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Contributions of Arbuscular Mycorrhizal Fungi to Growth, Biomass and Nutrient Status of Pistachio Seedlings under Saline Conditions

*S. Soleimanian[1], H. Abbaspour[*1], A.R.Mohammadi Nafchi[2]*

[1]Department of Biology, Damghan Branch, Islamic Azad University, Damghan, Iran

[2]Department of Food Science and Technology, Damghan Branch, Islamic Azad University, Damghan, Iran

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Abstract

Excessive salt accumulation in soil is a major ecological and agronomical problem, especially in arid and semiarid areas. Excessive soil salinity affects the establishment, development and growth of plants, resulting in major losses of production. A pot experiment was set up to examine the effects of arbuscular mycorrhizal fungus (*Glomus etunicatum* and *Glomus versiforme*) and salinity on the growth, pigment concentration, biomass and nutrient acquisition of pistachio (*Pistacia vera* L.) seedlings. Two-month-old pistachio seedlings colonized by *G. etunicatum* and *G. versiforme* were irrigated with 0 and 150 mM NaCl solution for 45 days to induce salt stress. The results showed that salt stress significantly reduced mycorrhizal colonization in pistachio seedlings, and *G. versiforme* was found to be more colonized than *G. etunicatum*. Mycorrhizal inoculation, especially *G. versiforme*, had higher plant growth, biomass and pigment content than non-mycorrhizal under control and salt stress treatments. Shoot Na concentrations were lower in mycorrhizal than in non-mycorrhizal seedlings under given salinity conditions. Total P, K, N, Ca macronutrients and micronutrients decreased with soil salinity in both mycorrhizal and non-mycorrhizal seedlings. These nutrients were higher in AM, especially *G. versiforme*, than in NM seedlings in control and salt stress treatment. The results suggested that mycorrhizal, especially *G. versiforme*, pistachio plants exhibited greater efficiency in alleviating salt stress, which resulted in better growth.

Keywords: *Glomus etunicatum*, *Glomus versiforme*, Growth, Mineral nutrition, *Pistacia vera*, Salinity.

Abbreviations:

AMF: arbuscular mycorrhizal fungi, AM: arbuscular mycorrhizal, NM: non mycorrhizal

Introduction

Excessive salinization of soil is a major ecological and agronomical problem as a result of its effects on the growth and development of plants, particularly in arid, and semiarid areas and in Mediterranean ecosystems (Evelin *et al*., 2009) About one billion hectares of the world's land area is not in use due to salinity stress. Estimations indicated that salinization of arable land will result in 30 percent land loss within the next 25 years and up to 50 percent within the next 40 years (Porcel *et al*., 2012). Excessive salts in soil adversely affects the availability of water and ionic imbalance by osmotic stress and / or ion cytotoxicity in plant cell and functioning, leading to a significant decrease in plant production and quality (Teakle *et al*., 2006). Thus, approaches to improve the tolerance of plants to salinity and increase the utilization of saline soil is becoming an emerging challenge.

Plants respond to salinity stress through morphological, physiological and metabolic modifications, which allow the

***Corresponding author**: Email: abbaspour75@yahoo.com

plant to avoid the stress or to increase its tolerance. In addition to intrinsic protective systems of plants against stress, plants in their natural environment are colonized both by external and internal microorganisms that can alleviate stress symptoms.

Arbuscular mycorrhizal fungi (AMF) that widely distribute soil microorganisms in the phylum Glomeromycota can form the Arbuscular mycorrhizal (AM) symbiosis in nearly 80 percent of plant species (Helgason *et al*., 2009). AM fungi are important soil organisms, fundamental for plant nutrition and soil fertility and represent a living bridge for the translocation of water and nutrients to host plants and plant assimilates to fungi (Van der Heijden *et al*., 2009). It is well documented that AMF can enhance plant tolerance to salinity (Abbaspour, 2010). By improving plant nutrient uptake, especially phosphorus (Asghari, 2008), ion balance (Giri *et al*., 2007), induction of antioxidant enzymes (Kholer *et al*., 2009), facilitating water uptake (Colla *et al*., 2008) and increasing the capacity of osmotic adjustment (Kumar *et al*., 2010). AM fungi have been considered as important bio-ameliorators for saline soils (Rabie and Almadini, 2005).

Pistachio (*Pistacia vera* L.) is a major orchard crop in Iran. The aim of this study is to evaluate the symbiotic efficiencies of two AMF in alleviating salt stress on pistachio plants, in terms of plant growth performance, biomass and nutrient uptake.

Materials and Methods

Plant growth conditions

The experiment was conducted in the greenhouse of the Department of Biology, Islami Azad Univesity, and Semnan Provinance, Iran. The minimum and maximum temperature and photosynthetic photon flux were 19 °C, 31 °C and 700-850 u mol m^{-2} s^{-1}, respectively. Seeds of pistachio (*Pistacia vera* L. cv. Badami) supplied by the pistachio research institute, Rafsanjan city, Kerman Province, Iran, 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. Thirty-day-old seedlings, uniform in size, were transplanted into (30 × 20 cm) plastic pots filled with a salinity clay soil mixed with washed sand (clay: sand, 1:5). After mixture with sand, the soil had the following physicochemical properties: electrical conductivity (EC) 1.3 dsm^{-1}, pH 7.2, 4.5% silt, 13% clay, 81% sand, 1.5% organic matter, 10.9 $mgkg^{-1}$ P, 153 $mgkg^{-1}$ K, and 43 $mgkg^{-1}$ N.

This soil was autoclaved at 121 ^{o}C for two hours. Either 100 g (dry wt) mycorrhizal (*G. etunicatum* or *G. versiforme*) inoculum (soil, spores, mycelium and infected root fragment) or autoclaved inoculum was placed at 5 cm depth of soil mixture. *G. etunicatum* or *G. versiforme* Becker and Gerdeman (Gec) were used as the AMF inoculum. Pure starter cultures were provided by the International Culture Collection of Arbuscular and Vesicular–Arbuscular Mycorrhizal Fungi (INVAM).

Salinity stress was induced by adding 350 ml of 150 mM NaCl solution after 60 days of sowing, and the control (0 mM NaCl) seedlings were irrigated with 350 ml of distilled water. To avoid osmotic shock, the soil was gradually salinized by 50 mM NaCl per day. Plants were watered with tap water until harvest. When leaching occurred, the leachate was collected and added back to the soil to maintain salinity treatments near target levels. Seedlings were harvested 45 days after salinity stress.

Parameter analysis

Leaf number per plant, number of branches, stem diameter, and plant height were recorded. Leaf area was determined using an AM-200 leaf area meter. The shoots and roots were separated, oven-dried for 48 hours at 70 ^{o}C, weighed and saved for mineral analysis. Mycorrhizal colonization was measured through the method of Phillips and Hayman (Phillips *et al*., 1970). The roots from each plant were cut into 1-cm-long pieces and dipped in KOH solution for 24 hours and then kept in HCl solution for 30 minutes. A staining solution containing cotton blue dye was added. The percent root infection was calculated as follows:

Percent root infection: A, Total number of infected roots: B and Total number of roots observed: C

$$A = B/C \times 100 \quad (1)$$

The entry points, vesicles and Arbuscules were counted from the colonized roots at the time of microscopical observation and expressed the number per centimeter of root. Potassium and sodium content in plant shoots were estimated using flame photometry. Nitrogen and phosphorus content was estimated by the colorimetric method of Linder using Nesslers reagent following digestion in a mixture of concentrated sulfuric acid and perchloric acid. Other mineral nutrients (Zn, Cu, Fe, Ca and Mg) were analyzed by atomic absorption. Leaf chlorophyll concentration (Chl a and Chl b) was measured on the second fully expanded leaf of pistachio. Fresh tissue (1g) was cut into small segments, extracted with 80% acetone and read using a UV-VIS spectrophotometer at 663 and 645 nm. The concentration of chlorophyll was calculated according to Arnon (Arnon, 1949) using the following formulas:

$$\text{Chl a (mg ml}^{-1}) = 11.64 \times (A663) - 2.16 \times (A645) \quad (2)$$

$$\text{Chl b (mg ml}^{-1}) = 20.97 \times (A645) - 2.16 \times (A663) \quad (3)$$

A663 and A645 are absorbance values read at 663 and 645 nm wavelengths, respectively. To determinate flavonoid content, 1 gram of fresh leaf sample was homogenized with pure methanol and centrifuged at 3000 × g for ten minutes. Chlorophylls and carotenoids were separated from flavonoid content with petroleum ether. Flavonoid concentration was calculated using the equation 4:

$$\text{Flavonoid} = A330 \times y / E^{1\%}_{1\ Cm} \times 100 \quad (4)$$

Where y is the volume of dilution and $E^{1\%}_{1\ Cm}$ is the coefficient of specific absorbance.

Statistical analysis

All data were statistically analyzed using analyses of variance (ANOVA) and least significant difference (LSD) using the SPSS windows version 16.0. Two ways ANOVA was performed considering NaCl and AMF as independent factors. Tukeys multiple-range tests were performed at $P \leq 0.05$ on each of the significant variables measured.

Results

After 105 days under mycorrhizal colonization, morphological and physiological structures of AM were observed in roots of seedlings after inoculation with mycorrhiza fungi. Extraradical hyphae were associated with the roots, and arbuscules and vesicles were observed in cortical cells. Plant injury was more severe in NM than AM plants at 150 mM NaCl level as was evident from observing the yellow leaves or plant lower growth.

There was no mycorrhizal colonization recorded in the non-inoculated seedlings. The seedlings inoculated with AMF showed root colonization of 32.4-68.7 percent. The highest colonization was in salt-free soils infected by *G. versiforme*, and the lowest colonization occurred in *G. etunicatum* colonized seedlings subjected to salt stress (Table 1). There was a significant negative correlation between AM fungal root colonization and salinity, indicating that salinity suppresses AM establishment. With salt stress, the root infection was significantly decreased from 68.7 to 42.1 in *G. versiforme* and from 53.2 to 32.4 in *G. etunicatum* (Table 1).

Changes in growth of pistachio as affected by NaCl salinity are shown in Table 1. Salinity has been shown to decrease growth of pistachio in saline soils. The seedlings inoculated with AMF had higher plant growth, biomass and pigment contents than non- AMF plants regardless of salinity stress (Table 1). The seedlings inoculated with *G. versiforme* had higher plant growth, biomass and pigment contents than seedlings inoculated with *G.etunicatum* in the control and salt stress treatment. No significant differences in pigment contents and root dry weight were recorded between *G.etunicatum* and *G. versiforme* colonized seedlings in the control treatment. Under the salt stress treatment, no significant differences in pigment contents, biomass and leaf

area and stem diameter were recorded between *G.etunicatum* and *G. versiforme* colonized seedlings (Table 1).

Table 1. Effect of salinity and mycorrhizal association on plant height (Cm), stem diameter (Cm), leaf number, number of branches, Leaf area (mm^2), total chlorophyll (mgg^{-1} fresh wt), flavonoids content (mgg^{-1} fresh wt), shoot dry wet (g), Root dry wet (g), and total dry wet (g) of pistachio seedlings.

Salinity	AMF	RLC percent	Plant height	Stem diameter	Leaf number	Number of branches	Leaf area	Total chlorophyll	Flavonoid content	Root DW	Shoot DW	Total DW
0	*G. etunicatum*	53.2 a	19.1 a	0.261 a	22.3 a	3.67 a	842 a	4.87 a	6.42 ad	1.85 a	2.75 b	4.60 a
	G. versiforme	68.7 b	22.8 b	0.284 b	25.6 b	4.71 b	931 b	5.09 a	6.21 ad	1.97 a	3.31 a	5.28 b
	Non-AMF	0 e	16.2 c	0.237 c	17.8 c	2.39 c	752 c	3.96 b	5.11 b	1.23 b	2.41 c	3.64 c
150	*G. etunicatum*	32.4 c	17.0 c	0.247 a	18.1 c	2.53 c	790 ac	4.13 b	7.34 c	1.19 b	2.39 c	3.58 c
	G. versiforme	42.1 d	21.9 b	0.257 a	21.2 a	3.46 a	802 a	4.21 b	7.26 c	1.31 b	2.63 bc	3.94 c
	Non-AMF	0 e	13.9 d	0.214 d	14.2 d	1.37 d	591 d	3.17 c	5.72 bd	0.79 c	1.86 d	2.65 d

Same letter within each column indicates no significant difference among treatments ($P < 0.05$).

The macro and micro nutrients content of pistachio's shoot as a function of salinity and mycorrhiza was shown in Table 2. Shoot Na concentration increased by soil salinity in both mycorrhizal (M) and non-mycorrhizal (NM) seedlings. Shoot Na concentrations were lower in M than in NM seedlings under given salinity conditions. No significant differences in Na content were recorded between *G. versiforme* and *G.etunicatum* colonized seedlings.

The Mg content in shoots were not affected by AM symbiosis and salinity stress (Table 2). Other nutrients concentrations decreased with soil salinity in both M and NM seedlings. Under the non-salinity and salinity conditions, the K, P, N, Ca, Fe, Cu and Zn concentrations were higher in M than in NM seedlings, but the differences for K, P and Fe were significant in control and salt stress treatment, for Ca and Cu were significant in salt stress treatment and for Zn was significant in control treatment. The seedlings inoculated with *G. versiforme* had higher nutrient contents than seedlings inoculated with *G.etunicatum* under non-salinity and salinity conditions, but a significant difference was only observed in the K and P concentration under total and control conditions, respectively (Table 2).

Table 2. Effect of salinity and mycorrhizal association on Na, P, K, N, Mg, Ca (mg/g dry wt), Zn, Fe and Cu (μgg^{-1} dry wt) contents in the shoots of pistachio seedlings.

Salinity	AMF	Na	K	P	N	Mg	Ca	Fe	Cu	Zn
0	*G.etunicatum*	171 a	1186 a	20.1 a	281 a	187 a	902 ab	2841 a	74 a	783 a
	G.versiforme	190 a	1274 b	22.3 b	286 a	191 a	931 a	2864 a	83 a	867 a
	Non-AMF	161 a	1061 c	15.9 c	273 a	182 a	889 ab	2201 b	71a	689 b
150	*G. etunicatum*	278 b	1093 c	18.9 a	234 b	183 a	861 b	2145 b	59 b	625 bc
	G.versiforme	292 b	1164 a	20.3 a	244 b	186 a	874 b	2361 b	71 b	643 bc
	Non-AMF	421 c	943 d	12.8 d	229 b	178 a	786 c	1584 c	39 c	567 c

Same letter within each column indicates no significant difference among treatments ($P < 0.05$).

Discussion

Mycorrhizal colonization is a key component in helping plants cope with adverse environmental conditions. Understanding plant responses at the seedling stage is particularly important for elucidating the mechanism of salt tolerance, sensitivity, and survival in plants (Arnon *et al*., 1992). In this study, we observed that the AM fungal colonization

rate decreased by salt stress. The decline in colonization under stress could be caused by inhibiting the germination of spores, inhibiting the growth of hyphae in soil and hyphal spreading after initial infection has occurred and reducing the number of arbuscules (Juniper and Abbott, 2006). The reduced colonization by the salt application was also reported by another study (Kumar *et al*., 2010). In this study, mycorrhizal colonization was greater in *G. versiforme* than in *G.etunicatum.* This is in agreement with several studies on maize and zucchini [Sheng *et al*., 2008; Colla *et al*., 2008]. It was shown that a symbiotic association between *G. versiforme* fungi and stress-tolerant pistachio plants was strengthened in the saline environment once the association was established.

Salinity has been shown to decrease growth of pistachio seedling in saline soil, and AM seedlings had higher plant growth, biomass and pigment content than non-AMF seedlings. These parameters were greater in *G. versiforme* than in *G.etunicatum* under non-saline control treatment. Generally, salinity inhibits plant growth due to water deficit and salt excess effects. Salt tolerance has usually been assessed as the biomass production. The beneficial effects of AM on growth may be related to mycorrhiza-mediated effects on water absorption, nutrient uptake and increased photosynthetic activity under salinity stress (Miransari *et al*., 2010). Mycorrhizal plants develop a more efficient carbon-use root system, which is more effective with the AM fungus assisting nutrient absorption (Schellenbaum *et al*., 1991). Similar results were also observed by other researchers (Kumar *et al*., 2010). AM symbiosis enhanced the chlorophyll content of pistachio plants, which correlates to the results of other research (Dudhane *et al*., 2011). AM colonization enhanced P and Mg uptake and reduced Na concentration in the plants. This, in turn, helped to increase the chlorophyll content and improve the overall performance of mycorrhizal plants (Giri *et al*., 2011). Flavonoids (as another pigment) are the most common secondary metabolites in vascular plants. In contrast to pharmacological properties of flavonoid pigments, numerous in vitro studies have indicated that flavonoids can directly scavenge molecular species of active oxygen (Yamasaki *et al*., 1996). The results of the current study suggest that flavonoids may contribute to the overall mechanism for protecting cells from oxidative damage in addition to their actions as optical filters (Gould *et al*., 1995).

The present study showed that shoot's Na concentration in seedlings was increased by soil salinity but other nutrients contents, exception of Mg, decreased by soil salinity. Mg content was not affected by soil salinity. Also, nutrient contents, with the exception of Na and Mg, were higher in M than in NM. Na contents were lower in M under salt stress, and Cu contents were not affected by AM symbiosis. Symbiosis can control the uptake of Na when it became toxic to plants (Allen *et al*., 1983).

Mycorrhizal colonization of host plant has been shown to prevent Na translocation to shoot tissues, while enhancing K absorption under saline conditions (Abbaspour *et al*., 2012). Thus, AM plants maintain a higher K/Na ratio, preventing the disruption of cellular enzymatic processes and inhibition of protein synthesis. K is a competitor of Na under saline conditions. Therefore, maintenance of a higher cytosolic K/Na ratio is a key feature of plant salt tolerance (Wu and Zou, 2009).

The phosphorus concentration in plant tissues rapidly lowered under salt stress because phosphate ions precipitate with Ca ions in saline soil and become unavailable to plants. AM fungi have been shown to positively influence the composition of mineral nutrients (especially poor mobility nutrients such as P) of plants under salt stress conditions (Al-Karaki and Clark, 1998). Several studies have showed that AMF plays a vital role in improving the P nutrition of the host plants under salt stress conditions [Abbaspour, 2010; Evelin *et al*., 2012). This is probably due to the extended network of AM fungal hyphae that allow them to explore more soil volume than NM plants. Indeed, mycorrhizal hyphae extend beyond that depletion zones around roots and acquire nutrients that are several centimeters away from the root surface and thus, suppress the adverse effects of salinity stress (Ruiz-Lozano and Azcon, 2000). Also, the mobility of Cu, Zn, and Fe in soils is low. AM plants could take up

more metal nutrients via extra radical hyphae, which provides larger surface areas than the roots alone and reduce the distance for diffusion, thereby enhancing the absorption of immobile metal nutrients. Enhanced acquisition of P, Zn, Cu and Fe by mycorrhizal plants has been reported by other researchers (Abbaspour, 2010; Abbaspour *et al*., 2012).

The Ca caption is an important factor in the resistance of plants to salinity. The higher Ca nutrient occurred in M than NM plants, and NaCl salinity reduced the uptake of Ca in pistachio plants. Salinity induced Ca deficiency has been reported in other studies (Evelin *et al*., 2012). Colonization and sporulation of AMF were enhanced by the higher levels of Ca, which contributes to maintain cellular homeostasis and plant growth under salt stress. Our study showed the negative effect of salinity on N content in seedling. Salinity affects total N uptake and soil N contribution, leading to reduced plant growth (Van Hoorn *et al*., 2001). Our results showed significant increase of K and P concentration in *G. versiforme* compared to *G.etunicatum.* Increased K and P concentration in pistachio plants by *G. versiforme* colonization help the seedling to improve plant growth and survive in salinized soil.

Conclusions

In summary, our results suggested that although salinity significantly inhibited the symbiosis establishment of pistachio plants the inoculated plants showed increased plant growth and were more tolerant to salt stress than the uninoculated ones. This can attributed to the improvement of nutrient acquisition of roots, demonstrating the potential of AMF colonization for the protection against salt damage of pistachio plants. Reduction in shoot Na uptake and maintaining electrical conductivity of the soil may help mycorrhizal plants to survive in saline conditions. As a result, under our experimental conditions, *G. versiforme* was an effective fungus for applying mycorrhiza for vegetation of saline soils.

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