, placed in 10 nk% HCl solution for 10 min and then stained with glycerol–trypan blue solution (0.05%) at 90 °C for 20 min. Infection units originating from entry points were counted, at 45–100× magnification. The results were expressed as infection units per root length. Root colonization by AMF was estimated by the gridline intersection method (Bierman and Linderman, 1981) and expressed as percentage of root length colonized.

Phosphorus and nitrogen content were determined in dried shoots colorimetrically (Boltz and Lueck, 1958). K in plant shoots was determined by flam photometry. Other mineral nutrients (Zn, Cu, Fe, and Ca) were analyzed by atomic absorption.

Pigments were extracted using the method of Li (2000). Chlorophyll was extracted in 80% (v/v) acetone from 1 g of fresh leaf sample in the dark at room temperature. Absorbance was measured at 663 and 645 ηm in a UV/VIS spectrophotometer. Chlorophyll concentration was calculated using the equation:

$$Chl = 0.0202 \times A_{645} + 0.00802 \times A_{663}$$

For the determination of flavonoid content, 1 g of fresh leaf sample was homogenized with pure methanol and centrifuged at 3000 × *g* for 10 min. Chlorophylls and carotenoides were separated from flavonoid content with petroleum ether. Flavonoid concentration was calculated using the equation:

$$\text{Flavonoid} = A_{330} \times \frac{y}{E^{1\%}_{1\,cm}} \times 100$$

where *y* is the volume of dilution and $E^{1\%}_{1\,cm}$ is the coefficient of specific absorbance.

For the determination of free proline and total soluble sugar content, fresh roots and leaves were collected and proline content was assessed by spectrophotometric analysis at 515 ηm of the ninhydrin reaction using the protocol of Bates et al. (1973). Soluble sugar content was determined by 0.1 mL of the alcoholic extract reacting with 3 mL freshly prepared anthrone (200 mg anthrone + 100 mL of 72% H_2SO_4) and placed in a boiling water bath for 10 min according to Irigoyen et al. (1992). After cooling, the absorbance was read at 620 ηm.

To prepare the extraction of enzymes and soluble proteins, fresh leaves and roots were homogenized in 5 mL phosphate buffer (0.1 mol/L, pH 7.8), centrifuged at 10,000 × *g* for 20 min at 4 °C