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Effect of Carbon and Nitrogen Source and Concentration on Rock Phosphate Dissolution Induced by Fungi

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Abstract

Inorganic Phosphate (Pi) is an essential nutrient for plant growth and development, however that most soils have in low availability. To overcome this problem it is necessary to apply high amounts of Pi fertilizers, however this is inefficient and costly. Recently, there is increasing interest in the use of rock phosphate (RP) and microorganisms capable of increasing its agronomic effectiveness as crop fertilizer. However, it is unclear the impact that some critical nutrients such as carbon (C) and nitrogen (N) may have on the effectiveness of the microbial RP dissolution. The aim of this study was to evaluate the effect of C and N source and concentration on the RP dissolution by the fungus Mortierella sp. under in vitro conditions. The results indicate that the efficiency to dissolve RP was significantly higher with glucose, followed by arabinose; other sources were ineffective (fructose, sucrose, maltose, cellulose and molasses). On the other hand, NH4Cl was significantly the most effective N source, followed by NH_4NO_3. By contrast, KNO3 was ineffective to promote RP dissolution. The best concentration of glucose was 10 g L^{-1}, while for NH4Cl it was 1 g L^{-1}. These findings show that by varying nutrient supply, the RP dissolution reactions can be significantly enhanced.

Keywords: Solubilization, Phosphorus, Phosphorus solubilizing microorganisms

1. Introduction

Inorganic Phosphate (Pi) is an essential macronutrient for the growth and development of plants (Singh & Reddy, 2011); however, many tropical soils have low availability of it (Osorio & Habte, 2013). In order to increase its availability it is necessary to apply high amounts of soluble Pi fertilizer (Reddy et al., 2002). Only a small amount of the applied Pi fertilizer is used by plants (5-10%) and the remainder fraction is hold in the soil solid phase in insoluble forms (Vassileva et al., 2000; Osorio & Habte, 2009; Batti & Yamar, 2010). As a result of that, the Pi fertilization in these soils is inefficient, expensive practice, and potentially polluting of water (Shigaki et al., 2006).

An alternative for soluble Pi fertilizers is the use of rock phosphate (RP); however, this has low solubility and low agronomic effectiveness (Pramanik et al., 2009). A strategy to increase its effectiveness as Pi fertilizer, the RP is partially acidulated with sulfuric acid or phosphoric acid (Batti & Yamar, 2010). Unfortunately, this process is expensive and potentially contaminating of the environment (Zapata & Roy, 2007; Xiao et al., 2008). Recently, there is an increasing evidence that some microorganisms are capable of accelerate the dissolution of RP and have been termed phosphate-solubilizing microorganisms (PSM) (Relwani et al., 2008; Singh & Reddy, 2011). Among the effective bacterial PSM are *Pseudomonas* (Bar Yosef et al. 1999), *Enterobacter* (Vasquez et al., 2000), and *Bacillus* (Chen et al., 2006). In the case of fungal PSM *Penicillium* (Wakelin et al., 2004), *Aspergillus* (Bojinova, 2008), and *Mortierella* (Zhang et al., 2011; Osorio & Habte, 2013) are outstanding.

In order to improve the biotechnological applications of PSM in the fertilizer industry to either produce more soluble Pi fertilizers (Bar Yosef et al., 1999) or enhance the effectiveness of the direct use of RP as Pi fertilizer (Osorio, 2011) it is necessary to know the impact of some critical factors (Cunningham & Kuiack, 1992). Among the most relevant factors that may control the organic acid production, responsible of RP dissolution, are the microbial C and N nutrition (Londoño, 2010; Nisha & Venkateswaran, 2011; Habte & Osorio, 2012). We consider that a more efficient use of these natural and finite resources is mandatory since the global P crisis suggests a shortage of the easily mining RP reserves in the next 60-100 years (Dibb, 2004; Gilbert, 2009). The aim of this study was to determine the effect of C and N concentration and source of C and N to improve the *in vitro* dissolution of RP by the soil fungus *Mortierella* sp.

2. Materials and Methods

In this study the PSM tested was the fungus *Mortierella* sp., which is known for its ability to dissolve efficiently RP (Qin et al., 2009; Zhang et al., 2011; Osorio & Habte, 2013). This fungus was originally isolated from an Andisol of Hawaii by Osorio and Habte (2001). For all studies, the fungus was previously grown on PDA medium for 5 days at 28 °C and then suspended in sterile distilled water and stored in a refrigerator at 4 °C for further use at the Laboratory of Ecology and Biogeochemistry the Universidad Nacional de Colombia at Medellin. Samples of commercial RP from Huila, Colombia, were employed after sieving at 250 μm. This RP contains a Pi concentration of 12%. Its empirical formula is $Ca_{9.69}Na_{0.22}Mg_{0.09}(PO_4)_{5.14}(CO_3)_{0.86}F_{2.34}$ (Hamond & Chien, 1979).

The *in vitro* medium consisted of (g L^{-1}): glucose 10, NH_4Cl 1.34, KCl 1.87, $CaCl_2.2H_2O$ 0.2, $MgSO_4.7H_2O$ 0.4, and Huila RP 3.5 as the sole source of Pi (Osorio, 2008). Seventy five mL of this medium was brought to 250 mL Erlenmeyer flasks and sterilized in autoclave at 120 °C, 0.1 MPa for 20 minutes. The flasks were inoculated and then continuously shaken at 100 rpm, 28 ° C for 7 days.

2.1 Activity No.1. Amount of Inoculum

The effect of the amount of inoculum on the RP dissolution was evaluated with grading amounts of a fungal suspension (0, 1, 2, 4, and 7 mL per flask) that contained 10^7 colony forming units (CFU) of *Mortierella* sp. per mL. Other experimental conditions were as described above.

2.2 Activity No.2. Concentration of Glucose

Grading concentrations of glucose (5, 10, 15, 20, and 25 g L^{-1}) were prepared in the Erlenmeyer flask contained the culture medium described above. One mL of the suspension of *Mortierella* sp. was added (Act. No 1). Other culture conditions were those described above.

2.3 Activity No.3. Carbon Source

The effects of C source on microbial RP dissolution were tested with monosaccharides (glucose, fructose or arabinose), disaccharides (sucrose or maltose), and polysaccharides (cellulose, carboxymethylcellulose or molasse). Based on the results of activity No. 2, C was applied at a concentration of 4 g L^{-1}. One mL of the suspension of *Mortierella* sp. was added (based on Act. No. 1). Other culture conditions were those described above.

2.4 Activity No.4. Concentration of Ammonium

Grading concentrations of NH_4Cl (0.5, 1.0, 1.5, and 2.0 g L^{-1}), as the only N source, were established in the culture medium. One mL of the suspension of *Mortierella* sp. was added (based on Act. No. 1); glucose was applied as the only C source (based on Act. No. 3) at a concentration of 10 g L^{-1}. The other culture conditions were those described above.

2.5 Activity No.5. Nitrogen Source

The effects of N sources on microbial RP dissolution were tested with NH_4NO_3 (0.61 g L^{-1}), NH_4Cl (0.5 g L^{-1}), or KNO_3 (0.93 g L^{-1}). In all treatments N was applied at a rate of 0.12 g of N L^{-1}. Glucose was applied as the only C source (based on Act. No. 3) at a concentration of 10 g L^{-1}. One mL of the fungal was added (based on Act. No. 1). Other culture conditions were those described above.

2.6 Variables

After the incubation period, the medium pH was determined by a potentiometer (WTW Sentix electrode 81) immerging directly the electrode in the medium. The concentration of Pi (mg L^{-1}) in the culture medium was determined by the blue-molybdate method (Murphy & Riley 1962) at 890 nm wavelength with Genesys 20 Thermo Spectronic spectrophotometer after filtration through Whatman No. 42 filter paper and centrifugation (centrifuge Jouan MR 1812) at 4000 rpm (1500 × g) for 10 minutes.

2.7 Experimental Design

The experiments were arranged in a completely randomized design (Table 1). Each activity

had four replicates, including uninoculated controls. 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 STATGRAPHICS centurion version XVI.

Table 1. Experimental design

Activity No.	Treatment	Details	Replicates	Statistical Analysis
1	*Amount of inoculum*	0, 1, 2, 4, and 7 mL per flask	4	Independent Anova and Duncan multiple range test were used to evaluate the significance of treatment effects (P-value ≤ 0.05).
2	*Concentration of glucose*	5, 10, 15, 20, and 25 g L^{-1}		
3	*Carbon source*	glucose, fructose, arabinose, sucrose, maltose, cellulose, carboxymethylcellulose and molasses		
4	*Concentration of ammonium*	0.5, 1.0, 1.5, and 2.0 g L^{-1}		
5	*Nitrogen source*	NH_4NO_3, NH_4Cl and KNO_3		

3. Results

3.1 Amount of Inoculum.

Uninoculated flasks exhibited a solution pH of 7.80, which was significantly higher than the solution pH of inoculated flasks (Figure 1A). The inoculation with *Mortierella* sp. decreased significantly ($P \leq 0.05$) the solution pH. The lowest pH values were obtained when the medium was inoculated with 1 and 2 mL of the fungal suspension per flask (pH: 3.15 and 3.11, respectively), while with the inoculation of 4 and 7 mL the solution pH was significantly higher (3.34 and 3.5, respectively). Consequently, the uninoculated flasks had a solution Pi concentration of 1.22 mg L^{-1} (Figure 1B). Inoculated flasks had solution P levels significantly higher; the magnitude of this effect was greater when the medium was inoculated with 1 mL of the fungal inoculum per Erlenmeyer (70.89 mg L^{-1}). Larger amounts of inoculum (2-7 mL) generated significantly less soluble Pi and did not differ significantly to each other in this regard (45.00 mg L^{-1}).

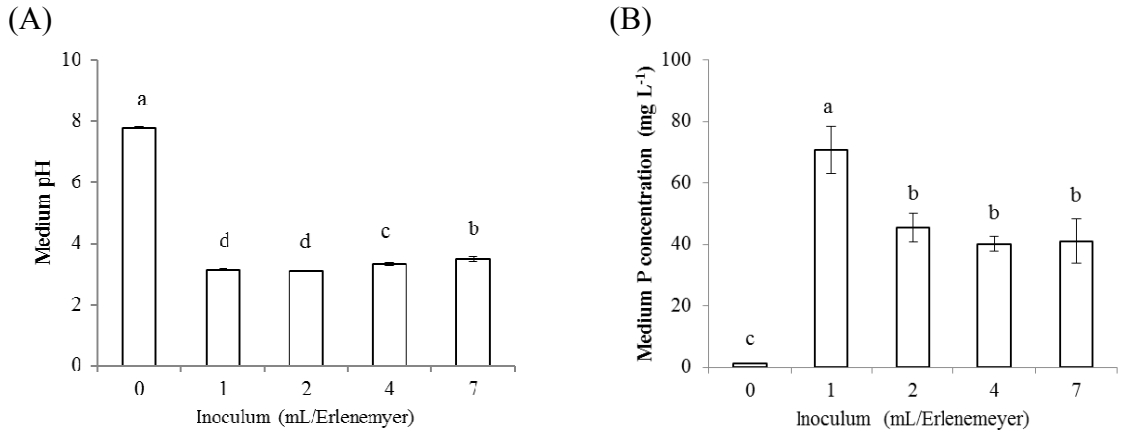


Figure 1. (A) Medium pH and (B) medium Pi concentration (mg L^{-1}) as a function of the amount of inoculum of *Mortierella* sp. added into Erlenmeyer flask. Each value is the mean of four replicates. The bars indicate the standard error. Columns with different lowercase letters indicate significant difference between treatments according to Duncan's test ($P \leq 0.05$)

3.2. Concentration of Glucose

Uninoculated flasks had solution pH values (=7.8) significantly higher than inoculated flasks (Figure 2A). The lowest medium pH were obtained with 10-25 g of glucose per L (3.1), with concentration 5 g per L of glucose in medium pH was significantly higher. Uninoculated flasks had a medium Pi concentration that ranged between 0.24 and 0.30 mg L^{-1} and did not differ to each other regardless the glucose concentration (Figure 2B). The highest solution Pi concentration was obtained with 10 g of glucose per L (118.26 mg L^{-1}); glucose concentration below o above this level produced a solution Pi concentration significantly lower.

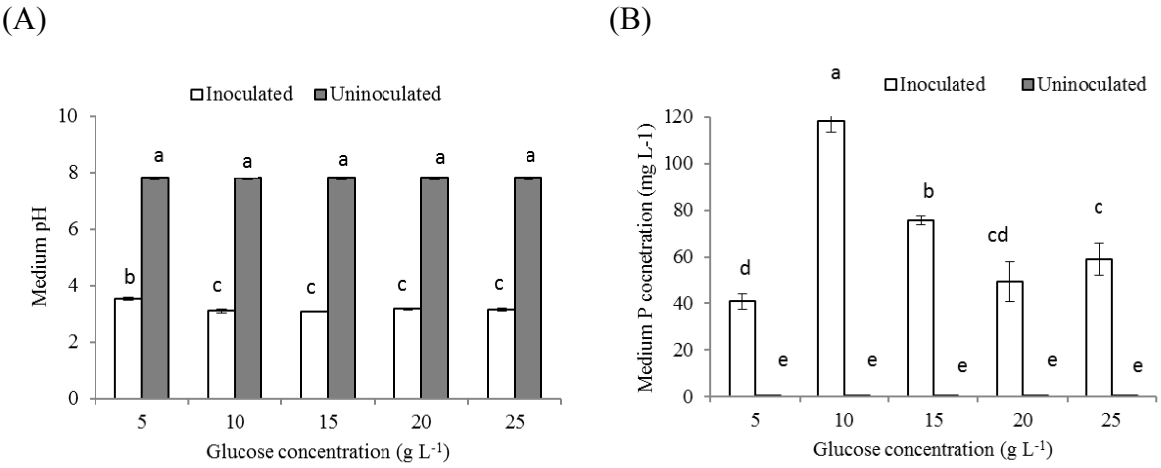
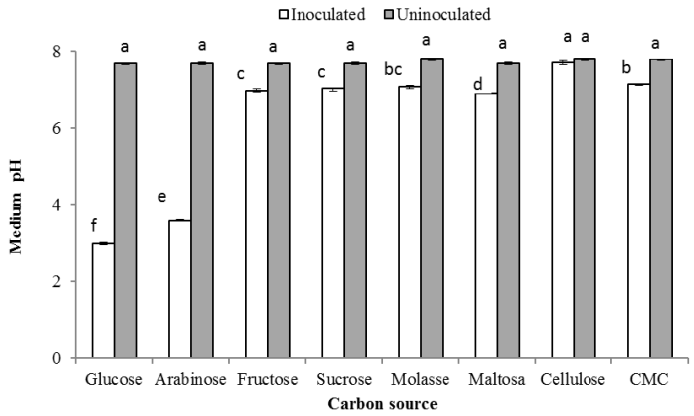


Figure 2. (A) pH and (b) Pi concentration in solution (mg L^{-1}) in function of the amount of glucose (g L^{-1}) added. Each value represents the average of four replicates. The bars indicate standard deviation. Columns with different small letters indicate significant difference of treatment according to Duncan's test ($P \leq 0.05$)

3.3 Carbon source

Uninoculated flasks had medium pH values (7.7-7.8) significantly higher than inoculated flasks, which were unaffected by the C source (Figure 3A). By contrast inoculated flasks had significantly lower pH values, however, the effect in lowering the medium pH was controlled by the C source. The decreased in the medium pH followed the next order: glucose (2.99) < arabinose (3.6) <= fructose = maltose = sucrose = (6.9) < molasses (7.07) < carboxymethyl cellulose (7.15) <cellulose (7.7) (Figure 3A). As a result of that, uninoculated flasks had significantly lower Pi concentration (0.2 mg L^{-1}). The most effective C source to promote RP dissolution by *Mortierella* sp. was glucose, the solution Pi concentration was 81.05 mg L^{-1}, followed by arabinose (45.7 mg L^{-1}) (Figure 3B). The use of the other C sources was ineffective to promote RP dissolution.

(A)



(B)

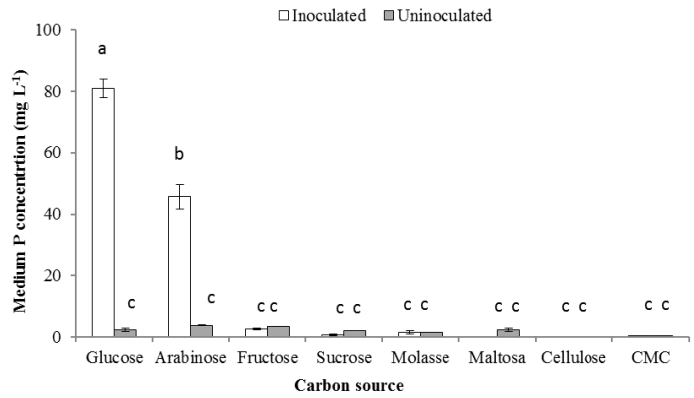


Figure 3. (A) pH and (b) Pi concentration in solution (mg L^{-1}) depending on the source of C. Each value represents the average of four replicates. The bars indicate standard deviation. Columns with different small letters indicate significant difference of treatment according to Duncan's test ($P \leq 0.05$). CMC: carboxymethyl cellulose

3.4 Concentration of Ammonium

Uninoculated flasks had medium pH values (mean 7.6) significantly higher than inoculated flasks (Figure 4A). In inoculated flasks the pH decreased but the effect was affected by the level of NH_4Cl; concentrations of NH_4Cl of 0.5-1.5 g L^{-1} had pH value of 3.2, which were significantly lower than that obtained at 2 g L^{-1} (3.3). Consequently, uninoculated flasks had medium Pi concentrations were 0.08 mg L^{-1} and did not differ to each other regardless the NH_4Cl concentration (Figure 4B). In the inoculated flasks the highest medium Pi concentration was obtained with 0.5 g of NH_4Cl per L (82.5 mg L^{-1}). The tendency observed was that as NH_4Cl concentration increased, the medium Pi concentration obtained via RP dissolution significantly decreased. Thus, with NH_4Cl concentration of 1.0 and 1.5 g L^{-1} the medium Pi concentrations were 66.1 and 68.3 mg L^{-1}, respectively. At 2.0 g of NH_4Cl per L the medium Pi concentration was the lowest (45.5 mg L^{-1}).

(A) (B)

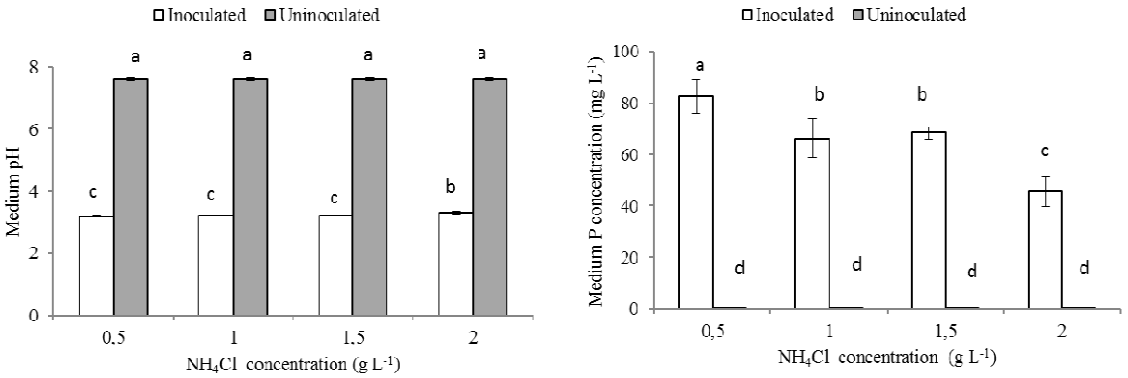


Figure 4. (A) pH and (b) Pi concentration in solution (mg L^{-1}) in function of the amount of NH_4Cl (g L^{-1}). Each value represents the average of four replicates. The bars indicate standard deviation. Columns with different small letters indicate significant difference of treatment according to Duncan's test ($P \leq 0.05$)

3.5 Nitrogen Source

Uninoculated flasks had medium pH values (7.5-7.6) significantly higher than inoculated flasks, which did not differ to each other as a function N source (Figure 5A). By contrast inoculated flasks had significantly lower pH values; nevertheless, the magnitude of lowering the medium pH depended on the N source. In this way, the medium pH detected with NH_4Cl (pH=3.2) was significantly lower than with NH_4NO_3 (pH= 4.01) and this, in turn, significantly lower than with KNO_3 (pH= 6.5). As a consequence of this, uninoculated flasks had significantly lower medium Pi concentration regardless of the N source (0.04-0.07 mg L^{-1}) (Figure 5B). In inoculated flasks, medium Pi concetration depended on the N source being NH_4Cl the most effective N source to promote RP dissolution and increase Pi concentration (73.41 mg L^{-1}) followed by NH_4NO_3 (33.74 mg L^{-1}). On the other hand, KNO_3 was ineffective to promote RP dissolution (0.2 mg L^{-1}).

(A) (B)

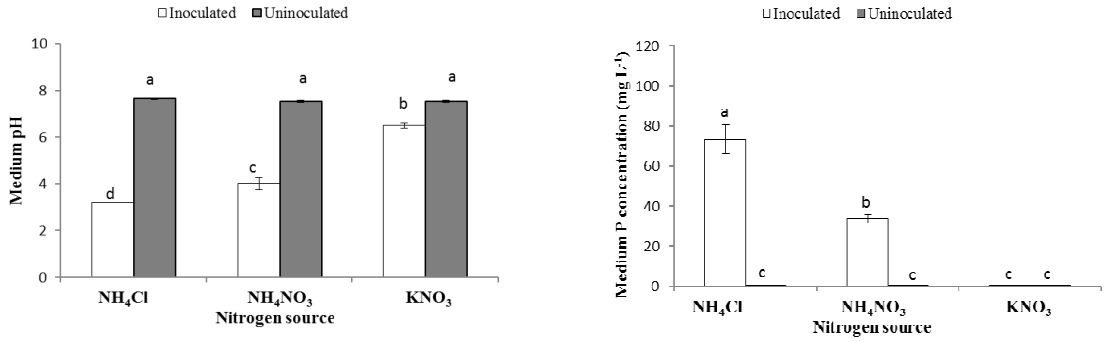


Figure 5. (A) pH (b) Pi concentration in solution (mg L^{-1}) in function of the N source. Each value represents the average of four replicates. Bars indicate standard deviation. Columns with different small letters indicate significant difference of treatment according to Duncan's test ($P \leq 0.05$)

4. Discussion

The results clearly show that by changing the amount and source of C and N in the culture medium the ability of *Mortierella* sp. to dissolve RP was improved. Calculations to estimate the maximal efficiency in doing that [EDPR (%) = (Pi soluble ÷ Pi total in the RP) × 100] indicate that *Mortierella* sp. has a value of 18.1%, while in the uninoculated control it was only 0.02%.

Furthermore, the fact that the maximum capacity of *Mortierella* sp. was detected with the lowest population level (1×10^7 CFU) (EDPR = 16.9%) suggest that this fungus is effective using nutrients and producing the required acidity to dissolve RP. By increasing the population density of *Mortierella* sp. their effectiveness, which may be due to intraspecific competition.

From previously published results (Nahas, 1996; Osorio & Habte, 2001) and from this study, it is clear that there is an inverse relationship between the medium pH and the concentration of Pi in solution via RP dissolution. Also, the results indicate that the most effective source of C for the microbial RP dissolution was glucose, which agrees with results obtained by other researchers in comparable studies (Hameeda et at., 2006; Sharan et al., 2008; Nisha & Venkateswaran, 2011). For heterotrophic organisms as *Mortierella* sp. is important to have an adequate source of C and energy that would meet their metabolic need and produce metabolites like organic acids. In the same way, the amount of glucose in the culture medium appears to be a critical factor in the microbial dissolution of RP, being the EDPR low (9.7%) at the lowest level of glucose used (5 g L^{-1}), which increased to 28.1% at the level of 10 g L^{-1}, but surprisingly decreased down to 13% with the highest level of glucose. For *Mortierella* sp. the oxalic acid production seems to be induced by environmental conditions and non constitutive as in other fungi such as *A. niger* (Madigan, 2004).

Xiao et al. (2008) evaluated the effect of the amount of C as glucose to optimize the RP dissolution capacity of *Pseudomonas expansum*, *Mucor ramoissimus,* and *Candida krissii;*

they found that the three microorganisms improve its performance but with different amounts of C. Hameeda et al. (2006) found that the type of source C affected the RP dissolution of *Serratia marcescens* and *Pseudomonas* sp., being glucose the best source. Reyes et al. (2006) compared the effect of the C sources in the RP dissolution capacity of *Pseudomonas* sp. and *Azotobacter* sp. and found better results when using sucrose and dextrose. Cerezine et al. (1988) found that the dissolution of fluorapatite by *A. niger* was favored with fructose and glucose, whereas sucrose, galactose, maltose, and starchdid not promote it. Sharan et al. (2008) evaluated the effect of C source on the arachidonic acid production by *M. Alpine* and found that glucose was the best source, other C sources improved the fungal growth but not the production of this acid.

The increase in the microbial dissolution of RP by raising the concentration of NH_4^+ in the culture has been reported by some authors (Nahas, 2007; Habte & Osorio, 2012). In this study we consider that NH_4Cl increases the activity of the proton pump at the cell membrane (Cooke & Whipps, 1993; Illmer & Schinner, 1995), which lowered the medium pH, which is required to dissolve RP. It is worth noting that the efficiency of *Mortierella* sp. to produce acidity was higher with the lowest level of NH_4Cl (0.5 g L^{-1}), at this level the EDPR was 20%. However, *Mortierella* sp. was ineffective in reducing the medium pH and dissolving RP when the nitrogen source was KNO_3. Similar results with this source of N have been obtained with the same fungus by Habte and Osorio (2012) at the medium pH of 6.5, where the EDPR was only 0.05%. As the authors reported, this situation may be promoting Pi immobilization by the cells of *Mortierella* sp. On the other hand, Lu et al. (2011) affirmed that inorganic N sources favor the production of organic acids, whereas organic N sources favor cell growth.

In addition to the N source, it is clear that in this study the amount of N in the medium also had a significant effect on the microbial dissolution of RP. Reyes et al. (1999) found that low concentrations of NH_4Cl in the culture medium decreased the citric acid production by the fungus *P. rugulosum* and hence the RP dissolution diminished. Kara & Bozdemir (1998) reported that 1.5 g NH_4Cl per liter is the optimal amount for *A. foetidus* to produce gluconic acid in the RP dissolution. The results of the current study coincide with those reported by Cerezine et al. (1988); they found that among several N sources [NH_4NO_3, $(NH_4)_2SO_4$, NH_4Cl, $NaNO_3$, urea, and peptone], NH_4Cl was the most effective N source in the RP dissolution by *A. niger*.

The dissolution of RP is a process that depends on several factors such as the microorganisms used (Narsia & Patel, 2000) and nutritional, physiological and growth conditions in the culture medium (Adham, 2002; Haq & Iqbal, 2003; Nahas, 2007). The high concentration of soluble Pi in the culture medium generated during the incubation is related to the good adaptation of the RP dissolving fungi to the growth environment (Xiao et al., 2008). The use of PSM is as biotechnological approach effective to enhance the agronomic effectiveness of RP for direct use in Pi deficient soils (Osorio & Habte, 2013) as well as a strategy to obtain more soluble phosphate fertilizers via bioacidification of RP (Stewart & Howell, 2003; Smith & Moore, 2005).

5. Conclusion

From this study it can be concluded that the *in vitro* bioacidification of rock phosphate by *Mortierella* sp. is controlled by the carbon and nitrogen source and amount applied to the culture medium. The best results for this *in vitro* bioacidification model were obtained with glucose as carbon source (10 g per L) and ammonium chloride as nitrogen source (0.5 g per L).

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