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INORGANIC PHOSPHATE SOLUBILIZATION BY TWO *PENICILLIUM* SPECIES IN SOLUTION CULTURE AND SOIL

P. E. A. ASEA,[1] R. M. N. KUCEY[2] and J. W. B. STEWART[1]

[1]Department of Soil Science, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N 0W0 and [2]Agriculture Canada Research Station, Lethbridge, Alberta, Canada T1J 4B1

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Summary—Two fungal isolates, *Penicillium bilaji* and *Penicillium* cf. *fuscum* were found to solubilize different amounts of rock phosphate in liquid culture. Inorganic P solubilization was directly related to the pH drop generated by each isolate. Nitrogen in the ammonium form in the medium was necessary for increased P solubilization by *P. bilaji*. *Penicillium* isolates and the form of nitrogen affected the duration of the lag before each isolate began to solubilize P, the rate at which inorganic P was solubilized and the net amount of P solubilized and maintained in solution. In a greenhouse experiment, wheat, *Triticum aestivum* L. cv. Neepawa, was grown in a calcareous Chernozemic soil which had low available P. When this soil was inoculated with *P. bilaji*, plant dry matter yield increased by 16%, and total plant P uptake by 14%. *P. bilaji* also increased the proportion of P derived from native P sources by 11% even in the presence of added rock phosphate.

INTRODUCTION

Many soil fungi and bacteria isolated from Canadian prairie soils can solubilize inorganic phosphorus compounds such as rock phosphate (Kucey, 1983). Inorganic P solubilization by microbes has been attributed to processes involving acidification, chelation and exchange reactions in the growth environment (Sperber, 1958; Molla and Chowdhury, 1984; Raper and Thom, 1968; Roos and Luckner, 1984). Sperber (1958) identified organic acid metabolites as the primary means of inorganic P solubilization by *Aspergillus niger* and an unidentified *Penicillium*. The particular acids released differed between the fungi as did the rate of P solubilization. Several processes can work in conjunction with each other; i.e. organic acids can act as chelating agents and have a direct acidifying effect on the surrounding environment. Because of the multiplicity of effects, it is not surprising that the ability to acidify the surrounding media is only weakly correlated with the ability to solubilize inorganic phosphate (Gaur *et al.*, 1973; Surange, 1985).

The mechanisms used by fungi for inorganic solubilization have not been tested. In fact, few studies have rigorously addressed factors that could affect the rate at which microbially-mediated inorganic phosphate solubilization occurs (Beever and Burns, 1981). Although some studies have shown an increase in P uptake by plants in response to the addition of free-living fungi, there have been few attempts to quantify the source of the extra P absorbed.

In this study the effects of NH_4 and NO_3 sources of N on the ability of two species of *Penicillium* to produce acid and solubilize P are examined. The effect of inoculating a sterilized soil with *Penicillium bilaji* and vesicular–arbuscular mycorrhizal fungi on the growth and P nutrition of Neepawa wheat grown under greenhouse conditions, was also evaluated.

MATERIALS AND METHODS

The interactions of the following treatments: P-solubilizing fungi, rock phosphate and nitrogen form were tested using liquid cultures. The fungi used were: *P. bilaji* Chalabuda and *P. fuscum* (Sopp) Biorge sensu. Kucey (1983) had shown that *P. bilaji* was an effective P-solubilizer, whereas *P. fuscum* was less effective and was therefore used for comparison. Pure cultures of these isolates were maintained on soil extract agar slants, and fresh inoculum was prepared by inoculating the individual isolates onto potato dextrose agar plates and incubating at 30°C for 3 days prior to use. Pieces of agar supporting growing sporulating hyphae were transferred aseptically from the growing colonies and added to the experimental units.

Idaho rock phosphate (10.3% P) was ground (63 mesh) and dry-autoclaved. Levels of rock P used were 0, 0.1 and 0.2 g 100 ml^{-1} media. The media used differed in the chemical form of nitrogen supplied. Medium A contained 0.1 g NaCl, 0.4 g NH_4Cl, 0.78 g KNO_3, 0.5 g $MgSO_4$, 0.1 g $CaCl_2.2H_2O$ and 10.0 g dextrose l^{-1}. Medium B was the same as A less the NH_4Cl. Sterile solutions (100 ml) were measured into 250 ml screw-top polyproplene centrifuge bottles and sterilized by autoclaving before addition of rock phosphate or *Penicillium* isolates. All transfers of fungal inocula, rock phosphate and subsequent sampling of culture solutions were carried out aseptically in a laminar flow cabinet.

Incubation of the experimental units (2 media × 2 fungi × 3 rock phosphate × 4 reps) was conducted on a bench-top rotary shaker (200 rotations min^{-1}, 24°C). The screw caps of the bottles were loosened to allow gas exchange but minimize airborne contamination. Once daily, for 12 days, a 5 ml subsample of growth medium was withdrawn from each bottle. The subsample was centrifuged (10,000 rev min^{-1} for

5 min) and the supernatant decanted and filtered. The pH of the supernatant was measured using a glass electrode and the P in solution was determined using the colorimetric molybdate blue method (Watanabe and Olsen, 1965). The P solubilized by the fungi was compared to the P dissolved by the addition of HCl to separate uninoculated media. Increasing amounts of 0.1 N HCl were added to bottles of media containing 0.2 g rock phosphate. The pH was measured at the beginning of the experiment and again after 240 h. The P in solution was measured after 240 h by colorimetric methods. The P in solution and the corresponding solution pH after 240 h incubation were used for comparison with the maximum P in solution and minimum pH of the fungal cultures, taking into consideration the volume of solution culture remaining on the day of maximum P concentration.

The P nutrition of wheat (*Triticum aestivum* L. cv. Neepawa) under greenhouse conditions was examined in a completely randomized block experiment utilizing 8 replications of three main effects and their combinations: *P. bilaji*, Idaho rock phosphate and a mixed culture of vesicular–arbuscular mycorrhizal fungi (VAM). The rock phosphate was ground to pass through a 63-mesh sieve. Where used, the rock phosphate was added at a rate of 0.35 g pot^{-1}. The VAM used was a mixed culture of unidentified species isolated from southern Alberta soils and maintained in pot culture (Kucey, 1987). The VAM inoculum consisted of 5 g of dried strawberry roots and adhering soil from a culture maintained by one of the authors (R.M.N.K.). The VAM inoculum in this form contained 24–28 VAM spores g^{-1} of inoculum as determined by direct counts. The *P. bilaji* inoculum was raised for 3 days at 30°C on moistened sterilized wheat chaff amended with 1% dextrose (w/w). The wheat chaff acted as a carrier for the hyphae growing on its surface and the spores produced; 5 g wet wt of chaff were used per pot where appropriate.

The test soil used was the top 0–15 cm (Ap horizon) of a Cavendish loamy sand, an Orthic Brown Chernozem, with a pH of 8.0 (1:1 water paste) containing 3 μg $NaHCO_3$-extractable P g^{-1} (Olsen *et al.*, 1954). Bulk soil was autoclaved to kill the native VAM and incubated in an uncovered condition for 7 weeks before use to allow for re-equilibration of soil nutrients and microbial populations. The soil was uniformly labeled with ^{32}P using the method of Kucey and Bole (1984). For this experiment, 200 ml of solution containing 222 MBq ^{32}P with 9 mg P as KH_2PO_4 were added to 230 kg soil 6 weeks before sowing seed. Labeled soil, equivalent to 1.8 kg dry soil, was placed into 15 cm dia ceramic pots. The *P. bilaji*, when required, was placed in a hole bored in the center of each pot and covered with the soil from the hole. Rock phosphate, when required, was mixed with the soil from the center hole before replacing it in the pot. VAM inoculum was placed beneath each seed located between the edges of the pot and the center hole.

Six wheat seeds were planted per pot and thinned to four plants per pot after emergence, 50 μg N g^{-1} soil was added as NH_4NO_3. The plants were grown in a greenhouse under controlled conditions (16/8 h day/night, 20/16°C). Pots were weighed daily and water added to maintain the soil at field capacity. Plant tops were harvested at the early heading stage, oven-dried (70°C) to a constant weight, weighed and ground to pass a 40-mesh sieve. A 0.5 g subsample of ground plant material was wet digested using the Bray and Kurtz (1945) method. Aliquots of the digest were colorimetrically analyzed for P content (Olsen *et al.*, 1954) and for ^{32}P using liquid scintillation counting methods. The specific activities of plant P in the treatments were compared to determine the proportion of P in the plant coming from unlabeled sources. Statistical interpretation of the various measurements made were carried out using analysis of variance (ANOVA) and other standard methods outlined in Snedecor and Cochrane (1967).

RESULTS AND DISCUSSION

Solution studies

The pH of the uninoculated treatments for both media without rock phosphate remained constant throughout the duration of the 12-day experiment (Fig. 1). The addition of 0.1 or 0.2 g rock phosphate to the uninoculated media, raised the pH of the media by 0.5–1.0 pH units, where it remained. The media pH subsequently remained constant (Fig. 1). This was attributed to the addition of carbonates contained in the rock phosphate. Inoculation of either medium with either of the two *Penicillium* isolates but without the addition of rock phosphate, resulted in poor but visible growth of the fungi. Addition of rock phosphate plus the fungi resulted in visibly greater fungal growth.

In the absence of rock phosphate, inoculation of the media with either fungus resulted in a drop in the pH of the media (Table 1). Both fungi significantly altered the pH of media A (data for *P. bilaji* is shown in Fig. 1). Figures 2 and 3 show the pH of medium B as affected by the two isolates. With the addition of rock phosphate, both isolates were able to cause a drop in solution pH in medium A (Fig. 1), but only *P. bilaji* was able to do so in medium B (Figs 2 and 3). This indicates that different mechanisms were utilized by these fungi and that the effectiveness of the mechanisms varied. One mechanism relied on the presence of ammonium ions in the medium. With regard to the two isolates, *P. bilaji* was only marginally better at reducing solution pH than *P. fuscum* using this first mechanism (Table 1). A second mechanism did not require the presence of ammonium in the media. In this case, *P. bilaji* was significantly more adept at reducing pH and solubilizing P than *P. fuscum*, which could not generate enough acidity to overcome the alkaline effect of the rock phosphate. Under the optimum conditions of solution culture, the first mechanism, that relying on ammonium, was able to effect greater amounts of P solubilization.

The amount of P solubilized by *P. fuscum* was directly related to the drop in media pH produced (Table 1), showing that this isolate relied primarily on the first mechanism to release P. The P solubilized by *P. bilaji*, however, did not show this relationship. Obviously the acidity generated by the second mechanism, i.e. that not requiring NH_4 was not as effective at solubilizing rock phosphate since the pH

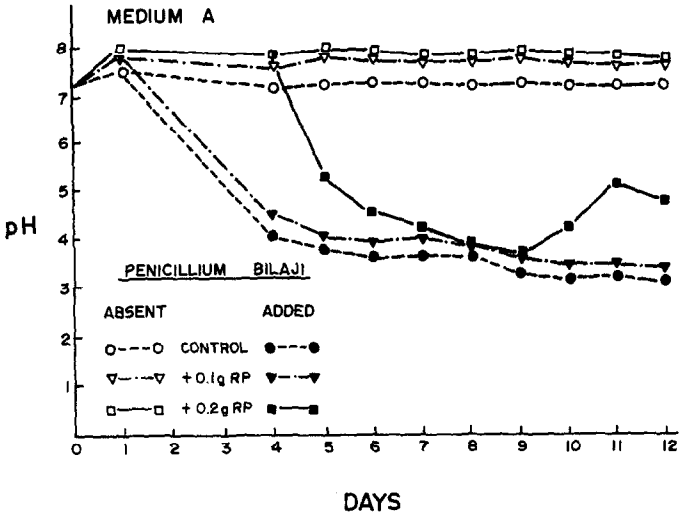

Fig. 1. Media pH values during 12 days following the addition of rock phosphate (RP) and *P. bilaji* (F_1) to solution medium A. Maximum SD of any one value was < 0.045 pH. Individual SD not shown.

Table 1. Minimum solution pH and maximum P in solution caused by two *Penicillium* isolates with and without NH_4^+

Penicillium isolate	Min. soln pH	Max. P conc. ($\mu g\ ml^{-1}$)	P in soln (mg)	P in soln by 0.1 NHCl at same pH (mg)[1]	P (fungi) / P (acid)
		Medium A[2]			
P. bilaji	3.7^{a}	298^{a}	14.2^{aA}	4.5^{bB}	3.2
P. fuscum	4.1^{b}	203^{b}	10.2^{bA}	1.6^{bB}	6.4
		Medium B[3]			
P. bilaji	4.0^{c}	46^{c}	2.5^{cA}	2.1^{cA}	1.2
P. fuscum	6.2^{a}	7^{d}	0.4^{cA}	0.1^{dB}	6.7

[1] After 10 days exposure to acid.
[2] Medium A—equimolar NH_4^+ and NO_3^--N.
[3] Medium B—NO_3^--N only.
[a–d] In either medium, means with the same superscript letter are not statistically different ($P < 0.01$).
[A, B] Comparison of means of P in solution (due to fungal action and to added HCl), means with the same superscript letter are not statistically different ($P < 0.01$).

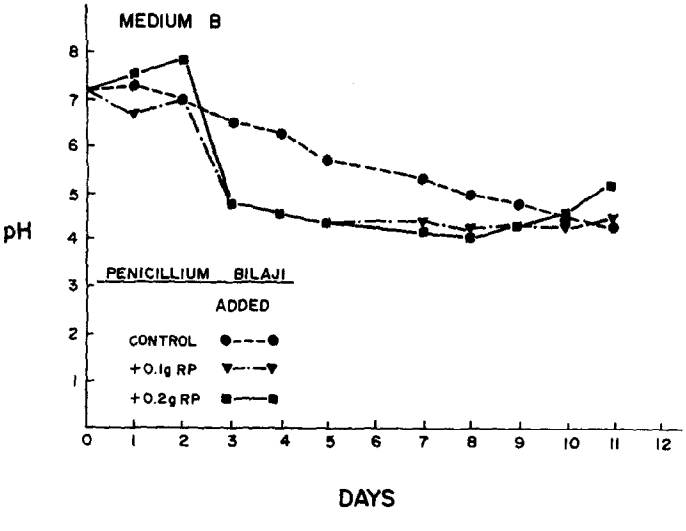

Fig. 2. Media pH values during 12 days following the addition of rock phosphate (RP) and *P. bilaji* (F_1) to solution medium B. Maximum SD of any one value was < 0.045 pH. Individual SD not shown.

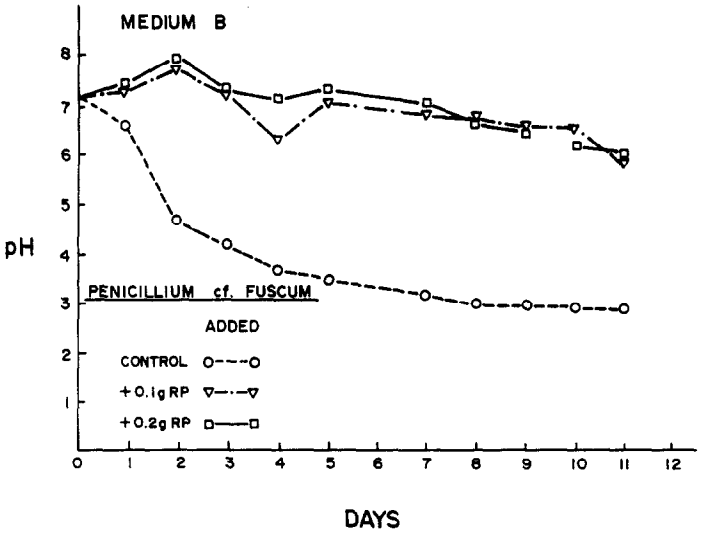

Fig. 3. Media pH values during 12 days following the addition of rock phosphate (RP) and *P. fuscum* (F_2) to solution medium B. Maximum SD of any one value was < 0.050 pH. Individual SD not shown.

of media A and B were not solubilizing rock phosphate since the pH of media A and B were not significantly different but the amounts of P released were (Table 1). This lack of correlation between the ability to reduce media pH and the ability to solubilize rock phosphate substantiates the work of Gaur *et al.* (1973) and Surange (1985).

The P solubilized by the fungi in medium A was greater than that released by acid dissolution of rock phosphate at equivalent solution pHs (Table 1), even after 10 days exposure to the acid. This indicates that both fungi, in the presence of NH_4, were not strictly relying on the production of acid to dissolve P, but were relying on another mechanism, possibly excretion of organic acid metabolites, to cause P release, and that this system was more effective at solubilizing P than strict acid dissolution. In medium B, the P released by *P. bilaji* was equivalent to that released by exposure to HCl, giving evidence that the mechanism not requiring NH_4 was possibly a strict acidifying effect (Table 1). The P released by *P. fuscum*, while exceptionally low, was still greater than that released by HCl, indicating that the same mechanism was being utilized by this isolate in the presence and absence of ammonium ions. Roos and Luckner (1984) established that an isolate of *Penicillium cyclopium* utilized an NH_4 /H exchange mechanism to reduce media pH while maintaining media electrical neutrality. In our study, it is probable that a NH_4/H exchange mechanism was employed by both *Penicillium* isolates, with the result that P solubilization was markedly accentuated. The quantities of P found in the media could not be attributed to acid alone.

The technique of Hue and Adams (1984) and Sabey *et al.* (1959) for measuring the effects of environmental factors on microbially-mediated processes was modified and applied to P solubilization. In both media, P concentration curves were typically sigmoid (Fig. 4). Using the aforementioned techniques, the maximum rate of P solubilization and the delay period to the onset of P solubilization were calculated. *P. bilaji* exhibited a shorter lag period than *P. fuscum*. Increasing the rock phosphate addition rate from 0.1 to 0.2 g in medium A adversely affected *P. bilaji* more than *P. fuscum* (Table 2, Fig. 4), indicating that the mechanism employed by *P. bilaji* to effect P solubilization was more sensitive to the increase in buffering ability of the rock phosphate. It could be inferred that even in the presence of ammonium ions, the two fungi employed different mechanisms for generation of acidity, or that possibly different organic acids were being excreted by the two fungi. Even at the reduced rate, however, *P. bilaji* showed greater ability to solubilize P than was observed for *P. fuscum*. Similar increases in the rock phosphate addition to medium B resulted in increased rates of P solubilization and decreased periods of delay (Table 2), however, the overall rates were of smaller magnitude than found for medium A.

In general, these results confirm the work of Kucey (1983, 1987) showing that *P. bilaji* has a greater ability to solubilize P than *P. fuscum*, although under the optimum conditions of solution culture and availability of NH_4^+, the differences were not large. *P. bilaji* appears to employ two mechanisms to lower the media pH, one mechanism relying on the presence of NH_4^+, while the second does not require ammonium. *P. fuscum* appears to employ only an ammonium requiring system which may be different from that used by *P. bilaji*. The mechanism requiring NH_4 is more effective than straight acid dissolution.

Soil studies

A significant ($P < 0.01$) increase in dry matter production and P content of wheat was measured as a response to inoculation of soil with *P. bilaji*. When all treatments involving *P. bilaji* are averaged and compared to the control (Table 3), Neepawa wheat plant dry matter yields increase by 30% and there is a 12% increase in P uptake. When all treatments involving *P. bilaji* are compared to the averages of all the treatments lacking *P. bilaji*, Neepawa wheat

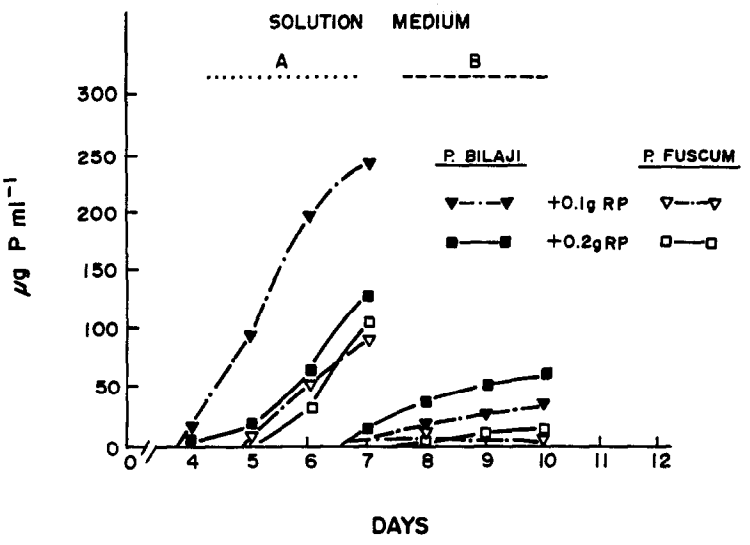

Fig. 4. Changes in solution P concentration with time following inoculation of medium A and medium B with two *Penicillium* spp at two concentrations of rock phosphate (RP). Maximum SD of any one value = 1.33 μg P ml^{-1}. Individual SD not shown.

Table 2. Variability in onset and rate of phosphate solubilization induced by presence and absence of ammonium supplied to two *Penicillium* isolates

Penicillium isolate	Rock phosphate (g 100 ml^{-1})	Maximum rate of P (μg P day^{-1})	Time to onset of P solubilization (days)
		Medium A[1]	
P. bilaji	0.1	77.4	3.7
	0.2	57.8	4.8
P. fuscum	0.1	41.9	4.8
	0.2	51.4	5.1
		Medium B[2]	
P. bilaji	0.1	9.6	6.3
	0.2	11.2	4.5
P. fuscum	0.1	−0.6	12.3
	0.2	6.9	7.8

[1]Medium A—equimolar NH_4^+ and NO_3^--N.
[2]Medium B—NO_3^--N only.

exhibited an overall 18% increase in plant dry matter yield and this was matched by an overall 5% ($P < 0.01$) increase in total P uptake (Table 3).

Microscopic examination of wheat roots showed that only VAM inoculated plants were colonized by VAM. VAM colonization resulted in a slight (3%) but non-significant reduction in wheat dry matter production and did not significantly affect plant P uptake (Table 3). These results concur with those of Gianinazzi-Pearson and Gianinazzi (1983) and others which indicate that not all combinations of host and VAM result in plant growth increases.

The addition of rock phosphate did not affect wheat dry matter production nor plant P uptake. Comparison of the specific activities of the plant P showed a significant effect of *P. bilaji* and of the addition of rock phosphate on the source of P absorbed by the plants (Table 3). The addition of rock phosphate to the soil resulted in ^{32}P isotopic dilution of the plant-absorbed P which could be calculated to mean that the rock phosphate supplied roughly 10% of the plant P both with and without VAM inoculation (Fried and Dean, 1952; Kucey and Bole, 1984). The rock phosphate did not, however, increase P supply in the soil as a whole, as indicated

Table 3. Dry matter, P uptake and sources of P for wheat inoculated with *P. bilaji* and VAM under greenhouse conditions

	Dry matter (g pot^{-1})	Total P uptake (mg pot^{-1})	% P derived from pool (labeled)	% P derived from pool (unlabeled)
Control	4.27	6.32	100.0	0.0
P. bilaji	5.75	7.99	82.3	17.7
VAM	4.36	6.67	96.6	3.4
Rock phosphate	4.59	6.47	89.7	10.3
Rock phosphate + *P. bilaji*	5.88	8.06	87.9	12.1
Rock phosphate + VAM	4.98	6.87	91.2	8.8
P. bilaji + VAM	5.43	7.98	88.3	11.7
Rock phosphate + VAM + *P. bilaji*	5.08	8.53	89.2	10.8
Analysis of variance[1]				
Rock phosphate	NS	NS	*	*
VAM	NS	NS	NS	NS
P. bilaji	**	**	**	**
VAM + *P. bilaji*	*	NS	NS	NS

[1]Only significant interactions shown (*$P < 0.05$, **$P < 0.01$).

by a lack of increase in plant P uptake for plants receiving rock phosphate alone. Possibly, isotopic exchange between the labeled soil P and the rock P resulted in a net tie-up of ^{32}P without increasing P availability in general. If so, then it is debatable whether the rock phosphate is available to the plant. VAM inoculation alone did not have an effect on the plant P specific activity. Bolan *et al.* (1984) also observed that the specific activities of P in both VAM and non-VAM colonized plants were the same when grown in ^{32}P-labeled soil, indicating that the VAM in that study were not supplying P from a source unavailable to the roots themselves.

Inoculation of the soil with *P. bilaji* resulted in ^{32}P isotopic dilution even in the absence of added rock phosphate. This indicates that the *Penicillium* fungus was able to cause the release of P from unlabeled sources, which in this case could only be from native soil P that was isotopically exchangeable. This can be stated with confidence since plant P uptake was increased as well (Table 3). When the fungus was added along with rock phosphate, no further isotopic dilution and only small increases in total P uptake were observed. This could mean that the fungus did not have a siginficant effect on solubilization of added rock phosphate or, if isotopic exchange did occur between the rock phosphate and soil P, then the fungus could be acting to release the exchangeable portion of the P in rock phosphate, which would result in the re-release of the ^{32}P. The high pH of soil (pH 8.1) and calcareous nature of rock phosphate makes it unlikely that any reaction occurred in the pot during 42 days growth. Hence we had no direct effect of rock phosphate. Double inoculation of pots with VAM and *P. bilaji* was not more effective than addition of *P. bilaji* alone, but was superior to the addition of VAM alone (Table 3). The highest total P uptake occurred in the treatment with all three factors treatment. The highest dry matter production was greater with *P. bilaji* than in corresponding treatments without *P. bilaji*. This indicates solubilization of rock phosphate since the total P uptake was increased above levels observed for treatments with *P. bilaji* and VAM alone. Azcon *et al.* (1976) reported similar findings for their work with VAM and a P-solubilizing bacterium. This indicates the complexity of P release in a soil system and a reliance of the plant on the interaction of all factors involved in the dynamics of P release; i.e. a source of P, a mechanism of causing its release and a mechanism for its uptake.

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