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Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress

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ABSTRACT

This study investigated the influence of inoculation with a plant growth-promoting rhizobacterium, *Pseudomonas mendocina* Palleroni, alone or in combination with an arbuscular mycorrhizal (AM) fungus, *Glomus intraradices* (Schenk & Smith) or *Glomus mosseae* (Nicol & Gerd.) Gerd. & Trappe, on antioxidant enzyme activities (catalase and total peroxidase), phosphatase activity, solute accumulation, growth and mineral nutrient uptake in leaves of *Lactuca sativa* L. cv. Tafalla affected by three different levels of salt stress. Salinity decreased lettuce growth, regardless of the biological treatment and of the salt stress level. The plants inoculated with *P. mendocina* had significantly greater shoot biomass than the control plants at both salinity levels, whereas the mycorrhizal inoculation treatments only were effective in increasing shoot biomass at the medium salinity level. At the highest salinity level, the water content was greater in leaves of plants treated with *P. mendocina* or *G. mosseae*. At the medium salinity level, *G. intraradices*- or *G. mosseae*-colonised plants showed the highest concentrations of foliar P. The *P. mendocina*- and *G. mosseae*-colonised plants presented higher concentrations of foliar K and lower concentrations of foliar Na under high salt conditions. Salt stress decreased sugar accumulation and increased foliar proline concentration, particularly in plants inoculated with the PGPR. Increasing salinity stress raised significantly the antioxidant enzyme activities, including those of total peroxidase and catalase, of lettuce leaves compared to their respective non-stressed controls. The PGPR strain induced a higher increase in these antioxidant enzymes in response to severe salinity. Inoculation with selected PGPR could serve as a useful tool for alleviating salinity stress in salt-sensitive plants.

1. Introduction

Saline soils and saline irrigation constitute a serious production problem for vegetable crops as saline conditions are known to suppress plant growth, particularly in arid and semiarid areas (Parida and Das, 2005). Lettuce (*Lactuca sativa*) is an important crop of semiarid Mediterranean agroecosystems in South-East Spain and lettuce growth is sensitive to salinity (Ruiz-Lozano and Azcón, 1996). Salt induces osmotic stress by limiting absorption of water from soil, and ionic stress resulting from high concentrations of potentially toxic salt ions within plant cells. Salt stress is also linked to an oxidative stress as a consequence of the generation of reactive oxygen species, such as superoxide ion, hydrogen peroxide and hydroxyl radicals, which are detrimental to plant survival under salt stress. Salt-stressed plants display a complex oxidative defence strategy, catalase (CAT, E.C. 1.11.1.6) and peroxidases (POX, E.C. 1.11.1.7) enzymes being involved in scavenging of the hydrogen peroxide generated in response to oxidative stress. Saline stress is also known to affect many physiological activities related to the accumulation of ions and osmolytes such as proline (Lee et al., 2008). The accumulation of these compounds plays a major role in the process of osmotic adjustment, limiting water loss and ion toxicity. Biochemical alterations in plants due to salt stress may affect the nutritional balance and consequently growth and development. Inorganic phosphate uptake and transport appear to be sensitive to salinity (Ehsanpour and Amini, 2003) and this may have severe consequences for the acid phosphatase involved in inorganic phosphate assimilation in plants.

The use of plant growth-promoting bacteria (PGPR) and symbiotic microorganisms, especially arbuscular-mycorrhizal (AM) fungi, may prove useful in developing strategies to facilitate plant growth in saline soils. More specifically, the soil-borne pseudomonads have

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received particular attention because of their catabolic versatility, excellent root-colonising ability and capacity to produce a wide range of enzymes and metabolites that help the plant withstand varied biotic and abiotic stress conditions (Vessey, 2003). Relatively few mechanisms have been demonstrated that explain the increased resistance to environmental stresses of plants treated with bacteria of the genus *Pseudomonas*. PGPR can facilitate plant growth indirectly by reducing plant pathogens, or directly by facilitating the uptake of nutrients from the environment, by influencing phytohormone production (e.g. auxin, cytokinin and gibberellins), by enzymatic lowering of plant ethylene levels and/or by production of siderophores (Glick et al., 1997; Kohler et al., 2006). Many studies have demonstrated that inoculation with AM fungi improves growth of plants under salt stress (Cho et al., 2006). The improved growth of AM plants has been attributed to enhanced nutrient uptake, particularly of N and P and subsequent increased growth (Jeffries et al., 2003). However, in some cases plant salt tolerance was not related to P concentration (Ruiz-Lozano and Azcón, 2000). Thus, it has been proposed that salt-tolerance mechanisms, such as enhanced osmotic adjustment and leaf hydration, increased intrinsic water use efficiency, reduced oxidative damage or improved nutritional status, can explain the contribution of AM symbioses to the salinity resistance of host plants (Augé, 2001).

Nothing is known about the interaction of PGPR with AM fungi and their effect on the physiological response of lettuce plants under different conditions of soil salinity. We hypothesise that inoculation with a PGPR and an AM fungus, alone or in combination, can confer salinity tolerance to lettuce and that such tolerance is correlated with changes in the activity of antioxidant enzymes (catalase and total peroxidase activities), the accumulation of solutes (proline and soluble sugars), mineral nutrient uptake or the acid phosphatase activity related to assimilation of P.

2. Materials and methods

2.1. Soil and plant

An agricultural soil, used to cultivate lettuce was collected near Murcia (SE Spain). The climate is semi-arid Mediterranean with an average annual rainfall of 300 mm and a mean annual temperature of 19.2 °C; the potential evapo-transpiration reaches 1000 mm year^{-1}. The analytical characteristics of the agricultural soil used, determined by standard methods (Page et al., 1982), were: pH (1:5) 8.89; electrical conductivity 0.180 dS m^{-1}; TOC 1.80%; total N 2.01 g kg^{-1}; N-NO_3^- 68 μg g^{-1}; N-NH_3 16 μg g^{-1}; available P, 70 μg g^{-1}; extractable K, 440 μg g^{-1}; cationic exchange capacity, 15 cmol kg^{-1}.

The plant used in the experiment was lettuce (*L. sativa* L. cv. Tafalla). Seeds of lettuce were grown for 15 days in peat substrate under nursery conditions, without any fertilization treatment.

2.2. Microorganisms

The mycorrhizal fungi used were either *Glomus intraradices* (Schenk & Smith) or *Glomus mosseae* (Nicol & Gerd.) Gerd. & Trappe, obtained from the collection of the experimental field station of Zaidín, Granada. The two *Glomus* species were multiplied in pots using a mixture of sterile sepiolite/vermiculite (1:1, v:v) as growing substrate and Sorghum sp. as host plant. Trap cultures were maintained under greenhouse controlled conditions for 4 months. AM fungal inoculum consisted of a mixture of rhizospheric soil from the trap cultures containing spores, hyphae and mycorrhizal root fragments and was stored in polyethylene bags at 5 °C. Both inocula were subjected to a most probable number test (Sieverding, 1991) to determine potential infectivity and to equalize application doses. Both sources of inoculum had a potential infectivity of about 35 infective propagules g^{-1} inoculum.

The *Pseudomonas mendocina* Palleroni strain was obtained from Probelte, S.A., Murcia, which was selected on the basis of its ability to produce siderophores. *P. mendocina* was grown in a medium (nutrient broth, Scharlau Chemie, Spain) composed of meat and yeast extracts, peptone and sodium chloride, for 2 days at room temperature on a Heidolph Unimax1010 shaker. The bacterial culture was centrifuged at 4000 rpm for 5 min at 2 °C and the sediment was re-suspended in sterilized tap water. The bacterial suspension contained 10^9 CFU ml^{-1}.

2.3. Microbial inoculation and salt stress treatments

The experiment was a mesocosm assay, conducted as a factorial design in randomised blocks with two factors and fivefold replication. The first factor had seven levels: control soil, soil inoculated with the AM fungus *G. intraradices* or *G. mosseae*, soil inoculated with the bacteria *P. mendocina*, the combination of soil inoculated with the bacteria *P. mendocina* and with *G. intraradices* or *G. mosseae* and a soil fertilized with inorganic fertilizer, and the second one had three levels of salt stress: non-salt stress, moderate salt stress and severe salt stress. Five replicates per treatment were set up, making a total of 105 pots.

Seven hundred grams of substrate, consisting of soil and vermiculite at a ratio of 2:1 (v:v) sterilized by autoclaving at 105 °C for 60 min in three consecutive days, were placed in 1-l pots. In April 2006, *L. sativa* seedlings were transplanted to the pots (one per pot). The AM inoculum was mixed with the potting substrate, at a rate of 5% (v/v). The same amount of the autoclaved inoculum was added to non-AM plants, supplemented with a filtrate (Whatman no. 1 paper) of the culture to provide the microbial populations accompanying the mycorrhizal fungi. *P. mendocina* was inoculated two times during the growth period. The dose of inoculum applied corresponded to 10^{10} colony forming units (CFUs) per plant. Fertilized plants received 10 ml of Long Ashton Nutrient Solution on two occasions (μg ml^{-1}): 175.9 N, 156.2 K, 160.2 Ca, 98.4 S, 11.7 Na, 5.4 Cl, 5.0 Fe-chelate sequestrene, 0.54 Mn, 0.54 B, 0.10 Cu, 0.06 Zn, 0.006 Mo and 40 P. Two concentrations (2 and 4 g NaCl kg^{-1} soil) of saline solution were applied to the saline pots. To avoid osmotic shock, the NaCl concentration was gradually increased for four consecutives days, until the desired concentration was attained. A plastic bag was put underneath each pot to collect excess water due to drainage. This water was reapplied to the respective pot. All seedlings were grown for 4 weeks without any fertilizer treatment (except fertilized seedlings). At the end of experiment, the electrical conductivity of the non-saline pots and the pots cultivated under medium and high salinity level was about 0.340, 0.620 and 0.920 dS m^{-1}, respectively. The experiment was conducted in a greenhouse (average temperature ranged from 20 to 25 °C and the relative humidity 70 to 80%), located in the SACE service at Campus of Espinardo (Murcia, Spain). Midday photosynthetically active radiation (PAR) averaged 260 μE m^{-2} s^{-1}.

2.4. Plant analyses

One month after planting five plants per treatment were harvested and basal stem diameters were measured with callipers. The roots were washed free from soil under a stream of cold tap water and fresh and dry (105 °C, 5 h) weights of leaves and roots were recorded. Plant tissues were ground before chemical analysis. The foliar contents of phosphorus were determined, after digestion in nitric–perchloric acid (5:3) for 6 h, by colorimetry (Murphy

and Riley, 1962) and the plant K was estimated by flame photometry. The N concentration was determined by colorimetry after the Kjeldahl digestion.

Roots were subsampled in three 2-cm cross-sections of the upper, middle, and lower root system. To assess colonisation, roots were cleared with 10% KOH and stained with 0.05% trypan blue (Phillips and Hayman, 1970). The percentage of root length colonised by AM fungi was calculated by the gridline intersect method (Giovannetti and Mosse, 1980). Positive counts for AM colonisation included the presence of vesicles or arbuscules or typical mycelium within the roots.

Acid phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP, 0.115 M) as substrate. Two millilitres of 0.5 M sodium acetate buffer at pH 5.5 using acetic acid and 0.5 ml of substrate were added to 100 mg of fresh root tissue and incubated at 37 °C for 90 min. The reaction was stopped by cooling at 2 °C for 30 min. Then, 0.5 ml of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were added, and the mixture was centrifuged at 4000 rpm for 5 min. The *p*-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm (Tabatabai and Bremner, 1969). Controls were made in the same way, although the substrate was added after incubation.

2.5. Proline and total soluble sugars

Free proline and total soluble sugars were extracted from 1 g of fresh leaves (Bligh and Dyer, 1959). The methanolic phase was used for the quantification of both substances. Proline was estimated by spectrophotometric analysis at 515 nm of the ninhydrin reaction, according to Bates et al. (1973). Soluble sugars were analysed by 0.1 ml of the alcoholic extract reacting with 3 ml freshly prepared anthrone (200 mg anthrone + 100 ml 72% (w:w) H_2SO_4) and placed in a boiling water bath for 10 min according to Irigoyen et al. (1992). After cooling, the absorbance at 620 nm was determined in a spectrophotometer. The calibration curve was made using glucose in the range of 20–400 $\mu g\ ml^{-1}$.

2.6. Leaf enzyme extraction

All operations were performed at 4 °C. Leaves (2 g) were homogenised with a mortar and pestle in 4 ml of ice-cold 50 mM Tris-acetate buffer pH 6.0, containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM cysteine, 2% (w/v) polyvinylpyrrolidone (PVP), 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 0.2% (v/v) Triton X-100. The homogenate was centrifuged at 14,000 × *g* for 20 min and the supernatant fraction was filtered through Sephadex G-25 columns (NAP, Pharmacia Biotech AB, Uppsala, Sweden), equilibrated with the same buffer used for the homogenisation.

2.7. Assays performed

CAT activity was determined at 25 °C according to Aebi (1984). The reaction mixture contained 10 mM H_2O_2 in a 50 mM phosphate buffer pH 7.0, and 100 μl of leaf enzyme extract in a total volume of 3 ml. CAT activity was estimated by decreased in absorbance of H_2O_2 at 240 nm.

Total peroxidase was determined in assays containing 50 mm Tris-acetate buffer (pH 5.0) and 0.5 mm H_2O_2, using 1.0 mm 4-methoxy-α-naphtol as electron donor (E_{595} = 21,600 $m^{-1}\ cm^{-1}$; Ros-Barceló, 1998). The reaction was initiated by adding enzyme. Controls were carried out in the absence of H_2O_2.

2.8. Statistical analysis

Data were log transformed to achieve normality. Microbial inoculation, salinity level and their interactions effects on measured variables were tested by a two-way analysis of variance and comparisons among means were made using Tukey's test calculated at $P < 0.05$. Statistical procedures were carried out with the software package SPSS 10.0 for Windows.

Table 1
Effect of inoculation with *Glomus intraradices*, *G. mosseae* and *Pseudomonas mendocina* on growth parameters, shoot water content and colonisation of *Lactuca sativa* seedlings grown at three levels of salinity ($n = 5$).

	Shoot dry biomass (g dw)	Root dry biomass (g dw)	Shoot water content (%)	R/S ratio	Colonisation (%)
Without NaCl					
Control	1.48de	0.82f	80.0abc	0.55	0a
Fertilized	1.90f	0.80f	84.7def	0.42	0a
P. mendocina	1.92f	0.89f	86.4ef	0.46	0a
G. intraradices	1.42cde	0.64bcdef	80.6abcd	0.45	68.8f
G. mosseae	1.46de	0.73ef	77.6ab	0.50	63.6ef
P. mendocina + *G. intraradices*	1.44de	0.73ef	83.9bcde	0.51	68.2f
P. mendocina + *G. mosseae*	1.62ef	0.84f	84.7cdef	0.52	59.4def
2 g NaCl kg^{-1} soil					
Control	0.84a	0.42abc	83.0cde	0.50	0a
Fertilized	1.18bcd	0.66cdef	84.9def	0.56	0a
P. mendocina	1.18bcd	0.77f	86.8ef	0.65	0a
G. intraradices	1.30bcd	0.70def	84.7def	0.54	49.4cde
G. mosseae	1.21bcd	0.78f	87.6f	0.64	45.6bcd
P. mendocina + *G. intraradices*	1.18bcd	0.80f	86.8ef	0.68	39.8bc
P. mendocina + *G. mosseae*	1.29bcd	0.85f	85.8ef	0.66	37.6bc
4 g NaCl kg^{-1} soil					
Control	0.83a	0.26a	75.7a	0.31	0a
Fertilized	1.27bcd	0.44abcd	83.3cde	0.35	0a
P. mendocina	1.22bcd	0.41ab	82.5bcde	0.34	0a
G. intraradices	1.11abc	0.41ab	79.2abc	0.37	31.6b
G. mosseae	1.05ab	0.45abc	83.5cde	0.43	34.0b
P. mendocina + *G. intraradices*	1.29bcd	0.47abcd	82.2bcde	0.36	41.4bc
P. mendocina + *G. mosseae*	1.22bcd	0.50abcde	83.8cde	0.41	41.8bc

For each parameter, values in columns followed by the same letter are not significantly different (Tukey, $p < 0.05$).

Table 2
Two factors ANOVA (microbial inoculation and saline stress) for all parameters studied of *L. sativa*. *p* significance values.

	Microbial inoculation (*I*)	Saline stress (*S*)	Interaction (*I* × *S*)
Shoot	<0.001	<0.001	<0.001
Root	<0.001	<0.001	<0.001
Shoot water content	<0.001	<0.001	<0.001
N	<0.001	<0.001	<0.001
P	<0.001	<0.001	<0.001
K	<0.001	<0.001	<0.001
Fe	<0.001	<0.001	<0.001
Ca	<0.001	<0.001	<0.001
Colonisation	<0.001	<0.001	<0.001
Nitrate reductase	<0.001	<0.001	<0.001
Acid phosphatase	0.301	<0.001	<0.001
Proline	<0.001	<0.001	<0.001
Total soluble sugars	0.035	<0.001	0.003
POX	<0.001	<0.001	<0.001
CAT	<0.001	<0.001	<0.001

3. Results

3.1. Growth, shoot water content, nutrient assimilation and mycorrhizal colonisation

Under non-saline conditions, shoot dry biomass of lettuce was increased by the inoculation with *P. mendocina*, while no significant effect was found with the mycorrhizal inoculation treatments (Tables 1 and 2). The fertilization and bacterial inoculation produced similar increases in plant growth (about 30% greater than control plants). Neither of the microbial treatments had an effect on the root biomass. There was no interaction between the bacterial inoculation and mycorrhizal inoculation with either of the selected AM fungi regarding growth parameters, shoot water content or mycorrhizal colonisation (Table 2).

Salinity decreased the dry weight of the shoots and roots for all lettuce plants. However, the plants inoculated with *P. mendocina* had significantly greater shoot biomass than the control plants at both salinity levels, whereas the mycorrhizal inoculation treatments only were effective in increasing shoot biomass at the medium salinity level. Leaf hydration was significantly changed by the salt and microbial treatments (Table 2). Only the bacterial inoculation increased significantly the shoot water content in the non-salinised plants. At the highest salinity level, the water content was higher in leaves of plants treated with *P. mendocina* or *G. mosseae*.

In non-salinised plants, both mycorrhizal inoculation treatments produced active colonisation of the root system of the *L. sativa* seedlings although there were no significant differences between them with regard to the percentage of root length colonisation (Table 2). The level of colonisation in roots of mycorrhizal plants was not affected by bacterial inoculation but decreased significantly with increasing NaCl concentration (Table 1).

Except for Fe, the assayed microbial inoculations affected significantly the foliar nutrient concentrations of plants grown under non-stressing conditions (Table 2). The mycorrhizal inoculation treatments in combination with *P. mendocina* were the most effective treatments for increasing the foliar N concentration (Table 3). Likewise, the highest foliar concentrations of phosphorus were seen in the plants inoculated with *G. intraradices*, alone or in combination with *P. mendocina*, followed by the fertilized plants, under non-saline conditions. In contrast, the lowest concentrations of Na and Ca were in the leaves of plants inoculated with the bacterial strain, alone or in combination with *G. intraradices*.

Salinity increased significantly the foliar N, Na and Ca concentrations and decreased the foliar K concentrations in lettuce plants (Table 2). The increases in foliar Na due to salt stress were higher in the control plants than in the plants treated with *P. mendocina* or AM fungi. At the medium salinity level, *G. intraradices*- or *G. mosseae*-colonised plants showed the highest concentrations of foliar P. The *P. mendocina*- and *G. mosseae*-colonised plants presented had higher concentrations of foliar K and lower concentrations of foliar Na than the control plants under high salt-stress conditions.

Table 3
Effect of inoculation with *G. intraradices*, *G. mosseae* and *P. mendocina* on foliar nutrients of *L. sativa* seedlings grown at three levels of salinity (n = 5).

	N (g kg^{-1})	P (mg kg^{-1})	K (mg kg^{-1})	Na (mg kg^{-1})	K/Na	Fe (mg kg^{-1})	Ca (mg kg^{-1})
Without NaCl							
Control	16.8a	1987ab	48,750d	7339b	6.6	341c	14,039b
Fertilized	16.7a	2535c	50,929de	6807ab	7.5	283bc	11,457ab
P. mendocina	18.6ab	2177b	50,536de	6039a	8.4	226bc	10,979a
G. intraradices	17.4a	2733d	49,250de	6743ab	7.3	253bc	12,032ab
G. mosseae	19.5ab	2257bc	52,929ef	8682b	6.1	371c	15,396bc
P. mendocina + *G. intraradices*	21.0b	3222e	53,250f	6396a	8.3	252bc	10,732a
P. mendocina + *G. mosseae*	20.0b	2420cd	57,321g	8043b	7.1	279bc	14,039b
2 g NaCl kg^{-1} soil							
Control	23.1bc	1886a	45,286bc	34,629d	1.3	321bc	17,293d
Fertilized	27.4de	2196b	45,286bc	33,307d	1.4	234bc	16,689cd
P. mendocina	22.9bc	2038ab	42,929bc	27,507c	1.6	403c	15,900c
G. intraradices	23.9bc	2325bc	43,571bc	25,121c	1.7	321bc	16,461cd
G. mosseae	22.2bc	2393bc	44,786bc	26,621c	1.7	272bc	16,821d
P. mendocina + *G. intraradices*	23.8bc	2166b	44,321bc	27,807c	1.6	190ab	16,843d
P. mendocina + *G. mosseae*	24.8cd	2294bc	44,714bc	27,479c	1.6	212b	16,796d
4 g NaCl kg^{-1} soil							
Control	24.1cd	2046ab	36,750a	48,393f	0.8	373c	18,875e
Fertilized	31.5e	2463cd	42,214ab	37,929de	1.1	157a	16,507cd
P. mendocina	26.3cd	2171b	42,393bc	41,500e	1.0	231bc	17,593de
G. intraradices	28.6de	2020ab	39,643a	38,500e	1.0	201b	17,521de
G. mosseae	26.2cd	2287bc	43,679bc	40,429e	1.1	248bc	17,311de
P. mendocina + *G. intraradices*	28.7de	2204bc	44,643bc	36,643de	1.2	135a	17,407de
P. mendocina + *G. mosseae*	29.0e	2602d	46,714c	37,643de	1.2	169ab	16,289cd

For each parameter, values in columns followed by the same letter are not significantly different (Tukey, $p < 0.05$).

Table 4
Effect of inoculation with *G. intraradices*, *G. mosseae* and *P. mendocina* on root acid phosphatase activity and proline and total soluble sugars contents in shoots of *L. sativa* seedlings grown at three levels of salinity ($n = 5$).

	Acid phosphatase (μmol PNP g^{-1} h^{-1})	Proline (μmol g^{-1} FW)	Total soluble sugars (mg g^{-1} FW)
Without NaCl			
Control	37abcd	6.0abc	44.5f
Fertilized	29a	2.6a	33.7def
P. mendocina	31ab	1.8a	25.5bcde
G. intraradices	33abc	6.0abc	32.7cdef
G. mosseae	39abcd	6.6abcd	41.3ef
P. mendocina + *G. intraradices*	34abc	9.2bcdef	40.6ef
P. mendocina + *G. mosseae*	38abcd	5.6abc	36.3def
2 g NaCl kg^{-1} soil			
Control	56ef	19.3fghi	22.3bcd
Fertilized	41abcde	19.0efghi	31.7cdef
P. mendocina	50def	15.3bcdefgh	27.2bcde
G. intraradices	41abcd	8.6abcde	34.6cdef
G. mosseae	37abcd	4.7ab	27.4bcde
P. mendocina + *G. intraradices*	39abcd	16.0cdefgh	28.5cdef
P. mendocina + *G. mosseae*	36abcd	19.5fghi	34.9cdef
4 g NaCl kg^{-1} soil			
Control	38abcd	20.4ghi	6.3a
Fertilized	60f	16.9defgh	13.0ab
P. mendocina	37abcd	11.1bcdef	13.2ab
G. intraradices	44abcde	28.6i	20.6abcd
G. mosseae	45bcdef	17.6efgh	14.7ab
P. mendocina + *G. intraradices*	46cdef	23.5hi	17.1abc
P. mendocina + *G. mosseae*	45bcdef	22.1hi	17.9abc

For each parameter, values in columns followed by the same letter are not significantly different (Tukey, $p < 0.05$).

3.2. Phosphatase activity and solute accumulation

Neither bacterial nor mycorrhizal inoculation had any significant effect on the phosphatase activity of non-salinised plants (Table 4). Phosphatase activity in roots of the *P. mendocina*-inoculated and control plants was increased significantly at the medium salinity level.

Table 5
Effect of inoculation with *G. intraradices*, *G. mosseae* and *P. mendocina* on shoot total peroxidase (POX) and catalase (CAT) activities of *L. sativa* seedlings grown at three levels of salinity ($n = 5$).

	POX (μmol min^{-1} g^{-1} FW)	CAT (mmol min^{-1} g^{-1} FW)
Without NaCl		
Control	122.0a	29.1cde
Fertilized	549.4ij	31.9de
P. mendocina	130.5a	13.1ab
G. intraradices	403.7efg	31.0de
G. mosseae	322.8cde	21.5bcd
P. mendocina + *G. intraradices*	445.8fgh	31.5de
P. mendocina + *G. mosseae*	366.9def	33.8e
2 g NaCl kg^{-1} soil		
Control	195.7ab	19.0abc
Fertilized	272.6bc	20.6bcd
P. mendocina	148.2a	18.6abc
G. intraradices	126.6a	9.0a
G. mosseae	151.5a	11.0ab
P. mendocina + *G. intraradices*	645.0k	13.5ab
P. mendocina + *G. mosseae*	467.8ghi	26.9cde
4 g NaCl kg^{-1} soil		
Control	318.4cd	58.2f
Fertilized	519.3hi	21.1bcd
P. mendocina	442.3fgh	30.8de
G. intraradices	393.1defg	34.3e
G. mosseae	440.8fgh	36.4e
P. mendocina + *G. intraradices*	608.1jk	15.2ab
P. mendocina + *G. mosseae*	440.0fgh	89.1g

For each parameter, values in columns followed by the same letter are not significantly different (Tukey, $p < 0.05$).

No significant differences among the different treatments were found for proline accumulation in lettuce leaves under non-saline conditions (Table 4). Proline concentrations increased as a consequence of salinity (Table 4). However, proline accumulation was considerably less for the mycorrhizal plants than for the non-mycorrhizal plants at the medium salinity level. In the case of *P. mendocina*-inoculated plants, the increase in proline concentration was higher than for the control plants at the highest salinity level.

Under non-saline conditions, only the inoculation with the PGPR gave differences in the total soluble sugar accumulation as compared with the control plants; the latter reached higher levels than the *P. mendocina*-inoculated plants (Table 4). Salt stress decreased sugar accumulation in all plants (Table 2). Under high salt stress conditions, the control plants accumulated the lowest concentration of total soluble sugars.

3.3. Antioxidant enzyme activities

The inoculation with *P. mendocina* decreased CAT activity (about 55% lower than for the control plants) and the inoculation with either of the selected AM fungi increased total peroxidase activity in leaves of non-salinised lettuce plants (about 198% higher than for the control plants), as shown in Table 5. Moderate salinity notably decreased the specific total peroxidase and CAT activities in the mycorrhizal plants. In contrast, these antioxidant enzymes were increased under high salt stress conditions, particularly the plants inoculated with the bacterial strain, with *G. mosseae* and the control plants. The total peroxidase activity of plants treated with *P. mendocina* in combination with either of the selected AM fungi was increased by all salt levels.

4. Discussion

Salinity adversely affected the growth of *L. sativa*, regardless of the biological treatment and the salt-stress level. However, when the plants were inoculated with an AM fungus or a PGPR the extent of growth suppression was decreased and these treated plants

had greater dry weights than untreated plants. Our results show that both AM fungi assayed stimulated significantly the growth of lettuce plants only under moderately saline conditions, while inoculation with *P. mendocina* increased plant biomass even under severely saline conditions. On the other hand, the effect of dual inoculation with PGPR and AM fungi on shoot biomass of *L. sativa* was similar to that of PGPR colonisation alone. These observations clearly suggest that the selected microorganisms can differentially alleviate some of the deleterious effects of salt stress.

Increased salt concentration decreases the osmotic potential of a growth medium, thus reducing the water availability. The use of water content is a good indicator of water stress (Mayak et al., 2004). In the control plants, leaf water content decreased as the external salt concentration increased. However, plants inoculated with *P. mendocina* were more hydrated than the control plants under saline conditions. These results demonstrated that the bacterial treatment influenced the extent of water stress and that *P. mendocina* efficiently protected the host plants against the detrimental effects of salt. Greater hydration induced by the PGPR strain might be attributable to increased water use efficiency and/or enzymatic lowering of plant ethylene concentrations thereby decreasing the ethylene inhibition of seedling root biomass (Saravanakumar and Samiyappan, 2007). The root biomass of control plants was decreased as a consequence of salinity, while those of inoculated and fertilized plants were not decreased at the medium salinity level and suffered a lesser reduction at the highest level.

The increased shoot biomass of seedlings inoculated with AM fungi could be partly related to the increase in water uptake that a mycorrhizal fungus provides under saline conditions (Ruiz-Lozano and Azcón, 1996): the higher shoot water content in plants inoculated with *G. mosseae* supports this possibility. Nutritional mechanisms may have played a role in the differential modulation of host water status by the different AMF species. Improved nutritional status can lead to specific stimulation of photosynthetic capacity over stomatal conductance, which, in turn, influences positively the plant water status (Querejeta et al., 2003). In particular, the protection of mycorrhizal plants against salt stress was related to the effect that the added endophytes had on increasing P uptake. The concentration of foliar P in mycorrhizal plants was particularly increased under moderately saline conditions. Enhanced P nutrition is considered as one of the major mechanisms by which AMF can improve the water status of their host plants (Augé, 2001). Non-nutritional mechanisms by which AMF can also improve the water status of their host plants include hormonal signalling, osmotic adjustment, changes in root hydraulic conductivity, direct contributions of extraradical hyphae to water uptake, and changes in the moisture characteristics of rhizosphere soil (Augé, 2001). It has been hypothesised that mycorrhizae can alter the morphology of the root system, yielding a more extensive absorbing area, which may be considered a mechanism of salinity tolerance (Ibrahim et al., 1990). Likewise, mycorrhizae are known to increase the xylem pressure potential, by increasing root biomass and therefore improving water uptake (Augé, 2001). It is worth noting that the root biomass of *G. mosseae*-colonised plants was about double that of the control plants under moderately saline conditions.

In many plants, salt stress has been shown to affect carbohydrate partitioning and metabolism, leading to the synthesis of new compounds (Sharma et al., 1990). In particular, various solutes have been shown to accumulate during salinity. Their accumulation might be of importance for the adjustment of the cellular water potential under conditions of reduced water availability, and they can act as scavengers of reactive oxygen species. In plants exposed to salinity, the total non-structural carbohydrates content in the leaves was reduced significantly compared with plants not exposed to salinity. This could indicate that salinity induces a preferential partitioning of carbohydrates to the roots, as demonstrated by Schellenbaum et al. (1998). The decrease in total soluble carbohydrates due to salinity could be related also to limited carbohydrate availability, as a consequence of a decline in photosynthesis (Goicoechea et al., 2005). The control plants presented the highest reductions in leaf total soluble sugars throughout the salt-stress experiment, which could indicate that these plants were less salt tolerant. The total soluble carbohydrates of plants inoculated with *P. mendocina* and/or either of the selected AM fungi were not affected under moderate salinity.

In contrast to soluble carbohydrates, moderate and severe salinity increased shoot proline accumulation in non-mycorrhizal plants. A high level of proline enables the plants to maintain an osmotic balance when growing under low water potentials. It is reported that proline protects higher plants against salt/osmotic stresses, not only by adjusting osmotic pressure, but also by stabilising many functional units such as complex II electron transport, membranes and proteins and enzymes such as RUBISCO (Mäkelä et al., 2000). However, no changes in proline concentrations were observed in plants inoculated with either of the selected AM fungi in response to moderate salinity. This could indicate that moderate salinity affected to a lesser extent the mycorrhizal plants, so they accumulated less proline. The concentrations of proline are always not high enough to adjust the osmotic potential in some plants under stress (Hoque et al., 2007). In fact, the inhibition of growth of lettuce was higher in non-inoculated plants than in inoculated plants in spite of the fact that the levels of proline were similar for all plants under severe salinity. Thus, our data suggest that the accumulation of proline itself cannot confer salt tolerance in lettuce plants.

Salinity dominated by Na^+ and Cl^- not only reduces Ca^{2+} and K^+ availability, but also reduces Ca^{2+} and K^+ mobility and transport to the growing parts of plants, affecting the quality of both vegetative and reproductive organs. Moreover, many studies have shown that high concentrations of NaCl in the soil solution may increase the ratios of Na^+/Ca^{2+} and Na^+/K^+ ratios in plants, which would then be more susceptible to osmotic and specific ion injury as well as to nutritional disorders that result in reduced yield and quality (Sivritepe et al., 2003). Salt tolerance in plants has been related to their ability to decrease leaf osmotic potential, which has been linked to Na^+ and/or Cl^- ion exclusion mechanisms or to the retention of salt ions in roots, preventing the accumulation of Na^+ and/or Cl^- in the shoot. The results of this study show that salinity caused an increase in Na concentration and a decrease in K^+ and Ca^{2+} regardless of the microbiological treatment. However, both microbiological treatments reduced the Na^+ uptake of plants and/or increased the K^+ uptake, compared with the control plants under salt stress, thus increasing the K^+/Na^+ ratio. Previous studies indicate that an increase in concentration of K^+ in plants under salt stress could ameliorate the deleterious effects of salinity on growth and yield (Giri et al., 2007). Potassium plays a key role in plant water stress tolerance and has been found to be the cationic solute responsible for stomatal movements in response to changes in bulk leaf water status (Caravaca et al., 2004). This means that the *P. mendocina* strain and selected AM fungi could alleviate the effects of salinity stress in lettuce. Reduced Na concentration in lettuce plants exposed to salinity, due to AM inoculation, may have helped the plants prevent accumulation of cellular Na to a toxic concentration. There are several reports of lower Na^+ concentrations in AM plants, compared to non-AM plants, under salinity (Giri and Mukerji, 2004; Ashraf et al., 2004; Sharifi et al., 2007). In the case of *P. mendocina*, this PGPR strain can produce exopolysaccharides that bind cations, including Na^+ (Kohler et al., 2006), thus decreasing the content of Na^+ available for plant uptake. We have shown the beneficial effect of this PGPR strain on soil aggregate

stabilisation (Kohler et al., 2006). Previous studies have described a negative relationship between soil aggregation and percentage of Na^+ saturation in the exchange complex (Lax et al., 1994). In this context, the reduced Na^+ concentrations recorded in leaves of *P. mendocina*-colonised plants could be due to the lower percentage of exchangeable Na^+ in the soil cultivated with such plants.

It has been indicated that salt stress affects the physiology and biochemistry of plant cells under in vitro and in vivo conditions. In this context, increased acid phosphatase has been reported in plants grown under salt stress (Stephen et al., 1994). We have observed that moderate salinity enhanced the phosphatase activity of non-mycorrizal lettuce plants. Ehsanpour and Amini (2003) also demonstrated that osmotic and salt stresses increase acid phosphatase activity in *Medicago sativa*. Acid phosphatase is known to act under stress by maintaining a certain level of inorganic phosphate in plant cells (Olmos and Hellin, 1997). The fact that mycorrhizal plants showed the highest levels of foliar P could explain why the salinity did not affect the phosphatase activity of such plants. However, our results show that acid phosphatase did not play a role in responses of inoculated and non-inoculated lettuce to severe salinity.

Salt stress may induce a combination of negative effects on salt-sensitive plants including osmotic stress, ion toxicity and oxidative stress. The induction of antioxidant enzyme such as catalase and peroxidase can be considered as one mechanism of salt tolerance in plants (Hernández et al., 2003). These antioxidant enzymes are involved in eliminating H_2O_2 from salt-stressed roots (Kim et al., 2005). Catalase, which is localized in peroxisomes, decomposes hydrogen peroxide to water and molecular oxygen without consuming reductants and, thus, may provide plant cells with an energy-efficient mechanism to remove hydrogen peroxide (Scandalios et al., 1997). Hydrogen peroxide can be removed also by "non-specific" peroxidases in the apoplast of lignifying tissues (Ros-Barceló et al., 2006), which use hydrogen peroxide as an electron donor to metabolise phenolic compounds. These latter enzymes are ubiquitous and are involved in various processes such as cell growth control and tolerance of environmental stress (Quiroga et al., 2000). Thus, decreased activity of POX can prevent or reduce lignification processes caused by salinity, thereby enabling continuity of water uptake through the cell walls (Yazici et al., 2007). In our study the CAT activity of mycorrhizal plants experienced a reduction as a consequence of moderate salinity. Reduced CAT activity under stress conditions has been reported in other plants, such as sunflower (Quartacci and Navari-Izzo, 1992), this reduction being parallel to a rise in the H_2O_2 content. Khedr et al. (2003) found that salt stress inhibited the activities of catalase and peroxidase, but the activities of these enzymes were significantly higher in the presence of proline than in its absence. These authors suggested that proline acts as a free radical scavenger to alleviate salt stress. This could explain why the POX and CAT activities of non-mycorrhizal plants did not experience changes under moderate salinity, whereas the mycorrhizal plants showed a suppression of such antioxidant enzymes possibly due to lower levels of accumulated proline. As indicated above, this decrease in antioxidant enzymes in mycorrhizal plants could be explained partially by the fact that these plants may have been submitted to a lower oxidative stress under moderately saline conditions. It is worth noting that this behaviour was also observed in the POX activity of fertilized plants, which probably support less stress. Increasing salinity stress significantly increased the antioxidant enzyme activities of lettuce leaves, including those of POX and CAT, of lettuce leaves compared to their respective non-stressed controls. Remarkably, the PGPR strain induced a higher increase in these antioxidant enzymes in response to severe salinity.

Based on these findings, a PGPR, *P. mendocina*, was more effective than an AM fungus with respect to alleviating the negative effect of salinity on the growth of lettuce. Co-inoculation with PGPR and AM fungus had no additive effect on the plant growth. The contribution of the PGPR and AM fungi to plant salt tolerance was closely related to salinity avoidance mechanisms, including increased water uptake and restricted Na uptake. The protection of mycorrhizal plants against moderate salt stress was also related to the effect that the added endophytes had on increasing P uptake. Induction of antioxidant enzyme activities was involved in the ability of the PGPR strain to increase the tolerance of lettuce grown under severe salt stress. Therefore, inoculation with selected PGPR could serve as a useful tool for alleviating salinity stress in salt-sensitive plants.

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