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Serna Posso, Eduardo José; Sánchez de Prager, Marina; Cisneros Rojas, Carlos Adolfo
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Organic acids production by rhizosphere microorganisms isolated from a Typic Melanudands and its effects on the inorganic phosphates solubilization

Producción de ácidos orgánicos por microorganismos rizosféricos aislados de un Typic Melanudands y sus efectos en la solubilización de fosfatos inorgánicos

Eduardo José Serna Posso, Marina Sánchez de Prager and Carlos Adolfo Cisneros Rojas*

Facultad de Ciencias Agropecuarias. Departamento de Ciencias Biológicas. Universidad Nacional de Colombia sede Palmira, Colombia. Author for correspondence: ejsernap@unal.edu.co

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Abstract

It has been established that organic acid secretion by rhizosphere microorganisms is one of the mechanisms to solubilize the phosphorus (P) attached to insoluble mineral compounds in soil. This action is an important biotechnological alternative, especially in those soils where high fixation of this nutrient occurs, a very common situation in the tropics. This research evaluated the ability performed by five bacterial and five fungal isolates from Typic Melanudands soil to produce organic acids and generate available phosphorus from insoluble P sources. Given these concerns, the selected microorganisms were replicated for 7 days in liquid medium Pikovskaya (PVK) modified sources tricalcium phosphate (P-Ca), aluminum phosphate (P-Al) and iron phosphate (P-Fe). The results indicated that phosphorus availability in the media, correlates positively with the organic acids production in each of the sources used (P-Ca (0.63), P-Al (0.67) and P-Fe (0.63). In turn, the chemical processes linked to the phosphates solubilization (e.g., Ca availability) affected the development of the microorganisms tested. Both, fungi and bacteria varied in their ability production and type of metabolized organic acids, the most frequent were as follows: citric and gluconic acid.

Keywords: Available phosphorus, insoluble P sources, phosphorus dynamics, *Penicillium ochrochloron.*

Resumen

Se ha establecido que la secreción de ácidos orgánicos por microorganismos rizosféricos constituye uno de los mecanismos para solubilizar el fósforo (P) unido a compuestos minerales insolubles en el suelo. Esta acción es una alternativa biotecnológica importante, especialmente en aquellos suelos donde ocurre alta fijación de este nutrimento, situación muy frecuente en el trópico. Este estudio evaluó la capacidad que presentan cinco aislamientos bacterianos y cinco fúngicos provenientes de un suelo *Typic Melanudands* para producir ácidos orgánicos y generar fósforo disponible a partir de fuentes insolubles de P. Para ello, los microorganismos seleccionados se sembraron por 7 días en medio líquido Pikovskaya (PVK) modificado con fuentes de fosfato tricálcico (P-Ca), fosfato de aluminio (P-Al) y fosfato de hierro (P-Fe). Los resultados indicaron que la disponibilidad del fósforo en los medios correlaciona positivamente con la producción de ácidos orgánicos en cada una de las fuentes utilizadas (P-Ca (0.63), P-Al (0.67) y P-Fe (0.63)). A su vez, los procesos químicos ligados a la solubilización de fosfatos (por ejemplo, disponibilidad al mismo tiempo de Ca) afectó el desarrollo de los microorganismos evaluados. Tanto hongos como bacterias variaron en su capacidad de producción y tipo de ácidos orgánicos metabolizado, siendo los más frecuentes ácido cítrico y glucónico.

Palabras clave: Dinámica del fósforo, fósforo disponible, fuentes insolubes de fósforo, *Penicillium ochrochloron.*

Introduction

Phosphorus (P), is uptaked by plants as $H_2PO_4^-$ and HPO_4^{2-} and is key in plant metabolism with consequent impact on yield, nutritional quality and resistance to plant pathogens and pests (Ashley *et al.,* 2011).

Given the high sensitivity of P to be fixed in different ways, this can lead to soil becoming deficient in this nutrient and physiology and crop production is negatively affected. Examples of rainfall, in calcareous soils reacts with Ca^{2+} and Mg^{+2} and form insoluble salts such as tricalcium phosphate [$(PO_4)_2Ca_3$], in acid soils major cations that determine this reaction are the Fe^{3+} and Al^{+3} producing compounds such as strengita ($FePO_4.2H_2O$) and Variscite ($AlPO_4.2H_2O$). It can also adsorb on the surface of clays and oxides of iron and aluminum. The P can be immobilized in the processes of microbial and plant biosynthesis. All these transformations make this element a part of organic and inorganic materials in soil (parent material), becoming its main natural deposits (Jones & Oburger, 2011).

Farmers counteract daily the situation of P deficiency with the addition of high amounts of inorganic fertilizer with negative consequences on the cost of crop production and environmental degradation, among others (Jones & Oburger, 2011).

A higly interesting biotechnological alternative to solve this problem is phosphorus solubilization by rhizosphere microorganisms, which convert insoluble forms of P available to plants, increasing soil fertility and producing, social, economical and environmental benefits. The most abundant P solubizing microorganisms present in soil are bacteria and fungi. Some of the bacterial genera that often perform this activity are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium, Mesorhizobium, Azotobacter and Azospirillum.* Among the fungi, *Aspergillus* and *Penicillium* predominate (Sindhu *et al.,* 2014).

When P is linked to organic material and humus, the main mechanism of solubilization of soil microorganism is the production of enzymes (different types of phosphatases), carrying out mineralization and making it available to plants and microorganisms (Jones & Oburger, 2011).

When P is stored in inorganic molecules, their availability occurs by a solubilization processes related to the dissolution properties of mineral and medium acidification. Microorganisms influence soil pH by absorption of NH^{4+}, where these protons ejected to compensate the potential difference in the membrane and acidify the soil. Acidification has also been associated with the presence of CO_2 generated during respiration of both roots and microorganisms (Jones & Oburger, 2011).

In addition, other known mechanism, is the production of organic acids by microorganisms, including, gluconic, citric, malic, malonic, oxalic, succinic, lactic and tartaric. These acids provide both protons and organic anions that serve as chelating agents. The anions have a negative charge, so that possess the ability to form complexes with the positively charged ions (eg Ca^{+2}, Al^{+3}, Fe^{+3}) present in the soil and thus, release the phosphorus which is precipitated or occluded (Mardad *et al.,* 2013).

In addition to solubilizing P, these organic acids are carbon sources for microorganisms, those consuming and reduce. Therefore, its solubilizing effect have allowed to estimate the life span of these acids is between 0.5 and 12 h, suggesting that they must be continuously produced and secreted down (Xiao & Wu, 2014).

Based on the above mentioned, the aim of this research was to evaluate qualitatively and quantitatively, the production of organic acids and soluble phosphorus by metabolic activity of five fungi and five bacteria solubilizing phosphate from a *typic Melanudands*.

Material and methods

This research was carried out at the Uhiversidad Nacional de Colombia, Palmira, campus. The microorganisms were obtained accordingly to Cisneros & Prager, (2015), who selected five bacterial and five fungal strains for its high solubilising efficiency in vitro in P-Ca, P-Al and P-Fe, isolated from a *typic Melanudands* from Cajibío, Cauca department of Colombia.

Production of organic acids and phosphorus solubilized by the selected microorganisms

The production of organic acids and soluble phosphorus generation was performed using a liquid fermentation process. The fermenters used were 250 ml Erlenmeyer flasks under ambient conditions of light, temperature and relative humidity, and constant agitation of 120 rpm. Pikovskaya (PVK) was peformed as the culture medium, varying the phosphorus source among P-Ca, P-Al and P-Fe, (Cisneros & Prager, 2015). 99 experimental units were performed from these treatment combinations: 3 sources P, 10 microbial strains, plus 3 uninoculated, controls with 3 replications/treatment. Microorganisms are inoculated at 3 x 10^8 cfu.ml-1 bacteria and 1x10^6 spores.ml-1 in fungi. Bacteria were identified B1 to B5 and H1 to H5 fungi. The

fermentations were carried out for 7 days. 10 ml of sample in each treatments on days 1, 3, 5 and 7, and the analysis were performed as follows:

Evaluation of the growth of fungi and bacteria

Was estimated by plating and serial dilution. A qualitative and quantitative identification of organic acids, were performed, respectively. High Efficiency Liquid Chromatography (HPLC) under the conditions used by Mardad *et al.,* (2013), was used as the analytical method.

Soluble phosphorus generated by fungi and bacteria. The blue molybdovanadate was performed as the spectrophotometric technique (Mardad *et al.,* 2013)

Microorganisms identification

It was performed using molecular techniques. In fungi, primers ITS1 and ITS4, were performed, and the FD1 and RD1 in bacteria (Sasso *et al.,* 2012).

Experimental design and statistical analysis

The arrangement of the experiments correspond to a completely randomized design, and the response variables were as follows: concentration of soluble phosphorus, organic acids quantified and total acidity generated in the medium by each microorganism. Analysis of variance with a 95% significance was established, and in the required cases, comparison of means by Duncan test. Pearson correlation was analyzed to establish relation among found organic acids and soluble phosphorus. The coefficient of variation (CV) was determined for each of the estimated acids for each source of P used. All analyzes were performed using SAS statistical package version 9.1.3. ®.

Results and discussion

Monitoring microbial cultures have allowed to define the seventh day as the most suitable for evaluation of the production for organic acids and their statistical analysis, due to the evaluated microorganisms on this stage, performed the largest acid concentration.

Production of organic acids by evaluated microorganisms and soluble phosphorus

In general, both bacterial and fungal strains produced D-malic, D-lactic, L-malic, L-lactic, acetic, citric and gluconic acids. Separate analysis of cultivated strains show that in all five bacteria, predominated gluconic acid secretion with concentrations between 500 and 3000 ppm, especially P-Ca, in which B5, showed the highest value. In the other two P sources, the contents of these organic acids were below 50 ppm and in some cases invaluable. Regarding fungi citric acid predominated with concentrations between 100 and 300 ppm, mainly P-Ca. In P-Al H3 and H5, only citric acid metabolised in the order of 100 and 170 ppm, respectively. P-Fe lower concentrations were recorded (0 to 50 ppm).

When performing the appropriate statistical analysis, CV were found in the evaluated organic acids, fluctuated considerably, presenting equal quantities or greater than 80% in each of the P sources (Table 1). In contrast, Ghaly & Kamal (2004), and Rathi *et al.* (2002), who in biological systems and fermentation processes found CV between 3.9% and 39.05%. Low and/or negative Peason correlations are also evidence, no significantly response among the estimated acids and soluble phosphorus (Table 2). However, citric acid, showed the highest correlation in the three sources. These findings raise doubts in terms of the normal distribution in the fermentative processes.

Table 1. Coefficient of variation (CV) expressed in %, of each organic acids metabolised by the microorganisms per the phosphate sources

Source	Organic acids determined by HPLC					
	Citric	Gluconic	D- Malic	D- Lactic	L- Lactic	L- Malic
$AlPO_4$	107.73	121.5	361.21	229.98	226.3	108.21
$Ca_3(PO_4)_2$	80.18	175.65	80.66	321.48	314.2	129.37
$FePO_4$	387.5	113.81	248.03	346.27	142.58	150.82

Table 2. Pearson correlation coefficients between the organic acid and soluble phosphorus in each of the sources of phosphates, generated during fermentation

Source	Organic acids determined by HPLC						
	Citric	Gluconic	D- Malic	D- Lactic	L- Lactic	L- Malic	Ac. Total
$AlPO_4$	0.58	-0.39	0.22	0.17	0.2	0.29	0.67
	0.15	0.03	0.24	0.37	0.28	0.16	<.0001
$Ca_3(PO_4)_2$	0.53	-0.18	-0.68	0.21	0.22	0.44	0.63
	0.03	0.34	0.72	0.25	0.25	0.015	0.03
$FePO_4$	0.5	0.32	0.18	-0.083	0.048	0.28	0.63
	0.11	0.009	0.35	0.66	0.8	0.04	0.0008

Conventions: The Pearson correlation coefficients is accompanied by its significance, which it corresponds to the value found in a smaller size. Ac. = Acidity.

The search for an explanation to this situation have allowed establishing one of the possible causes, and is the way of the interpretation

results. Jones *et al.* (2003), analyzed several studies which underestimated the concentration of organic acids in the rhizosphere due to the dynamics that occur in soil.

Based on the above, the organic acid monitoring scheme during the bioprocess was carried out in this research (Figure 1). As can be shown, from the culture medium and microbial inoculation (Figure 1 A), the organic acids (B), are produced by part of them, in addition, participate in the solubilization reaction (C) and the remaining, is soluble in the solution (D), the latter, which is determined by HPLC and analyzed statistically, produce the unfavorable findings above mentioned.

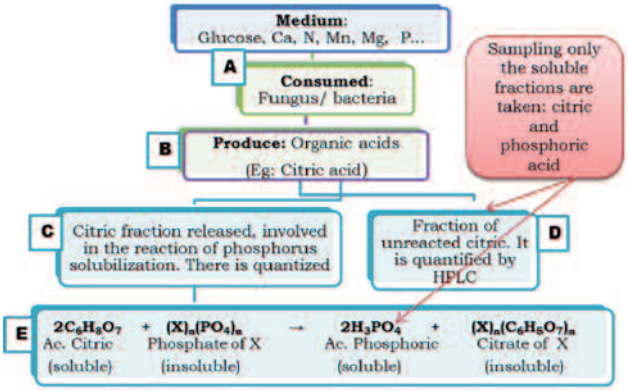


Figure 1. Production scheme and reaction of organic acids during the fermentation process

Conventions: Ac = acid, X could be Ca, Al or Fe, n = 1, 2.3, which corresponds to the amount of ions present in the molecule.

In addition, the solubilized phosphorus should be related to the total acids produced by each microorganism, not individualities, as mentioned by Oburger *et al.* (2009), the phosphorus release was higher when citric, malic, oxalic and malonic acids were combined. Mardad *et al.* (2013), take similar approaches and relate total acids with soluble phosphorus, finding positive, highly significat correlations ($p < 0.01$, $r = 0.93$). Therefore, a decision was established to determine the total acidity, from converting the units in which express concentrations each estimated acid by HPLC, transforming figures from $mg.L^{-1}$ to $meq.L^{-1}$, since this last unit, allows concentrations that are equivalent and can be added. In turn, the amount of soluble phosphorus in this unit ($meq.L^{-1}$) is the amount of acids, which were involved in the solubilization process (Figure 1 E). Therefore, total acids produced by each microorganism correspond to the sum of the acids determined by HPLC plus the acids involved in the solubilization of phosphate (Table 3).

Table 3. Determination of total acidity (Ac Total) produced by each microorganism (Mic) in each source of phosphate. The units are expressed in $meq.L^{-1}$

a) Source: P-Ca

Mic	Organic acids determined by HPLC							P-Sol	Ac Total
	Citric	Gluconic	D- Malic	D- Lactic	L- Lactic	L- Malic	Acetic		
H1	4.30	0.60	0.30	0.00	0.50	0.90	0,00	5,50	12,1
H2	4,90	0,70	0,70	0,40	1,00	0,10	1,20	6,50	15,5
H3	4,90	0,20	0,50	0,30	1,80	2,70	1,10	7,40	18,9
H4	0,90	0,50	1,10	1,20	3,10	1,00	0,00	6,70	14,5
H5	3,90	2,50	0,90	0,00	0,70	4,80	1,00	7,00	20,8
B1	0,60	1,40	0,20	0,00	0,00	0,00	0,00	3,90	6,1
B2	0,30	4,90	0,00	0,00	0,00	0,00	0,00	5,10	10,3
B3	0,90	1,70	0,00	0,00	0,50	0,40	0,00	4,20	7,7
B4	0,30	5,50	0,10	0,00	0,10	0,10	0,00	4,80	10,9
B5	0,40	15,30	0,80	0,00	0,20	0,40	1,50	4,90	23,5

b) Source: P-Al

Mic	Organic acids determined by HPLC							P-Sol	Ac Total
	Citric	Gluconic	D- Malic	D- Lactic	L- Lactic	L- Malic	Acetic		
H1	0,00	1,80	0,16	0,00	1,07	0,22	0,00	1,92	5,2
H2	0,00	0,00	0,00	0,00	0,00	0,68	0,00	2,49	3,2
H3	2,70	0,12	1,36	0,23	4,00	0,00	0,00	2,74	11,2
H4	0,00	0,88	0,00	0,00	0,00	0,33	0,00	2,16	3,4
H5	1,82	0,00	0,00	0,00	0,00	5,93	0,00	3,49	11,2
B1	0,51	0,10	0,00	0,46	0,00	0,00	0,00	2,67	3,7
B2	0,24	0,48	0,10	0,00	0,00	0,20	0,00	0,86	1,9
B3	0,26	0,31	0,00	0,00	0,00	0,30	0,00	0,90	1,8
B4	0,47	0,74	0,11	0,00	0,20	0,13	0,00	0,91	2,6
B5	0,00	0,20	0,00	0,28	2,16	0,10	0,00	1,08	3,8

c) Source: P-Fe

Mic	Organic acids determined by HPLC							P-Sol	Ac Total
	Citric	Gluconic	D- Malic	D- Lactic	L- Lactic	L- Malic	Acetic		
H1	0,00	0,49	0,37	0,37	0,44	0,17	0,41	0,74	3,0
H2	0,00	0,00	0,00	0,00	0,00	0,28	0,00	0,73	1,0
H3	0,49	0,41	0,22	0,00	0,00	0,19	0,89	1,77	4,0
H4	0,00	0,09	0,28	0,55	0,32	0,10	0,00	0,57	1,9
H5	0,00	0,45	0,00	0,00	0,29	3,88	0,00	1,18	5,8
B1	0,00	0,00	0,08	0,00	0,00	0,19	0,00	0,36	0,6
B2	0,00	0,44	0,00	0,00	0,00	0,00	0,00	0,46	0,9
B3	0,00	0,04	0,00	0,00	0,00	0,08	0,00	0,69	0,8
B4	0,09	0,14	0,00	0,00	0,22	0,28	0,00	0,68	1,4
B5	0,00	0,21	0,05	0,36	0,17	1,07	0,00	0,56	2,4

With this methodological adjustment, the statistical results changed considerably since the correlation among total acidity and soluble phosphorus (Table 2), and the minor variation coefficient (P-Al: 39.53%, P-Ca: 18.53%, P-Fe: 38.76%). Determining the correlation among the total acidity and soluble phosphorus have allowed understanding the solubilization process as a biotechnological dynamics induced by microorganisms (Figure 2). From which, the phosphate source used, is critical and solubilized, can stimulate or inhibit the organic acids production of microbial origin in this case. Both, the source of P, as the type of acid metabolised and its concentration, influence the generation and amount of available phosphorus. (Figure 3). The phosphate source of microorganisms, showed higher production of organic acids (P-Ca> P-Al> P-Fe), which coincides with the greatest amount of soluble phosphorus.

Normally, when the P solubilization process is analyzed, the result emphasizes in the availability of this element. However, the biotechnological dynamics for solubilizing phosphates "*in vitro*" (Figure 2), can display other important components. The release of Ca, Al and Fe as organic chelates and/or free ions, depending on the used source, which introduce additional changes that will affect, for example, the growth of microbial populations in environmental conditions that may be positive or negative for the whole process, among others.

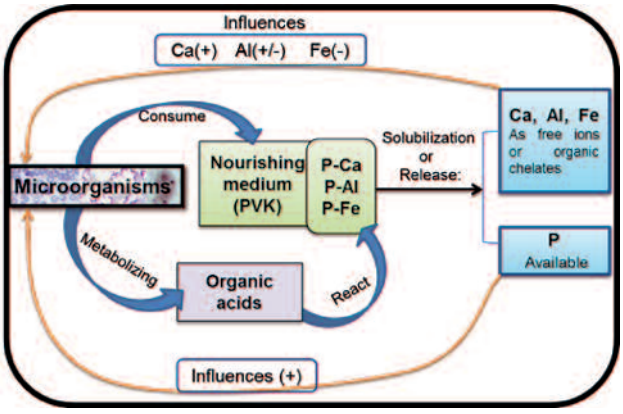


Figure 2. *in vitro* dynamics biotechnology solubilizing phosphates

Conventions: The sign represents the effect of ion solubilized or available on microbial growth (+) positive effect (+/-) that the effect is not as positive or negative in some cases, and (-) negative effect

Microbial growth and organic acids production in different evaluated P sources

Both fungi and bacteria, showed the highest growth population (Figures 4a and c) when the source used was P-Ca, coincident with the production of acids (Figure 3). Papagianni (2004), argues that one of the determining factors in the generation of secondary metabolites (in this case, organic acids) is the development of microorganisms. In the P-Ca medium, fungi remained in exponential phase, while bacteria achieved twice the population obtained by fungi between days 3 and 5. However, 4 of the 5 bacteria showed a population decrease in day 7, except the B3 which took almost 5 days to begin growth and reached only the exponential phase (Figure 4a and c). On the contrary, fungi with smaller populations remain longer, which is a sustained ability to produce acids that are more organic and therefore, solubilize more phosphorus.

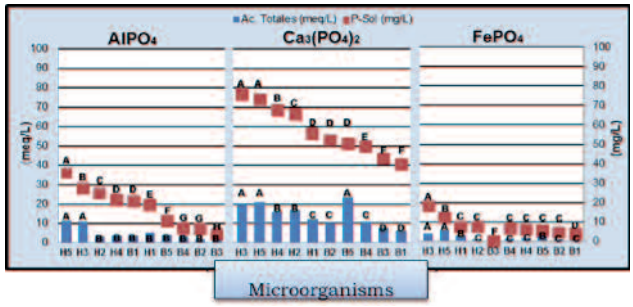


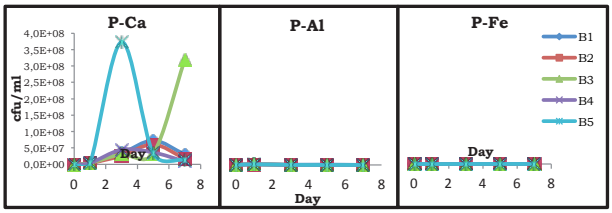
Figure 3. Organic acids expressed as total acidity (Ac. Total) and soluble phosphorus (P-Sol) generated fungi and bacteria, in each of the phosphate sources.

Conventions: meq = milliequivalent, H = fungus, B = Bacteria, letters on the bars represent the grouping Duncan (α = 0.05)

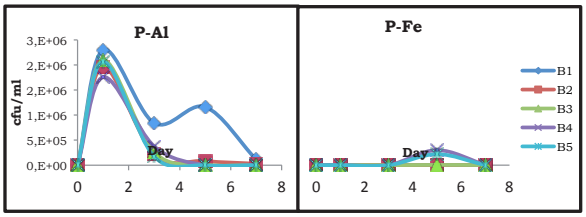
In concordance to Papagianni (2004), the initiation of growth population is matched by the release of the first traces of acids, which react with the phosphate source to start P solubilization, vital for cell development. Besides this soluble phosphorus, other nutrients remaining availables (Figure 2), become in food sources for the involved microorganisms. Naveena (2011), established that the available Ca induces fungal growth.

The P-Al source influence negatively the production of organic acids in fungi and bacteria mainly (Figure 3). The toxic effect of solubilized Al is initially invisible and appreciated only through bacterial incubation time, to the extent that their concentration increases in the culture medium (Figure 4b). Similar results have been obtained by Illmer & Erlebach (2003), who found the aluminum concentration exceeded 30 uM, which is a harmful effect is seen on bacteria of the genus *Arthrobacter sp.* The fungi in P-Al had a similar growth as the one presented in P-Ca (Figure 4c). This behavior could be explained by an internal tolerance mechanism in the cell wall, which has the ability to adsorb the Al^{3+} by functional groups such as hydroxyls, amines and carboxyls present in the polysaccharide. Although the increase

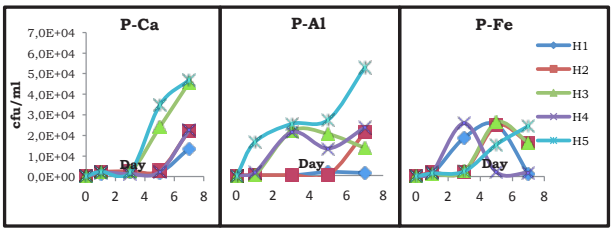
in biomass remains stable, acid secretion is decreased (Chao *et al.* 2013).



a) Population growth of bacteria expressing the scale of the orderde 10^8 cfu.ml^{-1}



b) Population growth of bacteria in P-A and P-Fe, expressing the scale of the order of 10^6 cfu.ml^{-1}



C) Population growth of fungi expressing the scale of the order of 10^4 cfu.ml^{-1}

Figure 4. Growth 10 microorganisms in culture media with different phosphate sources: P-Ca: tricalcium phosphate, P-Al: aluminum phosphate P-Fe: iron phosphate.

The P-Fe, negatively affected the production of organic acids in both microbial groups (Figure 3), with emphasis on bacteria, Chamnongpol *et al.* (2002), argue that the Fe^{3+} as part of $FePO_4$ can permeabilize the bacterial membrane from the outside, resulting in lysis and death of the organism, explaining the few or no populations found in this research (Figure 4b).

In this P-Fe medium, fungi grew even less than in P-Ca and the evaluated concentration of organic acids was drastically affected. Philpott (2006), argues that the iron in ferric form is not absorbed, due to the fungi cell wall using a reducing system which involves in two stages: the first ferric iron (Fe^{3+}) is reduced to ferrous (Fe^{2+}) in the plasma membrane by proteins designated FRE reductases, and then is transported into the cell by specialized proteins called FIT.

When the intracellular concentration of Fe^{2+} is increased, it becomes toxic as it reacts with oxygen and generates free radicals that attack macromolecules such as DNA leading to cell death (de Freitas & Meneghini, 2001). Apparently, this explains that the H1, H2, H3 and H4 fungi would reduce their population (Figure 4c) among the fifth and seventh day of fermentation. The only fungus that did not present this decline was H5, considering that it possibly has a greater number of heme proteins that store intracellular iron (Philpott, 2006), counteracting the toxic effect and having increased tolerance to high concentrations of iron.

In general, the ten evaluated microorganisms were able to grow in greater and/or lesser degree, in the presence of P-Ca, P-Al and P-Fe, while solubilizing these sources. The H5 and H3 fungi, showed the greatest solubilizing capacity, although to a different extent, on the three sources used in this study. H3, was identified as *Penicillium ochrochloron*, widely recognized as phosphate solubilizing by producing citric and malic acid (Coutinho *et al.,* 2012). Conversely, bacteria solubilized especially P-Ca, the most efficient B5, identified as *Kocuria sp*, which is registered in the group of plangt growth promoters by different mechanisms, including the phosphates solubilization of (Goswami *et al.,* 2014).

These results, indicates a very important biotechnological potential for the use of microorganisms whicht solubilize P and this can lead to reduce the economic costs coupled with social and ecosystem effects, which is involved in meeting the P needs in crops with environmental friendly alternatives.

Conclusion

This research established that the phosphorus source used, significantly influenced the following order: P-Ca> P-Al> P-Fe, on variables of the evaluated growth population microorganisms, the production of organic acids and soluble phosphorus. The action of these microorganisms on the three P evaluated sources, also manifested in the production of different organic acids (citric, malic, gluconic and lactic, mainly), contribute to provide P available in the culture medium, liberates cations, which create environmental conditions for microbial growth. This research also identify *Penicillium ochrochloron* and *Kocuria sp.,* as highly phosphorus solubilizing microorganisms, provides theoretical and practical elements from their condition "*in vitro*", which explains processes that occur in the rhizosphere and contribute in plant nutrition.

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