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ORIGINAL PAPER

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Influence of a *Bacillus* sp. on physiological activities of two arbuscular mycorrhizal fungi and on plant responses to PEG-induced drought stress

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Abstract The effects of bacterial inoculation (*Bacillus* sp.) on the development and physiology of the symbiosis between lettuce and the arbuscular mycorrhizal (AM) fungi *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe and *Glomus intraradices* (Schenck and Smith) were investigated. Plant growth, mineral nutrition and gas–exchange values in response to bacterial inoculation after PEG–induced drought stress were also evaluated. In AM plants, inoculation with *Bacillus* sp. enhanced fungal development and metabolism, measured as succinate dehydrogenase (SDH) and alkaline phosphatase (ALP) activities, more than plant growth. Under non-stressed conditions, *G. intraradices* colonization increased all plant physiological values to a higher extent when in dual inoculation with the bacterium. Under stress conditions, the bacterium had an important stimulatory effect on *G. intraradices* development. Under such conditions, the effects of the bacterium on photosynthetic rate, water use efficiency (WUE) and stomatal conductance of lettuce plants differed with the fungus species. Plant-gas exchange was enhanced in *G. intraradices*- and reduced in *G. mosseae*-colonized plants when co-inoculated with *Bacillus* sp. Thus, the effects of each fungus on plant physiology were modulated by the bacterium. Stress was detrimental, particularly in *G. intraradices*-colonized plants without the bacterium, reducing intra and extraradical mycelium growth and vitality (SDH), as well as plant-gas exchange. Nevertheless, *Bacillus* sp. inoculation improved all these plant and fungal parameters to the same level as in non-stressed plants. The highest amount of alive and active AM mycelium for both fungi was obtained after co-inoculation with *Bacillus* sp. These results suggest that selected free-living bacteria and AM fungi should be co-inoculated to optimize the formation and functioning of the AM symbiosis in both normal and adverse environments.

Keywords Alkaline phosphatase · AM fungus · Bacterium co-inoculation · PEG-induced drought stress · Succinate dehydrogenase

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Introduction

The rhizosphere is a site at which symbiotic and non-symbiotic microorganisms can interact (Jeffries and Barea 1994). Ubiquitous arbuscular mycorrhizal (AM) fungi and plant-growth-promoting rhizosphere bacteria are components of natural systems that benefit the host plant (Barea and Jeffries 1995). As a result of microbial-plant associations, crop production can be increased and mixed inocula have been recommended for re-vegetation purposes (Bowen and Rovira 1999). The cooperation of AM fungi and bacteria in nutrient uptake by plants may be due to specific attributes of microorganisms, such as the ability of certain bacteria to stimulate mycorrhizal formation and development (Azcón-Aguilar and Barea 1985; Garbaye 1994), as well as mycorrhizal effects on the associated bacterial population (Amora-Lazcano and Azcón 1997; Amora-Lazcano et al. 1998). There is a growing interest in improving understanding of the diversity and significance of microbial populations in soil and their involvement in nutrient cycling. The manipulation of certain bacteria and AM fungi is important with regard to sustainability issues (Bowen and Rovira 1999; Barea et al. 2002).

Many authors have correlated the plant growth responses to mycorrhization with high amounts of AM roots and fungal physiological activity, which is closely related to mycorrhizal functioning (Tisserant et al. 1993;

Guillemin et al. 1995). It is well known that microbial activities can affect the formation of mycorrhiza (Azcón et al. 1978; Azcón 1989), but there is no information about the effect of bacterial inoculation on metabolic characteristics of AM fungi.

AM colonization is normally quantified by assessing the level (frequency and intensity) of fungal infection in plant roots after trypan blue (TB) staining (Phillips and Hayman 1970). However, Vierheilig and Ocampo (1989) reported that TB staining was not a good indicator of AM efficiency and, in AM studies related to the functional aspect of symbiosis, vital staining techniques have been suggested (Tisserant et al. 1993; Boddington and Dodd 1998). Succinate dehydrogenase (SDH) and alkaline phosphatase (ALP) activities, linked to active metabolic fungal performance, have been proposed as useful indices for the analysis of efficient arbuscular endomycorrhizal infection (Smith and Gianinazzi-Pearson 1990; Tisserant et al. 1993; Vázquez et al. 2000).

Mycorrhizal symbiosis is considered a key factor in helping plants cope with adverse environmental conditions (Jeffries and Barea 1994). Although an effect of AM endophytes on drought alleviation has been reported (Ruiz-Lozano et al. 1995a, 1995b, 2001), no information is available on the ability of plants dually inoculated with AM fungi and mycorrhiza-helper bacteria to support drought stress. The bacterium assayed here (*Bacillus* sp.) was isolated from a desert area (Alicante, Spain) and shown to stimulate growth of red clover plants (Vivas et al., unpublished results). An experimental area was chosen within a desertification-threatened region in southeast Spain for studying the role of the most common natural saprophyte and symbiotic microbial isolates in sustaining vegetation cover in such stressed environments. The AM fungi selected for this study were *Glomus mosseae* and *G. intraradices*, which are well adapted to the dry conditions of Mediterranean areas (Ruiz-Lozano et al. 1995a, 1995b).

The aim of this study was to determine the effect of inoculation with a *Bacillus* sp. from a desert area on the growth, vitality and activity of the above-mentioned fungi. An additional objective was to determine the possible importance of the bacterium in combined bacterial and mycorrhizal inocula for plant tolerance to drought stress and to define the possible mechanisms involved.

Materials and methods

Experimental design and statistical analysis

At sowing time, plants were inoculated with either *G. mosseae* or *G. intraradices* and/or *Bacillus* sp. The bacterium and each of the mycorrhizal fungi were assayed alone or in dual fungus-bacterium combinations. Thus, there were five microbial treatments plus one control treatment without inoculation. Ten replicates of each treatment were made giving a total of 60 pots. Half of the pots were cultivated without PEG application and half exposed to PEG prior to harvesting.

Data were subjected to an analysis of variance (ANOVA) followed by Duncan's multiple range test (Duncan 1955). Percentage values were arcsin transformed before statistical analysis.

Soil and biological material

Lettuce (*Lactuca sativa* L. cv Romana) was grown for 2 months in 300 g sand/vermiculite/sepiolite (1:1:1) inert medium, previously washed and sterilized by autoclaving.

The bacterial inoculum was isolated from a desert soil in Alicante province (Spain) using standard techniques following serial dilutions of the soil. The inoculum was grown on a rotary shaker (150 rpm) at 28°C for 28 h in a 250-ml flask containing 50 ml of nutrient broth (8 g l^{-1}) solution. Aliquots (1 ml) of the bacterial culture containing 10^8 CFU ml^{-1} were added to each pot.

Mycorrhizal fungal inoculum from each endophyte was multiplied in an open-pot culture of *Lactuca sativa* L. and consisted of soil, spores, hyphae and AM root fragments. The AM species were *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe, isolate BEG 122 and *G. intraradices* (Schenck and Smith) isolate BEG 121. Aliquots (5 g) of each inoculum, with similar infective characteristics (an average of 50 propagules g^{-1} according to the most probable number test), were placed below the seeds of *Lactuca sativa* L. This amount of inoculum was selected in preliminary tests as producing an optimal infection level according to the total amount of soil in the pot. Non-mycorrhizal treatments received the same quantity of autoclaved inoculum together with a 2-ml aliquot of a filtrate (less than 20 μm) of the AM inoculum, in an attempt to provide a general microbial population free of AM propagules. Two seeds were sown and thinned after emergence to one seedling per pot.

Growth conditions

The plants were grown in a controlled-environment chamber under conditions of 50% relative humidity, day and night temperatures of 27°C and 18°°C, respectively, and a photoperiod of 14 h. Photosynthetic photon flux density (PPFD) was 500 μmol m^{-2} s^{-1} as measured with a light meter (LICOR, model LI-188B).

Drought stress was induced by adding PEG solution (15% PEG) to half of the pots at two growing stages (1 week and 24 h before harvesting) as described elsewhere (Ruiz-Lozano and Azcón 1997), so that the two PEG applications had accumulative effects. A PEG solution (purified PEG 6,000 Merck, molecular weight 5,000–7,000) was used as osmoticum to induce stress. The rest of the pots were kept as controls without PEG.

The plants were given a nutrient solution (Hewitt 1952) at half-P concentration with the pH adjusted to 6.8–7. This mineral solution has been found to give high levels of infection in plants inoculated with *G. mosseae* and *G. intraradices* (Ruiz-Lozano and Azcón 1996). Mineral solution was supplied twice a week (25 ml per pot) to maintain the required water and nutrient levels throughout the experiment. A rock phosphate source from Morocco with 19 mg P_2O_5 kg^{-1} (Olsen and Dean 1965) was applied to each pot by mixing 1 g with the sand/vermiculite/sepiolite medium before sterilizing. This soil-less substrate was selected as growth medium since it provides little benefit to the AM host plant and thus allows a more direct interpretation of the mechanisms of the bacterial-fungal interaction.

Parameters measured

Before harvest, the CO_2 exchange rate, transpiration rate, substomatal cavity CO_2 concentration and instantaneous water use efficiency (WUE) were measured on the fourth leaf below the apex of each plant. Atmospheric CO_2 was measured 5 m above ground level. PPFD was 1,180 mmol m^{-2} s^{-1}, which ensured that no limitation in photon irradiance occurred (Long and Hallgren 1987). Light was provided by a halogen lamp (General Electric 300 PAR 56/WFL). A model LCA-3 portable integrated infrared CO_2

analyzer (Analytical Development Co., Hoddesdon, UK) was used for these determinations. Measurements were made 2 h after the light was turned on.

At harvest (8 weeks after planting), the root system was separated from the shoot and the fresh and dry weights of each were recorded. The roots were carefully washed and then divided into three batches: one was stained by the normal non-vital TB staining of all fungal tissues (Phillips and Hayman 1970) and the others were used for histochemical vital staining of the mycorrhizal roots to measure living (SDH) and functional (ALP) mycorrhizal fungal development.

SDH activity was revealed by the procedure described by Smith and Gianinazzi-Pearson (1990). The roots were immersed in a freshly made solution of 0.2 M Tris-HCl pH 7.0, 2.5 M sodium succinate 6-hydrate, 4 mg ml^{-1} nitro blue tetrazolium, 5 mM $MgCl_2$. Root fragments were stained overnight at room temperature and then rinsed for 15–20 min in a 3% active chlorine solution of sodium hypochlorite.

ALP was determined according to the procedure described by Tisserant et al. (1993). The roots were immersed in a freshly made solution containing 50 mM Tris-citric acid, pH 9.2, 1 mg ml^{-1} α-naphthyl acid phosphate (monosodium salt), 0.05% $MgCl_2$ anhydro, 0.05% $MnCl_2$ tetrahydrate and 1 mg ml^{-1} fast blue RR salt. Root fragments were stained overnight at room temperature and then rinsed for 15–20 min in a 1% active chlorine solution of sodium hypochlorite.

Mycorrhizal development, after either non-vital or vital staining procedures, was evaluated by the method of Trouvelot et al. (1986) (for more information, see http://www.dijon.inra.fr/bbceipm/Mychintec/Mycocalc-prg/). An estimate of the length of root colonized by the fungus (the colonization frequency, F%) is given as the ratio between colonized root fragments and the total number of root fragments observed. The colonization intensity (m%) is an estimate of the amount of root cortex that became mycorrhizal, relative only to the mycorrhizal root fraction, while M% is the colonization intensity relative to the whole root system. Arbuscule abundance (a% and A%) is an estimate of arbuscule richness in the mycorrhizal root fraction and in the whole root system, respectively.

The extraradical mycelium in the soil was determined by an adaptation of the method described by Jones and Mollison (1948). Briefly, 1 g of dry soil was treated with sodium hexametaphosphate and trypan blue (0.05%) in lactic acid. The sample was heated in a water bath at 90°C for 30 min and then sieved through a 50-µm-mesh sieve. The remaining mycelium was mixed with bacteriological agar for quantification using a gridline intersection method described by Newman (1966).

Proline content in leaves and roots was determined by colorimetry (Bates et al. 1973). Concentrations of N (micro-Kjeldahl), P (Olsen and Dean 1965) and K (Lachica et al. 1973) in the shoots were measured.

Molecular identification of the bacterial strain

Total DNA from the bacterial isolate was obtained as described by Giovannetti et al. (1990) and characterized by 16S ribosomal DNA sequence analysis. PCR was carried out with the eubacterial primers 27f and 1495r (Lane 1991), located at the 5' and 3' ends of the ribosomal rDNA sequence, respectively, which enabled us to amplify almost the entire gene. Amplification reactions were done in a 20-µl volume containing 0.5 µM of each primer, 100 µM dNTPs, 1× PCR buffer (Sigma, St. Louis, Miss., USA), 2.5 mM $MgCl_2$, 10 ng of genomic DNA and 0.25 U Taq DNA polymerase (Sigma). A Perking-Elmer/Cetus DNA Thermal Cycler was used with the following parameters: initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s, elongation at 72°C for 1 min and a final elongation at 72°C for 5 min. The amplified DNA was purified following electrophoresis through a 1.2% agarose gel with the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany) and cloned into the pGME plasmid (Promega) for sequencing. Database searches for 16S rDNA sequence similarity, using FASTA and BLAST algorithms, unambiguously identified the bacterial isolate as a member of the genus *Bacillus*. However, the exact species must still be elucidated by complementary studies.

Results

The inert mixture used in this study as rooting medium was a good substrate for mycorrhizal performance. Mycorrhizal plants grew better than non-mycorrhizal plants and developed abundant AM (Fig. 1, Table 1). Inoculation with *Bacillus* sp. alone also increased shoot and root growth and proved to be the most effective treatment for enhancing root growth in stressed plants (Fig. 1). Plants colonized by *G. intraradices* alone showed the highest shoot growth, particularly under non-stressed conditions (Fig. 1). In PEG treatments, the two AMF increased shoot biomass with similar efficiencies when inoculated independently. *Bacillus* sp. increased plant growth more effectively in non-mycorrhizal plants (Fig. 1).

In the absence of bacteria and PEG, the total infective parameters (TB staining) were highest in plants colonized by *G. intraradices* (Table 1). Nevertheless, the same infective values were attained when *Bacillus* sp. and *G. mosseae* were co-inoculated. Greatest infectivity was not

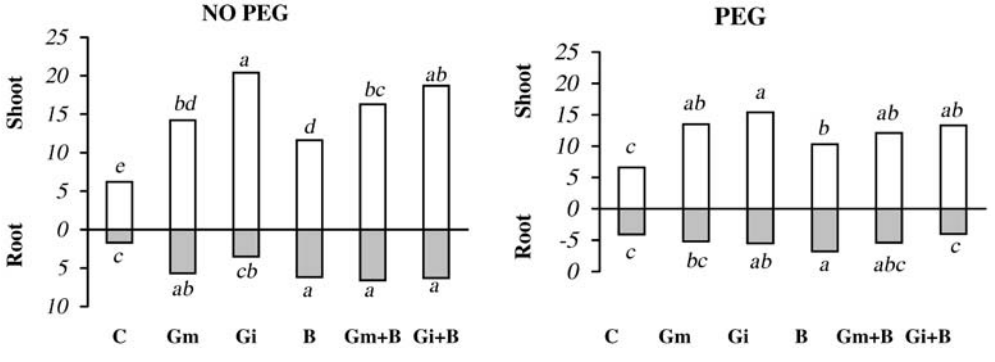

Fig. 1 Shoot and root fresh weight (g) in lettuce plants subjected or not to PEG-induced drought stress. Treatments are described as *C* (control), *B* (*Bacillus* sp.), *Gm* (*G. mosseae*), *Gi* (*G. intraradices*), *Gm+B* (*G. mosseae* + *Bacillus* sp.) and *Gi+B* (*G. intraradices* + *Bacillus* sp.). Means not followed by a common letter differ significantly (P=0.05) from each other according to Duncan's multiple range test (n=5)

Table 1 Effect of *Bacillus* sp. on arbuscular mycorrhizal colonization as measured by trypan blue (*TB*), succinate dehydrogenase (*SDH*) or alkaline phosphatase (*ALP*) staining in lettuce roots subjected or not to PEG-induced drought stress. Treatments were *Gm* (*G. mosseae*), *Gi* (*G. intraradices*), *Gm+B* (*G. mosseae* + *Bacillus* sp.) and *Gi+B* (*G. intraradices* + *Bacillus* sp.).Within each parameter, means with the same letter are not significantly different according to Duncan's multiple range test (P=0.05, n=3) (*%F* the colonization frequency, given as the ratio between colonized root fragments and the total number of root fragments observed, *%m* colonization intensity, as an estimate of the amount of root cortex that became mycorrhizal, relative only to the mycorrhizal root fraction, *%M* the colonization intensity relative to the whole root system, *%a* arbuscule abundance as an estimate of arbuscule richness in the mycorrhizal root fraction, *%A* arbuscule abundance as an estimate of arbuscule richness in the whole root system)

Staining	%F		%M		%m		%a		%A	
TB										
No PEG										
Gm	98	a	46.4	c	47.3	c	68.0	c	31.5	c
Gm+B	97	a	87.6	a	90.3	a	98.0	a	85.8	a
Gi	99	a	73.4	ab	74.1	ab	84.0	b	61.6	b
Gi+B	99	a	87.6	a	88.5	a	99.0	a	86.7	a
PEG										
Gm	97	a	44.1	c	45.5	c	88.0	ab	38.8	bc
Gm+B	97	a	69.9	b	72.0	b	89.8	ab	62.8	b
Gi	99	a	42.4	c	42.8	c	84.0	b	35.6	c
Gi+B	97	a	72.0	ab	74.2	ab	100.0	a	72.0	a
SDH										
No PEG										
Gm	99	a	33.0	c	33.3	c	67.4	c	22.2	d
Gm+B	99	a	73.0	a	73.7	a	97.1	a	70.9	a
Gi	98	a	69.7	a	71.1	a	80.5	b	56.1	b
Gi+B	97	a	76.7	a	79.0	a	97.7	a	75.0	a
PEG										
Gm	97	a	42.9	c	44.2	c	87.7	b	37.4	d
Gm+B	98	a	60.4	b	61.6	b	83.1	b	50.2	b
Gi	98	a	34.7	c	35.4	c	83.8	b	29.1	cd
Gi+B	99	a	71.7	a	72.4	a	96.1	a	68.9	a
ALP										
No PEG										
Gm	97	a	18.3	c	18.8	c	61.4	c	11.2	d
Gm+B	97	a	34.3	b	35.3	b	71.4	b	24.5	b
Gi	99	a	18.5	c	18.7	c	84.8	a	15.7	c
Gi+B	97	a	48.2	a	49.6	a	82.2	a	39.7	a
PEG										
Gm	99	a	17.5	c	17.6	c	60.8	c	10.6	d
Gm+B	98	a	47.4	a	48.3	a	76.7	b	36.4	a
Gi	98	a	30.0	b	30.6	b	62.1	c	18.5	c
Gi+B	98	a	42.2	a	43.0	a	80.9	ab	34.1	a

always related to mycorrhizal response (Table 1, Fig. 1). Bacterial inoculation did not increase shoot growth in mycorrhizal plants but did enhance mycorrhizal infective parameters compared with those of roots colonized by *G. mosseae* or *G. intraradices* alone (Table 1). The bacteria enhanced both live (SDH) and active (ALP) mycorrhizal fungal biomass (Table 1) in all treatments. Bacterial inoculation almost doubled ALP activity in both types of mycorrhizal root. Highest ALP activity was found in the absence of PEG in plants co-inoculated with *Bacillus* sp. and *G. intraradices*.

With regard to AM-colonizing parameters, *G. intraradices*-colonized roots showed higher ALP activity than *G. mosseae*-colonized roots under stress conditions, despite the fact that vital SDH activity was higher in *G. mosseae*- than in *G. intraradices*-colonized roots. Thus, the ratio of activity (ALP) to vitality (SDH) in *G. intraradices*-colonized plants was higher than in *G. mosseae*-colonized plants. Bacterial inoculation enhanced the metabolic characteristics of *G. intraradices* under stress conditions to a greater extent than in the absence of PEG. Most noticeable was that *Bacillus* sp. widely enhanced AM colonization and fungal metabolic activity in the mycorrhizal tissue (Table 1). Lettuce roots colonized by either of the AMF reached maximum infection and live and active mycelium in the presence of *Bacillus* sp.

Concerning plant-gas exchange, the transpiration rate was hardly affected by the microbial treatment, both under stress and non-stress conditions (Table 2). In contrast, the photosynthetic rate, WUE and stomatal conductance were considerably increased by both mycorrhizal fungi under non-stress conditions. The bacterial treatment had different effects on these parameters depending upon the associated *Glomus* species. Co-inoculation with *Bacillus* sp. increased photosynthesis, WUE and stomatal conductance in *G. intraradices*-colonized plants but not in *G. mosseae*-colonized ones. However, under stress conditions, *G. mosseae*-colonized plants showed the highest gas-exchange values (Table 2).

Drought stress decreased plant-gas exchange to a higher extent in plants colonized by *G. intraradices* than in those colonized by *G. mosseae*. Under such conditions, considerable differences were found in CO_2 exchange and WUE between the least effective (*G. intraradices*) and the most effective (*G. mosseae*) fungal species (Table 2). Co-inoculation with *Bacillus* sp. increased photosynthesis, WUE and stomatal conductance in *G. intraradices*-colonized plants relative to mycorrhizal inoculation alone, but not in the case of *G. mosseae*-colonized plants.

Under non-stress conditions, the proline content in leaves reached its maximum value in plants dually inoculated with *G. intraradices* and *Bacillus* sp. Under stress conditions, maximum proline accumulation was reached in the leaves of plants inoculated with *G. mosseae* alone (Fig. 2).

The highest amount of extraradical mycelium produced by *G. intraradices* in the absence of PEG correlated with the highest ability of this fungus for intraradical colonization. *Bacillus* sp. did not affect extraradical mycelium development. In contrast, under stress conditions, the bacterium increased the length of the mycelium produced by *G. intraradices* (Fig. 3).

The N, P and K contents were also affected by the microbial treatments (Fig. 4). Under non-stress conditions, N was only significantly increased by dual *G. mosseae-Bacillus* sp. inoculation, while no effect was observed under stress conditions. Plant P content was

Table 2 Effect of *Bacillus* sp. and PEG-induced drought stress on transpiration rate (mmol H_20 m^{-1} s^{-1}), photosynthetic rate (μmol CO_2 m^{-2} s−1), water use efficiency (mmol CO_2 mol H_20^{-1}) and stomatal conductance (mol H_2O m^{-1} s^{-1}) of lettuce plants. Treatments as in Table 1 plus *C* (control) and *B* (*Bacillus* sp.). Within each parameter, means with the same letter are not significantly different according to Duncan's multiple range test (P = 0.05, n = 5)

Microbial treatments	Transpiration		Photosynthetic rate		Water use efficiency		Stomatal conductance	
No PEG								
C	2.59	a	8.92	e	3.45	d	7.43	d
B	2.36	b	5.79	f	2.46	e	12.14	c
Gm	2.32	b	10.48	c	4.52	c	14.26	ab
Gi	2.34	b	12.35	b	5.28	b	13.45	b
Gm+B	2.32	b	9.81	d	4.22	c	14.09	ab
Gi+B	2.25	b	12.86	a	5.75	a	15.19	a
PEG								
C	2.22	b	4.52	bc	1.79	c	7.95	d
B	2.31	a	3.59	d	1.56	d	12.33	c
Gm	2.06	e	5.50	a	2.67	a	16.15	a
Gi	2.23	b	2.52	e	1.23	e	14.22	b
Gm+B	2.17	cd	4.22	c	1.95	c	15.04	ab
Gi+B	2.12	de	4.73	b	2.23	b	15.95	a

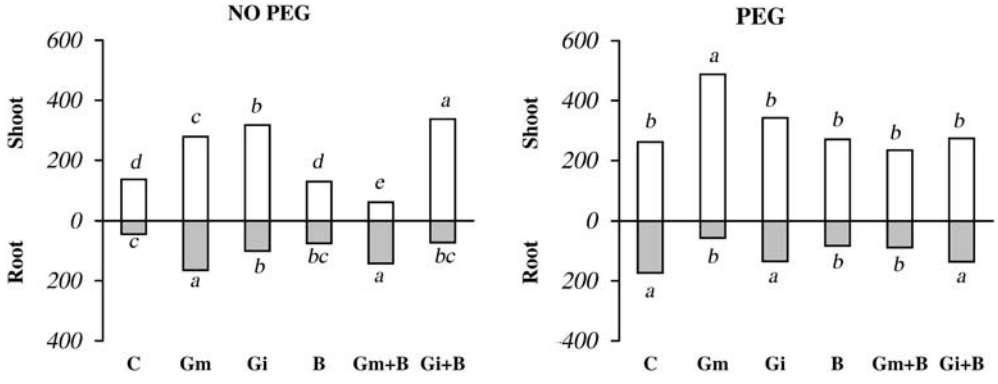

Fig. 2 Proline content (mg g FW^{-1}) in lettuce plants subjected or not to PEG-induced drought stress. Treatments as in Fig. 1. Means not followed by a common letter differ significantly (P=0.05) from each other according to Duncan's multiple range test (n=5)

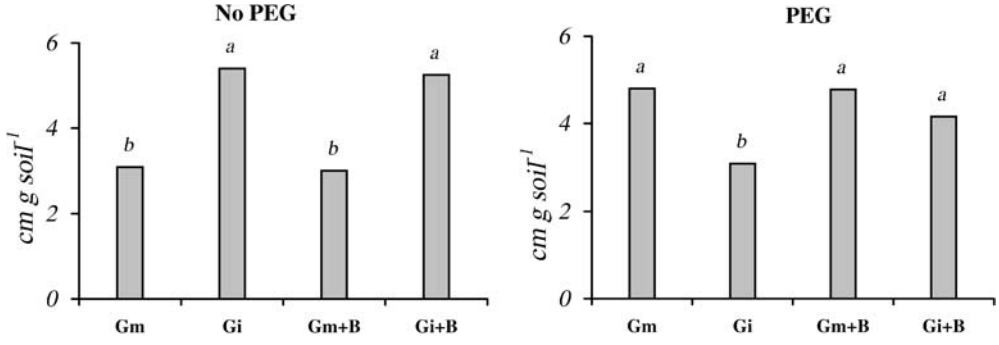

Fig. 3 Extraradical mycelium production (cm g $soil^{-1}$) by mycorrhizal plants subjected or not to PEG-induced drought stress. Treatments as in Fig. 1. Means not followed by a common letter differ significantly (P=0.05) from each other according to Duncan's multiple range test (n=5)

increased by all the microbial treatments compared with the uninoculated controls under non-stress conditions. Again, no effect on P was found under stress conditions. The K content under non-stress conditions was also increased by all treatments. In contrast, under stress conditions, plants inoculated with *G. intraradices* alone or those dually inoculated with *G. mosseae* and *Bacillus* sp. had the highest K content.

Discussion

We have shown that a rhizospheric bacterium isolated from a desert soil and identified as *Bacillus* sp. can influence the development and activity of two *Glomus* species. No information is available about the mode of action of mixed inocula on plant and/or fungal physiological status or effects on plant performance under drought stress. Under our experimental conditions, the bacterium was as effective as *G. mosseae* in increasing shoot growth and nutritional status. It also stimulated

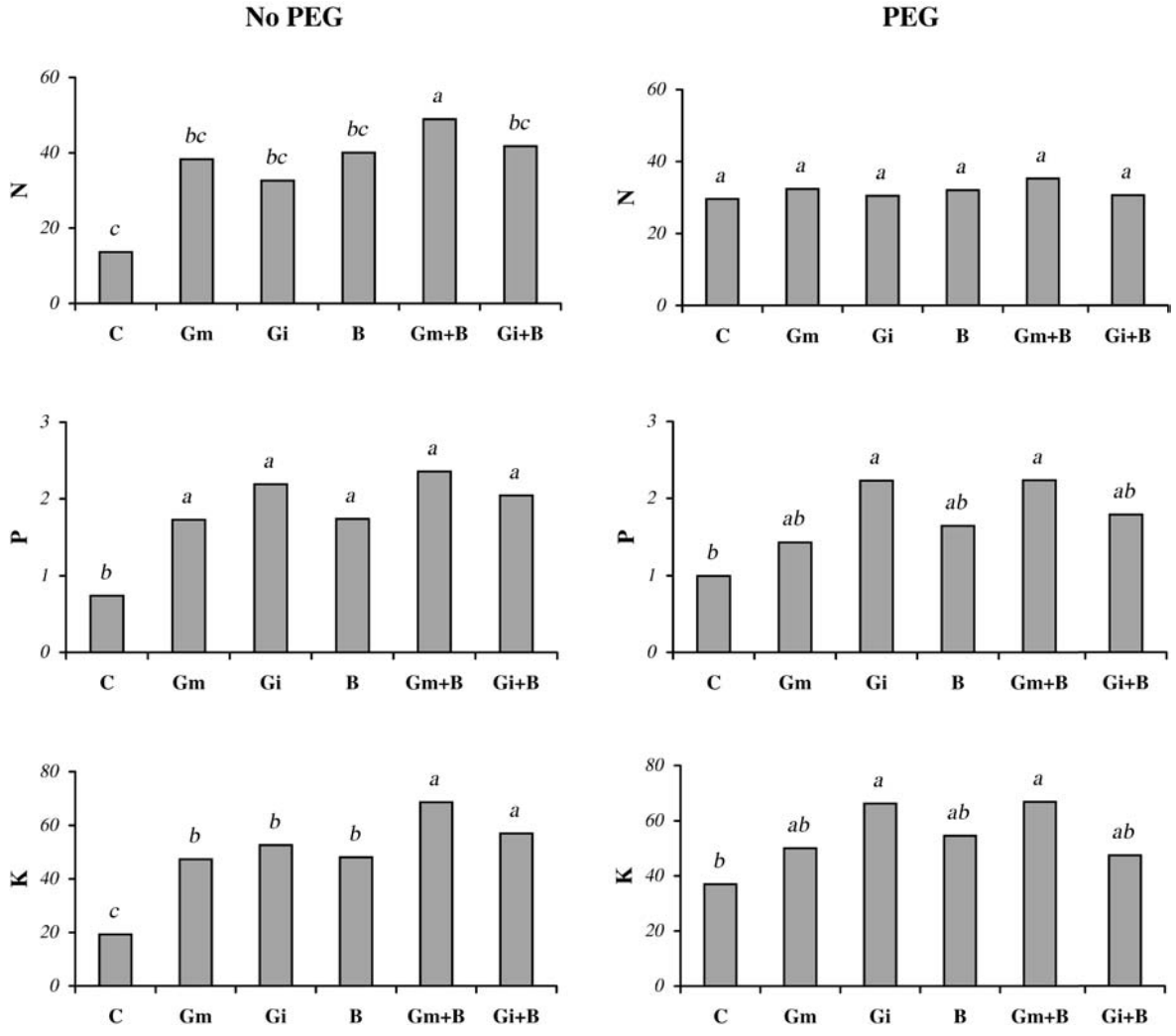

Fig. 4 N, P and K shoot contents (mg plant^{-1}) in lettuce plant subjected or not to PEG-induced drought stress. Treatments as in Fig. 1. Means not followed by a common letter differ significantly (P=0.05) from each other according to Duncan's multiple range test (n=5)

considerably the metabolic activity of the intraradical mycelium developed in *G. mosseae*- and *G. intraradices*-colonized roots, as well as the development of the extraradical mycelium of *G. intraradices*. In fact, the inoculation of AM plants with *Bacillus* sp. enhanced fungal development and metabolism (in terms of SDH and ALP) more than plant growth. The ability of *Bacillus* sp. to increase the proportions of both alive and active intraradical mycelium suggests a direct bacterial effect on the metabolic status of these fungi. Thus, the production of active metabolites such as vitamins, amino acids and growth substances (e.g. indoleacetic acid) by this bacterium (Vivas et al., unpublished results) could directly stimulate the growth and metabolic capacity of AM endophytes. The bacterium appears to act as a mycorrhiza-helper microorganism (Garbaye 1994; Barea 1997).

Another possibility is that the enhanced photosynthetic rate found in plants dually inoculated with *G. intraradices* and the bacterium affects the translocation of soluble sugars to host roots, thus increasing fungal growth and activity in the root (Amijee et al. 1989; Hetrick 1989). Nevertheless, co-inoculation of this bacterium with *G. mosseae* did not increase plant gas-exchange parameters and, curiously, it enhanced intraradical fungal growth and metabolism. The specific interactions between the bacterium and each *Glomus* sp. on plant physiology and metabolism were independent of drought stress and, thus, seem not to be related to fungal growth.

The increases in photosynthetic rate, WUE and stomatal conductance induced by bacterial inoculation of *G. intraradices*-colonized plants, and the contrasting effects on *G. mosseae*-infected plants, cannot be attributed to general stimulation by this bacterium of mycelial growth and metabolic activity in the intraradical fungal biomass developed by each endophyte. The effect seems to be due to a specific microbe-microbe interaction that modulates the effectivity of each AMF on plant physiology. The specific microbial compatibilities determined

were not translated into a higher shoot biomass under the experimental conditions used. We only found an increased plant capacity for nutrient uptake, perhaps because of higher fungal metabolic activity.

The lack of response by plants co-inoculated with *G. mosseae* and *Bacillus* sp. may be explained by the fact that the AM fungus plus the bacterium used plant carbohydrates during early plant development and, thus, created a carbon drain on the plant, as reported by Johnson et al. (1997). In plants colonized with specific microbial groups, the microbial carbon requirements and below-ground respiration seem to be higher (Pang and Paul 1980) and represent an important carbohydrate cost for the plant. However, in treatments without PEG, the mycorrhizal association stimulated photosynthetic rate (see Table 2) and compensated the C cost, as pointed out by Tinker et al. (1994). Thus, the assimilation, translocation and utilization of fixed C are integrated processes with a more complex interpretation in mycorrhizal plants (Smith and Gianinazzi-Pearson 1990).

The decrease in photosynthetic activity as a consequence of drought stress was greater in mycorrhizal plants, particularly in those colonized by *G. intraradices.* This photosynthetic depression caused by stress negatively affected both extraradical fungal development and intraradical activity (SDH). Wright et al. (1998) estimated that 4–20% of the total photosynthesized C was used by the AM endophyte, and the fungal consumption of carbohydrates can be critical for plant physiological processes when environmental conditions are limiting. The present results suggest that, under stress conditions, either the fungal respiration rate increased in *G. intraradices*-colonized roots or the high C requirements in these roots (Olsson and Johansen 2000) decreased more than the amount of C fixed in tissues colonized by this fungus (Smith and Smith 1996). Here, the differences in intraradical colonization levels do not explain differences in the photosynthetic rate between the mycorrhizal treatments under stress and non-stress conditions. However, plants would respond differently to colonization by AM fungi having different carbon requirements (Dodd et al. 2000).

The difference in the interactions between the bacterium and each AM fungus was also evident in proline accumulation in plant leaves. Proline accumulation is involved in osmotic cellular adaptation, but no clear relationship between proline, AM colonization and environmental stress has been found (Ruiz-Lozano et al. 1995a). In this study, the increasing proline content under PEG-induced drought stress is an indication of adjustment of leaf osmotic potential, required for enhanced intracellular osmotic balance. Plants exposed to drought stress usually reduce the stomatal aperture to avoid water loss, but we found no significant effect of PEG on this parameter. In contrast, stressed plants increased drought resistance by maintaining high levels of proline, photosynthetic activity and WUE. All these processes are mechanisms by which plants can cope with drought stress (Gale and Zeroni 1985; Ruiz-Lozano et al. 1995a).

The relative effectiveness of the two AM fungi results from the development of arbuscules for nutrient transfer between the symbionts (Varma and Hoock 1998). According to our study, both the quantity and activity of these arbuscules were increased by bacterial inoculation. As a result, changes in nutrient transfer to the plant and the equilibrium in the transfer of carbohydrates to the fungus must occur in dually inoculated plants. But in the soil-less medium used, with no nutrient limitation, nutrient acquisition via AM fungus was not significant for the plant. However, the effect of *Bacillus* sp. enhancing intra- and sometimes extraradical fungal growth and activity resulted in enhancement of the nutrient content of *G. intraradices*-colonized plants.

In conclusion, it has been proposed that plants must be mycorrhizal to thrive in degraded nutrient-poor and arid soils (Barea 1991, 2000) and that mycorrhizal effects can be improved by co-inoculation with mycorrhiza-helper bacteria, which can play an important role in stressed areas (Requena et al. 1996; Barea 1997). Results from this present study show that co-inoculation of selected free-living bacteria isolated from adverse environments and AM fungi can improve the formation and function of the AM symbiosis, particularly when the conditions for plants growth are also adverse. Hence, to restore a self-sustaining vegetation cover and to combat desertification, we recommend dual AM fungus-bacterium inoculation.

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