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Effect of Nitrogen Form on the Effectiveness of a Phosphate-Solubilizing Fungus to Dissolve Rock Phosphate

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Abstract

An *in vitro* experiment was carried out to evaluate the effect of nitrogen (N) form (NH_4+ and/or NO_3-) on the dissolution of rock phosphate (RP) by a phosphate solubilizing fungus (PSF) identified as *Mortierella* sp. In the presence of NH_4Cl or NH_4NO_3, the solution of pH significantly decreased from an initial value of 7.6 to 3.4 and 3.7 respectively. In the presence of KNO_3, the pH went down only to 6.7. As a result, significantly more P was detected in solution in the presence of NH_4Cl (129.65 mg/L) than in the presence of NH_4NO_3 (109.25 mg/L). The concentration of P in solution in the presence of KNO3 was only 0.08 mg/L. The excess of NH_4+ adversely affected the growth of *Mortierella* sp. However, this may have promoted a more active H+-pumping that decreased solution's pH. In the presence of NO_3- as the only source of N, *Mortierella* sp. not only dissolved a small amount of Pi from the RP but also immobilized most of it into its mycelia. In contrast, in the presence of NH_4Cl, *Mortierella* sp. was effective to dissolve RP and the Pi released remaining in solution while only a little portion was immobilized by the fungal mycelia.

Keywords: *Mortierella* sp; Phosphorus; Nitrate; Ammonium

Introduction

Soil phosphate deficiency is globally a major constraint for agriculture, particularly in the tropical regions [1-3]. An alternative to overcome this limitation is the use of rock phosphate (RP) available in many countries; unfortunately this material is quite insoluble, which impairs its use [4,5].

There is an increasing interest in developing strategies to improve the effectiveness of RP as a direct source of Pi for plant growth in parts of the tropics endowed with local deposits of the material [4,6-10]. One of the biotechnological strategies involve the use of phosphate-solubilizing microorganisms (PSM) [2,11-17]. Microbial dissolution of RP is brought about by a number of mechanisms including (i) release of organic acids [18,19], (ii) formation of complexes between organic anions and cations such as Al^{3+} and Ca^{2+} [20,21], and (iii) excretion of protons due to NH_4+ uptake [12,22]. The participation of the last mechanism in the microbial solubilization of RP has not been fully investigated for fungal P solubilizers [18]. Given the limited N supply of most soils [23,24], the N applied either as NO_3- or NH_4+ fertilizers can control the extent of acid production by PSM, which is crucial in the efforts to evaluate the suitability of *Mortierella* sp. as an effective RP solubilizer in the rhizosphere or for its use in the biotechnological production of bio-acidulated RP [25].

The hypothesis of this experiment is that the effectiveness of PSM in dissolving RP may be influenced by the N form present in the growth medium. This effectiveness may be enhanced if the PSM is supplied with NH_4+ as the sole source of N. The objective of the current investigation was to asses the effects of N form (NH_4+ or NO_3-) on RP solubilization activity of *Mortierella* sp. under *in vitro* conditions.

Materials and Methods

Mortierella sp. was originally isolated from an Andisol of Hawaii [13] and maintained on Yeast malt agar (YMA) slants at 4°C. For this study, the fungus was multiplied in petri dishes on YMA medium for three days at 28°C. Mycelia were removed from the surface of the agar with a sterile loop and suspended in sterile deionized water and shaken by hand until the clumps were dispersed.

1 mL of a Mortierella sp. suspension containing $5.9x10^5$ CUF was aseptically transferred into 250 mL Erlenmeyer flasks containing 75 mL of an autoclaved (30 minute, at 120°C and 0.1 MPa) liquid medium. The medium consisted of 1.0 g NaCl, 0.2 g $CaCl_2.2H_2O$, 0.4 g $MgSO_4.7H_2O$, 28 mg Fe-EDTA, 28 mg Cu-EDTA, 28 mg Mn-EDTA, 14 mg Zn-EDTA, 10.0 g glucose, and 3.5 g of Huila RP per liter. The RP was passed through a 0.5-mm aperture sieve. The P content of the Huila RP was 130 g kg^{-1}, and its empirical formula is $Ca_{9.69}Na_{0.22}Mg_{0.09}(PO_4)_{5.14}(CO_3)_{0.86}F_{2.34}$ [26]. The medium contained 0.35 g N/liter, the source of N was NH_4NO_3 (1.0 g L^{-1}), NH4Cl (1.34 g L^{-1}), or KNO_3 (2.53 g L^{-1}). The flasks containing NH_4NO_3 and NH_4Cl also received KCl (1.87 g L^{-1}) in order to maintain similar amounts of potassium in all the treatments. The initial solution pH was adjusted with 0.1 *M* NaOH to pH 7.6. Flasks were continuously shaken at 150 rpm on an orbital shaker (model Innova 4400, New Brunswick Scientific Co, Inc., Edison, NJ) at 25°C for seven days at the Soil Microbiology Laboratory of the University of Hawaii at Manoa (Honolulu, HI, USA).

After the incubation period, 50 mL of the suspension was pippeted into plastic tubes for centrifugation at 5000xg for 15 minutes. The supernatant was filtered through a Whatman No. 42 filter paper followed by filtration through a membrane filter (0.45 µm). Solution pH was measured with a pH-meter. Solution P concentration was determined using the molybdate-blue method [27]. The fungal mats were transferred onto a filter paper, oven-dried (60°C for 48 h), and weighted for fungal dry weight (FDW) determination after removal of remaining RP particles. Fungal P concentration was determined by the

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molybdate-blue method after dry-ashing samples in a muffle furnace at 500°C for 3 hours and dissolving the ash in one mL of 1 M HCl and then bringing up the solution to 10 mL with deionized water. Total P solubilized (TPS) by *Mortierella* sp. consisted of the sum of soluble-P and fungal-P.

Treatments were arranged in a completely randomized design and consisted of three different N sources (KNO_3, NH_4NO_3, or NH_4Cl), and there were four replicates per treatment. Analyses of variance and Duncan multiple range test were used to evaluate the significance of treatment effects (P value ≤ 0.05). Data were analyzed by means of the software package Statgraphics, version 4.0 (Statpoint, Inc., Herdon, Virginia).

Results

The effect of N source was significant for all measured variables. Solution pH was significantly lower when *Mortierella* sp. was supplied with N as ammonium than as nitrate (Figure 1). When the fungus grew in the presence of NH_4Cl or NH_4NO_3, the pH went down to 3.4 and 3.7, respectively; while in the presence of KNO_3 the pH went down only to 6.7. There was an inverse relationship between pH and P concentration in the culture medium inoculated with the fungus. In the presence of Mortierella sp., the concentrations of P in solution were 0.08, 109.25, and 129.65 mg/L if the N source was KNO_3, NH_4NO_3, and NH_4Cl, respectively (Figure 2).

Despite the very high RP solubilizing activity noted in the presence of NH_4+ compared to that observed in the presence of NO_3-, fungal dry mass was significantly higher when KNO_3 was the sole source of N (Figure 3). By contrast, fungal P concentration (%) was significantly higher in the presence of NH_4Cl or NH_4NO_3 than in the presence of KNO3 (1.9, 2.0, and 1.7%, respectively) (Figure 4). That is, Mortierella sp. absorbed more P when the sole source of N was NH4Cl or NH_4NO_3. The total amount of P solubilized by *Mortierella* sp. (P in fungal mycelium and P remaining in solution) clearly showed that significantly more P was solubilized if N was supplied as NH_4+ than if it was supplied as a mixture of the two ions or with NO_3- (Figure 5).

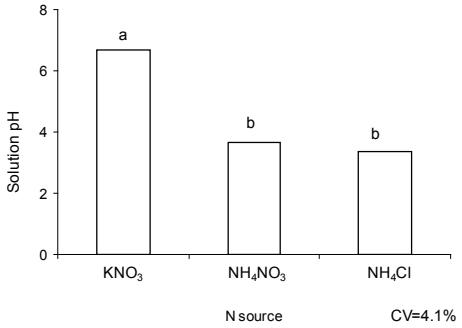

Figure 1: Solution pH of a liquid medium inoculated with *Mortierella* sp. as a function of N source. Columns with different lower-case letters are significantly different from each other (P ≤ 0.05).

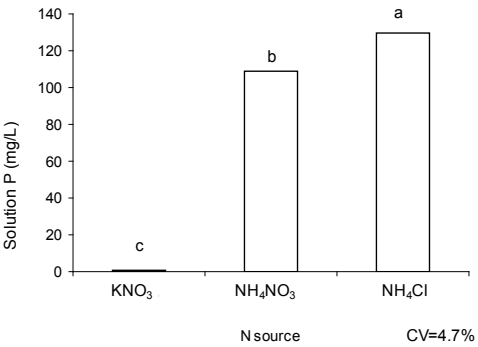

Figure 2: Solution P concentration (mg/L) of a liquid medium inoculated with *Mortierella* sp. as a function of N source. Columns with different lower-case letters are significantly different from each other (P ≤ 0.05).

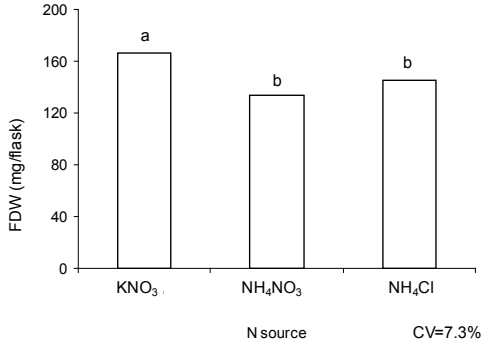

Figure 3: Fungal dry weight (FDW) of *Mortierella* sp. as a function of N source. Columns with different lower-case letters indicate are significantly different from each other (P ≤ 0.05).

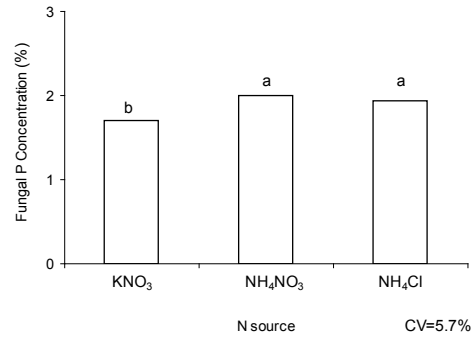

Figure 4: Fungal P concentration of *Mortierella* sp. as a function of N source. Columns with different lower-case letters are significantly different from each other (P ≤ 0.05).

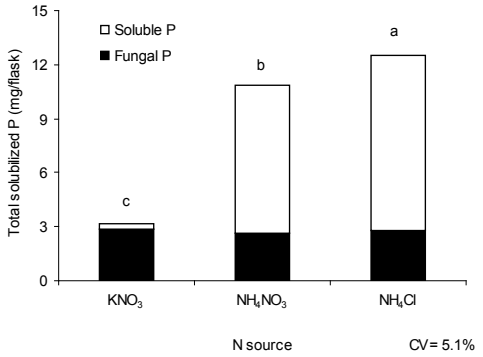

Figure 5: Total P solubilized by *Mortierella* sp. as a function of N source: Solution-P in white columns and fungal-P in black columns. Columns with different lower-case letters are significantly different from each other (P ≤ 0.05).

The relative proportion of soluble P and fungal P significantly varied with N source. When *Mortierella* sp. was supplied with NH_4Cl, the P remaining in solution represented 78% of the total amount of P solubilized by *Mortierella* sp., compared to only 0.2% if the N source was KNO_3. If N was supplied as NH_4NO_3 75% of the P solubilized remained in solution (Figure 5). The fraction of Pi immobilized by *Mortierella* sp. in the presence of the different N sources was in the order KNO_3>NH_4NO_3>NH_4Cl.

Discussion

The results of this study clearly showed that the presence of NH_4+ in *Mortierella* sp. acidified the growth medium to a greater extent than in the presence of NO_3-; most likely by increasing proton-pumping in the fungal cell membrane [28,29]. The fraction of RP dissolved by *Mortierella* sp. varied with the N form in the liquid medium, which was 37, 38 and 32% when NH_4+, NO_3- and both ions were supplied, respectively (Figure 5). The positive effect of NH_4+ as N source on RP solubilization observed has been reported previously by some authors [12,30].

It is well known that microbial cells must keep an internal balance of electrical charges in order to maintain a functional cell membrane [31]. This is achieved by maintaining a near-neutral pH and a more negatively charged cytoplasm than the external solution [28,31]. Since N is a major nutrient for fungi [32], the form in which it is taken up by fungal cells can shift the electrical charge in the cytoplasm. Since the net charge in the cytoplasm must remain negative, imbalance caused by the uptake of excess cations must be countered by a very active proton-pumping, which expels H^+ into the external medium through likely a K^+/H^+ antiport mechanism [32].

Furthermore, the assimilation of NH_4+ in the fungal cell for amino acid synthesis could reduce the cytosolic pH because NH_4+ is converted to NH_3 and the excess H^+ is introduced into the cytoplasm [33]. This H^+ is released into the external solution to maintain cytoplasmic pH, acidifying the medium surrounding the fungal cells and thereby favoring RP dissolution. Although NH_4+ is a nutrient for fungi, it can become toxic at high concentrations [33]. One problem due to an excessive level of NH_4+ is its interference with the uptake of other fungal nutrients (Mg^{2+}, Na^+, Ca^{2+}, etc.) as clearly seen in plants [34]. However, the most negative effect of NH_4+ is probably its interference with the electrochemical gradient that must be maintained between the cytoplasm and the external medium [28,33].

It is possible that excess positive charge in the cytoplasm may also trigger the release of organic anions in the cytoplasm in order to balance charges [35]. In this study, it was detected that *Mortierella* sp. produced oxalic acid/oxalate. This organic acid/anion is commonly synthesized in the Kreb's cycle or from compounds formed from it to be used for normal metabolic functions and to maintain fungal growth [11,32]. However, during periods of stress imposed by an excess of positive charges, these compounds could be used for the purpose of charge-balancing. This hypothesis may explain the lower dry mass of *Mortierella* sp. observed when the fungus was supplied with NH_4+ compared to when it was supplied with NO_3- as a sole source of N (Figure 3). It seems that the fungal cell was utilizing proton extrusion as well as organic anion synthesis concurrently.

The final outcome of the decrease in pH and the release of oxalic acid or oxalate associated with NH_4+ assimilation by *Mortierella* sp. is an increase in RP dissolution. The phenomenon is explained by the apatite dissolution reaction as presented by Lindsay [36] (equation 1):

$$Ca_5(PO_4)_3OH + 7H^+ \leftrightarrow 3H_2PO_4^{2-} + 5Ca^{2+} + H_2O \ (K=10^{14.5}) \quad (1)$$

If the formation of Ca-oxalate complex is included, the reaction is thermodynamically more favorable, as discussed by Osorio [37] (equation 2):

$$Ca_5(PO_4)\ 3OH + 7H^+ + 5\ Oxalate \leftrightarrow 3H_2PO_4^{2-} + 5Oxalate\text{-}Ca^{2+} + H_2O \ (K=10^{31.68}) \quad (2)$$

Increasing H^+ drives the reaction to the right, thus apatite (RP) dissolves and releases H_2PO_4-. The release of oxalate into the growth medium by *Mortierella* allows Pi to remain available by tying up Ca^{2+}, a Pi-fixing cation, as an organic complex (log K=3.44). Welch et al. [20] found that the dissolution of apatite was favored by the formation of Ca^{2+}-oxalate complex due to production of oxalate by microorganisms. This reaction occurred not only in solution but also at the mineral surface.

The potential practical implications of these results could be appreciable if farmers manage N fertilizer application with the view of enhancement of RP solubilization by PSM and prevention of re-adsorption of Pi. Increasing the efficacy of nitrification inhibitors will be part of the management strategy. This effort, of course, must take into account the fact that excess NH_4+ can affect plant growth negatively [38]. This negative effect on plant growth should reduce the amount of root exudates on which the RP solubilization activity is dependent [39].

On the other hand, an excess of H^+ in the external medium could reduce the fungal Pi uptake since fungal Pi uptake is accomplished by an H^+-symport mechanism [32]. As a result of this, more Pi will remain in solution. Sugar uptake might be affected in a similar manner [33], which is also a likely explanation for lower growth of *Mortierella* sp. when grown with NH_4Cl as N source.

The benefit that a plant could derive from a PSM lies in the ability of the microorganism to increase the soil solution P in the rhizosphere. This ability will hardly occur if *Mortierella* sp. is supplied with NO_3- as the sole N source because not only the fungus is ineffective in dissolving RP, but also immobilizes the small amount of Pi released in solution under this condition. From the standpoint of plant P nutrition this relationship is as important as P solubilization *per se*. Also, the results suggest that N form must be taken into account for any biotechnological use of phosphate solubilizing microorganisms.

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