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SOLUBILIZATION OF $CAHPO_4$ AND $ALPO_4$ BY *ASPERGILLUS NIGER* IN CULTURE MEDIA WITH DIFFERENT CARBON AND NITROGEN SOURCES

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ABSTRACT

The solubilization of inorganic phosphates by microorganisms supplies phosphates for plant nutrition and increases their growth. The solubilization of $CaHPO_4$ (Ca-P) and $AlPO_4$ (Al-P) by *Aspergillus niger* using several carbon and nitrogen sources was studied. Solubilization of Ca-P was enhanced when the carbon sources were mannitol, maltose, galactose and glucose (in that order). Galactose, sucrose and maltose were the carbon sources that enhanced the solubilization of Al-P. More extensive growth, acid production, and decrease in pH were obtained in the Al-P medium than in the Ca-P medium, however, the quantity of solubilized phosphate was 12% less. Phosphate solubilization was related to acid production, pH drop and fungal growth in the culture medium. The results of a study carried out under abiotic conditions showed that organic acids solubilize more Ca-P than Al-P. Evaluating the effect of the nitrogen source, the solubilization of Ca-P or Al-P decreased in the following order: glycine > NH_4Cl > $NaNO_3$ and NH_4NO_3 > urea > $(NH_4)_2SO_4$, respectively. Ammoniacal nitrogen (NH_4^+-N) sources were the most effective in the production of acids and in lowering of the pH.

Key-words: nutrition, phosphorus, phosphate source, phosphate solubilization, titratable acidity

INTRODUCTION

The availability of phosphorus in the soil is somewhat limited, notwithstanding its ample distribution in nature, explaining the need for the application of soluble fertilizers for adequate plant growth (26). Many insoluble forms of calcium, iron and aluminum phosphates occur in soil, however, in Brazilian soils, a predominance of Fe and Al phosphates over Ca phosphate has been found (3).

The ability of microorganism to solubilize different forms of calcium phosphate has been reported (17), however, few studies are reported related to the solubilization of other cations such as Al and Fe (12,24).

Aspergillus niger is a fungus that has been studied because of its ability in solubilization of inorganic phosphates through the production of acids (citric, gluconic, glicolic, succinic, and oxalic acids) and pH drop (18,25). A number of factors have been considered due to its effect in acid production and pH lowering by microorganisms, such as the C and N sources (8,20). The solubilizing ability has also been found to be influenced by the P source in the culture medium (17). A soil isolate of the fungus *Aspergillus niger* showed high ability to solubilize both calcium and aluminum phosphates in culture medium (3). Thus, seems to be important to evaluate this ability growing the fungus in a medium added with different C or N sources. The objective of this study was to examine the effect of carbon and nitrogen sources on fungal growth and solubilization of calcium phosphate and aluminum phosphate by *Aspergillus niger*.

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MATERIALS AND METHODS

The fungus *Aspergillus niger* F111 was grown on Sabouraud agar slants for 7 days at 30ºC, stored at 4ºC until use, for a maximum period of 30 days when was transferred to fresh medium.

Solubilizing activity was determined in liquid medium, containing per liter: 0.1g NaCl, 1.0g NH_4Cl, 0.2g KCl, 1.2g $MgSO_4.7H_2O$, 0.1g $CaCl_2$, 10.0g glucose and 0.5 g de yeast extract (19). $AlPO_4$ (Al-P) and $CaHPO_4$ (Ca-P) were added in a quantity of 1.26 g l^{-1} PO_4^{3-} and 1.36 g l^{-1} PO_4^{3-}, respectively. $CaHPO_4$ has been precipitated by the addition of 1.5 ml sterile 10% $CaCl_2$ solution and 1.0 ml sterile 10% K_2HPO_4 solution for each 50 ml of culture medium (25).

When the effect of C sources on phosphate solubilization was evaluated, D-arabinose, D-xylose, D-fructose, D-galactose, D-glucose, D-mannose, maltose, sucrose, soluble starch or mannitol were used in quantities of 4 g C l^{-1}. Each C source was dissolved in distilled water, sterilized separately and added to the culture medium prior to inoculation. The effect of N sources on solubilization was evaluated by replacing the ammonium chloride by ammonium nitrate, ammonium sulfate, sodium nitrate, potassium nitrate, glycine, L-glutamic acid or urea in quantities of 262 mg N l^{-1}. When the effect of N sources on phosphate solubilization was evaluated, sucrose was the carbon-source used.

Two ml of a suspension containing 13.4 x 10^6 spores ml^{-1} was inoculated in 30 ml of medium contained in a 250 ml Erlenmeyer flask. The fungus was incubated without agitation at a temperature of 30ºC for 8 days. At the end of the incubation period, the culture medium was filtered with a Buchner funnel and the mycelium washed with a solution of NaOH 0.5 M and distilled water. The dry weight of the mycelium was determined after 24 hours at 105ºC.

In the filtrate, the levels of soluble phosphates were determined by the Ames method (1), the pH and titratable acidity using an automatic titrator (5).

The effect of citric, lactic, maleic, oxalic and tartaric acids on Ca-P and Al-P solubilization under abiotic conditions was evaluated. $AlPO_4$ (1.26 g l^{-1} PO_4^{3-}) and $CaHPO_4$ (1.36 g l^{-1} PO_4^{3-}) were added to 1 mM organic acid solutions and maintained for three days at room temperature. After this period, the mixtures were centrifuged at 9000 x *g* for 10 minutes and soluble phosphate was determined in the supernatant by the Ames method (1).

The results were submitted for variance analysis and the means compared by the Tukey test ($p<0.05$). All statistical analysis was performed using the SAS program (22).

RESULTS

The C sources which resulted in the most extensive solubilization of Ca-P were maltose and mannitol with no significant differences ($p<0.05$) between them (Fig. 1A). Galactose was most effective in solubilizing Al-P. Mannose and galactose, in media added with Al-P, and sucrose with Ca-P yielded the greatest fungal growth. Fungal growth was 100% greater, on average, with Al-P than that with Ca-P (Fig. 1B). Because of this growth, acid production increased 139% with Al-P in relation to Ca-P and pH decreased 51%, although the quantity of solubilized phosphate was only 12% greater.

The C sources which produced the greatest quantities of acids were maltose with Ca-P and mannose in Al-P medium (Fig. 1C). The lowest pH values were found when mannitol and

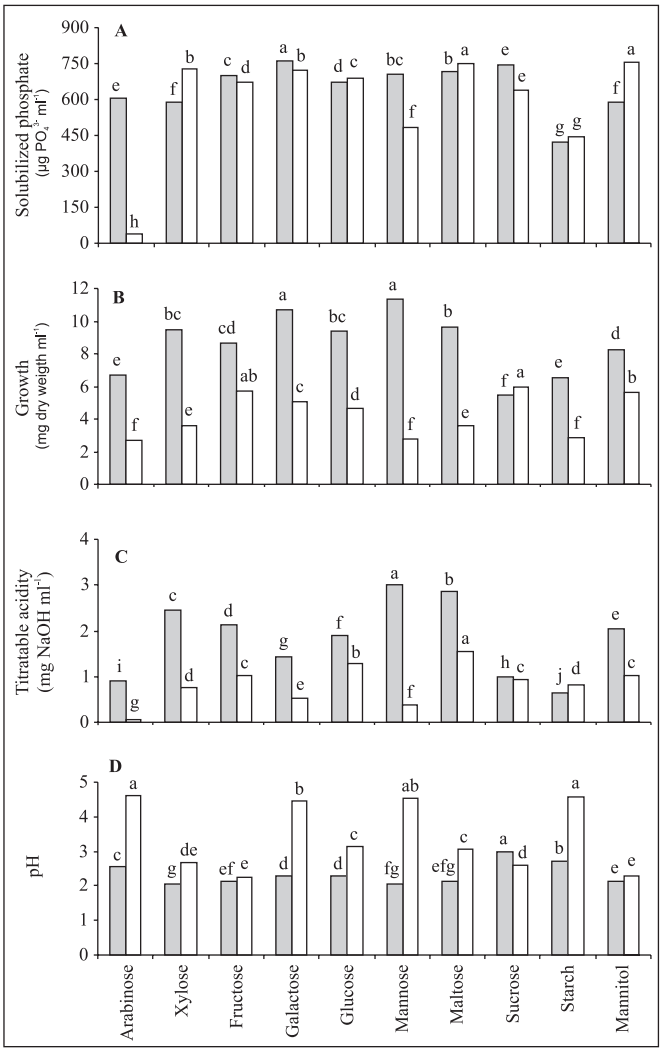

Figure 1. Effect of carbon sources on the solubilization (A), biomass (B), acids production (C) and pH (D) in media added with $AlPO_4$ ▭ or $CaHPO_4$ □. Different letters above the bars indicate significant differences between carbon source, according to an LSD value ($P < 0.05$).

fructose were added to Ca-P medium, with no significant difference between them (P<0.05), and xylose in Al-P medium (Fig. 1D).

The effect of the organic acids under abiotic conditions showed that Ca-P solubilization was almost twice that obtained with Al-P (Fig. 2).

The maximum quantity of solubilized Ca-P (888.31 µg PO_4^{3-} ml^{-1}) was found when glycine was added to the growth medium as the N source, followed by ammonium chloride and sodium nitrate (Fig. 3A). The maximum quantity of Al-P was solubilized with ammonium nitrate (705.50 µg PO_4^{3-} ml^{-1}) followed by ammonium chloride and ammonium sulfate (Fig. 3A). Fungal growth was 1.6-fold greater with Ca-P (5.81 to 11.30 mg of mycelium dry weight ml^{-1}) than with Al-P (3.53 to 8.03 mg of mycelium dry weight ml^{-1}) and glutamic acid provided the greatest growth for both P sources (Fig. 3B). Ammonium chloride was the best N source for the acid production of both P sources (Fig. 3C). The lowest value of final pH was found with glutamic acid in Ca-P medium and ammonium chloride in Al-P medium (Fig. 3D).

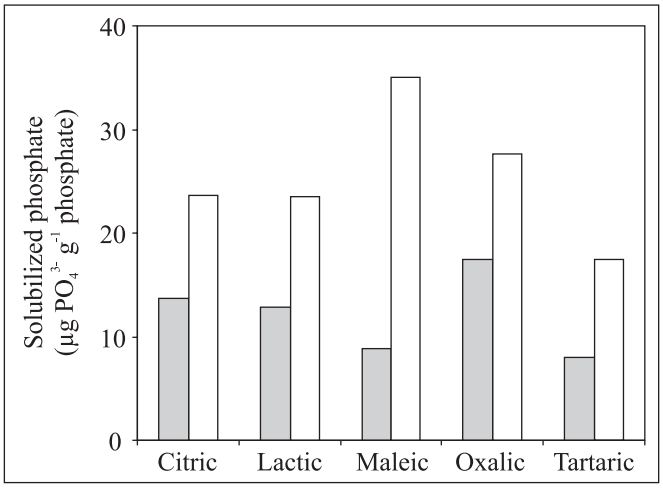

Figure 2. Effect of organic acids on the solubilization of $AlPO_4$ and $CaHPO_4$ under abiotic conditions.

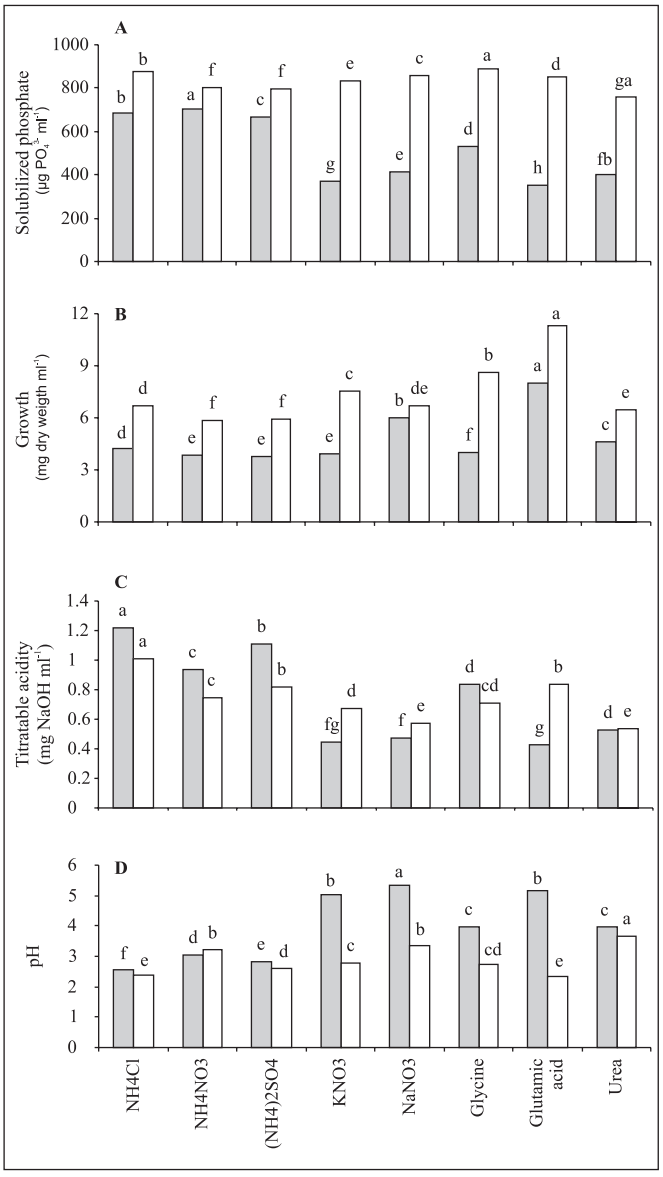

Figure 3. Effect of nitrogen sources on the solubilization (A), biomass (B), acids production (C) and pH (D) in media added with $AlPO_4$ or $CaHPO_4$. Different letters above the bars indicate significant differences between nitrogen source, according to an LSD value (P < 0.05).

DISCUSSION

Fungal growth was influenced as much by the C and N sources as by the phosphate sources added to the culture medium. As a consequence of fungus growth, the production of acids and pH decrease in the medium varied influencing the amounts of Ca-P and Al-P solubilized, as was made clear in this work. The production of acids by *A. niger* is an important mechanism for Al-P solubilization (11). In this study, greater growth, acid production and pH reduction were obtained in the medium containing Al-P than that containing Ca-P, although, the quantity of solubilized phosphate was only 12% greater in medium with Al-P than with Ca-P. These results can be analyzed according to the mycelium growth, where the soluble phosphate produced in the Al-P medium must have been absorbed by the fungus to sustain its growth, which shows that the P source influenced the solubilization capacity of the fungus.

Another possibility could be related to acid production. *A. niger* has been well studied for its ability to produce citric acid

(7, 27). In most studies citric acid was found the largest product in the solubilization of phosphates (23). Other acids like oxalic, glycolic, succinic and gluconic acid are also secreted by the fungus (25). These acids form complexes with metallic ions like Ca, Fe and Al, liberating soluble phosphate. Under abiotic conditions, more extensive solubilization of Ca-P than Al-P was obtained using the organic acids citric, lactic, maleic, oxalic or tartaric acid. The formation of complexes with metallic cations depends on the number and position of the functional carboxylic and phenolic groups of organic acids (4). However, for the same C source, it is possible that the nature of the organic acid varies with the phosphate used in the culture medium influencing the solubilization mechanism. In addition, the nature and quantity of organic acid secreted by the fungus were influenced by the C source and the balance between N and P sources (15).

Maltose, sucrose, glucose, mannose and fructose were the sugars which most stimulated citric acid production by *A. niger* (27). With the exception of mannose, in the present study these were also the sugars which proportioned the greatest quantity of acids in the Ca-P medium, whereas a greater quantity of soluble phosphate was obtained with mannitol, maltose, galactose and glucose. Of the sugars which most enhanced the production of acids, fructose and sucrose were those that stimulated the greatest decrease in pH, followed by mannitol and xylose. In the Al-P medium, the sugars which most enhanced the production of acids and decreased the pH were mannose, maltose and xylose and greatest solubilization was obtained with galactose, sucrose and maltose.

Most extensive growth of *A. niger* was found with maltose when compared to sucrose, lactose, glucose, and fructose (14). In the present study, maltose was not the preferred C source of the fungus. With Ca-P as the P source, growth yield decreased in the following order, sucrose > mannose > galactose and with Al-P, mannose > galactose > maltose and xylose. Interestingly, the sugars most efficiently used by *A. niger* for phosphate solubilization include hexoses (galactose, glucose and mannose), disaccharides (maltose and sucrose) and a sugar alcohol (mannitol). Presumably, depending on the C and P sources, the fungus uses alternative metabolic pathways and different organic acids can be secreted (16). Other acids besides citric acid must be produced with an ability of phosphate solubilization. The lowest pH was obtained with fructose (Ca-P) and xylose (Al-P), but the quantities of solubilized phosphates were low when compared with other C sources. Arabinose was the least effective C source for all the variables analyzed.

In response to different N sources, fungal growth, phosphate solubilization and decrease in pH were greater on average with Al-P than with Ca-P, although the production of acids had been greater with Al-P than with Ca-P. Glutamic acid allowed for the most extensive fungal growth whereas ammonium chloride allowed for highest acid production by the fungus in the presence of either Al-P or Ca-P.

Cerezine *et al*. (5) reported that ammoniacal sources increased the solubilization of fluorapatite by *A. niger* more than organic sources of N. An increase in rock phosphate solubilization by *Penicillium bilaji* was also found when NH_4^+-N was added to the medium (2). Similar results have also been reported by Whitelaw *et al.* (28) that found a higher acid production and P solubilization from ammonium assimilation by *Penicillium radicum.* In contrast, this study demonstrated that both inorganic and organic sources of N enhanced phosphate solubilization. While Ca-P solubilization was stimulated by amino acid and inorganic nitrogen salts in decreasing order as follows: glycine > ammonium chloride > sodium nitrate, Al-P solubilization was enhanced by the use of ammonium salts in the following order: ammonium nitrate > ammonium chloride > ammonium sulfate.

One of the mechanisms that explains solubilization activity results from the secretion of protons associated with the uptake of ammonia (21). However, citric and gluconic acids were produced by *A. niger* in the absence of ammonium nitrate; however, the addition of this salt stimulated citric acid production and decreased gluconic acid to undetectable levels (10). Evidence from this study indicated that Ca-P and Al-P solubilization was related to the production of acids and pH drop. In addition, as pointed out above, organic acids can be an important factor complexing the cation which is bound to P of poorly soluble phosphate (28). Ca-P solubilization was maximal in the medium added with glycine, which yielded one the most extensive fungal growth. Therefore, phosphate solubilization was also related to *A. niger* growth.

Sodium nitrate was one of the sources which stimulated Ca-P solubilization. Nitrate was found to be a worst N source than ammonium (6). However, Dixon-Hardy *et al*. (9) reported that nitrate increased solubilization of several phosphates. Lapeyrie (13) suggested that nitrate uptake by the cell stimulates acid secretion to compensate the cellular ionic potential; however, such a stimulus was not observed in the present study.

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RESUMO

Solubilização de $CaHPO_4$ e $AlPO_4$ por *Aspergillus niger* em meio de cultura com diferentes fontes de carbono e nitrogênio

A solubilização de fosfatos inorgânicos por microrganismos disponibiliza fosfato para a nutrição das plantas e aumenta seu crescimento. A solubilização de $CaHPO_4$ (Ca-P) e $AlPO_4$ (Al-P)

por *Aspergillus niger* na presença de várias fontes de carbono e de nitrogênio foi estudada. A solubilização de Ca-P foi aumentada quando as fontes de carbono foram manitol, maltose, galactose e glicose (nesta ordem). Galactose, sacarose e maltose foram as fontes de carbono que aumentaram a solubilização de Al-P. Maior crescimento, produção de ácidos e diminuição do pH foram obtidos em meio contendo Al-P do que Ca-P, porém, a quantidade de fosfato solubilizado foi apenas 12% maior. A solubilização dos fosfatos foi relacionada à produção de ácidos, diminuição do pH e crescimento do fungo no meio de cultura. Resultados de um estudo conduzido sob condições abióticas mostraram que os ácidos orgânicos solubilizaram mais Ca-P do que Al-P. Avaliando-se o efeito da fonte de N, a solubilização de Ca-P ou Al-P decresceu na seguinte ordem: glicina > NH_4Cl > $NaNO_3$ ou NH_4NO_3 > Uréia > $(NH_4)_2SO_4$, respectivamente. As fontes de N amoniacal (NH_4^+-N) foram as mais efetivas na produção de ácidos e diminuição do pH.

Palavras-chave: acidez titulável, fonte de fosfato, fósforo, nutrição, solubilização de fosfato

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