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ORIGINAL ARTICLE

Role of Mycorrhizal Symbiosis in Growth and Salt Avoidance of Pistachio Plants

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In a greenhouse experiment, plant growth and rates of uptake and root to shoot transport of chloride and sodium were investigated in seedlings of pistachio (*Pistacia vera* cv. Badami-Riz-Zarand) inoculated with *Glomus mosseae* and exposed for 21 and 42 days with four salinity levels (0.5, 3.0, 6.0 and 9.0 dSm^{-1}). Mycorrhizal (+M) plants maintained greater root and shoot biomass at all salinity levels compared to non-mycorrhizal (-M) plants. In -M plants, salt intensity had no significant effect on shoot dry weight (SDW) and leaf dry weight (LDW) on each of harvesting dates but root dry weight (RDW) showed a significant decrease at the highest salinity level 42 days after the start of salt treatment (DAT) in comparison with control (EC of 0.5 dSm^{-1}). In +M plants, SDW was increased with an increase in salt intensity especially in the first harvesting date. The same increase was observed in RDW of +M plants while LDW was not affected by salt stress levels. Rates of uptake and root to shoot transport of Cl^- and Na^+ were markedly lower in +M than in -M plants leading to decrease in accumulation of them. In conclusion, the study indicates that pistachio tolerance to salt stress is improved by mycorrhizal colonization, although the salinity levels used in this work could not induce biomass reduction in -M pistachio plants, higher levels of salinity should be investigated in order to optimize the effect of this symbiosis.

Key words: Glomus mosseae, Mycorrhiza, Pistacia vera, Salt stress

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Key words: Glomus mosseae, Mycorrhiza, Pistacia vera, Salt stress

The significance of soil salinity for agricultural yield is enormous (Tester and Davenport, 2003) as it affects the establishment, growth and development of plants leading to huge losses in productivity (Giri *et al.*, 2003; Mathur *et al.*, 2007). The direct effects of salt on plant growth may involve: (a) reduction in the osmotic potential of the soil solution that reduces the amount of water available to the plant causing physiological drought; (b) toxicity of excessive Na^+ and Cl^- ions towards the cell which may lead to disruption in the structure of macromolecules, damage to cell organelles and plasma membrane, disruption of photosynthesis, respiration and protein synthesis (Feng *et al.*, 2002), however, a major impact of sodium chloride (NaCl) salinity on growth inhibition is excess sodium (Na^+)

which may compete with potassium (K^+) in membrane transport, interferes enzyme activity and impairs the ability of plant to grow (Giri *et al.*, 2007); and (c) nutrient imbalance in the plant caused by nutrient uptake and/or transport to the shoot leading to ion deficiencies (Adiku *et al.*, 2001).

Salinization of soil is a serious problem and is increasing steadily in many arid and semi-arid parts of Kerman province especially in Rafsanjan region that is thought to be the largest center of pistachio production in Iran and in the world (Bagheri *et al.*, 2011). High levels of soil salinity in Rafsanjan region is mainly due to the soluble salts in irrigation water and fertilizers used by pistachio producers annually, low precipitation and high temperature in this region and over-exploitation of available water resources (e.g. ground water). Although pistachio plants are known to be tolerant to salts (Behboudian *et al.*, 1986; Picchioni *et al.*, 1990; Ferguson *et al.*, 2002), adverse effects of salinity on growth, photosynthetic rates and morphological changes in the leaves of pistachio have been shown (Behboudian *et al.*, 1986; Picchioni *et al.*, 1990; Munns *et al.*, 2002; Ranjbarfordoei *et al.*, 2001). A range of symptoms have been described, with chlorosis on the tips of older leaves, followed by necrosis and finally death of leaves (Xu *et al.*, 1999). Walker *et al.* (1987) reported highest chloride concentrations were in laminae and petioles of salt-treated pistachio plants, whereas sodium concentrations were highest in roots, especially the proximal roots, indicating retention of sodium in the lower part of the plant. However, an important characteristic of *Pistacia spp.* is their ability to store large quantities of Na^+ in roots, which might make pistachio to be resistant to Na^+ (Picchioni *et al.*, 1990).

AM fungi widely occur in salty soils (Wang and Liu, 2001). Recently, many researchers reported that AM fungi could enhance the ability of plants to cope with salt stress (Yano-Melo *et al.*, 2003; Rabie, 2005; Jahromi *et al.*, 2008) by improving plant nutrient uptake (Cantrell and Linderman, 2001; Asghari *et al.*, 2005) and ion balance (Zandavalli *et al.*, 2004; Giri *et al.*, 2007), protecting enzyme activity (Rabie and Almadini, 2005; Giri and Mukerji, 2004), and facilitating water uptake (Ruiz-Lozano and Azcón, 1995).

We are not aware of any studies on salt tolerance of mycorrhizal *Pistacia vera* and so the present study was conducted to define the effect of an established AM association on the uptake rate, root to shoot transport and distribution of chloride, sodium and potassium and further to estimate root and shoot Na^+/K^+ ratios in order to improve understanding of the mechanisms regarding the alleviation of salt toxicity in AM pistachio plants.

MATERIALS AND METHODS

Experimental site:

A greenhouse experiment was conducted in 2010 at the Agri-college of Vali-e-Asr university of Rafsanjan (30°23′06″ N, 55°55′30″ E), at 1523 m a.s.l.

AM inoculum production

Glomus mosseae (Nicolson & Gerdemann) [kindly supplied by Dr. E. Sedaghati (Plant Protec. Dept., Faculty of Agri-college, Vali-e-Asr Uni.)] was propagated in a sterile potted soil cropped with *Zea mays* L. between May and Aug. 2010. AM fungal inoculum consisted of a mixture of rhizospheric soil from trap cultures containing spores, hyphae and mycorrhizal root fragments.

Soil preparation and seed sowing

The soil used was an autoclaved (121°C for 2 h)

sandy loam with the following characteristics: sand 72.2%, silt 14.2%, clay 13.6%, pH 7.6, P 16 mg kg^{-1} soil, K 76 mg kg^{-1} soil, Fe 1.3 $\mu g.g^{-1}$, Zn 1.35 $\mu g.g^{-1,}$ Mn 1.3 $\mu g.g^{-1}$, Cu 1.35 $\mu g.g^{-1}$ and cation exchange capacity 1.8 $dS.m^{-1}$. Adequate amount of fertilizers (NH_4NO_3, K_2SO_4, $MnSO_4$ and Fe EDDHA) were added to soil, based on soil analysis results.

Seeds of P. *vera cv.* Badami-Riz-Zarand, were surface sterilized in 10% sodium hypochlorite for 10 min. and then incubated at 25°C on sterile moist cloth for one week. Five germinated seeds were sown in each pot containing 5 kg of autoclaved soil. The number of seedlings per pot was reduced to 3 within 21 days of germination.

Mycorrhizal inoculation

One hundred gram (fresh mass) of inoculum having on average of 80% of infected roots was placed on the soil surface immediately before planting and after placing the germinated seeds on it, seeds were covered with sterilized sand. Control plants received the same amount of an autoclaved inocula. The growth of seedlings continued for 180 days in greenhouse (T_{max}: 38±3°C; T_{min}: 22±4°C; RH: 35±3%; 12–14 h day light) before the start of salt treatments and during this period, the seedlings were watered every two days up to FC level with distilled water. At the end of this stage, sampled roots showed an average of 55% colonization.

Salt treatments

Four salt levels including EC of 0.5 (tap water as control), 3.0, 6.0 and 9.0 dSm^{-1}, achieved by adding a mixture of NaCl and $CaCl_2$ in the ratio of 2:1 in irrigation water. To avoid osmotic shock, salt solution in two higher levels (EC of 6.0 and 9.0 dSm^{-1}) was introduced gradually by 2 and 3 steps respectively. Plants were harvested 21 and 42 days after the commencement of salt treatments and during this period, they were irrigated every two days 20% more than predetermined FC level to avoid salt accumulation in the soil.

Root sampling and assessment of arbuscular mycorrhizal colonization

The experiment was terminated by separating shoots from roots 21 and 42 days after treatments commencement. Roots were carefully rinsed with running tap water and then the roots of 3 plants in each pot was mixed and cut into 1cm in length segments. Samples for mycorrhizal assessment were prepared according to method of Phillips and Hayman (1970). Roots were boiled for 1 h in 15 % KOH and then washed with tap water. Staining was performed in 0.05 % trypan blue by autoclaving the samples for 15 min. Thereafter, Samples were stored in lacto glycerol [mixture of lactic acid, glycerol, 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 according to Giovanetti and Mosse (Giovanetti and Mosse, 1980) at 100× magnification using 50 root segments of each sample. Roots used to determine AMF colonization were dried, weighed, and added to the total.

Growth parameters

At each harvest, root, leaf and stem biomass were determined after oven drying at 70°C for 72 h.

Ion analysis

Chloride was estimated by silver ion (0.05 N) titration of aqueous extracts (Chapman and Pratt, 1962). Sodium and Potassium concentrations were measured by flame photometry.

Rates of net ion uptake (J) and root to shoot transport (J_s) were calculated as described by

Pitman (Pitman, 1975), viz.:

$$J = \frac{(M_2 - M_1)}{\overline{W}_R(T_2 - T_1)}; \quad J_S = J.\frac{X_S}{X_S + X_R}$$

M_2 and M_1 are ion contents of the plant at times T_2 and T_1 and $\overline{W_R}$ is expressed as the logarithmic mean root weight between T_2 and T_1.

$$\overline{W}_R = \frac{W_{R2} - W_{R1}}{\log e(W_{R2}/W_{R1})};$$ X_S and X_R are ion contents of the shoot and root respectively, between T_2 and T_1.

Experimental Design

The experiment consisted of a randomized block design with three factors: (1) mycorrhizal inoculation with *G. mosseae* plus a non-mycorrhizal treatment, (2) four salinity levels (EC 0.5, 3.0, 6.0 and 9.0 dS.m^{-1}) and (3) two harvesting dates [21 and 42 day after treatment (DAT)] with 4 replications per treatment. Data were analyzed using MSTATC software and means were compared by Duncan's test at the 5% level.

RESULTS

Root colonization

None of the pistachio plants in the non-inoculated treatments were colonized by *G. mosseae*. Plants inoculated with *G. mosseae* had AMF root colonization of 53–76%. The highest colonization level was in salt level of 3.0 dSm^{-1}, 21 DAT and the lowest was in EC of 9.0 dSm^{-1}, 42 DAT (Table 1). There was significantly a negative correlation between AMF root colonization and salinity levels beyond EC of 3.0 dSm^{-1} which means salinity suppresses AM establishment.

Growth parameters

Dry weight of shoot and root was higher in +M than -M plants regardless of salinity intensity and duration (Fig. 1). Mycorrhizal symbiosis caused about 3, 2 and 2 fold increase in stem, root and leaves mean dry weight respectively in compare with -M plants. In -M plants, salt intensity had no significant effect on SDW (Fig. 1A,B) and LDW (Fig. 1C,D) on each of harvesting dates but RDW showed a significant decrease at the highest salinity level 42 DAT (Fig. 1F) in comparison with control (EC of 0.5 dSm^{-1}). In +M plants, SDW was increased with an increase in salt intensity especially in the first harvesting date (Fig. 1A). The same increase was observed in RDW of +M plants (Fig. 1E, F) while LDW was not affected by salt stress levels (Fig. 1C, D).

Rate of Uptake and root to shoot transport of Cl^- and Na^+

The rate of uptake and root to shoot transport of Na^+ was significantly different between +M and -M plants (Fig. 2). In -M plants, Na^+ uptake rate was not influenced by different salt treatments whereas in +M plants, the rate of Na^+ uptake was lower than -M plants at all salinity levels but this difference was significant just with EC of 0.5 dS m^{-1} where it was nearly half the rate of uptake by -M plants (Fig. 2A). The rate of Na^+ root to shoot transport was increased in -M plants up to EC of 6.0 dS m^{-1} and then decreased significantly. Transport rate of Na^+ between root and shoot was lower in +M plants in comparison with -M plants at all salinity levels except with EC of 9.0 dS m^{-1} (Fig. 2B). Rates of uptake and root to shoot transport of Cl^-, however, were markedly higher in -M than in +M pistachio plants. Uptake rate of Cl^- in +M plants showed about 50% decrease in EC of 6.0 and 9.0 dS m^{-1} in comparison with -M plants. Whereas in -M plants, from lowest to highest levels of salinity, uptake rate of Cl^- showed a 50% increase, in +M plants, no remarkable change was observed (Fig. 2C). The rate of root to shoot transport of Cl^- in -M plants was higher than +M plants at all salinity levels except

with EC of 3.0 dS m^{-1}, as in highest level of salinity, it was doubled in -M plants (Fig. 2D).

Distribution of Cl^-, Na^+ and K^+

In both harvesting date, the minimum K^+ content of shoot was obtained in maximum salt level whereas the highest amount of shoot K^+ was recorded in EC of 3.0 dS m^{-1}. Applied mycorrhizae had no significant effect on shoot K^+ (Fig. 3A, B). In -M plants, increase in salt intensity during first harvesting date caused a significant increase in K+ accumulation in root up to EC of 6.0 dS m^{-1} whereas in second harvesting date increase in salinity levels had no significant effect on root K^+. Same as shoot, mean K^+ content of root also was not affected by applied Mycorrhizae (Fig. 4A, B).

The Cl^- content of shoot was increased in -M plants parallel to increase in salinity level as it was 148% more in EC of 9.0 dS m^{-1} in compare with control. Applied mycorrhizae was not able to exclude Cl^- from shoot at all salinity levels up to the EC of 6.0 dS m^{-1} and then reduced the shoot Cl^- content during the highest salinity treatment when the influx of Cl^- into shoot was significantly lower than into shoot of -M plants (Fig. 3C, D). At first harvesting date, root Cl^- content of -M plants was lower than +M plants but in second harvesting date, there was no significant difference between root Cl^- content of -M and +M plants. However, irrespective of harvesting date and salt levels, mean Cl^- content of root in -M plants was 27% lower than +M plants (Fig. 4C, D).

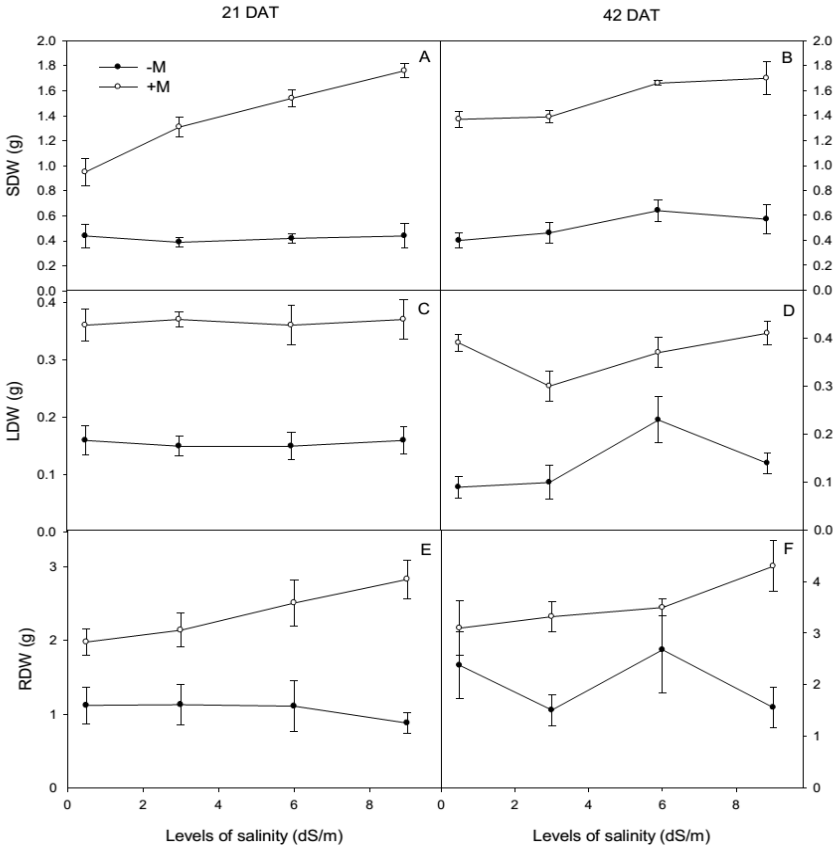
Mean shoot Na^+ content in +M plants was about 18% lower than -M plants and in maximum salt intensity (EC of 9.0 dS m^{-1}) the difference was reached to 29%. Regardless of mycorrhizal treatments and salt stress duration, increase in salinity level up to EC of 9.0 dS m^{-1} caused 55% increase in mean Na^+ content of shoot compare with control. Increase in salt stress duration leaded to a significant increase in shoot Na^+ content at all salinity levels (Fig. 3E, F). In the first harvesting date, mycorrhizae application in EC of 6.0 and 9.0 dS m^{-1} caused a decrease in Na^+ content of roots while in second harvesting date, it reduced Na^+ content of roots at all salinity levels (Fig. 4E, F).

Shoot tissues of +M plants had lower Na/K ratio at all salinity levels 21DAT and the magnitude of Na/K ratio increased as levels of soil salinity increased whereas at second harvesting date, no differences was found between +M and -M plants in this regard (Fig. 5A, B). Na/K ratio was much more in root tissues in comparison with shoot. In -M plants, this ratio showed a positive linear relationship with salinity levels especially at second harvesting date while in +M plants, it decreased sharply at the highest level of salinity (Fig. 5C,D).

Table 1: Arbuscular mycorrhizal root colonization (%) of Pistacia vera plants inoculated with *Glomus mosseae* at four salinity levels. Six months old plants were watered every two days 20% more than predetermined FC level with a salt solution containing a mixture of NaCl and $CaCl_2$ in the ratio of 2:1 during 21 and 42 days. (n=4)

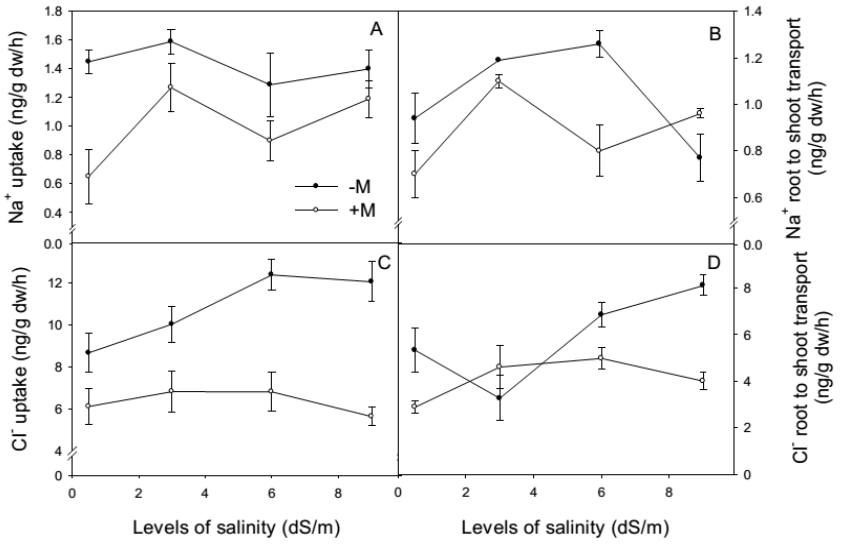
	Electrical conductivity of salt solutions (dSm^{-1})			
Harvesting time	0.5	3.0	6.0	9.0
21 DAT	53^{d}	76^{a}	55^{c}	54^{c}
42 DAT	55^{c}	57^{b}	53^{d}	47.5^{e}

Different letters within a column indicate significant differences at P=0.05 by Duncan's multiple Range test.
DAT = Day after salt treatment



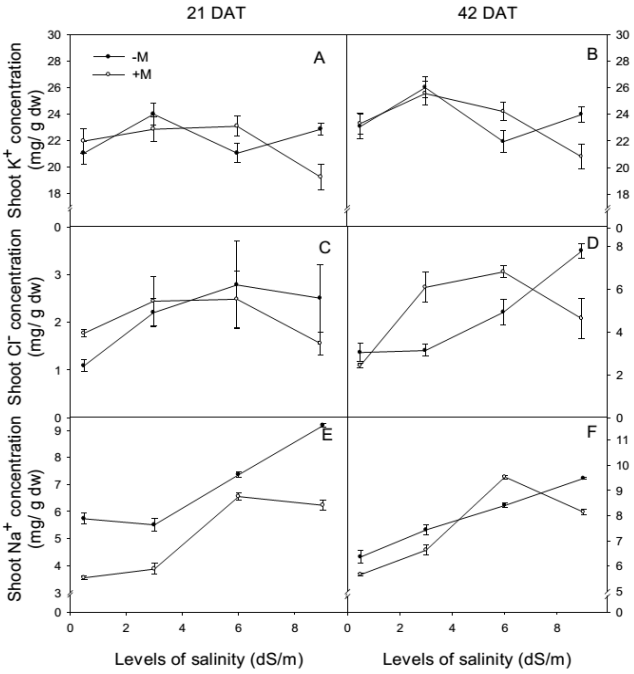
SDW = Shoot dry weight, LDW = Leaf dry weight, RDW = Root dry weight, DAT = Day after salt treatment, +M= Inoculated with *Glomus mosseae*, -M= Non-inoculated

Figure 1: Influence of *Glomus mosseae* (AMF) on stem, leaf and root dry weight of *P. vera* plants at four levels of salinity. Six months old plants were watered every two days 20% more than predetermined FC level with a salt solution containing a mixture of NaCl and $CaCl_2$ in the ratio of 2:1 during 21 and 42 days. Data are means ± SE of 4 replicates.



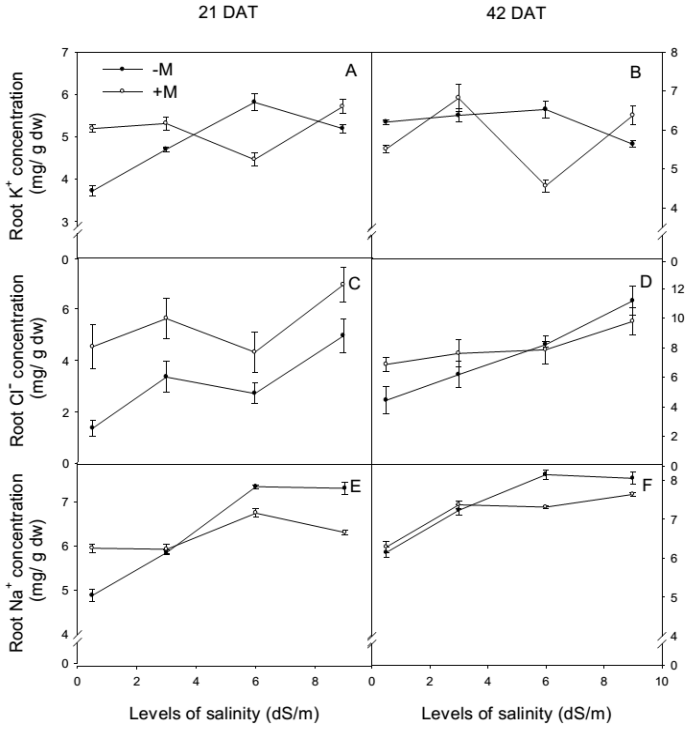
+M= Inoculated with *Glomus mosseae*, -M= Non-inoculated

Figure 2: Influence of *Glomus mosseae* (AMF) on uptake and root to shoot transport rate of Cl^- and Na^+ ions by *P. vera* plants treated with four levels of salinity during a 21 day period. Six months old plants were watered every two day 20% more than predetermined FC level with a salt solution containing a mixture of NaCl and $CaCl_2$ in the ratio of 2:1. Data are means ± SE of 4 replicates.



DAT = Day after salt treatment, +M= Inoculated with *Glomus mosseae*, -M= Non-inoculated

Figure 3: Influence of *Glomus mosseae* (AMF) on K^+, Cl^- and Na^+ concentrations (mg/g dw) in shoot of *P. vera* plants at four levels of salinity. Six months old plants were watered every two days 20% more than predetermined FC level with a salt solution containing a mixture of NaCl and $CaCl_2$ in the ratio of 2:1 during 21 and 42 days. Data are means ± SE of 4 replicates.



DAT = Day after salt treatment, +M= Inoculated with *Glomus mosseae*, -M= Non-inoculated

Figure 4: Influence of *Glomus mosseae* (AMF) on K^+, Cl^- and Na^+ concentrations (mg/g dw) in root of *P. vera* plants at four levels of salinity. Six months old plants were watered every two days 20% more than predetermined FC level with a salt solution containing a mixture of NaCl and $CaCl_2$ in the ratio of 2:1 during 21 and 42 days. Data are means ± SE of 4 replicates.

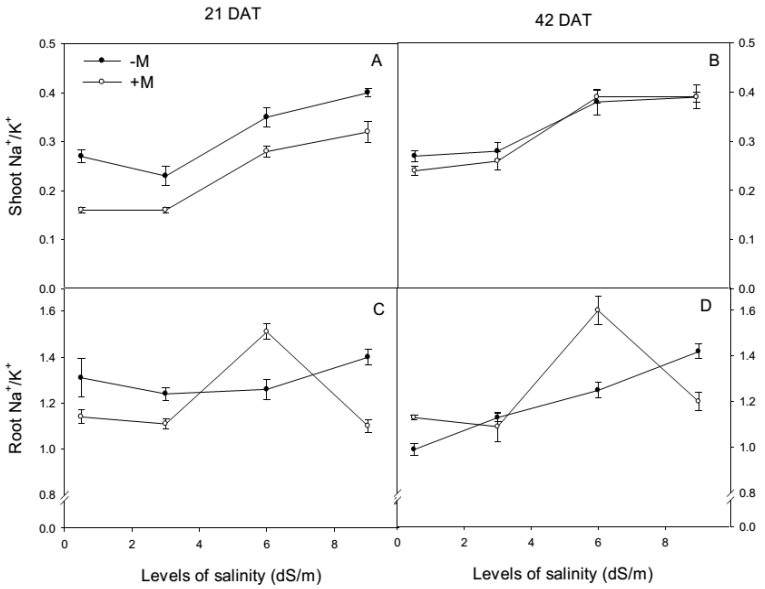


Figure 5: Na^+/K^+ ratio of root and shoot tissues of AM inoculated and Non-inoculated *P. vera* plants grown under four salinity levels. Six months old plants were watered every two days 20% more than predetermined FC level with a salt solution containing a mixture of NaCl and $CaCl_2$ in the ratio of 2:1 during 21 and 42 days. Data are means ± SE of 4 replicates.

DISCUSSION

A reduction in mycorrhizal fungus colonization of pistachio plants grown under saline condition was expected since soil salinity can hamper colonization capacity, spore germination and growth of hyphae of the fungus (Tian *et al.*, 2004; Juniper and Abbott, 2006; Giri *et al.*, 2007; Sheng *et al.*, 2008).

We found no evidence of salt-induced reduction in biomass of un-inoculated *P. vera* over the 42-day period. Although it has been suggested that the salt tolerance of the pistachio is similar to that of the date palm, the most salt tolerant of the horticulturally important woody perennials but prolonged treatment with high salinities will cause growth reduction. For example, Sepaskhah with collaborators (Sepaskhah *et al.*, 1985) observed a 50% reduction in shoot dry matter of *P. vera* seedlings treated for 20 weeks with a soil solution salinity of 159 mM Cl^-. So it can be assumed that the salinity levels and duration used in this experiment were not high enough to induce biomass reduction. Moreover, the data of this study showed a positive correlation between salinity and the enhancement of SDW and RDW in +M plants. Several studies investigated the role of AMF in protection against salt stress have demonstrated that the symbiosis often results in increased nutrient uptake, accumulation of an osmoregulator, an increase in photosynthetic rate and water-use efficiency, suggesting that salt-stress alleviation by AMF results from a combination of nutritional, biochemical and physiological effects and owing to the importance of AMF under salt stress conditions, they have been considered as bioameliorators of saline soils (Feng *et al.*, 2002; Sannazzaro *et al.*, 2007; Zuccarini and Okurowska, 2008). If we accept that the applied levels of salinity were under detrimental threshold then the positive effects of low Na ion concentration in permeability of root cell walls in absorbance of nutritional elements and also the

positive effects of Ca ion in growth may be responsible for enhancing of biomass. This finding is supported by Pessarakli (Pessarakli, 2001) who showed that the low levels of salts stimulate the growth and increases the yield of cotton. At the moment, it is not possible to describe more deeply, in the presence of *G. mosseae* how salinity induced the growth, since the salt tolerance mechanism in pistacia species has not been yet elucidated itself.

Sodium transport is largely suggested as a unidirectional flow and thus results in progressive accumulation of Na in the stem and leaf tissues with age of the plant. In this study, +M plants had lower concentration of Na in shoot tissues even at a high salinity level (EC of 9.0 dS m^{-1}), which increased significantly in -M plants at same salinity level (Fig.3E, F). On the other hand, reduction in Na concentration of root tissue and root to shoot transport rate was also observed in +M in comparison with -M plants (Fig. 2B, Fig. 4E and F). This suggests that AM fungus in *P. vera* roots accumulated more salt and thus prevented transport of Na to shoot tissues and this may be a strategy whereby AM fungi alleviate the detrimental effect of salinity. Earlier, Cantrell & Lindermann (Cantrell and Linderman, 2001) suggested that Na might have been retained in intraradical AMF hyphae. However, further investigations are required to find out this mechanism.

In our experiment, Cl^- accumulation in shoot tissue of -M plants was increased with increasing salinity levels which can be attributed to the enhanced rate of uptake and root to shoot transport. The concentration of Cl^- increased in +M plants shoot with increasing salinity up to a certain level (EC of 6.0), and subsequently decreased at higher salinity. This suggests that AMF induce a buffering effect on the uptake of Cl^- when the content of Cl^- goes beyond the permissible limit. However, lower shoot Cl^- concentrations in +M plants in the highest salinity level is presumably because of dilution within the more rapidly expanding shoot biomass. Although Cl^- uptake rate was much lower in +M plants especially at higher salinity levels, mean root Cl^- content of –M plants was about 27% lower than +M plants which can be related to a lower transport rate in +M. The high tissue Cl^- concentrations can be toxic to crop plants and may restrict agriculture in saline regions (Xu *et al.*, 1999). This problem can be tackled to some extent by the application of arbuscular mycorrhiza, which can reduce the uptake of Cl^- ions (Zuccarini and Okurowska, 2008). The Cl^- ions can be compartmentalized in vacuolar membranes, thereby preventing them from interfering with the metabolic pathways in the plant (Cantrell and Linderman, 2001). The similarity in salt tolerance and Cl^- exclusion ability between +M and –M plants indicates that from a salinity tolerance viewpoint, the presence of Cl^- ion in top of pistachio plants plays a more important role in growth suppression than Na^+.

When Na^+ or salt concentration in the soil is high, plants tend to take up more Na^+ resulting in decreased K^+ uptake. Na^+ ions compete with K**+** for binding sites essential for various cellular functions. In our study, K^+ content of root and shoot was not influenced by mycorrhiza which is in contrast with some previous reports that AMF enhances K^+ uptake suggesting that mycorrhizal responses may only be significant beyond a certain level of salinity. However, it is noticeable that Na^+/K^+ ratio was significantly lower in shoot of +M plants. The lower Na^+/K^+ ratio helps to prevent the disruption of various K-mediated enzymatic processes and

inhibition of protein synthesis. Low Na^+/K^+ ratios are also beneficial in influencing the ionic balance of the cytoplasm or Na^+ efflux from plants (Founoune *et al.*, 2002; Colla *et al.*, 2008).

In conclusion, the study indicates that pistachio tolerance to salt stress is improved by mycorrhizal colonization, although the salinity levels used in this work could not induce biomass reduction in un-inoculated pistachio plants. +M pistachio plants showed a greater ability for Cl^- and Na^+ exclusion than -M plants that is related to lower uptake and root to shoot transport rate especially at the highest level of salinity. The lower Na^+/K^+ ratios in shoot and to some extent in root tissues of +M plants may help in protecting disruption of K-mediated enzymatic processes under salt stress conditions. However, higher levels of salinity should be investigated in order to optimize the effect of this symbiosis.

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