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Water stress responses of two Mediterranean tree species influenced by native soil microorganisms and inoculation with a plant growth promoting rhizobacterium

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Summary Soil microorganisms, such as plant growth-promoting rhizobacteria (PGPR), play crucial roles in plant growth, but their influence on plant water relations remains poorly explored. We studied the effects of native soil microorganisms and inoculation with the PGPR strain Aur6 of *Pseudomonas fluorescens* on water stress responses of seedlings of the drought-avoiding *Pinus halepensis* Mill. and the drought-tolerant *Quercus coccifera* L. Plant growth, nutrient concentrations and physiology (maximum photochemical efficiency of photosystem II (PSII; F_v/F_m), electron transport rate (ETR), stomatal conductance (g_s) and predawn shoot water potential (Ψ_{PD})) were measured in well-watered plants, and in plants under moderate or severe water stress. Inoculation with PGPR and native soil microorganisms improved tree growth, and their interactions had either additive or synergistic effects. Both F_v/F_m and ETR were significantly affected by PGPR and native soil microorganisms. Marked differences in g_s and Ψ_{PD} were found between species, confirming that they differ in mechanisms of response to water stress. A complex tree species × treatment interactive response to drought was observed. In *P. halepensis*, F_v/F_m and ETR were enhanced by PGPR and native soil microorganisms under well-watered conditions, but the effects of PGPR on Ψ_{PD} and g_s were negative during a period of water stress. In *Q. coccifera*, F_v/F_m and ETR were unaffected or even reduced by inoculation under well-watered conditions, whereas Ψ_{PD} and g_s were increased by PGPR during a period of water stress. Our results indicate that microbial associates of roots can significantly influence the response of tree seedlings to drought, but the magnitude and sign of this effect seems to depend on the water-use strategy of the species.

Keywords: Pinus halepensis, plant growth promoting rhizobacteria, Quercus coccifera, rhizosphere microorganisms, water deficit.

Introduction

The influences of abiotic factors on plant ecophysiology have received sustained attention, whereas the effects of biotic factors and species interactions have been less studied, although interest in the topic has recently increased (Valladares et al. 2007). There is ample evidence that soil microorganisms influence plant growth, nutrient uptake and overall performance, although their effects on other plant properties, including water relations and responses to drought, have been less explored (Grayston et al. 1996, Barea et al. 2005). Different microbial types (mainly symbiotic and saprophytic bacteria and fungi) cohabit and compete for root colonization sites and for exudates in the rhizosphere, and directly affect the development of plants (Walker et al. 2003, Barea et al. 2005, Brimecombe et al. 2007). Because of their dependence on root exudates, the composition and functioning of rhizospheric microbial communities are directly affected by the same environmental factors that influence the physiology of plants (Brimecombe et al. 2007).

In semi-arid Mediterranean ecosystems, water and nutrient availabilities are the main constraints on the productivity of plants and the functional diversity of their root-associated microflora (Valladares et al. 2005, Bréda et al. 2006, Marulanda et al. 2006). Plants in semi-arid environments have developed a range of physiological mechanisms to cope with drought, including morphological adaptations, tight stomatal control, osmotic adjustment and photoprotective mechanisms for energy dissipation (Yordanov et al. 2000, Valladares and Pearcy 2002, Martínez-Ferri et al. 2004). Co-occurring Mediterranean tree species often show different water-use strategies in response to drought (mainly avoidance or tolerance, Martínez-Ferri et al. 2000). Drought-avoiding species prevent damage by closing their stomata before a sharp decline in leaf water potential occurs, whereas drought-tolerant species show simultaneous decreases in stomatal conductance and leaf water potential (Guehl et al. 1991, Martínez-Ferri et al. 2000, Baquedano and Castillo 2006). Biotic factors, such as the association of plants with symbiotic soil microorganisms, mainly mycorrhiza-forming fungi and nitrogen-fixing bacteria, can alleviate drought stress (Augé 2001, Morte et al. 2001, Aranjuelo et al. 2005). However, few studies have focused on the effects of saprophytic, free-living rhizospheric microorganisms on the amelioration of water stress in plants (Grayston et al. 1996, Timmusk and Wagner 1999).

We compared effects of inoculation with a selected strain of a free-living, plant growth-promoting rhizobacterium (PGPR) with those of native soil microorganisms on the ecophysiological responses to water stress of two co-occurring Mediterranean tree species with contrasting water-use stratgies: the drought-avoiding *Pinus halepensis* Mill. and the drought-tolerant *Quercus coccifera* L. Among free-living bacteria, PGPR are known to participate in many important ecosystem processes such as the biological control of pathogens, nutrient cycling and plant growth mainly by mobilizing soil nutrients and producing antibiotics and phytohormones (Badaluco and Nannipieri 2007, Lugtenberg and Leveau 2007, Van Loon 2007). Inoculation of nursery planting stock with selected PGPR can affect the physiological state of tree seedlings (Chanway 1997, Lucas-García et al. 2004, Enebak 2005). We hypothesized: (1) that inoculation with a PGPR improves the growth of *P. halepensis* and *Q. coccifera*; and (2) that native soil microorganisms enhance seedling development and interact with inoculated PGPR. Inoculation with PGPR or native soil microorganisms can improve field survival and growth of planted trees in semi-arid Mediterranean areas (Zaady and Perevolotsky 1995, Roldán et al. 1996, Medina et al. 2003). Therefore, we tested the hypothesis that inoculated PGPR and native soil microorganisms affect the physiological responses of seedlings to severe water stress.

Materials and methods

Plant material and seedling culture

Seeds of *Pinus halepensis* Mill. provenance La Mancha (Spain) and *Quercus coccifera* L. provenance Serranía de Cuenca (Spain) were imbibed in water overnight, surface disinfected by shaking for 20 min in 30% (v/v) H_2O_2 and washed in five changes of distilled water. Seeds were germinated in 330-ml containers (two seeds per container) filled with a 2:2:1 (v/v) mixture of soil:vermiculite:perlite. The soil was obtained from a *Q. coccifera* forest at Chinchón (Madrid, Spain). Soil was homogenized, sieved (2 mm) and, depending on treatment, autoclaved or not at 120 °C for 30 min. Soil characteristics, which were unaffected by autoclaving, were as follows: pH (1:20, H_2O), 7.8 ± 0.1, total N (%), 0.12 ± 0.01, organic matter (%), 2.9 ± 0.2; electrical conductivity (mS cm^{-1}), 2.87 ± 0.07; total CO_3 (%), 25 ± 2.5; bio-available P_2O_5 (mg $(100\ g)^{-1}$), 8.5 ± 0.3; K (mg $(100\ g)^{-1}$), 22.3 ± 2.2; Ca (mg $(100\ g)^{-1}$), 3045 ± 146.2; and Mg (mg $(100\ g)^{-1}$), 68 ± 1.6. The vermiculite and perlite medium was autoclaved (120 °C, 20 min) for all treatments before mixing with soil. Seedlings were thinned to one per container after germination. No additional nutrients were supplied. Seedlings were raised in a greenhouse in a 16-h photoperiod (minimum 200 µmol s^{-1} m^{-2}). Greenhouse temperature oscillated between 18 and 25°C and relative humidity was close to 40%.

Bacterial inoculum

The PGPR bacterial strain Aur6 of *Pseudomonas fluorescens* was originally isolated from the rhizosphere of *Lupinus hispanicus* Boiss. et Reut. (Gutiérrez-Mañero et al. 2003) and identified by FAMEs (Microbial ID Inc. Newark, NJ). To obtain the bacterial inoculum, a single colony was transferred to 3 ml of Luria-Bertani (LB) medium and incubated at 28 °C with shaking at 200 rpm overnight. This pre-inoculum was diluted with fresh liquid LB medium (1:20, v/v) and incubated at 28 °C and 200 rpm. Bacteria were collected by centrifugation (10,000 *g*, 10 min) and washed twice with sterile distilled water. Bacteria were suspended in PBS buffer (140 mM NaCl, 2.6 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4), to achieve a final concentration of 10^8 cfu ml^{-1}.

Experimental design

For each tree species, the following factors were analyzed: (1) inoculated (I+) and uninoculated (I–) with PGPR; (2) with (S+) or without (S–) native soil microorganisms; and (3) with zero (T0), moderate (T1) or severe (T2) water stress.

One month after germination, I+ seedlings were inoculated with *P. fluorescens* Aur6 by adding 10 ml of inoculum per seedling (final dose 10^9 $seedling^{-1}$). Bacterial inoculation was repeated twice in a month. Uninoculated control (I–) seedlings received the same volume of PBS buffer without bacteria. Native microorganisms were eliminated from the soil by autoclaving (S–). After five months of seedling growth, water stress treatments were applied to all four inoculation treatments (I+S–, I–S–, I+S+ and I–S+). A third of the seedlings in each treatment were kept well watered (T0) by irrigating to field capacity every 3 days. Water was withheld from the remaining seedlings in each of the treatments for either 10 (T1) or 24 days (T2). The water stress treatments mimicked the timing, duration and severity of drought typically experienced by woody seedlings in the field as reported by Valladares et al. (2005). Greenhouse environment simulated late-summer Mediterranean conditions with 40% humidity and air temperature of 25–34 °C. Water loss of the T1 and T2 seedlings was controlled by weighing the containers every 3 days (Figure 1). Based on water loss per container, we calculated the volume of water to be added to maintain a severe but not lethal water stress level (Figure 1).

Seedling growth and nutrient concentration in shoots

Before the start of the water-stress treatment, seedling height was measured, and a sample of 15 seedlings per species and treatment was harvested, weighed and dried at 60 °C for 2 days to determine fresh and dry shoot biomass. Dry shoots were ground to powder, and a subsample used to determine N concentration by the Kjeldahl method. The remainder of the ground tissue sample was digested with 5:3 (v/v) nitric acid:perchloric acid and the concentrations of P, K, Mg and Mn in the digest were determined by inductively coupled plasma (ICP) spectrometry (Optima 43000DV, Perkin-Elmer).

Physiological measurements

All physiological variables were recorded in well-watered seedlings (T0) and in seedlings subjected to water stress for 10 (T1) or 24 (T2) days.

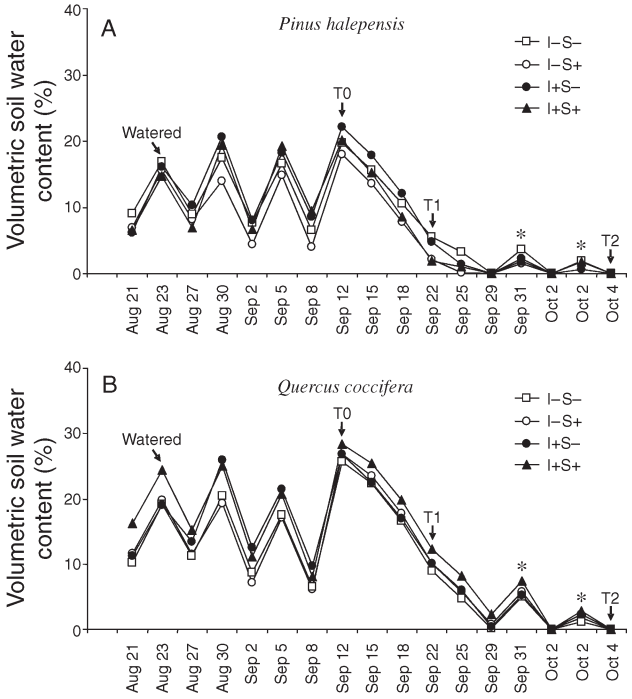

Figure 1. Time course of mean volumetric soil water content in containers with (A) *Pinus halepensis* and (B) *Quercus coccifera* seedlings during the water stress experiment. Treatments: I– and I+ = uninoculated and inoculated with the PGPR *Pseudomonas fluorescens* Aur6, respectively; S– and S+ = without and with native soil microorganisms, respectively; T0 = beginning of water withholding; and T1 and T2 = 10th and 24th day without irrigation, respectively. Asterisks (*) indicate additional watering to prevent seedling death (5 and 6 ml per container for *P. halepensis* and *Q. coccifera* respectively).

Predawn shoot water potentials (Ψ_{PD}) of 15 seedlings per species and treatment were determined with a Scholander pressure chamber (Scholander et al. 1965). Water loss from the excised shoots was prevented by enclosing the seedlings in a plastic bag and humidifying the pressure chamber with a wet paper towel before excision (Valladares and Pearcy 2002).

Stomatal conductance to water vapor (g_s) was measured with a portable steady-state porometer (Li-1600, Li-Cor, Lincoln, NE). Chlorophyll fluorescence emission was measured with a portable pulse-modulated fluorometer (PAM-2000, Walz, Effeltrich, Germany). Leaves were held in the leaf clip holder of the fluorimeter for 1 h and then minimal fluorescence (F_o) was measured. After F_o determination, maximal fluorescence yield (F_m) of the dark-adapted leaves was recorded after exposing leaves (photosystem II (PSII)) to a saturating pulse of white light (800 ms at about 6000 µmol photons m^{-2} s^{-1}). Values of F_o and F_m were used to calculate maximal photochemical efficiency of PSII (F_v/F_m), where F_v is variable fluorescence ($F_v = F_m - F_o$). Apparent electron transport rate through PSII (ETR) was estimated as ETR = $0.5(F_v'/F_m')$(PPF)0.84, where F_v' is ($F_m' - F_s$), F_m' is maximal fluorescence of light-adapted leaves, F_s is the steady state fluorescence yield in ambient light, and assuming a leaf absorbance of 0.84 and an equal distribution of excitation between PSI and PSII (Valladares and Pearcy 2002). Stomatal conductance, F_v/F_m and ETR were measured on the same 15 seedlings per species and treatment throughout the water-stress experiment.

Statistical analysis

Data were analyzed by two-way ANOVA taking into account the factors (1) inoculation with PGPR (I+ versus I–) and (2) presence of native soil microorganisms (S+ versus S–) (see Table 1). Data from the water-stress experiment were analyzed by three-factor ANOVA taking into account the additional factor water stress (see Table 2). Before ANOVA, data were square-root or log transformed to satisfy the normality and homocedasticity assumptions (tested by Kolmogorov-Smirnov and Levene tests, respectively). When interactions among factors were detected, one-way ANOVA was performed separately for each factor. The relationship between leaf water potential and stomatal conductance was assessed by Pearson's correlation analysis.

Results

Responses to inoculation with PGPR and the presence of native soil microorganisms

In both species, PGPR inoculation had significant effects on height, P and K concentrations, F_v/F_m and ETR (Table 1). Additionally, PGPR inoculation significantly affected fresh mass and Mg concentration in *P. halepensis* and dry mass in *Q. coccifera*. Soil microorganisms significantly affected almost all measured variables in *P. halepensis*, whereas they significantly affected only height, Mn concentration and ETR in *Q. coccifera*. Neither Ψ_{PD} nor g_s was affected by inoculation and soil microorganisms. In both species, height, dry mass, N concentration and ETR showed interactions between PGPR inoculation and soil microorganisms (Table 1). For *P. halepensis*, interactions were also found for fresh mass, Mg concentration and F_v/F_m (Table 1).

Seedling growth and photosynthetic capacity In the absence of soil microorganisms (autoclaved soil) and for both tree species, PGPR inoculation significantly increased height and dry mass by over 37 and 50%, respectively (Figures 2A–D). In *P. halepensis* seedlings, this growth stimulus was suppressed by the presence of soil microorganisms, whereas in *Q. coccifera* seedlings, only the stimulation of height growth by PGPR inoculation was maintained in the presence of soil microorganisms (Figures 2A–D). In seedlings uninoculated with PGPR, the presence of native soil microorganisms significantly increased height and dry mass by about 50 and 60%, respectively, in *P. halepensis* and by 20 and 40% in *Q. coccifera* (Figures 2A–D).

In autoclaved soil, PGPR inoculation significantly increased F_v/F_m and ETR in *P. halepensis*, whereas it had no effect on F_v/F_m and caused a significant decrease in ETR in *Q. coccifera* (Figures 2E–H). For *P. halepensis*, the significant positive effect of PGPR inoculation on photosynthetic capacity was maintained in the presence of soil microorganisms

Table 1. Results of two-way ANOVA for morphological and physiological variables of *Pinus halepensis* and *Quercus coccifera*, according to the factors inoculation with the PGPR, *Pseudomonas fluorescens* Aur6 (I), and the presence of native soil microorganisms (S). For each factor, the proportion of the explained variance (*F*-values), the interaction between factors and the significance level (*; $P < 0.05$) are indicated; R^2 = proportion of total variance accounted for by the model. Abbreviations: N = nitrogen; P = phosphorus; K = potassium; Mg = magnesium; Mn = manganese; Ψ_{PD} = predawn shoot water potential; g_s = stomatal conductance; F_v/F_m = maximal photochemical efficiency of PSII; and ETR = apparent electron transport rate through PSII.

	I	S	I × S	R^2
Pinus halepensis				
Height	59.5 *	192.1 *	71.3 *	81.3
Fresh mass	42.1 *	88.5 *	65.4 *	75.4
Dry mass	3.5	22.7 *	24.9 *	45.0
N	2.3	22.6 *	10.6 *	64.5
P	130.8 *	36.2 *	1.8	90.6
K	39.1 *	166.4 *	1.0	92.3
Mg	23.5 *	40.1 *	41.3 *	83.5
Mn	0.5	210.2 *	1.5	92.1
Ψ_{PD}	4.2	0.24	6.7	12.0
g_s	1.2	0.08	0.3	0.2
F_v/F_m	50.0 *	67.2 *	4.4 *	67.5
ETR	152.7 *	73.5 *	34.4 *	81.7
Quercus coccifera				
Height	4.4 *	5.5 *	0.8	0.1
Fresh mass	0.4	0.7	1.8	1.4
Dry mass	14.9 *	3.0	11.1 *	30.6
N	2.5	0.1	6.2 *	16.7
P	66.1 *	0.8	0.6	71.7
K	5.6 *	2.2	0.9	20.1
Mg	1.5	0.1	0.1	5.3
Mn	3.1	304.9 *	1.1	93.0
Ψ_{PD}	0.3	0.7	3.1	0.06
g_s	1.4	3.3	2.5	0.06
F_v/F_m	8.9 *	0.07	1.0	0.1
ETR	37.7 *	9.0 *	6.5 *	46.0

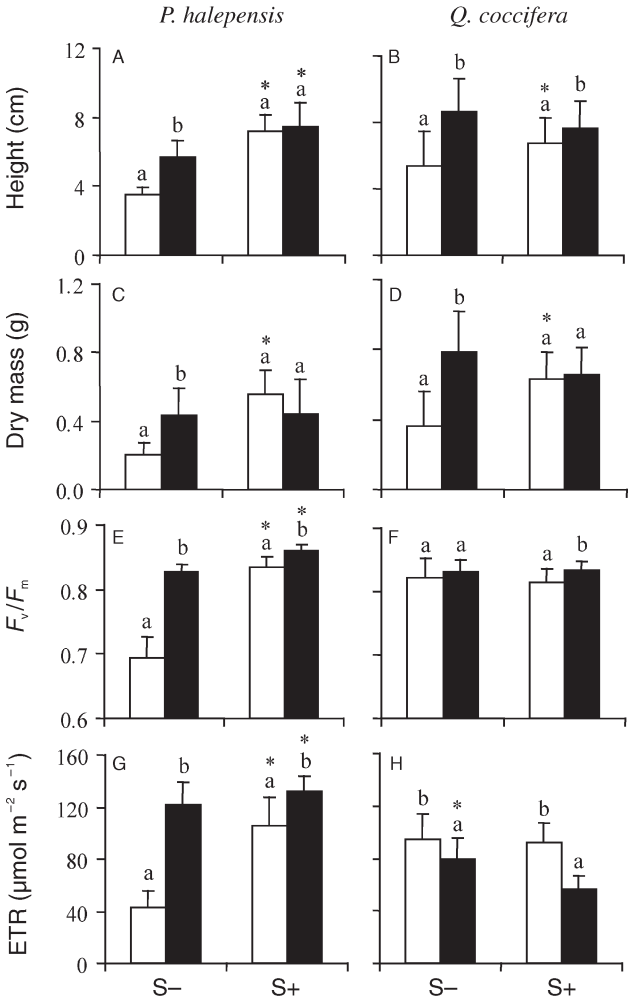

Figure 2. Effects of inoculation with the plant growth-promoting rhizobacterium (PGPR), *Pseudomonas fluorescens* Aur6 (filled bars versus open bars), and the presence of native soil microorganisms (S+ versus S–) on plant height, dry mass, photochemical efficiency of PSII (F_v/F_m) and apparent electron transport rate (ETR) of *Pinus halepensis* and *Quercus coccifera* seedlings. The effect of each factor was evaluated by one-way ANOVA. Different letters within the same native soil microorganisms treatment denote significant differences between PGPR inoculation treatment, whereas asterisks denote significant differences between treatments with (S+) or without (S–) native soil microorganisms for the same PGPR inoculation treatment ($P < 0.05$). Values are means + SD.

(Figures 2E and 2G). For inoculated *Q. coccifera* grown in the presence of soil microorganisms, F_v/F_m increased slightly but significantly and ETR decreased compared with uninoculated seedlings (Figures 2F and 2H). Independently of PGPR inoculation, *P. halepensis* showed significantly higher F_v/F_m and ETR when grown in the presence of soil microorganisms compared with autoclaved soil (Figures 2E and 2G). In *Q. coccifera* seedlings uninoculated with PGPR, the presence of soil microorganisms had no effect on F_v/F_m and ETR, although ETR was significantly decreased when inoculated seedlings were grown in presence of soil microorganisms (Figures 2F and 2H). Neither Ψ_{PD} nor g_s of either species was significantly affected by PGPR inoculation or the presence of soil microorganisms (Figures 3G–J).

Nutrient concentration In both tree species, PGPR inoculation did not increase shoot N concentration in either of the native soil microorganism treatments (Figures 3C and 3D). For *P. halepensis*, Mg concentration was significantly increased by PGPR inoculation only in seedlings grown in autoclaved soil, whereas neither PGPR inoculation nor the presence of soil microorganisms affected Mg concentration in *Q. coccifera* seedlings (Figures 3E and 3F). Inoculation with PGPR significantly improved P concentration in both tree species (Figures 4A and 4B). The presence of native soil microorganisms significantly increased this effect in *P. halepensis* but not in *Q. coccifera* (Figures 4A and 4B). Similarly, a significant effect of PGPR inoculation was observed for K concentration in *P. halepensis*, whereas K concentration was significantly in-

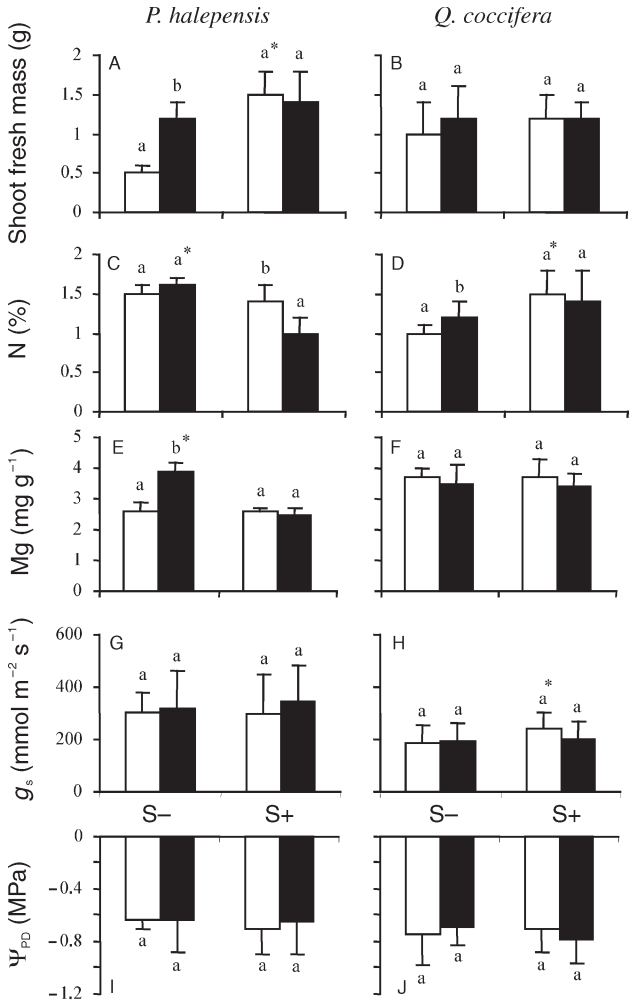

Figure 3. Effects of inoculation with the plant growth-promoting rhizobacterium (PGPR), *Pseudomonas fluorescens* Aur6 (filled bars versus open bars), and the presence of native soil microorganisms (S+ versus S–) on shoot fresh mass, nitrogen (N) and magnesium (Mg) concentrations, stomatal conductance (g_s) and predawn shoot water potential (Ψ_{PD}) of *Pinus halepensis* and *Quercus coccifera* seedlings. Each factor was separately analyzed by one-way ANOVA. Different letters within the same S treatment denote significant differences between PGPR inoculation treatments, whereas asterisks denote significant differences between treatments with (S+) or without (S–) native soil microorganisms for the same PGPR inoculation treatment ($P < 0.05$). Data are means + SD.

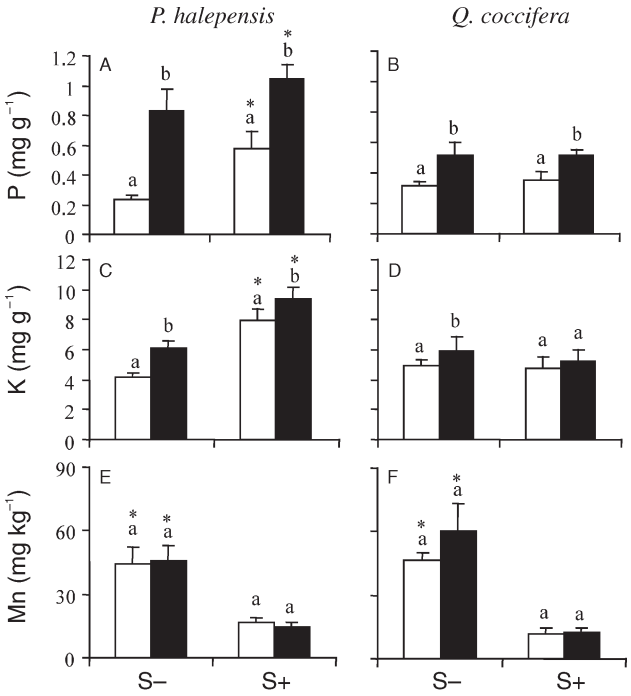

Figure 4. Effects of inoculation with the plant growth-promoting rhizobacterium, *Pseudomonas fluorescens* Aur6 (filled bars versus open bars), and the presence of native soil microorganisms on nutrient concentrations of *Pinus halepensis* and *Quercus coccifera* seedlings. Factors were separately analyzed by one-way ANOVA. Different letters within the same treatment of native soil microorganisms denote significant differences between inoculation treatments, whereas asterisks denote significant differences between treatments with (S+) or without (S–) native soil microorganisms for the same PGPR inoculation treatment ($P < 0.05$). Data are means + SD.

creased by PGPR inoculation only in *Q. coccifera* seedlings grown in autoclaved soil (Figures 4C and 4D). In *P. halepensis*, K concentration was significantly higher in seedlings growing in the presence of soil microorganisms compared with seedlings in autoclaved soil, whereas there was no significant effect of soil microorganisms on K concentration in *Q. coccifera* (Figures 4C and 4D). For both tree species, inoculation with PGPR had no effect on Mn concentration, whereas the presence of soil microorganisms significantly reduced Mn concentration in both inoculated and uninoculated seedlings (Figures 4E and 4F).

Responses to water stress

For *P. halepensis*, PGPR inoculation, soil microorganisms and water stress had significant effects on almost all variables tested (Table 2). For *Q. coccifera*, water stress significantly affected all variables, PGPR inoculation significantly affected Ψ_{PD} and g_s, but native soil microorganisms affected none of the variables tested. Interactions among factors were observed for all variables except F_v/F_m (Table 2).

Water stress and stomatal conductance Severe water stress (T2) significantly decreased the shoot Ψ_{PD} of inoculated *P. halepensis* seedlings compared with uninoculated seedlings in both the presence and absence of soil microorganism (Figure 5). In contrast, shoot Ψ_{PD} was lower in uninoculated *Q. coccifera* seedlings than in PGPR inoculated seedlings in both the presence and absence of soil microorganisms. In both tree species, the decrease in shoot Ψ_{PD} in PGPR inoculated seedlings was significantly higher in the presence of soil microorganisms than in their absence.

Stomatal conductance decreased progressively with increasing severity of water stress, and this effect was more pronounced in *P. halepensis* than in *Q. coccifera* (Figure 5). When well-watered, *P. halepensis* seedlings had higher g_s than

Table 2. Results of the three-way ANOVA for measured physiological variables of *Pinus halepensis* and *Quercus coccifera*, according to the factors inoculation with the PGPR *Pseudomonas fluorescens* Aur6 (I), presence of native soil microorganisms (S) and water stress (W). The proportion of the explained variance (*F* values) and the significance level (*, $P < 0.05$) are indicated for each factor and the interactions; R^2 = proportion of total variance absorbed by the model.

	I	S	W	I × S	I × W	S × W	I × S × W	R^2
Pinus halepensis								
Ψ_{PD}	163.2 *	21.3 *	392.3 *	3.0	118.2 *	11.6 *	9.9 *	89.1
g_s	12.6 *	1.8	249.1 *	0.9	29.0 *	1.7	5.5 *	78.0
F_m/F_v	221.6 *	294.2 *	31.2 *	159.1 *	2.9	5.2 *	24.8 *	83.2
ETR	135.6 *	53.9 *	237.0 *	152.9 *	24.8 *	20.6 *	0.3	83.3
Quercus coccifera								
Ψ_{PD}	11.3 *	0.1	156.4 *	3.2	15.3 *	0.8	2.6	68.0
g_s	6.7 *	0.05	34.6 *	3.5	13.0 *	2.5	9.0 *	43.0
F_m/F_v	2.0	1.2	18.6 *	0.4	0.5	0.8	0.02	15.9
ETR	1.3	0.02	51.3 *	2.8	15.5 *	5.1 *	8.1 *	46.4

Q. coccifera seedlings, although this pattern was reversed during water stress. In both species, the presence of soil microorganisms had no significant effect on g_s in seedlings in the severe water stress treatment. In response to severe water stress, *P. halepensis* seedlings inoculated with PGPR had significantly lower g_s than uninoculated seedlings, whereas the opposite was observed in *Q. coccifera* whether native soil microorganisms were present or not (Figure 5). For *P. halepensis*, a significant correlation between Ψ_{PD} and g_s was observed only in PGPR inoculated seedlings, whether in the presence or absence of soil microorganisms, whereas in *Q. coccifera*, the highest correlations between Ψ_{PD} and g_s was observed in uninoculated seedlings, whether in the presence or absence of soil microorganisms (Figure 6).

Photosynthetic capacity In both tree species, F_v/F_m and ETR progressively decreased with increasing water stress (Figure 5). Well-watered *P. halepensis* seedlings showed significantly higher F_v/F_m and ETR in response to PGPR inoculation and to the presence of native soil microorganisms (Figure 5). However, under severe water stress, the positive effects of PGPR inoculation on F_v/F_m and ETR were maintained only in

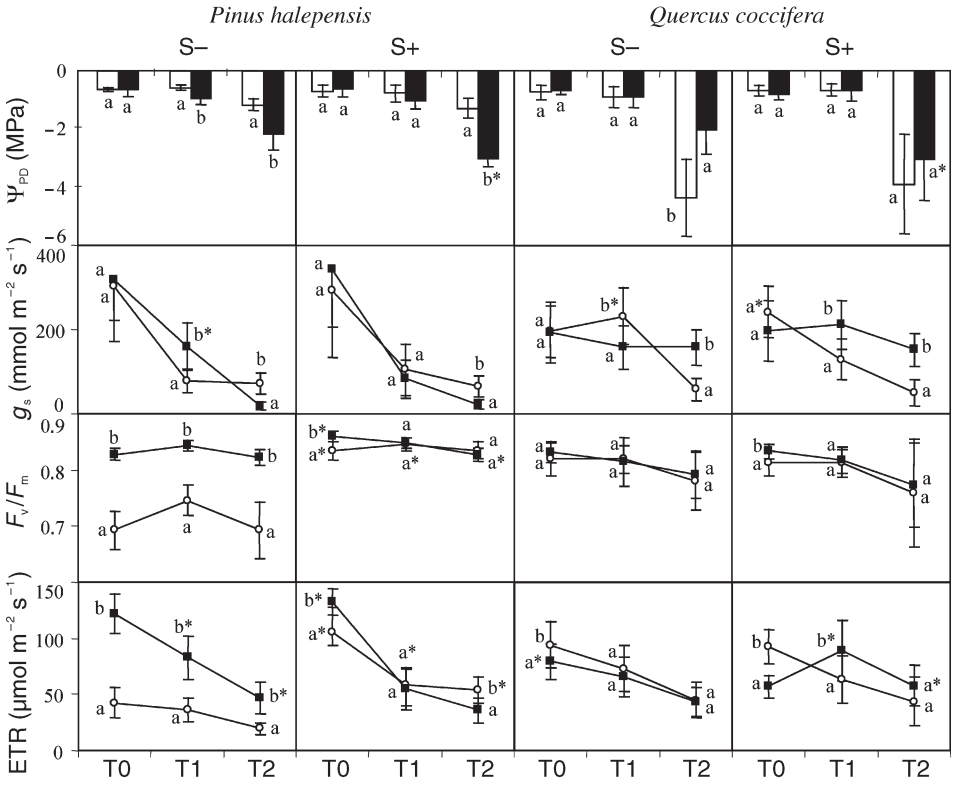

Figure 5. Effects of water stress, inoculation with the plant growth-promoting rhizobacterium (PGPR), *Pseudomonas fluorescens* Aur6 (filled bars versus open bars), and the presence (S+) or absence (S–) of native soil microorganisms on predawn shoot water potential (Ψ_{PD}), stomatal conductance (g_s), photochemical efficiency of PSII (F_v/F_m) and apparent electron transport rate (ETR) of *Pinus halepensis* and *Quercus coccifera*. At each time throughout the water stress treatment, different letters within the same S treatment denote significant differences between PGPR inoculation treatments, whereas asterisks denote significant differences between S treatments for the same PGPR inoculation treatment ($P < 0.05$). Data are means ± SD. Treatments: T0 = well-watered, and T1 and T2 = 10 and 24 days after water withholding, respectively. Symbols: ○ = control uninoculated treatment; and ■ = PGPR.

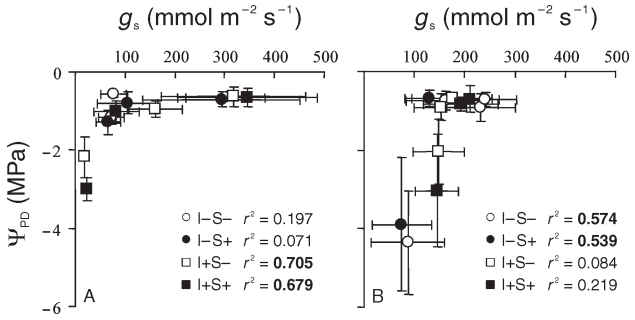

Figure 6. Correlation between predawn shoot water potential (Ψ_{PD}) and stomatal conductance (g_s) of (A) *Pinus halepensis* and (B) *Quercus coccifera* in the different combinations of PGPR inoculation with native soil microorganisms. Treatments: I– and I+ = uninoculated and inoculated with the PGPR *Pseudomonas fluorescens* Aur6, respectively; S– and S+ = without and with native soil microorganisms, respectively.

seedlings grown in autoclaved soil. In the presence of soil microorganisms, PGPR inoculation had no effect on F_v/F_m but significantly decreased ETR compared with uninoculated seedlings. Both F_v/F_m and ETR were highly dependent on the presence of native soil microorganisms and were greatly reduced by severe water stress in uninoculated *P. halepensis* seedlings grown in autoclaved soil compared with seedlings grown in the presence of soil microorganisms. In seedlings grown in autoclaved soil, the effect of PGPR inoculation was comparable with that of native soil microorganisms. The PGPR inoculated *P. halepensis* seedlings had similar F_v/F_m and significantly grater ETR when grown in autoclaved soil than when grown in the presence of native soil microorganisms (Figure 5).

In *Q. coccifera*, F_v/F_m was unaffected by either PGPR inoculation or native soil microorganisms and, as expected, F_v/F_m decreased with increasing plant water stress (Figure 5). Well-watered *Q. coccifera* seedlings without PGPR inoculation showed significant higher ETR than inoculated seedlings, both in the presence and absence of soil microorganisms. However, PGPR inoculated seedlings had a higher ETR than uninoculated seedlings when grown in the presence of soil microorganisms under conditions of severe water stress.

Discussion

Responses to inoculation with PGPR and to the presence of native soil microorganisms

Pinus halepensis and *Q. coccifera* seedlings inoculated with PGPR and grown in sterilized soil had similar growth rates to uninoculated seedlings grown in the presence of native soil microorganisms, highlighting the importance of root-microbial associates for early tree development. Although originally isolated from lupin, the PGPR strain Aur6 is highly efficient in promoting the growth of different forest tree species (Lucas-García et al. 2004, Rincón et al. 2005*a*). Similarly, other PGPR strains within the genera *Pseudomonas*, *Bacillus* and *Azospirillum* have been used successfully in the production of nursery conifer and oak seedlings (Zaady and Perevolotsky 1995, Shishido et al. 1996, Chanway et al. 2000, Lucas-García et al. 2004, Enebak 2005).

Interactions of the inoculated PGPR strain with native soil microorganisms were observed for many variables, especially in *P. halepensis*, probably reflecting the competitive relationships of rhizospheric microorganisms for root colonization sites and nutrient resources. However, niche overlap between an inoculant and native soil microorganisms is likely to be limited, and spatial separation and versatility in nutrient use are important aspects that can define the respective niches (Castro-Sowinski et al. 2007). In this sense, microbial interactions are not necessarily detrimental to the plant, and often have cooperative synergistic effects (Barea et al. 2005, Rincón et al. 2005*a*, Castro-Sowinski et al. 2007).

Although PGPR inoculation did not increase seedling growth to rates observed in inoculated seedlings grown in the presence of native soil microorganisms, a positive synergism between the factors was observed for K and P concentrations in *P. halepensis*. A significant increase in seedling K concentration could positively affect plant tissue hydration because K is an important solute involved in the regulation of cell turgor and stomatal opening (Benlloch-González et al. 2008). Many bacteria are able to solubilize inorganic soil P, usually by releasing chelating organic acids, which make P available to plants (Vessey 2003, Barea et al. 2005). Phosphorus concentrations were significantly increased in both *P. halepensis* and *Q. coccifera* by inoculation with the PGPR *Pseudomonas fluorescens*, suggesting that this nutrient is a limiting factor for tree growth. The effect is of particular relevance to afforestation on alkaline and calcareous soils where P availability may be severely limited (Marschner 1986). The restrictive role of P during the initial growth phases of *P. halepensis* and *Quercus* spp. has been indicated in field experiments performed in different Mediterranean areas (Leonardi and Rapp 1990, Roldán et al. 1996, Sardans et al. 2006).

In both species, tissue Mn concentrations were strongly reduced in the presence of native soil microorganisms, regardless of PGPR inoculation. The availability of Mn depends on oxidation–reduction processes driven by soil bacteria whose proliferation can be inhibited by mycorrhizal fungi (Grayston et al. 1996). Seedling mycorrhization, which was not investigated, may have been responsible for the low Mn concentrations observed in our study in the presence of native soil microorganisms.

Another synergistic effect of PGPR inoculation and native soil microorganisms was observed on the photosynthetic capacity of *P. halepensis*. However, the photosynthetic capacity of *Q. coccifera* was unaffected by native soil microorganisms and inoculation with PGPR decreased ETR. These opposing responses on nutrition and photosynthetic capacities may be related to divergent microbe-induced modifications of seedling rhizo-deposition. The tree-root-associated microbial community can determine the quantity and composition of root exudates and, in a similar manner, an introduced PGPR can trigger the roots to change the composition of root

exudates, thereby altering the structure and functioning of the native soil microbial population (Bent et al. 2001, Bertin et al. 2003, Marulanda et al. 2006, Lugtenberg and Leveau 2007).

Although the density of an introduced bacterium usually declines with time, the threshold bacterial concentration needed to promote plant performance can be low (Frey-Klett et al. 1997). We found that the development of *Q. coccifera* seedlings was less dependent on PGPR inoculation and the presence of native soil microorganisms than that of *P. halepensis* seedlings, probably because of the larger seed reserves of *Q. coccifera*, which are sufficient to support seedling development during the first year of growth.

Responses to water stress

Under severe water stress, the presence of native soil microorganisms did not affect either Ψ_{PD} or g_s. However, *P. halepensis* seedlings subjected to water stress and inoculated with PGPR showed a significant decrease in Ψ_{PD} and g_s, whereas the opposite effect was observed in water-stressed PGPR-inoculated *Q. coccifera* seedlings. Overall these results indicate that PGPR inoculation reinforced the respective hydric strategies of these species under conditions of severe water stress, although the mechanisms by which these effects were produced are uncertain. We suggest that the induced nutritional benefits of PGPR inoculation resulted in improved osmotic adjustment of seedlings. Non-nutritional hormonal effects could also be involved because certain bacteria can produce abscisic acid (ABA), a phytohormone directly related to plant responses to water stress (Boiero et al. 2007, Perrig et al. 2007). Although the physiological role of ABA in plant–microbe interactions is unclear, it has been proposed that beneficial microorganisms regulate plant ABA homeostasis helping to alleviate water stress (Augé 2001, Rincón et al. 2005*b*, Boiero et al. 2007). Alternatively, PGPR inoculation may have altered root morphology through the production of auxin (IAA) (Gutiérrez-Mañero et al. 2003), a phytohormone that induces lateral root formation and root branching. Branch root junctions are structures that may facilitate radial transport of water, increasing the root hydraulic conductivity of seedlings (Kothari et al. 1990): further experiments are needed to test this hypothesis.

Under conditions of water stress, ETR decreased in *P. halepensis* seedlings inoculated with PGPR and grown in the presence of native soil microorganisms, whereas there was a positive synergistic effect of inoculation and presence of soil microorganisms on ETR in water-stressed *Q. coccifera* seedlings. These results suggest that for interactions among the effects of the inoculated and native soil microorganisms under water-stress were dependant on the hydric strategy of each tree species.

Our results provide insight into the complexity of microbial interactions within the rhizosphere of juvenile trees, and indicate that root-associated microorganisms not only influence the physiological status of trees but can mediate their response to water stress. The major finding of our study concerning free-living PGPR inoculants were that: (1) *Pseudomonas fluorescens* strain Aur6 can reinforce the hydric strategy of its associated tree species, and (2) this can improve the drought adaptation of drought-tolerant *Q. coccifera* but not of drought-avoiding *P. halepensis*. We conclude that use of the *Pseudomonas fluorescens* strain Aur6 can improve tree performance in the nursery, although its effects in the field, where multiple environmental factors could substantially modify its influence, remain to be determined.

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References

Aranjuelo, I., P. Pérez, L. Hernández, J.J. Irigoyen, G. Zita, R. Martínez-Carrasco and M. Sánchez-Díaz. 2005. The response of nodulated alfalfa to water suply, temperature and elevated CO_2: photosynthesis downregulation. Physiol. Plant. 123:348–358.

Augé, R.M. 2001. Water relations, drought and vesicular–arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42.

Badaluco, L. and P. Nannipieri. 2007. Nutrient transformations in the rhizosphere. *In* The Rhizosphere. Biochemistry and Organic Substances at the Soil–Plant Interface. Eds. R. Pinton, Z. Varanini and P. Nannipieri. CRC Press, Taylor and Francis Group, New York, pp 111–133.

Baquedano, F.J. and F.J. Castillo. 2006. Comparative ecophysiological effects of drought on seedlings of the Mediterranean water-saver *Pinus halepensis* and water-spenders *Quercus coccifera* and *Quercus ilex*. Trees 20:689–700.

Barea, J.M., M.J. Pozo, R. Azcón and C. Azcón-Aguilar. 2005. Microbial co-operation in the rhizosphere. J. Exp. Bot. 56: 1761–1778.

Benlloch-González, M., O. Arquero, J.M. Fournier, D. Barranco and M. Benlloch. 2008. K^+ starvation inhibits water-stress-induced stomatal closure. J. Plant Physiol. 165:623–630.

Bent, E., S. Tuzun, C.P. Chanway and S.A. Enebak. 2001. Alterations in plant growth and in root hormone levels of lodgepole pines inoculated with rhizobacteria. Can. J. Microbiol. 47:793–800.

Bertin, C., X. Yang and L.A. Weston. 2003. The role of root exudates and allelochemicals in the rhizosphere. Plant Soil 256:67–83.

Boiero, L., D. Perrig, O. Masciarelli, C. Pena, F. Cassán and V. Luna. 2007. Phytohormone production by strains of *Bradyrhizobium japonicum* and possible physiological and technological implications. Appl. Microbiol. Biotechnol. 74:874–880.

Bréda, N., R. Huc, A. Granier and E. Dreyer. 2006. Temperate forest trees and stands under severe drought: a review of ecophysiological responses, adaptation processes and long-term consequences. Ann. For. Sci. 63:625–644.

Brimecombe, M.J., F.A.A.M. De Leij and J.M. Lynch. 2007. Rhizodeposition and microbial populations. *In* The Rhizosphere. Biochemistry and Organic Substances at the Soil–Plant Interface. Eds. R. Pinton, Z. Varanini and P. Nannipieri. CRC Press, Taylor and Francis Group, New York, pp 73–109.

Castro-Sowinski, S., Y. Herchkovitz, Y. Okon and E. Jurkevitch. 2007. Effects of inoculation with plant growth-promoting rhizobacteria on resident rhizosphere microorganisms. FEMS Microbiol. Lett. 276:1–11.

Chanway, C.P. 1997. Inoculation of tree roots with plant growth promoting soil bacteria: an emerging technology for reforestation. For. Sci. 43:99–112.

Chanway, C.P., M. Shishido, J. Nairn, S. Jungwirth, J. Markham, G. Xiao and F.B. Holl. 2000. Endophytic colonization and field responses of hybrid spruce seedlings after inoculation with plant growth-promoting rhizobacteria. For. Ecol. Manage. 133:81–88.

Enebak, S.A. 2005. Rizobacteria isolated from lobolly pine seedlings mediate growth-promotion of greenhouse-grown lobolly, slash, and longleaf pine seedlings. For. Sci. 51:541–545.

Frey-Klett, P., J.C. Pierrat and J. Garbaye. 1997. Location and survival of mycorrhizal helper *Pseudomonas fluorescens* during the establishment of ectomycorrhizal symbiosis between *Laccaria bicolor* and Douglas fir. Appl. Environ. Microbiol. 63:139–144.

Grayston, S.J., D. Vaughan and D. Jones. 1996. Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability. Appl. Soil Ecol. 5:29–56.

Guehl, J.M., G. Aussenac, J. Boucachrine, R. Zimmerman, J.M. Pennes, A. Ferhi and P. Grieu. 1991. Sensitivity of leaf gas exchange to atmospheric drought, soil drought, and water use efficiency in some Mediterranean *Abies* species. Can. J. For. Res. 21:1507–1515.

Gutiérrez-Mañero, F.J., A. Probanza, B. Ramos, J.J. Colón-Flores and J.A. Lucas-García. 2003. Effects of culture filtrates of rhizobacteria isolated from wild Lupine on germination, growth and biological nitrogen fixation of lupine seedlings. J. Plant Nutr. 26: 1101–1115.

Kothari, S.K., H. Marchner and E. George. 1990. Effect of VA mycorrhizal fungi and rhizosphere microorganisms on root and shoot morphology, growth and water relations in maize. New Phytol. 116:303–311.

Leonardi, S. and M. Rapp. 1990. Biomass production and nutrient requirement during restoration of a holm oak coppice. Acta Oecol. 11:819834.

Lucas-García, J.A., J. Domenech, C. Santamaría, M. Camacho, A. Daza and J.F. Gutiérrez-Mañero. 2004. Growth of forest plants (pine and holm-oak) inoculated with rhizobacteria: relationship with microbial community structure and biological activity of its rhizosphere. Environ. Exp. Bot. 52:239–251.

Lugtenberg, B. and J. Leveau. 2007. Biocontrol of plant pathogens principles, promises, and pitfalls. *In* The Rhizosphere. Biochemistry and Organic Substances at the Soil–Plant Interface. Eds. R. Pinton, Z. Varanini and P. Nannipieri. CRC Press, Taylor and Francis Group, New York, pp 267–296.

Marschner, H. 1986. Mineral nutrition in higher plants. Academic Press, San Diego, 674 p.

Martínez-Ferri, E., L. Balaguer, F. Valladares, J.M. Chico and E. Manrique. 2000. Energy dissipation in drought-avoiding and drought-tolerant tree species at midday during the Mediterranean summer. Tree Physiol. 20:131–38.

Martínez-Ferri, E., E. Manrique, F. Valladares and L. Balaguer. 2004. Winter photoinhibition in the field involves different processes in four co-occurring Mediterranean tree species. Tree Physiol. 24: 981–990.

Marulanda, A., J.M. Barea and R. Azcón. 2006. An indigenous drought-tolerant strain of *Glomus intraradices* associated with a native bacterium improves water transport and root development in *Retama sphaerocarpa*. Microbiol. Ecol. 52:670–678.

Medina, A., A. Probanza, F.J. Gutiérrez-Mañero and R. Azcón. 2003. Interactions of arbuscular-mycorrhizal fungi and *Bacillus* strains and their effects on plant growth, microbial rhizosphere activity (thymidine and leucine incorporation) and fungal biomass (ergosterol and chitin). Appl. Soil Ecol. 22:15–28.

Morte, A., G. Díaz, P. Rodríguez, J.J. Alarcón and M.J. Sánchez-Blanco. 2001. Growth and water relations in mycorrhizal and nonmycorrhizal *Pinus halepensis* plants in response to drought. Biol. Plant. 44:263–267.

Perrig, D., M.L. Boiero, O.A. Masciarelli, C. Penna, O.A. Ruiz, F.D. Cassán and M.V. Luna. 2007. Plant-growth-promoting compounds produced by two agronomically important strains of *Azospirillum brasilense*, and implications for inoculant formulation. Appl. Microbiol. Biotechnol. 75:1143–1150.

Rincón, A., B. Ruíz-Diez, S. García-Fraile, M. Fernández-Pascual, J.A. Lucas-García, J.J. Pueyo and M.R. de Felipe. 2005*a*. Colonisation of *Pinus halepensis* roots by *Pseudomonas fluorescens* and interaction with the ectomycorrhizal fungus *Suillus granulatus*. FEMS Microbiol. Ecol. 51:303–311.

Rincón, A., O. Priha, M.A. Lelu-Walter, M. Bonet, B. Sotta and F. Le Tacon. 2005*b*. Shoot water status and ABA responses of transgenic hybrid larch *Larix kaempfieri* × *L. decidua* to ectomycorrhizal fungi and osmotic stress. Tree Physiol. 25:1101–1108.

Roldán, A., I. Querejeta, J. Albaladejo and V. Castell. 1996. Survival and growth of *Pinus halepensis* Miller seedlings in a semi-arid environment after forest soil transfer, terracing and organic amendments. Ann. For. Sci. 53:1099–1112.

Sardans, J., F. Rodá and J. Peñuelas. 2006. Effects of a nutrient pulse supply on nutrient status of the Mediterranean trees *Quercus ilex* subsp. *ballota* and *Pinus halepensis* on different soils and under different competitive pressure. Trees 20:619–632.

Scholander, P.F., H.T. Hammel, E.D. Bradstreet and E.A. Hemmingsgten. 1965. Sap pressure in vascular plants. Science 148: 339–346.

Shishido, M., D.J. Petersen, H.B. Massicotte and C.P. Chanway. 1996. Pine and spruce seedling growth and mycorrhizal infection after inoculation with plant growth promoting *Pseudomonas* strains. FEMS Microbiol. Ecol. 21:109–119.

Timmusk, S. and E.G.H. Wagner. 1999. The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: a possible connection between biotic and abiotic stress responses. Mol. Plant-Microbe Interact. 12:951–959.

Valladares, F. and R.W. Pearcy. 2002. Drought can be more critical in the shade than in the sun: a field study of carbon gain and photo-inhibition in a Californian shrub during a dry El Niño year. Plant Cell Environ. 25:749–759.

Valladares, F., I. Dobarro, D. Sánchez-Gómez and R.W. Percy. 2005. Photonhibition and drought in Mediterranean woody saplings: scaling effects and interactions in sun and shade phenotypes. J. Exp. Bot. 56:483–494.

Valladares, F., E. Gianoli and J.M. Gómez. 2007. Ecological limits to plant phenotypic plasticity. New Phytol. 176:749–763.

Van Loon, L.C. 2007. Plant responses to plant growth-promoting rhizobacteria. Eur. J. Plant Pathol. 119:243–254.

Vessey, J.K. 2003. Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255:571–586.

Walker, T.S., H.P. Bais, E. Grotewold and J.M. Vivanco. 2003. Root exudation and rhizophere biology. Plant Physiol. 132:44–51.

Yordanov, I., V. Velikova and T. Tsonev. 2000. Plant responses to drought, acclimatation and stress tolerance. Photosynthetica 38: 171–186.

Zaady, E. and A. Perevolotsky. 1995. Enhancement of growth and establishment of oak seedlings (*Quercus ithaburensis* Decaisne) by inoculation with *Azospirillum brasilense*. For. Ecol. Manage. 72:81–83.