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The Amount of Phosphate Solubilization Depends on the Strain, C-Source, Organic Acids and Type of Phosphate

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ABSTRACT

Although production of organic acids (OAs) is usually mentioned as the main mechanism of phosphate solubilization, the relationship between carbon sources (C-sources) and OAs produced during phosphate-solubilization by microorganisms is still poorly understood. We evaluated the influence of different C-sources on $FePO_4{\cdot}2H_2O$ and $Ca_3(PO_4)_2$ solubilization by bacteria and on the identity/quantity of the OAs produced. Our results showed that the amount of phosphate solubilization depends on the strain, C-source, OAs, and type of phosphate. Among the five strains under study isolated from cowpea nodules (*Rhizobium tropici* strain UFLA 03-08, *Acinetobacter* sp. strain UFLA 03-09, *Paenibacillus kribbensis* strain UFLA 03-10, *P. kribbensis* strain UFLA 03-106, and *Paenibacillus* sp. strain UFLA 03-116), three of them solubilized $Ca_3(PO_4)_2$ in all C-sources. The influence of C-sources on $Ca_3(PO_4)_2$-solubilization increased in the following order: cellulose < lactose < mannitol < glucose. A significant positive correlation between the amount of phosphorus solubilized from $Ca_3(PO_4)_2$ and the concentration of total OAs in the presence of glucose and mannitol was observed for these three strains. In the presence of glucose, the highest solubilization rates are associated with high concentrations of tartaric acid, and in the presence of mannitol, are associated with maleic acid. Only one strain produced OAs in the medium with lactose and $Ca_3(PO_4)_2$, but there was no OAs in the medium containing cellulose. Despite the production of OAs, albeit in small concentrations, in all the C-sources investigated, $FePO_4{\cdot}2H_2O$-solubilization was not observed. Thus, a relationship among C-sources, OAs, and phosphate solubilization was not always verified.

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Acinetobacter Rhizobium tropici; glucose; maleic acid; *rhizobium*; tartaric acid; *Paenibacillus*

Introduction

Although a large amount of phosphorus is available in tropical soils (Novais and Smyth 1999), the lack of available phosphorous is one of the most limiting factors for plant yield. Therefore, high rates of phosphate fertilizers are needed to increase crop yield. Low availability is mainly due to immobilization of phosphorus with other soil components through precipitation, which forms insoluble calcium, aluminum, and iron phosphates (Tan 1993).

Microorganisms can solubilize phosphate precipitates and increase phosphorus availability. Some bacteria and fungi are capable of solubilizing phosphate from rocks, increasing phosphorus for plant growth (da Costa et al. 2015; Collavino et al. 2010; Gerretsen 1948). The ability of these microorganisms to solubilize insoluble inorganic phosphates, such as $CaHPO_4$, $AlPO_4$, and $FePO_4$, has been attributed to their ability to reduce the surrounding pH (Braz and Nahas 2012; Farhat et al. 2009; Stumm and Morgan 1995; Whitelaw 2000). The most cited mechanism of solubilization is production of organic acids (Hariprasad and Niranjana 2009; Gulati et al. 2010; Marra et al. 2015; Sperber 1958), which can lower the pH and act as chelators of the elements that are bound to the phosphate, preventing reprecipitation (Bolan et al. 1994). Furthermore, Alexander (1961) reported that the amount of phosphate solubilized by microorganisms in the soil solution varies according to which carbon sources (C-sources) are present. Plant exudates represent the main C-source in the rhizosphere compartment that modulates P-solubilization and bioavailability. In the soil-root system, glucose is the carbohydrate that is secreted in the largest quantities (Kraffczyk et al. 1984), and cellulose is the most common natural polysaccharide as it contains most of the CO_2 fixed by plants.

Studies using insoluble inorganic phosphates with different C-sources have reported that the C-source is the key factor in the efficiency of solubilization by both bacteria

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Supplemental data for this article can be accessed on the publisher's website

(Nautiyal 1999; Sridevi and Mallaiah 2009) or fungi (Ahuja et al. 2007; Relwani et al. 2008). However, few studies have combined qualitative and quantitative results and evaluated the production of organic acids, and only a few acids have been evaluated in these studies.

Thus, the objectives of this study were to evaluate the influence of four C-sources (cellulose, glucose, lactose, and mannitol) on biosolubilization of $Ca_3(PO_4)_2$ and $FePO_4{\cdot}2H_2O$ in solid and liquid culture medium by five bacterial strains isolated from cowpea nodules and to verify the relationship of strains, C-sources, organic acids, and type of phosphate produced in this liquid medium with phosphate solubilization.

Materials and methods

Growth of strains in different C-sources with and without insoluble phosphate

We individually tested the ability of the strains UFLA 03-08 (*Rhizobium tropici*), UFLA 03-09 (*Acinetobacter* sp.), UFLA 03-10 (*Paenibacillus kribbensis*), UFLA 03-106 (*Paenibacillus kribbensis/P. peoriae/P. polymyxa* according to the NCBI-National Center for Biotechnology Information), and UFLA 03-116 (*Paenibacillus* sp.) to grow in solid culture medium 79 (Fred and Waksman 1928) with different C-sources without insoluble phosphate. These strains were isolated from cowpea nodules using medium 79 with mannitol (Marra et al. 2012). The GenBank accession numbers (16S rRNA sequences) of the strains under the study were JQ041883 (UFLA03-08), JQ041884 (UFLA03-09), JQ041885 (UFLA03-10), JQ041894 (UFLA 03-106), and JQ041897 (UFLA 03-116). The growth of the strains was evaluated in National Botanical Research Institute's phosphate (NBRIP) culture medium containing different carbon sources, including cellulose, glucose, lactose, and mannitol, all added to the medium at the concentration of 10 g L^{-1}. The strains were subcultured by streaking them onto plates containing the culture medium, and then, they were incubated at 28 °C for 10 days. The growth or lack of growth was determined at the end of this period. The experimental design was completely randomized with three replicates.

To evaluate the different C-sources in solid NBRIP medium (supplemented with $Ca_3(PO_4)_2$ or with $FePO_4{\cdot}2H_2O$ at a concentration of 1000 mg L^{-1} of phosphorus), four 20 µl aliquots of each culture (strain) with an optical density (OD) of 0.5 were inoculated in a separate Petri dish. The controls consisted of NBRIP media containing each phosphate and C-source but without inoculation with microorganisms. All treatments had three replicates. The culture plates were incubated at 28 °C, and the diameter of the solubilization halos (translucent area around the colony) was measured using a digital caliper at the beginning of solubilization and after 15 days of incubation. From these measurements, we determined the solubilization index (SI), which is equal to the diameter of the halo (mm) divided by the diameter of the colony (mm) (Berraquero et al. 1976). Based on this SI, the strains were classified as having low ($SI < 2.00$), moderate ($2.00 \leq SI > 4.00$), or high ($SI > 4.00$) solubilization ability.

Solubilization of phosphates and production of organic acids in liquid media with different C-sources

Three experiments were conducted to test the capacity of the UFLA 03-08 (*R. tropici*), UFLA 03-09 (*Acinetobacter* sp.), UFLA 03-10, UFLA 03-106 (*P. kribbensis*), and UFLA 03-116 (*Paenibacillus* sp.) strains to solubilize insoluble inorganic calcium and iron phosphates in liquid NBRIP culture medium (Nautiyal 1999) that originally contained glucose and the following components (L^{-1}): 5 g of $MgCl_2{\cdot}6H_2O$, 0.25 g of $MgSO_4{\cdot}7H_2O$, 0.2 g of KCl, and 0.1 g of $(NH_4)_2SO_4$. These media were supplemented with $Ca_3(PO_4)_2$ or $FePO_4.2H_2O$ at a concentration of 100 mg L^{-1} of phosphorus. Different C-sources were evaluated: cellulose, glucose, lactose, and mannitol (at a concentration of 10 g L^{-1}). The initial pH of the media was adjusted to 7.0.

To obtain and standardize the inocula, strains were inoculated in liquid medium 79 (Fred and Waksman 1928) containing the following components: 0.5 g L^{-1} K_2HPO_4, 0.2 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.1 g L^{-1} NaCl, 10.0 g L^{-1} mannitol, and 0.4 g L^{-1} yeast extract, at pH 6.8. The strains were incubated under shaking (100 rpm) and aerobic conditions at room temperature. The measurements were taken over time using a spectrophotometer at the wavelength of 560 nm until the bacteria reached an OD of 0.5, which indicates that there were approximately 10^9 cells per ml. If the OD was greater than 0.5, 0.85% saline was added to dilute and adjust the culture to the desired cell concentration.

For the liquid NBRIP medium, evaluation was performed by inoculating a 1 ml aliquot of the cultures in liquid 79 medium, with OD of 0.5 at 560 nm, into Erlenmeyer flasks (125 ml) containing 50 ml of NBRIP medium with each insoluble inorganic phosphate and C-source. The flasks were incubated at 28 °C on a shaker at 130 rpm for 10 days. The samples were then centrifuged (19,187*g* for 5 min). After separation of the pellet, the pH and concentration of soluble phosphorus in the supernatant were measured using the phosphomolybdic method (Murphy and Riley 1962). The organic acids produced in the medium were also quantified and identified. The control, without inoculation, for each phosphate and C-source was also tested. The ability of each strain to solubilize phosphate was determined by the difference between the concentration of soluble phosphorus found in the culture medium and the concentration of the control treatment without inoculation.

High performance liquid chromatography (Agilent HP 1100 Series) was used to identify and quantify the organic acids. After the samples were collected, they were filtered through a cellulose membrane with a pore diameter of 0.45 µm before being injected into a Supelcogel C-610H 9 µm chromatographic column measuring 30 cm × 7.8 mm. The eleven Merck® pro-analysis organic acids reported in the literature as being involved in solubilization were used as analytical standards. The mobile phase consisted of 0.1% H_3PO_4 (pH 1.81), with a flow of 0.5 ml min^{-1} and an

injection of 100 μl per sample. The method used was according to instructions of the manufacturer (SUPELCO/SIGMA ALDRICH) of the Supelcogel column. The acquisition time of the chromatograms was set at 30 min, with a 30 min interval between runs. Components were detected by ultraviolet at 210 nm using a diode array detector. The molecules identified and the standard retention times for the acids were as follows: oxalic (10.10 min), 2-keto-gluconic (12.10 min), citric (12.40 min), gluconic (13.04 min), maleic (13.33 min), tartaric (13.45 min), malic (14.85 min), malonic (15.23 min), lactic (17.89), succinic (17.91 min), and propionic (25.08 min). The quantity of the acids was measured using calibration curves with analytical standards. Some chemical characteristics of the organic acids studied are found in Table S1 (supplementary material).

Statistical analysis

The standard errors based of the evaluated solubilization indexes in solid media were calculated. The experiments in the liquid NBRIP culture medium were designed as independent assays according to the phosphate source. Each assay was conducted in a completely randomized design 6×4 factorial scheme (six treatments involving the strains and four carbon sources in the culture medium) with two replicates for each carbon source. Data were preliminarily submitted to normality tests (Shapiro-Wilk) and homogeneity of variances (Cochran) and, afterwards, the means were clustered by the Scott-Knott algorithm (Scott and Knott 1974) at 5% probability, using the statistical program SISVAR version 4.6 (Ferreira 2011). Pearson correlation coefficients between the concentration of P-soluble, pH, and the concentration of total or individual organic acids were estimated using SigmaPlot version 12.

Results

Growth of strains in different C-sources and solubilization in solid media with $Ca_3(PO_4)_2$

All the strains grew in solid culture medium 79 with all the C-sources tested. The UFLA 03-116 (*Paenibacillus* sp.) strain grew in the solid NBRIP culture medium but it was unable to solubilize $Ca_3(PO_4)_2$ in any of the C-sources studied. The other strains also grew but were unable to solubilize $Ca_3(PO_4)_2$ when cellulose was used as the C-source. All strains that were able to solubilize $Ca_3(PO_4)_2$ had a low SI after 15 days of incubation, except for strain UFLA 03-08 (*R. tropici*), which showed a moderate SI (2.51) when glucose was used (Table 1).

Growth and solubilization in liquid media with $Ca_3(PO_4)_2$

The strain UFLA 03-116 (*Paenibacillus* sp.) did not solubilize $Ca_3(PO_4)_2$ in liquid NBRIP culture medium containing any of the C-sources. Additionally, when this bacterium was cultured, the pH of the medium did not change compared to the pH at the start of the experiment (Figure 1). In contrast, the UFLA 03-08 (*R. tropici*), UFLA 03-10, and UFLA 03-106 (*P. kribbensis*) strains were able to solubilize $Ca_3(PO_4)_2$ in the presence of all of the C-sources studied. The highest levels of soluble phosphorus were found in the presence of glucose, followed by mannitol, lactose, and finally cellulose. The UFLA 03-09 (*Acinetobacter* sp.) strain was able to solubilize $Ca_3(PO_4)_2$ in the medium with glucose and cellulose (Figure 1). Notably, the UFLA 03-106 strain provided higher levels of soluble phosphorus in glucose (70.09 mg L^{-1}) than did the UFLA 03-08 (35.58 mg L^{-1}), UFLA 03-09 (29.72 mg L^{-1}), and UFLA 03-10 (23.42 mg L^{-1}) strains. Additionally, it also provided higher levels of soluble phosphorus than did the other strains for mannitol and lactose (Figure 1).

There was no change in the pH of the medium containing cellulose for strains that were able to solubilize phosphate (Figure 1). Therefore, in this case, there was no correlation between the pH and the amount of soluble phosphorus. However, there was a reduction in the pH of the media that contained glucose, lactose, and mannitol (Figure 1), causing a significant negative correlation between the amount of soluble phosphorus and the pH in the culture media that contained glucose ($r = -0.79^*$), lactose ($r = -0.84^*$), and mannitol ($r = -0.94^*$) (Figure 2).

In relationship to identification and quantification of organic acids in the liquid media containing $Ca_3(PO_4)_2$, the types of organic acids produced varied depending on the C-source and generally varied according to the strain (Table 2). The UFLA 03-08 (*R. tropici*), UFLA 03-09

Table 1. Solubilization index (SI) of $Ca_3(PO_4)_2$ in solid NBRIP medium containing different carbon sources for strains isolated from cowpea nodules after 3 and 15 days of cultivation at 28 °C.

	Cellulose		Glucose		Lactose		Mannitol	
Strains	3 days	15 days	3 days	15 days	3 days	15 days	3 days	15 days
UFLA 03-08[a]	GNS[b]		1.41[c] (±0.10)[d]	2.51 (±0.11)	1.25 (±0.04)	1.88 (±0.07)	1.18 (±0.03)	1.05 (±0.01)
UFLA 03-09[e]	GNS		1.32 (±0.06)	1.61 (±0.05)	1.03 (±0.01)	1.11 (±0.03)	1.03 (±0.01)	1.06 (±0.02)
UFLA 03-10[f]	GNS		1.29 (±0.05)	1.02 (±0.01)	1.21 (±0.05)	1.05 (±0.02)	1.13 (±0.02)	1.04 (±0.01)
UFLA 03-106[f]	GNS		1.23 (±0.06)	1.41 (±0.07)	1.26 (±0.03)	1.86 (±0.09)	1.26 (±0.06)	1.03 (±0.01)
UFLA 03-116[g]	GNS		GNS		GNS		GNS	

[a]*Rhizobium tropici*.
[b]GNS: Grew and did not solubilize.
[c]SI: halo diameter (mm)/colony diameter (mm), evaluated after 3 (beginning the solubilization) and 15 days of incubation.
[d]±Standard error.
[e]*Acinetobacter* sp.
[f]*Paenibacillus kribbensis*.
[g]*Paenibacillus* sp.

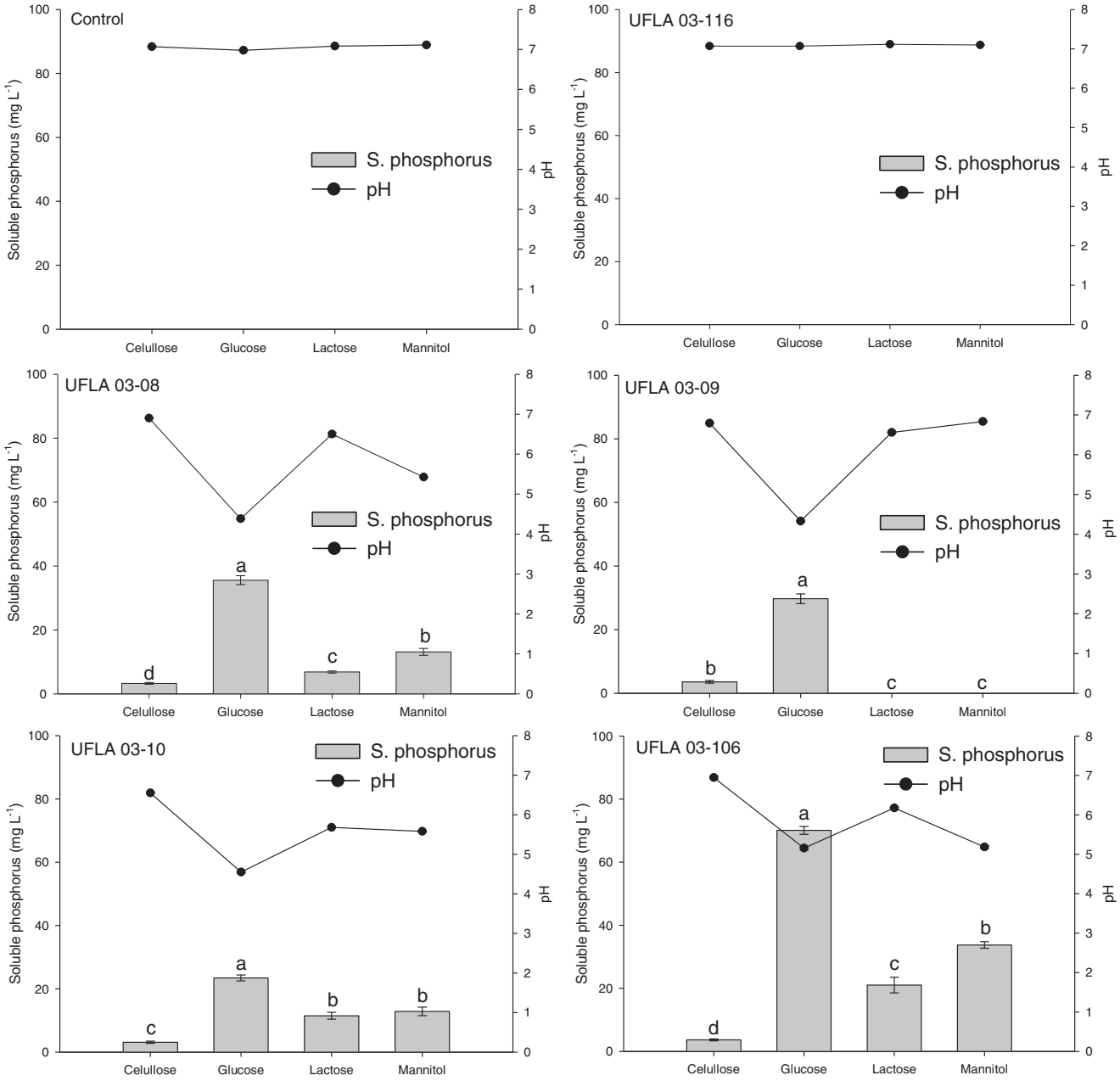

Figure 1. Soluble phosphorus (mg L^{-1}) and pH in liquid NBRIP medium containing $Ca_3(PO_4)_2$ and different C-sources after 10 days of bacterial cultivation. Error bars represent the standard error of the means, $n = 2$. Letters above the bars refer to grouping of means comparison by the Scott-Knott test at 5% probability.

(*Acinetobacter* sp.), UFLA 03-10, and UFLA 03-106 (*P. kribbensis*) strains produced organic acids when the C-source was glucose or mannitol (Figure 3).

In the medium containing glucose, gluconic, and succinic/lactic acids were observed for the UFLA 03-08 strain, and tartaric acid was observed for the UFLA 03-09 strain (Table 2). Furthermore, 2-ketogluconic, propionic, succinic/lactic, and tartaric acids were observed for the UFLA 03-106 strain. Maleic acid was observed in the medium containing mannitol after incubation with the UFLA 03-08, UFLA 03-09, and UFLA 03-106 strains. Citric and tartaric acids were found in the media after incubation with the UFLA 03-10 strain in the medium containing glucose and in the medium containing mannitol, but different concentrations of citric and tartaric acids were found (Table 2).

UFLA 03-10 was the only strain to produce 2-ketogluconic (0.28 mmol L^{-1}) and tartaric (0.32 mmol L^{-1}) organic acids in the medium containing lactose. None of the strains produced organic acids in the medium containing cellulose. UFLA 03-116 (*Paenibacillus* sp.) was the only strain that did not produce acid in media containing $Ca_3(PO_4)_2$, and the strain did not solubilize this inorganic compound in any C-source. The highest concentration of acids was observed for the UFLA 03-106 strain (47.76 mmol L^{-1}), followed by the strains UFLA 03-09 (32.95 mmol L^{-1}), UFLA 03-10 (25.90 mmol L^{-1}), and UFLA 03-08 (23.46 mmol L^{-1}). More than 94% of the acids were detected in the medium containing glucose for all strains (Figure 3; Table 2).

A significant positive correlation between the amount of soluble phosphorus in the liquid NBRIP medium and the

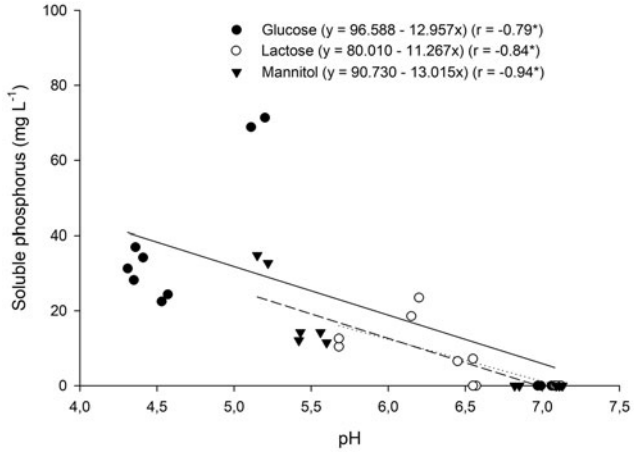

Figure 2. Pearson's correlation between the pH and the concentration of soluble phosphorus in liquid NBRIP medium containing $Ca_3(PO_4)_2$ and glucose, lactose, and mannitol after 10 days of bacterial cultivation ($n = 12$).

concentration of total organic acids produced in the presence of $Ca_3(PO_4)_2$ was observed only for the media containing glucose ($r = 0.96^*$) and mannitol ($r = 0.96^*$) (Figure 4(A)). The highest solubilization rates of $Ca_3(PO_4)_2$ were found at high concentrations of gluconic or tartaric acids when the carbon source was glucose. However, for this carbon source, there was a positive correlation ($r = 0.83^*$) between the soluble phosphorus and the tartaric acid concentrations found, which did not occur for gluconic acid ($r = 0.4^{NS}$) (Figure 4(B)). There was also a positive correlation between solubilization and maleic acid concentration when the carbon source was mannitol ($r = 0.91^*$) (Figure 4(C)).

Growth and solubilization in solid and liquid media with $FePO_4{\cdot}2H_2O$

None of the strains were able to solubilize $FePO_4{\cdot}2H_2O$ in the presence of any of the C-sources tested in either solid or liquid NBRIP culture medium. However, there was a greater reduction in pH in the media in which the UFLA 03-10 and UFLA 03-106 strains were cultured, regardless of the C-source. The same results were found for the UFLA 03-08 strain, except for when it was grown in the medium containing lactose which presented a low pH reduction, similar to strain UFLA 03-09 in all carbon sources. UFLA 03-116 also had a low pH reduction in lactose and mannitol media but did not change pH in media with cellulose and glucose (Figure 5).

All the strains produced organic acids in the medium containing $FePO_4{\cdot}2H_2O$, regardless of the C-source (Table 3). The types of organic acids produced varied depending on the C-source, and the same acid was not produced in different C-sources. In addition, all strains produced the same acids in the same C-source. The acid concentration was higher in the medium containing glucose than in the media containing the other C-sources (Figure 6). Gluconic and 2-ketogluconic acids were detected for all strains in the medium containing glucose (Table 3). Maleic and malonic acids were also observed in the medium containing mannitol for all the strains. All the strains produced succinic/lactic, citric, and tartaric acids in the medium containing lactose. All the strains produced propionic acid in the medium containing cellulose (Table 3), especially UFLA 03-116, which produced the highest concentration of total acids when cultured in the medium containing cellulose (0.27 mmol L^{-1}) (Figure 6).

The highest acid concentration was detected for the UFLA 03-106 strain (7.29 mmol L^{-1}), followed by UFLA 03-116 (5.55 mmol L^{-1}), UFLA 03-08 (2.38 mmol L^{-1}), UFLA 03-10 (1.99 mmol L^{-1}), and UFLA 03-09 (1.34 mmol L^{-1}). More than 54% of organic acids were detected for all the strains when they were grown in the medium containing glucose (Figure 6).

Notably, the total concentration of organic acids was 6 to 26 times higher in the medium containing $Ca_3(PO_4)_2$ than in the medium containing $FePO_4{\cdot}2H_2O$ for the different strains; this phenomenon was found for all the C-sources. In addition, the type of organic acids varied depending on the type of phosphate in the medium.

Discussion

The C-source directly influences the growth of heterotrophic microorganisms and affects some biochemical processes in both positive and negative ways. The use of more readily available sources favors the solubilization of insoluble inorganic phosphates in some groups of microorganisms (Halder et al. 1990; Silva-Filho and Vidor 2000; Son et al. 2006; Sridevi et al. 2007).

The results demonstrate that the C-source does not influence the ability of the strains to solubilize $FePO_4{\cdot}2H_2O$ in either solid or liquid NBRIP medium because none of the strains was able to solubilize phosphates under these conditions, despite the fact that they produced organic acids, though at much lower concentrations than with $Ca_3(PO_4)_2$. Studies performed with bacterial and fungal isolates in solid GEL (glucose and yeast extract) medium (Sylvester-Bradley et al. 1982) also demonstrated the inability of the microorganisms to solubilize $FePO_4{\cdot}2H_2O$ (Silva-Filho and Vidor 2000). In a previous study, the same strains evaluated here were inoculated on solid GELP medium (Sylvester-Bradley et al. 1982) with a single C-source (glucose), and $FePO_4.2H_2O$ was not solubilized (Marra et al. 2012). However, the UFLA 03-116 (*Paenibacillus* sp.), UFLA 03-10, and UFLA 03-106 (*P. kribbensis*) strains solubilized 10–20 mg of $FePO_4{\cdot}2H_2O$ per liter of solution (Marra et al. 2012) in liquid GELP medium. Therefore, it is noteworthy that use of glucose as the C-source in the GELP medium can promote solubilization of $FePO_4{\cdot}2H_2O$, depending on the strain. The composition of the media regarding other components, such as yeast extract of the GELP medium, probably also influences solubilization.

Reduction in pH was observed in the liquid medium containing $FePO_4{\cdot}2H_2O$ for the UFLA 03-10 and UFLA 03-106 strains compared to the control treatment for all the C-sources tested, and for the UFLA 03-08 strain in the media containing cellulose, glucose, and mannitol. In addition, organic

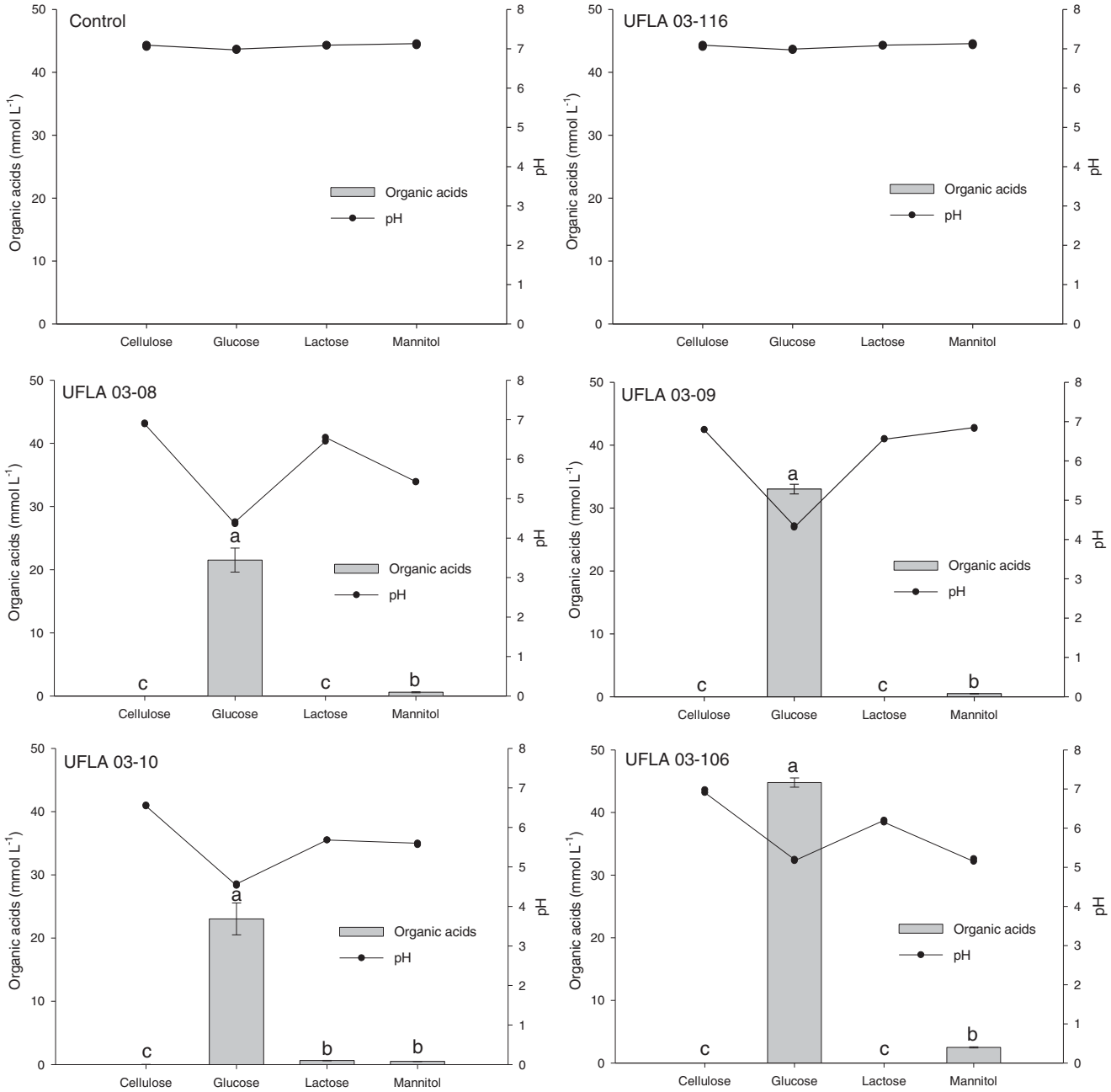

Figure 3. Total organic acids (mmol L^{-1}) and pH in liquid NBRIP medium containing $Ca_3(PO_4)_2$ and different C-sources after 10 days of bacterial cultivation. Error bars represent the standard error of the means, n = 2. Letters above the bars refer to means grouping by the Scott-Knott test at 5% probability.

acids were detected for all the sources of carbon for all strains. However, there was no solubilization of that inorganic phosphate source. These results indicate that the type of organic acid depends on the C-source, and their production does not result in $FePO_4{\cdot}2H_2O$ solubilization. The possible influence of the strong energy bond between iron and phosphorus should be highlighted, which may require higher organic acid concentrations for solubilization than the concentrations observed in our study. Thus, the point of solubilization of iron phosphate is higher than calcium phosphate, that means that the pH necessary to reach the solubilization is lower than reached in this experimentation (Marra et al. 2012; Massenssini et al. 2015).

UFLA 03-116 did not solubilize $Ca_3(PO_4)_2$ or produce organic acids, regardless of the C-source. No strain produced organic acids with cellulose. In contrast, the highest $Ca_3(PO_4)_2$ solubilization rates were associated with high concentrations of tartaric and/or gluconic acids. In addition, tartaric acid was the only acid identified in three carbon sources for the same strain (UFLA 03-10). Chen et al. (2016) observed that phosphate solubilizing activity was associated with the release of organic acids produced from glucose, while the composition of the organic acids produced was dependent on the phosphorus forms. However, no relationship with the type of organic acid was reported.

Cellulose was the only C-source studied that did not promote solubilization of $Ca_3(PO_4)_2$ in the solid medium. The same result was observed in GES (glucose and soil extract) medium inoculated with bacterial and fungal isolates using

Table 2. Identification and quantification of organic acids produced in liquid NBRIP medium containing $Ca_3(PO_4)_2$ and different C-sources after 10 days incubation inoculated with different bacterial strains.

			(mmol L^{-1})		
Strains	Cellulose	Glucose	Lactose	Mannitol	Total
UFLA 03-08	nd	Gluconic (21.87) Succinic/lactic (0.99)	nd	Maleic (0.60)	23,46
UFLA 03-09	nd	Tartaric (32.47)	nd	Maleic (0.48)	32.95
UFLA 03-10	nd	Citric (2.72) Tartaric (10.22)	2-ketogluconic (0.28) Tartaric (0.32)	Citric (0.20) Tartaric (0.28)	25.90
UFLA 03-106	nd	2-ketogluconic (1.2) Propionic (8.80) Succinic/lactic (1.98) Tartaric (33.47)	nd	Maleic (2.49)	47.76
UFLA 03-116	nd	nd	nd	nd	nd
Total	nd	Citric (2.72) Gluconic (21.87) 2-ketogluconic (1) Propionic (8.80) Succinic/lactic (2.97) Tartaric (88.04)	2-ketogluconic (0.28) Tartaric (0.32)	Citric (0.20) Maleic (3.57) Tartaric (0.28)	130.07

nd: not detected. The value within parenthesis indicates the concentration of the organic acid produced by the respective bacterial strain. The column 'Total' presents the sum of the concentration of all organic acids, irrespective of the C source, for each bacterial strain.

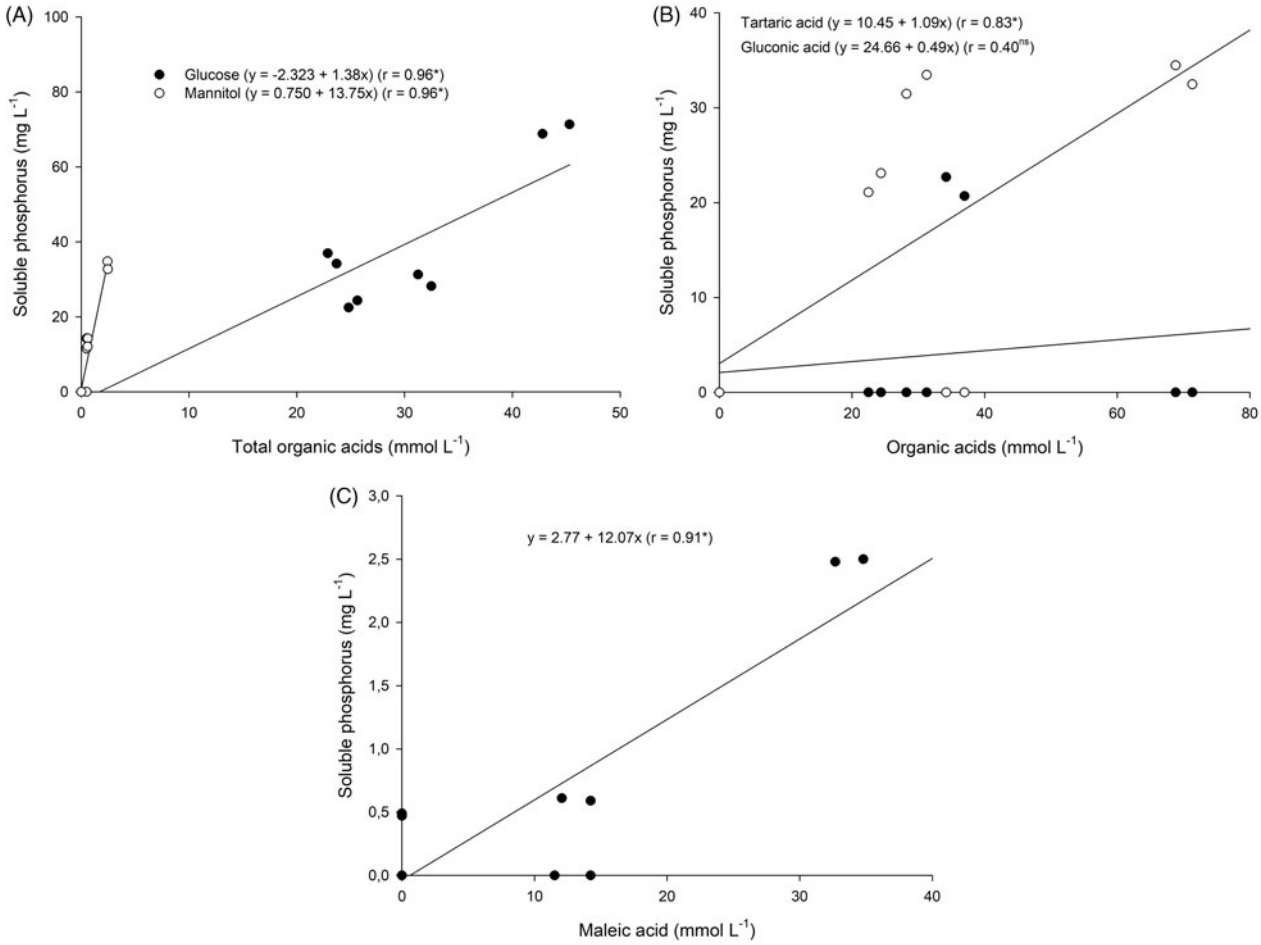

Figure 4. Pearson's correlation after 10 days of bacterial cultivation ($n = 12$) between (A) the concentration of soluble phosphorus and the concentration of total organic acids in liquid NBRIP medium containing $Ca_3(PO_4)_2$ with glucose or mannitol, (B) the concentration of organic acids (gluconic and tartaric) and the concentration of soluble phosphorus in liquid NBRIP medium containing $Ca_3(PO_4)_2$ and glucose, and (C) the concentration of maleic acid and the concentration of soluble phosphorus in liquid NBRIP medium containing $Ca_3(PO_4)_2$ and mannitol.

cellulose, starch, glucose, fructose, sucrose, and xylose as the C-source (Silva-Filho and Vidor 2000). Moreover, varying the C-source did not influence the solubilization capacity of the UFLA 03-116 strain, even in liquid NBRIP medium supplemented with $Ca_3(PO_4)_2$.

The use of a C-source depends on the presence of transport mechanisms, metabolic pathways, and specific enzymes that are capable of breaking down the substance. The oxidation of glucose to organic acids by the glucose dehydrogenase and gluconic acid dehydrogenase enzymes results in

acidification of the region around the cell, which provides an efficient environment for phosphate solubilization by some Gram-negative bacteria (Kpomblekou and Tabatabai 1994; Rodríguez and Fraga 1999). This explains the high solubilization rates with glucose by all strains except UFLA 03-116.

UFLA 03-08 (*R. tropici*) was the only strain that was able to solubilize $Ca_3(PO_4)_2$ in solid medium with a moderate SI, which occurred in the medium containing glucose, indicating that glucose promotes greater solubilization. This SI was higher than the values found for the species of *Rhizobium* derived from *Crotalaria retusa* and *C. verrucosa* that were inoculated in solid Pikovskaya culture medium (Pikovskaya 1948), containing glucose as a C-source (Sridevi et al. 2007).

It is well known in the literature that in liquid media generally the strains have the capacity to present greater potential of solubilization, since they are in contact with greater amount of insoluble phosphate, which does not happen in the solid medium. In other words, liquid medium favors greater diffusion of organic acids, which results in higher solubilization. In the solid medium, the phosphate precipitates and most of it become distant from the area of action of the strains, since the colonies stay on the surface of the medium. Hence the importance of also including liquid medium in the process of selection of solubilizing strains. Indeed, in the liquid medium containing glucose, a greater amount of organic acids and, consequently, acidification of the medium, was observed, which contributed to the increased solubilization. Son et al. (2006) also observed that the bacterial species *Pantoea agglomerans* reduced the pH of the medium when glucose was used as the C-source, which resulted in greater solubilization of $Ca_3(PO_4)_2$. However, these authors did not evaluate the production of organic acids. Studies with *Pseudomonas* sp., *Xanthomonas campestris*, and *Rhizobium* sp. also demonstrated that growing bacteria in the presence of glucose results in greater solubilization of insoluble phosphates than when they are grown in the presence of arabinose, fructose, galactose, mannitol, maltose, sucrose, or xylose (Nautiyal 1999; Sharan et al. 2008; Sridevi and Mallaiah 2009). When glucose or sucrose was used as the C-source, fungi caused reduction in the pH of the media, which resulted in greater solubilization of phosphate (Relwani et al. 2008) than when fructose, galactose, lactose, or xylose was used. In contrast, Ahuja et al. (2007), who were also working with fungi, demonstrated greater solubilization when fructose was used as the C-source rather than glucose, galactose, lactose, sucrose, glycerol, maltose, or mannose.

After glucose, mannitol was the C-source that resulted in the greatest solubilization of $Ca_3(PO_4)_2$; this was also related to a reduction in pH (Figure 2) and production of organic acids (Figure 4A). Sridevi et al. (2007) and Son et al. (2006) also found that the greatest solubilization of phosphate occurred in a medium containing mannitol, followed by a medium containing glucose.

It has been suggested in the literature that the reduction in pH, caused by the production of organic acids and the release of protons, is a basic factor in phosphate

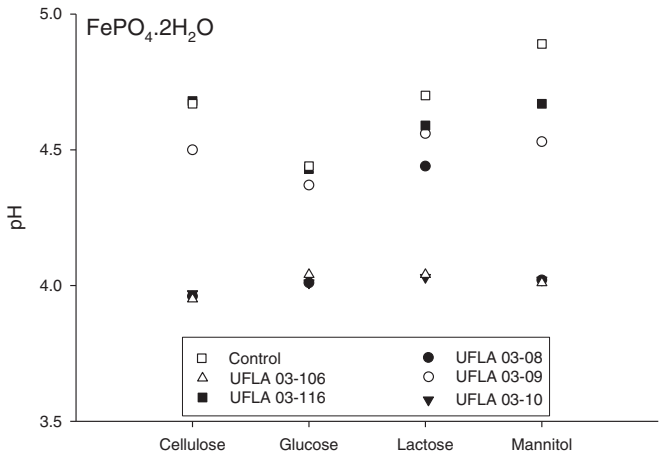

Figure 5. pH of the liquid NBRIP medium containing $FePO_4{\cdot}2H_2O$ and the different C-sources after 10 days of bacterial cultivation.

Table 3. Identification and quantification of organic acids produced in liquid NBRIP medium containing $FePO_4{\cdot}2H_2O$ and different C-sources after 10 days incubation inoculated with different bacterial strains.

	(mmol L^{-1})				
Strains	Cellulose	Glucose	Lactose	Mannitol	Total
UFLA 03-08	Propionic (0.03)	Gluconic (1.8) 2-ketogluconic (0.20)	Maleic (0.40) Malonic (0.23)	Succinic/lactic (0.19) Citric (0.11) Tartaric (0.14)	2.38
UFLA 03-09	Propionic (0,02)	Gluconic (0.35) 2-ketogluconic (0.14)	Maleic (0.29) Malonic (0.20)	Succinic/lactic (0.14) Citric (0.10) Tartaric (0.10)	1.34
UFLA 03-10	Propionic (0.01)	Gluconic (0.71) 2-ketogluconic (0.17)	Maleic (0.27) Malonic (0.19)	Succinic/lactic (0.24) Citric (0.15) Tartaric (0.25)	1.99
UFLA 03-106	Propionic (0.06)	Gluconic (3.25) 2-ketogluconic (1.24)	Maleic (1.51) Malonic (0.94)	Succinic/lactic (0.11) Citric (0.07) Tartaric (0.11)	7.29
UFLA 03-116	Propionic (0.27)	Gluconic (2.13) 2-ketogluconic (0.80)	Maleic (1.2) Malonic (0.80)	Succinic/lactic (0.24) Citric (0.10) Tartaric (0.19)	5.55
Total	Propionic (0.39)	Gluconic (7.52) 2-ketogluconic (2.55)	Maleic (3.49) Malonic (2.36)	Succinic/lactic (0.92) Citric (0.53) Tartaric (0.79)	18.55

The value within parenthesis indicates the concentration of the organic acid produced by the respective bacterial strain. The column 'Total' presents the sum of the concentration of all organic acids, irrespective of the C source, for each bacterial strain.

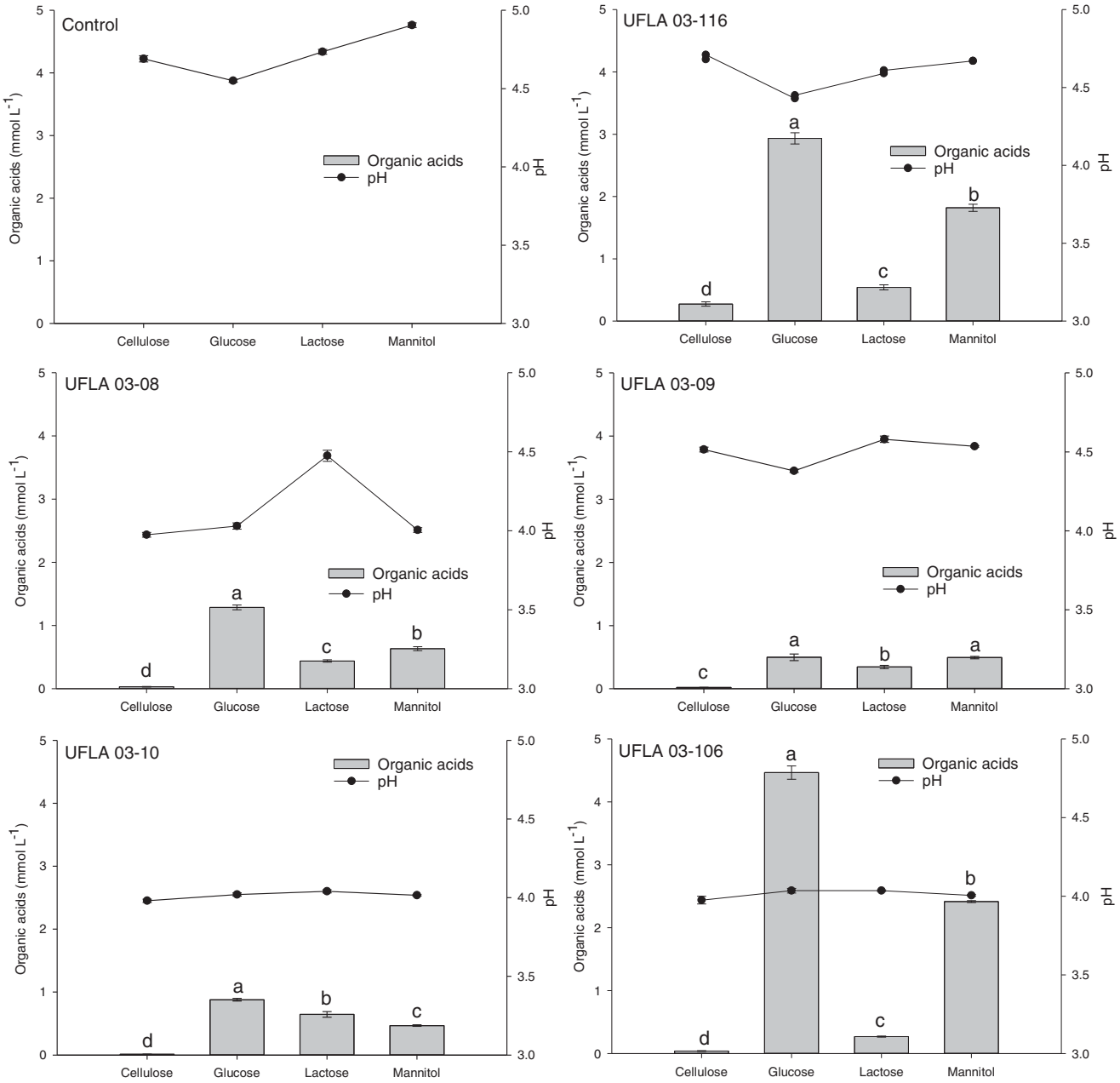

Figure 6. Total organic acids (mmol L^{-1}) and pH in liquid NBRIP medium containing $FePO_4{\cdot}2H_2O$ and different C-sources after 10 days of bacterial cultivation. Error bars represent the standard error of the means, n = 2. Letters above the bars refer to means grouping by the Scott-Knott test at 5% probability.

solubilization (Chen et al. 2006, 2016; Viruel et al. 2011; Whitelaw 2000). Indeed, Marra et al. (2012; 2015) and Nautiyal et al. (2000) observed that acid production may have contributed to phosphate solubilization; however, it was not the only reason for phosphate release into the medium. Although a negative correlation between pH and phosphate solubilization was observed for lactose, it was not correlated with the production of organic acids. Only the UFLA 03-10 strain (*P. kribbensis*) produced 2-ketogluconic (0.28 mmol L^{-1}) and tartaric (0.32 mmol L^{-1}) acids when grown in the presence of this C-source. In another study, it was observed that acidification is not the mechanism of $Ca_3(PO_4)_2$ solubilization at an initial pH of 5.0 (Marra et al. 2015). Interestingly, the presence of soluble phosphorus, even in small quantities, did not result from reduction in pH or from the presence of organic acids. This finding shows that another mechanism of solubilization is involved (Hamdali et al. 2008; Illmer and Schinner 1992), and that these strains have different solubilization mechanisms that act depending on the C-source.

Notably, the UFLA 03-08 (*R. tropici*), UFLA 03-10, and UFLA 03-106 (*P. kribbensis*) strains were able to solubilize $Ca_3(PO_4)_2$ in the presence of all the four C-sources studied. In another study, the UFLA 03-10 strain (*P. kribbensis*), combined with rock phosphate, was indicated as an economical and sustainable strategy for improving the growth and nutrient accumulation of rice plants (da Costa et al. 2015). Plants secrete a variety of carbohydrates, and glucose is the most highly secreted (Kraffczyk et al. 1984). Furthermore, cellulose is the most common natural polysaccharide, and it contains most of the CO_2 that is fixed by plants. Therefore, these strains can increase and maintain

the availability of phosphorus for plants in soils with diverse C-sources as a result of their ability to promote phosphorus solubilization under variable conditions.

Conclusions

The amount of phosphate solubilization depends on the strain, C-source, organic acids, and type of phosphate. The strains varied in their ability to solubilize phosphate and both qualitative on and quantitative organic acid production.

The use of glucose as a C-source favors the production of organic acids and contributes to an increase in solubilization of calcium phosphate.

Acidification of the medium is not the mechanism of calcium phosphate solubilization when cellulose is the only C-source present.

The presence of organic acids in a medium containing iron phosphate in the pH range of 4–5 does not favor phosphate solubilization, regardless of the C-source.

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Disclosure statement

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