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PLANT MICROBE INTERACTIONS

Differential Effects of *Pseudomonas mendocina* and *Glomus intraradices* on Lettuce Plants Physiological Response and Aquaporin PIP2 Gene Expression Under Elevated Atmospheric CO_2 and Drought

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Abstract Arbuscular mycorrhizal (AM) symbiosis and plant-growth-promoting rhizobacterium (PGPR) can alleviate the effects of water stress in plants, but it is unknown whether these benefits can be maintained at elevated CO_2. Therefore, we carried out a study where seedlings of *Lactuca sativa* were inoculated with the AM fungus (AMF) *Glomus intraradices* N.C. Schenk & G.S. Sm. or the PGPR *Pseudomonas mendocina* Palleroni and subjected to two levels of watering and two levels of atmospheric CO_2 to ascertain their effects on plant physiological parameters and gene expression of one PIP aquaporin in roots. The inoculation with PGPR produced the greatest growth in lettuce plants under all assayed treatments as well as the highest foliar potassium concentration and leaf relative water content under elevated [CO_2] and drought. However, under such conditions, the PIP2 gene expression remained almost unchanged. *G. intraradices* increased significantly the AMF colonization, foliar phosphorus concentration and leaf relative water content in plants grown under drought and elevated [CO_2]. Under drought and elevated [CO_2], the plants inoculated with *G. intraradices* showed enhanced expression of the PIP2 gene as compared to *P. mendocina* or control plants. Our results suggest that both microbial inoculation treatments could help to alleviate drought at elevated [CO_2]. However, the PIP2 gene expression was increased only by the AMF but not by the PGPR under these conditions.

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Introduction

Plants in the field are exposed constantly to several environmental stress conditions, water deficit, salinity, and elevated temperatures being the most common environmental stress factors experienced by plants [43]. Also, the atmospheric [CO_2] is expected to double in the next 100 years and may accompanied by a 1.5–4.5°C increase in temperature [17]. Such a climate change would result in a substantially warmer planet with altered weather patterns [18], and it is expected that temperate areas will become warmer and drier [6]. All these stresses cause dehydration of plant tissues, decreasing their development and diminishing the growth and production of crops [44]. These effects will be particularly enhanced in semi-arid agricultural conditions [8] such as those predominant in Mediterranean areas.

Despite the importance of soils and their biota to terrestrial ecosystem functioning and global carbon cycles [15], little is known about how the soil biota will respond to climate change [47]. A key component of soil ecosystems is the community of arbuscular mycorrhizal (AM) fungi, which are obligate symbionts of plants: the fungus receives plant photosynthates, while the plants improve their nutrient uptake and their tolerance to biotic and abiotic stresses [16, 41]. It is currently accepted that AM symbioses protect host plants against the detrimental effects of water deficit and that the contribution of the AM symbiosis to plant drought tolerance results from a combination of physical, nutritional and cellular effects [38]. The cellular effects include modulation of some genes involved in the response of plants to osmotic stresses, such as genes encoding aquaporins. Aquaporins are membrane

intrinsic proteins that form pores in all cell membranes of living organisms, facilitating and regulating the passive movement of water molecules down a water potential gradient [26]. In plants, four classes of aquaporins have been identified so far: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins, nodulin-like intrinsic proteins, and small basic intrinsic proteins [29]. Studies have suggested that some aquaporins play a role in hydraulic conductivity of cell membranes and that PIP proteins seem to play an important role in regulating transcellular water transport [22, 31]. In fact, they are expressed mainly in roots, where they carry out most of the water uptake from the soil [21], although aquaporins are also expressed in leaves [32].

Until now, there have been few studies in which the effects of AM symbiosis on PIP gene expression in roots and/or shoots under drought stress or saline conditions have been evaluated. Aroca et al. [3] found differential expression of several PIP genes in the roots of *Phaseolus vulgaris* plants under different types of stress, depending on the AM fungal presence. On the other hand, Ouziad et al. [34] found that inoculation with a mixture of AM fungi decreased the expression of one PIP gene in the roots of tomato plants under saline conditions. In the study by Porcel et al. [36], AM plants responded to drought stress by downregulating the expression of two PIP genes and anticipating its downregulation, as compared with non-AM plants. However, how the AM symbiosis affects PIP gene expression under elevated CO_2 (eCO_2) conditions, combined with induced water stress, has never been investigated. Between the different PIP aquaporins, we selected *PIP2* gene in lettuce's roots, since there are evidences that *PIP2* are more active in water flow across plasma membranes than other PIP genes [36].

At eCO_2 concentrations, plant responses include increased photosynthetic rates. AM fungi may help alleviate the increased plant nutrient limitation associated with higher photosynthetic rates [11]. Although many laboratory-based studies have shown that there is no direct effect of eCO_2 on AM fungi [9, 13, 48], in others, such as that of Staddon et al. [47], it was found that production of extraradical mycorrhizal hyphae was stimulated by eCO_2. Unfortunately, little data are currently available on the physiological properties of AM plants.

Other microorganisms beneficial to plants are the plant-growth-promoting rhizobacteria (PGPR), a group of free-living bacteria that can exert a beneficial effect on plant growth due probably to a combination of N_2 fixation, increased availability of nutrients in the soil, and excretion of plant hormones [50]. Kohler et al. [23] found that specific strains of *Pseudomonas* increased the growth and biomass production of lettuce plants under field conditions. Moreover, PGPR alleviated the oxidative damage produced under water shortage [24]. However, there is no information available about the effect of PGPR on the physiological properties of *Lactuca sativa* plants under elevated CO_2 conditions or about how PGPR affect PIP gene expression under the combination of induced water stress and eCO_2.

We hypothesize that in the scenery of eCO_2, the beneficial effects of AM fungi or PGPR to alleviate drought stress in plants can be modified. Therefore, the main objective of the present work was to evaluate the influence of the application of an AM fungus (AMF) or a plant growth-promoting rhizobacterium on plant development, physiology, and PIP gene expression in roots of *L. sativa* grown in a controlled environment under drought stress, elevated CO_2, or the combination of both factors.

Material and Methods

Soil and Plants

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 L m^{-2} and a mean annual temperature of 19.2°C; the potential evapotranspiration reaches 1,000 L m^{-2} $year^{-1}$. The main characteristics of the agricultural soil used were: pH (1:5) 8.89; electrical conductivity 0.18 dS m^{-1}; TOC 1.80%; total N 2.01 g kg^{-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.

Microorganisms

The mycorrhizal fungus used was *Glomus intraradices* N. C. Schenk & G.S. Sm., obtained from the collection of the experimental field station of Zaidín, Granada. AM fungal inoculum consisted of a mixture of rhizospheric soil from pot cultures (*Sorghum* sp.) containing spores, hyphae, and mycorrhizal root fragments. The inocula were subjected to a most probable number test [46] to determine potential infectivity and to equalize application doses. *G. intraradices* had a potential infectivity of about 35 infective propagules per gram of 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 2,287×*g* for 5 min at 2°C, and the sediment was resuspended in sterilized tap water. The bacterial suspension contained 10^9 CFU mL^{-1}.

Microbial Inoculation and Water Stress Treatments

The experiment was a mesocosm assay, conducted as a randomized factorial design with three factors and 6-fold replication. The first factor had three levels: control soil, soil inoculated with the AMF *G. intraradices*, and soil inoculated with the bacteria *P. mendocina*; the second factor was the application of water stress or not; and the third factor was the application of an elevated CO_2 atmosphere or not.

Seven hundred grams of substrate, consisting of soil and vermiculite at a ratio of 2:1 (*v*/*v*) were placed in 1-L pots. In April 2007, *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 plants noninoculated with *G. intraradices*, supplemented with a filtrate (Whatman no. 1 paper) of the culture to provide the microbial populations accompanying the mycorrhizal fungus. *P. mendocina* was inoculated two times during the growth period. The dose of inoculum applied corresponded to 10^{10} CFU/plant. All seedlings were grown for 9 weeks without any fertilizer treatment. Six replicates per treatment were set up, making a total of 72 pots. One half of the pots were watered regularly with decalcified water maintaining substrate water potential equivalent to field capacity (−0.03 MPa). The other half of the pots were cultivated under a soil water potential of −0.3 MPa. Soil water potential was determined by a pressure plate apparatus, and soil water content was measured by weighing the soil before and after drying at 110°C for 24 h. A characteristic soil moisture curve was constructed and used to correlate soil water content and soil water potential (Ψ) by gravimetric measurement of soil water content in the pots. The experiment was conducted in two growing chambers located in the SACE service at the Campus of Espinardo (Murcia, Spain), one with a pCO_2 of 380 ppm and the other with a pCO_2 of 760 ppm. During the experiment, the lettuce plants were subjected to a photoperiodic cycle of 13 h light with a light intensity of 270 μmol m^{-2} s^{-1} and 11 h dark. The day temperature of the growth medium was kept at 24°C, the night temperature at 18°C while the relative humidity was hold constant at 60%. Soil moisture was monitored gravimetrically before each watering.

Biomass Production and Nutrient Content

Nine weeks after planting, six plants per treatment were harvested. The roots were washed free from soil under a stream of cold tap water. 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 (*v*/*v*) for 6 h, by colorimetry [33], and the plant K was estimated by flame photometry. The foliar N was determined with the Kjeldahl method, consisting of titration after sample digestion and distillation [5].

Shoot water content was calculated as a percentage of the dry-fresh weight ratio.

Symbiotic Development

Roots were subsampled in three 2-cm cross-sections of the upper, middle, and lower root system. To assess AM colonization, roots were cleared with 10% KOH and stained with 0.05% trypan blue [35]. The percentage of root length colonized by AM fungi was calculated by the gridline intersect method [14]. Positive counts for AMF colonization included the presence of vesicles or arbuscules or typical mycelium within the roots.

Leaf Relative Water Content

The relative water content (RWC) in plant leaves was determined at the harvest time as previously described by Ruiz-Lozano and Azcón [39].

Stomatal Conductance

Before harvest, the stomatal conductance was measured on one fresh leaf of the youngest part of the plant for each plant with a steady-state porometer LI-COR, LI-1600 (Lincoln, NE, USA). Measurements were taken at 1,000 mmol m^{-2} s^{-1} light intensity, previously defined as being the light saturation under greenhouse conditions.

RNA Isolation and Synthesis of First Strand cDNA

Total RNA was extracted from lettuce roots using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). DNase treatment of total RNA was performed according to Promega´s recommendations. Total RNAs (2.5 μg) from lettuce roots were reverse transcribed to first strand cDNA using AMV-RT enzyme (Finnzymes, Espoo, Finland) and oligo$(dT)_{15}$ primer (Promega, Madison, WI), in a final volume of 25 μl with the buffer and temperature recommended by the enzyme supplier.

Quantitative Real-Time RT-PCR

PIP2 gene expression was studied by real-time PCR by using iCycler (Bio-Rad, Hercules, CA, USA). cDNAs were

obtained from 2.5 μg of total DNase-treated RNA in a 20-μl reaction containing 500 ng oligo$(dT)_{12-18}$ primer, 0.5 mM each dNTP, 10 mM DTT, 40 U of RNase inhibitor, 1× first strand buffer (Invitrogen, Carlsbad, CA, USA), and 200 U of superscript ii reverse transcriptase (Invitrogen). The primer sets used to amplify *PIP2* genes in the synthesized cDNA amplified a 304 bp DNA product (PIP2For: 5′-CAAATGGTCCTTCTACAGAGC-3′; PIP2Rev: 5′-CAAACACTGTGCAATCATGTATCC-3′) [36]. Each 25-μl reaction contained 1 μl of a dilution 1:10 of the synthesized cDNA, 200 nM dNTPs, 400 nM each primer, 3 mM $MgCl_2$, 2.5 μl of 1× SYBR Green (Molecular Probes, Eugene, OR, USA), and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in 1× PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl).

The PCR program consisted in a 4-min incubation at 95°C to activate the hot-start recombinant Taq DNA polymerase, followed by 30 cycles of 45 s at 95°C, 45 s at 56°C, and 45 s at 72°C, where the fluorescence signal was measured. The specificity of the PCR amplification procedure was checked with a heat dissociation protocol (from 70°C to 100°C) after the final cycle of the PCR. The results obtained on the different treatments were standardized to the 18S rRNA levels, which were amplified with the primers 18S [36]. Real-time PCR experiments were carried out at least five times, with the threshold cycle (C_T) determined in triplicate. The relative levels of transcription were calculated by using the $2^{-\Delta\Delta CT}$ method [28]. Negative controls without cDNA were used in all PCR reactions.

Statistical Analysis

Data were log-transformed to achieve normality. CO_2 level, water regime, microbial inoculations, and their interactions effects on measured variables were tested by a three-way analysis of variance, and comparisons among means were made using Duncan′s test calculated at $P<0.05$. Correlation analysis between all the parameters measured was carried out using Pearson′s rank correlation coefficients. Statistical procedures were carried out with the software package SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Shoot and Root Dry Biomass

Shoot and root dry biomass were affected by microbial inoculation, water stress, and CO_2 level (Tables 1, 2). Under elevated CO_2 with drought stress or not, the shoot dry biomass was increased by inoculation with *P. mendocina* or, to a lesser extent, the AMF; however, only the treatment with *P. mendocina* at −0.03 MPa and 380 CO_2 showed the highest increase with respect to control plants (Table 1). The drought decreased the shoot dry biomass at both CO_2 levels, although this decrease was more pronounced at the ambient CO_2 level than at the elevated CO_2 level. There was a significant CO_2 level × water stress interaction for shoot and root dry biomass (Table 2). Root dry biomass was increased by *P. mendocina* at atmospheric CO_2 for both watering levels. The CO_2 treatment decreased the root biomass of plants grown under well-watered conditions. *G. intraradices* also slightly decreased the root biomass at elevated CO_2 under stressing and non-stressing conditions (Table 1).

Mycorrhizal Colonization

The plants inoculated with *G. intraradices* showed the highest AMF colonization at both water and CO_2 levels (Table 1). The percentage of colonized roots decreased with water stress at normal CO_2; however, at elevated CO_2, the colonization increased, particularly in *G. intraradices*-inoculated plants. Only the interaction between water stress and CO_2 level had a significant effect on the colonization of lettuce roots (Table 2).

Shoot Water Content and Leaf Relative Water Content

The microbial inoculation treatments had no significant effect on the shoot water content at both CO_2 and watering levels (Fig. 1A; Table 2). Shoot water content was decreased under drought-stress conditions, this decrease being more pronounced at the atmospheric CO_2 level than at the eCO_2 level. There was a significant CO_2 × water stress interaction for shoot water content (Table 2).

The leaf RWC was influenced by microbial inoculation and water stress but not by CO_2 level (Table 2). Under well-watered conditions, no significant differences ($P>0.05$) in RWC were observed between inoculated and noninoculated plants, at either level of CO_2 (Fig. 1B). Drought diminished significantly the RWC in control and *P. mendocina*-inoculated plants grown at atmospheric CO_2; however, at eCO_2, the plants inoculated with *P. mendocina* showed the highest value while the RWC of control plants was significantly decreased (Fig. 1B).

Leaf Stomatal Conductance

The stomatal conductance (g_s) was influenced significantly by both factors: microbial inoculation and water stress (Table 2). In nonstressed plants, g_s was increased significantly in plants inoculated with *G. intraradices* and decreased in plants inoculated with *P. mendocina* (Table 1).

Table 1 Effect of inoculation with *G. intraradices* and *P. mendocina* on physiological parameters and colonization of *L. sativa* seedlings grown at two levels of irrigation and two levels of CO_2 concentration after 9 weeks of plantation (n=6)

	CO_2	Watering (MPa)	Shoot dry biomass (g dw)	Root dry biomass (g dw)	Colonization (%)	Stomatal conductance (mmol m^{-2} s^{-1})
Control	380	−0.03	1.08 (0.03)	0.21 (0.01)	37.8 (0.8)	49.5 (1.8)
		−0.3	0.63 (0.02)	0.20 (0.01)	18.4 (1.3)	21.6 (1.1)
P. mendocina	380	−0.03	1.48 (0.02)	0.26 (0.01)	39.0 (1.5)	18.4 (0.6)
		−0.3	0.69 (0.02)	0.28 (0.00)	32.5 (2.9)	15.0 (0.4)
G. intraradices	380	−0.03	1.21 (0.05)	0.18 (0.00)	54.5 (0.6)	99.4 (4.1)
		−0.3	0.63 (0.02)	0.21 (0.01)	37.7 (1.3)	39.4 (1.6)
Control	760	−0.03	0.98 (0.03)	0.12 (0.01)	26.0 (0.7)	144.9 (5.9)
		−0.3	0.64 (0.02)	0.24 (0.01)	31.2 (1.4)	22.3 (0.7)
P. mendocina	760	−0.03	1.15 (0.04)	0.14 (0.01)	36.6 (1.2)	57.1 (2.2)
		−0.3	0.89 (0.02)	0.28 (0.02)	45.2 (3.7)	20.7 (1.1)
G. intraradices	760	−0.03	1.03 (0.01)	0.09 (0.00)	45.2 (1.3)	41.1 (1.4)
		−0.3	0.77 (0.02)	0.19 (0.01)	65.7 (1.5)	18.6 (1.2)

For each parameter, values represent the mean followed by the standard errors in brackets

The drought diminished g_s in all treatments assayed, although *G. intraradices*-inoculated plants showed the highest g_s values. Under eCO_2, the noninoculated plants showed increases in g_s, but there were strong decreases under drought stress.

Nutrient Concentration

The microbial inoculation treatments had no significant effect on the foliar N, P, or K concentrations under either watering level at atmospheric CO_2 (Fig. 2). Water deficit induced a significant decrease in all these foliar nutrients, for all plants, under normal CO_2. Under well-watered conditions, the elevated CO_2 treatment increased significantly the foliar N and P concentrations in plants inoculated with either microbial inoculant; however, the foliar K concentration was not affected by any treatment. Under stressing conditions at eCO_2, the highest foliar P concentration, significantly so, was in the plants inoculated with *G. intraradices* (Fig. 2B). In the case of foliar N, its concentration was not affected by any treatment (Fig. 2A). However, the foliar K concentration was increased signif-

Table 2 Three factors ANOVA (microbial inoculation, water stress, and CO_2) for all parameters studied

	Microbial inoculation (M)	Water stress (W)	CO_2 level (C)	Interaction (M × W)	Interaction (M × C)	Interaction (W × C)	Interaction (M × W × C)
Shoot biomass	0.008	<0.001	0.049	n.s.	n.s.	0.001	n.s.
Root biomass	0.037	<0.001	0.005	n.s.	n.s.	0.004	n.s.
Colonization	<0.001	n.s.	n.s.	n.s.	n.s.	<0.001	n.s.
Shoot water content	n.s.	<0.001	<0.001	0.022	n.s.	0.013	n.s.
Relative water content	0.005	<0.001	n.s.	<0.001	0.002	n.s.	0.002
Stomatal conductance	0.002	<0.001	n.s.	n.s.	<0.001	n.s.	n.s.
Foliar N	n.s.	<0.001	0.009	n.s.	n.s.	0.005	n.s.
Foliar P	0.025	<0.001	<0.001	0.003	n.s.	n.s.	n.s.
Foliar K	n.s.	<0.001	<0.001	n.s.	0.010	<0.001	0.005
PIP2 gene expression	<0.001	<0.001	<0.001	0.007	<0.001	<0.001	<0.001

Significance is indicated by *P* values

n.s. not significant

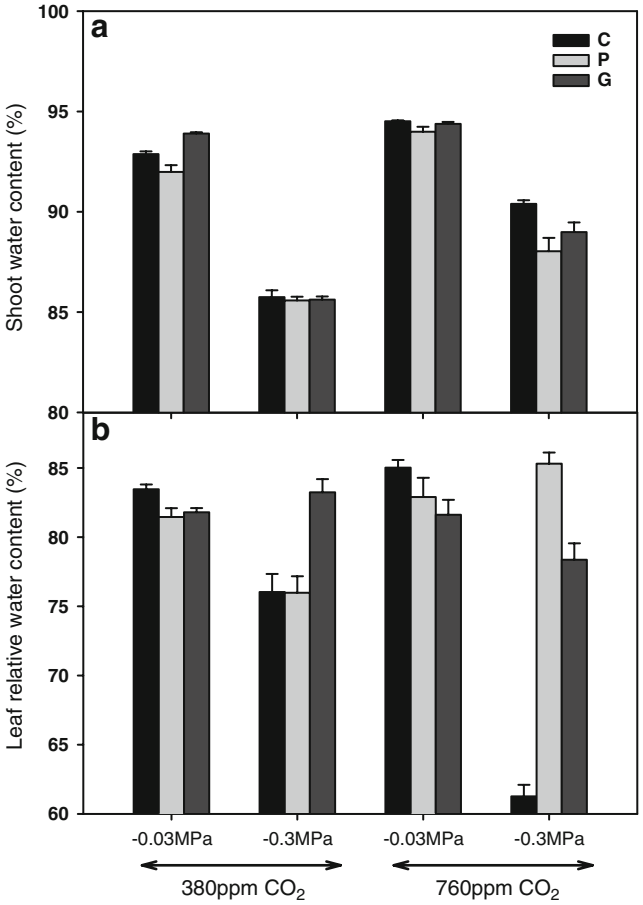

Figure 1 Effect of inoculation with *G. intraradices* and *P. mendocina* on shoot water content (**a**) and leaf relative water content (**b**) of *L. sativa* seedlings grown at two levels of irrigation and two levels of CO_2 concentration after 9 weeks of plantation. *C* uninoculated plants; *P* plants inoculated with *P. mendocina*; *G* plants inoculated with *G. intraradices*. *Columns* represent mean ± SE (n=6)

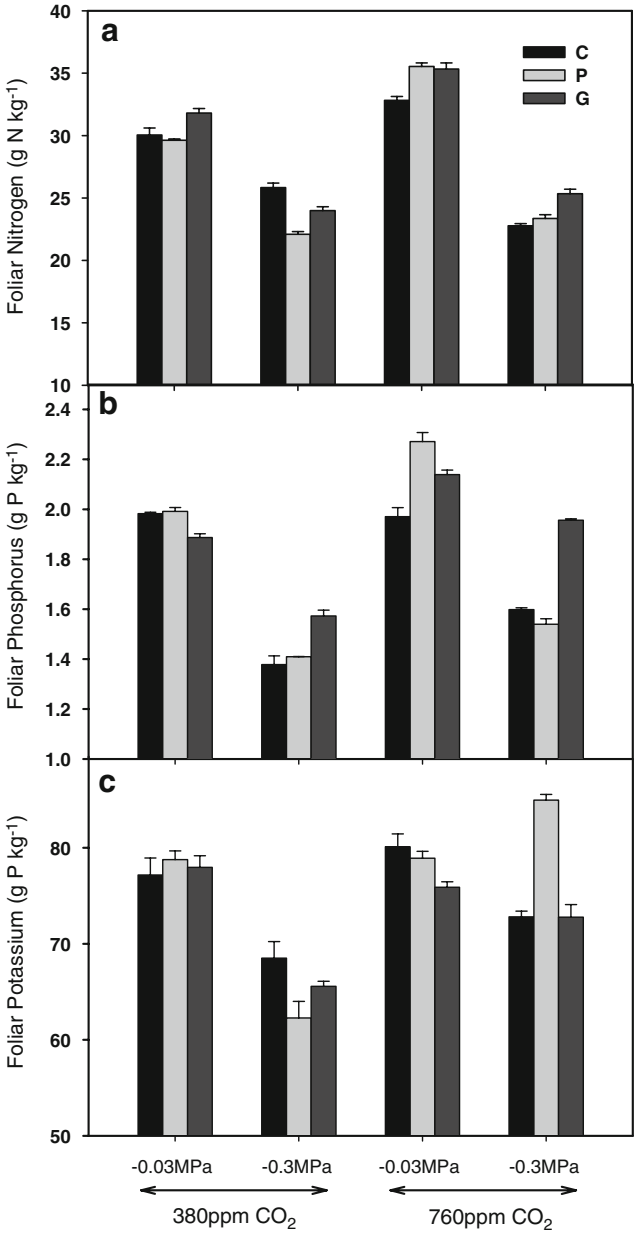

Figure 2 Effect of inoculation with *G. intraradices* and *P. mendocina* on foliar nitrogen (**a**), foliar phosphorus (**b**), and foliar potassium (**c**) of *L. sativa* seedlings grown at two levels of irrigation and two levels of CO_2 concentration after 9 weeks of plantation. *C* uninoculated plants; *P* plants inoculated with *P. mendocina*; *G* plants inoculated with *G. intraradices*. *Columns* represent mean ± SE (n=6)

icantly by *P. mendocina* (Fig. 2C), and a significant microbial inoculation × water stress × CO_2 level interaction was observed for this nutrient (Table 2).

PIP Gene Expression

In nonstressed plants at ambient CO_2, the *PIP2* gene expression was significantly higher in roots inoculated with *G. intraradices*, followed by *P. mendocina* (Fig. 3). The microbial inoculation had a significant effect on *PIP2* gene expression (Table 2). The water stress decreased significantly the gene expression in all treatments assayed. There was a significant interaction between CO_2 level, water stress, and microbial inoculation (Table 2). The eCO_2 concentration decreased the *PIP2* gene expression in both watering conditions; however, under such conditions the roots of the *G. intraradices*-inoculated lettuce plants showed the significantly highest expression of the *PIP2* gene (Fig. 3).

Discussion

Plant Growth and Physiology

Elevated CO_2 maintained the shoot biomass of lettuce plants, although the root biomass was decreased, this effect being more pronounced in *G. intraradices*-inoculated plants. According to Gavito et al. [12], at eCO_2, although the plant photosynthesis rate increases and a larger amount

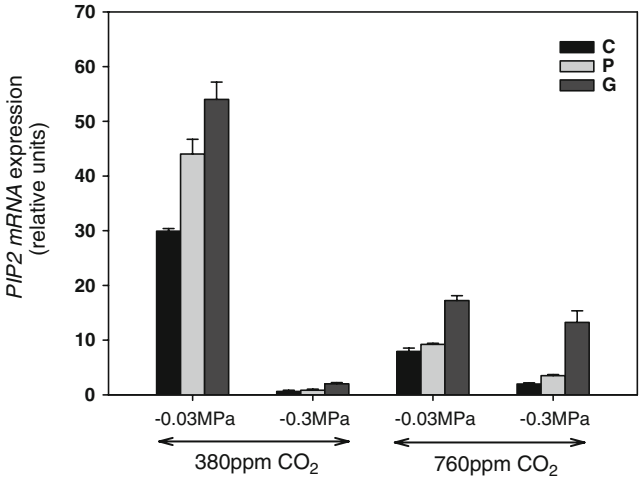

Figure 3 Effect of inoculation with *G. intraradices* and *P. mendocina* on mRNA levels of the *PIP2* gene determined by quantitative real-time PCR in *L. sativa* roots grown at two levels of irrigation and two levels of CO_2 concentration after 9 weeks of plantation. *C* uninoculated plants; *P* plants inoculated with *P. mendocina*; *G* plants inoculated with *G. intraradices*. *Columns* represent mean ± SE (n=6)

of photosynthates could be sent below-ground, the additional C might not be sufficient to overcome an increased nutrient demand in both noninoculated and inoculated plants; therefore, shoot C could be used at the expense of root development. Moreover, the root biomass was also lower in *G. intraradices*-inoculated plants, possibly because shoot C was partitioned to the mycorrhizal fungi. Under such conditions, where nutrient demand was expected to be higher than under ambient CO_2, the inoculated plants of both treatments showed enhanced nitrogen and phosphorous uptake. In this sense, *P. mendocina* was more effective than *G. intraradices* with respect to increasing the P concentration and the growth of the plants. *P. mendocina* is a PGPR bacterium that can facilitate the growth of plants via several mechanisms [50]. Between them, the PGPR can increase the availability and the uptake of mineral nutrients by the plant in the rhizosphere, particularly of less-mobile nutrients like phosphorus. In addition, the CO_2 enhancement could alter in some way the plant nutrient uptake and plant–bacteria interactions due to higher availability of photosynthates below-ground. This could explain the bacterial effect obtained at eCO_2.

The elevated CO_2 produced a stimulatory effect on plant growth under drought conditions. This agrees with several studies [47, 51], suggesting that greater water-use efficiency under eCO_2 helps to alleviate the deleterious effects on plant growth that water stress produces [51, 52]. In fact, in our study, plant growth was correlated positively with percentage shoot water content (r=0.529; P<0.01). Moreover, we observed a significant interaction between elevated CO_2 and drought regarding the effect on shoot water content (Table 2). Under such conditions, the plants inoculated with *P. mendocina* showed higher shoot and root biomass than control plants and those inoculated with *G. intraradices*. In addition, a positive interaction between these two factors with respect to root biomass was found (Table 2). This effect could be due to an increase in carbon supply at the source, resulting in an increase in the translocation of carbon to developing sinks below-ground [51]. The root system of *P. mendocina*-inoculated plants grown under elevated CO_2 and drought, therefore, had an enhanced capacity to extract available soil water and nutrients. In fact, plants inoculated with *P. mendocina* had the highest leaf RWC. Moreover, we found that inoculation with *P. mendocina* increased significantly the K uptake in plants grown under drought stress and eCO_2 conditions, with respect to noninoculated and *G. intraradices*-inoculated plants. Potassium has been shown to be the cationic solute responsible for stomatal movement in response to changes in bulk leaf water status [40]. Also, in our study, the K concentration was correlated with the stomatal conductance (r=0.233; P<0.05). It is evident that *P. mendocina* was more effective than *G. intraradices* with respect to protecting the lettuce plants against the unfavorable effects of drought at eCO_2.

It is interesting to emphasize that some PGPR can enhance the relationship between the host plant and beneficial rhizospheric fungi, such as AM fungi, increasing plant biomass, and nutrient contents [37]. In our study, the PGPR strain used had a stimulatory effect, increasing the natural colonization by native endophytes in the plants under drought stress at eCO_2. Therefore, the bacteria could be acting in concert with the native AM fungi to improve growth of the host plants under such conditions.

The results of our experiments show that there was no effect of eCO_2 on colonization of roots by AM fungi. This lack of effect has been found also by other authors [9, 13, 47, 48]. However, a positive interaction between eCO_2 and drought stress regarding the colonization of the roots by AM fungi was observed in our study, the plants inoculated with *G. intraradices* being the most effective at increasing the colonization percentage (Tables 1, 2). Increased CO_2 assimilation has been considered a plant strategy for drought stress tolerance [10]. This could be due to the fact that elevated CO_2 increased the supply of fixed C to roots, thus, increasing C availability to the fungus and promoting its growth [9]. As a consequence, services performed by the fungus might be enhanced, the best characterized of which is the provision of P to the plant, as happened in our study. In fact, the effects of AM fungi on plant water status have been associated with improved host nutrition, particularly P nutrition [14, 40]. Also, the increase found in the leaf RWC of the *G. intraradices*-inoculated plants may be a consequence of improved water use efficiency, resulting from a

relative increase in intracellular CO_2 and partial stomatal closure [7, 42] to alleviate the drought conditions.

Gene Expression

PIP aquaporins play an essential role in adjusting the root hydraulic conductivity of plants [20, 53]. Previous studies have shown that aquaporins are also involved in plant adaptations to drought stress conditions [30]. However, the connection between the expression of aquaporins and plant responses to water stress remains unclear because of the different isoforms and the complexity in the regulatory mechanisms that aquaporins display. Several studies have shown the upward or downward regulation patterns produced by osmotic stresses with respect to mRNAs encoding the homologs of aquaporins in the roots of many plant species [21]. For example, the overexpression of a *PIP1* gene increased the drought tolerance of rice and tobacco plants [27, 54]; however, Aharon et al. [1] found that overexpression of a different *PIP1* gene had a negative effect on tobacco plants during drought stress, producing wilting.

In our study, *PIP2* gene expression was decreased by drought stress in both noninoculated and inoculated plants under both atmospheric CO_2 and eCO_2, as well as in well-watered plants at eCO_2. This response of the lettuce plants may have been due to downregulation of aquaporin transcript level as the plants attempted to avoid excessive water loss during periods of dehydration by decreasing membrane water permeability to conserve cellular water [45, 53]. Hence, the decreased expression of the *PIP2* gene experienced by the plants under elevated CO_2 may be a regulatory mechanism to limit the water lost from the cells [4]. In fact, in support of this hypothesis, the plants grown under elevated CO_2 had significantly higher shoot water contents than plants grown at atmospheric CO_2 (Fig. 1A, Table 2).

On the other hand, the mycorrhizal inoculation by itself increased expression of the *PIP2* gene for both levels of watering and at both CO_2 levels. The important role of the AM symbiosis in helping plants to survive in unfavorable conditions has been well documented [2]. Mycorrhizal plants show greater resistance to environmental stress conditions than nonmycorrhizal plants. Since AM fungi stimulate water uptake by plants, the water being transferred by the AM fungi to the roots of the host plants, it is expected that the plants will display greater membrane water permeability in order to facilitate the water transport. In this case, the expression of aquaporin genes is induced to allow a higher rate of transcellular water flow [4, 19, 21]. As can be seen in our data, the increased expression of aquaporins in roots inoculated with *G. intraradices* can be interpreted as a plant protection mechanism that helps them to survive under drought stress and/or elevated CO_2 conditions. In fact, the leaf relative water content was significantly higher in plants inoculated with the AM inoculum under drought stress, at both levels of CO_2, than in noninoculated control plants, indicating that the AMF exerts a control on the expression of this gene under such conditions. In accordance with our results, Krajinski et al. [25] observed AMF-enhanced expression of another aquaporin in roots of *Medicago trunculata*. By contrast, Porcel et al. [36] observed a downregulation of aquaporin genes in roots of AM soybean and lettuce plants under drought stress compared to non-AM plants. A similar result was obtained by Aroca et al. [3] in their study of the expression of PIP genes in roots of AM bean. Also, Ouziad et al. [34] observed a decreased expression of PIP genes in the roots of AM tomato plants grown under salt stress. However, no CO_2 treatments were included in any of these experiments.

The surprising result obtained in this study was that the inoculation with *P. mendocina* increased the expression of the *PIP2* gene only in plants grown under well-watered conditions without elevated CO_2. Porcel et al. [36] found that soybean plants inoculated with *Bradyrhizobium japonicum* had reduced expression of a PIP gene, also under well-watered conditions. Although the functional diversity and the mechanism of action differ greatly among different kinds of bacteria, and up to now the influence of bacteria on PIP gene expression has not been demonstrated, the data presented here could mean that bacteria can influence the regulation of the PIP gene expression under normal conditions also. Moreover, aquaporins not only facilitate passive water movement across biological membranes but also low molecular-weight gases (ammonia or CO_2) and neutral molecules (glycerol or urea) can pass through them [29]. Also, in zones of the plant where there is fast cell division and expansion or where the flow of solutes or water would be higher, such as biotrophic interfaces between plants and bacteria or fungi, aquaporin activity is increased [49]. Taken all together, the induction or inhibition of aquaporin genes by fungi or bacteria under different osmotic stresses could improve the regulation of plant water status, contributing to plant resistance under stressful environmental conditions.

In conclusion, the results obtained in this study show that both AMF and PGPR can help to alleviate drought stress imposed on lettuce plants at elevated CO_2. The inoculation with PGPR favored plant growth to a greater extent than the AMF for all assayed treatments. *G. intraradices* was more effective than *P. mendocina* or native AM fungi for stimulating the expression of the PIP gene studied under drought and elevated CO_2 conditions. This may be a protection mechanism to achieve cellular water conservation. However, the presence of *P. mendocina* had no effect on PIP gene expression under elevated CO_2

and/or drought stress. Therefore, it can be concluded that under elevated CO_2 and drought, only the AMF is able to modulate a *PIP2* gene expression while *P. mendocina* does not seem to have any effect. However, as PIPs are members of a multigene family, it is possible that other different PIP genes may be regulated differently.

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