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# Effect of nitrogen source on the solubilization of different inorganic phosphates by an isolate of *Penicillium rugulosum* and two UV-induced mutants

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**Abstract**

The mechanisms of action of mineral phosphate solubilization (MPS) were studied in the wild-type $Mps^{+}$ *Penicillium rugulosum* strain IR94-MF1 and in negative ($Mps^{-}$) and superpositive ($Mps^{++}$) mutants derived from it. MPS activities were measured in liquid media using sucrose as C source, four N (arginine, nitrate, nitrate+ammonium and ammonium) and P sources ($KH_2PO_4$, hydroxyapatite, $FePO_4$ and $AlPO_4$). Ammonium significantly ($P < 0.01$) decreased phosphate solubilization, and this activity was 1–66 times higher in the $Mps^{++}$ mutant than in the wild-type depending on the P and N sources used. The $Mps^{+}$ phenotype was strongly associated with the production of gluconic or citric acids. The results also suggest for the $MPS^{-}$ mutant the involvement of the $H^{+}$ pump mechanism in the solubilization of small amounts of phosphates. 

*Keywords:* *Penicillium rugulosum*; Phosphate solubilization; Citric acid; Gluconic acid; Nitrogen source; Phosphate source

## 1. Introduction

The role of soil microorganisms in the solubilization of inorganic phosphates in relation to soil phosphate mobilization has been the subject of an increasing number of studies in recent years [1–5]. In fact, several bacteria and fungi were isolated from soil and evaluated for their mineral phosphate solubilizing (MPS) activity with various P sources such as calcium phosphate [6,7], iron phosphate [8] and aluminum phosphate [9,10]. Results from studies carried out in liquid media indicated that some microorganisms active on calcium phosphates are poor iron and aluminum phosphate solubilizers [9,11].

Usually, in vitro MPS activity is associated with a drop in pH [1,11], however, some reports do not show such a trend [6]. Potential mechanisms for explaining MPS activity point to acidification either by proton extrusion associated with ammonium assimilation [12], or by organic acid production [13]. Therefore, P solubilizing microorganisms can be

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very effective in solubilizing calcium phosphate with [13–15] or without [7] organic acid production. MPS activity is usually measured by using glucose [1,6,16] or sucrose [11,13] as the sole carbon source. Furthermore, in most studies ammonium was found to be a better N source than nitrate [1,11]. These observations indicate that P solubilization is a complex phenomenon which depends on many factors such as the nutritional, physiological and growth conditions of the cultures [13].

*Penicillium rugulosum* IR-94MF1 is a fungus with a high hydroxyapatite (HA) solubilization capacity isolated from a pasture tropical soil present on the top of an unexploited apatite-rock-phosphate mine [17]. Mutants with a negative ($Mps^{-}$) or an amplified ($Mps^{++}$) MPS activity were developed and our results showed that P solubilization activities were significantly higher when sucrose was used as the C source as compared to glucose or maltose [17].

With the aim of elucidating the mechanisms of action involved in the MPS activity, in this work *P. rugulosum* IR-94MF1 and its $MPS^{-}$ and $MPS^{++}$ mutants were used to investigate the effect of different N sources on the solubilization of inorganic phosphates. We also report on the effect of the different N and P sources on the production of organic acids associated with MPS activity.

## 2. Materials and methods

### 2.1. Inoculum preparation

Inocula for the wild-type *P. rugulosum* IR-94MF1 and its mutants were prepared by using a synchronous 3-day-old vegetative mycelium prepared as described by Reyes et al. [17].

### 2.2. Phosphate solubilization experiments

To study MPS activity the basal medium (BM) used contained per liter of distilled water: NaCl, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.5 mg; $MnSO_4 \cdot H_2O$, 1.56 mg; $ZnSO_4 \cdot 7H_2O$, 1.40 mg; vitamin $B_{12}$, 2 μg; and sucrose, 30 g. The following N sources were used: arginine (3.7 mM with 0.025% N), nitrate (18 mM with 0.025% N), nitrate+ammonium (9 mM with 0.012% N for each N form) and ammonium (0.7–36 mM with 0.001–0.05% N). Inorganic phosphate sources were washed three times to remove any soluble P and added at a concentration of 30 mM P of HA ($Ca_5HO_{13}P_3$, Sigma), $FePO_4 \cdot 4H_2O$ (Fluka Chemika) or $AlPO_4$ (Fisher). A soluble phosphate source, $KH_2PO_4$ (3 mM P), was used as a control. For each inorganic phosphate and nitrogen source tested, triplicate 250-ml Erlenmeyer flasks containing 100 ml BM were used. Each flask received five disks (3 mm diameter) of the inoculum. Inoculated flasks and uninoculated controls were incubated at 28°C on a rotary shaker (150 rpm) in the dark. To inhibit any potential bacterial contaminant, all media were supplemented with 30 and 100 μg $ml^{-1}$ of chloramphenicol and streptomycin sulfate, respectively. At each sampling date (3, 5 and 7 days), a 3-ml subsample of the culture supernatant was aseptically withdrawn from each flask and filtered through a 0.22-μm Millipore filter. One half of the filtrate was used to measure pH with a flat surface Fisher electrode (because of its small volume) and the other half was used for colorimetric determination of P by the vanado-molybdate method [18]. At the end of the experiment, the 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 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 the adherence of phosphate to the mycelium. Values obtained with the uninoculated controls were always subtracted from their respective treatments.

### 2.3. Organic acid determination by ion chromatography

After 7 days incubation, the organic acids present in the culture filtrates of the different treatments were separated by the method of Baziramakenga et al. [19] modified as follows. A Dionex 4000i ion chromatograph (Dionex Corp.) equipped with an AS11 column and an AG11 guard column and a 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. Culture filtrate

samples and standard controls of citric and gluconic acids were incubated with citrate lyase [20] or gluconate kinase and ATP [21] in order to confirm the presence of citric and gluconic acids.

### 2.4. Data analyses

The homogeneity of the variance (ANOVA), comparison of treatment means (LSD) and regression analyses were conducted by the general linear models (GLM) of SAS using the rank procedure [22,23] because of the non-normal distribution of some data. Data were transformed with ranks for analysis and retransformed for presentation.

## 3. Results

### 3.1. Effect of ammonium concentration on growth and MPS activity

When the concentration of ammonium in the culture medium was increased from 3.6 to 7 mM, a significant ($P < 0.01$) decrease of HA solubilization by the wild-type IR-94MF1 and the $Mps^{++}$ mutant was observed (Table 1). The growth of both fungi was inhibited by an ammonium concentration of 36 mM. Although the $Mps^{-}$ mutant was able to grow on HA solid medium (results not shown), it did not show any detectable growth in HA liquid medium.

Table 1
Effect of ammonium concentration on HA solubilization and growth of *P. rugulosum* IR-94MF1 and its $Mps^{++}$ mutant after 7 days of incubation in liquid medium

| Isolate[a] | Ammonium (mM) | Soluble P (mM) | Biomass (mg 100 $ml^{-1}$) |
|---|---|---|---|
| Wild-type | 3.6 | 1.94 a | 17.4 a |
| | 7.0 | 1.24 b | 20.3 a |
| | 36.0 | 0.05 c | ng[b] |
| $Mps^{++}$ | 3.6 | 2.17 a | 17.0 a |
| | 7.0 | 1.31 b | 10.4 b |
| | 36.0 | traces | ng |

Data are means from experiments performed in triplicate. For each isolate, means in each column followed by the same letter are not significantly different ($P > 0.01$) according to the LSD test performed with the rank procedure.
[a]The $Mps^{-}$ mutant did not show detectable growth in the liquid culture medium.
[b]ng: no growth.

The highest HA solubilization rates were obtained only when ammonium was used as sole N source at a very low concentration (0.7 mM) as shown in Fig. 1. The solubilization of $AlPO_4$ was affected in a similar manner by ammonium. In fact, the rates of $AlPO_4$ solubilization obtained with 0.7 mM ammonium are comparable to those of HA (Fig. 1). A 10-fold (7 mM) increase in the concentration of this nitrogen source reduced $AlPO_4$ solubilization by 56% and 35% for the wild-type and the $Mps^{++}$ mutant respectively, whereas an increase of 19% was observed with the $Mps^{-}$ mutant (results not shown). In $AlPO_4$ medium fungal growth was also reduced 52%, 71% and 11%, for the wild-type, the $Mps^{++}$ and the $Mps^{-}$ mutants respectively, when the ammonium concentration was increased from 0.7 to 7 mM (results not shown).

### 3.2. Solubilization of poorly soluble inorganic phosphates

Although all inorganic phosphate sources were washed before use, the incubation of the flasks with agitation probably solubilized some P. In fact, according to the vanado-molybdate method (detection limit 0.03 mM P) some soluble P was detected in uninoculated control flasks with $FePO_4$ and $AlPO_4$, but not with HA (Fig. 1).

P solubilization activity measured as soluble P present in the medium was faster and more efficient for HA than for other P sources tested. The three P sources used presented quite different patterns of phosphate solubilization. Of all the N sources tested, ammonium caused a significantly ($P < 0.05$) lower HA solubilization for the wild-type and the $Mps^{++}$ mutant. The $Mps^{-}$ mutant was not able to grow or solubilize P in the HA liquid media with any of the N sources used (Fig. 1).

When P was supplied in the form of $FePO_4$ with all N sources, the $Mps^{-}$ mutant probably used for growth the soluble P present in the culture media but it did not show any MPS activity. In a similar manner, the wild-type also immobilized in its biomass phosphate from $FePO_4$. In the arginine treatments solubilization was very low and the soluble P concentrations measured after 7 days were slightly higher than those of the uninoculated control. A different pattern of $FePO_4$ solubilization was observed with

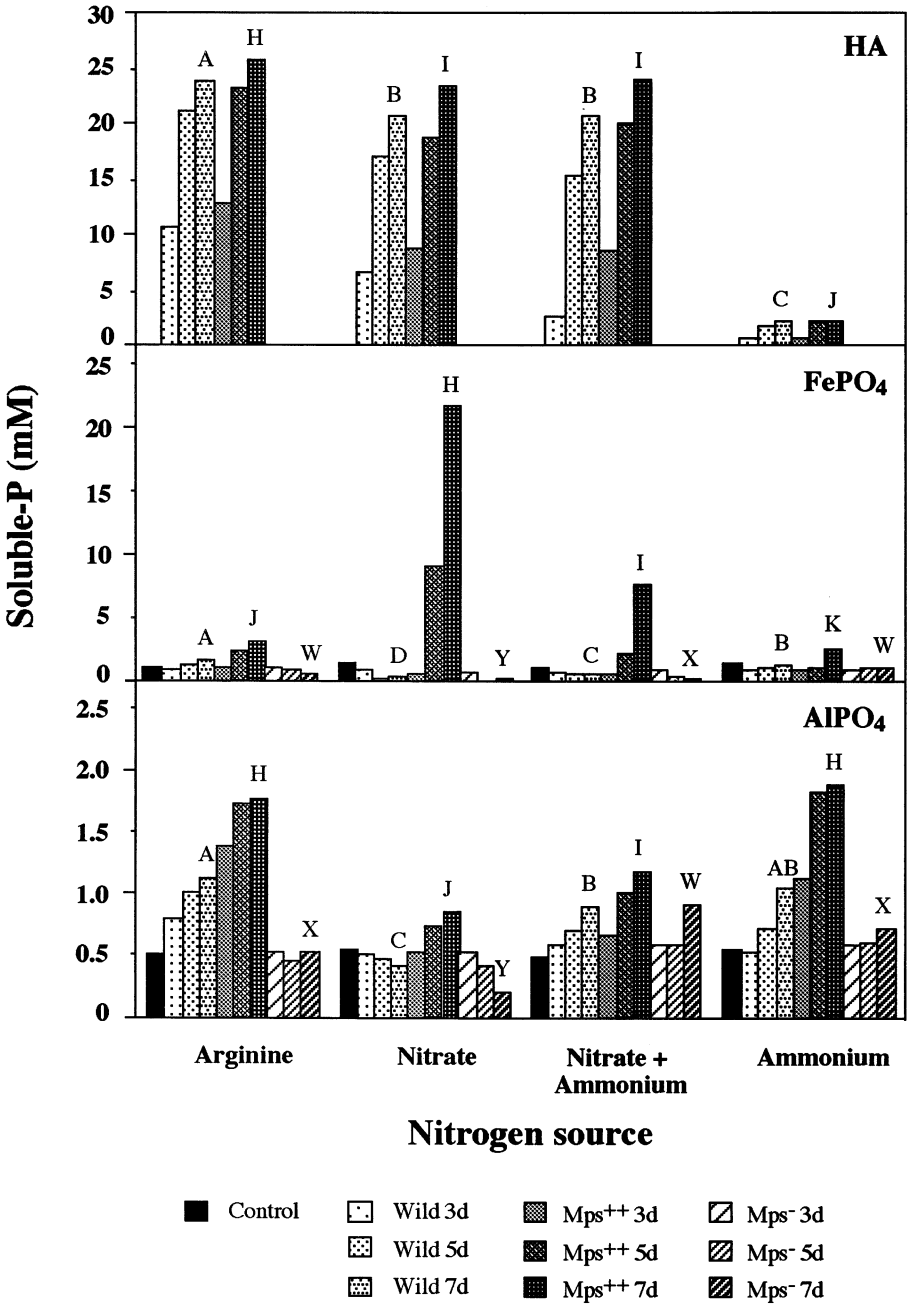


Fig. 1. Solubilization of HA, $FePO_4$ ad $AlPO_4$ by *P. rugulosum* isolate IR-94MF1 and its $Mps^{++}$ and $Mps^{-}$ mutants in liquid medium using sucrose as the C source and different N sources (3.7 mM arginine, 18 mM nitrate, 18 mM nitrate+ammonium and 0.7 mM ammonium) after 3 (3d), 5 (5d) and 7 (7d) days of incubation. Values are means of three replicates. Means labeled with the same letter are not significantly ($P > 0.01$) different according to the LSD test used with the rank procedure.

the $Mps^{++}$ mutant. In fact, nitrate when used as the sole N source induced the highest solubilization of $FePO_4$. The addition of ammonium to nitrate significantly ($P < 0.01$) decreased $FePO_4$ solubilization (Fig. 1).

The best $AlPO_4$ solubilization by the wild-type and the $Mps^{++}$ mutant was obtained when arginine or ammonium was used as the sole N source. Significantly ($P < 0.05$) lower activities were observed with nitrate or nitrate+ammonium.

### 3.3. Acidification of media

The results of the acidification of the culture media when HA was used as the P source are not presented in Fig. 2, because they were similar for the wild-type and the $Mps^{++}$ mutant, with all N sources. From initial values of 6.8 and 7.0, the pH dropped at day 3 to 3.9 and 4.2 and the values stayed between 3.7 and 3.8 after 5 and 7 days incubation, for both the wild-type and the $Mps^{++}$ mutant respectively. In general comparable drops in pH were observed with all isolates in the presence of soluble phosphate ($KH_2PO_4$; Fig. 2). With $KH_2PO_4$, $FePO_4$ and $AlPO_4$ in the presence of nitrate, the $Mps^{-}$ mutant significantly ($P < 0.01$) increased the pH while the $Mps^{++}$ mutant decreased it (Fig. 2B). The lowest pH values were recorded in $AlPO_4$ media containing nitrate+ammonium, inoculated with the wild-type or the $Mps^{-}$ mutant ($P < 0.01$, Fig. 2C). These observations indicate that the $Mps^{-}$ mutant like the wild-type is able to acidify the culture media in the presence of ammonium. Significant negative correlations ($P < 0.01$) were observed between P solubilization and the pH of the culture media. After 7 days of growth, Spearman coefficients ($r_s$) for HA, $FePO_4$, and $AlPO_4$ were respectively $-0.69$, $-0.52$ and $-0.86$ ($P < 0.01$).

### 3.4. Growth

Measurements of organic matter production indicated that growth of all isolates was affected by the N and P sources used. In the presence of poorly soluble phosphate sources (HA, $FePO_4$ and $AlPO_4$), when arginine or ammonium (0.7 mM) was used as the sole N source, the organic matter produced by all isolates was significantly ($P < 0.01$) lower than that

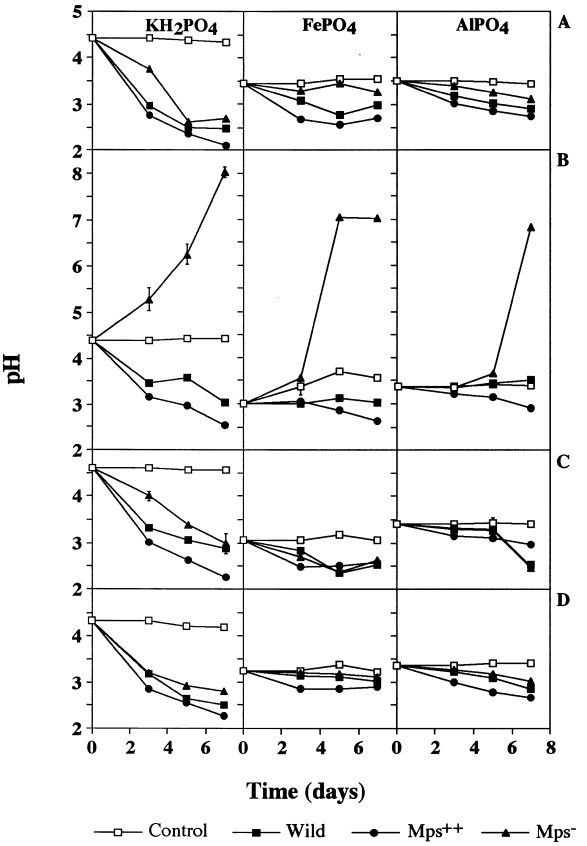


Fig. 2. Changes of the pH of the culture filtrate of *P. rugulosum* IR-94MF1 and its $Mps^{++}$ and $Mps^{-}$ mutants cultivated in liquid media containing sucrose as the sole C source and different N or P sources: (A) 3.7 mM arginine; (B) 18 mM nitrate; (C) 18 mM nitrate+ammonium; and (D) 0.7 mM ammonium. Values are means of three replicates. Error bars (±S.D.) are shown when larger than the symbol.

obtained with nitrate (Table 2). The highest organic matter production was obtained with the $Mps^{++}$ mutant grown in the $FePO_4$ medium containing nitrate as the N source. When phosphate was supplied as soluble $KH_2PO_4$, ammonium at 18 mM was as good as other N sources for the wild-type and the $Mps^{-}$ mutant, but not for the $Mps^{++}$ mutant.

Growth of *P. rugulosum* IR-94MF1 on poorly soluble inorganic phosphate sources increased in the following order: HA < $AlPO_4$ < $FePO_4$ (Table 2).

### 3.5. Organic acid production

When sucrose was used as the sole C source, the

Table 2
Growth of *P. rugulosum* IR-94MF1 and its mutants expressed as organic matter produced in liquid medium after 7 days of incubation with different P and N sources

| Isolate | N source[a] | Organic matter (mg 100 ml$^{-1}$) | | | |
|---|---|---|---|---|---|
| | | $KH_2PO_4$ | HA | $FePO_4$ | $AlPO_4$ |
| Wild-type | Arginine | 380.3 b | 21.4 b | 61.0 c | 14.3 d |
| | Nitrate | 348.3 b | 48.4 a | 291.5 b | 339.6 a |
| | Nitrate+ammonium | 377.2 b | 48.3 a | 481.2 a | 95.0 b |
| | Ammonium I | 63.9 c | 17.2 b | 52.5 c | 34.7 c |
| | Ammonium II | 461.2 a | ng[b] | nt[c] | nt |
| Mps$^{++}$ | Arginine | 390.5 a | 28.2 b | 47.6 b | 10.0 c |
| | Nitrate | 317.9 ab | 63.2 a | 585.0 a | 126.4 a |
| | Nitrate+ammonium | 250.2 bc | 74.5 a | 561.4 a | 27.8 b |
| | Ammonium I | 80.1 d | 31.3 b | 33.3 b | 13.9 c |
| | Ammonium II | 181.7 c | ng | nt | nt |
| Mps$^{-}$ | Arginine | 370.5 ab | ng | 25.6 c | 23.3 b |
| | Nitrate | 239.0 c | ng | 311.8 b | 187.3 a |
| | Nitrate+ammonium | 335.5 ab | ng | 451.5 a | 132.1 a |
| | Ammonium I | 79.7 d | ng | 14.8 c | 24.7 b |
| | Ammonium II | 404.9 a | ng | nt | nt |

[a]Arginine 3.7 mM, nitrate 18 mM, nitrate+ammonium 18 mM, ammonium I 0.7 mM (0.001% N) and ammonium II 18 mM (0.025% N). Means in the same column followed by the same letter are not significantly different ($P > 0.01$) according to the LSD test used with the rank procedure. Data are means from experiments performed in triplicate.
[b]ng: no growth.
[c]nt: not tested.

MPS activity was accompanied by the production of citric and gluconic acids in the culture media; however, only gluconic acid was found when glucose was used [17]. With sucrose, both acids were produced by the wild-type and the Mps$^{++}$ mutant, but they were not detected in filtrates from the Mps$^{-}$ mutant (Tables 3 and 4). The organic acids were determined in the culture filtrates after 3, 5 and 7 days of growth, but only data for 7 days are presented in Tables 3 and 4. In general, the concentration of citric and gluconic acids increased gradually in the culture filtrate after 3–7 days of incubation. This increase was

Table 3
Gluconic acid[a] (mM) present in the culture filtrate of *P. rugulosum* IR-94MF1 and its mutants after 7 days of incubation in the presence of different P and N sources

| N source | Isolate | $KH_2PO_4$ | HA | $FePO_4$ | $AlPO_4$ |
|---|---|---|---|---|---|
| Arginine | Wild-type | 10.33 ± 2.26 | 96.58 ± 8.41 | 0.62 ± 0.04 | 12.50 ± 2.20 |
| | Mps$^{++}$ | 32.99 ± 3.94 | 107.47 ± 3.67 | 16.95 ± 0.73 | 48.76 ± 3.41 |
| | Mps$^{-}$ | 0.23 ± 0.01 | ng[b] | nd[c] | nd |
| Nitrate | Wild-type | 16.25 ± 1.06 | 97.72 ± 1.01 | 19.94 ± 0.56 | 10.49 ± 0.53 |
| | Mps$^{++}$ | 32.66 ± 3.81 | 90.28 ± 2.86 | 7.72 ± 2.19 | 21.01 ± 1.49 |
| | Mps$^{-}$ | 0.43 ± 0.09 | ng | nd | 0.24 ± 0.13 |
| Nitrate+ammonium | Wild-type | 1.14 ± 0.34 | 95.56 ± 2.03 | 0.65 ± 0.11 | 4.93 ± 0.72 |
| | Mps$^{++}$ | 9.39 ± 2.70 | 87.92 ± 8.99 | 2.82 ± 1.07 | 10.57 ± 2.60 |
| | Mps$^{-}$ | 0.22 ± 0.04 | ng | nd | nd |
| Ammonium | Wild-type | 2.05 ± 0.36 | 0.18 ± 0.30 | 0.80 ± 0.19 | 13.46 ± 4.09 |
| | Mps$^{++}$ | 15.24 ± 0.55 | 5.66 ± 0.05 | 8.40 ± 1.30 | 49.09 ± 3.02 |
| | Mps$^{-}$ | 0.04 ± 0.01 | ng | nd | nd |

[a]Values are means of duplicate measurements (± S.D.).
[b]ng: no growth.
[c]nd: not detected.

Table 4
Citric acid[a] (mM) present in the culture filtrate of *P. rugulosum* IR-94MF1 and its mutants after 7 days of incubation in the presence of different P and N sources

| N source | Isolate | $KH_2PO_4$ | HA | $FePO_4$ | $AlPO_4$ |
|---|---|---|---|---|---|
| Arginine | Wild-type | 2.20 ± 0.65 | 0.05 ± 0.01 | 0.14 ± 0.01 | nd |
| | $Mps^{++}$ | 12.13 ± 3.80 | 0.09 ± 0.02 | 0.33 ± 0.05 | 0.11 ± 0.01 |
| | $Mps^{-}$ | nd[b] | ng[c] | nd | nd |
| Nitrate | Wild-type | 12.78 ± 2.55 | 0.11 ± 0.01 | 0.31 ± 0.06 | 2.28 ± 0.21 |
| | $Mps^{++}$ | 12.27 ± 1.12 | 0.28 ± 0.03 | 14.27 ± 1.72 | 3.28 ± 0.42 |
| | $Mps^{-}$ | nd | ng | 0.01 ± 0.01 | 0.06 ± 0.03 |
| Nitrate+ammonium | Wild-type | 1.30 ± 0.13 | 0.06 ± 0.01 | 0.33 ± 0.13 | 0.27 ± 0.01 |
| | $Mps^{++}$ | 14.32 ± 2.89 | 0.17 ± 0.01 | 10.87 ± 1.30 | 0.35 ± 0.07 |
| | $Mps^{-}$ | nd | ng | nd | nd |
| Ammonium | Wild-type | 0.51 ± 0.03 | 0.02 ± 0.02 | 0.10 ± 0.02 | 0.02 ± 0.01 |
| | $Mps^{++}$ | 1.25 ± 0.22 | 0.01 ± 0.01 | 0.52 ± 0.10 | 0.01 ± 0.04 |
| | $Mps^{-}$ | nd | ng | nd | nd |

[a]Values are means of duplicate measurements (± S.D.).
[b]nd: not detected.
[c]ng: no growth.

associated with an increase in the excess of soluble P found in the filtrate. After 7 days, the highest production of gluconic acid was observed when HA was used as phosphate source for both the wild-type and the $Mps^{++}$ mutant (Table 3). In general, for all other P and N sources the $Mps^{++}$ mutant exhibited higher rates of gluconic acid production than the wild-type, while the $Mps^{-}$ mutant presented traces of gluconic acid with $KH_2PO_4$ and undetectable amounts with other phosphate sources. The highest citric acid production by the wild-type was obtained with $KH_2PO_4$ only when nitrate was the N source, while the $Mps^{++}$ mutant was able to produce similar citric acid concentrations with all other N sources except ammonium (Table 4). Similar high concentrations were produced by the $Mps^{++}$ mutant with $FePO_4$ in the presence of nitrate.

## 4. Discussion

In tropical soils, inorganic phosphates are found in three poorly available fractions, calcium, iron and aluminum phosphates (Ca-P, Fe-P and Al-P). Transformations from one form to another occur and their solubilities with respect to soil acidity decrease in the following order: Ca-P > Al-P > Fe-P [24]. The increase of soil weathering and the enhancement of nutrients availability in soil are frequently associated with the production of organic acids. The chelation property of citric and oxalic acids enables them to form stable complexes with $Ca^{2+}$, $Fe^{3+}$ and $Al^{3+}$ liberating phosphates [25–27] and sulfates [28] into soil solution, whereas gluconic acid and 2-ketogluconic acid have been proposed to dissolve calcium phosphates by the release of acidic protons [29]. *P. rugulosum* IR-94MF1 was able to solubilize different poorly soluble inorganic phosphates such as HA, $FePO_4$, $AlPO_4$ and some rock phosphate ores (not shown). The comparative analysis of HA solubilization by the $Mps^{++}$ and $Mps^{-}$ mutants allowed us to suggest for the wild-type several mechanisms of action that can be implicated in the MPS activity.

Nutritional status, mainly the nature of P and N sources, can affect phosphate solubilization by *P. rugulosum* IR-94MF1 beyond their effect on the development of the fungal biomass (Fig. 1 and Table 2). At a low rate of P solubilization the fungus used for growth the little soluble P that can be present in the culture medium and most of the newly available P, solubilized from the poorly soluble phosphate sources. In the presence of a high rate of solubilization, in addition to the P used for growth, an excess of P was detected in the culture filtrates. The isolate IR-94MF1 of *P. rugulosum* grows better on nitrate than on ammonium, when relatively insoluble phosphate sources are used. A poor assimilation of ammonium has been reported and related to a low performance of the Krebs cycle recharge reaction for an isolate of the ectomycorrhizal fungus *Hebeloma cy-*

*lindrosporum* [30]. Phosphate solubilization by *P. rugulosum* IR-94MF1 appeared to be particularly sensitive to the presence of ammonium chloride, because the concentration used in this study was lower than those used with other fungi like *P. bilaii* [13] and *P. simplicissimun* [10] which were 37 and 9.3 mM, respectively. As citric and gluconic acids were found to be implicated in the MPS phenotype of the wild-type and its mutants, more work is required to verify if the ammonium reduction of growth and phosphate solubilization is caused by an insufficient $CO_2$ fixation at the level of the recharge reaction of the Krebs cycle (anaplerotic reactions), which in turn could affect the production of citric acid. The effect of the different N sources on the production of citric and gluconic acids for both the wild-type and the $Mps^{++}$ mutant was studied using the $KH_2PO_4$ treatments, used in this study as soluble phosphate control (Tables 3 and 4). The two isolates produced similar quantities of citric acid only with nitrate. Flasks of the $FePO_4$-nitrate treatment inoculated with the $Mps^{++}$ mutant revealed both enhanced growth and production of citric acid (14.3 mM). Furthermore, for this mutant a significant correlation ($r_s = 0.857$, $P < 0.01$) was observed between $FePO_4$ solubilization and citric acid production. After 7 days of growth, under similar conditions, *P. bilaii* [13] produced between 2.6 and 9 mM of citric acid. Iron dissolution by the chelating properties of organic acids has already been directly demonstrated with some organic acid solutions (such as pyruvate, oxalate, citrate) [27] and organic acids from the ectomycorrhizal fungus *Suillus granulatus* [31]. In the present study, the addition of ammonium to nitrate decreased $FePO_4$ solubilization and citric acid production by the $Mps^{++}$ mutant (Fig. 1 and Table 4), but it did not affect its growth (Table 2). It is known that ammonia blocks the induction of genes implicated in nitrate assimilation in certain fungi by catabolite repression [32]. Moreover, a repressive effect due to easily metabolized N sources is known to be the most common and effective negative control of secondary metabolic biosynthesis in some filamentous fungi, such as *P. urticae* [33]. Nevertheless, other factors may be implicated in the solubilization of $FePO_4$ by the wild-type of *P. rugulosum* in liquid media. Although the wild-type in the $KH_2PO_4$ liquid medium presented similar growth with arginine, nitrate and nitrate+ammonium (Table 2), citric acid was produced in larger concentrations only with nitrate (12.78 mM, Table 4). However, for $FePO_4$ a measurable solubilizing halo was previously reported for the wild-type isolate [17] grown in the presence of nitrate+ammonium and sucrose. Azcon et al. [34] reported that under dry conditions, mycorrhizal plants produced a higher yield than phosphate fertilized plants when nitrate was supplied as the only N source, in the absence of ammonium. It is suggested here that *P. rugulosum* IR-94MF1 may play a role in $FePO_4$ solubilization by the production of organic acids under dry conditions, when nitrate concentration in soil becomes important.

The use of different poorly soluble inorganic phosphate sources also influenced the patterns of growth and production of citric and gluconic acids. Even when gluconic acid was excreted in the presence of $FePO_4$ and $AlPO_4$ sources, the concentrations were generally smaller than those measured in the presence of HA with all nitrogen sources except ammonium (Table 3). In fact, significant correlations were found only between HA solubilization and the amounts of gluconic acid present in culture filtrates of the wild-type ($r_s = 0.762$, $P < 0.05$) and the $Mps^{++}$ mutant ($r_s = 0.929$, $P < 0.01$). Nevertheless, the wild-type *P. rugulosum* IR-94MF1 seems to have the metabolic capacity to produce relatively high amounts of both acids. It is known that citric acid is able to solubilize calcium phosphate [26]. Therefore, it is suggested here that an induction of a 'short' path (direct oxidation of the aldonic sugar by nonphosphorylating oxidation) could be selected by IR-94MF1 instead of a 'long' biosynthetic pathway [17], when HA is used as the phosphate source. This hypothesis could explain the low growth of IR-94MF1 on this phosphate source (Table 2). When sucrose is used as the carbon source, most of the glucose molecules can be converted to gluconic acid while fructose forms citric acid through the tricarboxylic acid cycle.

The $Mps^{-}$ mutant is apparently repressed for the production of both gluconic and citric acids. However, the low pH values observed with all N sources except nitrate and the $AlPO_4$ solubilization by $Mps^{-}$ grown with ammonium suggest that this mutant was still able to solubilize small amounts of the phosphates, possibly by using the $H^+$ pump mechanism.

The alkalinization of media of the $Mps^{-}$ mutant when nitrate was assimilated would thus come from the extrusion of $OH^{-}$ ions which are produced in the cytosol of cells during the reduction of nitrate to ammonium, as known for some plant cells [35]. To maintain the pH homeostasis, cells produce organic acids [32] which could be detected outside the cells if produced in high concentrations. Considering again $AlPO_4$ solubilization with arginine or ammonium used as the N source, the $Mps^{++}$ mutant as compared to the wild-type significantly ($P < 0.01$) increased solution P (Fig. 1), by releasing gluconic rather than citric acid (Tables 3 and 4). This is supported by the significant correlation ($r_s = 0.774$, $P < 0.05$) observed between the gluconic acid produced by the $Mps^{++}$ and the concentration of P released from the solubilization of $AlPO_4$. These results indicate that $AlPO_4$ and HA are solubilized by *P. rugulosum* IR-94MF1 mainly through gluconic acid production.

In this work the use of the MPS mutants allowed the identification of three possible phosphate solubilizing mechanisms which can be used by the isolate *P. rugulosum* IR-94MF1: the production of gluconic acid, of citric acid or the $H^{+}$ pump. These mechanisms are influenced by the N, P and C [17] sources. Further work is necessary to elucidate how these different mechanisms are selected and regulated in tropical soils.

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