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The use of isotopic dilution techniques to evaluate the interactive effects of *Rhizobium* genotype, mycorrhizal fungi, phosphate-solubilizing rhizobacteria and rock phosphate on nitrogen and phosphorus acquisition by *Medicago sativa*

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SUMMARY

A pot experiment was designed to evaluate the interactive effects of multiple microbial inoculation treatments and rock phosphate (RP) application on N and P acquisition by alfalfa plants using ^{15}N and ^{32}P isotopes. The microbial inocula consisted of a wild type (WT) *Rhizobium meliloti* strain, its genetically modified (GM) derivative, which had an enhanced competitiveness, the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, and a phosphate-solubilizing rhizobacterium (*Enterobacter* sp.). Inoculated micro-organisms became established in the root tissues and/or in the rhizosphere soil of alfalfa plants (*Medicago sativa* L.). The GM *Rhizobium* strain did not interfere with AM formation. Inoculated phosphate-solubilizing rhizobacteria established in the alfalfa rhizosphere, but the level of establishment was lower where the natural population of phosphate-solubilizing bacteria was stimulated by AM inoculation and RP application. The stimulation of these indigenous bacteria was also greater in the rhizosphere of alfalfa nodulated by the GM *Rhizobium*. Improvements in N and P accumulation in alfalfa corroborate beneficial effects of the improved GM *Rhizobium* on AM performance, in RP-amended plants. Inoculation with *Enterobacter* did not improve the AM effect on N or P accumulation in the RP-added soil, but it did in the non RP-amended controls. Measurements of the $^{15}N:^{14}N$ ratio in plant shoots indicated enhanced N_2 fixation rates in *Rhizobium*-inoculated AM-plants, over that achieved by the same *Rhizobium* strain in non-mycorrhizal plants. Regardless of the *Rhizobium* strain and of whether or not RP was added, AM-inoculated plants showed a lower specific activity ($^{32}P:^{31}P$) than did their comparable non-mycorrhizal controls, suggesting that the plant was using otherwise unavailable P sources. The phosphate-solubilizing, AM-associated, microbiota could in fact release phosphate ions, either from the added RP or from the indigenous 'less-available' phosphate. Deficiency in Ca concentration in soil solution in the neutral test soil might benefit P solubilization. The proportion of plant P derived either from the labelled soil P (labile P pool) or from RP was similar for AM inoculated and non-mycorrhizal controls (without *Enterobacter* inoculation) for each *Rhizobium* strain, but the total P uptake, regardless of the P source, was far higher in AM-plants. *Enterobacter* inoculation seems to improve the use of RP in the rhizosphere of non-mycorrhizal plants inoculated with the WT *Rhizobium*.

Key words: Arbuscular mycorhizas, *Rhizobium*, GMO, PGPR, phosphate-solubilizing bacteria, ^{32}P, ^{15}N, rock phosphate.

INTRODUCTION

The application of efficient microbial inoculants (Elliott & Lynch, 1995) and slow-release fertilizers, like rock phosphate (Khasawneh & Doll, 1978) are low-input technological practices which might lead to the development of sustainable soil–plant systems (Barea & Jeffries, 1995). Legumes have a particular significance in such a development (Bohlool *et al.*, 1992).

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Sustainability is dependent on natural resource management where soil micro-organisms are involved (Bethlenfalvay & Linderman, 1992; Kennedy & Smith, 1995). Indeed, some members of the microbiota population can be managed to improve their performance as biofertilizers or for biological control of plant pathogens (Barea, Azcón-Aguilar & Azcón, 1997). In this context, novel techniques involving microbial molecular ecology are facilitating research on biodiversity in soil environments, and on how to exploit it as a source of new microbial inoculants. Meanwhile, *ad hoc* biotechnological approaches are using genetic modifications to generate superior strains (GMOs), which must be appropriately tested for their biosafety release. The availability and success of microbial inoculants are therefore improving (O'Gara, Dowling & Boesten, 1994).

A genetically modified (GM) *Rhizobium meliloti* strain, with an enhanced nodulation competitiveness (Sanjuan & Olivares, 1991), has been tested for effectiveness on development and function of arbuscular mycorrhiza in *Medicago sativa*. Mycorrhizal performance was used as a biosafety model system based on a functional rhizosphere. It was concluded that this GM *Rhizobium* inoculant did not interfere with mycorrhiza formation or function (Barea, Tobar & Azcón-Aguilar, 1996; Tobar *et al*., 1996). Moreover, the dual inoculation of both types of plant symbionts (the GM *Rhizobium* and the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd and Trappe) improved N and P acquisition in *Medicago sativa*. The combined effect of these microbial inoculants on N acquisition in the legume crop was probably due to an increased P acquisition enhancing N_2 fixation in the symbiotic system.

Nitrogen fixation can be measured by means of ^{15}N isotope dilution, which is the only method for estimating accurately the relative contribution of N_2 fixation or endogenous soil N to plant N content (Hardarson & Danso, 1990). In addition, ^{15}N-based techniques make it possible to distinguish whether or not particular treatments, like AM inoculation, influence N nutrition by acting on N_2 fixation or N_2 uptake (Barea, Azcón & Azcón-Aguilar, 1992). When only qualitative estimates of the N_2 fixation level are needed, as in the ranking of the effects of several microbial or chemical treatments, the ^{15}N-aided methods do not require a non-fixing reference crop. A small amount of ^{15}N tracer is added to each pot (or plot) in all treatments. At harvest, lower ^{15}N:^{14}N ratio in plant tissue indicated enhanced N_2 fixation (Danso, 1988). This qualitative approach has been used by Azcón, Rubio & Barea (1991) in AM research.

In relation to saprophytic micro-organisms used as inoculants, current practices refer to plant-growth-promoting rhizobacteria (PGPR). These bacteria, which are able to establish themselves in plant rhizosphere, participate in many key ecosystem processes, such as biological control of plant pathogens, nutrient cycling, seedling establishment, etc. (Kloepper *et al*., 1991; Glick, 1995). One group of PGPR, the phosphate-solubilizing rhizobacteria, are particularly important for the present study.

A number of *in vitro* experiments has shown that many soil micro-organisms are able to solubilize phosphate ions from sparingly soluble inorganic or organic P compounds (Kucey, Janzen & Leggett, 1989). However, the effectiveness of this process in soil is unclear because of difficulties related to the translocation of phosphate ions to the root surface, if there is any solubilization. The microbiologically solubilized phosphate would be taken up by a mycorrhizal system, thereby developing synergistic microbial interactions (Barea *et al*., 1997).

Because plant-available P is scarce in soil, the phosphate pool must be restored in any agricultural system. The use of rock phosphate (RP) has been proposed for sustainability purposes (see Zapata & Axmann, 1995). The problem with this inexpensive, sparingly-soluble form of P is its low effectiveness, particularly in non-acidic soils (Khasawneh & Doll, 1978). To improve its agronomic performance integrated approaches involving mycorrhiza–bacteria interactions were proposed (Barea, Azcón & Azcón-Aguilar, 1983).

In spite of the number of studies already done, further research is needed to differentiate clearly the sources of soil P contributing to the bioavailable pool for AM- or non-mycorrhizal plants (Stribley, 1987; Bolan, 1991). The pool of available P can be evaluated using extractants, the $NaHCO_3$-extraction (Olsen *et al*., 1954) method being one of the most commonly used. However, it has been shown (Bolan *et al*., 1984; Bolan, Robson & Barrow, 1987; Li, George & Marschner, 1991) that fungal hyphae are able to exploit forms of P which were not considered 'available' by the Olsen method.

The use of radioactive P (^{32}P) allows us to evaluate the exchange rates governing phosphate equilibrium between the solution and the solid phases of the soil (Fardeau, 1993). It has also been used to measure P availability in RP materials (Zapata & Axmann, 1995) and to identify P sources for AM and non-mycorrhizal plants (Bolan, 1991). It could therefore be applied to determine the extent of the transformation of unavailable soil P into bioavailable P by the metabolic activities of soil micro-organisms, whether they act directly on P solubilization or by modifying root uptake properties (Gianinazzi-Pearson & Gianinazzi, 1989). The so-called 'isotopically exchangeable soil P' (Fardeau, 1993) can be labelled with phosphate ions labelled with ^{32}P. The isotopic composition, or specific activity (S.A.), i.e. the ^{32}P:^{31}P ratio, is then determined in the plant tissues. In this way it can be ascertained how P

uptake is affected by microbial treatments such as AM inoculation. Bolan (1991) reviewed the related information and concluded that, in most cases, no differences in specific activity were found, regardless of whether the plants were mycorrhizal or not. The only conclusions that can be drawn is that both mycorrhizal and non-mycorrhizal plants are using similarly-labelled P from soils. This does not exclude the possibility that AM-plants can use P forms which are not available to non-mycorrhizal controls (Bolan, 1991). The use of the S.A. parameter in AM relationships is still an open research topic. No definite conclusions have been reached regarding whether or not AM-plants are able to take up P from different pools than non-mycorrhizal plants (Joner, 1994).

Although RP solubilization rarely occurs in non-acidic soils, it might take place when these soils are deficient in exchangeable Ca (Barea *et al*., 1983), this being a condition conducive to solubilization (Khasawneh & Doll 1978; Rajan, Watkinson & Sinclair, 1996). Consequently, a pot experiment was designed to evaluate, by using ^{15}N and ^{32}P isotopic dilution approaches, the effects of multiple microbial inoculation treatments and RP application on N and P acquisition by alfalfa plants growing on a neutral, low-Ca, agricultural soil.

MATERIALS AND METHODS

The trial involved factorial combinations of two *Rhizobium* inocula (wild type, (WT) and its genetically modified derivative (GM)), four microbial treatments (uninoculated control, mycorrhizal inoculation (M), phosphate-solubilizing rhizobacterium inoculation (RB), and RB + M dual inoculation), and two fertilizer treatments (unamended control and rock phosphate (RP) application). These 16 treatments were replicated five times giving a total of 80 pots that were arranged in the glasshouse in a randomized block design.

The AM fungus used was *Glomus mosseae*. (BEG 12). The *Rhizobium meliloti* strains tested were the WT GR4 isolate, and its GM derivative GR4(pCK3), developed by Sanjuán & Olivares (1991) to improve the nodulation competitiveness of the WT strain. The phosphate-solubilizing rhizobacterium was an *Enterobacter* sp. (Toro, 1996), isolated in our laboratory (Azcón, 1993). The antibiotic resistance characteristics of this strain are streptomycin 150 and spectinomycin 150. The rhizobacterial strain maintained acquired streptomycin (150 μg ml^{-1}) and spectinomycin (150 μg ml^{-1})-resistant phenotype during several successive subculturing transfers in RC liquid medium (Ramos & Callao, 1967) and its growth rate did not differ from that of the parent strain.

Alfalfa (*Medicago sativa* L., cv. Aragón) was used as the test plant. Five-d-old seedlings obtained from surface sterilized seeds were transplanted into pots (500 ml) containing an agricultural soil, in which alfalfa had never been grown, collected in the province of Granada (Spain). The characteristics of this test soil, a Cambisol, were: pH (H_2O), 6·8; available P ($NaHCO_3$-extractable) (Olsen *et al*., 1954), 15 mg l^{-1}; total N, 2600 mg l^{-1}; organic C, 0·8 %; and a texture with sand, 58·7 %; silt, 26·4 % and clay, 14·9 %. This soil was deficient in exchangeable Ca and $CaCO_3$ (Toro, 1996).

The experimental soil was sieved (4 mm), steam-sterilized (100 °C for 1 h on three consecutive days) and then re-inoculated with a soil filtrate, obtained by shaking non-pasteurized soil with water then filtering it through a Watman® no 1 filter. The filtrate was assumed to contain the natural soil microbial population minus propagules of AM fungi.

The experimental soil was divided into two batches: one unamended control and one RP added. The RP originated from Riecito (Venezuela), and contained 11·4 % total P with 6·64 % of neutral ammonium citrate-soluble P (Casanova, 1995). The application rate was 100 mg kg^{-1} soil as finely (100-mesh) ground material.

At transplanting, seedlings (one per pot) were inoculated appropriately. The mycorrhizal inoculum, obtained from a pot culture of *Lactuca sativa* L. as the host plant, contained 20 spores g^{-1} together with mycelium and mycorrhizal root fragments. Ten g per pot of this mycorrhizal inoculum was thoroughly mixed with the soil in the pot. The rhizobial and the rhizobacterial inocula consisted of 1 ml per seedling of the corresponding culture. The rhizobial cultures were prepared following standard procedures (Azcón *et al*., 1991) and contained 10^8 cells ml^{-1}. The rhizobacterial cultures were obtained in RC (Ramos & Callao, 1967) liquid medium, also following standard procedures (Azcón, 1993), and contained 10^8 colony forming units (cfu) ml^{-1}. The plants were grown in a glasshouse under a day/night cycle of 16/8 h, 25/19 °C, 50 % r.h. A photosynthetic photon flux density of 400–500 μmol m^{-2} s^{-1} was applied as supplementary light. Plants were treated with a nutrient solution (5 ml wk^{-1} per pot) (Hepper & O'Shea, 1984) lacking N and P. The pots were weighed and watered to field capacity daily.

The qualitative approach (Danso, 1988) of the isotope ^{15}N dilution technique (Hardarson & Danso, 1990) was used for N_2 fixation studies. After 10 d of plant growth each pot received a solution of $(^{15}NH_4)_2SO_4$ with 10 % ^{15}N atom excess, which supplied 2 mg N kg^{-1} soil, equivalent to 10 kg N ha^{-1}.

The isotope dilution technique (Zapata & Axmann, 1995) was used for ^{32}P studies. An aliquot containing 925 kBq ^{32}P per pot was added to obtain sufficient activity in the plant material. To prepare the ^{32}P-labelled carrier solution the total activity

required for the experiments was added as ^{32}P carrier-free to a known volume of carrier solution (KH_2PO_4) with 10 ppm P (Zapata, 1990). Labelling was done by mixing thoroughly the soil, before potting and just before planting, with 10 ml per pot of the solution containing ^{32}P phosphate ions.

Plants were harvested after 55 d of growth. Shoot d. wt was recorded after drying at 70 °C to constant weight. Shoot N and P concentrations were measured after Kjeldahl digestion or molybdenum blue procedures respectively (Lachica, Aguilar & Yañez, 1973).

The N isotopic composition of plant shoot was determined by using an automated N analyser (Fisons NA 1500 NC) interfaced to a Finnigan MAT 251 continuous-flow isotope ratio mass spectrometer (ANA-MS method) (Jensen, 1991). It is assumed that when several treatments are being tested for their effects on N_2 fixation, under equal exposure to all the pots ^{15}N-labelled fertilizer with the same ^{15}N enrichment, treatments more effective to improve N_2 fixation will lower more the atom % ^{15}N excess in a sample of plant tissue (Danso, 1988).

The ^{32}P activity in the plant material was measured by liquid scintillation (Packard Tri-Carb 300) counting of the ^{32}P, by the Cerenkov effect. Counts were corrected by counting efficiency (50 %), and expressed in Bq. The specific activity (S.A.) of P was then calculated by considering the radioactivity per amount of total P content in the plant, and expressed in Bq mg P^{-1}. The percentage of plant P derived from the labelled (L) source (%PdfL) was calculated (Zapata & Axmann, 1995) as follows:

$$\%\text{PdfL} = \frac{\text{S.A. plant in presence of RP}}{\text{S.A. plant in absence of RP}} \times 100.$$

The percentage of P in plant derived from the RP was obtained directly using isotope dilution concepts (Zapata & Axmann, 1995), where:

$$\%\text{Pdf RP} = \left(1 - \frac{\text{S.A. plant in presence of RP}}{\text{S.A. plant in absence of RP}}\right) \times 100.$$

Three extra replicate pots per treatment were also prepared in the same way, except that the isotopes were not applied. These extra pots were maintained under similar conditions to those followed for the core experiment. The extent of AM colonization and the RB survival in rhizosphere soil were then assessed at harvest in the radioactivity [^{32}P]-free rhizospheres. The extent of root AM colonization was assessed by the staining method described by Phillips & Hayman (1970). The percentage of the total root length that became mycorrhizal was calculated by a gridline-intersect technique (Giovannetti & Mosse, 1980). *Enterobacter* survival was tested in serial dilutions of rhizosphere soil samples (Lalande *et al.*, 1991). The RC solid medium was used containing streptomycin (150 μg ml^{-1}) and spectinomycin (150 μg ml^{-1}). Numbers of established *Enterobacter* were recorded.

Data were processed by ANOVA and Duncan's test ($P = 0{\cdot}05$). In the case of values given in percentage, these were subjected to arcsin square-root transformation.

Number of indigenous phosphate-solubilizing bacteria were counted on dilutions of rhizosphere soil samples plated on hydroxyapatite-amended RC medium (Raj, Bagyaraj & Manjunath, 1981). The number of introduced phosphate-solubilizing *Enterobacter* calculated as above was subtracted.

RESULTS

Plant growth and nutrient acquisition

Tables 1–3 show that most of the microbial inoculations and RP application treatments increased shoot biomass, and N and P accumulation in alfalfa plants. The most effective combination of treatments was the dual inoculation (with AM fungi and the GM *Rhizobium* strain) and RP-addition. In spite of some exceptions it can be generalized that: (i) mycorrhizal inoculation was very important in improving plant growth and nutrient acquisition; (ii) inoculated *Enterobacter* (RB) interacted positively with AM fungi in the control soil, but the dual inoculation of AM fungi and RB did not enhance growth or nutrient uptake additionally to either AM fungi or RB on RP-amended plants; (iii) for non-mycorrhizal plants *Enterobacter* inoculation (RB) improved the use of added RP, but it was not effective in the control, non RP added, soil; (iv) the GM *Rhizobium* strain was more effective than its parent WT strain, but the effects were statistically

Table 1. *Shoot d. wt (mg per pot) of alfalfa plants grown for 55 d, as affected by microbial inoculation and rock phosphate application*

Microbial treatment	Chemical treatment	
	Control	Rock phosphate
Rhizobium (WT)		
Control	146a	205b
Rhizobacteria (RB)	156a	310c
Mycorrhiza (M)	218b	290c
RB + M	272c	284c
Rhizobium (GM)		
Control	164a	365d
Rhizobacteria (RB)	176a	406ed
Mycorrhiza (M)	232b	420e
RB + M	312c	397d

WT, *R. meliloti* wild type; GM, *R. meliloti* genetically modified; RB, *Enterobacter* sp.; M, *Glomus mosseae*. Means ($n = 5$) not sharing a letter in common differ significantly ($P = 0{\cdot}05$) from each other (Duncan's multiple range test).

Table 2. *Shoot N content (mg per pot) and atom per cent ^{15}N excess (^{15}N % a.e.) in alfalfa plants grown for 55 d, as affected by microbial inoculation and rock phosphate application*

Microbial treatment	Chemical treatment			
	Control		Rock phosphate	
	N content	^{15}N % a.e.	N content	^{15}N % a.e.
Rhizobium (WT)				
Control	6·9 a	0·87 a	8·5 b	0·94 a
Rhizobacteria (RB)	6·1 a	0·81 ab	13·3 c	0·56 cd
Mycorrhiza (M)	10·5 b	0·61 c	14·4 c	0·61 c
RB + M	14·6 c	0·63 c	13·6 c	0·52 d
Rhizobium (GM)				
Control	6·8 a	0·77 b	16·0 cd	0·53 cd
Rhizobacteria (RB)	6·9 a	0·75 b	19·1 de	0·54 cd
Mycorrhiza (M)	11·9 b	0·54 cd	21·5 e	0·52 d
RB + M	15·6 cd	0·60 c	17·5 de	0·55 cd

WT, *R. meliloti* wild type; GM, *R. meliloti* genetically modified; RB, *Enterobacter* sp.; M, *Glomus mosseae*. For each parameter, means ($n = 5$) not sharing a letter in common differ significantly ($P = 0{\cdot}05$) from each other (Duncan's multiple range test).

Table 3. *Shoot P content (mg per pot) and specific activity (S.A.), as Bq mg P^{-1}, in alfalfa plants grown for 55 d, as affected by microbial inoculation and rock phosphate application*

Microbial treatment	Chemical treatment			
	Control		Rock phosphate	
	P content	S.A.	P content	S.A.
Rhizobium (WT)				
Control	0·20 a	2200 a	0·34 b	1083 b
Rhizobacteria (RB)	0·16 a	2080 a	0·69 c	900 c
Mycorrhiza (M)	0·44 bc	1333 b	0·90 d	650 d
RB + M	0·56 c	1166 bc	0·85 d	666 d
Rhizobium (GM)				
Control	0·17 a	1233 b	0·96 d	650 d
Rhizobacteria (RB)	0·16 a	1066 b	1·10 de	555 d
Mycorrhiza (M)	0·70 c	950 c	1·20 e	500 e
RB + M	1·05 d	933 c	0·96 d	566 d

WT, *R. meliloti* wild type; GM, *R. meliloti* genetically modified; RB, *Enterobacter* sp.; M, *Glomus mosseae*. For each parameter, means ($n = 5$) not sharing a letter in common differ significantly ($P = 0{\cdot}05$) from each other (Duncan's multiple range test).

significant only in RP-amended plants; (v) RP application improved the effect of any microbial treatment with respect to the corresponding control (non RP-added soil).

Isotope uptake

At least in part, the effect of certain treatments at enhancing N nutrition in alfalfa plants appears to be exerted through N_2 fixation, as shown by ^{15}N enrichment measurements (Table 2). Lowering of the $^{15}N:^{14}N$ ratio in plants, which mainly occurred in mycorrhizal plants, might indicate a relative improvement of N_2 fixation, as induced by the treatment.

Similarly, the effects of certain combinations of treatments produced a lowering in the S.A. of P in alfalfa plants (Table 3). This lowering was more evident in AM-inoculated plants and in the GM *Rhizobium* treatments, particularly when RP was added. This might indicate that these treatments are inducing the plants to absorb P from soil which is labelled differently from the P used by the corresponding control plants.

Table 4. *Percentage and total amount (μg per pot) of plant P derived from the 'bioavailable' (labelled) P (PdfL) or from rock phosphate (PdfRP) in alfalfa plants grown for 55 d, given rock phosphate, as affected by microbial inoculation*

Microbial treatment	Rock-phosphate-added pots			
	PdfL		PdfRP	
	%	mg	%	mg
Rhizobium (WT)				
Control	49	0·166 a	51	0·173 a
Rhizobacteria (RB)	43	0·296 b	57	0·393 c
Mycorrhiza (M)	48	0·432 cd	52	0·468 d
RB + M	57	0·484 d	43	0·365 c
Rhizobium (GM)				
Control	53	0·508 d	47	0·451 d
Rhizobacteria (RB)	51	0·561 e	49	0·539 e
Mycorrhiza (M)	53	0·636 f	47	0·564 e
RB + M	60	0·576 e	40	0·385 cd

WT, *R. meliloti* wild type; GM, *R. meliloti* genetically modified; RB, *Enterobacter* sp.; M, *Glomus mosseae*. For each parameter, means ($n = 5$) not sharing a letter in common differ significantly ($P = 0{\cdot}05$) from each other (Duncan's multiple range test).

Table 5. *Colonization of the rhizosphere of alfalfa plants by inoculated rhizobacteria (cfu ($\times 10^2$) g^{-1} rhizosphere soil (cfuRB), natural population of phosphate-solubilizing bacteria (cfu ($\times 10^3$) g^{-1} (cfuPSB)), nodulation (nodule number (NN) per plant^{-1}) and AM formation (percentage mycorhizal root length (%AM)) following growth for 55 d, as affected by microbial inoculation and rock phosphate application*

Microbial treatment	Chemical treatment							
	Control				Rock phosphate			
	%AM	NN	cfu RB	cfu PSB	%AM	NN	cfu RB	cfu PSB
Rhizobium (WT)								
Control		25 b		0·6 a		26 b		4·8 d
Rhizobacteria (RB)		29 b	3·00 a	0·5 a		35 a	4·59 a	4·6 d
Mycorrhiza (M)	62 a	36 a		1·9 b	67 a	38 a		8·7 ef
RB + M	69 a	37 a	6·77 b	1·8 b	72 a	39 a	3·50 a	8·5 e
Rhizobium (GM)								
Control		19 c		3·1 c		23 bc		9·6 f
Rhizobacteria (RB)		17 c	2·53 a	3·4 c		26 b	3·33 a	8·9 ef
Mycorrhiza (M)	65 a	13 d		5·3 d	70 a	26 b		19·3 g
RB + M	70 a	15 cd	4·00 ab	5·2 d	75 a	25 b	0·20 c	18·7 g

WT, *R. meliloti* wild type; GM, *R. meliloti* genetically modified; RB, *Enterobacter* sp.; M, *Glomus mosseae*. For each parameter, means ($n = 5$) not sharing a letter in common differ significantly ($P = 0{\cdot}05$) from each other (Duncan's multiple range test). Numbers were rounded after statistical analyses.

Data recorded in Table 3 allow for the calculation of the origin of the P absorbed by the plants from RP-amended soil, and the results are summarized in Table 4. It is evident that plants are using both the 'bioavailable' endogenous soil P and that in the RP particles, as released to become available by the microbial treatments. The relative contribution of the different P sources to plant P content (%PdfL vs. %PdfRP) depends on the treatment involved. Mycorrhizal inoculation (*M*) did not change the proportion (PdfL vs. PdfRP) compared with control (non-mycorrhizal, non-RB-inoculated) plants, for each *Rhizobium* strain.

Establishment of inocula in the rhizosphere

The establishment of the nodular and mycorrhizal symbioses and that of the inoculated rhizobacteria was evaluated (Table 5).

For each microbial and chemical treatment, the GM rhizobial strain formed fewer total nodules than

the WT strain. Inoculated *Enterobacter* (RB) established in the rhizosphere of AM plants, but to a lesser extent in the RP-added rhizospheres. It is worth noting the low level of RB establishment in GM *Rhizobium*-inoculated and RP-added AM plants, and the stimulation of the indigenous phosphate-solubilizing bacteria, as induced by either AM inoculation or RP addition. Such a stimulation was more evident in the GM− than in the WT *Rhizobium*-inoculated plants (Table 5). The inoculation with *Enterobacter* (RB) did not change significantly the level of stimulation of naturally-present phosphate solubilizers (Table 5).

DISCUSSION

The impact of a biotechnological practice (microbial inoculation), combined with a low-input technology (rock phosphate application), has been studied, and its effectiveness in improving nutrient supply to plants is demonstrated. Such agrotechnological practices rely on optimizing the performance of soil microbiota (Glick, 1995; Kennedy & Smith, 1995; Elliot & Lynch, 1995; Barea & Jeffries, 1995). Both a pot bioassay, to mimic interactions which occur in living soil–plant situations, and isotopic approaches, were used to evaluate the effectiveness of microbiologically-mediated activities with regard to N and P supply.

Inoculated micro-organisms were established in the root and/or in the rhizosphere soil of alfalfa plants. The GM *Rhizobium* strain, which is known to form fewer, but more active, nodules (Sanjuán & Olivares, 1991) owing to its enhanced competitiveness, did not interfere with AM formation, as previously shown (Barea *et al*., 1996). Inoculated phosphate-solubilizing rhizobacteria were established in the alfalfa rhizosphere, but the level of establishment was lower where the natural population of such bacteria was stimulated by certain treatments, such as inoculation with AM fungi and RP application, supporting previous findings (Barea *et al*., 1983, 1997). The stimulation of indigenous bacteria was also higher in the rhizosphere of alfalfa nodulated by the GM *Rhizobium* (vs. the WT strain). This *Rhizobium* genotype effect might be due to changes in the root exudation patterns induced by the GM strain (Tobar *et al*., 1996).

Improvements in N and P accumulation due to the interaction between AM fungi and *Rhizobium* dual inoculation (RB inoculation not involved) were found in this study, a well documented effect (Barea *et al*., 1992) which requires no additional discussion. It has been accepted that the total N and P content are useful parameters, since they take into account well-balanced effects on N and P concentration in plant tissues and biomass production (Jarrel & Beverley, 1981). In fact it was the data on N and P content in alfalfa which allowed us to identify beneficial effects of the improved GM *Rhizobium* on AM performance in RP-amended plants.

Inoculation with selected rhizobacteria (RB) did not improve the AM effect on N or P accumulation in the RP-added soil, but it did in the non RP-amended, control pots. This might be related to the increased number of natural phosphate solubilizers in the combined treatment of AM fungi and GM *Rhizobium* inoculation, plus RP addition. The resulting effect of the microbial component on plant growth and nutrition was not greater than that of its appropriate control (GM *Rhizobium*+RP). One logical explanation of this result is that the stimulated bacteria might immobilize a certain amount of released P, since the bacteria need it for growth.

There are obvious advantages to using isotopes in the measurement of nutrient sources contributions to plant nutrition in plant–microbe interactions. However, for microcosm-type bioassays a compromise must be maintained between the rates of radioactivity application (^{32}P) and the harvest time, which in turn will affect the feasibility of ^{15}N isotope use for N_2-fixation measurements. In fact, in order to obtain sufficient radioactivity in plant material, without exceeding the safety level of the radioisotope, the plant growth period must be relatively short. This contrasts with the longer growth period which is required when measuring N_2-fixation with ^{15}N-based methods. The amounts of ^{32}P and ^{15}N used in this experiment, and the 55-d growth period, allowed us to obtain: (i) quantitative data regarding some of the P sources used by the plants and (ii) qualitative estimates of the N_2 fixation ability of the plant–microbe system.

Measurements of the $^{15}N:^{14}N$ ratio in plant shoots suggest an enhancement of the N_2 fixation rates in *Rhizobium*-inoculated AM-plants, over that achieved by the same *Rhizobium* strain in non-mycorrhizal plants. This AM effect is well documented (Barea *et al*., 1992).

Regardless of the *Rhizobium* strain and of whether or not RP was added, AM-inoculated plants showed a lower specific activity ($^{32}P:^{31}P$) than did their comparable non-mycorrhizal controls. This contrasts with previous findings (Bolan, 1991) supporting, in most cases, a similar specific activity in plant tissues for both AM and non-mycorrhizal plants. Differences in specific activity values mean that mycorrhizal plants are taking soil P which is labelled differently from that taken up by non-mycorrhizal controls (Bolan, 1991). It could be argued that the treatments which provoked the lower specific activity values suggest that the plant is using extra ^{31}P released from otherwise unavailable P sources. The phosphate solubilizing, AM-associated microbiota, could in fact release phosphate ions, either from the added RP or from the indigenous 'less-available' phosphate. Deficiency in Ca in soil solution might enhance the solubilization of P ions

from the RP particles (Khasawneh & Doll, 1987; Rajan *et al*., 1996).

If the $^{32}P:^{31}P$ ratio in soil solution is uniform both spatially and temporally, this will produce a similar $^{32}P:^{31}P$ ratio (specific activity) in the plant, whether mycorrhizal or not. In short-term experiments ^{32}P can be expected to exchange with only the 'labile' fraction of soil P (Bolan, 1991). Conversely, if a given microbial treatment involving mycorrhizas develops a process of P solubilization, the released P ions constitute a part of the total ^{31}P pool. Such microbial activity could result in a lower specific activity in the host plant than that in the appropriate controls which have not received the microbial treatment (responsible for the P solubilization process described above).

Previous discussions have suggested that the specific activity parameter alone does not allow us to reveal differences in plant uptake from P pools of varying availability (Bolan, 1991; Joner, 1994). In spite of this, it appears to be useful to evaluate P availability in the RP material by applying isotope dilution concepts (Zapata & Axmann, 1995). The present study obtained valuable information precisely by means of these conceptual approaches. First of all, the proportion of plant P according to its origin (%PdfL vs. %PdfRP) was about the same for AM inoculated and non-mycorrhizal controls (non RB inoculated), for each *Rhizobium* strain. However, the total P uptake from both sources, L (labelled soil P) and RP, was far higher in AM-plants. These data corroborated the well-known fact that mycorrhizas increase P uptake and that they do so because the symbiosis explores 'the soil volume more thoroughly to find more of the point sources of P' (Bolan, 1991). *Enterobacter* (RB) inoculation seems to improve the use of RP in the rhizosphere of non-mycorrhizal plants, but only in those inoculated with the WT *Rhizobium*.

The microbial inoculation treatments, particularly those involving rhizobacteria in mycorrhizal alfalfa nodulated with the GM *Rhizobium*, used proportionally more P from the labile fraction in soil than from RP. Again, the stimulated natural population of phosphate-solubilizing bacteria could be involved. These bacteria might solubilize endogenous P sources better than the added RP, and these P ions will be ^{32}P-labelled by the time they reach the soil solution. As is known, mycorrhizal hyphae can intercept some P immobilization by soil microorganisms, and by other soil components, like clay minerals (Joner & Jakobsen, 1994). AM hyphae will therefore transport to the plant ^{32}P labelled phosphate from the labile fraction of the soil. This might increase the use of P from soil labile pool (PdfL) when compared with the phosphate rock (PdfRP) by AM plants. As has been previously shown using labelled insoluble P sources, when AM hyphae still have soil microhabitats to be explored for P supply, they 'prefer' to tap P from these microhabitats (L fraction) than from the insoluble source (like RP) (Azcón-Aguilar *et al*., 1986).

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