Microbial Ecology

Influence of Salinity on the *In Vitro* Development of *Glomus intraradices* and on the *In Vivo* Physiological and Molecular Responses of Mycorrhizal Lettuce Plants

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Abstract

Increased salinization of arable land is expected to have devastating global effects in the coming years. Arbuscular mycorrhizal fungi (AMF) have been shown to improve plant tolerance to abiotic environmental factors such as salinity, but they can be themselves negatively affected by salinity. In this study, the first *in vitro* experiment analyzed the effects of 0, 50, or 100 mM NaCl on the development and sporulation of *Glomus intraradices*. In the second experiment, the effects of mycorrhization on the expression of key plant genes expected to be affected by salinity was evaluated. Results showed that the assayed isolate *G. intraradices* DAOM 197198 can be regarded as a moderately salt-tolerant AMF because it did not significantly decrease hyphal development or formation of branching absorbing structures at 50 mM NaCl. Results also showed that plants colonized by *G. intraradices* grew more than nonmycorrhizal plants. This effect was concomitant with a higher relative water content in AM plants, lower proline content, and expression of *Lsp5cs* gene (mainly at 50 mM NaCl), lower expression of the stress marker gene *Lslea* gene, and lower content of abscisic acid in roots of mycorrhizal plants as compared to nonmycorrhizal plants, which suggest that the AM fungus decreased salt stress injury. In addition, under salinity, AM symbiosis enhanced the expression of *LsPIP1*. Such enhanced gene expression could contribute to regulating root water permeability to better tolerate the osmotic stress generated by salinity.

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Introduction

Salinization of soil is a serious land degradation problem and is increasing steadily in many parts of the world, in particular in arid and semiarid areas [2, 22]. Increased salinization of arable land is expected to have devastating global effects, resulting in 30% land loss within next 25 years and up to 50% by the middle of 21st century [51]. This is mainly due to low precipitation in these regions, in addition to overexploitation of available water resources (e.g., ground water) [2], and leads to huge losses in terms of arable land and productivity as most of the economically important crop species are very sensitive to soil salinity [35].

Plants growing in saline soil are subjected to three distinct physiological stresses. First, the toxic effects of specific ions such as sodium and chloride, prevalent in saline soils, disrupt the structure of enzymes and other macromolecules, damage cell organelles, disrupt photosynthesis and respiration, inhibit protein synthesis, and induce ion deficiencies [28]. Second, plants exposed to the low osmotic potentials of saline soil are at risk of physiological drought because they must maintain lower internal osmotic potentials to prevent water moving from the roots into the soil. Finally, salinity also produces nutrient imbalance in the plant caused by decreased nutrient uptake and/or transport to the shoot [1, 36]. As a consequence, salt stress affects all the major processes, such as growth, photosynthesis, protein synthesis, and energy and lipid metabolisms [44].

Arbuscular mycorrhizal fungi (AMF) are ubiquitous among a wide array of soil microorganisms inhabiting the rhizosphere. These fungi constitute an important integral component of the natural ecosystem and are known to exist in saline environments [22]. Although AMF exist in saline soils, the growth and colonization of

plants may be affected by the excess of salinity, which can inhibit the growth of microbes due to osmotic and/or toxic effects of salts [29]. Therefore, in the study of the effects of salts, it is important to observe the effect of salinity on AMF growth and sporulation in relative isolation, preferably under *in vitro* conditions. *In vitro* studies of AMF growth, almost impossible in the past, are now possible by means of monoxenic cultures [16]. Such *in vitro* methods allow clear observation and quantification of the effects of salt on the hyphal growth, sporulation, and other features of AMF such as branched absorbing structures (BAS) [6]. In this study, the first experiment analyzed *in vitro* the effects of increasing concentrations of NaCl in the growth medium on the development and spore production by the AMF *Glomus intraradices*.

Arbuscular mycorrhizal fungi have been shown not only to help their host to absorb nutrients, but also to improve plant tolerance to abiotic environmental factors. In fact, AM symbiosis has been demonstrated to increase resistance to soil salinity in a variety of host plants such as maize, clover, tomato, cucumber, and lettuce [3, 20, 45, 48].

Although it is clear that AM fungi mitigate growth reduction caused by salinity, the mechanism involved remains unresolved. So far, studies on salt stress tolerance in mycorrhizal plants have suggested that AM plants grow better due to improved mineral nutrition and physiological processes like photosynthesis or water use efficiency, the production of osmoregulators or higher K^+/Na^+ ratios, and compartmentalization of sodium within some plant tissues [2, 22, 48]. To our knowledge, apart from Ouziad *et al.* [39], who studied the effect of mycorrhization and salt stress on the expression of Na^+/H^+ transporters and three aquaporin genes in tomato, the alteration of plant genes by AM symbiosis has never been investigated in relation to alleviation of salt stress by AM fungi.

In this work, we present a comprehensive study including key plant genes expected to be affected by salinity [38]. We included a gene encoding for Δ1-pyrroline-5-carboxylate synthetase (P5CS) that catalyzes the rate-limiting step in the biosynthesis of proline [32], one of the most widespread osmoregulator in plants [53]. We also analyzed a gene encoding for a late embryogenesis abundant protein. Late embryogenesis abundant proteins have a protective role under osmotic stress because they possess chaperone-like activity and act as stress markers [14]. A gene encoding for 9-*cis*-epoxycarotenoid dioxygenase (NCED), the key enzyme for the biosynthesis of the stress hormone abscisic acid (ABA) [49] was also studied. Abscisic acid plays a major role in plant responses to several stresses [54]. Abscisic acid promotes stomatal closure to minimize transpirational water loss. It also mitigates stress damage through the activation of many stress-responsive genes, which collectively increase plant stress tolerance [12]. Finally, we analyzed the expression of two genes encoding for two plasma membrane (PIP) aquaporins that are involved in the regulation of transcellular water flux [34] in plants and, hence, may have an important role in maintaining a proper plant water status under osmotic stress, such as salinity. In addition to the expression of these genes, some physiological markers such as proline and ABA contents, transpiration and relative water content (RWC) were also assayed for correlation with the molecular data. The study was conducted in uninoculated or mycorrhizal lettuce plants subjected or not to two salt levels.

Materials and Methods

In Vitro Experiment

Biological Materials. *Glomus intraradices* (Schenck and Smith, DAOM 197198, Biosystematic Research Center, Ottawa, Canada) was grown in monoxenic culture and subjected to 0, 50, or 100 mM NaCl. The specimens, kept in continuous monoxenic cultures, were provided by the culture collections of Zaidin Experimental Station, Granada, Spain. Five replicates of each salt level were incubated at 25°C.

Growth Conditions. Bicompartmental petri dishes (9 cm) were prepared as described by Cranenbrouk *et al.* [15]. Proximal compartment of each plate was filled with sterile modified Strullu–Romand medium [15], referred to as "M" throughout this report, to the top of the division wall. The medium had been autoclaved for 20 min at 121°C. The distal compartment of each plate was filled in the same manner, except that the M medium used did not contain any sucrose (referred to as "M-C" in this work). In addition, prior to the sterilization of the M-C medium, NaCl had been added to the medium to obtain 0-, 50-, or 100-mM concentrations of salt. The media were allowed to solidify at room temperature.

A piece of M agar (approximately 0.5 cm^2) was cut from the proximal compartment of each petri dish and replaced with fungal inoculum, which consisted of a piece of agar of the same size obtained from a stock monoxenic culture containing spores and hyphae of *G. intraradices* (DAOM 197198). Pieces of transformed carrot roots (2.5 cm in length), grown in M medium, were placed on top of the fungal inoculum in the proximal compartments. Two weeks later, inoculation plates were checked, and if roots were crossing onto the distal compartments, they were aseptically moved back to their proximal compartments. This check was subsequently repeated once a week throughout the experiment.

Parameters Measure and Statistical Analysis. Six weeks after inoculation, plates were examined under dissecting microscopes using the method described by Bago *et al.* [6]. A transparent 2-mm grid was used to determine the

hyphal length, the number of BAS [6], and the number of spores in three areas of 1 cm^2 per distal compartment of each plate.

Data of the total hyphal length was square root transformed prior to the analysis. Analysis of variance was used to analyze the data and if significant differences were found less significant difference at 5% of significance was applied to determine differences between treatments.

In Vivo Experiment

Soil and Biological Materials. This experiment consisted of a randomized complete block design with two inoculation treatments: (1) noninoculated control plants and (2) plants inoculated with the AM fungus *G. intraradices* (Schenck and Smith) BEG 121. There were 24 replicates of each treatment, totaling 48 pots (one plant per pot), so that eight of each microbial treatment were grown under nonsaline conditions throughout the entire experiment, while eight pots per treatment were subjected to 50 mM of NaCl and the remaining eight pots per treatment were subjected to 100 mM of NaCl.

Loamy soil was collected from the Zaidin Experimental Station (Granada, Spain), sieved (2 mm), diluted with quartz-sand (<1 mm) (1:1, soil/sand, v/v) and sterilized by steaming (100°C for 1 h on three consecutive days). The original soil had a pH of 8.1 (water); 1.81% organic matter, nutrient concentrations (mg kg^{-1}): N, 2.5; P, 6.2 ($NaHCO_3$-extractable P); K, 132.0. The electrical conductivity of the original soil was 0.7 dS m^{-1}. The soil texture was made up of 35.8% sand, 43.6% silt, and 20.5% clay. Three seeds of lettuce (*Lactuca sativa* L. cv. Romana) were sown in pots containing 750 g of the same soil/sand mixture as described above and thinned to one seedling per pot after emergence.

Mycorrhizal inoculum was bulked in an open-pot culture of *Zea mays* L. and consisted of soil, spores, mycelia, and infected root fragments. The AM species used was *G. intraradices* isolate BEG 121. Ten grams of inoculum with about 80 infective propagules per gram (according to the most probable number test) was added to appropriate pots at sowing time just below lettuce seeds. Uninoculated control plants received the same amount of autoclaved mycorrhizal inoculum together with a 2-mL aliquot of a filtrate (<20 μm) of the AM inoculum to provide a general microbial population free of AM propagules.

Growth Conditions. The experiment was carried out under glasshouse conditions with temperatures ranging from 19 to 25°C, 16/8 light/dark period, and a relative humidity of 70–80%. A photosynthetic photon flux density of 800 μE m^{-2} s^{-1} was measured with a light meter (LICOR, Lincoln, NE, USA, model LI-188B). Water was supplied daily to maintain soil at field capacity during the entire period of plant growth.

Plants were established for 1 month prior to salinization to allow adequate plant growth and symbiotic establishment. Three concentrations (0, 50, and 100 mM NaCl) of saline solution were reached in the pot soil on the basis of the soil volume in the soil by adding appropriate dilution of a 2-M saline solution. The concentration of NaCl in the soil was increased gradually by 20 mM per day on alternative days to avoid an osmotic shock. It took 6 and 10 days to reach the desired 50 and 100 mM NaCl, respectively. Plants were maintained under these conditions for additional 20 days.

Parameters Measured and Statistical Analysis

Biomass Production. At harvest (60 days after planting), the shoot and root systems were separated and the shoot dry weight was measured after drying in a forced hot-air oven at 70°C for 2 days. Six replicates were used, avoiding the biggest and the smallest plant of each treatment.

Symbiotic Development. The percentage of mycorrhizal root infection in lettuce plants was estimated by visual observation of fungal colonization after clearing washed roots in 10% KOH and staining with 0.05% trypan blue in lactic acid (v/v), as described by Phillips and Hayman [40]. The extent of mycorrhizal colonization was calculated according to the gridline intersect method [21].

Relative Water Content. The RWC in plant shoots was determined at the harvest time as previously described by Ruiz-Lozano and Azcón [46]. Six replicates per treatment were used.

Leaf Transpiration Rate. Leaf transpiration rate was determined by a gravimetric method [4] on four replicates per treatment. Surfaces of the pots were covered with aluminum foil. The pot–plant system was weighed and referred to as W_0. The pot–plant system was weighed again after 2 h and referred to as W_f. Leaf transpiration rate was calculated as $(W_0 - W_f)/(t \times A)$, where t is the time in seconds and A is the leaf area in square meters. Leaf area was calculated as follows: leaves of a whole plant were detached and scanned (hp scanjet 5550c, Hewlett Packard, Palo Alto, CA, USA). The corresponding images were analyzed with Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA).

Proline Content. Free proline was extracted from 1 g of fresh root that was ground with 6 mL of methanol and 6 mL of chloroform [10]. After that, 3 mL of a 0.9% NaCl solution was added and mixed. The resulting mixture was

centrifuged at 5000 rpm for 10 min at 1°C. The methanolic phase was used for quantification. Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates *et al.* [9]. Four replicates per treatment were used.

ABA Content. Abscisic acid was measured on 250 mg of frozen root tissues that were immersed in 2 mL distilled water and incubated for 24 h at 4°C in the dark [5]. Quantitative analysis was performed on crude aqueous extracts using an ELISA assay based on monoclonal antibody against (*S*)-*cis*,*trans*-ABA (BT-GB-252A, Babraham Biosciences Technologies, Cambridge, UK). A procedure described by Walker-Simmons [50] was followed. Briefly, microtitration plates were coated overnight at 4°C with bovine serum albumin (BSA)–ABA [11] and rinsed three times with 50 mM Tris–ClH (pH = 7.8), 1 mM $MgCl_2$, 150 mM NaCl, 0.1% (p/v) BSA, and 0.05% (v/v) Tween 20. Next, 200 µL of the overnight-incubated samples and standards with the antibody were added. Plates were incubated at room temperature for 150 min before they were rinsed again as described above and incubated for 2 h at room temperature with Anti-rat IgG (1:1000). Finally, plates were rinsed again and p-nitrophenyl phosphate was added. Plates were incubated until the absorbance at 405 nm of the non-ABA sample was 1. Absorbance was inversely proportional to the amount of ABA. Four independent plants were analyzed per treatment and all sample results were the average of three serial dilutions within the linear range of the ABA standard curve.

Northern Blot Analysis. Total RNA was isolated from lettuce roots by phenol/chloroform extraction according to the method described by Kay *et al.* [31]. Northern blots of total root RNA with *Lsp5cs*, *Lslea*, *Lsnced*, *LsPIP1*, and *LsPIP2* DNA probes were carried out as previously described [42]. Hybridizations were carried out overnight at 65°C under standard conditions. After washing twice for 5 min at room temperature in 2× standard saline citrate (SSC) and 0.1% sodium dodecyl sulfate (SDS), and twice for 15 min at 65°C with 0.5× SSC and 0.1% SDS, membranes were exposed to phosphorimager. The amount of 26S rRNA in the membranes was quantified using Quantity One software (Bio-Rad, Hemel Hempstead, UK) after ethydium bromide staining. Next, the signals on phosphorimager were analyzed and quantified using the same software. Transcript accumulations for each gene probe (in arbitrary units) were divided by the corresponding amount of 26S rRNA in the membrane. Each quantification of signals on screens and of 26S rRNA in the membranes was repeated three times, and the average value for each was used for normalization. Northern blot analyses were repeated two times with different sets of plants and the results obtained were similar, with low variability. We show representative results for each Northern blot.

Data were subjected to analysis of variance with microbial treatment, salt level, and microbial treatment–salt level interaction as sources of variation, and followed by Duncan's multiple-range test [18]. Percentage values were arcsin transformed before statistical analysis.

Results

Influence of Salinity on the In Vitro Development of G. intraradices. Table 1 shows the parameter of fungal growth with or without salinity in the medium. There was no significant difference in hyphal length between the control (no salt) and 50 mM NaCl, but these treatments had produced significantly longer hyphae than under 100 mM NaCl. Salinity in the medium significantly decreased the number of spores produced by *G. intraradices*. However, the numbers of spores produced under 50- and 100-mM levels of NaCl were not significantly different. There was no significant difference between the number of BAS produced in the control and 50-mM-NaCl treatments. The number of these structures was significantly reduced at 100 mM NaCl relative to the control.

Influence of Salinity on the In Vivo Responses of Mycorrhizal Lettuce Plants.

Mycorrhizal Colonization and Shoot Dry Weight. Uninoculated lettuce plants did not show mycorrhizal colonization. Plants inoculated with *G. intraradices* were well colonized. Under

Table 1. Total hyphal length and number of spores and BAS formed by *G. intraradices* grown in monoxenic culture and subjected to 0, 50, or 100 mM NaCl

	Salt level			
	0 mM	*50 mM*	*100 mM*	*LSD*
Hyphal length (sqrt mm cm^{-2})	48.4a	42.6a	30.7b	10.9
Number of spores cm^{-2}	59.5a	14.0b	11.0b	29.4
Number of BAS	29.1a	21.7ab	13.2b	11.4

Means followed by different letters are significantly different ($P < 0.05$).
LSD = less significant difference, sqrt = square root.

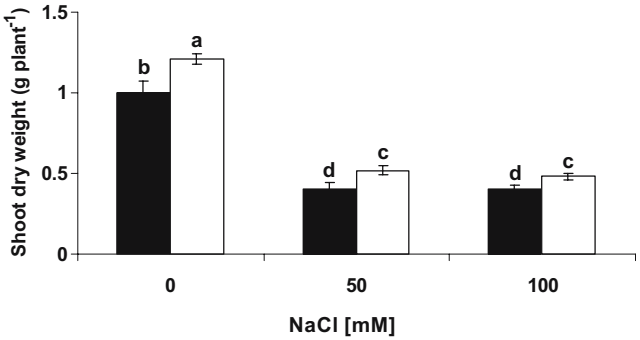

Figure 1. Shoot dry weight (g plant^{-1}) in lettuce plants. *Black bars* represent noninoculated control plants and *white bars* represent plants inoculated with *G. intraradices*. Plants were subjected to 0, 50, or 100 mM NaCl. Columns with *different letters* are significantly different ($P < 0.05$). Columns represent mean ± SE ($n = 6$).

nonsaline conditions or under 50 mM NaCl, the roots exhibited 84% of mycorrhizal colonization. The increase of salinity to 100 mM NaCl slightly but significantly decreased the percentage of mycorrhizal root length to 67%.

The highest plant development was achieved in plants grown under nonsaline conditions (Fig. 1). Plants colonized by *G. intraradices* showed 20% increased shoot dry weight as compared to nonmycorrhizal plants. Both salinity levels reduced shoot dry weight in mycorrhizal and nonmycorrhizal plants. However, under 50 mM NaCl, *G. intraradices*-colonized plants had 30% higher shoot dry weight than uncolonized control plants. Under 100 mM NaCl, the increase in mycorrhizal plants was 17%.

Proline Content. There was little proline accumulated in lettuce roots grown under nonsaline conditions (Fig. 2). In contrast, under 50 mM NaCl, plants considerably increased the proline accumulation in roots. This effect was remarkable in uninoculated control plants, whereas plants colonized by *G. intrarradices* accumulated 85% less proline than uninoculated plants. At 100 mM NaCl, uninoculated plants did not significantly change proline accumulation, as compared to 50 mM. In contrast, in plants colonized by *G. intraradices*, the level of proline increased notably,

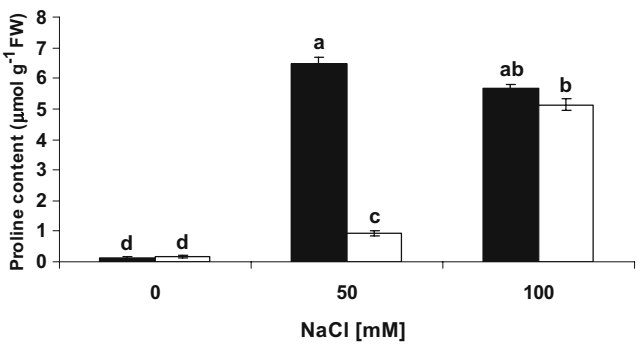

Figure 2. Proline content (µmol proline g fresh weight^{-1}) in lettuce roots. See legend of Fig. 1.

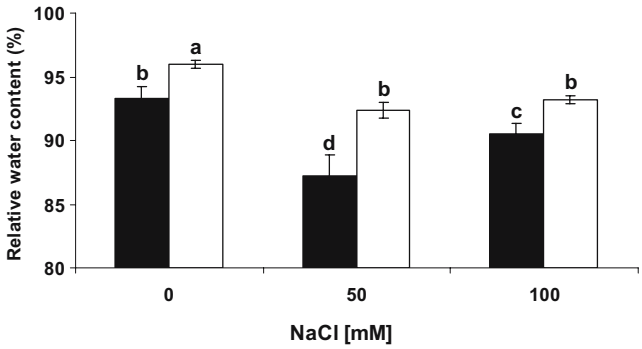

Figure 3. Relative water content (%) in lettuce shoots. See legend of Fig. 1.

reaching a level similar to that of uninoculated control plants.

Relative Water Content. The RWC of lettuce plants was always higher in mycorrhizal than in nonmycorrhizal plants (Fig. 3). Both salinity levels decreased RWC but mycorrhizal lettuce plants maintained higher water content than uninoculated control ones regardless of salt level.

Transpiration Rate. Under nonsaline conditions, uninoculated control plants exhibited a higher transpiration rate than *G. intraradices*-colonized ones (Fig. 4). At 50 mM NaCl, uninoculated control plants decreased the transpiration rate, whereas mycorrhizal plants did not show significant differences as compared to nonsaline conditions. In contrast, at 100 mM NaCl, all plants further decreased transpiration rate to a similar extent.

ABA Content. Under nonsaline conditions, roots colonized by *G. intraradices* had a considerably higher ABA content than nonmycorrhizal roots (Fig. 5). In contrast, under both salt levels, nonmycorrhizal plants enhanced their ABA content in roots, whereas AM plants decreased their ABA content at 50 mM NaCl and had a similar ABA content than under nonsaline conditions when they were subjected to 100 mM NaCl. In any case, at both salt levels, nonmycorrhizal roots accumulated more ABA than roots colonized by *G. intraradices*.

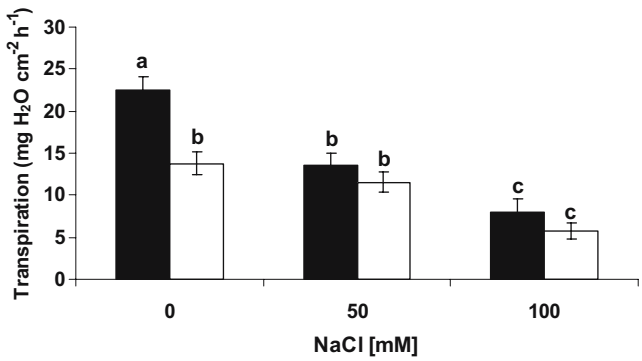

Figure 4. Transpiration rate (mg H_2O cm^{-2} h^{-1}) in lettuce plants. See legend of Fig. 1.

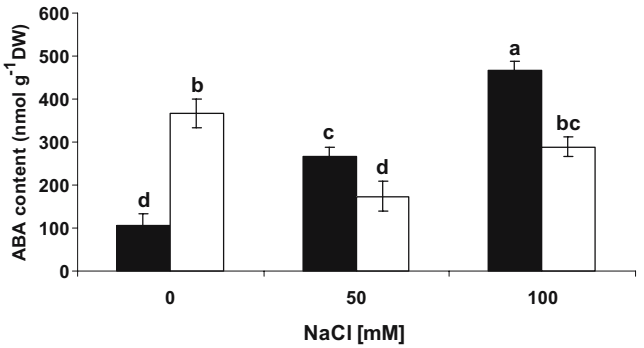

Figure 5. Abscisic acid content (nmol g dry weight^{-1}) in lettuce roots. See legend of Fig. 1.

Northern Blots. The gene *Lsp5cs* exhibited a low expression level under nonsaline conditions, mainly in plants colonized by *G. intraradices* (Fig. 6). Plants growing under 50 mM NaCl notably increased transcript accumulation for this gene. However, the up-regulation of *Lsp5cs* was considerably more intense in nonmycorrhizal plants than in those colonized by *G. intraradices*. Plants exposed to 100 mM NaCl maintained high levels of *Lsp5cs* gene expression as compared to plants under nonsaline conditions. At this salt level, however, the expression of this gene was similar in mycorrhizal and nonmycorrhizal plants.

The *Lslea* gene was only expressed under conditions of salt stress (Fig. 6). The highest induction of this gene

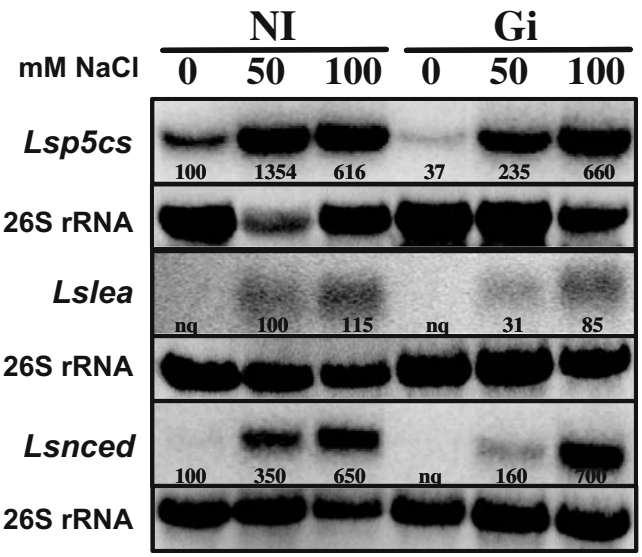

Figure 6. Northern blot of total RNA (15 μg) from lettuce roots using *Lslea*, *Lsp5cs*, and *Lsnced* gene probes. Treatments are designed as *NI*, noninoculated control, or *Gi*, plants inoculated with *G. intraradices*. Plants were subjected to 0, 50, or 100 mM NaCl. The lower panels show the amount of 26S rRNA loaded for each treatment. Numbers close to each Northern represent the relative gene expression (after normalization to 26S rRNA) as a percentage of the value for control plants cultivated under nonsaline conditions (except for *Lslea*, which were set at 100% in control plants subjected to 50 mM NaCl). *nq*, not quantifiable.

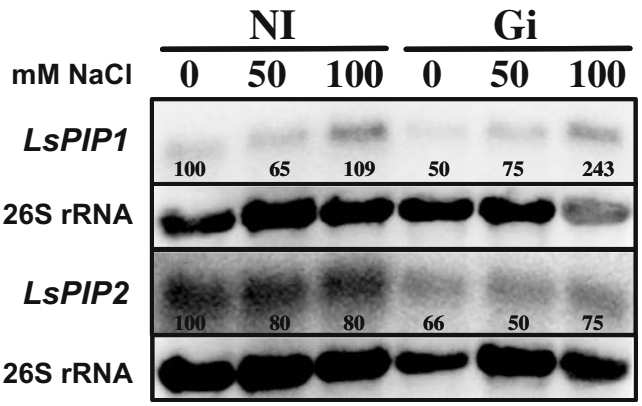

Figure 7. Northern blot of total RNA (15 μg) from lettuce roots using *LsPIP1* and *LsPIP2* gene probes. Treatments are designed as *NI*, noninoculated control, or *Gi*, plants inoculated with *G. intraradices*. Plants were subjected to 0, 50, or 100 mM NaCl. The *lower panels* show the amount of 26S rRNA loaded for each treatment. Numbers close to each Northern represent the relative gene expression (after normalization to 26S rRNA) as a percentage of the value for control plants cultivated under nonsaline conditions.

was found in control plants, whereas in plants colonized by *G. intraradices*, the induction of this gene was lower at both 50 and 100 mM NaCl.

The gene *Lsnced* showed a very low basal expression under nonsaline conditions in control plants and was unquantifiable in AM plants (Fig. 6). At 50 mM NaCl, nonmycorrhizal plants notably increased *Lsnced* transcript accumulation (by 250%), whereas plants colonized by *G. intraradices* increased it to a lesser extent (only 60%). At 100 mM NaCl, all plants further increased the expression of this gene (by about 550%), but at this salt level there were no differences between mycorrhizal and nonmycorrhizal plants.

Regarding aquaporins, in the absence of salinity in the medium, the mycorrhization *per se* inhibited the expression of *LsPIP1* and *LsPIP2* genes (Fig. 7). At 50 mM NaCl, nonmycorrhizal plants also inhibited the expression of *LsPIP1* gene. The expression of *LsPIP1* recovered in both plant treatments when salinity reached 100 mM NaCl in the soil. The recovery was considerably more intense in plants colonized by *G. intraradices*, which doubled the expression level of control plants in the absence of salinity. The expression of the *LsPIP2* gene showed little variation in mycorrhizal and in nonmycorrhizal plants as a consequence of the application of 50 or 100 mM NaCl (Fig. 7).

Discussion

Mycorrhizal symbiosis is a key component in helping plants to cope with adverse environmental conditions. It has been suggested that salt stress may decrease colonization by AM

fungi via a direct effect on fungal growth [28]. In this work, we used monoxenic cultural conditions to study salt effects on fungal development. Although monoxenic AMF cultures may be criticized for being too artificial, Bago and Cano [6] demonstrated that monoxenic cultures are accurate means of observing fungal developmental features, especially when they are carried out together with other studies such as physiological and molecular investigations. Importantly, some features of AMF, such as BAS, are very difficult to observe in axenic soil cultures [7]. However, these structures are very conspicuous in monoxenic cultures and can be easily observed and studied.

It is known that salts in the growth medium may induce changes in the length and in other morphological properties of the hyphae, thus affecting their symbiotic capacity [28]. Juniper and Abbott [29] studied the effect of different NaCl concentrations (0, 150, and 300 mM) on spore germination and growth of a range of AMF in soil substrate. They found that in some *Glomus* species the hyphal development under saline conditions was not significantly reduced over time. However, data from this study showed that, despite the fact that the fungal growth, in terms of hyphal development, was not affected under a relatively high concentration of NaCl (50 mM), the sporulation significantly declined. This decline suggests that if salinity conditions persist there can be a decrease in plant colonization by decreasing the availability of inoculum (i.e., spores). With further increase in salinity (100 mM in this study) the hyphal length is also reduced, which can further inhibit the colonization and symbiotic capability of the AMF. In addition, the number of BAS also declined significantly when subjected to 100 mM NaCl, which can further reduce the sporulation. Branched absorbing structures are thought to be associated with the formation of spores. It has been shown [7] that some BAS can gradually form spores in their ramifications.

The beneficial effects of different mycorrhizal fungi on plant growth under saline conditions have been demonstrated in various plant species [2, 3, 13, 20, 48, 52]. In this study, under both salt levels, mycorrhizal plants grew 30% (50 mM NaCl) and 17% (100 mM NaCl) more than nonmycorrhizal plants, indicating that the colonization by *G. intraradices* is effective in stimulating plant growth under salinity. To determine the possible cause of such an effect, we evaluated the effects of both salinity and mycorrhization on a variety of plant genes potentially involved in the plant response to salinity [39].

To avoid the osmotic stress caused by salinity, plants may accumulate inorganic ions such as K^+ and low-molecular-weight solutes such as proline to maintain the internal osmotic potential [24, 37]. Several studies have shown that AM symbiosis improved salt resistance through improved osmoregulation [20, 26].

In this study, at 50 mM NaCl, the expression of *Lsp5cs* gene was more up-regulated in nonmycorrhizal plants than in AM plants, and the amount of proline accumulated followed a similar trend. In contrast, when salinity in the soil increased to 100 mM NaCl, both groups of plants exhibited a similar *Lsp5cs* gene expression and proline content. Investigations carried out so far on osmorregulation in the AM symbiosis are scarce and somewhat contradictory. While some studies have shown an increase in proline accumulation in mycorrhizal plants subjected to drought [23, 47], the same studies also demonstrated that the increase in proline accumulation was quite variable depending on the AM fungus involved. On the contrary, other studies regarding drought [41, 43], osmotic stress [46], or salt stress [47] have shown a lower proline accumulation in AM plants than in non-AM ones, as we have found in this study at 50 mM NaCl.

Our results indicate that protection of AM plants against salinity was not mediated by increased accumulation of proline in these plants. They rather suggest that these plants were suffering less stress (at least under 50 mM NaCl) than nonmycorrhizal plants. In fact, under salt stress, AM plants had better water status, as revealed by the higher RWC than non-AM plants and the expression of the stress marker gene *Lslea* was higher in non-AM roots than in AM roots (mainly at 50 mM NaCl), indicating that the osmotic stress induced by salinity is also higher in nonmycorrhizal roots than in the mycorrhizal ones.

In this study, we measured ABA content in roots of lettuce plants and the expression of *Lsnced* gene. With the exception of AM plants under nonsaline conditions that showed increased ABA levels as compared to non-AM plants but reduced *Lsnced* gene expression, for the rest of the treatments, data on ABA content and *Lsnced* gene expression fit well. As expected, ABA content and *Lsnced* gene expression increased in non-AM plants as a consequence of salinity. In AM plants, *Lsnced* gene expression also increased with salinity, but AM roots showed higher ABA content under non-saline conditions than at 50 mM NaCl. In any case, at both salt levels, AM roots showed significantly lower ABA content than non-AM roots. Previous studies have shown that mycorrhization can alter the levels of ABA in the host plant and that, under drought stress, the levels of ABA are lower in AM than in non-AM plants [17, 19, 33], as we found here under salt stress. These results point in the same direction as those on proline content, indicating that AM plants were less strained than non-AM plants by salinity stress imposed; hence, they accumulated less ABA.

The role of aquaporins in plants under salt stress has not been fully explored. It is clear, however, that water passes to a large extent through the plasmalemma or the tonoplast thanks to the aquaporin activity [34]. Accordingly, a correlation between the expression or activity of aquaporins and the sensitivity or tolerance of plants to salinity is expected [27]. In this study, the expression of *LsPIP1* and *LsPIP2* genes was inhibited by mycorrhiza-

tion under non-saline conditions, which agrees with previous findings on these aquaporin genes and mycorrhizal lettuce [41]. Under saline conditions, the expression of both PIP genes followed a different pattern according to the salt level applied and the presence or absence of AMF in roots. In fact, mycorrhizal plants maintained the expression of *LsPIP2* gene, which was almost unaffected, whereas the expression of *LsPIP1* gene was up-regulated, mainly at 100 mM NaCl. A differential effect of AM symbiosis on PIP aquaporin isoforms under salinity has also been described by Ouziad *et al.* [39], who suggested that AM symbiosis exerts a differential control on expression of aquaporin genes. On the other hand, Kawasaki *et al.* [30] found that, in rice roots, aquaporins were upregulated after seven days under salt stress. In the present work, lettuce plants were subjected to salinity for 20 days, and we found a rise induced by mycorrhization in *LsPIP1* gene, in accordance with results by Kawasaki *et al.* [30]. The induction of particular aquaporins by AM symbiosis under osmotic stress should result in greater water permeability and facilitated water flux and should contribute to the global plant resistance to the stressful conditions [8, 25].

In conclusion, results from this study show that the isolate DAOM 197198 of *G. intraradices* can be regarded as a moderately salt-tolerant AMF because it did not significantly decrease hyphal development or BAS production at 50 mM NaCl. Results also show that *G. intraradices* stimulated the growth of lettuce plants under two levels of salinity. This effect was concomitant with a higher RWC in AM plants, lower proline content (mainly at 50 mM NaCl), lower expression of the stress marker gene *Lslea* gene, and lower accumulation of ABA in roots of AM plants as compared to non-AM plants, which suggest that AM plants were suffering from the salt stress less than nonmycorrhizal plants. This is likely to be due to primary salt-avoidance mechanisms, such as reduced Na^+ and Cl^- accumulation, as it has been evidenced in several studies [2, 22]. In addition, under salinity, AM symbiosis enhanced the expression of *LsPIP1* that could contribute to regulating root water permeability and, consequently, to better tolerating the osmotic stress generated by salinity.

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References

1. Adiku, G, Renger, M, Wessolek, G, Facklam, M, Hech-Bischoltz, C (2001) Simulation of dry matter production and seed yield of common beans under varying soil water and salinity conditions. Agric Water Manag 47: 55–68
2. Al-Karaki, GN (2006) Nursery inoculation of tomato with arbuscular mycorrhizal fungi and subsequent performance under irrigation with saline water. Sci Hortic 109: 1–7
3. Al-Karaki, GN, Hammad, R, Rusan, M (2001) Response of two tomato cultivars differing in salt tolerance to inoculation with mycorrhizal fungi under salt stress. Mycorrhiza 11: 43–47
4. Aroca, R, Tognoni, F, Irigoyen, JJ, Sánchez-Díaz, M, Pardossi A (2001) Different root low temperature response of two maize genotypes differing in chilling sensitivity. Plant Physiol Biochem 39: 1067–1073
5. Aroca, R, Vernieri, P, Irigoyen, JJ, Sánchez-Díaz, M, Tognoni, F, Pardossi, A (2003) Involvement of abscisic acid in leaf and root of maize (*Zea mays* L.) in avoiding chilling-induced water stress. Plant Sci 165: 671–679
6. Bago, B, Cano, C (2005) Breaking myths on arbuscular mycorrhizas *in vitro* biology. In: Declerck, S, Strullu, DG, Fortin, JA (Eds.) *In Vitro* Culture of Mycorrhizas, Springer, Berlin Heidelberg New York, pp 111–138
7. Bago, B, Azcon-Aguilar, C, Goulet, A, Piche, Y (1998) Branched absorbing structure (BAS), a feature of the extrarradical mycelium of symbiotic arbuscular mycorrhizal fungi. New Phytol 139: 375–388
8. Barrieu, F, Marty-Mazars, D, Thomas, D, Chaumont, F, Charbonnier, M, Marty, F (1999) Desiccation and osmotic stress increase the abundance of mRNA of the tonoplast aquaporin BobTIP26-1 in cauliflower cells. Planta 209: 77–86
9. Bates, LS, Waldren, RP, Teare, ID (1973) Rapid determination of free proline for water stress studies. Plant Soil 39: 205–207
10. Blig, EG, Dyer, WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917
11. Blintsov, AN, Gussakovskaya, MA (2004) Immunochemical approach to the problem of differential determination of natural forms of abscisic acid. Biochemistry (Mosc) 69: 1099–1108
12. Bray, EA (2002) Abscisic acid regulation of gene expression during water-deficit stress in the era of the *Arabidopsis* genome. Plant Cell Environ 25: 153–161
13. Cho, K, Toler, H, Lee, J, Ownley, B, Stutz, JC, Moore, JL, Augé, RM (2006) Mycorrhizal symbiosis and response of sorghum plants to combined drought and salinity stresses. J Plant Physiol 163: 517–528
14. Close, TJ (1996) Dehydrins: emergence of a biochemical role of a family of plant dehydration proteins. Physiol Plant 97: 795–803
15. Cranenbrouk, S, Voets, L, Bivort, C (2005) Methodologies for in vitro cultivation of arbuscular mycorrhizal fungi with root organs. In: Declerck, S, Strullu, DG, Fortin, JA (Eds.) *In Vitro* Culture of Mycorrhizas, Springer, Berlin Heidelberg New York, pp 341–375
16. Declerck, S, Strullu, DG, Fortin, JA (2005) *In Vitro* Culture of Mycorrhizas. Springer, Berlin Heidelberg New York
17. Duan, X, Newman, DS, Reiber, JM, Green, CD, Saxton, AM, Augé, RM (1996) Mycorrhizal influence on hydraulic and hormonal factors implicated in the control of stomatal conductance during drought. J Exp Bot 47: 1541–1550
18. Duncan, DB (1955) Multiple range and multiple F-tests. Biometrics 11: 1–42
19. Estrada-Luna, AA, Davies, FT (2003) Arbuscular mycorrhizal fungi influence water relations, gas exchange, abscisic acid and growth of micropropagated chile ancho pepper (*Capsicum annuum*) plantlets during acclimatization and post-acclimatization. J Plant Physiol 160: 1073–1083
20. Feng, G, Zhang, FS, Li, XL, Tian, CY, Tang, C (2002) Improved tolerance of maize plants to salt stress by arbuscular mycorrhizal is

related to higher accumulation of soluble sugars in roots. Mycorrhiza 12: 185–190
21. Giovannetti, M, Mosse, B (1980) An evaluation of techniques for measuring vesicular–arbuscular infection in roots. New Phytol 84: 489–500
22. Giri, R, Kapoor, R, Mukerji, KG (2003) Influence of arbuscular mycorrhizal fungi and salinity on growth, biomass and mineral nutrition of *Acacia auriculiformis*. Biol Fertil Soils 38: 170–175
23. Goicoechea, N, Szalai, G, Antolín, MC, Sánchez-Díaz, M, Paldi, E (1998) Influence of arbuscular mycorrhizae and *Rhizobium* on free polyamines and proline levels in water-stressed alfalfa. J Plant Physiol 153: 706–711
24. Hasegawa, PM, Bressan, RA, Zhu, KL, Bohnert, HJ (2000) Plant cellular and molecular responses to high salinity. Annu Rev Plant Physiol Plant Mol Biol 51: 463–499
25. Jang, JY, Kim, DG, Kim, YO, Kim, JS, Kang, H (2004) An expression analysis of a gene family encoding plasma membrane aquaporins in response to abiotic stresses in *Arabidopsis thaliana*. Plant Mol Biol 54: 713–725
26. Jindal, V, Atwal, A, Sekhon, BS, Rattan, S, Singh, R (1993) Effect of vesicular–arbuscular mycorrhizae on metabolism of moong plants under NaCl salinity. Plant Physiol Biochem 31: 475–481
27. Johansson, I, Karlsson, M, Johansson, U, Larsson, C, Kjellbom, P (2000) The role of aquaporins in cellular and whole plant water balance. Biochim Biophys Acta 1465: 324–342
28. Juniper, S, Abbott, L (1993) Vesicular–arbuscular mycorrhizas and soil salinity. Mycorrhiza 4: 45–57
29. Juniper, S, Abbott, LK (2006) Soil salinity delays germination and limits growth of hyphae from propagules of arbuscular mycorrhizal fungi. Mycorrhiza 16: 371–379
30. Kawasaki, S, Borchert, C, Deyholos, M, Wang, H, Brazille, S, Kawai, K, Galbraith, D, Bohnert, HJ (2001) Gene expression profiles during the initial phase of salt stress in rice. Plant Cell 13: 889–905
31. Kay, R, Chau, A, Daly, M (1987) Duplication of CaMV 35S promoter sequences creates a strong enhancer for plants genes. Science 236: 1299–1302
32. Kishor, PB, Hong, Z, Miao, GH, Hu, CA, Verma, DPS (1995) Overexpression of Δ^1-pyrroline-5-carboxilate synthetase increases proline production and confers osmotolerance in transgenic plants. Plant Physiol 108: 1387–1394
33. Ludwig-Müller, J (2000) Hormonal balance in plants during colonization by mycorrhizal fungi. In: Kapulnik Y, Douds, DD (Eds.) Arbuscular Mycorrhizas: Physiology and Function, Kluwer, The Netherlands, pp 263–285
34. Luu, DT, Maurel, C (2005) Aquaporins in a challenging environment: molecular gears for adjusting plant water status. Plant Cell Environ 28: 85–96
35. Mahajan, S, Tuteja, N (2005) Cold, salinity and drought stress: an overview. Arch Biochem Biophys 444: 139–158
36. Marschner, H (1995) Mineral Nutrition of Higher Plant, 2nd ed. Academic, New York
37. Morgan, JM (1984) Osmoregulation and water stress in higher plants. Annu Rev Plant Physiol 33: 299–319
38. Munns, R (2005) Genes and salt tolerance: bringing them together. New Phytol 167: 645–663
39. Ouziad, F, Wilde, P, Schmelzer, E, Hildebrandt, U, Bothe, H (2006) Analysis of expression of aquaporins and Na^+/H^+ transporters in tomato colonized by arbuscular mycorrhizal fungi and affected by salt stress. Environ Exp Bot 57: 177–186
40. Phillips, JM, Hayman, DS (1970) Improved procedure of clearing roots and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55: 159–161
41. Porcel, R, Azcón, R, Ruiz-Lozano, JM (2004) Evaluation of the role of genes encoding for Δ^1-pyrroline-5-carboxylate synthetase (P5CS) during drought stress in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants. Physiol Mol Plant Pathol 65: 211–221
42. Porcel, R, Aroca, R, Azcón, R, Ruiz-Lozano, JM (2006) PIP aquaporin gene expression in arbuscular mycorrhizal *Glycine max* and *Lactuca sativa* plants in relation to drought stress tolerance. Plant Mol Biol 60: 389–404
43. Ramakrishnan, B, Johri, BN, Gupta, RK (1988) Influence of the VAM fungus *Glomus caledonius* on free proline accumulation in water-stressed maize. Curr Sci 57: 1082–1084
44. Ramoliya, P, Patel, H, Pandey, AN (2004) Effect of salinization of soil on growth and macro- and micro-nutrient accumulation in seedlings of *Salvadora persica* (Salvadoraceae). For Ecol Manag 2002: 181–193
45. Rosendahl, CN, Rosendahl, S (1991) Influence of vesicular-arbuscular mycorrhizal fungi (*Glomus* spp.) on the response of cucumber (*Cucumis sativis* L.) to salt stress. Environ Exp Bot 31: 313–318
46. Ruiz-Lozano, JM, Azcón, R (1997) Effect of calcium application on the tolerance of mycorrhizal lettuce plants to polyethylene glycol-induced water stress. Symbiosis 23: 9–22
47. Ruiz-Lozano, JM, Azcón, R, Gómez, M (1995) Effects of arbuscular mycorrhizal *Glomus* species on drought tolerance: physiological and nutritional plant responses. Appl Environ Microbiol 61: 456–460
48. Ruiz-Lozano, JM, Azcón, R, Gómez, M (1996) Alleviation of salt stress by arbuscular–mycorrhizal *Glomus* species in *Lactuca sativa* plants. Physiol Plant 98: 767–772
49. Schwartz, SH, Qin, X, Zeevaart, JAD (2003) Elucidation of the indirect pathway of abscisic acid biosynthesis by mutants, genes, and enzymes. Plant Physiol 131: 1591–1601
50. Walker-Simmons, M (1987) ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. Plant Physiol 84: 61–66
51. Wang, W, Vinocur, B, Altman, A (2003) Plant responses to drought, salinity and extreme temperatures: toward genetic engineering for stress tolerance. Planta 218: 1–14
52. Yano-Melo, AM, Saggin, OJ, Costa-Maia, L (2003) Tolerance of mycorrhized banana (*Musa sp.* cv. Pacovan) plantlets to saline stress. Agric Ecosyst Environ 95: 343–348
53. Yoshiba, Y, Kiyosue, T, Nakashima, K, Yamaguchi-Shinozaki, K, Shinozaki, K (1997) Regulation of levels of proline as an osmolyte in plants under water stress. Plant Cell Physiol 18: 1095–1102
54. Zhang, J, Jia, W, Yang, J, Ismail, AM (2006) Role of ABA in integrating plant responses to drought and salt stresses. Field Crops Res 97: 111–119