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Interactions between a plant growth-promoting rhizobacterium, an AM fungus and a phosphate-solubilising fungus in the rhizosphere of *Lactuca sativa*

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Abstract

This study evaluated the interactions between the inoculation with an arbuscular mycorrhizal fungus, *Glomus intraradices* Schenck & Smith, a plant growth-promoting rhizobacterium, *Bacillus subtilis*, and a filamentous soil fungus, *Aspergillus niger*, with respect to their effects on growth of lettuce plants and on indicators of biological soil quality (microbial biomass C, water-soluble C and carbohydrates and dehydrogenase, urease, acid phosphatase and benzoyl argininamide hydrolyzing protease activities). Water-soluble carbohydrates and microbial biomass were increased only in the rhizosphere soil of *G. intraradices*-plants. Rhizosphere soil from all microbial inoculation treatments had significantly higher dehydrogenase activity than the control soil, particularly in the soil inoculated with *B. subtilis* (about 21% higher than control soil). Inoculation with *A. niger* or *B. subtilis* increased significantly the urease, protease and phosphatase activities of the rhizosphere soil of the lettuce plants. The foliar P and K contents increased significantly with the *B. subtilis* or *G. intraradices* inoculation, alone or in combination. The most effective co-inoculation was observed in the combined treatment of inoculation with *G. intraradices* and *B. subtilis*, which synergistically increased plant growth compared with singly inoculated (about 77% greater with respect to the control plants).

Keywords: Arbuscular mycorrhizal fungi; Enzyme activity; Microbial biomass C; Plant growth-promoting rhizobacteria; Sustainable agriculture

1. Introduction

Co-inoculations of beneficial rhizosphere microorganisms into soils, reducing the inputs of environmentally deleterious agro-chemicals required for optimal plant growth, are gaining increased attention in sustainable agroecosystems (Barea et al., 1997). It is known that microorganisms are activated in the soil–plant interface where a microcosm system, the rhizosphere, develops (Cordier et al., 2000). Carbon fluxes are critical for rhizosphere functioning. Many microbial interactions are responsible for key environmental processes, such as the biogeochemical cycling of nutrients and matter and the maintenance of plant health and soil quality. There are several groups of beneficial rhizosphere microorganisms. The bacteria that provide benefits to the plant either form symbiotic relationships with the plant or are free-living in the soil, but found near or even within the roots. Beneficial free-living soil bacteria are usually referred to as plant growth-promoting rhizobacteria or PGPR (Kloepper et al., 1989), a group that includes the genus Bacillus. PGPR participate in many key ecosystem processes, such as those involved in the biological control

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of plant pathogens (Weller and Thomashow, 1994), solubilisation of nutrients (Rodriguez and Fraga, 1999) and phytohormone synthesis (Gutiérrez-Mañero et al., 2001), and therefore deserve particular attention for sustainable agriculture.

In the case of ubiquitous rhizosphere arbuscular mycorrhizal (AM) fungi, the beneficial effect on plant development including crop plants has been well studied. Inoculation with AM fungi is an effective method of enhancing the ability of the host plants to become established and to cope with stress situations such as nutrient deficiency, drought and soil disturbance (Caravaca et al., 2003a). 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 (Jeffries et al., 2003), or by improving soil aggregation in eroded soils (Caravaca et al., 2002). 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. Conversely, soil microorganisms can affect AM formation and function. Particularly, the so-called mycorrhiza helper bacteria are known to stimulate mycelial growth of mycorrhizal fungi or to enhance mycorrhizal formation (Toro et al., 1997).

Another large group of beneficial microorganisms in the rhizosphere are the phosphate-solubilising filamentous soil fungi, such as *Aspergillus niger* (Caravaca et al., 2005). Their effects may be of great interest in soils with scarce assimilable P, such as those in semiarid agroecosystems. Nevertheless, the effectiveness of phosphate solubilisation by microorganisms inoculated directly into the soil under field conditions is unclear because of the possible re-fixation of phosphate ions on their way to the root surface. The microbiologically solubilised phosphate could, however, be taken up by a mycorrhizal mycelium, thereby developing a synergistic microbial interaction (Barea et al., 1997). The combined inoculation of selected rhizosphere microorganisms has been recommended for maximising plant growth and nutrition (Probanza et al., 2001). The study of the antagonic or synergic effects of the different microbial inoculants when co-inoculated is a crucial step in the development of effective host-microorganism combinations. In previous studies, it was shown that the presence of *A. niger* stimulated the growth of ectomycorrhizal shrub species (Caravaca et al., 2005). It has also been reported that dual inoculation with *Glomus intraradices* and *Bacillus subtilis* promoted the establishment of the introduced AM fungus and increased plant biomass and tissue P accumulation (Toro et al., 1997). However, reports on co-inoculation of the AM fungi with both phosphate-solubilising fungi (Aspergillus) and plant growth-promoting rhizobacteria (Bacillus) are uncommon.

The effectiveness of microorganisms as modifiers of soil fertility and facilitators of plant establishment classically has been ascertained by measuring changes in the nutritional and development status of the plant. The use of soil biological markers related to microbial activity, for instance microbial biomass, enzyme activities and labile carbon fractions, has been proposed (Naseby and Lynch, 1997; Caravaca et al., 2003b). This approach provides a comprehensive view of the impact of an inoculant on the functioning of the soil ecosystem. However, there are relatively few studies regarding the use of such parameters as a method of monitoring the effects of microbial inoculation.

The objectives of this study were: (1) to assess the interactions between three groups of microorganisms (AM fungi, PGPR, filamentous soil fungi) with respect to their effects on the promotion of plant growth in a typical crop species (lettuce) under greenhouse conditions, and (2) to determine their combined effects on soil properties considered to be indicators of soil quality, such as labile C fractions (microbial biomass C, water-soluble C and carbohydrates) and enzyme activities (dehydrogenase, urease, protease-BAA and acid phosphatase). The selected species were: *G. intraradices*, representing the AM fungi, *B. subtilis*, representing the PGPR, and *A. niger*, a typical filamentous soil fungus.

2. Materials and methods

2.1. Soil and plant

An agricultural soil, used to cultivate lettuce was collected near Murcia (SE Spain). The climate is semiarid Mediterranean with an average annual rainfall of 300 mm and a mean annual temperature of 19.2 °C; the potential evapo-transpiration reaches 1000 mm $year^{-1}$. The main characteristics of the agricultural soil used were: pH (1:5) 8.89; electrical conductivity 0.18 dS m^{-1}; TOC 1.80%; total N 2.01 g kg^{-1}; available P, 70 μg g^{-1}; extractable K, 440 μg g^{-1}; cationic exchange capacity, 15 cmol kg^{-1}.

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

2.2. Microorganisms

The AM fungus *Glomus intraradices* (Schenk & Smith) strain EEZ1 was obtained from the collection of the experimental field station of Zaidín, Granada, Spain. AM fungal inoculum consisted of a mixture of rhizospheric soil from trap cultures (*Sorghum* sp.) containing spores, hyphae and mycorrhizal root fragments.

The *B. subtilis* (Ehrenberg) Cohn strain DSM 8563 was obtained from Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. *B. subtilis* was grown in a synthetic medium (nutrient broth, Scharlau Chemie, Spain) composed of meat and yeast extracts, peptone and sodium chloride, for 2 days at room temperature on a Heidolph Unimax1010 shaker. After the incubation period the bacterial culture contained 10^9 CFU ml^{-1}.

The strain of *A. niger* van Tieghem used was isolated from the agricultural soil using standard techniques following serial dilution of the soil. For inoculum preparation, *A. niger* was grown on PDA (potato dextrose agar, Scharlau Chemie) dishes at room temperature for 8 days and spores were suspended in sterile NaCl (0.9%) with two drops of Tween 80 as surfactant. The suspension obtained had a concentration of 10^5 spores ml^{-1}.

2.3. Experimental design and layout

The experiment was a mesocosm assay, conducted as a completely randomised factorial design with three factors. The first factor was the inoculation or not of soil with *A. niger*. The second factor was the inoculation or not of soil with *B. subtilis*. The third factor was the inoculation or not of lettuce plants with *G. intraradices*. Five replicates per treatment were carried out, making a total of 40 pots.

Four hundred grams of substrate, consisting of soil and vermiculite at a ratio of 2:1 (v/v) sterilized by autoclaving at 120 °C for 60 min in three consecutive days, were placed in 0.6-l pots. In May 2004, *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). *B. subtilis* and *A. niger* were inoculated six times during the 2 months growth period. The doses of inoculum applied corresponded to 10^{10} CFU of *B. subtilis* and 10^6 spores of *A. niger* per pot and per application. The experiment was carried out in the nursery of the University of Murcia, in Murcia, without any fertiliser treatment. The plants were well watered and kept outdoors under ambient irradiance, temperature and air humidity.

Two months after planting, plants were harvested and soil samples were taken from the pots. The soil samples were air-dried and sieved to <2 mm and divided into two subsamples. One subsample was stored at 2 °C for biological and biochemical analyses and the other was allowed to dry at room temperature for physical-chemical analysis.

2.4. Plant analyses

Fresh and dry (105 °C, 5 h) mass of shoots and roots were recorded. Plant tissues were ground before chemical analysis.

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

The foliar concentrations of P and K were determined after digestion in nitric-perchloric acid (2:1) for 2 h (Plank, 1992). The foliar P was determined by colorimetry (Murphy and Riley, 1962) and foliar K was estimated by flame photometry (Schollemberger and Simon, 1954).

2.5. Soil chemical, biological and biochemical analyses

In a 1:5 (w/v) aqueous solution, water soluble carbon was determined with an automatic Carbon Analyser for liquid samples (Shimadzu TOC-5050A) and water soluble carbohydrates were determined by the method of Brink et al. (1960).

Microbial biomass C was determined using the fumigation-extraction method (Vance et al., 1987). Ten grams of soil at 60% of its field capacity were fumigated in a 125-ml Erlenmeyer flask with purified $CHCl_3$ for 24 h. After removal of residual $CHCl_3$, 40 ml of 0.5 M K_2SO_4 solution was added and the sample was shaken for 1 h before filtration of the mixture. The K_2SO_4-extracted C was measured as indicated for water soluble carbon. Microbial biomass C was calculated as the difference between the carbon of fumigated and non-fumigated samples divided by the calibration factor (K_{EC}).

Dehydrogenase activity was determined according to García et al. (1997). For this, 1 g of soil at 60% of its field capacity was exposed to 0.2 ml of 0.4% INT

(2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazolium chloride) in distilled water for 20 h at 22 °C in the dark. The INTF (iodo-nitrotetrazolium formazan) formed was extracted with 10 ml of methanol by shaking vigorously for 1 min and filtering through Whatman no. 5 filter paper. INTF was measured spectrophotometrically at 490 nm.

Urease and *N*-α-benzoyl-L-argininamide (BAA) hydrolyzing protease activities were determined in 0.1 M phosphate buffer at pH 7; 1 M urea and 0.03 M BAA were used as substrates, respectively. Two milliliters of buffer and 0.5 ml of substrate were added to 0.5 g of sample, which was incubated at 30 °C (for urease) or 40 °C (for protease) for 90 min. Both activities were determined as the NH_4^+ released in the hydrolysis reaction (Nannipieri et al., 1980).

Acid phosphatase activity was determined using *p*-nitrophenyl phosphate disodium (PNPP, 0.115 M) as substrate. Two milliliters of 0.5 M sodium acetate buffer at pH 5.5 using acetic acid (Naseby and Lynch, 1997) and 0.5 ml of substrate were added to 0.5 g of soil and incubated at 37 °C for 90 min. The reaction was stopped by cooling at 2 °C for 15 min. Then, 0.5 ml of 0.5 M $CaCl_2$ and 2 ml of 0.5 M NaOH were added, and the mixture was centrifuged at 4000 rpm for 5 min. The *p*-nitrophenol (PNP) formed was determined by spectrophotometry at 398 nm (Tabatabai and Bremner, 1969). Controls were made in the same way, although the substrate was added before the $CaCl_2$ and NaOH.

2.6. Statistical analysis

The effects of mycorrhizal inoculation and *B. subtilis* and *A. niger* inoculations and their interactions on measured variables were tested by a three-way analysis of variance. Pearson's correlation coefficients between all the soil parameters were assessed. Statistical procedures were carried out with the software package SPSS 10.0 for Windows.

3. Results

3.1. Labile C fractions and microbial biomass C

Inoculation with *A. niger*, *B. subtilis* or *G. intraradices* alone or in combination had no significant effect on soil water-soluble C (Table 1). The rhizosphere soil of *G. intraradices*-inoculated plants had higher water-soluble carbohydrates values than that of non-inoculated plants, while the soil inoculated with *B. subtilis* showed lower values (Table 1). The microbial biomass C of the lettuce rhizosphere soil did not increase after inoculation with *B. subtilis* and/or *A. niger* but increased significantly after inoculation with *G. intraradices* (Table 1). The biomass C of both the soil co-inoculated with *G. intraradices* and *B. subtilis* and the soil co-inoculated with the two fungal inocula was greater than that of the non-inoculated soil. However, only the interaction of *A. niger* × mycorrhizal inoculation had a significant effect on soil microbial biomass C.

Table 1
Labile C fractions and microbial biomass C of rhizosphere soil of the lettuce plants in response to microbial inoculation treatments 2 months after planting (means with standard errors in parentheses, $n = 5$)

	Water soluble carbon (μg g^{-1})	Water soluble CH (μg g^{-1})	Microbial biomass C (μg g^{-1})
C	216 (6)	27 (1)	241 (2)
A	192 (6)	24 (1)	232 (6)
B	195 (5)	19 (1)	253 (10)
G	205 (6)	34 (2)	309 (7)
AB	184 (4)	17 (1)	249 (7)
AG	209 (7)	27 (2)	376 (21)
BG	213 (6)	29 (1)	338 (6)
ABG	236 (9)	28 (2)	366 (10)
A	NS*	NS	NS
B	NS	0.044	NS
G	NS	0.002	< 0.001
AB	NS	NS	NS
AG	NS	NS	0.034
BG	NS	NS	NS
ABG	NS	NS	NS

NS: not significant; C: control soil; A: inoculation with *A. niger*; B: inoculation with *B. subtilis*; G: inoculation with *G. intraradices*; AB: inoculation with *A. niger* and *B. subtilis*; AG: inoculation with *A. niger* and *G. intraradices*; BG: inoculation with *B. subtilis* and *G. intraradices*; ABG: inoculation with *A. niger*, *B. subtilis* and *G. intraradices*; CH: carbohydrates.

* Significant *P* values from three factors ANOVA (*A. niger*, *B. subtilis* and *G. intraradices*) are included.

3.2. Biochemical properties

Rhizosphere soil from the treatments involving inoculation with *A. niger*, *B. subtilis* or *G. intraradices* had significantly higher dehydrogenase activity than the control soil (Table 2), particularly the soil inoculated with *B. subtilis* (about 21% higher than control soil). Dual- and multilevel inoculation treatments also increased dehydrogenase activity with respect to the non-inoculated soil but not with respect to each inoculum applied individually. The single inoculation with *A. niger* or *B. subtilis* increased significantly the urease and phosphatase activities of the rhizosphere soil. The dual inoculations of *B. subtilis* with *A. niger* or *G. intraradices*

Table 2
Enzyme activities of rhizosphere soil of the lettuce plants in response to microbial inoculation treatments 2 months after planting (means with standard errors in parentheses, $n = 5$)

	Dehydrogenase (μg INTF g^{-1} soil)	Urease (μmol NH_3 g^{-1} h^{-1})	Protease-BAA (μmol NH_3 g^{-1} h^{-1})	Phosphatase (μmol PNP g^{-1} h^{-1})
C	48 (1)	1.03 (0.04)	0.14 (0.01)	0.33 (0.02)
A	55 (1)	1.53 (0.03)	0.20 (0.01)	0.40 (0.03)
B	59 (1)	1.40 (0.12)	0.18 (0.01)	0.59 (0.04)
G	54 (1)	0.95 (0.01)	0.21 (0.01)	0.17 (0.01)
AB	80 (1)	1.12 (0.03)	0.18 (0.01)	0.45 (0.04)
AG	64 (4)	1.22 (0.05)	0.33 (0.02)	0.38 (0.04)
BG	72 (2)	1.35 (0.07)	0.35 (0.01)	0.57 (0.02)
ABG	80 (2)	1.37 (0.07)	0.38 (0.02)	0.63 (0.02)
A	0.002*	0.012	0.014	0.017
B	<0.001	0.010	0.011	<0.001
G	0.025	NS	<0.001	NS
AB	NS	<0.001	0.029	0.002
AG	NS	NS	NS	0.004
BG	NS	0.009	NS	0.002
ABG	NS	0.018	NS	NS

NS: not significant; C: control soil; A: inoculation with *A. niger*; B: inoculation with *B. subtilis*; G: inoculation with *G. intraradices*; AB: inoculation with *A. niger* and *B. subtilis*; AG: inoculation with *A. niger* and *G. intraradices*; BG: inoculation with *B. subtilis* and *G. intraradices*; ABG: inoculation with *A. niger*, *B. subtilis* and *G. intraradices*.
* Significant *P* values from three factors ANOVA (*A. niger*, *B. subtilis* and *G. intraradices*) are included.

were less effective than single inoculation with *B. subtilis* for increasing soil urease and phosphatase activities. The mycorrhizal inoculation only had a significant effect on protease activity in single inoculation (Table 2). Except for dual inoculation with *A. niger* and *B. subtilis*, the combined inoculations increased protease activity with respect to the non-inoculated soil but the effects of their interactions were not significant.

Table 3
Growth parameters, foliar nutrients and AM colonisation of the lettuce plants in response to microbial inoculation treatments 2 months after planting (means with standard errors in parentheses, $n = 5$)

	Shoot (g dw)	Root (g dw)	Shoot/root	Phosphorus (mg $plant^{-1}$)	Potassium (mg $plant^{-1}$)	Colonisation (%)
C	2.96 (0.05)	2.04 (0.10)	1.50 (0.07)	4.2 (0.1)	92 (2)	6.5 (2.0)
A	3.55 (0.09)	4.21 (0.32)	0.95 (0.08)	4.4 (0.1)	95 (4)	0.2 (0.1)
B	4.08 (0.05)	1.76 (0.05)	2.35 (0.06)	5.7 (0.2)	168 (2)	1.6 (0.7)
G	3.11 (0.04)	1.95 (0.07)	1.63 (0.06)	7.8 (0.2)	116 (5)	59.8 (2.0)
AB	3.78 (0.06)	1.66 (0.04)	2.40 (0.05)	4.7 (0.1)	147 (2)	5.5 (1.3)
AG	3.85 (0.10)	1.84 (0.05)	2.10 (0.03)	9.8 (0.3)	142 (4)	64.4 (1.0)
BG	5.23 (0.11)	1.89 (0.07)	2.83 (0.10)	13.1 (0.2)	239 (5)	62.4 (1.6)
ABG	4.44 (0.08)	1.85 (0.04)	2.42 (0.07)	10.1 (0.2)	189 (5)	65.6 (0.6)
	Shoot	Root	Shoot/root	P foliar	K foliar	Colonisation
A	0.050*	0.024	NS	NS	NS	NS
B	<0.001	<0.001	<0.001	<0.001	<0.001	NS
G	<0.001	0.011	<0.001	<0.001	<0.001	<0.001
AB	<0.001	0.006	NS	<0.001	0.001	NS
AG	NS	0.007	0.031	NS	NS	NS
BG	0.027	<0.001	0.007	NS	NS	NS
ABG	NS	0.002	0.001	NS	NS	NS

NS: not significant; C: control soil; A: inoculation with *A. niger*; B: inoculation with *B. subtilis*; G: inoculation with *G. intraradices*; AB: inoculation with *A. niger* and *B. subtilis*; AG: inoculation with *A. niger* and *G. intraradices*; BG: inoculation with *B. subtilis* and *G. intraradices*; ABG: inoculation with *A. niger*, *B. subtilis* and *G. intraradices*.
* Significant *P* values from three factors ANOVA (*A. niger*, *B. subtilis* and *G. intraradices*) are included.

3.3. Growth parameters, foliar nutrients and colonisation

Except for the mycorrhizal inoculation, all single microbial inoculation treatments significantly increased shoot dry weight in lettuce seedlings with respect to the control plants (Table 3). Dual- and multilevel inoculations also increased shoot biomass of the lettuce plants but only the interaction between bacterial strain and mycorrhizal fungus produced a positive synergism. Thus, the highest effect on plant growth was observed in the combined treatment of inoculation with *G. intraradices* and *B. subtilis* (about 77% higher with respect to the control plants). Only the inoculation with *A. niger* in single inoculation increased significantly the root dry weight of the lettuce seedlings. In contrast, inoculation with *B. subtilis* or *G. intraradices* alone or in combination decreased root biomass, particularly in the soil inoculated with *A. niger* and *B. subtilis*.

The foliar P and K contents were increased significantly by inoculation with *B. subtilis* or *G. intraradices* (Table 3), although the interaction of the two microbial inocula produced even higher values. The effectiveness of *B. subtilis* for increasing the foliar nutrients was reduced in the presence of introduced *A. niger*, although the values reached in the dual inoculation were higher than in the control soil.

Only the mycorrhizal inoculum increased significantly the extent of AM fungal colonisation of the root system compared with the non-inoculated control treatments (Table 3). Co-inoculation with *A. niger* or *B. subtilis* did not affect root colonisation by *G. intraradices*.

Mycorrhizal inoculation was positively correlated with shoot dry weight and foliar nutrient (PK) content (Table 4). The shoot biomass was positively correlated with the enzyme activities and microbial biomass of the rhizosphere soil of the lettuce plants. However, neither growth parameters nor foliar nutrients were related to the labile C fractions.

4. Discussion

The results of this study have demonstrated a clear synergistic effect of *B. subtilis* and *G. intraradices* and additive effects in plants co-inoculated with *G. intraradices* and *A. niger* and with the three microorganisms on the shoot biomass of the lettuce plants. Garbaye (1994) postulated that bacteria such as those of the genus Bacillus, producing phytohormones and cohabiting in the rhizosphere with AM fungi, could play a helper role in the plant-fungus interaction. Although the AM fungus G. intraradices hardly enhanced lettuce seedling growth when inoculated alone, it is noteworthy that the greatest growth was achieved by mycorrhizal plants grown with *B. subtilis*. Medina et al. (2003) observed that dual inoculation of *Bacillus* spp. and AM fungi did not always increase shoot biomass of *Medicago sativa* plants compared with single AM-colonised plants. Interactions between various groups of soil bacteria and AM fungi have been observed but the mechanism of interaction is still not completely understood. Most studies to date have dealt with such interactions in relation to AM colonisation enhancement (Toro et al., 1997). Dual-inoculated and *G. intraradices*-colonised plants showed similarly high levels of mycorrhizal colonisation. Although the presence of *Bacillus* spp. or other bacteria is not essential for AM establishment, infection by AM fungi may be aided by the better infection conditions created at or near the root surface by these common rhizosphere bacteria (Barea et al., 1997). Once roots are colonised, continued spread of the colonisation may be less influenced by rhizosphere microorganisms. This may explain why AM infection was not enhanced by *B. subtilis* in *G. intraradices*-inoculated plants at the end of growth period. In the earlier stages of the plant growth, more rapid formation of AM promoted by the bacterial strain could result in more rapid development of hyphae and hence increased ion uptake and earlier induction of

Table 4
Correlation coefficients between biological and biochemical parameters and foliar nutrients and growth parameters ($n = 40$)

	Shoot	Root	Shoot/root	P foliar	K foliar
Colonisation	0.349*	NS	0.342*	0.845***	0.457**
Water soluble C	NS	NS	NS	NS	NS
Water soluble carbohydrates	NS	NS	NS	NS	NS
Microbial biomass C	0.367*	NS	0.386*	0.680***	0.423**
Dehydrogenase	0.478**	NS	0.446**	0.353*	0.562***
Urease	0.422**	0.406**	NS	NS	NS
Protease-BAA	0.474**	NS	0.345*	0.698***	0.519***
Acid phosphatase	0.654***	NS	0.521***	0.310*	0.598***

*, **, ***Significant at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. NS: not significant.

other physiological benefits from mycorrhizal symbioses. Specialised bacterial activities may also be involved in these interactions. The bacterial effect on plant growth has been attributed to auxin and gibberellin production (Gutiérrez-Mañero et al., 2001), which regulate adventitious root formation. However, it seems that these mechanisms were not involved in the promotion of plant growth since root biomass was reduced by inoculation with *B. subtilis*. The increase in lettuce plant growth may be due to enhanced supply of P and K to the crop by *B. subtilis* (Weller and Thomashow, 1994). In previous studies, it was also shown that *B. subtilis* is a phosphate- and potassium-solubilising rhizobacterium, which may enhance mineral uptake by plants by solubilising insoluble P and releasing K from silicate in soil (Toro et al., 1997). Rodriguez and Fraga (1999) suggested that the inoculated rhizobacteria could have released phosphate ions from insoluble P sources, which were then taken up by the external AM mycelium because the rhizobacteria cannot transfer it into the root. Alternatively, the improved P uptake and growth of plants could be due to the production of CO_2 by soil microorganisms. Knight et al. (1989) reported that plants infected by mycorrhizas had elevated concentration of CO_2 in the root, which were highly correlated with soil total solution P. Our results showed that the combination of *B. subtilis* and *G. intraradices* enhanced the positive effect achieved by each individual microbial inoculum on the foliar nutrient contents. These results suggest that dual inoculation of beneficial bacteria and AM fungi could, at least to some extent, compensate for nutrient deficiency in soils. These results indicate also that the two microorganisms are compatible with each other in the rhizosphere. In contrast, the lack of additive or synergistic effects from the co-inoculation of *A. niger* and *B. subtilis* on plant growth suggests the presence of competition for root exudates, and this is supported by the concentrations of labile C fractions obtained. This type of filamentous fungus is able to excrete organic acids into soil and solubilise insoluble phosphate, making it available for plants (Caravaca et al., 2005). However, there were no differences in the foliar phosphorus content between inoculated and control plants. Of particular note also was the fact that the combination of phosphate-solubilising fungus and rhizobacterium caused an antagonistic effect on the foliar P and K contents. This might have been due to a low content of organic substrates in the soil for the production of organic acids by the fungus, which would increase the concentration of phosphorus in solution.

The effect of inoculants on microbial activity in the rhizosphere is decisive for maximising plant nutrient availability, since the soil microbial community in the rhizosphere plays a key role in plant nutrition and thus in plant growth. In this study, shoot biomass was highly significantly correlated ($p < 0.01$) with soil microbial activity and biomass. Mycorrhizal colonisation may alter roots physiologically, and may result in a change in the microbial population. A direct measurement of the reactivation of microbial populations is the C-biomass. In our experiment, the mycorrhizal inoculation in combination with *B. subtilis* increased the C-biomass, by 40%, with respect to the control. Enzyme activities are sufficiently sensitive to indicate perturbations caused by microbial inoculation (Naseby and Lynch, 1997). They give an indication of ecosystem function rather than just a measurement of perturbation. Oxidoreductases, such as dehydrogenase, are involved in oxidative processes in soils and their activity mainly depends on the metabolic state of soil biota; thus, they are considered as good indicators of the soil microbial activity (García et al., 1997). The increase in microbial activity is reflected by the increase in dehydrogenase activity in the rhizosphere soil of dual-inoculated plants. The measurement of hydrolases provides an early indication of changes in soil fertility, since they are related to the mineralisation of important nutrient elements required for both plant and microbial growth (Alguacil et al., 2005). The increases observed in urease, protease-BAA, and acid phosphatase may be related to increase in the rhizosphere microbial population as a consequence of the inoculation treatments. Secretion of phosphatases by phosphate-solubilising bacteria and/or by AM fungi is a common mode of facilitating the conversion of insoluble forms of P to plant-available forms and thus enhance plant P uptake and growth of the plants (Kim et al., 1998). Thus, there was a significant correlation ($p < 0.05$) between phosphatase activity and foliar P content. The fact that the highest phosphatase activity was recorded in the rhizosphere soil of lettuce plants inoculated with *B. subtilis* may be indicating a direct bacterial secretion of this enzyme. However, it does not seem probable that mycorrhizal phosphatases contribute to the P nutrition of AM plants.

In conclusion, the most effective treatment, with respect to improving the performance of lettuce plants under greenhouse conditions, was the combined inoculation of an AM fungus and a rhizobacterium (PGPR). The contribution of AM symbiosis to the nutrient status of plants may be increased by the presence of selected rhizobacteria. The increases in microbial biomass and enzyme activities suggest that the proliferation of introduced and/or native microflora could also have contributed to improvement in plant growth. However,

the level of AM root infection was not related to the ability of the co-inoculated treatments to improve plant growth. The effectiveness of the filamentous soil fungus for enhancing plant growth was based only on an improvement of biological quality, because it had no effect on the foliar phosphorus content of plants.

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