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Effects on yield and nutrition of mycorrhizal and nodulated *Pueraria phaseoloides* exerted by P-solubilizing rhizobacteria

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Abstract We studied the effect of bacteria involved in rock phosphate (four isolates), iron phosphate (two isolates), and aluminium phosphate (two isolates) solubilization, and two phytate-mineralizing bacteria in terms of their interaction with two *Glomus* spp. on *Pueraria phaseoloides* growth and nutrition. The plant – *Rhizobium* sp. – mycorrhiza symbiosis system may increase in yield and nutrition in association with specific rhizosphere bacteria that solubilize calcium, iron, and aluminium phosphates. No benefit from phytate-mineralizing bacteria was found under these experimental conditions. *P. phaseloides* growth responses were influenced in different ways by specific combinations of the selected bacteria and arbuscular mycorrhizal fungi. Considerable stimulation of nutrient uptake was observed with fungus-bacteria combinations of *Azospirillum* sp. 1, *Bacillus* sp. 1 or *Enterobacter* (spp. 1 or 2) associated with *G. mosseae*. The fact that *Bacillus* sp. 1, a calcium-phosphate solubilizing isolate, positively interacted with *G. mosseae* and negatively with *G. fasciculatum* is an indication of specific functional compatibility between the biotic components integrated in the system. From our results, the interactions between bacterial groups able to solubilize specific phosphate and mycorrhizal fungi cannot be interpreted as occurring only via P solubilization mechanisms since no generalized effect was obtained. Iron-phosphate solubilizing microorganisms were more active alone than in dual associations with *Glomus* sp., but the aluminium-phosphate dissolving isolates positively interacted in mycorrhizal plants. Further work is needed in this area in order to elucidate the mechanisms that affect rhizosphere microorganism interactions. *G. mosseae* was more effective but less infective than *G. fasciculatum* in most of the combined treatments.

Key words Mycorrhiza · *Glomus* sp. · P-solubilizing bacteria · Phytate-mineralizing rhizobacteria · *Pueraria phaseoloides* · Rhizosphere · Unavailable P

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Introduction

Pueraria spp. are used in tropical and subtropical regions as cover crops and fodder crops. During nodule formation these legumes have a high demand for phosphate. Much of the inorganic phosphate applied to soil as fertilizer is rapidly inmobilized and consequently unavailable to plants (Banik and Dey 1982); thus plants can become P-limited at times of high demand (Stevenson 1986). Soil P can be categorized as soil solution P, insoluble inorganic P, or insoluble organic P. Insoluble phosphates gradually release soluble P by a variety of solubilization reactions in which rhizosphere microorganisms are involved (Barea et al. 1983). Soil microorganisms such as mycorrhizal fungus and saprophytic bacteria able to solubilize insoluble sources of phosphate (Bolan et al. 1987) can play an important role in the cycling of soil P (Banik and Dey 1982; Datta et al. 1982), allowing an effective plant use of P under certain environmental conditions.

Mycorrhizal-dependent plants can take up more phosphate than non-mycorrhizal plants, mainly from the same soluble P source. Applying phosphate-solubilizing microbes may help to solubilize sources of P inmobilized as Ca, Fe, or Al salts. The products released by these microbes are more actively taken up by mycorrhizal roots, which are thus able to explore soil where phosphate ions have been liberated from insoluble sources (Azcón et al. 1976; Barea et al. 1983; Kucey et al. 1989).

Microsymbionts can differ in the degree of their functional compatibility with the host plant (Azcón 1989). Legume yields can be greatly increased by the selection of symbiotic and saprophyte organisms suited to a specific

situation (Azcón 1993). The concerted action of these biofertilizers may alleviate the problem of P deficiency in many soils with deposits of insoluble P sources (Barber 1984). Organic P, occurring in humified organic matter, can account for 2–80% of the P content of the soil (Dalal 1977). The objective of the present investigation was to select rhizosphere bacterial isolates able to solubilize different inorganic and organic phosphates, and to assess their contribution to the mycorrhizal effect and to the interaction with atmospheric N_2 fixation by nodulated *Pueraria phaseoloides*. We evaluated each of the bacterial isolates involved in rock phosphate, aluminium and iron phosphate, and phytate solubilization in terms of their interaction with the arbuscular mycorrhizal fungi *Glomus mosseae* or *G. fasciculatum*.

Materials and methods

Experimental design

The experiments consisted of assessing the influence of bacterial isolated, selected for their ability to solubilize rock phosphate (four isolates), iron phosphates (two isolates), or aluminium phosphate (two isolates), or to mineralize phytate (two isolates), on *Pueraria phaseoloides* in the presence or absence of inoculum of *G. mosseae* or *G. fasciculatum*. These treatments, replicated five times, were applied in three successive experiments: the first one for rock phosphate-solubilizing bacteria (75 pots), the second for iron- and aluminium-solubilizing bacteria (75 pots), and the third one for phytate-mineralizing bacteria (45 pots). Each experiment was set out in a random block design with one plant per pot.

Microbial isolation and inoculation

Rhizosphere bacteria were isolated from a Venezuelan tropical soil [pH (H_2O) 4.4, 12% organic matter] following the standard procedure (Barea et al. 1975). Appropriate dilutions were plated (0.2 ml) on the Ramos and Callao (1967) medium containing one an insoluble mineral phosphate (Bayovar rock phosphate, iron or aluminium phosphate) or sodium phytate. The solubilization of a precipitated calcium, iron, or aluminium phosphate in agar synthetic medium has been used as a criterion for the isolation of P-solubilizing microorganisms that produce a clear zone of P solubilization.

After 3–5 days of growth, bacteria were selected for their phosphate-solubilizing activity. Pure cultures were isolated, consisting of four rock phosphate-solubilizing isolates and two with each of aluminium, iron- and phytate-solubilizing activity.

Bacteria were identified in the microbiology laboratory in the Pharmacy Faculty (Granada University), using the standard methodology of the Gram test. The presence of spores, oxidation and fermentation of glucose, oxidase, reduction of nitrates to nitrites or to dinitrogen, catalase, motility, and growth on MacConkey's bile salt medium (Difco). The four rock phosphate-solubilizers were identified as an *Azospirillum* sp., *Bacillus* sp. 1 (*megaterium* group), *Pseudomonas* sp. 1, and an unidentified species; two phytate-mineralizers (*Pseudomonas* spp. 2 and 3), and four iron and aluminium phosphate-solubilizers (*Pseudomonas* spp. 4 and 5, and *Enterobacter* spp. 1 and 2).

One milliliter per pot (or plant) of each P-solubilizing bacterial isolate [10^8 colony-forming units (CFU) ml^{-1}] was applied to germinating seeds (inoculated at sowing time) and seedlings (15 days after sowing) in the appropriate treatments.

Inoculum (3 g) of a *G. mosseae* isolate (Nicol. and Gerd.) Gerd. and Trappe or *G. fasciculatum* (Thaxter *sensu* Gerd.) Gerd. and Trappe from a stock culture (*Allium cepa* L.) consisting of spores, mycelium, and mycorrhizal root fragments of each fungus with *Allium cepa* inoculum (5 g pot^{-1}) was placed directly in the planting hole. Both inocula were reproduced and maintained at the Estación Experimental del Zaidin, Granada, Spain. The inoculum potential was similar for both fungi.

Rhizobium cowpea (32 H 1) inoculum nodulating with *Pueraria phaseoloides* was cultured in Allen medium (1957). One milliliter (10^8 CFU ml^{-1}) was added to each pot.

A natural soil filtrate (equal soil : water volume filtered through Whatman no. 1 filter paper) containing a native microbial population, but lacking propagules of A-mycorrhiza, was added to each pot (5 ml pot^{-1}) to re-establish the soil microbiota.

Soil and plant growth conditions

The test soil was collected from Granada Province, Spain. The main characteristics of this calcareous soil were 2.9 ppm $NaHCO_3$-extractable P (Olsen), pH (H_2O) 6.7, 0.1% total N (Kjeldahl), and 0.87% organic matter. Soil was mixed with sand (5 : 2) and steam-sterilized (100 °C for 1 h on 2 successive days). Peat (1% v/v) was added to the sterilized soil-sand mixture and the new mixture was sterilized for 1 h. The peat used was an inert substrate which improved the soil-sand texture and lowered the pH of the mixture to a final pH (H_2O) of 6.3.

Pueraria phaseoloides (Kudzu) was used as the test legume plant. The seeds were scarified with 10% H_2SO_4, repeatedly washed with sterile water, and then germinated. One plant was placed in each pot.

Three different successive experiments were carried out, combining the different isolates of phosphate-solubilizing bacteria and each of the *Glomus* spp. Appropriate controls with no P solubilizers, or arbuscular mycorrhizal fungi, were also established. Each treatment was sampled five times. The pots were kept in a greenhouse under controlled conditions at 25 °C by day and 19 °C by night with a 16-h photoperiod (photosynthetic photon flux density 700 μmol m^{-2} s^{-1}) and a relative humidity of 70–90%. The plants were watered every day from below using a capillary system, and 10 ml of the Hepper and O'Shea (1984) nutrient solution without N and P was added weekly to each pot.

Yield measurements

After 10 weeks of growth (for the rock-phosphate experiment) and 7 weeks (for the iron, aluminium, and phytate experiments) the plants were harvested, and shoot and root dry weights were determined after drying for 20 h at 70 °C. Shoot tissues were analyzed for P, N, K, Ca, and Mg contents (Lachica et al. 1973). The roots were carefully observed, nodules were counted, and the central part (1 cm length) of the root system was cleared and stained for mycorrhizal infection according to the methods described by Phillips and Hayman (1970). Arbuscular mycorrhizal colonization was assessed using the gridline intersection method (Giovanetti and Mosse 1980).

The results were subjected to a random block analysis of variance. All analyses of variance were significant ($P<0.05$). The treatments (means of five replicates) were differentiated with Duncan's multiple range test by the least significance difference method ($P<0.05$).

Results

Under the experimental conditions used here, arbuscular mycorrhizal fungi and, in some cases, phosphate-solubi-

Table 1 Dry weight (mg pot^{-1}) and symbiotic colonization [% of arbuscular mycorrhizal (AM) infection and nodulation] of *Pueraria phaseoloides* inoculated with two AM fungi (*Glomus* spp.) and four rock phosphate-solubilizing bacteria. Values followed by the same letter do not differ significantly ($P = 0.05$). Nodulation: *L* less than 10 nodules; *M* between 10 and 20 nodules, *H* more than 20 nodules

Bacterial treatment	Mycorrhizal treatment	Dry weight (mg)	AM (%)	Nodules (no.)
No bacteria	None	462a	None	L
	G. mosseae	941d	36	H
	G. fasciculatum	790c	53	M
Azospirillum sp.	None	548ab	None	M
	G. mosseae	1167d	49	H
	G. fasciculatum	755bc	66	M
Bacillus sp. 1	None	590ab	None	M
	G. mosseae	1222f	58	H
	G. fasciculatum	564ab	59	M
Unidentified isolate	None	665b	None	M
	G. mosseae	1307f	60	H
	G. fasciculatum	1170df	66	H
Pseudomonas sp. 1	None	627ab	None	M
	G. mosseae	1166df	49	H
	G. fasciculatum	1024df	66	H

lizing bacteria increased the growth of *Pueraria phaseoloides* (Table 1). *G. mosseae* was the more effective endophyte. Plant growth responses were influenced in different ways by specific combinations of the selected fungi and bacteria.

Rock phosphate-solubilizing bacteria

The Rock phosphate-solubilizing isolates in dual inoculation with a mycorrhizal fungus affected the plants in different ways. The *Azospirillum* sp. did not alter plant growth significantly, whereas the unidentified isolate improved the growth of both non-mycorrhizal and mycorrhizal plants. *Bacillus* sp. 1 behaved differently, depending on the associated *Glomus* sp.; the dry weight of *G. mosseae*-colonized plants increased by 30% but those colonized with *G. fasciculatum* showed a 29% fall in weight. *Pseudomonas* sp. 1 interacted positively with *G. fasciculatum*, but did not affect control or *G. mosseae*-infected plants.

The most effective combination of biofertilizers was *G. mosseae* plus the unidentified bacterial isolate, which increased the growth of nodulated plants by 283% relative to the uninoculated controls.

Some bacterial isolates had a greater effect on nutrient acquisition than on growth (Fig. 1). For example, *Azospirillum* sp. 1, *Bacillus* sp. 1, and *Pseudomonas* sp. 1, each in dual inoculation with *G. mosseae*, increased N, P, K, and Ca contents. P and K contents were increased more than the N content by this mycorrhizal colonization.

A considerable stimulation of nutrient uptake was observed with specific fungus-bacteria inoculations. The maximum value for N uptake was reached in plants inoculated with *G. mosseae* plus *Pseudomonas* sp. 1, which contained 216% more N than the controls. For P and K acquisition, the most effective treatment was *G. mosseae* plus *Bacillus* sp., which resulted in increases of 416% P and 396% K compared with the control plants. Symbiotic colonization (Table 1) was also increased by microbial inoculation. The highest nodulation was recorded on *G. mosseae*-colonized roots and the highest percentage of arbuscular mycorrhizal fungal colonization in *G. fasciculatum*-infected roots.

Iron and aluminium phosphate-solubilizing bacteria

A significant beneficial effect on plant growth was observed following inoculation with iron phosphate-solubilizing isolates (Table 2). The growth of nodulated plants increased by 233% with *Pseudomonas* sp. 2 and by 289% with *Pseudomonas* sp. 3. The mycorrhizal fungi were also effective in stimulating plant growth. *G. mosseae* was the most effective endophyte in combination with both aluminium and iron phosphate-solubilizing bacteria. *G. mosseae* in dual inoculation with iron-solubilizing isolates gave a growth increase of 364% compared with non-inoculated plants. The aluminium phosphate-solubilizing bacterium *Enterobacter* sp. 2 gave better plant growth than *Enterobacter* sp. 1. A significant interaction was observed in dual inoculations, particularly with *G. mosseae*. The maximum growth benefit from these biological treatments was a yield increase of 543% relative to untreated controls. *G. mosseae* plus *Enterobacter* sp. 2 maximized P and K acquisition by the plant. The foliar N content was

Table 2 Dry weight (mg pot^{-1}) and symbiotic colonization (% of AM infection and nodulation) of *Pueraria phaseoloides* inoculated with two iron-solubilizing (*Pseudomonas* spp. 2 and 3) or two aluminium-solubilizing (*Enterobacter* spp. 1 and 2) bacteria. See Table 1 for further explanations

Bacterial treatment	Mycorrhizal treatment	Dry weight (mg)	AM (%)	Nodules (no.)
No bacteria	None	110a	None	L
	G. mosseae	213b	32	M
	G. fasciculatum	190b	23	M
Pseudomonas sp. 2	None	256b	None	L
	G. mosseae	396bc	63	M
	G. fasciculatum	172ab	31	M
Pseudomonas sp. 3	None	318bc	None	M
	G. mosseae	400bc	70	M
	G. fasciculatum	260bc	49	L
Enterobacter sp. 1	None	118a	None	L
	G. mosseae	565d	65	M
	G. fasciculatum	370bc	40	M
Enterobacter sp. 2	None	206b	None	M
	G. mosseae	597d	65	M
	G. fasciculatum	444bcd	53	L

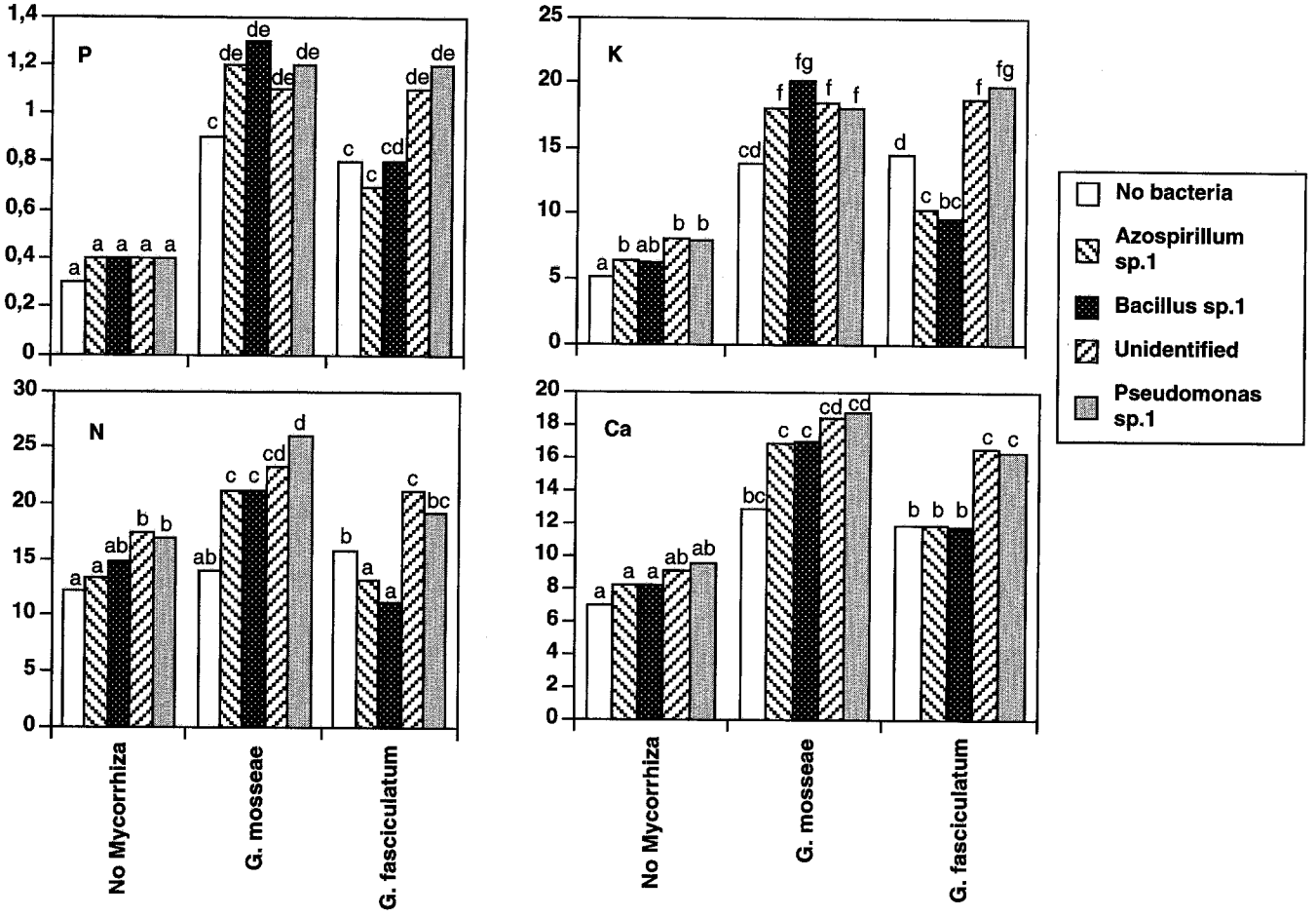

Fig. 1 P, N, K, and Ca foliar content (mg pot^{-1}) of nodulated *Pueraria phaseoloides* inoculated with one of two arbuscular mycorrhizal fungi (*Glomus* spp.) plus one of four rock phosphate-solubilizing bacteria. *Bars* surmounted with the same letter represents values that do not differ significantly ($P = 0.05$)

particularly increased by inoculation with *Pseudomonas* sp. 2 in *G. mosseae*-colonized plants.

A non-generalized effect of co-inoculation was observed with *Pseudomonas* sp. isolates 2 and 3 (Table 2), which increased the growth of non-mycorrhizal nodulated plants but did not significantly affect mycorrhizal plants. In contrast, *Enterobacter* sp. 1 considerably increased plant growth and nutrition in association with both *Glomus* spp., but not in non-mycorrhizal plants.

Iron and aluminium phosphate-solubilizing bacteria were particularly effective in stimulating colonization by *G. mosseae* (Table 2). The *G. mosseae-Enterobacter* spp. 1 and 2 interactions were the most efficient treatments for both growth and nutrition (Table 2, Figure 2).

Phytate-mineralizing bacteria

Following inoculation with phytate-mineralizing bacteria, no significant effects on plant growth or nutrition were observed (Table 3, Fig. 3). The stimulatory effect of *G. mosseae* was decreased by 50% in combination with *Pseudomonas* sp. 4. Nodule numbers and mycorrhizal colonization remained at low levels (Table 3).

Discussion

In all these experiments the growth of nodulated *Pueraria phaseoloides* plants benefited from inoculation with a mycorrhizal fungus. *G. mosseae* was the more effective endophyte. Since no significant differences were observed in the relative acquisition of nutrients by plants colonized with either endophyte, physiological aspects related to C assimilation may be involved in the different growth effects. Other direct or indirect biotic mechanisms were also operational in the tripartite symbiosis. Bacterial isolates with the property of solubilizing different inorganic (Al^{3+}, Fe^{3+}) rock phosphates or phytate had various active effects or no effect at all in association with either of two *Glomus* spp. on the growth and nutrition of nodulated plants. The results observed with the unidentified isolate (Table 1) and *Enterobacter* sp. 2 (Table 2) could be interpreted as the effects of the bacteria in dissolving the natural phosphates and thus stimulating the growth of nodulated plants (single inoculation), and in combination with more efficient P uptake from the

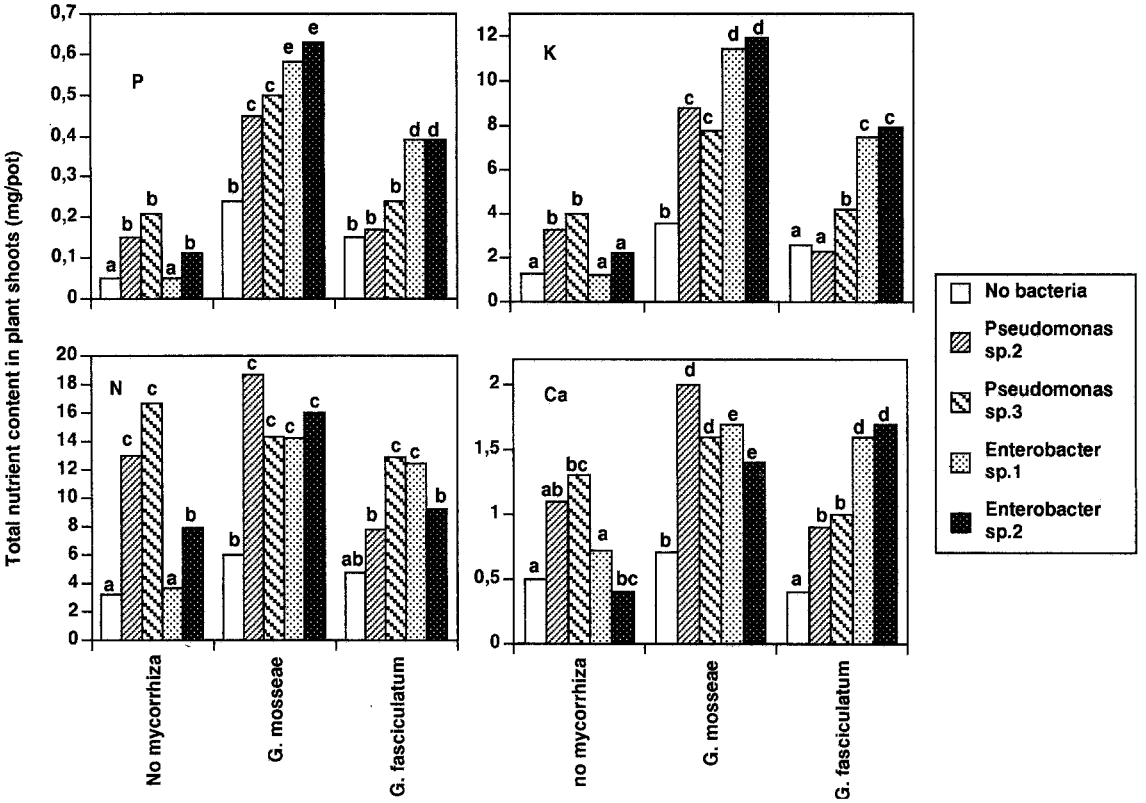

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Fig. 2 P, N, K, and Ca foliar content (mg pot^{-1}) of nodulated *Pueraria phaseoloides* inoculated with one of two arbuscular mycorrhizal fungi plus one of two iron or two aluminium phosphate-solubilizing bacteria. For further explanations see Fig. 1

Fig. 3 P, N, K, and Ca foliar content (mg pot^{-1}) of nodulated *Pueraria phaseoloides* inoculated with one of two arbuscular mycorrhizal fungi plus one of two phytate-mineralizing bacteria. For further explanations see Fig. 1

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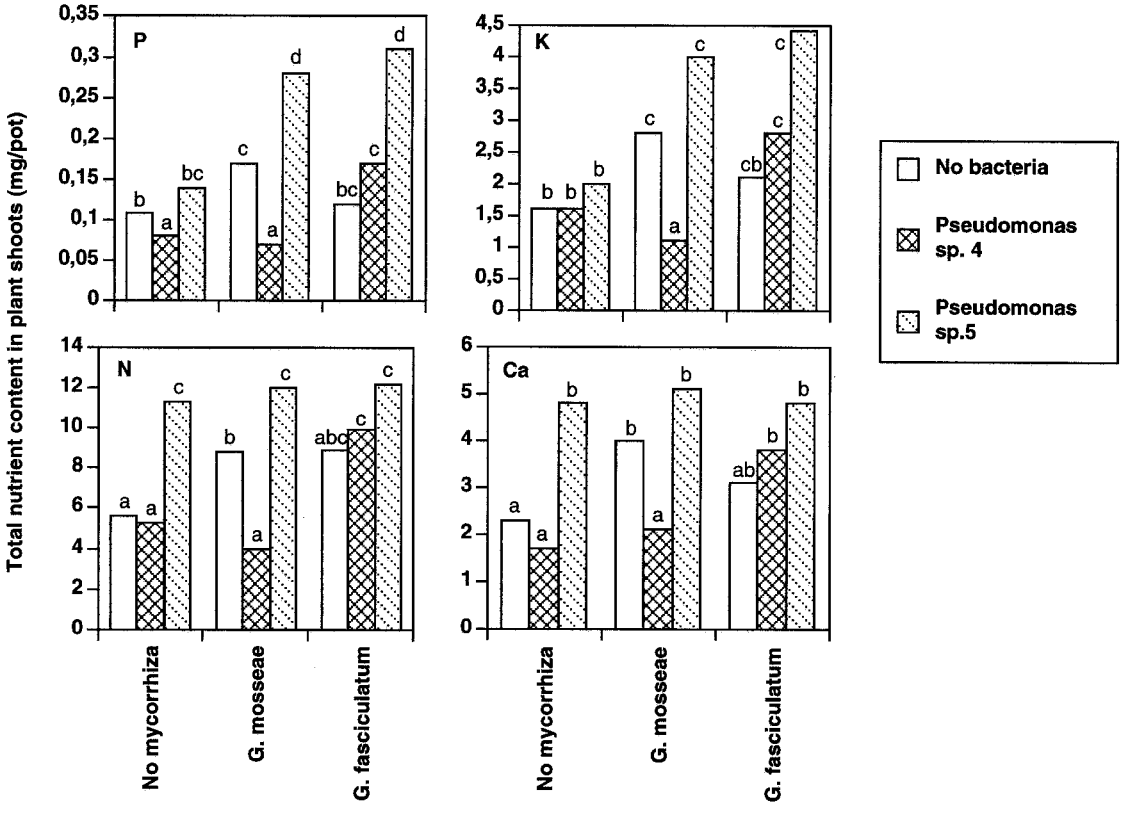

Table 3 Dry weight (mg pot^{-1}) and symbiotic colonization (% of AM infection and nodulation) of *Pueraria phaseoloides* inoculated with two phytate mineralizing bacteria (*Pseudomonas* spp. 4 and 5). See Table 1 for further explanations

Bacterial treatment	Mycorrhizal treatment	Dry weight (mg)	AM (%)	Nodules (no.)
No bacteria	None	110ab	None	L
	G. mosseae	190c	23	L
	G. fasciculatum	140bc	29	L
Pseudomonas sp. 4	None	110ab	None	L
	G. mosseae	90a	17	L
	G. fasciculatum	190c	9	M
Pseudomonas sp. 5	None	170bc	None	L
	G. mosseae	220bc	28	L
	G. fasciculatum	230c	30	L

solubilized form by mycorrhizal plants. These interactive effects have been discussed in previous studies (Barea et al. 1983).

The interactions between P-solubilizing bacteria and mycorrhizae in the present experiments cannot be generalized. The mechanisms of action involved are not likely to have consisted solely of P solubilization by the bacterial isolate in combination with increased P absortion by mycorrhizal roots. The effects observed here may be attributable on occasions to a direct interaction between the microsymbionts and the host plant (Bass et al. 1989). *Bacillus* sp. 1 (Table 1) interacted positively with *G. mosseae* and negatively with *G. fasciculatum* in terms of plant growth stimulation. Other examples of specific bacteria-*Glomus* sp. interactions were also seen (Table 3).

Bacteria belonging to the genera *Bacillus, Pseudomonas*, and others are known to bring about dissolution of insoluble phosphate compounds (Arora and Gaur 1979; Banik and Dey 1982) and may produce biologically active substances that contribute significantly to the symbiosis (mycorrhizal roots and nodule formation and/or function; (Azcón et al. 1976; Azcón 1993). But a major finding of the present study is that these microbial factors can affect plants colonized by different *Glomus* spp. in different ways (Azcón 1989). The efficiency and formation of symbiotic structures seems to be specifically affected by saprophyte microorganisms and depends on the particular groups involved. These observations indicate that the mechanisms involved in the stimulation of inhibition of the symbiosis are specific to each *Glomus* sp. This selective influence could be mediated by direct or indirect mechanisms that affect the establishment of infection (e.g. more rapid and extensive colonization) with earlier detrimental or beneficial effects (Abbott and Robson 1981). The effect could also comprise stimulation of elongation, distribution, or survival of the external infective hyphae (Azcón 1989). No specific negative interactions arose from a direct effect on infection as measured at the end of the experiment.

P-solubilizing organisms have been reported to dissolve inorganic, unavailable forms of P by excreting organic acids that directly chelate cationic partners of the P ions (Sperber 1958). Theoretical calculations have considered the quantities of acid required to release of P from phosphate materials (Sanders and Tinker 1971). It appears that the nature of acids released is likely to be more important than the quantity (Louw and Webley 1959) and their effectiveness depends upon the microenvironment produced (Piccini and Azcón 1987). If the medium has a high potential for complexing these ions no benefit from bacterial solubilization will be observed (Azcón-Aguilar et al. 1986).

The population and dynamics of taxonomic and functional groups may be particularly affected in rhizosphere soil, now appropriately called the mycorrhizosphere (Rambelli 1973). Microbial groups may vary with each arbuscular mycorrhizal fungal species since not only the extraradical mycelium of each mycorrhizal fungus may exude different metabolites but also photosynthetic rates and the partitioning of photosynthate to shoots and roots may differ in different mycorrhizal *Glomus* spp. One aspect that needs to be taken into account is the energy dynamics in the tripartite symbiosis. All these differential biochemical events can influence the interactions and the functional compatibility of the symbiotic components.

The response of the rhizosphere bacterial population to arbuscular mycorrhizal fungi may depend on the host response to the fungal species. Paulitz and Linderman (1989) found that *G. intraradices* colonized roots rapidly and reduced the overall rhizosphere population. This effect was most pronounced in the early growth stages (1- to 3-week-old-seedlings). At this time the fungus might act as a significant C sink, while the photosynthetic capacity of the plant is low. This reduction of bacterial populations was not evident in roots colonized by *G. etunicatum*, which colonizes roots more slowly. Thus, the plant response to microbial associations is difficult to generalize and will depend on the particular groups of microorganisms involved. Inherent physiological differences among fungal species and the nature of bioactive substances (beneficial or detrimental) produced by rhizospheric microorganisms may explain the effect exerted and the compatibility of associations.

The components of these tripartite microbial associations must function optimally for maximum plant growth to occur. As Linderman (1992) observed, the challenge of agrobiological studies is to characterize rhizosphere microbial relationships and to optimize all components so that they function compatibly in the system. The results of the present study show that plant growth can be improved by specific combinations of symbionts and saprophytic bacteria, which can help to obtain maximum yields. Although the benefits of using specific combinations of selected microorganisms was demonstrated only under greenhouse-controlled situations, their potential for use in sustainable agriculture promising and will be evaluated in future studies.

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