Nutrient Cycling in Agroecosystems **63:** 35–42, 2002.

The application of isotopic (^{32}P and ^{15}N) dilution techniques to evaluate the interactive effect of phosphate-solubilizing rhizobacteria, mycorrhizal fungi and *Rhizobium* to improve the agronomic efficiency of rock phosphate for legume crops

J.M. Barea[1,*], M. Toro[1], M.O. Orozco[3], E. Campos[2] & R. Azcón[1]
[1]*Departamento de Microbiología del Suelo y Sistemas Simbióticos;* [2]*Departamento de Ciencias de la Tierra y Química Ambiental Estación Experimental del Zaidín (CSIC) C/Profesor Albareda 1, 18008 Granada, Spain;* [3]*Instituto de Sistemática y Ecología, Academia Cubana de Ciencias Habana, Cuba (*Corresponding author: JMBAREA@EEZ.CSIC.ES)*

Key words: Arbuscular mycorrhiza, ^{15}N, ^{32}P, phosphate solubilizing rhizobacteria, *Rhizobium*, rock phosphate

Abstract

A pot experiment was designed to evaluate the interactive effects of multifunctional microbial inoculation treatments and rock phosphate (RP) application on N and P uptake by alfalfa through the use of ^{15}N and ^{32}P isotopic dilution approaches. The microbial inocula consisted of a wild type (WT) *Rhizobium meliloti* strain, the arbuscular mycorrhizal (AM) fungus *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe, and a phosphate solubilizing rhizobacterium (*Enterobacter* sp.). Inoculated microorganisms were established in the root tissues and/or in the rhizosphere soil of alfalfa plants (*Medicago sativa* L.). Improvements in N and P accumulation in alfalfa corroborate beneficial effects of *Rhizobium* and AM interactions. Inoculation with selected rhizobacteria improved the AM effect on N or P accumulation in both the RP-added soil and in the non RP-amended controls. Measurements of the ^{15}N/^{14}N ratio in plant shoots indicate an enhancement of the N_2 fixation rates in *Rhizobium*-inoculated AM-plants, over that achieved by *Rhizobium* in non-mycorrhizal plants. 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" soil phosphate. A low Ca concentrations in the test soil may have benefited P solubilization. Under field conditions, the inoculation with AM fungi significantly increased plant biomass and N and P accumulation in plant tissues. Phosphate-solubilizing rhizobacteria improved mycorrhizal responses in soil dually receiving RP and organic matter amendments. Organic matter addition favoured RP solubilization. This, together with a tailored microbial inoculation, increased the agronomic efficiency of RP in the test soil that was Ca deficient at neutral pH.

Introduction

Current developments in sustainability involve a rational exploitation of soil microbial activities (Barea et al., 1997) and the use of less expensive and bioavailable sources of plant nutrients like rock phosphates (Zapata and Axmann, 1995), which may be made available by microbiologically-mediated processes (Barea et al., 1997). Both saprophytes and mutualistic symbionts are involved in microbial management approaches (Barea et al., 1997). Among other microbial types, the saprophytes include the so-called plant growth-promoting rhizobacteria (PGPR) which participate in many key ecosystem processes such as those involved in the biological control of plant pathogens, nutrient cycling and seedling establishment (Glick, 1995; Kloepper et al., 1991). One group of PGPR, the phosphate-solubilizing rhizobacteria, are particularly important for the present study.

With regard to mutualistic symbionts, mycorrhizal fungi must be considered for sustainability issues (Barea et al., 1997). These fungi, upon root col-

onization, develop an external mycelium which is a bridge connecting the root with the surrounding soil microhabitats. Therefore, the mycorrhizal symbiosis, by linking the biotic and geochemical portions of the ecosystem, can contribute to nutrient capture and supply (Jeffries and Barea, 1994). Particularly, the arbuscular mycorrhizal (AM) symbiosis plays a direct role in nutrient cycling rates and patterns in both agroecosystem and natural environments (Bethlenfalvay and Linderman, 1992; Joner and Jakobsen, 1994).

It has been shown that many soil microorganisms are able to solubilize phosphate ions from sparingly soluble inorganic or organic P compounds in vitro (Barea et al., 1983). Nevertheless, the effectiveness of this process in soil is unclear because of the transient nature of the compounds released by these microorganisms, responsible for phosphate solubilization, and the possible re-fixation of phosphate ions on their way to the root surface, if any solubilization does take place. The microbiologically solubilized phosphate, however, would be taken up by a mycorrhizal mycelium, thereby developing a synergistic microbial (mycorrhizosphere) interaction (Barea et al., 1997).

Because assimilable P is scarce in soil, the phosphate stock must be restored in any agricultural system. Rock phosphate (RP) can be used but the problem with this sparingly soluble form of P is its low effectiveness in many cases (Rajan et al., 1996; Zapata and Axmann, 1995). Integrated approaches involving mycorrhizosphere interactions have been proposed to improve P bioavailability from RP sources, therefore, its agronomic performance (Toro et al., 1997). Although RP solubilization rarely occurs in non-acidic soils, it may take place when these soils are deficient in exchangeable Ca, because this characteristic facilitate P solubilization (Khasawneh and Doll, 1978). Organic matter additions, which also affect microbial interactions, may facilitate RP solubilization (Khasawneh and Doll, 1978).

Radioactive P (^{32}P) has been applied as a means of evaluating the exchange rates governing phosphate equilibrium between the soil solution and the solid phases of the soil (Fardeau, 1993). It can also be used to measure P availability in RP materials (Zapata and Axmann, 1995) and to identify P sources for AM and nonmycorrhizal plants (Bolan, 1991). The isotopic composition, or specific activity (SA), in plant growing in ^{32}P-labelled soil can be affected by treatments such as AM inoculation so that a lowering in the SA compared to that in control plants would indicate that the plant is using extra ^{31}P solubilized from microbial activity, from otherwise unavailable P sources (Toro et al., 1997). It is assumed that all 'labile' P attain isotopic exchange within the experimental period.

From the point of view of the crop plant species, legumes have a particular significance in sustainable development (Bohlool et al., 1992), but it is known that an adequate supply of available P is a critical constrait for nitrogen fixation (Barea and Jeffries, 1995). However P can be supplied by the AM symbiosis. Nitrogen fixation rates are measured by means of ^{15}N isotope dilution (Hardarson and Danso, 1990) technique that makes it possible to distinguish whether or not particular treatments, like AM inoculation, influence N nutrition by acting on N_2 fixation or N uptake (Toro et al., 1998). When only qualitative estimation of the N_2 fixation level is needed, as in the ranking of the effects of several microbial or chemical treatments, a non-fixing reference crop is not required. 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 tissues indicates enhanced N_2 fixation (Danso, 1988). It is accepted that all the available N pool in soil is labelled with the isotope added.

The aim of this work was to assess the impact of a biotechnological practice (microbial inoculation), in interaction with a low-input technology (rock phosphate application), and their effectiveness in improving sustainable nutrient supply to plants. The proposed agrotechnological practices rely on optimizing the performance of soil microbiota (Barea and Jeffries, 1995; Glick, 1995; Kennedy and Smith, 1995). Both a pot experiment, to mimic interactions which occur in living soil-plant situations, and isotopic approaches, are proposed to evaluate the effectiveness of microbiologically-mediated activities with regard to N and P supply. Accordingly, a pot experiment was designed to evaluate, by using ^{15}N and ^{32}P isotopic dilution approaches, the interactive effect of phosphate-solubilizing bacteria, *Rhizobium* and AM fungi on RP use in soil with neutral pH and low Ca. A field experiment was further carried out to validate results from the pot trial. In the field assay organic matter amendments were also applied.

Material and methods

Pot experiment

The experiment involved a factorial combination of four microbial treatments [*Rhizobium* WT inoculation, mycorrhiza inoculation (M), phosphate-solubilizing rhizobacteria inoculation (RB), and the M + RB dual inoculation], and two chemical treatments [unamended control without P application soil and rock phosphate (RP) application]. These 8 treatments were replicated five times giving a total of 40 pots units that were arranged in the greenhouse in a randomized block design.

The AM fungus used was *Glomus mosseae* (BEG 12). The *Rhizobium meliloti* strain tested was the WT GR4 isolate. The phosphate solubilizing rhizobacterium was an *Enterobacter* sp. (Toro et al., 1998).

Alfalfa (*Medicago sativa* L., cultivar Aragón) was the test plant. Five-day-old seedlings obtained from surface sterilized seeds were transplanted into 1 l pots containing an agricultural soil in which alfalfa had never been grown. The soil was collected in the province of Granada (Spain). The soil was a Cambisol with pH (H_2O) of 6.8, available ($NaHCO_3$ – extractable) P of 15 mg l^{-1}, total N of 2600 mg l^{-1}, 10 me exchangeable Ca l^{-1}, organic C of 0.8%, and a texture of 58.7% sand, 26.4% silt, and 14.9% clay. The soil does not contain active $CaCO_3$ [Toro et al., 1998].

The experimental soil was sieved through a 4 mm screen, steam-sterilized at 100 °C for 1h on 3 consecutive days, and then reinoculated with a soil filtrate. The soil filtrate was obtained by shaking 250g of non-pasteurized soil with 1l of water during 15 min and filtered through Whatman no. 1 filter paper. The filtrate contains the natural soil microbial population minus propagules of AM fungi which were retained on the filter paper.

The experimental soil was divided into two batches. One batch was an unamended control and the other batch was amended with RP. The source of RP was from Riecito (Venezuela) and contained 11.4% total P with 6.64% of neutral ammonium citrate-soluble P (Casanova, 1995). The RP was applied as finely ground material (less than 100 mesh) at a rate of 100 mg of total P per kg soil.

At transplanting, seedlings (two 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. Twenty 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 (Toro et al., 1998) and contained 10^8 cells ml^{-1}. The rhizobacterial cultures were also obtained following standard procedures (Toro et al., 1998) and contained 10^8 colony forming units (cfu) ml^{-1}. The plants were grown in a greenhouse under a day/night cycle of 16/8 h, 25/19 °C, 50% relative humidity. A photosynthetic photon flux density of 400–500 μmol m^{-2} sec^{-1} was applied as supplementary light during the 16 h day. Plants were fertilized (5 ml wk^{-1} pot^{-1}) with a nutrient solution (Toro et al., 1998) lacking N and P. Basal nutrients were supplied in the following amounts (μmol kg^{-1} growth medium): K_2SO_4 (2008), $MgSO_4.7H_2O$ (2029), $MnSO_4.H_2O$ (118), $CuSO_4.5H_2O$ (100), $ZnSO_4.7H_2O$ (35), $CoCl_2.6H_2O$ (21), H_3BO_3 (81) and $NaMoO_4.2H_2O$ (21). The pots were weighed and watered to field capacity (19 ml of water per 100 g of soil) daily.

The qualitative approach (Danso, 1988) of the isotope ^{15}N dilution technique (Hardarson and 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.

The isotope dilution technique (Zapata and Axmann, 1995) was used for ^{32}P studies. An aliquot containing 1850 K Bq ^{32}P pot^{-1} 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 KH_2PO_4 carrier solution with 10 ppm P. Labelling was done by mixing the soil thoroughly with 10 ml of the solution containing ^{32}P phosphate ions. Seedlings were transplanted one day after soil labelling.

Plants were harvested after 55 d of growth. Shoot dry weight 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 et al., 1973).

The N isotopic composition of plant shoots was determined in samples previously digested, according to the Dumas method, by using an automated N analyzer (Fisons NA 1500 NC) interfaced to a Finnigan MAT 251 continuous-flow isotope ratio mass spectrometer (ANA–MS method). It is assumed that when several treatments are being tested for their effects

on N_2 fixation, under equal exposure to all the pots with ^{15}N-labelled fertilizer having the same ^{15}N enrichment, treatments more effective in improving N_2 fixation lowers the atom % ^{15}N excess (^{15}N% a.e.) 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 for isotope decay and counting efficiency (50%), and expressed in Bq. The specific activity 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} (Zapata and Axmann, 1995).

Field experiment

The test plant (alfalfa) and soils, the microbial inocula and treatments, and rock phosphate source were the same as in the pot experiment. With regard to thephysico-chemical treatments, the RP was broadcast applied as a finely ground (100-mesh) natural product at a rate of 88 g of RP per m^2(equals 100 × kg P ha^{-1}). Vermicompost (VC) was used as an organic matter (OM) amendment to soil. The product was obtained from a commercial earthworn (*Eisenia andrei*) farm in the region. It was applied at a rate of 2 kg of commercial product per m^2. The total Kjeldahl N of the product was 15 g kg^{-1} and the total P 5.7 g kg^{-1} (Elvira et al., 1998).

Individual plots measured 3 × 6 m in which 30 groups of plants (10 seeds/group) were planted per m^2. Planting was done in groups to facilitate mycorrhizal inoculation. All plants received a *Rhizobium* inoculum as a seed treatment. Plants were grown for five months under irrigation by sprinkling and submitted to weeding practices. The harvested area measured 2 × 5 m to avoid edge effects.

The trial involved a 4 × 4 factorial combination of four microbial treatments [(i) (non-inoculated control) (ii) mycorrhizal soil inoculation (AM), (iii) rhizobacterium seed inoculation (RB) and (iv) RB + M dual inoculation]; and four fertilizer treatments [(i) unamended control, (ii) rock phosphate (RP) addition, (iii) vermicompost (OM) application, and (iv) RP + OM dual amendment]. These 16 treatments were replicated five times giving a total of 80 plots that were distributed in the experimental field following a complete randomized block design.

Plants grew for five months. Shoot dry weight was determined and shoot N and P concentration were measured (Lachica et al., 1973) as described for the greenhouse experiment.

In both the field and greenhouse experiments data were processed by ANOVA and Duncan's test ($P = 0.05$).

Results

Pot experiment

Tables 1–3 shows that most of the microbial inoculation and RP application treatments benefited shoot biomass and N and P accumulation in alfalfa plants. Mycorrhizal inoculation was very important in improving plant growth and nutrient acquisition. Inoculated *Enterobacter* (RB) interacted positively with AM fungi. For non-mycorrhizal plants, *Enterobacter* inoculation (RB) improved the use of added RP. The most effective combination of treatments was the inoculation with AM fungi and RP-addition. Rock phosphate application improved plant growth with respect to the corresponding control (non RP-added soil) for each microbial treatment.

Calculations on "microbial dependency" (yield for a given microbial treatment-yield for control/yield for such a microbial treatment) together with calculations of agronomic effectiveness for RP (yield for RP-yield for control/yield for RP) indicate that only rhizobacteria actually improve the use of RP.

At least in part, the effect of certain treatments at enhacing N nutrition in alfalfa plants appears to be exerted through N_2 fixation, as shown by ^{15}N enrichment studies (Table 2). Lowering of the $^{15}N/^{14}N$ ratio in

Table 1. Shoot dry weight (mg pot^{-1}) of alfalfa plants

Microbial treatment[a]	Chemical treatments	
	Control	Rock Phosphate
Rhizobium (WT)		
Control	269a[b]	390b [31][d]
Rhizobacteria (RB)	290[a] (7)[c]	535c (37) [68]
Mycorrhiza (AM)	405b (33)	570c (31) [29]
RB + AM	560c (52)	618d (66) [19]

[a] WT = *R. meliloti* wild type; RB = *Enterobacter* sp; AM = *G. mosseae*.
[b] For each parameter, means (five replicates) not sharing a letter in common differ significantly ($P = 0.05$) from each other (Duncan's multirange test).
[c] Microbial dependency (Duncan's multirange test).
[d] Agronomic effectiveness for rock phosphate under the different microbial treatments.

Table 2. Shoot N content (mg pot^{-1}) and atom percent ^{15}N excess (^{15}N % a.e.) in alfalfa plants

	Chemical treatment			
	Control		Rock Phosphate	
Microbial treatment[a]	N content	^{15}N % a.e.	N content	^{15}N % a.e.
Rhizobium (WT)				
Control	12.8a[b]	0.90a	15.1b	0.93a
Rhizobacteria (RB)	12.1a	0.81ab	25.3c	0.65c
Mycorrhiza (AM)	20.1b	0.60c	27.8c	0.62c
RB + AM	29.6c	0.59c	31.2d	0.50d

[a]WT = *R. meliloti* wild type; RB = *Enterobacter* sp; AM = *G. mosseae*.
[b]For each parameter, means (five replicates) not sharing a letter in common differ significantly ($P = 0.05$) from each other (Duncan's multirange test).

Table 3. Shoot P content (mg pot^{-1}) and specific activity (^{32}P/Total P-Bq mg P^{-1}) in alfalfa plants

	Chemical treatment			
	Control		Rock Phosphate	
Microbial treatment[a]	P content	^{32}P/Total P	P content	^{32}P/Total P
Rhizobium (WT)				
Control	0.39a[b]	2200a	0.65b	1083b
Rhizobacteria (RB)	0.34a	2080a	1.10c	900c
Mycorrhiza (AM)	0.81b	1333b	1.75d	650d
RB + AM	1.01c	1117c	1.85d	580e

[a]WT = *R. meliloti* wild type; RB = *Enterobacter* sp; AM = *G. mosseae*.
[b]For each parameter, means (five replicates) not sharing a letter in common differ significantly ($P = 0.05$) from each other (Duncan's multirange test).

plants, which mainly occurred in mycorrhizal plants, could be resulted from increased N fixation or greater exploration of soil nitrogen.

Similarly, the effects of certain combinations of treatments produced a lowering in the P specific activity in alfalfa plants (Table 3). This lowering was more evident in AM-inoculated plants, particularly in the RP added soil.

Field experiment

The results of the field trial are recorded in Figures 1–3. Inoculation with AM fungi significantly increased biomass and N and P accumulation in plant tissues. The supply of N and P with the vermicompost do not improved N and P content in plants (OM *vs* control treatment) for each microbial treatment as can be deduced from results in Figures 2 and 3. Phosphate-solubilizing rhizobacteria improved mycorrhizal responses in soil dually amended with RP and OM. Organic matter addition favoured RP solubilization.

Calculations of the agronomic effectiveness for rock phosphate, as affected by the microbial treatments, were done using the RP + OM treatment as basis, since this is the effective chemical treatment. Data are given in Figure 1.

Discussion

Improvements in N and P accumulation due to the interaction between AM fungi and *Rhizobium* dual inoculation (RB inoculation not involved) were found and is well documented (Toro et al., 1998). 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 (Toro et al., 1997; Toro et al., 1998), therefore these data were used to identify interactive beneficial effects of *Rhizobium* and AM fungi in RP-amended plants.

There are obvious advantages to using isotopes in the measurement of nutrient sources contributions to

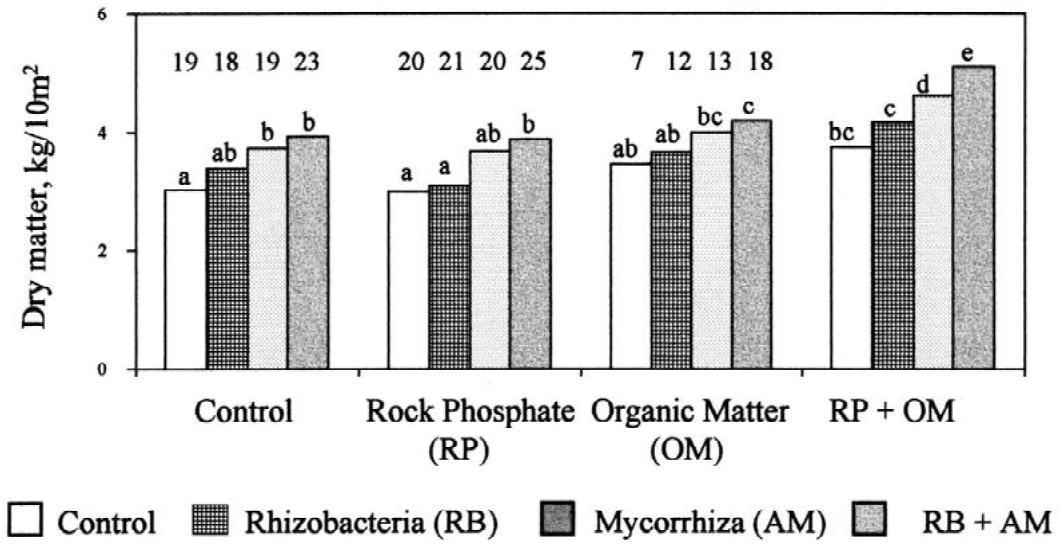

Figure 1. Dry matter production by alfalfa plants growing in the field for 5 months. For each experimental variable values means of (five replicates) not sharing a letter in common differ significantly ($P = 0.05$) from each other (Duncan's multirange test). Data of the Agronomic Efficiency of RP after comparing the RP+OM treatment with the rest of chemical treatments, for each microbial treatment are given over the corresponding column.

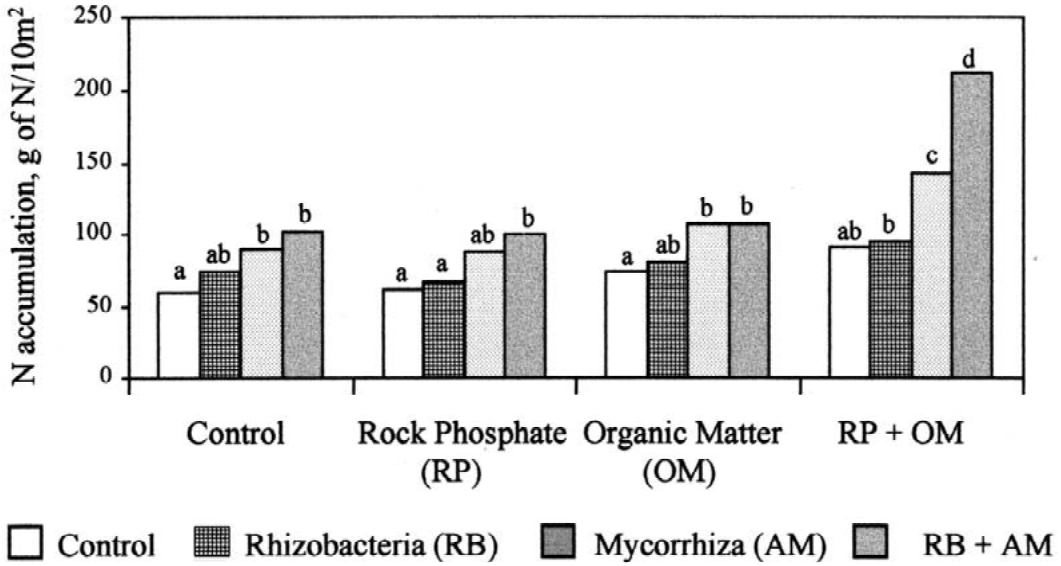

Figure 2. Nitrogen accumulation by alfalfa plants growing in the field for 5 months. For each experimental variable values means of (five replicates) not charing a letter in common differ significantly ($P = 0.05$) from each other (Duncan's multirange test).

plant nutrition in plant-microbe interactions. However, for the pot bioassays a compromise must be maintained between the rate at which ^{32}P was applied 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 day 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.

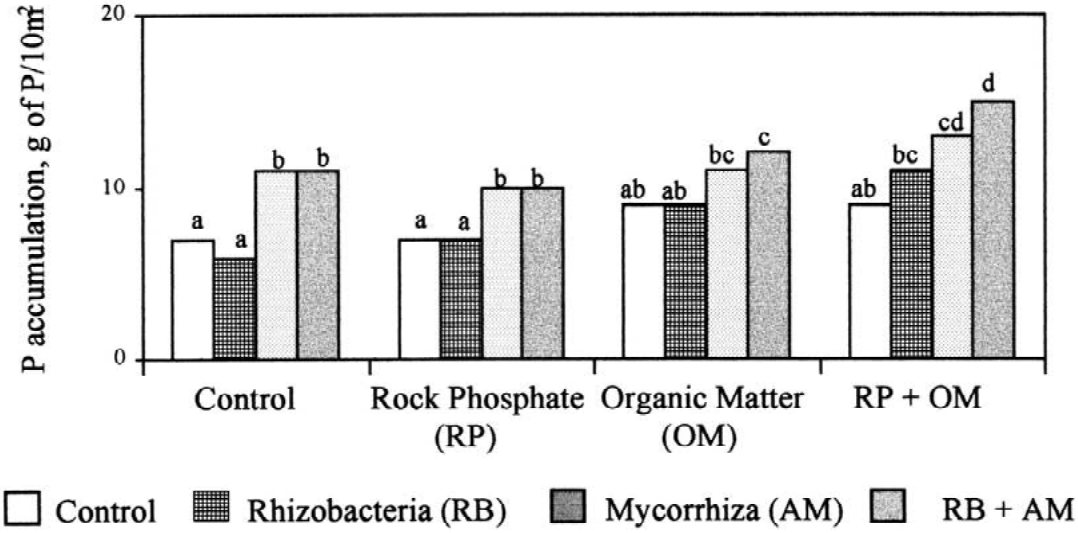

Figure 3. Phosphorus accumulation by alfalfa plants growing in the field for 5 months. For each experimental variable values means of (five replicates) not charing a letter in common differ significantly ($P = 0.05$) from each other (Duncan's multirange test).

Measurements of the $^{15}N/^{14}N$ ratio in plant shoots indicate 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 (Toro et al., 1998).

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) where a similar specific activity in plant tissues were found for both AM- and nonmycorrhizal plants. The treatments which provoked the lower specific activity values indicate the plants were 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. The soil was also low in exchangeable Ca which may benefit the solubilization of P ions from the RP particles (Khasawneh and Doll, 1978; Rajan et al., 1996).

If the ^{32}P/Total P ratio in soil solution is uniform both spatially and temporally, this will produce a similar 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 mycorrhiza develops a slow and late process of P solubilization, the release of P ions constitutes a part of the total P pool. Such microbial activity could give way to a lower specific activity in the host plant than that the appropriate controls which have not received the microbial treatment responsible for the P solubilization process described above.

Data from the field experiment, in which isotopes were not applied, allow us to validate the interactive effects of *Rhizobium*, mycorrhizal fungi and rhizobacteria inocula (Toro et al., 1997; Toro et al., 1998) to improve the agromic efficiency of RP. However, such an effect was not observed unless an organic matter amendment were applied. Calculations of agronomic efficiency of the treatment RP + OM *vs* either Control, RP or OM treatments corroborate this statement for each one of the microbial treatment with the dual inoculation (mycorrhiza + rhizobacteria) as the most effective one. It is noteworthy that the agronomic efficiency of RP in comparison the dual M + RB microbial inoculation *vs* Control in the RP+OM treatment is 27%, and that calculated comparing M+RB microbial treatment in the RP+OM treatment *vs* the "absolute" Control (no microbial inoculation no chemical amendements) is 41%. The reasons accounting for such microbial effects have been discussed before (Bolan, 1991; Toro et al., 1997; Toro et al., 1998) and are well-accepted, but the effect the organic matter need further discussion. Organic matter is usually a good preconditioner for increasing microbial activities to improve the plant use of RP (Khasawneh and Doll, 1978; Rajan et al., 1996). This has been corroborated in a pilot-scale experiment using the source

of vermicompost here used (Elvira et al., 1998). The supply of organic acids appears as the most effective mechanisms with regard to RP solubilization.

Conclusions

The use of isotopic (^{32}P and ^{15}N) dilution approaches allowed us to demonstrate that: (i) the interaction between mycorrhizal fungi and phosphate-solubilizing rhizobacteria improved plant P acquisition from rock phosphate materials in a neutral, low-Ca agricultural soil; and (ii) in *Rhizobium*-inoculated AM-plants, there was an enhanced N_2 fixation rate over that achieved in non-mycorrhizal alfalfa plants. It was also shown in the same soil, under field conditions, that inoculation with AM fungi significantly increased biomass and N and P accumulation in plant tissues. Phosphate-solubilizing rhizobacteria improved mycorrhizal responses of *Rhizobium*-inoculated alfalfa plants in soil dually amended with RP and OM. Organic matter addition favoured RP solubilization. This, together with microbial inoculation, increased the agronomic efficiency of rock phosphate.

Acknowledgements

The authors thank the Joint FAO/IAEA Division, United Nations, Vienna (Phosphate CRP), particularly Dr. F. Zapata for his comments and advice, and the EC Biotechnology Programme, IMPACT Project BIO4-CT96-0027.

References

Barea JM, Azcón R & Azcón-Aguilar C (1983) Interactions between phosphate solubilizing bacteria and VA mycorrhiza to improve plant utilization of rock phosphate in non acidic soils. In: 3rd International Congress on Phosphorus Compounds, pp 127–144. Brussels October 4–6

Barea JM, Azcón-Aguilar C & Azcón R (1997) Interactions between mycorrhizal fungi and rhizosphere microorganisms within the context of sustainable soil-plant systems. In: Multitrophic Interactions in Terrestrial Systems, pp 65–77. Cambridge: Blackwell Science

Barea JM & Jeffries P (1995) Arbuscular mycorrhizas in sustainable soil plant systems In: Mycorrhiza Structure Function Molecular Biology and Biotechnology, pp 521–559. Heidelberg: Springer-Verlag

Bethlenfalvay GJ & Linderman RG (1992) Mycorrhizae in Sustainable Agriculture. ASA Spec Publ Madison WI

Bohlool BB, Ladha JK Garrity DP & George T (1992) Biological nitrogen fixation for sustainable agriculture: a perspective. Plant Soil 141: 1–11

Bolan NSA (1991) critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. Plant Soil 134: 189–207

Casanova EF (1995) Agronomic evaluation of fertilizers with special reference to natural and modified phosphate rock. Fert Res 41: 211–218

Danso SKA (1988) The use of ^{15}N enriched fertilizers for estimating nitrogen fixation in grain and pasture legumes In: Nitrogen fixation by Legumes in Mediterranean Agriculture, pp 345–358, Dordrecht: Martinus Nijhoff Publishers

Elvira C, Sampedro L, Benítez E & Nogales R (1998) Vermicomposting of sludges from paper mill and dairy industries with *Eisenia andrei*: a pilot-scale study. Bio Tech 63: 205–211

Fardeau JC (1993) Le phosphore assimilable des sols: sa représentation par un modèle fonctionnel á plusieurs compartiments. Agron 13: 317–331

Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41: 109–117

Hardarson G & Danso SKA (1990) Use of ^{15}N methodology to assess biological nitrogen fixation In: Use of Nuclear Techniques in Studies of Soil-Plant Relationships, pp 129–160. Vienna International Atomic Energy Agency

Jeffries P & Barea JM (1994) Biogeochemical cycling and arbuscular mycorrhizas in the sustainability of plant-soil systems. In: Impact of Arbuscular Mycorrhizas on Sustainable Agriculture and Natural Ecosystem, pp 101–115. Basel: Brikhäuser Verlag

Joner EJ & Jakobsen I (1994) Contribution by two arbuscular mycorrhizal fungi to P uptake by cucumber (*Cucumis sativus* L) from ^{32}P-labelled organic matter during mineralization in soil. Plant Soil 163: 203–209

Kennedy AC & Smith KL (1995) Soil microbial diversity and the sustainability of agriculture soils. Plant Soil 170: 75–86

Khasawneh FE & Doll EC (1978) The use of phosphate rock for direct application to soils. In: Advances in Agronomy, pp 159–206. New York: Academic Press

Kloepper JW, Zablotowicz RM, Tipping EM & Lifshitz R (1991) Plant growth promotion mediated by bacterial rhizosphere colonizers. In: The Rhizosphere and Plant Growth, pp 315–326. Dordrecht: Kluwer Academic Publishers

Lachica M, Aguilar A & Yañez J (1973) Analisis foliar Métodos utilizados en la Estación Experimental del Zaidín. Anal Edafol Agrobiol 32: 1033–1947

Rajan SSS, Watkinson JH & Sinclair AG (1996) Phosphate rocks for direct application to soils. In: Advances in Agronomy, pp 78–159. New York: Academic Press

Toro M, Azcón R & Barea JM (1997) Improvement of arbuscular mycorrhizal development by inoculation with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (^{32}P) and nutrient cycling. Appl Environ Microbiol 63: 4408–4412

Toro M, Azcón R & Barea J M (1998) The use of isotopic dilution techniques to evaluate the interactive effects of *Rhizobium* genotype mycorrhizal fungi phosphate-solubizing rhizobacteria and rock phosphate on nitrogen and phosphorus acquisition by *Medicago sativa.* New Phytol 138: 265–273

Zapata F & Axmann H (1995)^{32}P isotopic techniques for evaluating the agronomic effectiveness of rock phosphate materials. Fert Res 41: 189–195