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Improvement of Arbuscular Mycorrhiza Development by Inoculation of Soil with Phosphate-Solubilizing Rhizobacteria To Improve Rock Phosphate Bioavailability (^{32}P) and Nutrient Cycling

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The interactive effect of phosphate-solubilizing bacteria and arbuscular mycorrhizal (AM) fungi on plant use of soil P sources of low bioavailability (endogenous or added as rock phosphate [RP] material) was evaluated by using soil microcosms which integrated ^{32}P isotopic dilution techniques. The microbial inocula consisted of the AM fungus *Glomus intraradices* and two phosphate-solubilizing rhizobacterial isolates: *Enterobacter* sp. and *Bacillus subtilis*. These rhizobacteria behaved as "mycorrhiza helper bacteria" promoting establishment of both the indigenous and the introduced AM endophytes despite a gradual decrease in bacterial population size, which dropped from 10^7 at planting to 10^3 CFU g^{-1} of dry rhizosphere soil at harvest. Dual inoculation with *G. intraradices* and *B. subtilis* significantly increased biomass and N and P accumulation in plant tissues. Regardless of the rhizobacterium strain and of the addition of RP, AM plants displayed lower specific activity (^{32}P/^{31}P) than their comparable controls, suggesting that the plants used P sources not available in their absence. The inoculated rhizobacteria may have released phosphate ions (^{31}P), either from the added RP or from the less-available indigenous P sources, which were effectively taken up by the external AM mycelium. Soluble Ca deficiency in the test soil may have benefited P solubilization. At least 75% of the P in dually inoculated plants derived from the added RP. It appears that these mycorrhizosphere interactions between bacterial and fungal plant associates contributed to the biogeochemical P cycling, thus promoting a sustainable nutrient supply to plants.

Current developments in sustainability involve a rational exploitation of soil microbial activities (5) and the use of less expensive, though less bioavailable, sources of plant nutrients, like rock phosphates (RP), which may be made available by microbiologically mediated processes (39). Microbial populations are key components of the soil-plant system where they are immersed in a framework of interactions affecting plant development (30). It is known that microorganisms are activated in the soil-plant interface where a microcosm system, the rhizosphere, develops (2, 28, 30). So-called plant-growth-promoting rhizobacteria in the rhizosphere participate in many key ecosystem processes such as those involved in the biological control of plant pathogens, nutrient cycling, and seedling establishment (18, 25, 37).

Mycorrhizal fungi, upon root colonization, 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 (20). Particularly, the arbuscular mycorrhizal (AM) symbiosis plays a direct role in nutrient cycling rates and patterns in agroecosystems and natural environments (8, 10, 22). The establishment of the AM symbiotic status affects the chemical composition of root exudates while the development of a mycorrhizal soil mycelium also introduces physical modifications in the environment surrounding the roots. These changes affect the rhizospheric microbial communities in the so-called mycorrhizosphere. Conversely, soil microorganisms can affect AM formation and function (2, 5, 28, 33). Particularly, the so-called mycorrhiza helper bacteria are known to stimulate mycelial growth of mycorrhizal fungi or to enhance mycorrhizal formation (5, 14, 15).

Many soil microorganisms are able to solubilize phosphate ions from sparingly soluble inorganic or organic P compounds in vitro (4). Nevertheless, the contribution of this process to plant nutrition is unclear because of the possible refixation of solubilized phosphate ions on their way to the root surface. The microbiologically solubilized phosphate could, however, be taken up by a mycorrhizal mycelium, thereby developing a synergistic microbial interaction (5).

Because assimilable P is scarce in soil, the phosphate stock must be restored in any agricultural system. The use of RP has been proposed for sustainability purposes (39). The problem with this less expensive but sparingly soluble form of P is its low effectiveness, particularly in nonacidic soils (23, 34). Integrated approaches involving mycorrhizosphere interactions have been proposed to improve P bioavailability in soil (4). It has been accepted that the soil P that is bioavailable is the P which leaves the solid phase of the soil and enters the soil solution (13).

Radioactive P (^{32}P) has been applied as a means of evaluating the exchange rates governing phosphate equilibrium between the solution and the solid phases of the soil (13). It can also be used to measure P availability in RP materials (39) and to identify P sources for AM and nonmycorrhizal plants (9). Labelling of the so-called isotopically exchangeable soil P (13) can be carried out with phosphate ions labelled with ^{32}P. The isotopic composition, or specific activity (SA), i.e., the ^{32}P/^{31}P ratio, is then determined in the plant tissues. The effects of treatments such as AM inoculation on P uptake can be ascertained by the determination of this ratio (36). If a given treat-

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ment lowers the SA compared to that of the control plants, it would indicate that the plant is using extra ^{31}P released, as a result of the treatment, from otherwise unavailable P sources. Isotope-based techniques could therefore be applied to determine the extent of the transformation of unavailable soil P into bioavailable P by the metabolic activities of soil microorganisms, whether they act directly on P solubilization or by modifying root uptake properties (16). Bolan (9) reviewed the topic and concluded that, in most cases, no differences in SA were found, regardless of whether the plants were or were not mycorrhizal. As Bolan (9) stated, the only conclusion that can be drawn from such studies is that both mycorrhizal and nonmycorrhizal plants use similarly labelled P from soils, but this does not exclude the possibility that AM plants can use P forms which are not available to nonmycorrhizal controls. The value of ^{32}P in evaluating AM relationships is still an open research topic because no conclusive evidence has yet been found regarding the ability of the technique to distinguish the P sources used by the plant (21).

Although RP solubilization rarely occurs in nonacidic soils, it may take place when these soils are deficient in exchangeable Ca (4) because this characteristic facilitates P solubilization (23, 34). In this study, a microcosm experiment was designed to evaluate, by using ^{32}P isotopic dilution techniques, the interactive effect of phosphate-solubilizing bacteria and AM fungi on P cycling in general and on RP use in particular in a neutral, low-Ca, agricultural soil.

MATERIALS AND METHODS

Experimental design. The experiment was microcosm based, and the design consisted of two blocks of AM inoculation treatments: inoculated (+Gi) and uninoculated (−Gi) but with the indigenous microbiota naturally present in the soil, including AM fungi. For each mycorrhizal block three bacterial treatments (uninoculated control and inoculation with either of two phosphate-solubilizing rhizobacterium isolates) and two fertilizer treatments (unamended control soil and RP application) were combined to give 12 treatments. These were replicated five times, giving a total of 60 unsterilized soil microcosm units that were arranged in the greenhouse in a randomized block design.

Microorganisms. The AM fungus used was *Glomus intraradices* (Schenck and Smith). Two phosphate-solubilizing rhizobacterium isolates were studied: *Enterobacter* sp. and *Bacillus subtilis* (36). These rhizobacterial strains maintained their induced streptomycin (150 mg ml^{-1})- and spectinomycin (150 mg ml^{-1})-resistant phenotypes during several successive subculturing transfers in Ramos-Callao (RC) liquid medium (35) and did not differ from their parent strains in growth rate.

Test plant and soil. Onion (*Allium cepa* L.) was the test plant. Five-day-old seedlings obtained from surface-sterilized seeds were transplanted to soil microcosms. An agricultural soil collected in the province of Granada (Spain) was used. The characteristics of the test soil, a Cambisol, were as follows: pH (H_2O), 6.8; available ($NaHCO_3$ extractable) P, 15 mg $liter^{-1}$; total N, 2,600 mg $liter^{-1}$; organic C, 0.8%; and a texture of sand (58.7%), silt (26.4%), and clay (14.9%). The soil was deficient in soluble Ca (36).

RP treatment. The experimental unsterilized soil was sieved (mesh, 2 mm) and divided into two batches: one unamended control and one with RP added. The RP source was from Riecito (Venezuela). This rock material has 11.4% total P (11) and was applied as a finely (100-mesh) ground natural product at a rate of 100 mg of total P per kg of soil. Soil from both batches was distributed into pots (300 ml).

Microbial inoculation. At transplanting, seedlings (one per pot) were inoculated appropriately. The mycorrhizal inoculum, obtained from a stock pot culture where *Lactuca sativa* L. was the host plant, contained 20 spores g^{-1} together with mycelium and mycorrhizal root fragments. Ten grams per pot of this mycorrhizal inoculum was thoroughly mixed with the soil in the pot. The rhizobacterial cultures were obtained in RC (35) liquid medium, following standard procedures (1), and contained 10^8 CFU ml^{-1} adjusted by optical density. Two milliliters of this inoculum was applied to the root system of each seedling in the planting hole.

Soil labelling. The isotope dilution technique (39) was used for ^{32}P studies. An aliquot containing 592 kBq of ^{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 carrier-free ^{32}P to a known volume of carrier solution (KH_2PO_4) with 10 mg of P kg^{-1} (38). Labelling was done by thoroughly mixing the soil with 10 ml pot^{-1} of the solution containing ^{32}P ions.

Growth conditions and measurements. The plants were grown in a greenhouse under a day/night cycle of 16/8 h and 25/19°C and 50% relative humidity. A photosynthetic photon flux density of 400 to 500 mmol m^{-2} s^{-1} was applied as supplementary light. Plants were fertilized (5 ml wk^{-1} pot^{-1}) with a nutrient solution (19) lacking P. The pots were weighed and watered to field capacity daily.

Plants were harvested after 60 days 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 and molybdenum blue procedures, respectively (26).

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 counting efficiency and expressed in becquerels (disintegrations per second). The SA of P in the plant material was then calculated by considering the radioactivity per amount of P in the plant material and expressed in becquerels $milligram^{-1}$ of P. The percentage of plant P derived from the labelled (L) source (% Pdf L) was calculated as indicated by Zapata and Axmann (39) as follows: % Pdf L = (SA in plant in presence of RP/SA in plant in absence of RP) ×100. The percentage of P in plant derived from the RP was obtained by using isotope dilution concepts (39), where: % Pdf RP = [1 − (SA in plant in presence of RP/SA in plant in absence of RP)] ×100.

Five extra replicate microcosm units per treatment were also prepared, except that the isotope was not applied, and were maintained under similar conditions to those for the core experiment. Root dry weight and length, the extent of AM colonization, and rhizobacterium survival in rhizosphere soil were assessed at harvest in the radioactivity (^{32}P)-free rhizospheres. In addition, rhizobacterium population densities were also assessed at 15 and 30 days after inoculation to monitor their population dynamics. The extent of AM root colonization was estimated by a staining method (32). The percentage of the total root length that became mycorrhizal was calculated by a gridline intersect technique (17). Rhizobacterial survival was tested in serial dilutions of rhizosphere soil samples (27). The RC solid medium used contained streptomycin (150 mg ml^{-1}), spectinomycin (150 mg ml^{-1}), and cycloheximide (150 mg ml^{-1}).

Root length was measured by using video images obtained with the Digital Image Analysis imager, version 1.10A (Delta T Devices Ltd). The specific root length (SRL) was calculated (7), and the results are given as root length (centimeters)/root dry weight (milligrams).

Statistical methods. Data were processed by analysis of variance and Duncan's test ($P \leq 0.05$). Values given as percentages were subjected to arc-sin square-root transformation. For bacterial densities, evaluation data were log transformed prior to statistical analyses.

RESULTS

Inoculum establishment in the plant rhizosphere. The mycorrhizal inoculum significantly increased the extent of AM colonization of the root system compared to roots colonized by the indigenous AM fungi (Fig. 1). The addition of RP and inoculation with *B. subtilis* increased root colonization by *G. intraradices*. The natural mycorrhizal potential of the test soil was low but the inoculation of *Enterobacter* sp. (in control soil) or *B. subtilis* (in RP-amended soil) increased the extent of AM colonization by indigenous endophytes (Fig. 1).

The population size of the inoculated rhizobacteria declined with time. Regardless of the isolate and whether RP was added or the rhizosphere was inoculated with AM, bacterial population dynamics followed the same pattern. There were no significant differences between treatments at any sampling time. The results of the log transformation of the CFU g^{-1} of rhizosphere soil were as follows (times in days after inoculation): Time zero, 6.3 a; time 15, 4.2 b; time 30, 4.1 b; and time 60, 2.9 c (values followed by the same letter are not significantly different).

Plant biomass and nutrient acquisition. Comparisons of the response variables in Fig. 1 show relationships between the level of AM colonization and plant response. Dual inoculation with an AM fungus and *B. subtilis* and RP addition resulted in the most effective treatment combination to improve AM colonization level, biomass accumulation, and N and P acquisition.

Root development. In general, AM inoculation increased root weight, did not affect root length, and decreased the SRL (Table 1). None of the rhizobacteria inoculated affected the root developmental parameters measured. RP addition im-

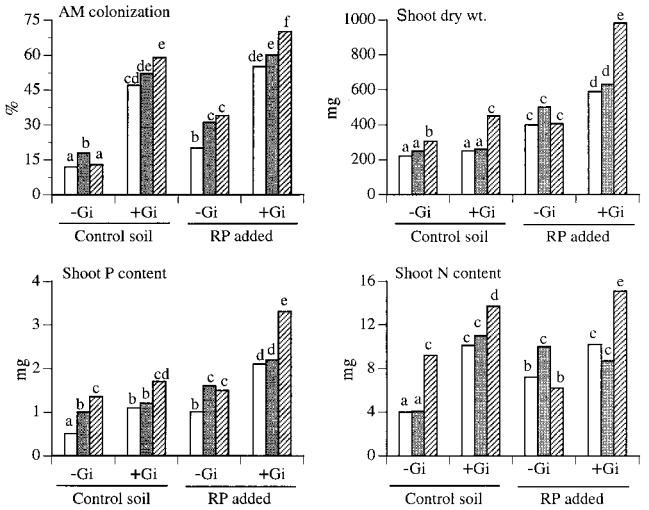

FIG. 1. AM colonization and biomass, N, and P accumulation in plants (shoots) grown for 60 days as affected by several microbial inoculation treatments and RP application. For each response variable, values (means of five replicates) not sharing a letter in common differ significantly ($P = 0.05$) from each other (Duncan's multirange test). Symbols: □, uninoculated control; ▨, *Enterobacter* sp.; ▨, *B. subtilis*.

proved root weight in non-AM-inoculated plant (−Gi) but not in AM-inoculated plants (+Gi) and improved root length but did not affect the SRL.

Isotope data. Certain combinations of treatments produced lower SA ($^{32}P/^{31}P$) in onion plants (Table 2). This was more evident in AM-inoculated plants, particularly in soil to which RP was added. Inoculation with *B. subtilis* and RP addition also tended to lower the SA in plant shoots. The dual inoculation of mycorrhiza and rhizobacteria in RP-treated plants was, therefore, the combined treatment which produced the lowest SA. These results may indicate that these treatments induced the plants to absorb from soil P which was differently labelled (a lower $^{32}P/^{31}P$ ratio) than the P used by the corresponding control plants.

Data recorded in Table 2 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 3. It is evident that

TABLE 1. Root dry weight (DW), root length (RL) and SRL in onion plants grown for 60 days as affected by several microbial inoculation treatments and RP application[a]

Microbial treatment	Chemical fertilization treatment					
	Control			RP added		
	RL (cm)	DW (mg)	SRL (cm mg^{-1})	RL (cm)	DW (mg)	SRL (cm mg^{-1})
−Gi						
Control	238 a	20 a	11.9 a	262 b,c	30 b	8.7 c
Enterobacter sp.	224 a	24 a	9.3 b	280 c	29 b	9.6 b
B. subtilis	222 a	23 a	9.6 b	277 c	32 b,c	8.7 c
+Gi						
Control	240 a	28 b	8.6 c,d	264 b,c	33 b,c	8.0 d
Enterobacter sp.	245 a	32 b,c	7.6 d	290 c	38 c	7.6 d
B. subtilis	250 a,b	30 b	8.3 c,d	218 c	36 c	7.8 d

[a] For each response variable, values (means of five replicates) not sharing a letter in common differ significantly ($P \leq 0.05$) from each other (Duncan's multirange test).

TABLE 2. SA ($^{32}P/^{31}P$) in the shoots of onion plants grown for 60 days as affected by several microbial inoculation treatments and RP application[a]

Microbial treatment	Chemical fertilization treatment	
	Control (Bq mg of P^{-1})	RP added (Bq mg of P^{-1})
−Gi		
Control	8.8 a	8.1 a
Enterobacter sp.	8.5 a	5.6 b,c
B. subtilis	6.6 b	5.8 b,c
+Gi		
Control	4.5 d	3.0 e
Enterobacter sp.	7.8 a,b	1.8 e,f
B. subtilis	5.6 c	1.3 f

[a] Values (means of five replicates) not sharing a letter in common differ significantly ($P \leq 0.05$) from each other (Duncan's multirange test).

plants are using both the bioavailable endogenous soil P and the P that was made bioavailable from the RP particles, as released by the microbial treatments. The relative contribution of the different P sources to plant P content (% Pdf L versus % Pdf RP) depended on the treatment involved, but in general, plants used PdfL preferentially except when dually inoculated with AM fungi and either *Enterobacter* sp. or *B. subtilis*, where a significant use of RP by the test plant was evident.

DISCUSSION

The effects of microbial activities on the biogeochemical cycling of plant nutrients are essential for sustainable ecosystems (20, 31). The results of this study of the interaction between a biotechnological practice (microbial inoculation) and a low-input technology (RP application) have demonstrated the effectiveness of such combined practices in improving sustainable nutrient supply to plants. This effectiveness relies on the improvement of soil microbiota performance (6, 12, 18, 24).

The phosphate-solubilizing rhizobacteria behaved as mycorrhiza helper bacteria (14, 15) because they promoted root colonization when associated with mycorrhizal fungi, confirming previous findings involving other AM fungi and phosphate-solubilizing bacteria combinations (3). In this study, the population of the bacteria introduced dropped to 10^3 CFU g^{-1} of

TABLE 3. Percentage and total amount (μg pot^{-1}) of plant P derived from the bioavailable (labelled) soil (Pdf L) or from RP (Pdf RP) in the shoots of onion plants given RP and grown for 60 days as affected by several microbial inoculation treatments[a]

Microbial treatment	Pdf L		Pdf RP	
	%	μg	%	μg
−Gi				
Control	92	929 e	8	81 a
Enterobacter sp.	65	975 e	35	525 c
B. subtilis	88	1,320 f	12	180 b
+Gi				
Control	66	1,386 f	34	714 c,d
Enterobacter sp.	23	506 c	77	1,694 f
B. subtilis	24	792 d	76	2,508 g

[a] Values (means of five replicates) not sharing a letter in common differ significantly ($P \leq 0.05$) from each other (Duncan's multirange test).

rhizosphere soil at harvest. Nevertheless, they were able to exert their mycorrhiza helper effect before their population densities declined. In another system, bacterial populations as low as 30 CFU g^{-1} still displayed a significant mycorrhiza helper effect (14). The mechanisms by which these bacteria stimulated AM colonization are still poorly understood. Apparently, the bacteria did not influence the AM effect on root weight or length. Specialized bacterial activities such as the production of vitamins, amino acids, and hormones, etc. (5), may be involved in these interactions.

Mycorrhizal inoculation is known to induce changes in root morphology (7), and our findings also show that AM colonization lowered the SRL, thus confirming previous findings which showed that *G. intraradices* increased root weight by increasing root diameter (7). Since no AM-induced changes in root length were found in this experiment, the AM effect on nutrient uptake may be due to the well-known ability of the external mycelium to extend the soil volume that the plants are able to explore for P (or N) uptake (9).

There are obvious advantages to using the ^{32}P isotope in the measurement of the P sources contributing to plant nutrition in plant-microbe interactions (9). Our study demonstrated that AM-inoculated plants showed a lower SA ($^{32}P/^{31}P$) than the controls. Bacterial inoculation and RP application also induced a lowering of the $^{32}P/^{31}P$ ratio, both in +Gi and in −Gi plants. The decrease in SA produced by these treatments contrasts with previous findings (9) according to which, in most cases, AM inoculation did not change the SA in plant tissues with respect to the comparable controls. Our data suggest that plants from the treatments with the lower SA values used otherwise unavailable P sources. The phosphate-solubilizing, AM-associated microbiota could be releasing phosphate ions, either from the added RP or from the less-available indigenous phosphate. Ca deficiency in the soil solution may facilitate the solubilization of P ions from the RP particles (23, 34).

If the $^{32}P/^{31}P$ ratio in the soil solution is uniformly maintained both spatially and temporally, a similar $^{32}P/^{31}P$ ratio (SA) will be produced in the plant, whether mycorrhizal or not (9). Conversely, when a well-established mycorrhizosphere system is able to develop a process of P solubilization, the released P ions constitute a part of the total ^{31}P pool. This would explain the lower SA found in AM-inoculated (+Gi) plants in our experiments.

The present study obtained valuable information by applying the conceptual approaches based on the SA parameter concerning RP use (39). Plants which were not inoculated with AM fungi (−Gi) but which received a bacterial inoculum used proportionally more P from the L fraction in soil than from RP. This indicates that these bacteria may solubilize the endogenous P sources more easily than the added RP. However, the dual AM and rhizobacteria inoculation improved the use of the RP versus the L fraction by the plants. The explanation for this may be that P ions released from RP are taken up by the AM mycelium, thereby maintaining a low soluble P concentration in the discrete soil microhabitats where the RP particles were attacked by the phosphate-solubilizing bacteria and thus favoring a continuous and sustained P release. The AM hyphae were, therefore, transfering to the plant either ^{32}P-labelled phosphate from the L fraction of the soil or ^{31}P released from RP particles.

In summary, it appears that the described interaction between AM fungi and phosphate-solubilizing bacteria contributed to the biogeochemical cycling of nutrients by more than just providing a greater surface area for scavenging mineral nutrients that may be relatively immobile in soil or in short supply (29). The established mycorrhizosphere system seems also to be able to retain nutrients by altering their concentration ratios in vegetation and by decreasing their mobility by retention within the biomass as previously shown (20).

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