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Effects of elevated CO_2, water stress, and inoculation with *Glomus intraradices* or *Pseudomonas mendocina* on lettuce dry matter and rhizosphere microbial and functional diversity under growth chamber conditions

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Abstract
Purpose In this study, we investigated the effects of elevated atmospheric CO_2, drought, and inoculation with a plant-growth-promoting rhizobacterium (PGPR) or an arbuscular mycorrhizal (AM) fungus on microbial community composition and functional diversity in the rhizosphere of *Lactuca sativa* L. cv. Tafalla.
Materials and methods The experiment was a mesocosm assay, conducted as a randomized factorial design with three factors. The first factor had three levels: control soil, soil inoculated with *Glomus intraradices* (Schenk and Smith), and soil inoculated with *Pseudomonas mendocina* Palleroni; the second one had two regimes of watering: adequate and inadequate water supply and the third factor had two concentrations of CO_2: ambient CO_2 and elevated CO_2. Six replicates per treatment were set up, making a total of 72 pots. Community structure was studied by PCR-DGGE (polymerase chain reaction–denaturing gradient gel electrophoresis), and functional properties of the microbiota were investigated by community-level physiological profiling (CLPP) with Microresp™.
Results and discussion DGGE banding patterns showed that water stress affected bacterial and fungal communities, while microbial inoculation had little effect. Under well-watered and normal CO_2 conditions, the fungal diversity increased clearly by PGPR and especially by AM inoculation. Elevated CO_2 concentration changed microbial communities, but did not increase the microbial diversity (Shannon diversity index (H′) based on PCR-DGGE data). A principal component analysis showed a significant effect of drought stress on CLPP. The inoculation with PGPR, especially, increased the functional diversity for different guilds of carbon source. In contrast, elevated CO_2 concentration had no influence on functional diversity.
Conclusions Our results revealed that elevated CO_2 decreased the negative effects of drought on soil structural microbial diversity as well as plant growth but without changes in functional microbial diversity. In addition, the synergistic effect of the PGPR on growth of lettuce plants might not only result from a direct PGP effect but also from an indirect shift of the fungal community in response to elevated CO_2 under water stress conditions.

Keywords CLPP · DGGE · Drought · Elevated CO_2 · *Lactuca sativa* · Microbial community · Microresp

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1 Introduction

Atmospheric CO_2 content is rising and is predicted to alter the global climate and ecosystem functioning (Fuhrer 2003). Such a climate change would result in a substantially warmer planet with altered weather patterns, and it is expected that temperate areas will become warmer and drier

(Cao and Woodward 1998). This has consequences for agricultural production in arid and semiarid Mediterranean regions, since water shortage limits the growth and production of crops (Harle et al. 2007). It is generally believed that stimulation of plant production, under increasing atmospheric CO_2 conditions, results in enhanced fluxes of organic compounds into the soil due to higher rates of plant litterfall, root turnover, and rhizodeposition (Suter et al. 2002). This organic carbon released into soil serves as substrate for heterotrophic soil microbes that drive nutrient cycles. Based on differences in rhizodeposition, the density, diversity, and function of the rhizosphere microbial community can vary under elevated atmospheric CO_2 levels (Weihong et al. 2000). Although relatively little is known about how soils and their biota will respond to climate change (Staddon et al. 2004), the effect of increases in CO_2 on plant–microorganism interactions has drawn considerable interest during the last decade.

The diversity of the soil microbial community has been suggested as a sensitive means of assessing soil quality (White and MacNaughton 1997; Lynch et al. 2004). While denaturing gradient gel electrophoresis (DGGE) analysis is a powerful and relatively easy way of comparing most of the species in different communities (Muyzer et al. 1993) and generally measures the diversity of the numerically dominant members of the community (Hedrick et al. 2000), functional diversity is popular in that it relates to the activity of the soil and gives information on the functioning of those members of the soil microflora involved in carbon cycling (Chapman et al. 2007). Community-level physiological profiles (CLPPs) are usually assessed by carbon substrate utilization, i.e., the ability of the soil microflora to metabolize a set of, usually, simple organic compounds.The relationship between microbial diversity and function in soil is largely unknown, but biodiversity has been assumed to influence ecosystem stability, productivity, and resilience towards stress and disturbance (Torsvik and Øvreås 2002). Therefore, changes in functional or structural microbial diversity could indicate changes in the whole ecosystem. Elevated CO_2 is likely to modify the ecosystem functioning including rhizosphere bacteria that are directly dependent on rhizodeposition (Tarnawski et al. 2006). This may include alteration of microbial populations that display phenotypic traits in relation with plant fitness because microorganisms can adapt rapidly to changing environmental conditions (Singer and Ewing 2000). Observations of the effects of increased atmospheric CO_2 levels on the structure of soil and rhizosphere microbial communities range from pronounced effects (Janus et al. 2005; Jossi et al. 2006) to subtle or undetectable effects (Bruce et al. 2000). However, these contrasting results have to be viewed in the light of the different systems studied and the different methods applied to assess community structure and diversity.

Mycorrhizal fungi are known to enhance the ability of plants to establish and cope with stress situations such as drought (Augé 2001). In fact, several authors have indicated that mycorrhizal fungi may improve the performance of seedlings, by stimulating water uptake (Augé 2001) or increasing nutrient uptake by the plant, particularly N and P (Hodge et al. 2001). In exchange, mycorrhizal plants provide the fungus with photosynthetic C, which in turn is delivered to the soil via fungal hyphae. As a consequence, mycorrhiza formation can affect the microbial population in the rhizosphere, directly or indirectly, through changes in root exudation patterns or through fungal exudates. It is known that elevated CO_2 tends to increase mycorrhizal indicators, such as amount of extraradical hyphae (Treseder and Allen 2000). Fitter et al. (2000) found direct and indirect effects of elevated atmospheric CO_2 on arbuscular mycorrhizal (AM) fungi. Direct effects of soil temperature and moisture on fungi affect growth and community structure in unknown ways and feedback on the plant community. Indirect effects act through plant C fixation. Both beneficial and deleterious effects on plant and fungal growth are potentially self-reinforcing, through positive feedback loops. Regulation of these loops might be achieved via alterations in C demand by the fungus. In one of the rare studies of the combined effect of elevated CO_2 and drought, Staddon et al. (2004) found that mycorrhizal fungi can take advantage of increased availability of plant photosynthate under elevated CO_2.

Plant-growth-promoting rhizobacteria (PGPR) are a group of bacteria that can actively colonize plant rhizospheres and increase plant growth. Because of the potential of PGPR for improving plant nutrition and health, the use of these rhizobacteria has been addressed in several investigations (Vessey 2003; Fuchs 2009). Specific strains of *Pseudomonas* have been shown to increase the yield of some agricultural crops (Kohler et al., 2006, 2009). Due to their competitiveness for nutrients and for suitable niches on the root surface, they are able to respond rapidly to environmental modifications (Stres et al. 2004). Tarnawski et al. (2006) showed that *Pseudomonas* strains associated with different plant/soil systems may respond differently to elevated CO_2 depending on the associated plant in the rhizosphere or on the different exposure time to elevated CO_2. They proposed a possible long-term selection of the best-adapted strains to the plant rhizosphere under rising CO_2.

To our knowledge, nothing is known about the effect of inoculation of PGPR and AM fungi on structural and functional diversity of rhizosphere microorganisms of a short-cycle crop under drought and elevated CO_2. In this study, it was hypothesized that AM fungi and PGPR would modify structure and function of soil-borne microbial

communities, particularly at elevated CO_2 where increased plant C supply was also expected. To gain further insight into the effects of CO_2 on soil-borne communities, we assessed the genetic composition of fungal, bacterial, and *Pseudomonas* communities and the functional diversity in the rhizosphere of *Lactuca sativa* grown under elevated CO_2 and water stress conditions. In addition, we tested whether the inoculation with an AM fungus, *Glomus intraradices* or a PGPR, *Pseudomonas mendocina,* changes these responses of rhizosphere microbial communities to elevated CO_2 and drought.

2 Materials and methods

2.1 Soil and plant species

An agricultural soil used to cultivate lettuce was collected near Murcia (SE Spain). The main characteristics of the agricultural soil used were: pH (1:5, H_2O) 8.5; electrical conductivity 2.88 dS m^{-1}; clay 20.1%; silt 43.3%; sand 36.6%; total organic C 8.5 g kg^{-1}; total N 1.03 g kg^{-1}; available P 42 μg g^{-1}; extractable K 550 μg g^{-1}; cationic exchange capacity 8 cmol kg^{-1}; and water holding capacity 32.8%. The climate is semiarid Mediterranean with an average annual rainfall of 300 mm and a mean annual temperature of 19.2°C; the potential evapotranspiration reaches 1,000 mm $years^{-1}$.

Seeds of lettuce (*L. sativa* L. cv. Tafalla) used in this study were grown for 15 days, in peat substrate under nursery conditions, without any fertilization.

2.2 Soil water potential

Soil substrate 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 (Richards 1941). 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.

2.3 Microorganisms

The mycorrhizal fungus used was *G. intraradices* (Schenk and Smith) obtained from the collection of the experimental field station of Zaidín, Granada. The inoculum consisted of a mixture of rhizospheric soil from pot cultures (*Sorghum* sp.) containing spores, hyphae, and mycorrhizal root fragments. It was subjected to a most probable number test (Sieverding, 1991) to determine potential infectivity. The source of inoculum had a potential infectivity of about 35 infective propagules per gram 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 liquid culture (nutrient broth, Scharlau Chemie, Spain) composed of meat and yeast extracts, peptone, and sodium chloride, by shaking at 75 rpm for 2 days at room temperature on a Heidolph Unimax1010 shaker. The bacterial culture was centrifuged (2,287×*g*, 5 min, 2°C), and the sediment was re-suspended in sterilized tap water. The bacterial suspension contained 10^9 colony-forming units (CFU) ml^{-1}.

2.4 Design of the experiment

The experiment was a mesocosm assay, conducted as a randomized factorial design with three factors. The first factor had three levels: control soil, soil inoculated with *G. intraradices*, and soil inoculated with *P. mendocina*; the second one had two regimes of watering: adequate and inadequate water supply and the third factor had two concentrations of CO_2: ambient CO_2 and elevated CO_2. Six replicates per treatment were set up, making a total of 72 pots.

Seven hundred grams of non-sieved soil were placed in 1.5-liter pots. *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 fungus. *P. mendocina* was inoculated two times during the growth period. The dose of inoculum applied corresponded to 10^{10} CFU per plant. Seedlings were grown without any fertilizer treatment under controlled environmental conditions for 9 weeks in two growth controlled CO_2 flow chambers (3.2×2.4×2.4 m), located in the SACE service at the Campus of Espinardo (Murcia, Spain). One chamber was continuously supplied with supplemental CO_2 from a compressed CO_2 cylinder; CO_2 concentration was monitored with an infra-red spectroscopic sensor as per VDMA 24772 (EGQ212, Fr. Sauter AG, Basel, Switzerland) equipped with an automatic switching solenoid to maintain the elevated CO_2 concentration at about twice ambient (760±20 parts/10^6). The other chamber maintained an ambient CO_2 concentration (380±20 parts/10^6). Within each of the growth chambers, one half of the pots were watered regularly with decalcified water maintaining substrate water potential equivalent to field capacity (–0.03 MPa), and the other half of pots were maintained under a soil water potential of –0.3 MPa. During the experiment, the lettuce plants were subjected to a photoperiodic cycle of 13 h light with a light intensity of 2 K Lux and 11 h dark. The average day/night temperature was 24°C/

18°C, and relative humidity was kept constant at 60%. Soil moisture was monitored gravimetrically before each watering.

2.5 Plant analyses

Nine weeks after planting, six plants per treatment were harvested, and soil samples were taken from the pots. Soil samples were immediately sieved at 2 mm and stored at –20°C.

Fresh and dry (105°C, 5 h) weight of shoots and roots were recorded. Roots were subsampled in three 2-cm cross-sections of the upper, middle, and lower root system. To assess colonization, roots were cleared with 10% KOH and stained with 0.05% trypan blue (Phillips and Hayman 1970). The percentage of root length colonized by AM fungi was calculated by the gridline intersect method (Giovanetti and Mosse, 1980). Positive counts for AM colonization included the presence of vesicles or arbuscules or typical mycelium within the roots.

2.6 Community-level physiological profiles

Analysis of CLPP was performed using the modified MicroResp™ method as described in Campbell et al. (2003). The used carbon sources were: *N*-acetyl-D-glucosamine, D-glucose, D-(+)-mannose, D-arabinose, D-(+)-cellobiose, D-trehalose, D-(+)-raffinose-pentahydrate, D-(+)-maltose-monohydrate, D-(+)-galacturonic acid monohydrate, D-xylose, D-ribose, L-arginine, L-lysine, L-proline, D-alanine, glycerol, citric acid, D-malic acid, 3-hydroxybutyric acid, α-cyclodextrin, and Tween 80. Each carbon source was dissolved in deionized water and prepared as a stock solution at a concentration designed to deliver 11 mg C g^{-1} soil water. Approximately 0.4 g of soil was placed in each well of a 96-deep-well microtiter plate. To estimate the evolved CO_2, a colorimetric method relying on the change in the pH of a gel-based solution of bicarbonate was used. The indicator dye with the gel detector plate consisted of cresol red dye (12.5 ppm, wt/wt), potassium chloride (150 mM), and sodium bicarbonate (10 mM) set in a 1% gel of Noble agar (150 µl per well). The absorbance at 595 nm was read with a Zenyth multimode reader (Anthos, Wals, Austria) immediately before and after 6 h of incubation at 22°C. The absorbance after 6 h was normalized for any differences recorded at time zero and then converted to the headspace CO_2 concentration by using a calibration curve obtained with an infra-red-based respiration measurement device (Heinemeyer et al. 1989) using five different soils with and without glucose addition (1% C). The best fit for the calibration curve, using the difference of absorbance between t0 and t1 (after 6 h) was: $y = 0.1229 \ln(x) + 0.2091$, $R^2 = 0.835$.

2.7 Analysis of the soil microbial community structure

2.7.1 DNA extraction

DNA was extracted with a bead beating procedure using the MO BIO PowerSoil DNA Isolation kit (MoBio Laboratories Inc., Carlsbad, CA, USA). To obtain suitable amplicons, tenfold dilutions of the extract were used as template for subsequent PCR. The DNA was quantified by fluorescent PicoGreen® dye (Invitrogen, Carlsbad, CA, USA).

2.7.2 Bacterial DNA amplification

Bacterial 16S rRNA gene fragments were amplified using the primer set F984 (5′-GC-clamp-AAC GCG AAG AAC CTT AC-3′) and R1378 (5′-CGG TGT GTA CAA GGC CCG GGA ACG-3′) (Heuer et al. 1997). For PCR, 2 µl of the DNA extract were added to 23 µl of PCR reaction mix composed of 0.625 U Bio Therm™ DNA Polymerase (Gene Craft, Germany), 0.4 mM dNTPs (Gene Craft, Germany), 1× PCR buffer (BioTherm™, Gene Craft, Germany), 0.2 µM of each primer, 2 mM $MgCl_2$, and filled up to the end volume with sterile ultrapure water. DNA was amplified in a Thermocycler using 35 cycles of 1 min denaturation at 94°C, 1 min at 55°C for primer annealing, and 2 min at 72°C for primer extension. The 35 cycles were followed by a final step of 10 min at 72°C and cooling down to 10°C (Heuer et al. 1997). Successful amplification was verified by electrophoresis in 1.5% (*w*/*v*) agarose gels with ethidium bromide (10 mg ml^{-1}). The DNA yields were quantified by fluorescent PicoGreen® dye (Invitrogen, Carlsbad, CA, USA).

2.7.3 Fungal DNA amplification

Fungal 18S rRNA gene fragments was amplified using the primer set FF390 (5′-CGA TAA CGA ACG AGA CCT-3′) and FR1GC (5′-CCC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GCC GAI CCA TTC AAT CGG TAI T-3′; Vainio and Hantula 2000). For PCR, 2 µl of the DNA extract was added to 23 µl of PCR reaction mix composed of 0.625 U Bio Therm™ DNA Polymerase (Gene Craft, Germany), 0.4 mM dNTPs (Gene Craft, Germany), 1× PCR buffer (BioTherm™, Gene Craft, Germany), 0.2 µM of each primer, 12 µg ml^{-1} BSA, and 2 mM $MgCl_2$ filled up to the end volume with sterile ultrapure water. The PCR program included an initial 8-min denaturation at 94°C and was followed by 35 thermal cycles of 30-s denaturation at 94°C, 45 s at 47°C for primer annealing, and 3 min at 72°C for primer extension. The 35 cycles were followed by a final step of 10 min at 72°C and cooling at 18°C. Successful amplification was verified by

electrophoresis in 1.5% (*w*/*v*) agarose gels with ethidium bromide (10 mg ml^{-1}). The DNA yields were quantified by fluorescent PicoGreen® dye (Invitrogen, Carlsbad, CA, USA).

2.7.4 Pseudomonas-specific DNA amplification

To amplify *Pseudomonas*-specific 16S rRNA gene fragments from soils, we used a nested PCR approach (Garbeva et al. 2004). For the first-round PCR, the *Pseudomonas*-specific primer set Ps_{for} (5′-TTA GCT CCA CCT CGC GGC-3′) and Ps_{rev} (5′-GGT CTG AGA GGA TGA TCA GT-3′; Widmer et al. 1998) were used in 25-µl reactions volumes containing 2 µl of the DNA extract, 0.2 µM of each primer, 2 mM $MgCl_2$, 1× enhancer, 0.4 mM dNTPs (Gene Craft, Germany), 12 µg ml^{-1} BSA, 1× PCR buffer, and 0.625 U Bio Therm™ DNA Polymerase (Gene Craft, Germany). DNA was amplified in a Thermocycler using 25 cycles of 45-s denaturation at 94°C, 45 s at 65°C for primer annealing, and 45 s at 72°C for primer extension. A final step followed with 15 min at 72°C and cooling at 10°C. Successful amplification was verified by electrophoresis in 1.5 % (*w*/*v*) agarose gels with ethidium bromide (10 mg ml^{-1}).

The PCR products served as templates for a second PCR with universal bacterial forward primer F984 GC and the *Pseudomonas*-specific primer Ps_{rev}. The program used for the second PCR included an initial denaturation at 94°C for 4 min, one cycle of 1 min at 94°C, 1 min at 60°C, and 2 min at 72°C, followed by ten times the same cycle with every subsequent one using a 0.5°C lower annealing temperature (until 55°C), 15 cycles of 94°C (1 min), 55°C (1 min), and 72°C (2 min), and final extension at 72°C (10 min). The PCR products were checked by electrophoresis in 1.5% (*w*/*v*) agarose gels and ethidium bromide staining (10 mg ml^{-1}) and stored at 4°C for further DGGE analysis. The DNA concentrations of the amplicons were quantified by fluorescent PicoGreen® dye (Invitrogen, Carlsbad, CA, USA).

2.7.5 Denaturing gradient gel electrophoresis

DGGE of bacterial communities and *Pseudomonas*-specific DGGE were performed with the Ingeny PhorU2 system (Ingeny International BV, The Netherlands). Sixty nanograms of each PCR amplificon were loaded onto polyacrylamide gels with a denaturing gradient of 40% to 70% [100% denaturant according to 7 M urea plus 40 % formamide in 1× TAE-buffer]. For fungal amplicons, 7% (*w*/*v*) polyacrylamide gels with a denaturing gradient of 30% to 60% were prepared. Gels were run at 60°C and 120 V (universal bacterial DGGE) resp. 100 V (fungal and *Pseudomonas*-specific DGGE) for 16 h. After electrophoresis, the gels were stained with silver nitrate using an automated gel stainer (Hoefer, Amersham Pharmacia Biotech), air-dried, and scanned. The gel images were analyzed with the software package GELCOMPAR II, version 4.0 (Applied Maths, Ghent, Belgium).

2.8 Statistical analysis

Data were log-transformed to achieve normality. Microbial inoculation, water regime, CO_2 concentration, and their interaction effects on measured variables were tested by a three-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 14.0 for Windows.

Similarity calculation of the DGGE bands was based on the Pearson correlation coefficient (Pearson 1926) and resulted in a distance matrix. Cluster analysis and dendrograms were calculated using the UPGMA algorithm (Legendre and Legendre 1998). The Shannon diversity index H′ (Shannon and Weaver 1963) was calculated to examine the structural diversity of the bacterial, fungal, and *Pseudomonas* community using the software program Primer 5.0.

Community-level physiological profiles were analyzed with a principal component analyses and by calculating a functional diversity index H'_f (Bradley et al. 2006).

3 Results

3.1 Effects of elevated CO_2, water stress, and microbial inoculation on lettuce dry matter yield and colonization

Shoot and root dry biomass were affected by microbial inoculation, water stress, and CO_2 level (Table 1 and Table 2). There was a significant CO_2 level *x* water stress interaction for shoot and root dry biomass (see Table 2). Under elevated CO_2, shoot dry matter was increased by inoculation with *P. mendocina* and, to a lesser extent, the AM fungus. However, only the treatment with *P. mendocina* at –0.03 MPa and 380 ppm CO_2 had higher shoot dry matter than the control plants (see Table 1). Drought stress decreased the shoot dry biomass and increased root dry matter at both CO_2 levels, although these effects were more pronounced at ambient CO_2 level. 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 (see Table 1).

The microbial inoculation treatments had no significant effect on the shoot water content, at both CO_2 and watering levels (see Tables 1 and 2). There was a significant CO_2× water stress interaction for shoot water content (see Table 2). Shoot water content was decreased under drought stress

Table 1 Effect of inoculation with *G. intraradices* and *P. mendocina* on growth parameters and colonization of *L. sativa* seedlings grown at two concentrations of atmospheric CO_2 and 2° of watering (n=6)

	CO_2	Watering	Shoot dry biomass (g dw)	Root dry biomass (g dw)	Shoot water content (%)	Colonization (%)
Control	380 ppm	−0.03 MPa	1.08±0.03	0.21±0.01	92.9±0.1	37.8±0.8
P. mendocina			1.48±0.02	0.26±0.01	92.0±0.3	39.0±1.5
G. intraradices			1.21±0.05	0.18±0.00	93.9±0.1	54.5±0.6
Control		−0.3 MPa	0.63±0.02	0.20±0.01	85.7±0.3	18.4±1.3
P. mendocina			0.69±0.02	0.28±0.00	85.6±0.2	32.5±2.9
G. intraradices			0.63±0.02	0.21±0.01	85.6±0.2	37.7±1.3
Control	760 ppm	−0.03 MPa	0.98±0.03	0.12±0.01	94.5±0.1	26.0±0.7
P. mendocina			1.15±0.04	0.14±0.01	94.0±0.2	36.6±±1.2
G. intraradices			1.03±0.01	0.09±0.00	94.4±0.1	45.2±1.3
Control		−0.3 MPa	0.64±0.02	0.24±0.01	90.4±0.2	31.2±1.4
P. mendocina			0.89±0.02	0.28±0.02	88.0±0.7	45.2±3.7
G. intraradices			0.77±0.02	0.19±0.01	89.0±0.5	65.7±1.5

Mean±standard errors

conditions, and this decrease was more pronounced at the atmospheric CO_2 level.

Only the interaction between water stress and CO_2 level had a significant effect on the colonization of lettuce roots (see Table 2). The plants inoculated with *G. intraradices* showed the highest AMF colonization, at both water and CO_2 levels (see 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.

3.2 Effects of elevated CO_2, water stress, and microbial inoculation on soil microbial community diversity

The microbial inoculations with *P. mendocina* or *G. intraradices* showed little influence on the bacterial communities patterns (Fig. 1). Cluster analysis based on Pearson's similarity matrix for all treated soils revealed that CO_2 level was the main factor grouping the soils, regardless of water regime.

The fungal community pattern showed a separate cluster for the samples inoculated with the AM fungus under water stress and elevated CO_2, which presented 51.8% similarity with the other patterns (Fig. 2). The second major cluster showed also a very clear separation between soils under elevated CO_2 and ambient CO_2. In the cluster comprising of samples under elevated CO_2, all samples of drought-stressed soil and inoculated with the PGPR was also distinguishable with 67.3% similarity with the other clusters. The inoculation with *P. mendocina* or *G. intraradices* and the environmental factors had no clear influence on the *Pseudomonas* communities (data not shown).

The Shannon–Weiner diversity indexes for bacteria ($H'_{bacteria}$), fungi (H'_{fungi}), and *Pseudomonas* ($H'_{Pseudomonas}$)

Table 2 Three factor-ANOVA (microbial inoculation, water stress, and pCO_2) for drought parameters, mycorrhizal colonization and Shannon diversity indexes (H′) for bacteria, *Pseudomonas*, and fungi communities and functional diversity index H'_f (*P* significance values)

	Microbial inoculation (M)	Water stress (W)	pCO_2 (C)	Interaction (M×W)	Interaction (M×C)	Interaction (W×C)	Interaction (M×W×C)
Shoot biomass	0.008	<0.001	0.049	NS	NS	0.001	NS
Root biomass	0.037	<0.001	0.005	NS	NS	0.004	NS
Shoot water content	NS	<0.001	<0.001	0.022	NS	0.013	NS
Colonization	<0.001	NS	NS	NS	NS	<0.001	NS
$H'_{bacteria}$	NS	0.036	NS	0.002	NS	NS	NS
H'_{fungi}	0.050	0.008	NS	0.025	NS	0.034	0.053
$H'_{Pseudomonas}$	NS	NS	0.007	NS	NS	0.002	NS
H'_f	<0.001	NS	NS	NS	NS	NS	NS

NS not significant

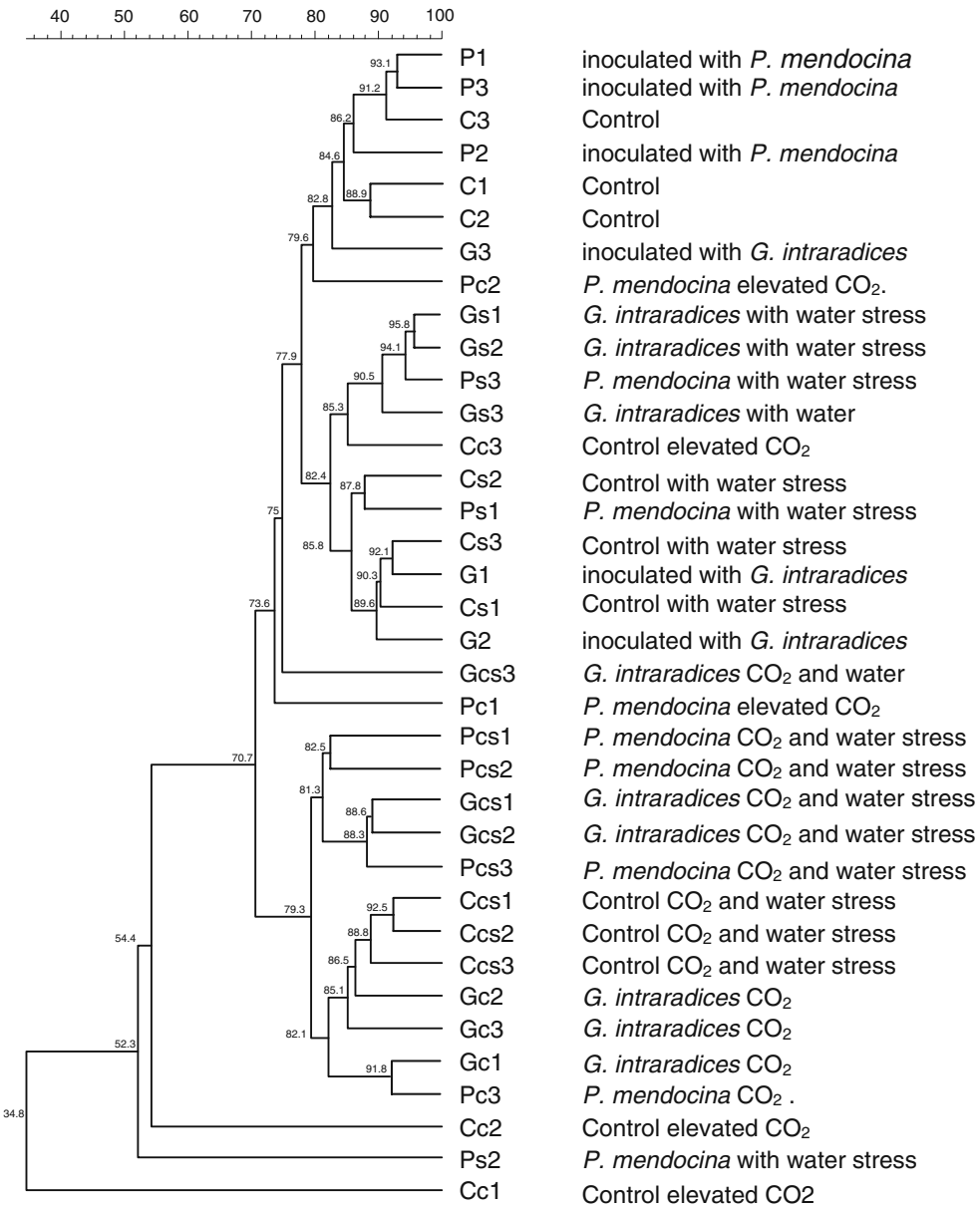

Fig. 1 Dendrogram constructed with UPGMA (position tolerance, 0.4; minimum height, 0.2; minimum surface, 0.2) representing the similarity between Bacteria-specific PCR-DGGE patterns obtained from soil samples under different treatments. Numbers *1*, *2*, and *3* indicate replicates. The similarity value is written at the *branch* of each cluster

were calculated from their DGGE patterns (Table 3). Microbial inoculation×water regime interaction had a significant effect on bacterial diversity (see Table 2). The $H'_{bacteria}$ decreased in the soils inoculated with the AM fungus or the PGPR in response to drought under both normal and elevated CO_2 conditions, whereas that of the non-inoculated soils increased with drought stress (see Table 3). In contrast, the CO_2 level did not have any effect on the bacterial diversity. The soils treated with *P. mendocina* or *G. intraradices* showed higher fungal diversity than the control soil. There was a significant negative water regime×CO_2 interaction for the index of fungal diversity. Drought strongly decreased this index under elevated levels of CO_2 compared with those soils under non-stressing conditions. The $H'_{Pseudomonas}$ was not influenced by inoculation with *P. mendocina*. The treatment with elevated CO_2 increased the diversity of *Pseudomonas* communities, except in the soil inoculated with *G. intraradices*. Under such conditions of CO_2, the $H'_{Pseudomonas}$ of all soils significantly decreased in response to drought. Band evenness (*E*) indicates the homogeneity of bands and ranged from 0.97 and 0.98 for the bacteria, from 0.87–0.93 for the fungi and from 0.77–0.96 for *Pseudomonas*.

3.3 Effects of elevated CO_2, water stress, and microbial inoculation on soil microbial community functional diversity

With the MicroResp™ system the functional diversity of the microbial communities were analyzed. Principal component analysis (PCA) identified two components that accounted for 75.3% of the total variance, being explained by axis 1 (44.3%) and by axis 2 (31.0%; see Fig. 3, Table 4). The PCA showed

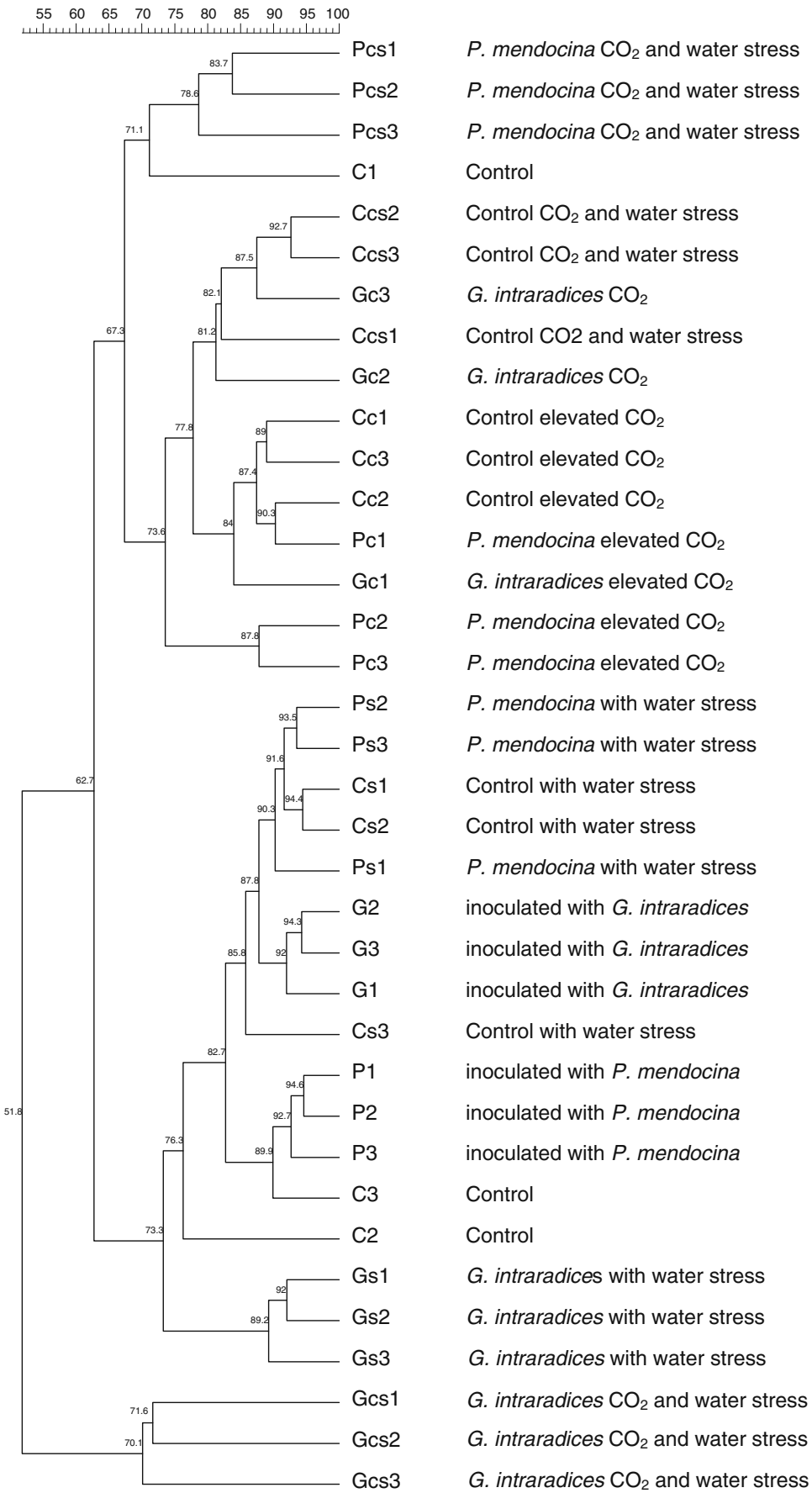

Fig. 2 Dendrogram constructed with UPGMA (position tolerance, 0.4; minimum height, 0.2; minimum surface, 0.2) representing the similarity between fungi-specific PCR-DGGE patterns obtained from soil samples under different treatments. Numbers *1*, *2*, and *3* indicate replicates. The similarity value is written at the *branch* of each cluster

Table 3 Effect of inoculation with *G. intraradices* and *P. mendocina* on Shannon diversity indexes (H′) for bacteria, *Pseudomonas*, and fungi communities and functional diversity index H'_f of rhizosphere soil of *L. sativa* seedlings grown at two concentrations of atmospheric CO_2 and 2° of watering (n=6)

	CO_2	Watering	$H'_{bacteria}$	H'_{fungi}	$H'_{Pseudomonas}$	H'_f
Control	380 ppm	–0.03 MPa	3.49	2.78	1.74	2.99
P. mendocina			3.53	3.02	1.59	3.02
G. intraradices			3.59	3.20	1.90	2.98
Control		–0.3 MPa	3.54	2.99	1.87	3.00
P. mendocina			3.40	3.06	1.88	3.00
G. intraradices			3.14	2.87	2.16	2.99
Control	760 ppm	–0.03 MPa	3.35	3.00	1.93	2.99
P. mendocina			3.43	3.04	1.87	3.01
G. intraradices			3.52	3.03	1.57	2.96
Control		–0.3 MPa	3.57	2.74	1.45	3.00
P. mendocina			3.32	2.91	1.38	3.01
G. intraradices			3.40	2.75	1.35	2.97

a clear influence of drought stress in the catabolic activity of microbes in the rhizosphere soil of *L. sativa* (see Fig. 3, Table 4). However, there was no shift in the CLPP as a result of elevated CO_2. Regardless of the environmental treatments, the PCA also revealed a change in the functional diversity with the inoculation with *P. mendocina*.

According to ANOVA (see Table 2) only the microbial inoculation had effect on the functional diversity (H'_f). A clear positive influence of the PGPR and a slight negative influence of mycorrhizal inoculation on H'_f were observed. No influence of drought or CO_2 concentration on the functional diversity H'_f could be found.

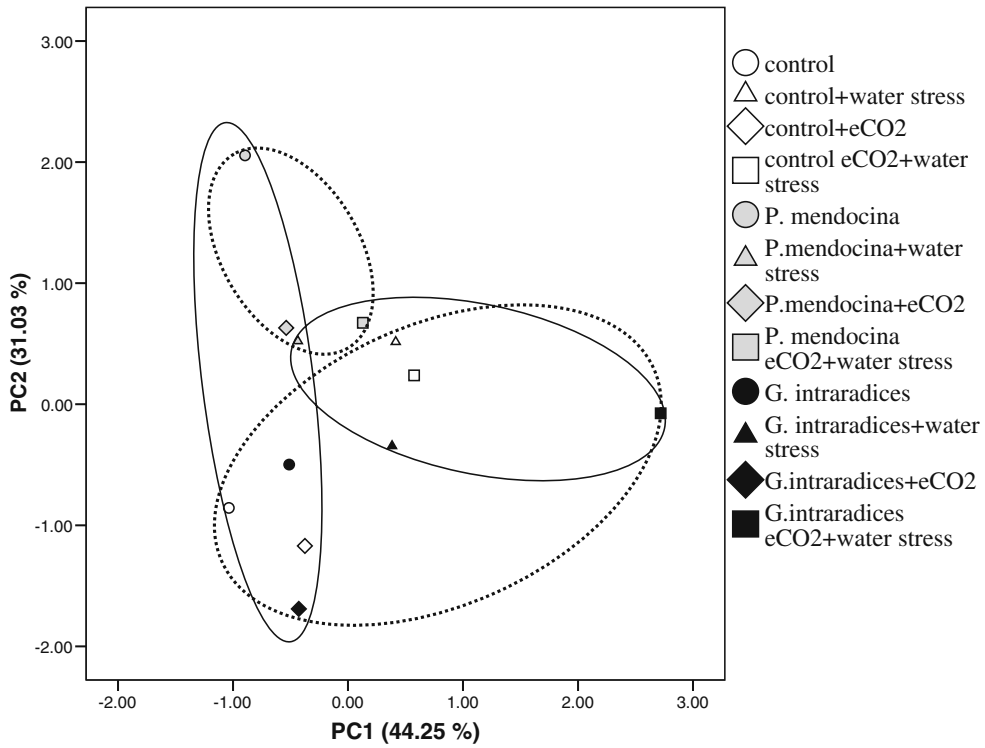

Fig. 3 First and second principal components derived from the substrates of the MicroResp™ system determined on the rhizosphere of *L. sativa* at two levels of irrigation and two levels of CO_2 concentration after 9 weeks of plantation (n=6). Values within the *full ellipse* are well-watered; values within the *dashed ellipse* are water-stressed treatments. *C* control, *C*+*WS* control+water stress, *C*+eCO_2 control+elevated CO_2, *C*+*WS*+eCO_2 control+water stress+elevated CO_2, *P P. mendocina*, *P*+*WS P. mendocina*+water stress, *P*+eCO_2 *P. mendocina*+elevated CO_2, *P*+*WS*+eCO_2 *P. mendocina*+ water stress+elevated CO_2, *G G. intraradices*, *G*+*WS G. intraradices*+ water stress, *G*+eCO_2 *G. intraradices*+elevated CO_2, *G*+*WS*+eCO_2 *G. intraradices*+water stress+elevated CO_2. *Solid line*: samples grouped according to water stress level; *dotted line*: samples grouped according to inoculation with *P. mendocina*

Table 4 Three factors ANOVA (microbial inoculation, water stress, and pCO_2) performed on PC1 and PC2 (*P* significance values)

	PC1	PC2
Microbial inoculation (M)	NS	0.010
Water stress (W)	<0.001	0.020
pCO_2 (C)	NS	0.046
M•W	0.001	NS
M•C	NS	0.017
W•C	0.036	NS
M•W•C	0.015	0.035

NS not significant

PC1 and *PC2* first and second principal components derived from the data

4 Discussion

4.1 Effects of elevated CO_2, water stress, and microbial inoculation on lettuce dry matter yield

Plant growth responded to all the environmental treatments, increasing shoot biomass with elevated CO_2, especially in soils inoculated with the PGPR strain, and decreasing with drought confirming previous studies (Nemani et al. 2003; Staddon et al. 2004). While under drought stress and normal CO_2, no increase of shoot biomass with the microbial inoculations could be observed, the negative effect of water stress under elevated CO_2 could be partly attenuated by the inoculation with *G. intraradices* and even more with *P. mendocina*, which indicates the importance of microbial inoculations for use in agriculture, if in a future elevated CO_2 scenario. Recently, Kohler et al. (2008) showed the potential use of PGPR inoculation to alleviate the oxidative damage produced under water stress in lettuce plants. Under severe drought plants inoculated with *P. mendocina* were more hydrated than the control plants. This demonstrated that *P. mendocina* efficiently protected the host plants against the detrimental effects of drought. Greater hydration induced by the PGPR strain might be attributable to increased water use efficiency and/or reduction of stress ethylene production via the action of ACC deaminase (Mayak et al. 2004).

The results of our experiments show that there was no effect of elevated CO_2 on colonization of roots by AM fungi. This lack of effect has been found also by other authors (Fitter et al. 2000; Staddon et al. 2004). Insam et al. (1999) also observed no effects of elevated CO_2 on percent mycorrhizal root colonization in an artificial tropical ecosystem. However, these authors found a reduced number of mycorrhizal spores under elevated CO_2. A positive interaction between elevated CO_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. 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 (Fitter et al. 2000). As a consequence, services performed by the fungus might be enhanced such as improved plant water status, as happened in our study. Previously, we have demonstrated that the contribution of mycorrhizal inoculation to lettuce plants drought tolerance at elevated CO_2 results from a combination of nutritional and cellular effects, such as modulation of genes encoding aquaporins (Alguacil et al. 2009).

4.2 Effects of elevated CO_2, water stress, and microbial inoculation on soil microbial community diversity

The changes observed in bacterial and fungal community structure due to elevated CO_2 are in agreement with previous studies conducted under field conditions in grassland plants (Marilley et al. 1999) confirming CO_2-related shifts in bacterial and *Pseudomonas* community composition. Drigo et al. (2007) assessed in a pot experiment of sandy dune soils with different *Poaceae* seedlings the effects of increased atmospheric CO_2 on bacterial, fungal, and nematode communities. They reported that effects of elevated CO_2 were soil-dependent, with greater influence observed in the highly organic soils that also supported higher levels of AM colonization. This is in agreement with our results because we found clear changes in bacterial and fungal communities in an agricultural soil with 1.8% TOC which is quite high for a Mediterranean soil.

Elevated CO_2 changed microbial community structure but did not increase Shannon diversity, which proved that, at the short-term, elevated CO_2 favored other strains: all fungi, total bacteria, and *Pseudomonas*, than under normal CO_2, but Drissner et al. (2007) proved in the long-term that bacterial diversity (particularly of Gram-negative bacteria) indeed increased under enriched CO_2.

The inoculation with *P. mendocina* had little effect neither on *Pseudomonas* populations nor on bacterial communities, which indicates that an inoculation of a PGPR did not take the place of soil-borne microorganisms in a stabile agroecosystem. A possible resilience of *Pseudomonas* populations during short-term CO_2 enrichment may also be possible.

Physiological stress, such as drought, tends to reduce microbial diversity, favoring those microbes best adapted to coping with the stress. This is consistent with the apparent reduction in bacteria diversity of the soils inoculated with the bio-inoculants, especially in the *G. intraradices*-inoculated soil. Probably, AM fungi deposition products

may also contain bacteriostatic or fungistatic agents, as suggested by Ravnskov et al. (1999). Filion et al. (1999) also showed that AM fungi exudates influence the abundance and activities of specific fungal and bacterial species. Fungal species may vary in their tolerance of low moisture conditions. In the current study, the unchanged fungal diversity recorded in the soil control and inoculated with the PGPR might indicate that fungi survived drought stress better than bacteria, possibly due to their ability to grow at lower matric potentials. However, it is worth noting that diversity of fungal and *Pseudomonas* populations were the most affected by elevated CO_2 and drought, with bacterial community being influenced to a somewhat lesser extent.

In our study, the bio-inoculants did not induce a significant modification in the bacterial community structure, contrasting with Roesti et al. (2006). They inoculated PGPR and AM fungi in wheat fields, and the PGPR treatment had greater impact on bacterial community structure than the presence of AM fungi, possibly due to the high-density inoculum of the PGPR strains. The fungal diversity increased clearly by PGPR and especially by AM inoculation under well-watered and normal CO_2 conditions. The reasons for this predominant effect of the AM inoculum are unclear. Staddon et al. (2004) found that mycorrhizal fungi can take advantage of increased availability of plant photosynthate under elevated CO_2 and may produce more external mycorrhizal hyphae. Growth of *Plantago lanceolata* responded to all the environmental treatments, positively to elevated CO_2 and soil warming, and negatively to drought. Fitter et al. (2000) concluded that an elevated atmospheric CO_2 may have only a minor impact on AM fungi beyond that mediated by vegetative change, but they respond positively to temperature changes. We also found a clear influence of the CO_2 and drought on fungal populations. There was evidence of interactive effects of the environmental variables (water regime and CO_2 concentration) on the response of the PGPR in relation to its effect on fungal diversity. Thus, it is noteworthy that the biggest differences in the DGGE dendrogram were found in rhizospheric soil inoculated with *P. mendocina*, supported by the Shannon diversity index H'_{fungi} which indicates the importance of a possible inoculation with PGPR under environmental stress situations in agricultural ecosystems. This suggests that the observed increase in *L. sativa* seedling growth inoculated with the PGPR may be related in part from the increase in fungal diversity in response to elevated CO_2 and water stress. In previous results, we have also recorded that the positive effect of both microbial inoculants on soil structural stability at elevated CO_2 was more pronounced after soil drying, which is one of the most important properties controlling plant growth (Kohler et al. 2009).

To our knowledge, this is the first time that the effect of drought and elevated CO_2 on *Pseudomonas* communities was investigated in a short-term experiment because the few studies which investigated shifts of *Pseudomonas* populations under elevated CO_2 were all long-term experiments. The approach of Tarnawski et al. (2006) investigated cultivable *Pseudomonas* associated with the soil and the rhizosphere of *Lolium perenne* and *Molinia caerulea* grown under ambient and elevated CO_2 conditions. *Pseudomonas* densities were not consistently influenced by CO_2 treatment after 8 years of exposure to elevated CO_2 concentrations. In contrast, with a cloning-sequencing approach, Marilley et al. (1999) showed a stimulation of *Pseudomonas* in the rhizosphere of *L. perenne* after 3 years of elevated CO_2. Such a difference could be explained by their different methodological approach (molecular vs. cultural). A possible resilience of *Pseudomonas* populations towards long-term elevated CO_2 may also be possible. In our experiment, which was established in a short-cycle crop, we did not measure the population densities, but we also found an influence of elevated CO_2 in the DGGE dendrogram of the *Pseudomonas* populations. This supports the findings of Marilley et al. (1999), although the Shannon diversity index for the *Pseudomonas* populations only showed significant interactions between microbial inoculation and CO_2 level. This implies that in the short-term elevated CO_2 does not change the *Pseudomonas* communities, unless they are influenced by other factors like microbial inoculation.

4.3 Effects of elevated CO_2, water stress, and microbial inoculation on soil microbial community functional diversity

In our study, microbial function was modified in response to drought without any changes in rhizosphere microbial community. The stress of drought results in reduced substrate diffusion in dry soils and increased microbial demands for C and N. Such a shift in microbial function likely altered nutrient cycling and feedbacks to plant growth, since rhizosphere microbes influence resource availability to the plant. Thus, the decreased shoot biomass in plants exposed to drought might be due to a lower availability of nutrients in soil.

Interestingly, the functional diversity of the microbial communities showed more influence of microbial inoculation than CO_2 level and contrasted with the results found with the community analysis of the DGGE. This could indicate that in microbial systems, functional stability is not necessarily related to community stability because the structure of the microbial community is dynamic (Fernández et al. 1999). On the other hand, Mayr et al. (1999) found a distinct effect of elevated CO_2 CLPPs in a grassland ecosystem, while several other soil

biological parameters did not show any response. The study of Griffiths et al. (2001) indicated that the effect of microbial diversity on microbial functions in soil depends on the measured function. According to Nannipieri et al. (2003), the functional aspects of a microbial community can be assessed only by functional assays, which integrate both microbial community and species composition. In ecology, the negative correlation between variation of functional measurements and diversity has been generally found in fairly uniform systems (Naeem and Li 1997). Redundancy of functions is believed to be typical in soil, and this could explain the reason for the lack of any observed relation between microbial diversity and soil functions. A reduction in any group of species has little effect on overall soil process since other microorganisms can carry out this function (Nannipieri et al. 2003).

5 Conclusions

Both the AM fungus, *G. intraradices*, and the PGPR, *P. mendocina*, seemed to attenuate the negative effect of drought stress to lettuce plants, especially under elevated CO_2, and to our knowledge, it is the first time to show that an PGPR inoculation may improve drought tolerance under elevated CO_2. Elevated CO_2 did slightly change microbial community structure but did not increase neither fungal nor bacterial microbial diversity. Drought provoked clear decreases in bacterial and fungal community structure. Elevated CO_2 decreased the negative effects of drought on soil structural microbial diversity but without changes in functional microbial diversity, which could indicate that they must be key organisms in soils which are resistant to drought and which maintain important soil functions. The beneficial effect of the PGPR on growth of lettuce plants might not only result from a direct PGP effect but also from an indirect shift of the fungal community in response to elevated CO_2 under water stress conditions.

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