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ORIGINAL ARTICLE

Mechanisms of phosphate solubilization by fungal isolates when exposed to different P sources

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Abstract The use of phosphate-solubilizing fungi is a promising biotechnological strategy in the management of phosphorus (P) fertilization, as it enables the utilization of rock phosphates (RP) or the recovery of P fixed in soil particles. The objective of our study was to evaluate fungal isolates for mechanisms of solubilization of P-bearing compounds, such as $AlPO_4$, $FePO_4$, $Ca_3(PO_4)_2$, Araxá RP, and Catalão RP. Four fungal isolates obtained from Brazilian soils were characterized in liquid media: *Aspergillus niger* FS1, *Penicillium canescens* FS23, *Eupenicillium ludwigii* FS27, and *Penicillium islandicum* FS30. *A. niger* FS1 was the only isolate able to solubilize all of the P sources, solubilizing 71, 36, 100, and 14 % of the P from $AlPO_4$, $FePO_4$, $Ca_3(PO_4)_2$, and RPs, respectively. Medium acidification was an effective solubilization mechanism, particularly for $Ca_3(PO_4)_2$. The other P sources were mainly solubilized through organic acids produced by the fungi. Oxalic acid, produced exclusively by *A. niger* FS1, and citric acid were decisive factors in the solubilization of $AlPO_4$ and $FePO_4$. *Penicillium* isolates produced more gluconic acid than *A. niger* FS1 in all treatments. However, this higher production did not result in higher solubilization for any of the P sources, showing that gluconic acid contributes little to the solubilization of the P sources evaluated. The higher capacity of medium acidification and the production of organic acids with stronger metal-complexation activity are characteristics that confer to *A. niger* FS1 a wider action on insoluble P sources. Consequently, this isolate qualifies as a promising candidate for application in the management of P fertilization.

Keywords *Aspergillus niger* · Filamentous fungi · Insoluble phosphates · Solubilization mechanisms · Phosphorus

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Introduction

Phosphorus (P) is an essential element for plant growth and development, playing an important role in energy transfer reactions, respiration, and photosynthesis. It is also an important constituent of biomolecules such as nucleic acids, coenzymes, phosphoproteins, and phospholipids. Despite its wide distribution in nature, most soils are deficient in P forms readily absorbable by plants due to its retention in soil particles. In tropical soils, phosphate anions can be adsorbed onto the surface of Al and Fe oxides and kaolinite (Fontes and Weed 1996). Furthermore, phosphate in soil solution can precipitate with Al^{3+} and Fe^{3+} in acidic soils or with Ca^{2+} in neutral or calcareous soils. For this reason,

frequent fertilization with P is mandatory to obtain good productivity in most soils (Novais and Smith 1999). The most widely used fertilizers at the present time are mostly obtained from the acidification of rock phosphates (RPs) with strong acids, a process that involves high costs and environmental damage (Vassilev et al. 2006b).

The rational management of P fertilization is increasingly important because P is a non-renewable resource, and according to the latest estimates, the global reserves of P can become depleted within 50–100 years. Moreover, the production costs of P fertilizers are increasing because of the lower availability of high-grade RP deposits (Cordell et al. 2009; Vaccari and Strigul 2011). In addition to the efficient use of the P reserves, it is also important to recover the P applied. However, the recovery of P from soils is not a simple task because of its irreversible fixation onto soil particles over time (Novais and Smith 1999).

P-solubilizing microorganisms are now recognized as a possible means to overcome some of the challenges in P fertilization management. Some microorganisms, especially fungal species belonging to *Aspergillus* and *Penicillium* genera, are able to release P from insoluble inorganic compounds through acidification of the medium and the production of organic acids (Banik and Dey 1982; Illmer et al. 1995). These microorganisms have been used in processes that aim to solubilize RP before the fertilizer is applied to the soil (Vassilev et al. 1996, 1998, 2006a) and represents a low-cost alternative to the industrial process. Ideally, for these purposes, P-solubilizing microorganisms should be able to grow vigorously using cheap agro-industrial wastes, such as sugarcane bagasse, as a carbon source. P-solubilizing microorganisms can be also applied directly to soils to improve the efficiency of P fertilization. The inoculation of fungi into soil in combination with high- or low-solubility P fertilizers improves plant growth and can increase the level of available P in soil by mobilizing P fixed in soil particles (Asea et al. 1988; Mittal et al. 2008; Jain et al. 2010).

The production of organic acids is the primary way by which fungi release P from insoluble inorganic compounds and soil. These acids can release P into the soil by replacing the P on the surface of metallic (hydro-) oxides through ligand-exchange reactions, by dissolving (hydro-) oxides that adsorb P, and by complexing with metals in solution, thereby preventing the precipitation of P with these metals (Nagarajah et al. 1970; Stumm 1986; Fox et al. 1990a, b; Bolan et al. 1994; Jones 1998). The effectiveness of a given organic acid is dependent on the chemical characteristics of the acid produced, such as the quantity and position of the carboxyl and hydroxyl groups, the stability constant of the metal–organic acid complex, the acid concentration, the concentration and type of metals in solution, and the pH of the solution (Bolan et al. 1994; Kpomblekou-A and Tabatabai 1994; Jones 1998; Whitelaw 1999). The production pattern of organic acids is a specific characteristic of a fungal isolate and is also dependent on the exposure to different cultivation conditions (Reyes et al. 1999; Reyes et al. 2001; Chuang et al. 2007). It is therefore reasonable to assume that studies on P solubilization mechanisms of distinct P compounds are necessary in the context of establishing new possibilities for the application of these microorganisms in processes aimed at improving P fertilization. Thus, the objective of our study was to isolate P-solubilizing fungi from soils and study their solubilization mechanisms when exposed to poorly soluble P compounds commonly found in soils or used in P fertilization.

Materials and methods

Isolation and selection of P-solubilizing fungi

Soil samples were collected under eucalyptus plantation (20° 46′ 14.2″ S, 42° 52′ 36.4″ W) and native forest (20° 46′ 4.2″ S, 42° 52′ 40.9″ W) in Viçosa, Minas Gerais, Brazil and used to obtain fungal isolates. The soil samples were mixed with non-sterile 2-cm sugarcane bagasse fragments in two ways: imbibition with the soil solution and burying in a tray. The soil solution was prepared by mixing 10 g of soil with 90 mL of saline solution (NaCl 0.85 %). The fragments of sugarcane bagasse were imbibed with 0.5 mL of this solution and placed into petri dishes containing moistened filter paper. The trays were set up to contain alternating layers of soil and sugarcane bagasse and were maintained at room temperature. The moisture was kept at about 60 % of soil water holding capacity. Fragments of sugarcane bagasse were collected after 7, 14, and 21 days and stored in petri dishes containing moistened filter paper. The petri dishes were incubated at 28 °C and checked daily for fungal growth.

The fungi observed on the bagasse were collected using a direct isolation method (Alfenas et al. 2007). The fungal structures formed on the sugarcane bagasse were observed through a stereomicroscope and the fungal structures were aseptically collected with a needle placed in a petri dish containing solid NBRIP (National Botanical Research Institute's phosphate growth medium) (Nautiyal 1999). The fungal isolates that produced a clear halo around the colony were transferred to petri dishes containing potato dextrose agar (PDA) and maintained at 28 °C.

A third methodology was used to isolate the fungi that showed no distinguishable structures on the fragments of sugarcane bagasse. Fragments of bagasse in the trays mentioned above were collected at 21 days and mixed with a 5 % (w/v) saline solution (NaCl 0.85 %). The mixture was shaken for 1 h at 200 rpm and serially diluted. The 10^{-3} and 10^{-4} dilutions were placed in petri dishes with solid NBRIP medium supplemented with chloramphenicol (30 μg mL^{-1}) and streptomycin (100 μg mL^{-1}) to avoid bacterial growth.

Fungi were chosen because they generally show a higher P solubilization ability than other microbial groups (Banik and Dey 1982) and are more tolerant to acidic conditions favorable to RP solubilization. The fungal colonies showing a clear halo were then transferred to petri dishes containing PDA.

The fungal isolates were identified based on morphologic and physiologic characteristics (Pitt 1979; Watanabe 2002). The isolates were submitted to a second step of selection based on the values of P solubilized in the liquid medium. For this, two disks (diameter 7 mm) containing 5-day-old mycelia cultivated on PDA were inoculated into 125-mL flasks with 50 mL of NBRIP medium supplemented with 5 g L^{-1} of hydroxyapatite [$Ca_5(PO_4)_3(OH)$] as the only P source. Non-inoculated flasks were used as a control. The flasks were incubated for 7 days at 28 °C and 150 rpm. The experiment was conducted in a completely randomized design with three replications. Based on the data on solubilized P and on the P/biomass yield ($Y_{P/X}$), calculated by the ratio between solubilized P (P, expressed in mg) and dry biomass (X, expressed in g), the following criteria were established to select four fungal isolates: the isolates should (1) belong to different species; (2) be in the two best groups of P solubilization according to the Scott–Knott cluster analysis (Scott and Knott 1974); (3) be in the two best groups of $Y_{P/X}$ according to the Scott–Knott cluster analysis.

Study of solubilization mechanisms

The selected isolates were inoculated in 250-mL flasks containing 100 mL of NBRIP medium modified by substitution of the P source. Five insoluble P sources were studied: $AlPO_4$, $FePO_4{\cdot}2H_2O$, $Ca_3(PO_4)_2$, and the RPs from Araxá and Catalão (Brazil); the composition of the RPs are shown in Table 1. Each P source was added at a concentration equivalent to 1 g of P L^{-1}. The pH of the medium was adjusted to 7 before the P sources were added. The addition of $AlPO_4$, $FePO_4{\cdot}2H_2O$, $Ca_3(PO_4)_2$, Araxá RP, and Catalão RP changed the pH to 5.0, 3.8, 6.8, 6.5, and 6.7, respectively. The flasks were inoculated with three mycelial disks, as described above, and incubated on an orbital shaker for 7 days at 28 °C and 150 rpm. Non-inoculated controls were also incubated. The treatments were arranged in a 4×5 factorial design, corresponding to four fungal isolates and five P sources. The experiment was conducted in triplicate according a completely randomized design. The data were submitted to analysis of variance and treatments were compared using the Tukey test ($p<0.05$). The Pearson correlations among the variables studied were also analyzed.

Simulation of the effect of pH on the solubilization process

The effect of pH on the solubility of $AlPO_4$, $FePO_4{\cdot}2H_2O$, $Ca_3(PO_4)_2$, and calcium oxalate was simulated using the software GEOCHEM-EZ (Shaff et al. 2010). Solubility data were obtained in pH intervals of 0.5 using NBRIP medium as the basal salts solution. The following configurations were set up: "solids can precipitate" and "calculate ionic strength" using 0.001 M L^{-1} as a calculated guess. The other options were maintained as program standards. The concentration of the P sources simulated was the same as that used in experiments, and calcium oxalate solubility was simulated for a concentration of 500 mg L^{-1}.

Analytical methods

The fungal biomass was collected and determined by weight loss after incineration at 500 °C for 6 h. This method avoids overestimation due to the adherence of phosphate particles on the mycelium (Reyes et al. 1999). Samples of the cultivation media were centrifuged at 10,000 *g* for 20 min, and the supernatant was used for the measurements of soluble P, pH, titratable acidity, and organic acids. Soluble P was quantified by the ascorbic acid method (Braga and Defelipo 1974). Titratable acidity was determined by titrating 1.5 mL of supernatant to pH 7 with 0.1 M NaOH using bromothymol blue as the indicator. Organic acids were determined by ionic chromatography as described by Silva et al. (2001). Before the chromatography, the samples were passed through OnGuard II Ag cartridges (Dionex, Sunnyvale, CA) to reduce chloride content.

Results

Isolation and selection of P-solubilizing fungi

In the first screening, we obtained 57 fungal isolates (with a clear predominance of *Penicillium* and *Aspergillus* species) able to form a clear halo in solid NBRIP medium containing hydroxyapatite as the only P source (Table 2). Due to the high number of isolates, we performed a second selection step based on quantitative variables. The data showed that P solubilization varied from 6 to 100 % among the isolates. *Aspergillus niger* was the species with the highest levels of solubilization. $Y_{P/X}$ was also used as selection criterion, varying from 84 to 628 mg P g^{-1} of dry biomass.

Based on the criteria established in the Materials and Methods, we selected four isolates for further study. *Aspergillus niger* FS1 was selected for achieving the highest value of solubilized P (Table 2). As the two best groups based on solubilized P consisted only of *A. niger* isolates, the third group was used to select the other isolates based on the values of $Y_{P/X}$ (criterion 3). Thus, *Penicillium canescens* FS23 and *P. islandicum* FS30 were randomly chosen within the group with the highest values of $Y_{P/X}$ (labeled with the letter "a" in the right-hand column of Table 2) and *Eupenicillium ludwigii*

Table 1 Chemical characterization of rock phosphates from Araxá and Catalão (Brazil)

RP	P_2O_5 (%)			Elemental composition (mg kg^{-1})										
	Total	NAC[a]	CA[b]	Al	Ca	Cd	Cr	Cu	Fe	Mg	Mn	Ni	Pb	Zn
Araxá	32	1	4	3,032	302,450	0.8	100.4	38.7	44,010	2,700	882	45.3	nd	171.5
Catalão	34	1	5	1,392[c]	339,900[c]	1	64.7	90.6	21,100	1,438[c]	708	44.1	nd	126.5

RP Rock phosphates; *nd* not detected

[a] Soluble in neutral ammonium citrate (NAC)

[b] Soluble in 2 % citric acid (CA)

[c] Schneider et al. (2010)

FS2, from the second-best group (labeled with the letter "b"). For this latter group, it should be noted that *E. ludwigii* is a teleomorph state of the genera *Penicillium*, possibly contributing to a wider physiological diversity in the whole experiment.

Study of solubilization mechanisms

Aspergillus niger FS1 demonstrated the highest rate of solubilization for all types of insoluble phosphates among the tested isolates (Table 3). The solubilization rate differed depending on the P source, with $Ca_3(PO_4)_2$ being completely solubilized, followed by $AlPO_4$ (71 %), $FePO_4$ (36 %), and the RPs (14 %). The other isolates showed low P solubilization capacity, attaining <10 % P solubilization, except in the treatment with $Ca_3(PO_4)_2$ when the soluble P concentration was 30 % lower than that obtained by the most active solubilizer, *A. niger* FS1. A negative value of solubilized P was observed in the medium with $FePO_4$ inoculated with *Eupenicillium ludwigii* FS27, indicating that the fungus consumed the P released by the abiotic processes. The P sources strongly influenced fungal metabolism and growth, as revealed by the analyses of pH, titratable acidity, and fungal biomass measured at the end of the fermentation process. In general, *A. niger* FS1 metabolism resulted in a higher titratable acidity and lower pH in media with $AlPO_4$, $FePO_4$, and $Ca_3(PO_4)_2$ compared to treatments with other P solubilizers.

The production of organic acids not only varied—as expected— among fungal isolates but also showed a strong dependency on the P source (Fig. 1). The method used to quantify organic acids is able to identify acetic, butyric, tartaric, isocitric, malic, citric, oxalic, and gluconic acids, but only the last three were produced by the filamentous fungi tested in this study. *Aspergillus niger* FS1 produced oxalic, citric, and gluconic acids. Oxalic acid was detected only in treatments with $AlPO_4$ (Fig. 1a) and $FePO_4$ (Fig. 1b), with the concentration being threefold higher in the latter case. Citric acid was produced in treatments with pure P sources [$AlPO_4$, $FePO_4$, and $Ca_3(PO_4)_2$; Fig. 1a–c] but was practically absent in treatments with RPs (Fig. 1d, e). Gluconic acid was produced in all treatments except that with $AlPO_4$ (Fig. 1a–e); however, the levels obtained were lower than those produced by the other fungal isolates. *Penicillium canescens* FS23 produced gluconic acid in all treatments (Fig. 1a–e) and citric acid in treatments with $AlPO_4$, $Ca_3(PO_4)_2$, and Araxá RP (Fig. 1a, c, d). Concentrations among treatments varied from 25 to 1,242 mg L^{-1} of gluconic acid, and from 25 to 813 mg L^{-1} of citric acid. *Eupenicillium ludwigii* FS27 and *Penicillium islandicum* FS30 produced only gluconic acid. The production of gluconic acid was almost inhibited in the treatment with $FePO_4$ (Fig. 1b) for both isolates. In the presence of $AlPO_4$, $Ca_3(PO_4)_2$, and the RPs (Fig. 1a, c–e), *E. ludwigii* FS27 accumulated the highest quantities of this acid among all isolates, reaching up to 3 g L^{-1} in the treatment with $Ca_3(PO_4)_2$. When all fungal isolates were taken into account, the production of all organic acids was reduced in the treatments with RPs (Fig. 1d, e) when compared to $Ca_3(PO_4)_2$ (Fig. 1c). The lowest concentrations of gluconic acid for all fungal isolates tested were obtained in the treatments with $AlPO_4$ (Fig. 1a) and $FePO_4$ (Fig. 1b).

The simulations of the effect of pH on solubility of the P sources showed that $Ca_3(PO_4)_2$ can be completely solubilized by lowering the pH from 7 to 4 (Fig. 2). When the data on pH and P solubilization of $AlPO_4$ obtained experimentally (Table 3) were compared with the corresponding simulation values (Fig. 2) no agreement was found. In the experiments with *Penicillium* species, when the final pH reached 2.9–3.2 about 60–80 % of solubilization of $AlPO_4$ was expected, while this value should increase to 100 % in the treatment with *A. niger* FS1 due to the observed pH of 2.3. On the other hand, according to the simulation data no solubilization could be attained using $FePO_4$ in the range of pH found in the respective experiments.

Discussion

The isolation procedure employed in this study was developed bearing in mind that the isolates should demonstrate

Table 2 Solubilized P, solubilization rate, biomass, and P/biomass yield of fungal isolates during the solubilization of hydroxyapatite [$Ca_5(PO_4)_3(OH)$] in liquid medium

Isolate	Identification	Solubilized P (mg L^{-1})	Solubilization rate[a] (%)	Dry biomass (mg $flask^{-1}$)	$Y_{P/X}$ (mg g^{-1})[b]
FS1	*Aspergillus niger*	1097 a	100 a	116 a	475 b
FS2	*Aspergillus niger*	1066 a	97 a	100 b	533 b
FS3	*Aspergillus niger*	1034 a	94 a	116 a	459 b
FS4	*Aspergillus niger*	1029 a	94 a	128 a	404 c
FS5	*Aspergillus niger*	985 a	90 a	110 b	454 b
FS6	*Aspergillus niger*	979 a	89 a	112 b	443 b
FS7	*Aspergillus niger*	970 a	88 a	107 b	457 b
FS8	*Aspergillus niger*	954 b	87 b	129 a	376 c
FS9	*Aspergillus niger*	936 b	85 b	126 a	375 c
FS10	*Aspergillus niger*	935 b	85 b	106 b	468 b
FS11	*Aspergillus niger*	924 b	84 b	100 b	487 b
FS12	*Aspergillus niger*	896 b	82 b	142 a	318 c
FS13	*Aspergillus niger*	838 b	76 b	109 b	401 c
FS14	*Aspergillus niger*	817 b	75 b	124 a	335 c
FS15	*Penicillium pinophilum*	741 c	68 c	78 c	477 b
FS16	Not identified	728 c	66 c	64 c	593 b
FS17	*Penicillium canescens*	707 c	64 c	94 b	377 c
FS18	*Penicillium pinophilum*	697 c	64 c	82 c	432 b
FS19	*Aspergillus* sp.	695 c	63 c	110 b	317 c
FS20	*Penicillium pinophilum*	685 c	62 c	79 c	432 b
FS21	*Penicillium* sp.	680 c	62 c	94 b	361 c
FS22	*Penicillium canescens*	669 c	61 c	59 c	587 b
FS23	*Penicillium canescens*	667 c	61 c	53 d	628 a
FS24	*Penicillium pinophilum*	659 c	60 c	92 b	359 c
FS25	*Penicillium* sp.	658 c	60 c	58 c	576 b
FS26	*Penicillium canescens*	657 c	60 c	59 c	567 b
FS27	*Eupenicillium ludwigii*	643 c	59 c	66 c	492 b
FS28	*Penicillium* sp.	640 c	58 c	55 d	589 b
FS29	*Penicillium* sp.	620 c	57 c	58 c	546 b
FS30	*Penicillium islandicum*	619 c	56 c	45 d	721 a
FS31	*Penicillium pinophilum*	604 c	55 c	97 b	323 c
FS32	*Penicillium decumbens*	601 c	55 c	47 d	684 a
FS33	Not identified	599 c	55 c	63 c	475 b
FS34	*Penicillium pinophilum*	595 c	54 c	76 c	392 c
FS35	*Penicillium solitum*	595 c	54 c	63 c	477 b
FS36	*Penicillium funiculosum*	594 c	54 c	80 c	378 c
FS37	*Penicillium melinii*	593 c	54 c	69 c	435 b
FS38	*Penicillium* sp.	583 c	53 c	67 c	438 b
FS39	*Penicillium* sp.	582 c	53 c	43 d	678 a
FS40	*Penicillium variable*	578 c	53 c	40 d	723 a
FS41	*Penicillium islandicum*	565 c	52 c	68 c	417 b
FS42	Not identified	499 d	45 d	55 d	470 b
FS43	*Penicillium* sp.	457 d	42 d	37 d	624 a
FS44	*Penicillium islandicum*	455 d	42 d	77 c	309 c
FS45	*Penicillium* sp.	453 d	41 d	61 c	377 c
FS46	*Penicillium* sp.	369 d	34 d	98 b	203 d
FS47	*Penicillium lividum*	295 e	27 e	125 a	119 d
FS48	*Penicillium* sp.	286 e	26 e	119 a	121 d

Table 2 (continued)

Isolate	Identification	Solubilized P (mg L^{-1})	Solubilization rate[a] (%)	Dry biomass (mg $flask^{-1}$)	$Y_{P/X}$ (mg g^{-1})[b]
FS49	*Penicillium* sp.	279 e	25 e	38 d	376 c
FS50	*Penicillium verruculosum*	279 e	25 e	95 b	146 d
FS51	*Penicillium purpurogenum*	275 e	25 e	79 c	179 d
FS52	*Penicillium* sp.	247 e	23 e	43 d	278 c
FS53	*Penicillium purpurogenum*	246 e	22 e	57 c	215 d
FS54	*Penicillium* sp.	179 f	16 f	118 a	77 d
FS55	*Penicillium purpurogenum*	142 f	13 f	71 c	111 d
FS56	*Penicillium rugulosum*	125 f	11 f	68 c	92 d
FS57	Not identified	61 f	6 f	37 d	84 d

Data are presented as the mean. In each column, means followed by the same lowercase letter are not significantly different at $p<0.05$ by Scott-Knott cluster analysis

[a] Percentage of the insoluble P added that was solubilized by the fungus

[b] P/biomass yield ($Y_{P/X}$) = Solubilized P (mg)/dry biomass (g), in 50 mL of medium

both P-solubilizing capacity and abundant growth on sugarcane bagasse. These characteristics were required in order to develop a system for microbial treatment of RP using sugarcane bagasse as substrate for fungal growth. The use of different isolation strategies enabled a great diversity of fungal isolates to be obtained. The direct isolation method, in particular, proved to be an efficient technique, enabling rapid attainment of pure cultures and, primarily, to access a large number of fungal groups. A great variety of *Penicillium* species showing P-solubilizing capacity was obtained (Table 2). The capacity of P solubilization is widespread among *Penicillium* species and their teleomorphs (Wakelin et al. 2004). However, all of the *Penicillium* species obtained in this work were less effective P solubilizers than the *Aspergillus niger* isolates. Moreover, it is also important to note that there are differences among isolates within the same species, such as in *A. niger*, *Penicillium islandicum*, and *P. purpurogenum*, in their solubilizing P capacity (Table 2), which demonstrates the importance of planning the isolation and screening procedures while bearing in mind the necessity of evaluating a large number of isolates.

Based on the results of hydroxyapatite solubilization in the liquid medium, we selected four isolates for further study on the mechanisms of microbial solubilization of different P sources. Our results reveal that the solubilization process was affected by intrinsic characteristics of both the P sources and the fungi evaluated. The differences among fungal isolates can generate a complex group of responses even when the cultivation conditions are only slightly modified. Because of this, specific isolation and characterization procedures will be always necessary to obtain optimized systems. In our study we observed that P sources, in addition to their intrinsic solubility characteristics, can modulate fungal metabolism. This modulation can be consequence, for instance, of the acidic or basic nature of the compound, of the chemical elements released during the solubilization process, or even of P availability during the process. These factors are closely involved in regulating the production of the organic acids (Kubicek and Röhr 1985; Mischak et al. 1985; Gadd 1999; Papagianni et al. 1999) that play an important role in the P solubilization process.

$Ca_3(PO_4)_2$ was the source with the highest solubilization levels. A reduction in pH is the most important mechanism for solubilizing pure P–Ca sources (Illmer and Schinner 1995; Whitelaw et al. 1999). Our simulation data showed that $Ca_3(PO_4)_2$ can be completely solubilized by lowering the pH from 7 to 4 (Fig. 2). In accordance with this, a high negative correlation (−0.93; $p<0.01$) between solubilized P and pH was observed in the treatment with $Ca_3(PO_4)_2$, while no significant correlation was observed between solubilized P and titratable acidity. The role of low pH is more evident in the analysis of solubilized P, pH, titratable acidity (Table 3), and citric acid production (Fig. 1c) by *Penicillium canescens* FS23, *P. islandicum* FS30, and *Eupenicillium ludwigii* FS27. The values of P solubilization and pH are equal for all isolates, while *P. canescens* FS23 produced more citric acid and, consequently, higher titratable acidity than the other strains, suggesting that the solubilization of $Ca_3(PO_4)_2$ results solely from lowering of the pH. Furthermore, correlations between biomass and pH (−0.97, $p<0.01$), and between biomass and solubilized P (0.93; $p<0.01$), were also observed. This is in accordance with the hypothesis of Illmer and Schinner (1995) who proposed that the solubilization of P–Ca sources results from processes depending on biomass production, such as the release of protons accompanying respiration or NH_4^+ assimilation.

The RPs, despite being composed mainly of P–Ca, were poorly solubilized when compared to $Ca_3(PO_4)_2$ (Table 3). The most likely explanation is that the P of the RPs is linked in a more complex structure than the pure $Ca_3(PO_4)_2$ used in the study. The RPs used in this study are of igneous origin

Table 3 Solubilized P, titratable acidity, pH, and biomass after cultivation of fungal isolates in liquid media containing different insoluble P sources

Fungal isolate	P source				
	$AlPO_4$	$FePO_4$	$Ca_3(PO_4)_2$	Araxá RP	Catalão RP
P solubilized (mg L^{-1})					
A. niger FS1	713 bA[a]	362 cA	1075 aA	147 dA	127 dA
P. canescens FS23	60 bB	32 bB	754 aB	72 bB	29 bB
E. ludwigii FS27	68 bcB	−3 cB	746 aB	96 bAB	61 bcAB
P. islandicum FS30	11 bB	0 bB	747 aB	62 bB	36 bB
Titratable acidity (mmol H^+ L^{-1})					
A. niger FS1	22.0 bA	37.9 aA	25.6 bA	8.5 cA	11.4 cA
P. canescens FS23	2.8 bB	3.4 bB	26.9 aA	5.7 bA	3.8 bA
E. ludwigii FS27	6.3 aB	4.9 aB	11.6 aB	11.4 a A	10.4 aA
P. islandicum FS30	4.0 bB	2.3 bB	15.8 aB	5.7 bA	4.7 bA
pH					
A. niger FS1	2.3	2.5 bC	2.8 aB	2.7 aB	2.5 bBC
P. canescens FS23	3.1	3.3 bA	4.2 aA	3.4 bA	3.0 cA
E. ludwigii FS27	2.9 bB	3.0 bB	4.1 aA	2.8 bB	2.4 cC
P. islandicum FS30	3.2 bA	3.2 bAB	4.2 aA	3.2 bA	2.7 cB
Dry biomass (mg $flask^{-1}$)					
A. niger FS1	111 bA	174 aA	170 aA	98 bcA	81 ca
P. canescens FS23	124 bA	163 aA	87 cB	100 cA	101 bcA
E. ludwigii FS27	66	124 aB	80 bB	53 cdB	34 dB
P. islandicum FS30	45 bB	94 aC	77 aB	83 aA	46 bB

[a] For each variable, data are presented as the mean. Means followed by the same lowercase letter in the row or by the same uppercase letter in the column are not significantly different at $p<0.05$ according the Tukey test

and, because of this, they have a compact structure with a low specific surface; as such, they are characterized as low-reactivity RPs (Novais and Smith 1999). However, it is important to note that there was a strong reduction in organic acids production in the treatments with RPs (Fig. 1d, e). In addition to containing P, the RPs contain other elements (Table 1), and it is possible that the reduction in organic acids production could be caused by the release of some chemical elements, such as Mn, Zn, Fe, and Cu, which admittedly affect organic acid production even in low concentrations (Shu and Johnson 1948; Gadd 1999; Papagianni 2007). Another possibility which may explain the lower percentage of solubilization is the RP dose used. As all P sources were added in a quantity corresponding to 1 g of P L^{-1}, the RPs with a low percentage of P were added in higher quantities. The RP dose is known to affect the final concentration of soluble P and the rate of solubilization (Xiao et al. 2008; Mendes et al. 2013). Thus, the high RP doses used in this work likely also had an inhibitory effect on solubilization. Finally, it is important to mention that the sugar content of NBRIP medium (10 g L^{-1} glucose) is below the optimal concentration for the production of citric acid (Xu et al. 1989). This medium has played an important role in isolation and screening procedures of P-solubilizing microorganisms, but further studies aimed at the optimization of P solubilization must consider the optimization of the production of organic acids.

Our comparison between experimental and simulation data showed that the solubilization of $AlPO_4$ and $FePO_4$ is dependent on factors other than pH. Organic acids are known for their capacity to form stable complexes with metals and, because of this, to solubilize P (Fox et al. 1990a, b; Jones 1998). Oxalic and citric acids are notable for their capacity to release P from RPs (Kpomblekou-A and Tabatabai 1994) and from soil particles (Fox et al. 1990b). The stability constants (log K) show that there is a strong interaction between these acids and Al^{3+} or Fe^{3+} ions (Table 4). Thus, these organic acids can increase the concentration of soluble P by ligand exchange reactions or by the complexation of metal ions released to solution, thereby preventing the precipitation of P with those ions and lowering the saturation state of the solution (Stumm 1986; Jones 1998; Fox et al. 1990a; Welch et al. 2002). Thus, the production of citric acid and, in a higher proportion, oxalic acid by *Aspergillus niger* FS1 explains the solubilization of $AlPO_4$ and $FePO_4$.

Aspergillus niger FS1 was superior to the other fungal isolates in terms of its ability to solubilize all of the P

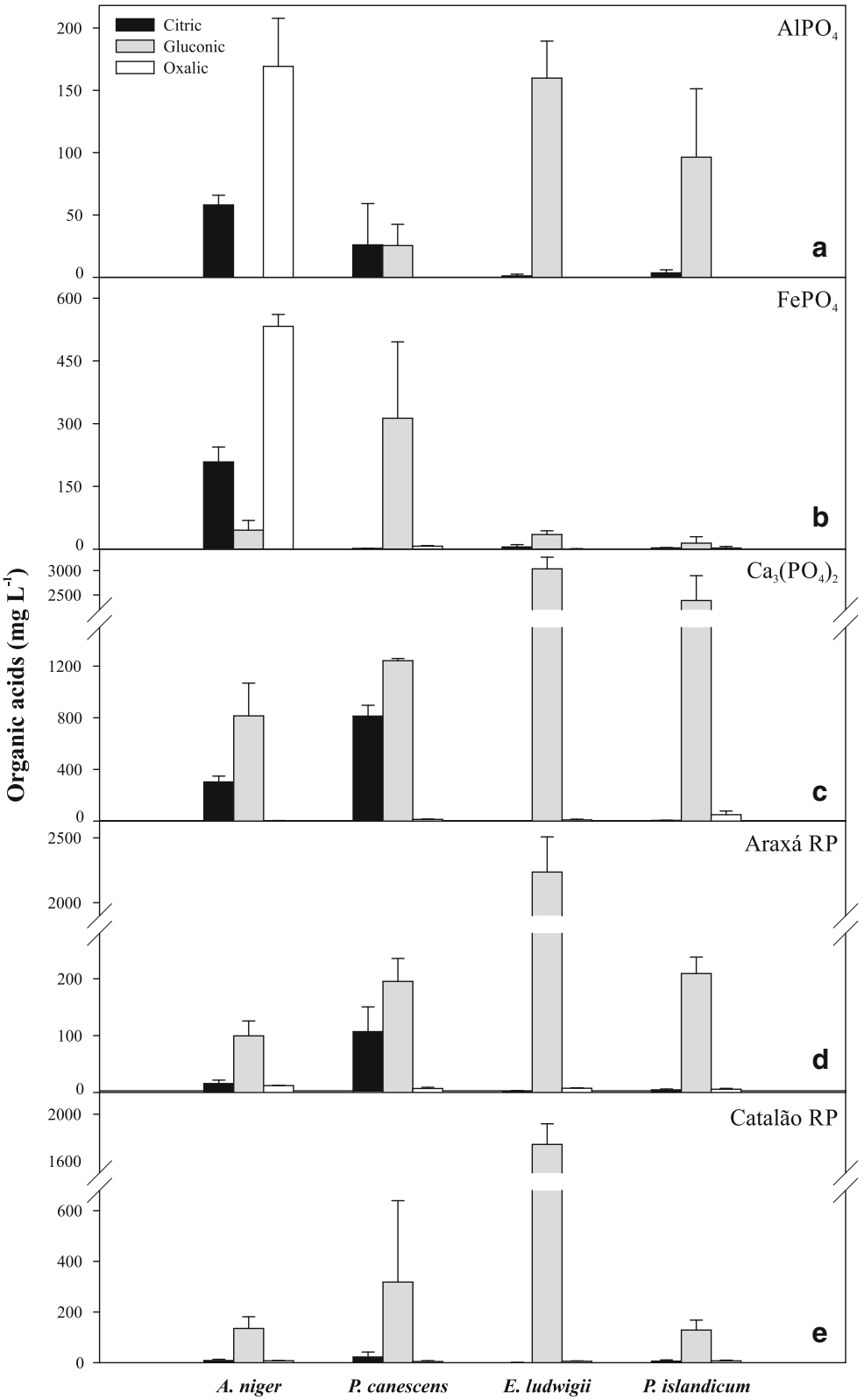

Fig. 1 Organic acids production by *Aspergillus niger* FS1, *Penicillium canescens* FS23, *Eupenicillium ludwigii* FS27, and *Penicillium islandicum* FS30 in media containing $AlPO_4$ (**a**), $FePO_4$ (**b**), $Ca_3(PO_4)_2$ (**c**), Araxá rock phosphate (*RP*) (**d**), or Catalão RP (**e**) as insoluble P source. *Error bars* Standard deviation

sources evaluated. It produced oxalic acid in the experimental conditions evaluated, which could explain its higher capacity of P solubilization compared to the other microorganisms. Interestingly, oxalic acid was detected only in the treatments with $AlPO_4$ and $FePO_4$. Using scanning electron microscopy, Schneider et al. (2010) demonstrated that the absence of oxalic acid in media containing RP when analyzed by high-performance liquid chromatography was

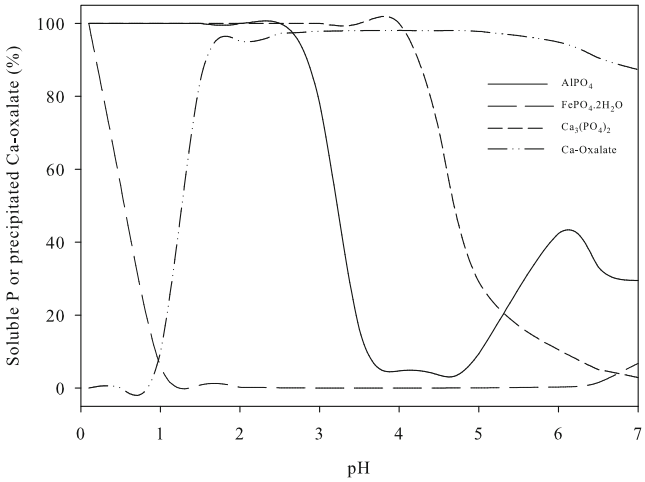

Fig. 2 Simulation of the effect of pH on solubility of P sources and calcium oxalate using the software GEOCHEM-EZ

due to the precipitation of calcium oxalate crystals. These crystals are solubilized only in extremely acid conditions (see Fig. 2). At the end of our experiment, the pH in the media with P–Ca sources (Table 3) and the concentration of approximately 2 g L^{-1} Ca ions [calculated from the solubilized $Ca_3(PO_4)_2$] would be sufficient to precipitate all the oxalic acid produced. Thus, *A. niger* FS1 probably also produces oxalic acid in media with P–Ca sources, which would be especially important for the solubilization of RPs because of the ability of this acid to precipitate with various metallic ions (Gadd 1999).

Gluconic acid production was favored in the media containing P–Ca sources. The pH of the medium was adjusted to 7 prior to adding the P sources. The addition of P–Ca sources had little effect on the pH, lowering it to 6.5–6.8. However, with $AlPO_4$ and $FePO_4$ the pH was reduced to 5.0 and 3.8, respectively. Furthermore, during the cultivation of the fungi the pH fell to below 3.3 for both $AlPO_4$ and $FePO_4$. The synthesis of gluconic acid is catalyzed by the extracellular enzyme glucose oxidase (GOD), which converts glucose into gluconic acid. GOD activity is pH dependent and is strongly inhibited when the pH is reduced from 5 to 3 (Mischak et al. 1985). Thus, the lower gluconic acid production in the media with $AlPO_4$ and, particularly with $FePO_4$, could be explained by the lower pH in these media. Moreover, *Penicillium* species produced, in general, more gluconic acid than *Aspergillus niger* (Fig. 1); this may also be pH dependent since the *Penicillium* species in most cases presented a higher pH at the end of the cultivation period (Table 3). Nevertheless, the results show that gluconic acid apparently plays only a minor role in P solubilization. Generally, monocarboxylic acids, such as gluconic acid, have a low complexing ability (Fox et al. 1990b; Jones 1998), as can be seen by the stability constants of this acid with Al^{3+} and Ca^{2+} (Table 4). Evidence of this is that *A. niger* FS1, even when producing less gluconic acid than the other fungi (Fig. 1), was able to solubilize equal or higher quantities of P (Table 3). Tests of abiotic P solubilization have shown that the addition of gluconic acid to samples of insoluble P–Ca (Illmer and Schinner 1995; Whitelaw et al. 1999) and P–Al (Whitelaw et al. 1999) is not effective in releasing P. It is likely that gluconic acid would be useful in solubilizing $FePO_4$, since the stability constant between this acid and Fe^{3+} is high (Table 4). However, the acidic nature of $FePO_4$ interferes with the production of gluconic acid. Alternatives, such as a buffered medium or the production of the acid prior the application of $FePO_4$, should be further studied to take advantage of *Eupenicillium ludwigii* FS27, which presented high gluconic acid production.

Table 4 Stability constants (log K) between organic acids and metallic cations

Metals (right) Organic acids (below)	Al^{3+}	Ca^{2+}	Fe^{+3}
Citric acid	7.98[a]	3.5[b]	11.85[b]
Gluconic acid	1.98[a]	1.21[c]	6.35[d]
Oxalic acid	7.26[b]	2.3[e]	8.2[e]

[a] Motekaitis and Martell (1984)
[b] Furia (1972)
[c] Cannan and Kibrick (1938)
[d] Bechtold et al. (2002)
[e] Smith and Martell (1987)

Conclusions

Our data show that P-solubilization mechanisms not only differ among fungal isolates but are also dependent on the applied P sources. Medium acidification is an effective solubilization mechanism, particularly for $Ca_3(PO_4)_2$. For compounds such as $AlPO_4$ and $FePO_4$ or more complex structures, like the RPs, the production of organic acids is probably the main P-solubilizing mechanism. *Aspergillus niger* FS1 produced oxalic acid in the experimental conditions evaluated, which could explain its higher capacity for P solubilization. The production of this acid, along with citric acid, is the key mechanism for the solubilization of $AlPO_4$ and $FePO_4$. On the other hand, gluconic acid, in comparison with oxalic and citric acids, demonstrated a lower ability to solubilize P-bearing sources at the concentrations detected in the cultivation media with the tested microorganisms. This fact is important for the development of biotechnological schemes for microbial treatment of RP, since the cultivation conditions can be manipulated to favor the production of organic acids with high P-solubilizing efficiency. Of the isolates included in our study, *A. niger*

FS1 was the only fungal isolate able to solubilize all of the P sources tested and should be further studied under fermentation and soil conditions.

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