



Solubilization of phosphate rocks and minerals by a wild-type strain and two UV-induced mutants of *Penicillium rugulosum*

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Abstract

Two Venezuelan phosphate rocks (PRs), apatite deposits from Monte-Fresco and Navay areas, and two minerals, Florida apatite and Utah variscite were used to investigate phosphate solubilization by the wild type strain IR-94MF1 of *Penicillium rugulosum* initially selected for its high mineral phosphate activity (Mps⁺) and two of its mutants Mps⁺⁺ and Mps⁻. In liquid cultures, the three fungal strains showed better growth on the Navay PR than on Monte Fresco PR. The Utah variscite was the best phosphorus (P) source for the growth of the wild type and the Mps⁺⁺ mutant. Solubilization of the various P sources by the wild-type IR-94MF1 and the Mps⁺⁺ mutant resulted mostly from the action of organic acids. Citric acid seemed to be more active agent for the solubilization of the Utah variscite while gluconic acid appeared to be responsible for the solubilization of the Florida apatite and the Monte Fresco PR. Both organic acids are likely involved in the solubilization of the Navay PR. The Mps⁻ mutant did not produce any organic acid when grown on all the P sources used. © 2001 Elsevier Science Ltd. All rights reserved.

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1. Introduction

In South America, phosphate rocks (PRs) should provide a cheap source of phosphorus (P) fertilizer for crop production. However, most PR deposits have low reactivity (León et al., 1986) and cannot be used successfully as P sources for crop production (Kpombekou and Tabatabai, 1994). It has been shown that organic acids can greatly increase the concentration of P in soil solution through chelation and an exchange reaction (Gadd, 1999). Therefore, the application of P solubilizing microorganisms (Kucey et al., 1989; Muchovej et al., 1989) is a promising approach for increasing P availability in PR amended soils. Production of carboxylic acids like citric and oxalic acids was associated with calcium phosphate solubilization by *Penicillium bilaii* (Cunningham and Kuiack, 1992). Gluconic acid was implicated in the solubilization of a rock phosphate by *Penicillium variabile* P16 (Vassilev et al., 1996), the solubilization

of Ca and Al phosphate minerals by *Penicillium radicum* (Whitelaw et al., 1999), and the release of Ca²⁺, Al³⁺ and Fe³⁺ from rock samples by *Penicillium frequentans* (De La Torre et al., 1993). However, the release of toxic concentrations of some metals during PR solubilization can affect fungal growth, physiology and metabolism (Karamushka et al., 1996). We have previously described *Penicillium rugulosum* IR-94MF1 which was isolated from a Venezuelan soil and selected for its high mineral phosphate solubilization activity (Mps⁺) with hydroxyapatite, and two of its UV-induced mutants with very high (Mps⁺⁺) or negative (Mps⁻) activity (Reyes et al., 1999b). These mutants allowed the elucidation of the mechanisms of action involved in the Mps⁺ activity of isolate IR-94MF1. The Mps⁺ phenotype was mainly associated with the production of gluconic and citric acids, and it responded differently to calcium, iron and aluminum phosphate salts when used as P-sources and to ammonium, nitrate or arginine as nitrogen sources (Reyes et al., 1999a). Our long-term aim is the bioactivation of poorly soluble PR using *P. rugulosum* IR-94MF1. In this paper, we evaluated the solubilizing activity of IR-94MF1 on two sedimentary PR apatites obtained from

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Table 1
Chemical composition of the different phosphate sources used

Rock phosphate	mg g ⁻¹										
	P	Ca	Mg	Na	K	Mn	Fe	Cu	Al	Zn	Si
Florida	128	305	2.5	6.2	0.9	0.20	8.6	0.02	9.3	0.05	40.4
Utah	123	129	1.7	1.2	0.6	0.10	11.9	0.04	136.6	0.08	24.4
Navay	93	200	0.2	1.6	0.4	0.03	2.0	0.02	3.5	0.08	193.7
M. Fresco	99	264	1.5	1.9	2.5	0.04	7.3	0.04	16.2	0.31	86.2

the Monte Fresco and San Joaquin de Navay deposits in the southwest region of Venezuela. The activity of IR-94MF1 (isolated from a soil in contact with the Monte Fresco deposit) on the two Venezuelan PRs was also compared with its activity on an apatite mineral from Florida. As IR-94MF1 was also able to dissolve AlPO₄ (Reyes et al., 1999b) we further investigated its effect on a variscite mineral from Utah.

2. Materials and methods

Four forms of P were used: two Venezuelan PRs, apatite deposits, from Monte-Fresco and Navay areas; and two minerals, Florida apatite and Utah variscite (Ward's Natural Science Establishment, Rochester, NY). All the P sources were finely ground (100% through a 100-mesh Tyler screen, 0.149-mm opening) prior to use. Following digestion in HNO₃/HClO₄, the amount of P was determined by the vanado-molybdate method (Tandon et al., 1968), and Ca, Mg, Na, K, Mn, Fe, Cu, Al and Zn were determined by atomic absorption spectrophotometry (Gaines and Mitchell, 1979). Total Si was determined by lithium metaborate fusion as described by Suhr and Ingamells (1966). Phosphate material reactivity was measured according to Bolland and Gilkes (1997). Briefly, a 1 g sample of each phosphate material was extracted for 1 h at 23°C in 2% citric acid or 2% formic acid. After centrifugation and filtration, the P concentration was measured as described by Tandon et al. (1968).

Inocula of isolate IR-94MF1 and its mutants were prepared by using synchronous 3-day-old vegetative mycelia (Reyes et al., 1999b). The composition of the incubation medium was (per liter of distilled water): NH₄Cl, 0.4 g; KNO₃, 0.78 g; NaCl, 0.1 g; MgSO₄·7H₂O, 0.5 g; CaCl₂·2H₂O, 0.1 g; FeSO₄·7H₂O, 0.5 mg; MnSO₄·H₂O, 1.56 mg; ZnSO₄·7H₂O, 1.40 mg; vitamin B₁₂, 2 µg and sucrose, 30 g. The different P sources were used at a concentration of 96 mg P per 100 ml. With the Monte-Fresco PR, preliminary assays indicated that 2.5 mg per ml of soluble P (KH₂PO₄) had to be added to initiate fungal growth. Triplicate 250-ml Erlenmeyer flasks containing 100 ml of medium were inoculated with five disks (3 mm diameter) of the inoculum. Inoculated and uninoculated controls flasks were incubated at 28°C on a rotary shaker (150 rpm) in the dark (Reyes et al., 1999a). After 7 days, fungal biomass was collected by centrifugation (10 min, 8000 rpm, 4°C), washed with distilled water and oven-dried (90°C for 48 h). Fungal growth was expressed as organic matter produced per flask containing 100 ml of medium and was determined by weight loss after incineration at 500°C for 6–8 h. This method was chosen to avoid weight overestimation due to adherence of phosphate to the mycelium (Reyes et al., 1999b). A sub-sample of the culture supernatant was filtered through a 0.22-µm Millipore filter for pH and P determinations (Tandon et al., 1968). The amounts of organic acids in the filtrate were determined by the method of Baziramakenga et al. (1995) modified as follows. A Dionex 400i ion chromatograph (Dionex Corp.) equipped with an AS11 column and an AG11 guard column and a

Table 2
Extractability of P (mg g⁻¹) and microelements (µg g⁻¹) in different phosphate sources using 2% citric acid or 2% formic acid. Data are mean of triplicates ± standard deviation

P source	P	Cu	Zn	Fe	Mn	Al
2% Citric acid						
Florida	24.3 ± 0.6	4.6 ± 0.7	13.7 ± 0.3	1318.9 ± 62.6	30.4 ± 0.9	869.0 ± 7.1
Utah	14.0 ± 1.7	6.4 ± 0.3	15.9 ± 0.3	4577.9 ± 242.2	17.9 ± 1.0	4330.9 ± 96.2
Navay	10.3 ± 2.0	4.4 ± 0.4	20.3 ± 0.3	302.9 ± 6.1	0.8 ± 0.1	390.6 ± 15.8
M. Fresco	9.7 ± 1.0	8.6 ± 2.5	51.8 ± 5.1	530.3 ± 80.8	5.6 ± 1.4	398.1 ± 39.9
2% Formic acid						
Florida	24.2 ± 1.3	4.0 ± 0.2	16.9 ± 3.4	1086.0 ± 29.6	32.0 ± 0.4	761.5 ± 17.7
Utah	18.0 ± 0.5	8.5 ± 0.2	20.2 ± 0.9	6195.6 ± 670.2	29.8 ± 2.3	4337.7 ± 86.2
Navay	11.0 ± 0.9	4.4 ± 0.1	22.3 ± 1.1	169.5 ± 1.9	1.1 ± 0.1	383.9 ± 20.0
M. Fresco	11.0 ± 1.3	5.4 ± 0.1	57.3 ± 1.4	195.4 ± 19.8	6.4 ± 0.3	386.8 ± 27.3

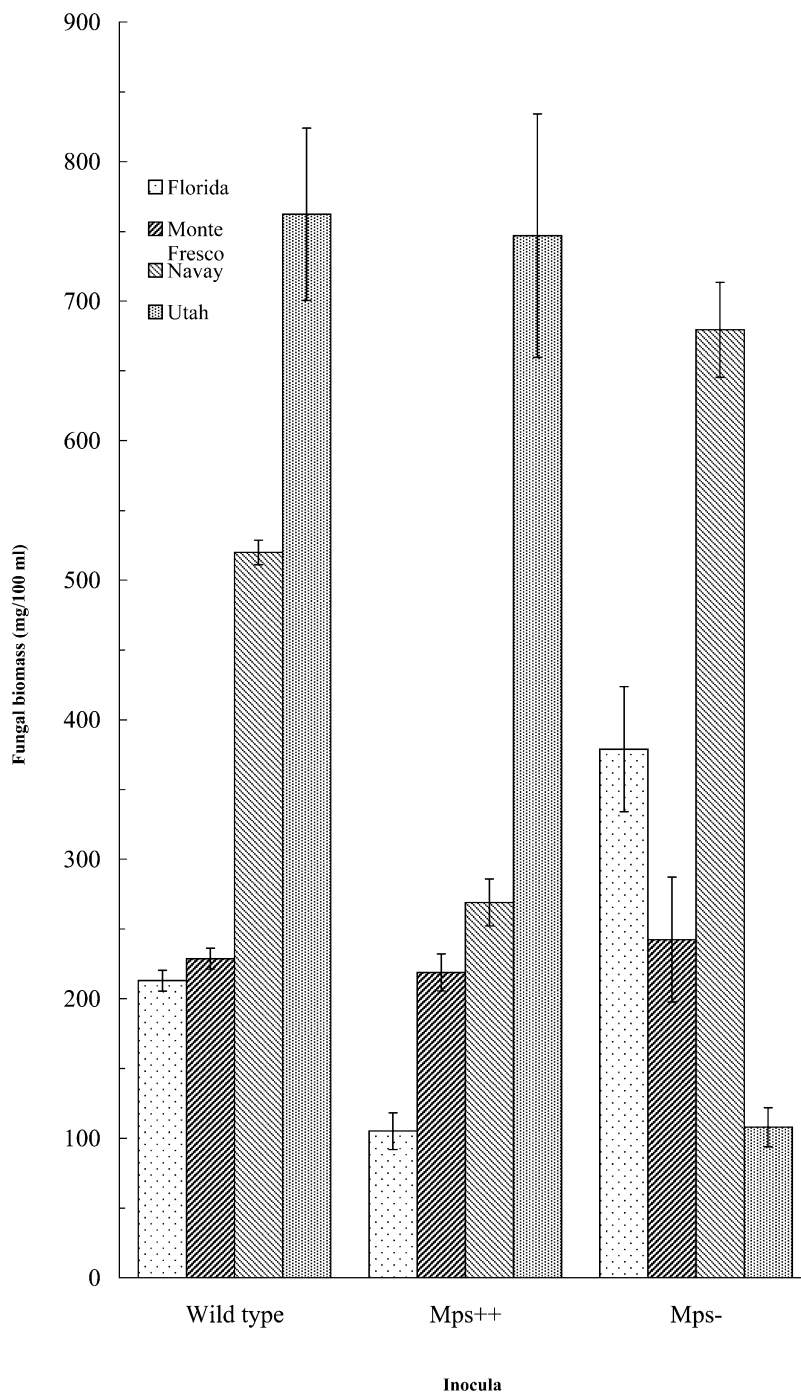


Fig. 1. Fungal biomass of the phosphate solubilizing (Mps^+) *P. rugulosum* IR-94MF1 and its Mps^{++} and Mps^- mutants after 7 days growth in liquid medium containing different P-sources. Error bars are \pm standard error ($n = 3$).

CDM-II conductivity detector was used. The elution was performed in 22 min, with a gradient that started with 2 mM and ended with 32.45 mM NaOH containing 18% methanol.

All the data (except pH) were \log_{10} -transformed to better normalize distributions. Analysis of variance (ANOVA) and the comparison of treatment means (LSD) were conducted by the General Linear Models of SAS (proc GLM; SAS, 1990).

3. Results and discussion

The chemical composition of the four phosphate materials used is presented in Tables 1 and 2. The total P contents ranged from 93 to 128 mg P g⁻¹. Florida apatite and Utah variscite contained higher amounts of P than Navay and Monte-Fresco apatites. Utah variscite contained lower amounts of Ca and higher amounts of Al and Fe as compared to other phosphate materials. The solubility of P

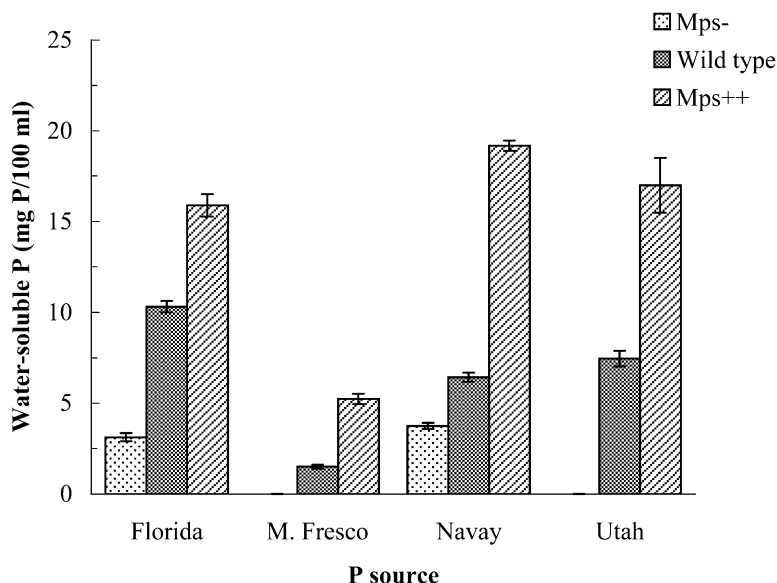


Fig. 2. Amount of P solubilized by *P. rugulosum* IR-94MF1 and its Mps⁺⁺ and Mps⁻ mutants, after 7 days incubation in liquid cultures. Error bars are \pm standard error ($n = 3$). Uninoculated control values were subtracted from their respective treatments.

in the phosphate materials, expressed as percentage of total P soluble in 2% citric acid and 2% formic acid, ranged from 10 to 19%.

After 7 days incubation in liquid media, a significant ($P < 0.01$) interaction was observed between the P-source used and the fungal inoculants, for all parameters studied. After 7 days the pH of the medium in the uninoculated flasks was 6.6, 6.8, 4.2 and 6.7 for the Florida apatite, the Monte Fresco and Navay PRs and the Utah variscite, respectively. A decrease in the pH of the medium was observed with the wild-type and Mps⁺⁺ mutant for all P sources (Table 3). In contrast, the pH of the medium was unaffected in the presence of the Mps⁻ mutant.

When used as the sole source of P, the Utah variscite and the Navay PR supported the greatest growth (as measured by organic matter production) of the wild-type *P. rugulosum* IR-94MF1 (Fig. 1). The best growth ($P < 0.01$) of the Mps⁺⁺ mutant occurred also on the Utah variscite, and that of the Mps⁻ mutant ($P < 0.01$) on Navay PR. All of the fungi tested exhibited similar growth with the Monte Fresco PR. However, in this case fungal growth occurred only when a minimum of 2.5 mg soluble P per 100 ml was added to the medium to initiate growth, and it was in

average one-third of that obtained on Utah variscite. In comparison to the other P-sources used, the Utah variscite mineral had higher Al and Fe content (Table 1), and produced higher Al and Fe concentrations in 2% citric or formic acid extracts (Table 2). Therefore, *P. rugulosum* IR-94MF1 seems to grow well under high Fe concentrations as previously reported (Reyes et al., 1999b) and to tolerate relatively high Al concentrations.

The greatest P ($P < 0.05$) solubilization was obtained with the Navay PR inoculated with the Mps⁺⁺ mutant (Fig. 2). This mutant gave also comparable solubilization values with Florida apatite and the Utah variscite. However, when the specific P solubilization activity (P solubilized g^{-1} fungal biomass) was considered, the Florida apatite allowed the highest values for the three strains. In fact, the amount of soluble P found in the culture filtrate of the Florida material may be attributed to the poor growth of the Mps⁺⁺ mutant on this P source (Fig. 1) in addition to the substantial amount of gluconic acid produced (Fig. 3). Otherwise, the P solubilization by the wild-type IR-94MF1 in the presence of the Florida apatite yielded the highest values compared to other P sources (Fig. 2).

When grown in the presence of the Utah variscite, the wild-type *P. rugulosum* IR-94MF1 produced a high quantity of citric acid (Fig. 3) but this was not translated into a significant accumulation of soluble P in the culture filtrate (Fig. 2). Like the wild-type, the Mps⁺⁺ mutant in the presence of Utah variscite produced a similar quantity of citric acid but a higher amount ($P < 0.05$) of gluconic acid, which could account for the increase in P solubilization by the Mps⁺⁺ mutant in the culture filtrate (Figs. 1 and 2). The amount of P that accumulated in the culture filtrate of the Mps⁺⁺ mutant in the presence of the Navay PR (Fig. 2) is probably the result of the amount of citric and gluconic

Table 3

pH of the culture filtrates of *P. rugulosum* IR-94MF1 and its two mutants after 7 days growth in liquid medium containing different P sources. Data are mean of triplicates. LSD were calculated for $P = 0.05$

Inocula	Florida	M. Fresco	Navay	Utah	LSD
Wild-type	3.51	3.25	4.40	3.28	0.13
Mps ⁺⁺	3.21	3.02	2.88	2.88	0.13
Mps ⁻	6.95	6.09	6.68	6.40	0.19
LSD	0.17	0.08	0.14	0.23	

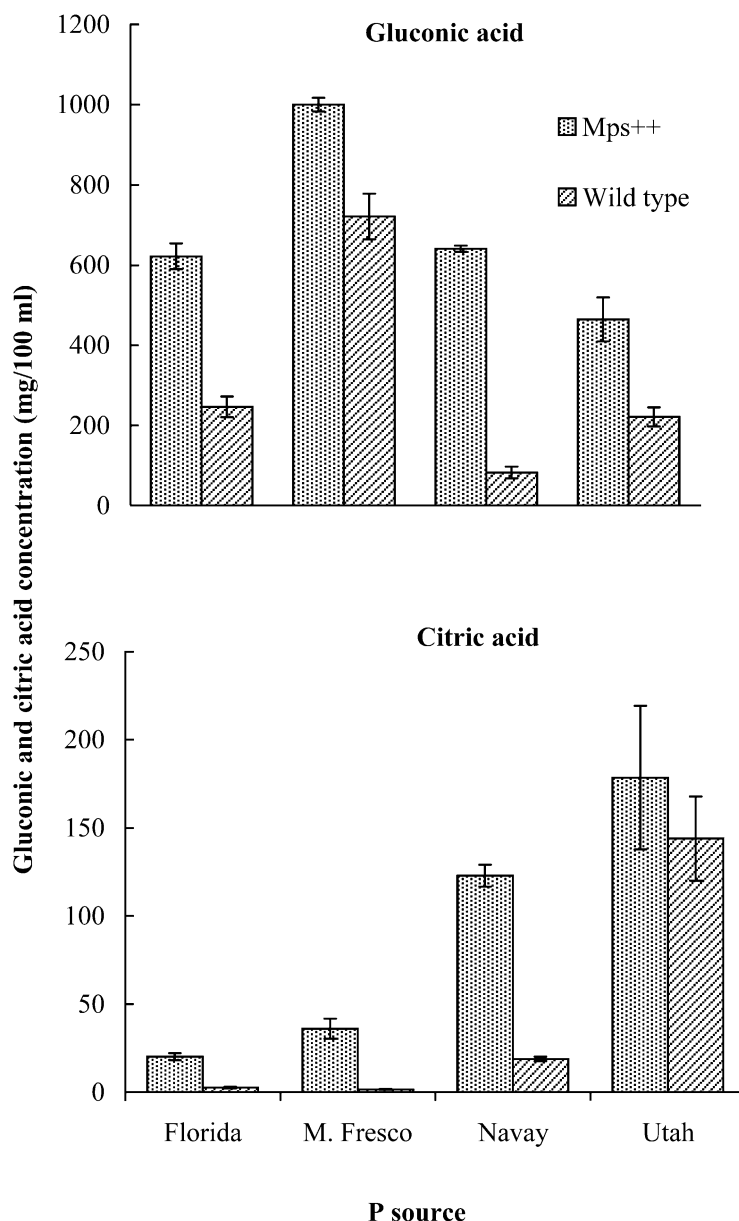


Fig. 3. Production of gluconic and citric acids by *P. rugulosum* IR-94MF1 and its Mps⁺⁺ mutant. After 7 days of growth in liquid medium no acid production was detected with Mps⁻ mutant. Error bars are \pm standard error ($n = 3$).

acids produced (Fig. 3) combined with the weak growth supported by this PR (Fig. 1). With the Monte Fresco PR, the wild-type IR-94MF1 and the Mps⁺⁺ mutant produced large quantities of gluconic acid (Fig. 3). However, this did not result in a better growth (Fig. 1) or a greater amount of soluble P in the medium (Fig. 2). No production of organic acid was detected with the Mps⁻ mutant, and its growth on the different P sources did not lower the pH of the culture filtrates as was observed with the two other fungi (Table 3). Our previous results obtained with less complex phosphate sources suggested the presence of a H⁺ pump mechanism involved in the solubilization of small amounts of P by the Mps⁻ mutant (Reyes et al., 1999a). In the present work this mechanism of action probably allowed the fungus to

develop a high biomass, in media containing either Florida apatite or Navay PR (Fig. 1). On the other hand, as less biomass was produced by the wild-type and the Mps⁺⁺ mutant with the apatite, more soluble-P was left in the medium (Fig. 2). This could be explained by the translocation of carbon to produce different types and amounts of organic acids instead of fungal biomass. With the Utah variscite as well as with iron phosphate (Reyes et al., 1999a), the high quantities of biomass produced by the wild-type and the Mps⁺⁺ mutant were accompanied with high levels of soluble-P in the medium. Further work is required to investigate these metabolic differences which could be related to the type of metabolic route used during the production of specific organic acids. Accordingly, the

mechanisms of action employed by the wild-type and Mps^{++} phenotypes could be potentially useful in soils containing iron phosphate or amended with variscite.

The intrinsic reactivity of PR based upon both their mineralogy and chemical composition (León et al., 1986) might also affect microbial solubilization. The presence of heavy metal ions in the different P sources can also influence the growth and probably the subsequent P-solubilization by fungi. In fact, in the Monte Fresco PR as compared to the other P sources tested, the Zn content was very high (Tables 1 and 2). This could explain in part the observed poor production of fungal biomass. Several authors have reported, antagonistic or synergistic interactions between toxicity of different metals in fungi (Hartley et al., 1997; Krantz-Rülcker et al., 1996). For example, Zn was reported to inhibit K^+ uptake, H^+ efflux and growth in fungi (Gadd, 1993). It has also been suggested that microorganisms may produce metal complexing agents (some organic acids, chitin and chitosan, phenolic polymers and melanins) to reduce the free metal ion concentration in solutions as a detoxification mechanism (Krantz-Rülcker et al., 1996; Gadd, 1993). Therefore, the presence of high concentrations of available toxic metals might decrease phosphate solubilization activity.

As previously documented, solubilization of different inorganic phosphates by microorganisms results from at least two mechanisms of action: release of organic acids (citric and gluconic acid), and release of protons accompanying respiration or NH_4^+ assimilation (De Freitas et al., 1997; Reyes et al., 1999b). In this work the wild-type IR-94MF1 and its Mps^{++} mutant solubilized the different P sources tested mainly by producing organic acids. Citric acid appeared to be more effective with the Utah variscite and gluconic acid was responsible for the solubilization of the Florida apatite and the Monte Fresco PR. Both organic acids are likely involved in the solubilization of the Navay PR. Thus, both the mineralogy and the chemical composition of the different phosphate sources seem to affect the growth and the expression of the Mps^+ phenotype in *P. rugulosum*. The over-expression of the Mps^+ phenotype in the Mps^{++} mutant caused an important increase in the P solubilization activity. Future work with this mutant will be aimed at the evaluation of its potential use in combination with different PRs to increase soil P availability for different crops. Finally, changes in the microbial metabolism related to the presence of metals, different phosphate and nitrogen sources should be considered in any biological phosphate solubilization assessment.

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