

Effects of arbuscular mycorrhiza inoculation on plant growth, biological and physiological parameters and mineral nutrition in pepper grown under different salinity and p levels

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Abstract

A study was conducted in a greenhouse, to investigate the effects of arbuscular mycorrhizal fungi (*Glomus intraradices*), soil salinity and P availability on growth (leaf area and dry weight), nutrient absorption and ion leakage, chlorophyll, soluble sugar and proline content and alkaline phosphatase activity of pepper plants (*Capsicum annuum* L.). Plants were grown at four levels of salinity (0, 50, 100 and 200 mM NaCl) and two P levels (10 and 40 mg kg⁻¹). Colonisation was 80% to 51% in non-stressed and high salt-stressed plants, respectively. The mycorrhizal dependency was high and only reduced at the higher salinity level. Mycorrhizal plants maintained greater root and shoot biomass at all salinity levels compared to non-mycorrhizal plants, regardless the P level. Interactions between salinity, phosphorous and mycorrhizae were significant for leaf area, root and shoot dry mass. Non-mycorrhizal plants accumulated higher Na and lower K and P compared to mycorrhizal plants. The cell membrane integrity was greater in mycorrhizal plants than in non-mycorrhizal ones. The proline content increases with increasing salt stress and was significantly higher in leaves than in roots. The results indicate that the mycorrhizal inoculation is capable of alleviating the damage caused by salt stress conditions on pepper plants, to maintaining the membranes stability and plant growth, and this could be related to P nutrition.

Keywords: *Capsicum annuum* L., *Glomus intraradices*, cell membrane stability, phosphorus availability.

1. Introduction

Salinity in soil or water is of increasing importance to agriculture because it causes a stress condition to crop plants. Salt-affected soil is one of the serious abiotic stresses that cause reduced plant growth, development and productivity worldwide. The salt-affected soils occupy approximately 7% of the global land surface (Sheng *et al.*, 2011).

Salt stress reduces plant growth, leaf expansion and induces mineral deficiency, like other stresses, destabilizes cell membranes, alters selective permeability (leakage of cell solutes), fluidity, microviscosity, and affects the solubility of many essential substrates and ions. AM is a biological strategy (Cekic *et al.*, 2012), arbuscular mycorrhizal

fungi (AMF) widely exist in salt affected environments, and form roots symbiotic associations, in most plant species. This association allows plants to explore larger volumes of soil to absorb more water and nutrients uptake and transport, and increases absorption of immobile mineral elements such as phosphorus (P), provides resistance to soil pathogens and drought, and improves water-use efficiency (Al-Karaki, 2000; Beltrano *et al.*, 2003). Therefore, AM symbiosis increases leaf area, delays senescence (Beltrano *et al.*, 2003; Beltrano and Ronco, 2008), and increases salinity resistance in several host plants, such as maize and tomato (Al-Karaki, 2000; Feng *et al.*, 2000) and pepper (Kaya *et al.*, 2009; Cekic *et al.*, 2012).

Many researchers reported that AMF could enhance the ability of plants to cope with salt stress (Ruiz-Lozano *et al.*, 1996; Jahromi *et al.*, 2008) by improving mineral nutrient absorption (Cantrell and Linderman, 2001), maintaining ion balance (Giri *et al.*, 2007), protecting enzyme activities, and facilitating water uptake (Colla *et al.*, 2008). Moreover, free amino acids, such as proline, is a contributor to osmotic adjustment in salt-stressed plants. Some studies have shown a reduction in proline levels in AM plants under salt stress (Ruiz-Lozano *et al.*, 1996; Jahromi *et al.*, 2008). On the contrary, some studies have presented an increase in proline accumulation in mycorrhizal plants subjected to salt stress (Kaya *et al.*, 2009). The accumulation of sugars induced by the AM symbiosis is a positive response to salt stress, since it can prevent structural changes in soluble protein, maintain the osmotic equilibrium in plant cells, and protect membrane integrity (Feng *et al.*, 2000).

Inorganic phosphorus often occurs in the soil at low concentrations and moves to roots primarily by diffusion. As phosphorus is absorbed rapidly, phosphorus depletion zones can form around roots and hyphae (Ruiz-Lozano *et al.*, 1996). Several studies have suggested that improvement of plant P status is the most important mechanism through which AMF confers salinity tolerance to plants (Al-Karaki *et al.*, 2006). Fungal succinate dehydrogenase (SDH) and

alkaline phosphatase (ALP) activity were used as index of viability and activity of mycorrhizal fungi, within the phosphate metabolism (Tisserant *et al.*, 1993). However, the mechanisms by which the AM symbiosis influences the metabolism of host plants under salinity stress are not clear (Kaya *et al.*, 2009, Cekic *et al.*, 2012).

Symbiotic interactions (especially in terms of growth and cell membrane stability) between AMF and host plants need to be studied under salt-stress conditions. Cellular membrane stability, measured as the conductivity of electrolytes leaking from leaf disks at high temperature, has been suggested as a screening technique to determine heat tolerance in plants. Various studies have suggested the effectiveness of this technique in salt stressed, heat tolerance and in drought stressed crops (Feng *et al.*, 2000; Beltrano *et al.*, 2003; Beltrano and Ronco, 2008). Little is known, about the effect of the AM symbiosis on the regulation of organic solute levels in pepper plants under saline conditions. Incorporating factors that enable plants to tolerate salt stress could improve plant growth under saline conditions.

Horticulture is the most promising area for practical use of AMF for nursery. There are two main benefits from introducing mycorrhizal fungi to horticultural crops: stronger growth in the nursery and improved performance after planting in the field. Pepper (*Capsicum annuum* L.) is one of the most common crops produced in nurseries, and one of the most important in Argentinean horticultural regions (Ronco *et al.*, 2008). Davies *et al.* (2000) observed that mycorrhizae can improve P absorption in pepper and increase shoot and root dry weight. The aim of this study was to determine - the effect of the AMF (*Glomus intraradices*) and phosphorus availability on the accumulation of organic solutes, plant growth, cell membrane stability, and mineral nutrient acquisition in pepper plants at different levels of soil salinity. For these purpose the content of proline, total soluble and reducing sugars, chlorophyll and SDH and ALP activity and interpreted modifications related to

Table 1. Weather records: air temperature and solar irradiation during September 2010-December 2010 period (Asborno and Pardi 2010).

Months	Air temperatura (°C)			Daily average solar irradiation (W . m ⁻²)
	Average	Max	Min	
Sept.	13.1	18.2	8.5	3171.4
Oct.	14.8	20.3	9.1	4621.2
Nov.	18.0	24.7	11.7	5302.2
Dec.	22.6	29.2	15.7	6128.9

possible mechanisms involved in the mitigation of salt stress by the AMF in pepper were measured.

2. Materials and Methods

2.1. Plant material and growth conditions

Experiments were conducted in a greenhouse at Instituto de Fisiología Vegetal (INFIVE), La Plata, Argentina (34° 52' S; 57° 58' W) under natural photoperiod, between September and December of 2010. Weather records as air temperature and solar irradiation were provided by Estación Experimental Ing. Agr. J. Hirschhorn, during September 2010-December 2010 period. The temperatures during the experiments were between the maximum 29,2°C in December and the minimum 8,5°C in September (Asborno and Pardi, 2010) (Table 1).

Seeds of pepper (*C. annuum* L. 'California Wonder 300') were germinated in a mix of soil, perlite and vermiculite (2:1:1 v/v) tinaldized, for excluding native AMF propagules. Half of the pots received the AMF (*Glomus intraradices*, Schenck & Smith GA4 isolation; Banco de Glomeromycota In Vitro BGI, Buenos Aires, Argentina) by placing 10% (w/w) of inoculum in the soil at the sowing time. The AM inoculum consisted of soil, spores (50 spores g⁻¹ inoculum), hyphae and infected clover root fragments. Sterilized inoculum was

added to "non-mycorrhizal" pots, in order to provide the same soil conditions. When root colonisation with *Glomus intraradices* (*Glomus intraradices* Schenck & Smith DAOM 197198 (recently reassigned to *G. irregulare* and then *Rhizophagus irregularis* (Błaszk., Wubet, Renker & Buscot) C. Walker & A. Schüßler comb. nov.; Stockinger *et al.*, 2009) was approximately 50% and non-inoculated pepper plants (50-day-old) were transplanted to 1L plastic pots, filled with an argiudol vertic soil mixed with sand (2:1 v/v). Soil properties before mixture were pH 5.5, 6 mg kg⁻¹ total P, 3.5% organic matter and 0.24% total N. The soil mix was tinaldized at 100°C for 60 minutes, during 3 consecutive days. Phosphorous as K₂HPO₄ was added at a rate of 10 or 40 mg kg⁻¹ soil, at the moment of transplanting.

Plants were established for 5 weeks before being subjected to four NaCl levels (0, 50, 100 and 200 mM NaCl) by addition of a salt solution to soil with the irrigation water and supplemented once a week with 50 mL of a Hoagland's solution per pot. The soil was salinized step-wise to avoid subjecting plants to an osmotic shock, NaCl concentration was gradually increased by 25 mM at one day intervals until reaching the required salinity of each NaCl treatment. Pots were weighed every day to estimate the amount of water loss, which was replaced with deionized water in order to avoid percolation and maintain the soil water potential at field capacity ($\Psi = -0.03$ MPa).

2.2. Measurements

AMF colonisation

Mycorrhizal colonisation was assessed according to Trouvelot *et al.*, (1986) and expressed as rate of mycorrhization (MC%) and relative arbuscules and vesicles abundances (A% and B% respectively). Roots were cleared with 10% KOH (p/v) and stained with trypan blue in lacto-phenol (Phillips and Hayman, 1970). The viability of hyphae was determined by measuring succinate dehydrogenase activity (SDH) (Schaffer and Peterson, 1993).

Mycorrhizal dependency (MD) was calculated according to the following formula:

$$MD = [(DW \text{ inoculated plants} - DW \text{ non inoculated plants}) / DW \text{ inoculated plants}] \times 100$$

2.3. Growth parameters

The experiment was concluded after 8 weeks of growth under salt conditions and all parameters were measured. Leaf area (LA) per plant (Li 3000 leaf area meter, LICOR, Lincoln, NE, USA) was measured. Dry weight (DW) of shoot (leaves and stems) and roots were obtained by oven-drying at 80°C until constant weight; the plants had no fruits for evaluations.

2.4. Biochemical and physiological parameters

Leaf chlorophyll content was determined at harvest ($n = 5$), on one leaf disc (1 cm diameter) per plant according to Moran and Porath (1980).

Cell membrane stability (CMS) in leaves and roots was determined in 500 mg of leaves or roots per treatment, according to Sullivan and Ross (1979), using a Conductivity meter (DIGICOND IV, Buenos Aires, Argentina). CMS was determined according to

to the following equation:

$$CMS = [(1 - (T1/T2)) / (1 - (C1/C2))] \times 100$$

T and C refer to conductivity of treated and control sampled. T1 and C1 represent the electrolyte leakage ($dS m^{-1}$) after incubating at 25 °C for 4 h, and T2 and C2 represent the total electrolyte concentration measured after heating in boiling water for 60 min and cooled to room temperature. T1 and T2 correspond to the first and second solution conductivity determinations of treated samples, and C1 and C2 are the respective values for the controls.

Alkaline phosphatase (ALP) activity was determined in roots that were washed with cold distilled water to remove soil particles and stored at 4°C until analysis. Root fragments were ground in 29 mM 4-aminoantipyrine buffer in 3 M amino-methylpropanol, pH 10, and incubated at 37°C for 10 min. Potassium ferricyanide (10 mM) was added and the homogenate was centrifuged at 2.000 x g for 10 min. The supernatant was used for ALP determination at 520 nm using a kit for alkaline phosphatase determination (Lab Wiener, Rosario, Argentina). ALP activity was expressed in mU mL⁻¹ root extract. One unit (U) of ALP was equivalent to the amount of enzyme releasing 1 μmol product min⁻¹ under assay conditions.

Free proline content was determined from 1 g leaf or root fresh weight (FW), according to Bates *et al.*, (1973). Extraction was made with an aqueous solution of 3% sulfosalicylic acid and the extract obtained reacted with ninhydrin acid and glacial acetic acid. Proline concentration was measured in a spectrophotometer (Shimadzu UV-160, -Kyoto, Japan) at 520 nm absorbance. Proline content was calculated per unit of FW according to:

$$\mu\text{mols proline g}^{-1} \text{ FW} = [(\mu\text{g proline} / \text{mL} \times \text{mL toluene}) / 115.5 \mu\text{g} / \mu\text{mols}] / (\text{g FW} / 5)$$

Soluble sugars (total and reducing) were extracted from 500 mg leaf or roots tissues in hot 80% (v/v) ethanol. Total sugar in leaves and roots was determined colorimetrically using Cronin and Smith, (1979) alkaline copper method. Absorbance was recorded at 510 nm. Reducing sugar content was determined from a standard curve against pure glucose.

2.5. Mineral nutrition

Dried shoots (stems+leaves) were ground to pass through a 0.5 mm sieve in a cyclone laboratory mill and stored for determination of mineral nutrients. Phosphorus content was measured by the molybdovanado phosphate method (Kitson and Mellon, 1944). Nitrogen (N) was determined by micro-Kjeldahl method. Sodium (Na), potassium (K) and calcium (Ca) contents were measured by flame photometry as described by Haddad and Higginson (1990).

2.6. Experimental design and statistical analysis

The experiment consisted of a randomized block design with three factors: (1) AMF treatments (with (M) and without *G. intraradices* (NM)), (2) two P levels (P1, 10 mg kg⁻¹ and P2, 40 mg kg⁻¹) and (3) four salinity levels (S0: 0 mM NaCl; S1: 50 mM NaCl; S2: 100 mM NaCl and S3: 200 mM NaCl). Each treatment was replicated three times and each replicate comprised six plants (i.e., 18 plants per treatment). Differences between treatments were analyzed for main effects (AMF, phosphorous and salinity) and their interactions by ANOVA using SigmaStat 3.5 software (Systat Software, Inc, San Jose, CA, USA). The Tukey's test ($p < 0.05$ or 0.001) was used to evaluate the differences between treatments and interaction means. For the statistical analysis all inoculation percentage values were arcsine transformed to improve homogeneity. For growth data ($n=10$), for mycorrhizal observations ($n=3$ replicates of 30 root fragments).

3. Results

3.1. AMF colonisation

No AMF colonisation was noted in roots of control plants. Peppers grown in non-saline soil and low phosphorous level had high AMF root colonisation, which decreased as soil salinity increased (Table 2).

At low phosphorous level, under the high salt condition (200 mM NaCl), roots colonisation was reduced by 28%. At high phosphorous level no significant changes in AMF colonisation was observed, regardless NaCl concentration. Arbuscules abundance showed a significantly lower percentage only at high P level and 200 mM NaCl. Vesicle abundance decreased by 75% with 200 mM NaCl, regardless P availability. Viability of hyphae, expressed by SDH activity, was reduced by increased NaCl concentration. The highest concentration of salt reduced viability over 50%, regardless phosphorous level (Table 2).

At low P the MD was 77% in the absence of salinity stress, and was reduced by 54% at the highest salinity stress conditions (200 mM). At high P level the MD was nearly 60% and was not modified by salinity stress treatments, the interaction P \times NaCl was positive ($p < 0.001$) (Table 2).

3.2. Plant growth

At low P level, shoot and root DW declined in both mycorrhizal and non-mycorrhizal plants as soil salinity increased. At high P level there were no significant differences on DW (Figure 1). Mycorrhizal plants under control and salinity conditions showed a higher growth in shoot and root DW and LA than non-mycorrhizal plants (Table 3). Leaf area was higher at high P level than at low P level in both mycorrhizal and non-mycorrhizal plants and decreased with salinity stress (Table 3).

There was a statistically significant interaction between P and NaCl ($p < 0.001$) for shoots and roots DW and leaf area.

Table 2. Percentage of mycorrhizal colonisation (MC), abundance of arbuscules (A) and vesicles (B), viable hyphae (SDH), and mycorrhizal dependence (MD) in pepper plants inoculated with AMF grown at different salinity and P conditions.

P Level (mg kg ⁻¹)	NaCl (mM)	MC (%)	A (%)	B (%)	SDH (%)	MD (%)
10	0	80a	61a	75a	68a	77.47a
	50	75a	60a	74a	52b	75.07a
	100	65b	64a	38b	49b	74.70a
	200	58b	56ab	20c	29c	54.49b
40	0	65b	59ab	42b	51b	62.91b
	50	63b	51b	38b	48b	58.36b
	100	60b	50b	22c	34c	60.18b
	200	58b	40c	10d	26c	58.92b
Main Effects						
	P	<0.001	<0.001	ns	ns	<0.001
	NaCl	<0.05	<0.001	<0.001	<0.001	<0.001
Interaction Effects						
	PxNaCl	<0.05	<0.05	ns	ns	<0.001

Within each column, means followed by different letter are significantly different ($p < 0.05$). ns: Not significant

3.3 Biochemical and physiological parameters

Chlorophyll content

In non-stressed conditions, chlorophyll content was not affected by AMF inoculation or by P availability, and decreased with salinity stress regardless of P

level. In the treatments with 200 mM NaCl, mycorrhizal plants had 15% higher chlorophyll content at the lower P level and 25% at the higher P level than non-mycorrhizal plants (Table 3). There was a statistically significant interaction between AMF and NaCl ($p < 0.001$).

Table 3. Effect of salinity concentration and P levels on leaf area (LA), chlorophyll content, root and leaf cell membrane stability (CMS) and alkaline phosphatase activity (ALP) in pepper plants non-inoculated or inoculated with AMF.

AMF Status	P Level (mg kg ⁻¹)	NaCl (mM)	LA (cm ² pl ⁻¹)	Chlorophyll (mg g ⁻¹ FW)	Rroot CMS (%)	Leaf CMS (%)	ALP (UI mg ⁻¹)
NM	10	0	80.8c	1.54a	99.3a	98.5a	3.55a
		50	72.9cd	1.27b	99.2a	36.6d	2.44b
		100	61.9d	1.15bc	94.6a	20.2e	2.57b
		200	53.8d	1.08c	77.6b	15.4e	2.02c
	40	0	101.0b	1.36ab	99.8a	99.1a	2.25b
		50	91.3bc	1.35ab	99.7a	93.5a	2.20b
		100	82.3c	1.02c	87.8b	77.1b	2.65b
		200	67.1d	0.96c	80.5b	55.1c	2.22b
M	10	0	105.4b	1.57a	99.4a	99.2a	2.61b
		50	103.7b	1.39ab	99.2a	91.7a	2.13bc
		100	100.9b	1.29b	96.2a	87.2a	1.84c
		200	80.5c	1.24b	95.4a	65.4b	1.81c
	40	0	132.9a	1.43a	99.3a	99.4a	2.02c
		50	117.9ab	1.23b	99.0a	93.9a	2.04c
		100	114.1ab	1.25b	98.4a	72.3b	2.27bc
		200	100.8b	1.20b	87.6b	59.3bc	2.50b
Main effects							
		AMF	<0.001	<0.001	<0.05	<0.001	<0.001
		P	<0.001	ns	ns	ns	<0.001
		NaCl	<0.001	<0.001	<0.05	<0.001	<0.001
Interaction effects							
		AMF×P	ns	ns	ns	<0.001	<0.05
		AMF×NaCl	ns	<0.05	<0.05	<0.001	<0.05
		P×NaCl	<0.001	ns	ns	ns	<0.05
		AMF×P×NaCl	<0.001	ns	ns	<0.001	<0.05

Within each column, means followed by different letter are significantly different ($p < 0.05$). ns: Not significant

3.4. Cell membrane stability (CMS)

The cell membranes stability was affected by salt stress (Table 3). There were no significant differences in CMS of roots or leaves plasma membrane of mycorrhizal and non-mycorrhizal plants grown in the absence of NaCl, regardless of the P level. The leaves CMS of salinity-stressed mycorrhizal plants was significantly greater than that of non-mycorrhizal ones at low P soil level (10 mg kg⁻¹). In the treatments with 50, 100 and 200 mM NaCl, non-mycorrhizal plants had 60%, 76% and 76% lower CMS than mycorrhizal plants, respectively. There was a statistically significant interaction between AMF_x NaCl ($p < 0.001$) for leaves CMS. With high P availability, there was no significant difference between non-mycorrhizal and mycorrhizal plants, and the CMS decreased slowly with increasing concentration of NaCl (Table 3).

There was no significant difference in electrolyte permeability of the root plasma membrane of mycorrhizal plants, irrespective of NaCl soil concentration or P level. In non-mycorrhizal plants only at 200 mM NaCl was observed significant differences, regardless of P level (Table 3).

3.5. Alkaline phosphatase (ALP) activity

The ALP activity was lower in mycorrhizal plants compared with non-mycorrhizal plants, regardless P availability and salinity ($p < 0.001$).

At low P availability, saline stress reduced significantly ALP activity in non-mycorrhizal and mycorrhizal roots and combine with high salt condition (200 mM) ALP activity was reduced by 44%. At high P availability (40 mg kg⁻¹); in high NaCl concentration (200 mM) ALP activity in non mycorrhizal roots decreased by 59% compared with non-saline and low P. In mycorrhizal plants at high P the ALP activity was increased. There was a statistically significant interaction between AMF_xP_x NaCl ($p < 0.05$) (Table 3).

3.6. Proline contents

Salt treatment induced proline accumulation in leaves and roots independently of P level in soil. With low P content in soil, proline content in leaves was higher in mycorrhizal than in non-mycorrhizal plants, regardless of the salt concentration, while the opposite was observed at high P level (40 mg kg⁻¹). At high P level in non-saline conditions non-mycorrhizal plants accumulated three fold more proline in leaves than mycorrhizal ones and with 200 mM NaCl in soil, the proline content was 42% higher in non mycorrhizal plants. There was a statistically significant interaction between AMF_xP and P_x NaCl ($p < 0.001$). (Table 4).

Accumulation of proline increased significantly in roots as a consequence of saline stress, regardless of P level. At low P level, at 200 mM NaCl, mycorrhizal plants accumulated twofold more proline than non-mycorrhizal plants (Table 4). At high P level, mycorrhizal plants accumulated 34% more proline as compared to non-mycorrhizal ones. There was a statistically significant interaction between AMF_xP, AMF_xNaCl and P_xNaCl, and AMF_xP_xNaCl.

3.7. Sugars contents

Total sugar content in leaves and roots was modified by salinity stress. Total sugar content decreased with increasing salinity. In leaves, mycorrhizal formation increased significantly total soluble sugar content regardless of salinity and P levels (Table 4). At higher concentrations of NaCl (200 mM) and at 10 mg kg⁻¹ P, the total sugar content decreased by 30% and 36% compared with control plants in non mycorrhizal and mycorrhizal plants, respectively. Increasing P (40 mg kg⁻¹ P) reduced the concentration of total soluble sugars in leaves by 11% compared with control plants in non mycorrhizal and mycorrhizal plants. The reducing sugar content shows the same trend as the total sugar content. The content of total and reducing sugars decreased in the roots compared with leaves, regardless of P levels and salinity.

Table 4. Leaf and root proline content, root and leaf total sugar (TS) and reducing sugar (RS) content of pepper plants non-inoculated (NM) or inoculated (M) with AMF grown under different salinity and P conditions.

AMF status	P level (mg kg ⁻¹)	NaCl (mM)	Proline (mM g ⁻¹ FW)		TS (mg g ⁻¹ FW)		RS (mg g ⁻¹ FW)	
			Leaf	Root	Leaf	Root	Leaf	Root
NM	10	0	1.02e	0.34e	6.19b	4.91b	4.51 ^a	3.08b
		50	2.17d	0.39e	6.04b	4.69bc	4.50 ^a	2.68c
		100	2.32cd	0.39e	5.53bc	4.52c	4.45a	2.64c
		200	3.28b	0.48d	4.28e	4.16d	3.89c	2.63c
	40	0	1.70d	0.09g	5.05d	5.07ab	3.60cd	3.41a
		50	1.94d	0.51d	4.78d	4.89b	3.41d	3.39bc
		100	3.99a	0.58d	4.72d	4.31cd	3.38d	3.15b
		200	4.23a	0.65c	4.47e	3.82d	3.43d	2.64c
M	10	0	1.56d	0.20f	7.43a	5.25a	4.32b	3.00b
		50	2.43cd	0.36e	6.69b	4.69bc	3.95c	2.83b
		100	3.94a	0.85b	6.48b	4.32d	3.74c	2.75b
		200	3.98a	1.07a	4.77d	4.18d	3.04e	2.51c
	40	0	0.59f	0.21f	6.00b	4.54c	4.52a	2.80b
		50	0.59f	0.31e	5.59bc	3.96d	3.79c	2.56c
		100	2.76c	0.68c	5.57bc	3.27e	3.70c	2.49c
		200	2.98bc	0.87b	5.39bc	3.41e	3.62c	2.42c
Main effects								
		AMF	<0.001	<0.001	<0.001	<0.05	<0.05	<0.05
		P	<0.001	<0.001	<0.001	<0.05	<0.05	<0.05
		NaCl	<0.001	<0.001	<0.001	<0.05	<0.05	<0.05
Interaction effects								
		AMF x PHOS	<0.001	<0.001	ns	<0.05	<0.05	<0.05
		AMF x SALIN	ns	<0.001	ns	ns	<0.05	ns
		PHOS. x SALIN	<0.001	<0.05	<0.05	ns	<0.05	ns
		AMF x PHOS X SALIN	ns	<0.001	ns	ns	<0.05	<0.05

Within each column, means followed by different letter are significantly different ($p < 0.05$). ns: Not-significant.

3.8. Mineral nutrition

The concentration of P was higher in shoot tissues of mycorrhizal plants, than in non-mycorrhizal plants at all salinity stress levels, and values decreased with increasing levels of salinity. An increase in P contents was caused by P addition in soil. With low P content in soil (10 mg kg⁻¹), at 100 and 200 mM NaCl, shoot tissue phosphorous content was more than 2-fold higher in mycorrhizal than in non-mycorrhizal plants. At high P level (40 mg kg⁻¹) uptake of P increased, in all salinity treatments, regardless mycorrhizal treatments.

The high NaCl content in soil, significantly increased the Na content, as much in the non-mycorrhiza as in mycorrhizal plants, regardless of P availability (Table 5). However, higher Na content in shoots was observed in non-mycorrhizal than in mycorrhizal plants regardless of salinity or P level.

Potassium uptake in non-saline treatment was not modified by mycorrhization or phosphorus availability. However, shoot tissue K contents decreased in non-mycorrhizal plants as soil salinity increased. In mycorrhizal plants no significant differences were determined for K content regardless of salinity and P level. Under high salt stress (200 mM NaCl) shoot tissue K concentration in mycorrhizal plants were 60% higher in presence of 10 mg kg⁻¹ P and 20% at 40 mg kg⁻¹ P level, relative to non-mycorrhizal plants. There was a statistically significant interaction between AMF, P and salt for Na, P ($p < 0.001$) and K ($p < 0.05$) (Table 5).

Shoot Ca contents of both mycorrhizal and non-mycorrhizal plants increased as soil salinity increased, regardless of P availability. Nitrogen uptake was not modified by the treatments. The Na/K ratio increased with the increasing Na in soil, and in non-mycorrhizal plants the ratio was higher than in mycorrhizal plants (Table 5).

4. Discussion

Plants exposed to salt stress undergo changes in their environment. The ability of plants to tolerate salt is determined by the multiple biochemical pathways that facilitate retention and/or acquisition of water, protect chloroplast functions, and maintain ion homeostasis. Mycorrhizal symbiosis is a key component in helping plants to cope with adverse environmental conditions. Salt stress inhibits photosynthetic ability and induces physiological drought in plants and a decrease in crop production. Arbuscular mycorrhizal colonization is reported to promote plant growth and salinity tolerance (Evelin *et al.* 2011). It has been suggested that salt stress may reduce colonisation by arbuscular mycorrhizal fungi via a direct effect on fungal growth (Giri *et al.*, 2007).

In the present study, roots of pepper plants were highly colonized by *G. intraradices* and were higher than other reports, as Kaya *et al.* (2009) with *Glomus clarum*, as Ruscitti *et al.* (2011) and Cekic *et al.* (2012), with *Glomus mosseae* and *Glomus intraradices*, and as Ronco *et al.* (2008) with *Glomus mosseae*.

The ability of *G. intraradices* to colonize the roots of pepper plants declined with increasing NaCl levels. This is in agreement with earlier studies reporting that addition of salt to soil inhibits hyphal growth, which subsequently reduces the spread of mycorrhizal colonisation (Jahromi *et al.*, 2008; Evelin *et al.*, 2011).

With 10 mg kg⁻¹ P, AMF colonisation declined with increasing NaCl level in agreement with results obtained by Al-Karaki (2006), while with 40 mg kg⁻¹ P, colonisation was lower and was not affected by salinity, in accordance with findings by Braunberger *et al.* (1991). The adverse effect of high soil P levels on AMF is well documented. Our results show that the percentage of vesicles and the hyphae viability was higher with no NaCl addition and with 10 mg kg⁻¹ P and declined with increasing NaCl level. Plants do not have a constant phosphatase activity, but respond to environmental conditions such as variation in soil

P availabilities. Generally, root phosphatase activity increases as soil P availability decreases (Fujita *et al.*, 2010).

We have investigated the influence of phosphate fertilization on the physiological activity of arbuscular mycorrhizal infection using fungal alkaline phosphatase activity (ALP) as a potential marker of efficiency of the symbiosis and succinate dehydrogenase (SDH) as a vital stain of metabolically active fungus. Under non stressed or low stress salinity (50 mM NaCl) conditions, phosphate fertilization reduced ALP activity in conjunction with a decrease in mycorrhizal infection and hyphae viability.

The more stable ALP behavior in mycorrhizal plants shows the effectiveness of mycorrhization regarding the provision of P to the plant. In non-mycorrhizal roots, the response is related to P deficiency and the detrimental effect of stress.

The beneficial effects of mycorrhizal fungi on plant growth under saline conditions have been demonstrated in various plant species, by Al-Karaki (2006) in tomato, Feng *et al.* (2000) in maize, Kaya *et al.* (2009) and Cekic *et al.* (2012) in pepper plants. Also, mycorrhizal symbiosis delays senescence, increases leaf area, modifies root architecture (Beltrano *et al.*, 2003), improves the growth of plants under a range of salinity stress conditions (Cekic *et al.*, 2012), and enhances phosphorous (P) absorption, especially when P availability is limited. The growth of pepper plants was reduced in the presence of NaCl. This finding may be explained by nutritional imbalances in the plant due to the salt stress. Previous studies show the positive effects of mycorrhiza on plant growth (Cekic *et al.*, 2012). In our study AMF positively affected the growth of pepper plants, improved shoot and root dry weight and leaf area compared with non-mycorrhizal plants at all salinity levels, in agreement with earlier reports on other species (Tain *et al.*, 2004 ; Al-Karaki, 2006) and with Kaya *et al.* (2009) and Cekic *et al.* (2012) in pepper plants.

We suggest that improved growth, nutrients uptake and the ionic favorable ratios in mycorrhizal plants are an indication of enhanced tolerance to salt stress, compared with the non-mycorrhizal plants.

In this study, the values of MD clearly demonstrated that pepper has a high MD in agree with Ronco *et al.* (2008). At low P level (10 mg kg⁻¹ P), in the absence of salinity stress, the MD was 77%, reaching 75%, 74% and 54% for 50 mM, 100 mM and 200 mM, respectively; which demonstrates the favorable relationship between pepper and *G. intraradices*, and shows that when roots were associated with AM fungi, the detrimental effect of the salinity stress decreased significantly. Taken together, although salinity reduced mycorrhizal colonization, the dependency of pepper plants on mycorrhizal fungi was high. This may be a sign showing the ecological importance of AM association for plant survival and growth under salinity stress. At high P level (40 mg kg⁻¹ P), the MD was nearly 60% and was not modified by salinity stress, and shown, that soil P availability is an important factor for determining the effects of mycorrhiza (Cekic *et al.*, 2012).

The AM symbiosis may improve nutrient uptake by improving exploration soil. O'Keefe and Sylvia (1993) observed that external hyphae adhere to soil particles, which would improve contact with the soil solution, and can access smaller pores than plant roots and root hairs. In this symbiotic association with AMF, the plants acquire water and nutrients by extraradical hyphae that extend beyond root depletion zones to new regions of soil. The beneficial effect of AM symbiosis on plant growth has been largely attributed to higher uptake of phosphorus. In this study AM plants showed higher values of phosphorus at all salinity levels (Table 5). This result indicates that the effect of AM fungi on phosphorus uptake constitutes one of the main mechanisms for increasing plant tolerance of salinity. High NaCl levels (100 and 200 mM NaCl) reduced P concentration in non-inoculated plants (Table 5). It is known that salt-stress induces P deficiency in plants by reducing P uptake or translocation.

Table 5. Shoot content of Na, K, Ca, P, N and Na/K relation of pepper plants, non-inoculated (NM) or inoculated (M) with AMF grown at different salinity and P levels.

AMF Status	P Level (mg kg ⁻¹)	NaCl (mM)	Na	K	Ca	P	N	Na/K
			mg 100 g ⁻¹ DW					
NM	10	0	332c	2058a	1320b	230b	3291	0.16
		50	332c	2058a	1320b	230b	3291	0.16
		100	1196b	1583b	1640a	110d	3151	0.76
		200	1196b	1319b	1680a	96d	2941	0.91
	40	0	340c	1847a	1400b	265a	3571	0.18
		50	340c	1847a	1400b	265a	3571	0.18
		100	950b	1583b	1560a	185c	3221	0.60
		200	2280a	1583b	1600a	166c	2871	1.44
M	10	0	124d	2005a	1480b	260a	3081	0.06
		50	243d	2216a	1400b	257a	3011	0.11
		100	386c	2216a	1560a	224b	3431	0.17
		200	908b	2110a	1680a	205b	3501	0.43
	40	0	106d	1988a	1360b	280a	2871	0.05
		50	280d	2269a	1480b	272a	3151	0.13
		100	440c	2269a	1720a	260a	3011	0.19
		200	990b	1899a	1720a	237b	2801	0.52
Main effects								
AMF			<0.001	<0.05	ns	<0.001	ns	-
P			ns	ns	ns	<0.001	ns	-
NaCl			<0.001	<0.05	<0.05	<0.001	ns	-
Interaction effects								
AMF x P			<0.001	<0.05	ns	<0.001	ns	-
AMF x NaCl			<0.001	<0.05	ns	<0.001	ns	-
P x NaCl			<0.001	<0.05	ns	<0.001	ns	-
AMF x P x NaCl			<0.001	<0.05	ns	<0.001	ns	-

Within each column, means followed by different letter are significantly different ($p < 0.05$). ns: Not significant

Under non salinity or low salinity (0 mM and 50 mM NaCl) conditions inoculated and non-inoculated plants had similar P content, although the dry weight of the mycorrhizal plants was significantly higher than the non-mycorrhizal plants. This suggests that AMF enhances the plant growth by mechanisms that may not be related to improvement of P nutrition (Ruiz-Lozano *et al.*, 1996; Feng *et al.*, 2000). The higher P content in mycorrhizal plants, at high salinity stress (100 and 200 mM NaCl) suggests that alleviation of salt stress by AMF includes improvement of P nutrition, in agreement with findings by Al-Karaki (2000), and Giri *et al.* (2007) who have suggested that improvement in phosphorus plant status is an important mechanism of salinity stress tolerance in mycorrhizal plants. However, other studies have shown that mycorrhizal plants grow better than non-mycorrhizal plants under salt stress even when both have similar P status (Ruiz-Lozano *et al.*, 1996; Feng *et al.*, 2000), implying that the advantages of mycorrhizal for plant growth under salt stress are not always related to P status improvement.

In the last decade, researchers have attempted to address the problem of salinity and nutrient disorder. The emphasis has been put on the effect of mycorrhizal colonisation on enhance K uptake and Na/K ratio in plants (Colla *et al.*, 2008), and few studies have addressed Ca and Mg uptake (Giri *et al.*, 2007) under salinity stress. Moreover, Ruiz-Lozano *et al.* (1996) concluded that the mechanisms underlying mycorrhizal plant growth improvement under saline conditions are based on physiological processes (increase carbon dioxide exchange, transpiration, stomatal conductance and water use efficiency) rather than on nutrient uptake (N or P). In our study, Na concentrations in shoots increased proportionately to the level of NaCl added to the soil. At high salinity treatments, non-mycorrhizal plants show more than double Na content compared to mycorrhizal plants. Moreover, Al-Karaki (2006) observed high Na retention by roots, without transport to shoots in inoculated plants, and suggests that the Na could be retained in the intraradical hyphae or compartmentalized in the root cell vacuoles.

Moreover, Hammer *et al.* (2011) propose that, AMF excludes Na by discrimination in its uptake from the soil or during its transfer to plants. These findings substantiate that AMF induces a regulatory effect on the translocation of Na to the aerial parts, maintaining a higher Na/K ratio in shoots of non-mycorrhizal plants. Sodium transport is largely suggested as a unidirectional flow, and results in a progressive Na accumulation in shoots and leaf tissues, mainly accumulated in older leaves because of its poor translocation (Lazof and Lauchli, 1991). Meanwhile, Al-Karaki (2006) suggested that lower Na content in mycorrhizal plants tissues might be explained by a dilution effect due to growth enhancement by AMF. This suggests that AMF in pepper roots are capable of accumulating more Na reducing the transport to shoot tissues, in concordance with findings by Cantrell and Linderman (2001). This event could be another strategy whereby AMF would alleviate the detrimental effects of salinity. Further investigations are required to find out this mechanism. Results of this work shown that in non-stressed or with 50 mM NaCl stressed plants; K content was not modified by AMF or by P availability. Under high salinity conditions, higher K contents were determined in mycorrhizal plants, in agreement with results by Colla *et al.* (2008) in zucchini. In resume, the high NaCl stress modifies the absorption of Na, P, K and Ca significantly. Meanwhile, N was not significantly affected by salinity, by inoculum, or by P addition.

Salinity stress induced nutritional disorder in the plant, and could produce damage in macromolecules such as chlorophyll, resulting in a loss of photosynthetic activity and in membrane integrity, and might accelerate senescence processes. Prevention of lipid peroxidation and maintenance of membrane integrity has been considered as one of the key processes in salinity tolerance (Garg and Manchanda 2009). The measurement of electrolyte leakage from plant tissues is a classical method to estimate membrane integrity in response to environmental stresses, senescence, fruit ripening, etc. The cell membrane is the first organelle to be affected by salt stress. The integrity

of the membrane is disrupted due to peroxidation of lipids and result in increased membrane permeability (Kaya *et al.*, 2009). Our data show that under low P level and salinity stress, AM symbiosis improves the cell membrane stability. In non-mycorrhizal plants, the salinity stress caused a significant decrease in CMS, compared to non-stressed plants, and confirm the findings by other authors, who showed that salinity-stressed pepper plants inoculated with AMF had lower membrane permeability than non-inoculated pepper (Kaya *et al.*, 2009). In the present study, leaf cells membranes stability of non-mycorrhizal plants at lower P level was severely affected by all salinity stress treatments. Meanwhile, in mycorrhizal plants, leaf cell membrane stability decreased moderately only at high salinity level (100 mM and 200 mM NaCl), regardless of P conditions. The root cell membrane stability of non-mycorrhizal plants at lower P level was affected only under higher salinity stress conditions, and the AMF inoculation maintained the integrity of root cell membranes. Our results show that membrane stability was associated with AMF colonisation, and may contribute to salinity stress tolerance. Thus, we propose that, AMF improved mineral nutrition of plants under saline conditions, and reduce the negative effects of Na by maintaining membrane integrity, in agreement with Cantrell and Linderman (2001) and Beltrano and Ronco (2008). Decreased electrolyte leakage in leaves of M plants may be related to P-induced changes in membrane phospholipid levels and associated changes in permeability properties (Evelin *et al.* 2011).

Moreover, in agree with these authors, our results show that mycorrhizal colonisation improved chlorophyll content under moderate (100 mM NaCl) and severe (200 mM NaCl) stress conditions, compared with non-mycorrhizal plants, but did not modify the chlorophyll content under non-stressed (0 Mm NaCl) or low stressed (50 mM NaCl) conditions, different results were obtained by Kaya *et al.* (2009), who showed detrimental effects even at 50 mM NaCl in pepper plants.

Some plants resist salinity by regulating the osmotic

potential of cells, as a defense strategy. They can accumulate higher concentrations of organic solutes of low-molecular weight, such as proline, betaine, soluble sugars or amino acids, which are generally at low concentration when plants are not under stress. The accumulation of proline under salt stress protects the cell by balancing the osmotic pressure of cytosol with external environment. A positive correlation between the proline accumulation and salt tolerance has been suggested (Giridara Kumar *et al.*, 2003). In agreement with these authors, our results show that the proline content increase with increasing salt stress and is significantly higher in leaves than in roots. At low P and at all salinity levels, the leaves of mycorrhizal plants accumulated more proline than non-mycorrhizal, while the roots of M accumulated more proline, only at higher levels of stress (100 and 200 mM NaCl). Meanwhile, at high P level, the leaves of non-mycorrhizal plants accumulated more proline compared with those of mycorrhizal plants. The roots of mycorrhizal plants accumulated more proline compared with those of non mycorrhizal plants. Higher proline content in non-mycorrhizal leaves at high P level is related with the higher effects on cell membranes stability and with higher Na content. Some authors did not observe any appreciable increase in free proline content (Jain *et al.*, 1987), even as others consider enhanced proline level merely a stress effect, rather than a cause of stress tolerance (de Lacerda *et al.*, 2003).

In our work, the proline content was significantly increased in the stressed of non-mycorrhizal and mycorrhizal plants, at all stress levels. The most pronounced increase was observed in the leaves compared to roots. Hence, the role of proline accumulation and its metabolism related to tolerance to salinity need to be critically examined. Moreover, the total sugar leaf content was higher in mycorrhizal plants, regardless of the stressful situation and the P availability, and decreased with severe salinity stress. In roots, the total soluble sugar content decreased under high salinity stress conditions (100 and 200 mM NaCl).

However, with low P level there were no differences between the non-mycorrhizal and mycorrhizal plants. With high P level, the total sugar content of non-mycorrhizal roots was greater than that of mycorrhizal ones.

Under salt stress the sugar content in roots was similar in both non-mycorrhizal and mycorrhizal plants, suggesting that osmotic adjustment can occur. In contrast, in shoots the sugar content of high salt stress in mycorrhizal plants was considerably lower than that in non-stressed mycorrhizal plants. Only a small increase in the soluble sugar content in salinity stressed plants indicates that soluble sugars do not play a significant role in pepper stress response.

Our work underscores the importance of P availability and uptake, and their interaction with salt-stress. The enhancement of growth in mycorrhizal plants, under high saline conditions, has been partially related to the mycorrhization, and the P plant nutrition. In the present study, at higher salinity treatments (100 and 200 mM NaCl), mycorrhizal pepper plants had higher leaf P than non-mycorrhizal plants. It is evident that, AMF hyphae can avoid phosphorus depletion zones roots, or facilitate absorb phosphorus in salinity stress conditions.

Beneficial microbial inoculants, such as AM fungi are attractive to farmers in the context of sustainable agriculture. Better nutrient balance, higher dry weight, chlorophyll concentration and cell membrane stability in leaves of mycorrhizal plants, under severe salinity stress conditions, showed that root colonisation by *G. intraradices* can alleviate the deleterious effects of NaCl stress. Most native plants and crops of arid and semi-arid areas are mycorrhizal and it has been suggested that AM fungal colonization might enhance salt tolerance of some plants (Tain *et al.*, 2004) and has been largely attributed to higher uptake to phosphorus. To some extent, these AM fungi have been considered as bio-ameliorators of saline soils (Tain *et al.*, 2004). Moreover, our results show that, under saline conditions, pepper plants need mycorrhizae for

acclimatization and also for continued nutrient uptake. According to these results and those of other authors, it is possible to recommend mycorrhizal inoculation to attain reasonable growth of pepper plants under high salinity conditions. Although, different AM fungal species can differ in their ability to minimize stress effects and to promote plant growth. It has also been suggested that establishment of mixed communities by different AM fungal species may be more beneficial to the growth of plants than any of individual species (Alkan *et al.*, 2006).

The mechanism(s) by which the mycorrhizae increases the tolerance to salt stress, remains unresolved, although a variety of mechanisms have been proposed to determine how reduces the effects of salinity stress in plants. Pre-inoculating in transplants, could be an economically feasible means of growing crops in intensive agricultural systems or in restoration/reclamation of saline environments. Our results and the findings reported by Cantrell and Linderman (2001) support that pre-inoculated plants grow better than non-mycorrhizal plants under saline conditions, and this has been related to host plant P nutrition.

5. Conclusion

Arbuscular mycorrhizal fungi alleviate detrimental effects of salinity on growth, improve nutrition (higher K and P and lower Na concentrations in leaf tissue) and alleviate salinity impacts on cell membrane stability, at high P concentration and high saline conditions. Thus, use of AMF provides a sustainable and environmentally safe treatment to improve salinity tolerance. In conclusion, root colonisation by *G. intraradices* could be an adequate strategy to alleviate the deleterious effects of salinity stress and retard the senescence syndrome in pepper plants.

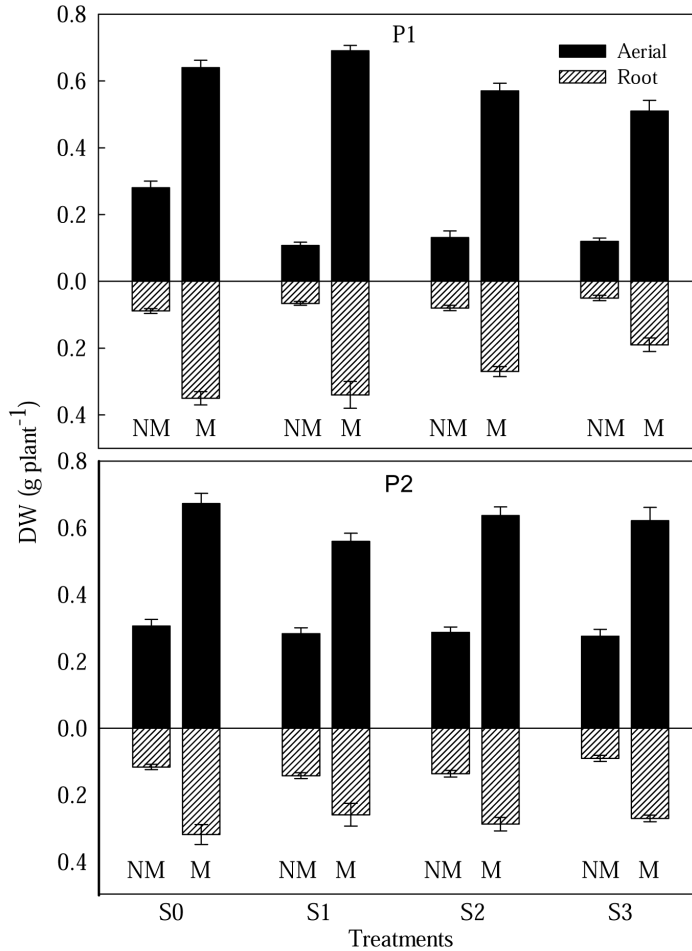


Figure 1. Effect of different salinity (S0: 0 mM NaCl; S1: 50 mM NaCl; S2: 100 mM NaCl; S3: 200mM NaCl) and P levels (P1: 10 mg kg⁻¹; P2: 40 mg kg⁻¹) on biomass partition in pepper plants inoculated (M) or non-inoculated (NM) with AMF .

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