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Synergistic Accumulative Effects between Exogenous Salicylic Acid and Arbuscular Mycorrhizal Fungus in Pistachio (*Pistacia Vera* cv. Abareqi) Seedlings under Drought Stress

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Abstract
This study was conducted to determine the combined effects of salicylic acid (SA) and arbuscular mycorrhizal fungi (AMF) (*Glomus mosseae*) symbiosis on the growth of pistachio seedlings (*Pistacia vera* 'Abareqi') grown in the greenhouse under different drought stress (DS) levels. The arbuscular mycorrhizal fungi (AMF) colonization or exogenous SA treatment could increase 'Abareqi' pistachio seedlings tolerance to DS. Application of SA on AMF-inoculated seedlings further promoted drought tolerance, as indicated by an alleviated plant biomass and water relations compared to the respective treatments. The analysis of proline and soluble carbohydrates showed that the increased drought tolerance in the treated plants may be associated, at least in part, with increasing of proline accumulation in the leaves of stressed plants.

Key words: drought, proline, soluble carbohydrates, stress.

Introduction

Pistachio (*Pistacia vera* L.) is a major orchard crop in Iran which is produced mainly in Kerman Province, especially in Rafsanjan Region, one of the largest pistachio production centers in the world (Bagheri *et al.*, 2012). Increased establishment of irrigated pistachio orchards during the last two decades in this region has decreased the availability of under-ground water resources and pistachio trees' productivity (Bagheri *et al.*, 2012). Under these circumstances, the development of methods that can induce drought stress tolerance and improve water use efficiency is vital.

AMF symbiosis can protect host plants against detrimental drought effects (for reviews see Augé, 2001; Ruiz-Lozano, 2003). Although mycorrhizal effects on plant water relations are not as dramatic and consistent as those on P acquisition and host growth, it is accepted that modest changes, if sustained, can have meaningful effects on plant fitness (Augé, 2001). To our knowledge, there are a few reports on AM symbiosis with pistachio (Ferguson *et al.*, 1997; Kafkas and Ortas, 2009) but our studies on this topic have demonstrated that the contribution of the AM symbiosis

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to pistachio drought tolerance results from a combination of nutritional (Bagheri *et al.*, 2012) and echo physiological effects (Bagheri *et al.*, 2011).

Salicylic acid (SA) is a common plant-produced signal molecule of phenolic nature which influences various physiological and biochemical functions in plants. There is evidence that externally applied salicylic acid increases plant's tolerance to several abiotic stresses, including osmotic stress (Wang *et al.*, 2010), drought (Azooz and Youssef, 2010), heavy metal stress (Moussa and El-Gamal, 2010) and also influences a range of diverse processes in plants, including seed germination, stomatal closure, ion uptake and transport, membrane permeability, photosynthesis and plant growth rate (Aftab *et al.*, 2010). However, to our knowledge, no information is available for SA involving in pistachio response to drought stress. Moreover, the role of SA in plant-mycorrhizal symbiosis is not yet known (for a review, see Zhao and Qi, 2008).

The objective of this study was to determine the synergistic effects of SA and AMF on drought tolerance of pistachio plants and to provide a new strategy to maintain plant growth under such conditions.

Materials and Methods

Preparation of AMF inocula

The AMF used in this study was *Glomus mosseae* (Nicolson and Gredemann) originally recovered from pistachio (*Pistacia vera* L.) orchards grown in different sites of Rafsanjan, Kerman, Iran and propagated in sorghum (*Sorghum bicolor*) pot cultures using autoclaved soil (as it is described below) in a greenhouse (T_{max}: 30±3°C; T_{min}: 22±2°C; RH: 58±3%) and adequate amount of sterilized water was supplied for 120 d. At maturity, the shoots of the sorghum plants were removed and the substrate was allowed to dry for a week at 30±5° C. The roots were finely chopped and the dried root/soil mixture was thoroughly mixed to obtain a homogenous inoculum.

Soil preparation, seed sowing and AMF inoculation

A sandy loam soil was sterilized by autoclaving (121°C, 2 h) on 3 consecutive days in order to eliminate the indigenous endophytes. The major characteristics of the soil were as follows: sand 79.5%, silt 12%, clay 8.5%, pH 7.8, P 11.7 mg kg^{-1} soil, K 15 mg kg^{-1} soil, Fe 1.1 µg g^{-1}, Mn 1.4 µg g^{-1} and cation exchange capacity 1.1 dS m^{-1}.

Seeds of 'Abareqi' pistachio were disinfected in a 10% (v/v) sodium hypochlorite solution for 2 min to eliminate possible seed-borne microorganisms and then rinsed 3 times under running water prior to soaking for 24 h in distilled water at room temperature. The seeds were then incubated at 30° C on sterile moist cloth for one week. Five germinated seeds were sown into plastic pots (25×35 cm) containing 4 kg of autoclaved soil. Before sowing, 100 g of fresh mass of inoculum having an average of 72% of infected roots was placed on the soil surface and then seeds were covered with sterilized sand. Control plants received the same amount of autoclaved inocula. The number of seedlings per pot was reduced to 3 within 21 d of germination. The seedlings were watered every 3 d up to field capacity (FC) level with distilled water for 90 d (to determine the water content of soil mixture at FC level, it was calculated based on the pot weight, soil dry weight and soil wet weight after watering and ceasing the runoff). At the end of this period and before starting the treatments, roots were sampled randomly from AMF-inoculated plants and an average of 75% of colonization was recorded (as described below).

Drought and SA treatments

One month before drought stress treatments (90 d after planting), the seedlings were sprayed three times (at 10 day intervals) with 0 (control), 0.50 or 1.0

mM SA solution with a hand sprayer until both sides of the leaves were completely wet. Tween-20 at amount of 1% (v/v) was added to SA solution as a surfactant. One day after the last spray, all plants were subjected to drought stress.

Drought stress was imposed for two months using different irrigation intervals (1as control, 3, 6 and 10 d) and in each irrigation level, individual pots were weighed and water added to bring the soil to the predetermined field capacity as described previously.

During the experiment, the average PAR measured at noon ranged from 916 to 1,760 $\mu molm^{-2}$ s^{-1} (no additional artificial lighting was used), the maximum temperature was 30 ± 4° C, the minimum temperature was 22 ± 2°C and the relative humidity was 55 ± 5%.

Measurements and data collection

Leaf, stem and root dry weight. At the end of the experiment, plants were separated into leaf, stem, and roots and dried for at least 48 h at 65°C and weighed.

AMF colonization percentage. To determine AMF colonization percentage, the whole root system with soil was excavated from each plant and carefully rinsed with running tap water then the root samples of 3 plants in each pot were mixed and cut into 1 cm long segments. Samples for mycorrhizal assessment were prepared according to the method of Phillips and Hayman (1970). Roots were boiled for 1 h in 10% KOH and then washed with tap water. Staining was performed in 0.05% trypan blue for 5 min followed by washing with tap water. Samples were stored in lactoglycerol [mixture of lactic acid, glycerol, and water 1:1:1 (v/v/v)]. Root segments were mounted on glass slides and examined under a compound microscope (CHS, Olympus Optical Co., Ltd., Japan). Mycorrhizal colonization (abundance of hyphae, vesicles, and arbuscules) was estimated (Giovanetti and Mosse, 1980) at 100 × magnification using 40 root segments of each sample. The dry weight of used samples were measured and added to the rest.

Water relations parameters. Relative water content (RWC) of leaves was determined following the Turner method (Turner, 1981). Using a punch, 15 leaf discs (6-mm diameter) were collected from fully-expanded leaves in each pot. After fresh weights were measured (FW), the leaf discs were floated on distilled water for 6 h at 4° C in the dark and then blotted to measure turgid weight (TW). Samples were then oven-dried at 85° C for 12 h and the dry weight was determined (DW). The relative water content of leaf tissues was calculated by the formula below:

$$RWC = \frac{FW - DW}{TW - DW} \times 100$$

Water use efficiency (WUE) was calculated as total plant dry weight (mg) per used water (ml) during the experiment.

Leaf proline and soluble carbohydrates. Leaf proline content was measured using the Bates method (Bates *et al.*, 1973). Extraction procedure and colorimetric determination with acidic ninhydrin reagent (2.5 g ninhydrin 100 mL^{-1} of a solution containing glacial acetic acid, distilled water and ortho-phosphoric acid 85% at a ratio of 6:3:1) were carried out as follows: Samples of 0.5 g leaf FW were ground in a mortar and 10 mL of a 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate was centrifuged at 3500 rpm for 10 min and the clear supernatant was then used in the assay. Glacial acetic acid and ninhydrin reagent (1 mL each) were added to 1 mL of the extract. The closed test tubes with the reaction mixture were kept in a boiling water bath for 1 h, and the reaction was terminated in a water bath of room temperature (21°C) for 5 min. Readings were taken immediately at a wavelength of 520 nm. The proline concentration was determined from a standard curve and calculated on a fresh weight basis (mmol proline g FW^{-1}).

To determine the leaf total soluble carbohydrates, 0.1 mL of the above extract was taken and mixed with 3mL of fresh anthrone (200mg anthrone + 100 mL sulphuric acid 72%) and then was kept in a water bath for 10 min. After cooling, readings were taken at a 625nm and a standard curve of glucose was used to calculate the concentration of total soluble carbohydrates.

Experimental design

A completely randomized design method was adopted in the experiment with two mycorrhizal treatments (with or without mycorrhizae), four irrigation intervals (1, 3, 6 and 10 d) and three salicylic acid levels (0, 0.5 and 1.0 mM) arranged in a factorial design. The data were statistically analyzed by three-way analysis of variance using MSTATC software (Michigan State University, USA) and the means were separated by Duncan's multiple range test (P<0.05).

Results

Leaf, stem and root dry weight

After 150 d of inoculation, the formation of AMF symbiosis had an obvious effect on the growth of 'Abareqi' pistachio seedlings. The biomass production of all the +M (with mycorrhiza) seedlings was faster than that of -M (without mycorrhizal) seedlings (Fig. 1). The mean leaf, stem and root dry weight of +M seedlings were 61, 37 and 44% more than -M seedlings respectively. In -M-SA (no mycorrhizal inoculation + no SA applied) treatment, drought levels had no significant effect on leaf, stem and root dry weight whereas in -M+SA (no mycorrhizal inoculation+ SA applied) treatments, leaf and root dry weight was increased at 0.5 and 1mM of SA with the increase of drought severity in comparison with control well-watered seedlings (Fig.1 A, C). In +M seedlings, mean dry weight of leaf, stem and roots was reduced at irrigation levels of 6 and 10 d significantly. In +M seedlings, SA at 0.5mM could improve leaf dry weight under stress conditions and also kept stem dry weight as the level of control up to irrigation level of 6 day (Fig.1 A, B). Root dry weight was increased significantly at 0.5 and 1mM of SA with irrigation level of 3 day compared with control (Fig.1 C). However, the mean stem and root dry weight was not affected by SA.

Proline and soluble carbohydrates

Stressed seedlings always exhibited a significantly higher proline content in the leaves, independently from mycorrhiza formation although proline accumulation was lower in +M seedlings (Fig. 2). Under each drought level, the proline content of +M pistachio leaves were not affected by the SA treatments but in -M seedlings, SA at 1mM increased proline content of seedlings in the highest level of drought stress (Fig. 2).

Drought stress induced soluble sugars accumulation in leaves of both +M and -M seedlings as well as SA treated seedlings just at the highest level (Fig. 3 A, B). At every level of drought, SA treatment had no effect but the mycorrhiza reduced soluble sugars (Fig. 3 A, B). The interesting point was that in irrigation levels of 3 and 6 day, +M plants had lower soluble sugars in comparison with control well-watered seedlings (Fig. 3 B). SA treatment had no effect on soluble sugars of +M as well as -M seedlings (Fig. 3 C).

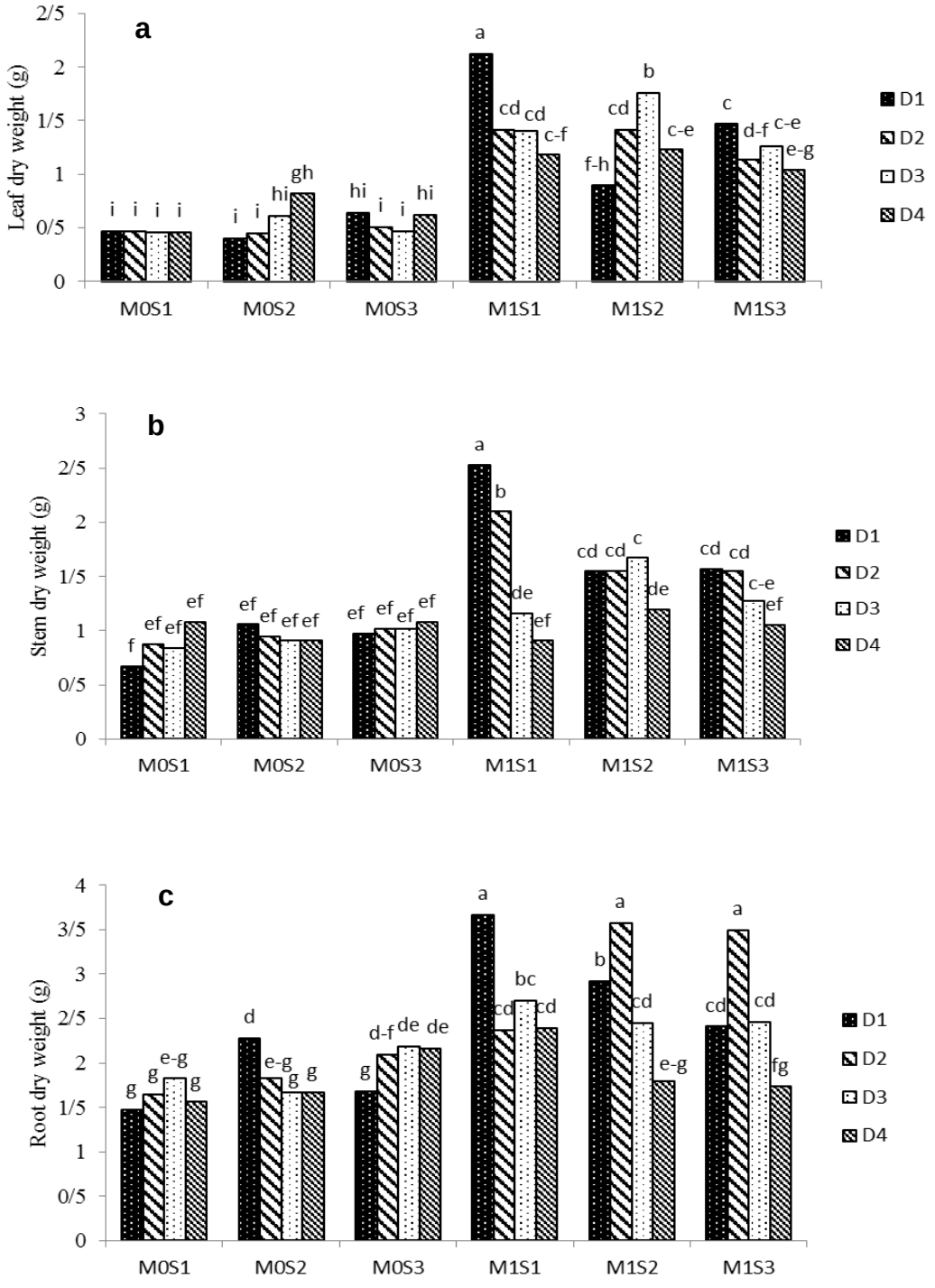

Fig. 1. Effects of mycorrhiza (M0 and M1 indicate with or without mycorrhizae), different water stress levels (D1, D2, D3 and D4 indicate irrigation interval of 1, 3, 6 and 10 d, respectively) and SA application (S1, S2 and S3 indicate 0, 0.50 or 1.0 mM, respectively) on leaf (a), stem (b) and root (c) dry weight of 'Abareqi' pistachio seedlings. Different letters indicate significant differences using Duncan's multiple range test at 5% significant level.

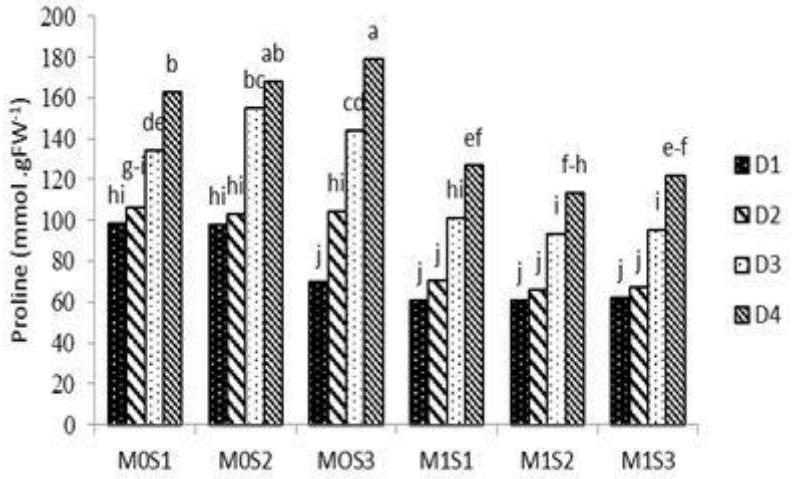

Fig. 2. Effects of mycorrhiza (M0 and M1 indicate with or without mycorrhizae), different water stress levels (D1, D2, D3 and D4 indicate irrigation interval of 1, 3, 6 and 10 d, respectively) and SA application (S1, S2 and S3 indicate 0, 0.50 or 1.0 mM, respectively) on leaf proline content of 'Abareqi' pistachio seedlings. Different letters indicate significant differences using Duncan's multiple range test at 5% significant level.

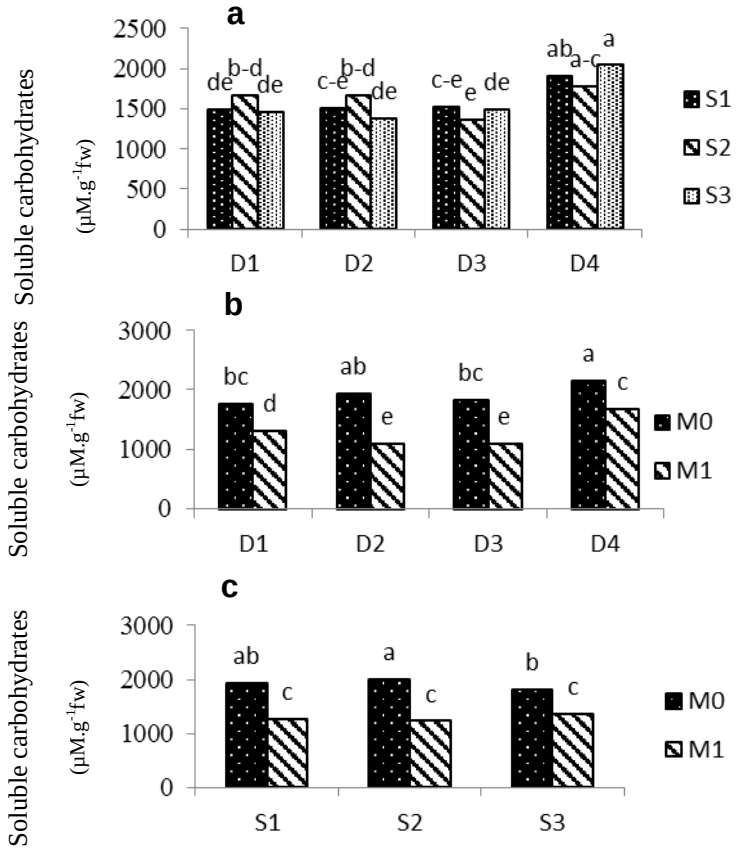

Fig. 3. Interaction effects of drought and SA (a), drought and mycorrhiza (b) and SA and mycorrhiza (c) on leaf soluble carbohydrates. Different letters indicate significant differences using Duncan's multiple range test at 5% significant level.

RWC and WUE

The data illustrated that drought stress, mycorrhiza and SA treatments significantly increased WUE and RWC of pistachio seedlings. In -M seedlings, use of SA at 0.5 and 1.0 mM had a positive significant effect on RWC under irrigation of 6 and 10 d interval respectively, while in +M seedlings, the same result was found just with 1.0 mM of SA under 3 and 6 d irrigation interval. RWC of control well-watered seedlings was not affected by mycorrhiza and SA treatments (Fig. 4 A). The +M plants used less water to produce one unit of plant dry weight than -M plants; in addition, with increasing the severity of drought stress and salicylic acid application, water use efficiency was increased. In -M seedlings, use of SA at 0.5 and 1.0 mM led to 16 and 49% increase in WUE while in +M seedlings, the same concentrations of SA resulted in 18 and 10% increase in WUE. The highest value of WUE was obtained in +M seedlings treated with 1.0 mM of SA and exposed to the highest level of drought stress (Fig. 4 B).

Mycorrhizal development

Drought stress significantly reduced the AMF colonization in pistachio seedlings (Fig. 5). Use of SA had neither positive nor negative effect on the level of root colonization of inoculated plants under unstressed or drought-stressed conditions.

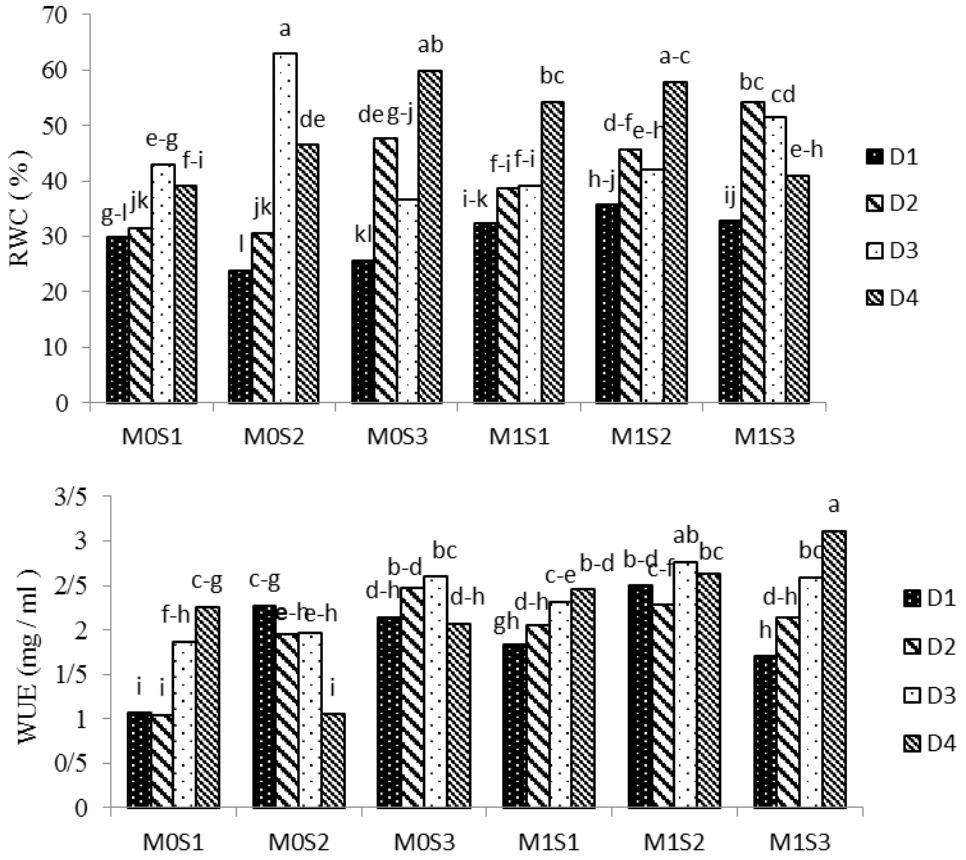

Fig. 4. Effects of mycorrhiza (M0 and M1 indicate with or without mycorrhizae), different water stress levels (D1, D2, D3 and D4 indicate irrigation interval of 1, 3, 6 and 10 d, respectively) and SA application (S1, S2 and S3 indicate 0, 0.50 or 1.0 mM, respectively) on RWC (a) and WUE (b) of 'Abareqi' pistachio seedlings. Different letters indicate significant differences using Duncan's multiple range test at 5% significant level.

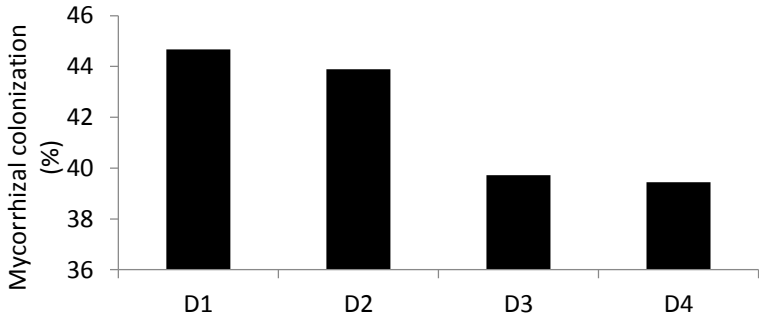

Fig. 5. Effect of different water stress levels (D1, D2, D3 and D4 indicate irrigation interval of 1, 3, 6 and 10 d, respectively) on mycorrhizal colonization percentage of 'Abareqi' pistachio seedlings. Different letters indicate significant differences using Duncan's multiple range test at 5% significant level.

Discussion

As it was expected, the data presented in this study indicated that pistachio seedlings can tolerate severe drought stress since drought at any level could not induce biomass reduction in -M-SA seedlings (Fig. 1). However, the drought tolerance of pistachio trees refers to their ability to survive under severe water stress conditions (Goldhamer, 1995; Kanber *et al.*, 1993). It has been stated that pistachio roots exploit soil moisture effectively at highly negative soil water potentials (Spiegel Roy *et al.*, 1977). This statement is in agreement with our results where -M-SA seedlings had higher RWC at irrigation intervals of 6 and 10 d (Fig. 4 A). The RWC is a useful measure of the physiological water status of plants (González and González-Vilar, 2003). Improvement of RWC in -M-SA seedlings with increasing drought stress can be related to leaf accumulation of proline (Fig. 2) and soluble carbohydrates (Fig. 3). Furthermore, pistachio resistance to drought is, at least in part, due to its deep root system and leaf structure (Bagheri *et al.*, 2011).

Use of mycorrhiza (*Glomus mosseae*) increased drought tolerance in 'Abareqi' pistachio seedlings. Mycorrhizal colonization by *Glomus mosseae* improved growth and water status of 'Abareqi' pistachio seedlings when exposed to varying intensities of drought stress. These findings agree with our previous results on other pistachio cultivars (Bagheri *et al.*, 2012). In this experiment, +M seedlings had RWC and WUE of 43.7% and 2.36 mg mL^{-1}, respectively, in comparison with 39.7% and 1.89 mg mL^{-1} of -M seedlings. The improved WUE in +M seedlings (especially at severe drought stress levels) as compared to -M, may indicate that AMF increased the ability of roots to absorb soil moisture, thus maintaining more open stomata in leaves and enhancing dry mass production. Mycorrhizal colonization may increase root length density or alter root system morphology, enabling colonized plants to explore more soil volume and extract more water than uncolonized plants during drought (Berta *et al.*, 1995). Our findings support this view because mean root dry weight of +M seedlings was 2.66g against 1.83 g of -M seedlings. One more reason for this is the fact that the shoots of +M seedlings had a larger biomass (more evaporative leaf surface area) than -M treatment.

In -M-D (no mycorrhizal inoculation + no drought stress) seedlings, use of SA had no significant effect on plants' biomass production except on root dry weight at 0.5 mM whereas in +M-D (mycorrhizal inoculation + no drought stress) seedlings, it decreased the whole growth of seedlings significantly at both concentrations. This may not be related to the effect of SA on mycorrhizal colonization of pistachio seedlings. In addition to this, SA treatments had no phytotoxic effect on these seedlings since those treated with SA had almost no

visible injury symptoms as it is shown in previous studies (War *et al.*, 2011). However, the cause of these reductions and their putative relationship with SA treatment remains elusive and needs to be further investigated. In -M+D (no mycorrhizal inoculation + drought stressed) treatments, stem dry weight was not affected by SA application but leaf and root dry weight were increased significantly under the highest level of drought with 0.5 and 1.0 mM of SA respectively, while in +M+D (mycorrhizal inoculation + drought stressed) treatments, leaf and stem dry weight were increased in combination with SA treatment at 0.5 Mm under irrigation interval of 6 d and root dry weight also increased significantly with SA at 0.5 and 1.0 mM under irrigation interval of 3 d. This fact that SA plays a key role in providing tolerance for the plants which are exposed to drought stress has been shown previously (Hayat *et al.*, 2008; Kadioglu *et al.*, 2011; Baninasab, 2010). SA was found to enhance the activities of antioxidant enzymes such as POD, SOD and CAT when sprayed exogenously on the drought stressed plants of tomato (Hayat *et al.*, 2008) or on the salinity stressed plants (Szepesi *et al.*, 2008). In -M+D seedlings, applied SA at 0.5 and 1.0 mM increased proline level at the highest drought stress level but not soluble carbohydrates. In the same treatments, RWC was also increased showing the role of proline in improving pistachio water relations under drought stress.

In our experiment, root colonization was not affected by SA treatment. Transient accumulation of SA during the early stage of AM root colonization, reminiscent of the early activation of other plant defense responses (García-Garrido and Ocampo, 2002), has been reported (Blilou *et al.*, 2000) and recently enhanced SA levels were linked to the inability of *Pisum sativum* mutants to form the AM symbiosis (Blilou *et al.*, 1999). Also, it has been shown that during the early stages of colonization, the level of mycorrhization was higher in NahG tobacco plants (unable to accumulate free SA) than in wild-type plants. These findings suggest a link between SA accumulation and fungal infectivity (Jahromi *et al.*, 2008). In our experiment, SA exogenously applied to leaves of AMF-inoculated pistachio seedlings showed no effect on mycorrhization extent. This means that changed SA levels in plants have an effect on AMF during the establishment of the fungus, but they do not affect the symbiotic potential of plants in terms of changes in maximal threshold of root colonization.

In conclusion, to our knowledge, there is no report yet to indicate the existence of a synergistic action of exogenous SA treatment and AM symbiosis in response to drought neither in pistachio nor in any other plants. In this experiment, although mycorrhizal treatment had the main role in alleviating the adverse effects of drought stress, some positive interactions were found when AMF-inoculated pistachio seedlings were exposed to SA in terms of biomass production and water relations.

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