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Solubilization of inorganic phosphates and plant growth promotion by *Aspergillus niger*

Chun-Chao Chuang · Yu-Lin Kuo · Chen-Ching Chao · Wei-Liang Chao

Abstract Two of 187 fungal isolates (*Aspergillus niger* 1B and 6A) displaying superior phosphate (P) solubilization and hydrolytic enzyme secretion were studied using P forms of calcium (Ca-P), iron (Fe-P), and aluminum (Al-P). Phosphate solubilization in a sucrose-basal salt (SB) broth was increased and pH decreased by both isolates. In Ca-P medium, solubilization for 6A was approximately 322 μg P mL^{-1} and pH decreased by 4.2 units to 2.3 in 72 h. However, when pH value of the SB broth was lowered to 2.5 using HCl, 65.3 ± 0.4 μg mL^{-1} of P was released from Ca-P, whereas trace amounts of P were released from Fe-P and Al-P. Both isolates displayed enhanced Al-P solubilization using NH_4Cl rather than KNO_3 as the N source; final pH values were not significantly different. With Ca-P, gluconic acid was predominantly produced by 1B and 6A, whereas oxalic acid predominated with Fe-P and Al-P. Addition of gluconic acid (final concentration of 8.5 μmol mL^{-1}) to Ca-P-supplemented SB lowered pH (2.9) and solubilized phosphate (146.0 ± 1.0 μg mL^{-1}). Similarly, addition of oxalic acid (final concentration 6.6 μmol mL^{-1}) to Ca-P- and Fe-P-amended media solubilized P (60.2 ± 0.9 and 21.6 ± 2.1 μg mL^{-1}, respectively), although these quantities were significantly lower than those detected in unamended SB. The presence of unidentified P solubilized compound(s) in the dialyzed (MW>500) supernatant warrants further study. In pot experiments, significant increases in plant (*Brassica chinensis* Linn.) dry weight and N and P contents were observed with the addition of isolate 6A, when a small amount of organic fertilizer together with either rock phosphate (South African apatite) or Ca-P served as the main P sources.

Keywords *Aspergillus niger* · Phosphate solubilization · pH · Organic acids · Yield

C.-C. Chuang · C.-C. Chao
Department of Soil Environmental Science,
National Chung-Hsing University, Taichung, Taiwan,
Republic of China

Y.-L. Kuo · W.-L. Chao (✉)
Department of Microbiology,
Soochow University, Shih Lin,
Taipei, Taiwan, Republic of China
e-mail: wlchao@mail.scu.edu.tw

C.-C. Chuang
Taoyuan District Agricultural Research and Extension Station,
Taoyuan, Taiwan, Republic of China

Introduction

Phosphorus (P) is one of the most important nutrients that limit crop production, and on average, soil contains 0.02–0.5% total P, which generally exists as poorly soluble inorganic P, with only 1% of soil P readily available for plant utilization (Barber 1984). In soils, the poorly accessible P typically comprises calcium phosphate (Ca-P), iron phosphate (Fe-P), and aluminum phosphate (Al-P), with their solubility decreasing in the following order: Ca-P>Al-P>Fe-P (Ahn 1993).

Phosphorus availability in soil is of concern in Taiwan. The country has roughly 865,000 ha of arable land, with 22.1% comprising soil that is strongly acidic (pH<5.5). The annual application of calcium superphosphate, the most commonly used phosphorus fertilizer in Taiwan, is approximately 200,000 metric tons (Fertilizer Digest 2001). In acidic soils, a large portion of the introduced phosphorus is fixed by free oxides and hydroxides of aluminum and iron, and under these forms is unavailable to plant (Jones et al. 1991). However, many soil bacteria and fungi have the

capacity to solubilize inorganic P and to increase P availability (Nahas 1996; Puente et al. 2004; Rodríguez and Fraga 1999; Vazquez et al. 2000). Kucey (1983) found significant correlation between the numbers of P-solubilizing fungi and the levels of total P in the soil and demonstrated that fungi were superior to bacteria in solubilizing Ca-P. Several studies have shown that increase in yield or plant growth can be achieved through the inoculation of P-solubilizing fungi in either pot experiments or under field conditions (Kucey and Leggett 1989; Wahid and Mehana 2000).

Solubilization of inorganic P by fungi has been linked to the production of various organic acids, including citric acid (Cunningham and Kuiack 1992; Illmer et al. 1995; Reyes et al. 2001), gluconic acid (Reyes et al. 2001; Whitelaw et al. 1999), and oxalic acid (Cunningham and Kuiack 1992; Gharieb 2000). However, disparate observations have been reported. Illmer and Schinner (1992) reported that when growing a P-solubilizing *Penicillium* sp. in an inorganic P-amended medium, none of the 24 organic acids assayed were produced in significant amounts. In a subsequent study, using $AlPO_4$ as P source, Illmer et al. (1995) detected a higher soluble Al concentration when NH_4^+ was used instead of NO_3^- as the N source. They proposed that proton excretion accompanied NH_4^+ uptake as the possible mechanism causing P solubilization. On the contrary, when NH_4^+ was used with hydroxyapatite, there was a significant decrease in P solubilization (Reyes et al. 1999). The same study reported that a small amount of P was solubilized by a *Penicillium rugulosum* (Mps⁻) mutant (i.e., the production of gluconic acid and citric acid was repressed) grown with NH_4^+, possibly via the H⁺ pump mechanism.

Generally, fungi are inferior to bacteria in their ability to colonize plant root (Chao et al. 1986; Puente et al. 2004), but they are more acid tolerant. Therefore, fungi may have a much better potential to be used as an agent to convert insoluble inorganic P into a form (e.g., $H_2PO_4^-$) usable by plants in acid soils. The aim of this research was to study the P-solubilizing mechanisms of P-solubilizing fungi isolated from various subtropical and tropical soil samples. The plant growth-stimulating capacity of a selected fungal isolate (i.e., isolate 6A) was also studied in an acidic soil.

Materials and methods

Isolation of P-solubilizing fungi

A total of 53 soil samples, including 21 tropical soils and 32 subtropical soils, were collected from the superficial layer (0–10 cm) and stored at 25±1°C. About 500 g of soil was collected from each sampling site, and their locations

are listed in Table 1. Ten-fold dilutions of these soil samples were made using sterile saline (0.85% NaCl). Aliquots (0.1 mL) of the dilutions were individually plated onto Ca-P-amended sucrose-basal salts (SB) medium containing the following compounds (expressed per liter of distilled water): sucrose, 10 g; $Ca_3(PO_4)_2$, 3 g; NH_4NO_3, 0.27 g; KCl, 0.2 g; $MgSO_4.7H_2O$, 0.1 g; yeast extract, 0.1 g; $FeSO_4.7H_2O$, 1 mg; $MnSO_4.H_2O$, 1 mg; agar, 15 g (Young et al. 2000). The final pH was adjusted to 6.8, and streptomycin sulfate (100 μg mL⁻¹) and Tween 80 (0.5%) were added to inhibit bacterial growth and restrict the overdevelopment of fungal colonies, respectively. The inoculated plates were incubated at 28°C for 5 days. Phosphorus-solubilizing fungi were shown by the formation of a clear zone around fungal colonies. Comparisons of P-solubilizing capabilities were provided by measurements of the clear zone diameter/colony diameter of each isolate.

Abiotic leaching by organic and inorganic acids

Each selected organic acid was added to the above medium containing Ca-P, Fe-P, or Al-P as the P source to give a concentration similar to that detected in the cultured broth. To study the importance of acidity in P solubilization, in another set of P-amended media, 1 M HCl was added to bring the final pH to 2.5. Each flask was inoculated and incubated at room temperature for 2 h prior to subsampling. Insoluble materials were removed by centrifugation (20,000×g) and the amount of soluble P in each supernatant was measured as described above.

Pot experiment

Clay loam (red soil) was collected from the experimental field located at the Taoyuan District Agricultural Research and Extension Station, Taoyuan, Taiwan (24°57′14″N; 121°01′03″E). Some of the soil properties are listed in Table 2. Portions (1.5 kg) of the sieved (10 mesh) soil were added to 15-cm clay pots. The amounts of ammonium sulfate, calcium superphosphate (or South African apatite), and potassium chloride added to each pot were 3.1, 4.4 (or 2.8 g), and 0.27 g, respectively, which were equivalent to 0.66 g of N, 0.8 g of P_2O_5, and 0.16 g of K_2O. In addition to the aforementioned chemical fertilizers, 15 g of composted plant residues (pea plant/rice husk/saw dust in a ratio of 1:1:2, representing 2% N, 0.4% P_2O_5, 1.9% K_2O; pH=6.3) was also added to each pot, as recommended by the agricultural extension service. Three replicates, each consisting of five pots, were set up for the control (no chemical P-fertilizer was added) and for each of the different P treatments (rock phosphate and calcium superphosphate as P source) with and without the addition of P-solubilizing fungi. The pots were placed in the field,

Table 1 Sampling location

Site	Location	Crop	Site	Location	Crop
Ba Li	121°23′15″E 25°09′55″N	Pomelo	Hou Bi	120°20′85″E 23°21′02″N	Corn
Jhu Wei	121°14′22″E 25°06′54″N	Cassava	Sin Ying	120°19′09″E 23°18′71″N	Green manure
Guan Yin	121°04′31″E 25°01′69″N	Cauliflower	Liou Ying	120°18′97″E 23°16′53″N	Custard apple
Fong Bi	120°57′37″E 24°53′92″N	Bamboo	Long Tian	120°18′31″E 23°12′29″N	Litchi
Chi Ding	120°52′85″E 24°44′44″N	Acacia	Guan Tian	120°19′74″E 23°10′94″N	Night-bloom gereus
Hou Long	120°45′66″E 24°37′45″N	Peanut	Shan Hua	120°19′24″E 23°08′88″N	Vegetable soybeans
Tong Siao	120°40′06″E 24°29′14″N	Sweet potato	Sin Shih	120°18′61″E 23°07′24″N	Cabbage
San Yi	120°43′43″E 24°20′57″N	Rice	Sin Shih	120°17′39″E 23°04′46″N	Banana
Tai Chung	120°35′51″E 24°05′44″N	Rice	Shan Shang	120°19′80″E 23°03′97″N	Pineapple
Fen Yuan	120°38′98″E 23°58′26″N	Chinese broccoli	Zuo Jhen	120°22′08″E 23°04′43″N	Mango
Chung Hsing	120°40′30″E 23°55′33″N	Lemon	Zuo Jhen	120°24′54″E 23°03′36″N	Litchi
Ming Jian	120°40′28″E 23°53′09″N	Fallow field	Yu Jing	120°25′33″E 23°05′40″N	Mango
Da Jhuang	120°38′69″E 23°52′55″N	Pineapple	Yu Jing	120°28′72″E 23°08′92″N	Mango
Chih Shuei	120°37′32″E 23°51′61″N	Chinese yam	Nan Si	120°28′89″E 23°10′75″N	Carambola
Tian Jhong	120°33′98″E 23°52′92″N	Cabbage	Mi Jhih	120°29′85″E 23°11′54″N	Carambola
Bei Dou	120°29′03″E 23°51′93″N	Guava	Mi Jhih	120°30′68″E 23°12′32″N	Mango
Si Jhou	120°28′98″E 23°50′55″N	Sugar cane	Ma Dou	120°17′86″E 23°11′43″N	Sugar cane
Si Luo	120°27′76″E 23°47′70″N	Corn	Ma Dou	120°14′93″E 23°11′61″N	Orange
Cih Tong	120°29′04″E 23°45′86″N	Cabbage	Chung Hsing-hsintsun	120°39′53″E 23°54′29″N	Litchi
Dou Nan	120°28′62″E 23°42′18″N	Bell pepper	Yang Sing	120°38′30″E 23°53′91″N	Betel nut
Dou Nan	120°27′97″E 23°39′98″N	Rice	Fu Shan	120°37′75″E 23°54′07″N	Tea tree
Da Lin	120°27′47″E 23°37′28″N	Orange	Fu Shan	120°37′80″E 23°54′47″N	Tea tree
Min Syong	120°25′09″E 23°35′55″N	Corn	Fong Ming	120°37′40″E 23°55′17″N	Pineapple
Min Syong	120°25′60″E 23°32′44″N	Mango	Fong Ming	120°37′81″E 23°55′73″N	Ginger
Bei Huei village	120°24′28″E 23°27′34″N	Banana	Fong Shan	120°37′73″E 23°56′61″N	Tea tree
Shuei Shing	120°22′93″E 23°25′38″N	Green manure	Fen Yuan	120°37′64″E 23°57′06″N	Chinese yam
Hou Bi	120°22′22″E 23°23′73″N	Pickled			

Table 2 Soil properties before and after the pot experiment with rock phosphate amendment

Treatment	pH	EC[a] (dS/m)	OM[c] (g/kg)	Available P (mg/kg)	Exchangeable		
					K (mg/kg)	Ca	Mg
Trial I							
−P[c], −Asp[d]	5.3	0.14	26	45	98	658	109
+P, −Asp	5.4	0.24	34	93	319	2,058	289
+P, +Asp	5.3	0.53	32	84	433	2,877	238
Trial II							
−P[c], −Asp[d]	4.8	0.49	27	90	655	1,633	233
+P, −Asp	5.2	0.31	36	125	386	6,629	374
+P, +Asp	5.0	0.58	30	106	586	8,384	306
Trial III							
−P[c], −Asp[d]	5.3	0.32	35	78	403	1,057	126
+P, −Asp	5.5	0.59	36	73	449	4,075	223
+P, +Asp	5.4	0.50	34	95	436	5,250	226

[a] *EC* electrical conductivity
[b] *OM* organic matter
[c] P source: rock phosphate
[d] *Aspergillus niger* 6A

arranged randomly and watered everyday, except when it rains. The weather conditions during the growth experiments are listed in Table 3. The roots of 3-week-old seedlings (about 10 cm high) of Chinese mustard (*Brassica chinensis* Linn.) obtained from a commercial nursery were treated with 5 mL of the spore suspension (5–6×10^{7} spores mL^{-1}) of selected P-solubilizing fungi and then transplanted in each pot. After four more weeks, plants were harvested and analyzed for their dry weight and N and P contents.

Effect of dialysis on the P-solubilization capacity of culture supernatant

Fungal isolates were initially cultured in the SB medium amended with Ca-P (0.05 g/L) for 28 h. Culture supernatants were collected and suspending mycelium debris was removed by centrifugation ($20,000\times g$). Supernatants were divided into two parts, with one serving as undialyzed control, whereas the other one was placed in a Spectra/Por membrane tubing (MWCO:500; Spectrum Medical Indus-

tries, Inc., Houston, TX) and dialyzed against 2 L of reverse osmosis-purified water at 4°C. The water was changed every 2 h for the initial 6 h and then after 12 h. The final volume of both the dialyzed and undialyzed supernatants was adjusted to 15 mL with purified water, mixed with equal volume of Ca-P-amended SB medium, and the mixture was incubated at room temperature for 2 h. The quantity of soluble P in each supernatant was measured as described below.

Analyses

The amount of soluble P was measured as described by Olsen and Sommers (1982). Phosphorus concentrations were determined colorimetrically at 882 nm by the formation of a molybdophosphate complex in the acid solution (H_2SO_4) with subsequent reduction by ascorbic acid. A standard curve was generated with KH_2PO_4.

The concentrations of oxalic acid, citric acid, tartaric acid, gluconic acid, malic acid, succinic acid, formic acid, and fumaric acid in the broth medium were determined by

Table 3 Weather conditions for plant trials

Weather conditions	Plant trial		
	I (June 2004)	II (August 2004)	III (October 2004)
Mean air temperature (minimum–maximum)	27.3°C (17.4–35.2°C)	28.2°C (23.5–35.2°C)	21.7°C (15.5–28.9°C)
Average relative humidity	77.8%	84.6%	78.5%
Accumulated rainfall	2 mm	293 mm	58.5 mm
Accumulated sunshine hours	138 h	192 h	204 h
Accumulated solar radiation	596 MJ/m^2	612 MJ/m^2	452 MJ/m^2

high-pressure liquid chromatography (HPLC) analysis. The HPLC system (HP-1100; Hewlet-Packard Co., Palo Alto, CA) consisted of a quaternary pump, a diode array detector, an autosampler, and a Transgenomic organic acid column (ORH-801; Transgenomic, Inc., Omaha, NE). The mobile phase (0.005 M H_2SO_4) was run at 0.7 mL/min at 40°C.

The abilities of the tested fungi to dissolve the poorly soluble Ca-P, Fe-P, and Al-P were determined by the medium described above without the addition of agar and inhibitors. Fungal cultures were grown for 5 days, and 5-mm-diameter agar plugs, which contained actively growing mycelium, were taken from the edge of the colony and used as inoculants. For each organism, three mycelium-containing agar plugs were added to a 250-mL Erlenmeyer flask with 50 mL of the sterile broth medium. Triplicate flasks were prepared for each organism and all were incubated at 30°C on a rotary shaker (120 rpm). Samples were taken daily for three consecutive days. Suspended mycelia were removed by centrifugation ($20,000 \times g$) and the supernatant was collected to be analyzed for pH, soluble phosphate, and organic acids.

To study the effect of N sources on the subsequent P solubilization, fungal isolates were cultured in the Al-P-amended growth medium in the presence of NH_4Cl or KNO_3 (final concentration, 3.4 mM) at 30°C for 72 h. Culture supernatant was sampled from each medium for P analysis as described above.

The pH of the soil–water mixture (1:5) was determined by a glass electrode (McLean 1982), and electrical conductivity (EC) was determined by a conductivity meter (Rhoades 1982). The amount of organic matter (OM) in soil was determined by the Walkley–Black procedure (Nelson and Sommers 1982). Available P was determined by Bray No.1 method (Olsen and Sommers 1982), exchangeable potassium by flame photometry (Knudsen et al. 1982), whereas atomic absorption spectrophotometry was used to measure exchangeable calcium and magnesium (Lanyon and Heald 1982).

The harvested plants were dried at 70°C; weights were measured and then ground to powder. Powder samples were wet digested with an acid mixture (Parkinson and Allen 1975), and their N and P contents were determined by the Kjeldahl and Vanadate–Molybdate–Yellow methods, respectively.

Statistical analyses

Student's *t* test was used to compare the differences of plant dry weight, the relative N and P content of plant tissue, and the amount of phosphate released through biotic and abiotic solubilization. Linear regression analyses were carried out using data showing rapid increases in phosphate and organic acid concentrations in the broth medium.

Results and discussion

A total of 187 fungal isolates with the ability to solubilize Ca-P were isolated. Sixteen displayed a ratio of clear zone diameter/colony diameter larger than two. Of these 16 potentially usable fungi, isolates 1B and 6A were selected for further study based on their abilities to excrete amylase, protease, and lipase. One of the final goals of this research project is to produce compost with enhanced P-solubilization capacity, and we hope to achieve this result by inoculating the starting materials with our selected P-solubilization organism prior to the composting process. By selecting fungi that can excrete these extracellular enzymes, we hope that they can utilize the respective nutrients in the starting materials and reach higher inoculant density in the final product. Subsequent morphological and microscopic observations suggested that the characteristics of both isolates were consistent with those of *Aspergillus niger*. For both isolates, the accumulation of soluble P in the medium was associated with a concomitant decrease in pH value regardless of the inorganic P used (Figs. 1, 2, and 3). For example, isolate 6A in Ca-P containing SB medium produced a maximum solubilized P of approximately 322 µg mL^{-1} and a decrease in pH value of about 4.2 units to a final pH value of 2.3 after 72 h. Others have also observed P solubilization with decrease in pH values (Cerezine et al. 1988; Puente et al. 2004; Reyes et al. 1999). The increased proton content responsible for the pH decrease can arise from the production of organic acid (Cunningham and Kuiack 1992) or H$^+$-excretion that accompanies NH_4^+-assimilation (Beever and Burns 1980). By adding 0.1 M HCl to the medium, Illmer and Schinner (1992) reached the conclusion that mechanisms other than acid production are also responsible for solubilizing rock phosphate. Likewise, in the present study, when the pH values of the test media containing Ca-P, Fe-P, or Al-P were adjusted to 2.5 using 1 M HCl, only the Ca-P-containing medium demonstrated P accumulation (about 65.3 µg mL^{-1}), but the solubilized P was significantly smaller than the amount detected when the medium was incubated with the test fungi (Table 4). Roos and Luckner (1984) reported that the acidification observed in cultures of *Penicillium cyclopium* results mainly from the uptake of NH_4^+, which is stoichiometrically coupled with H$^+$ excretion. Cunningham and Kuiack (1992) reported that *Penicillium bilaii* causes a significantly larger reduction of pH in NH_4^+-supplemented medium as compared to a medium supplemented with NO_3^-. In the present study, with AlPO$_4$ as the P source, we failed to observe any significant differences in the final pH of media supplemented with either NH_4^+ or NO_3^- (Table 5). On the other hand, better P solubilization was observed when NH_4^+ was used as the N source. Reyes et al. (1999) have also shown that AlPO$_4$ solubilization by *P. rugulosum*

Fig. 1 Relationships between pH, organic acid production, and solubilization of calcium phosphate by *Aspergillis* spp. **a** *Aspergillus* sp. 1B. **b** *Aspergillus* sp. 6A. Phosphate concentration (-■-), pH (-♦-), gluconic acid (-△-), malic acid (-◊-), oxalic acid (-□-), citric acid (-○-)
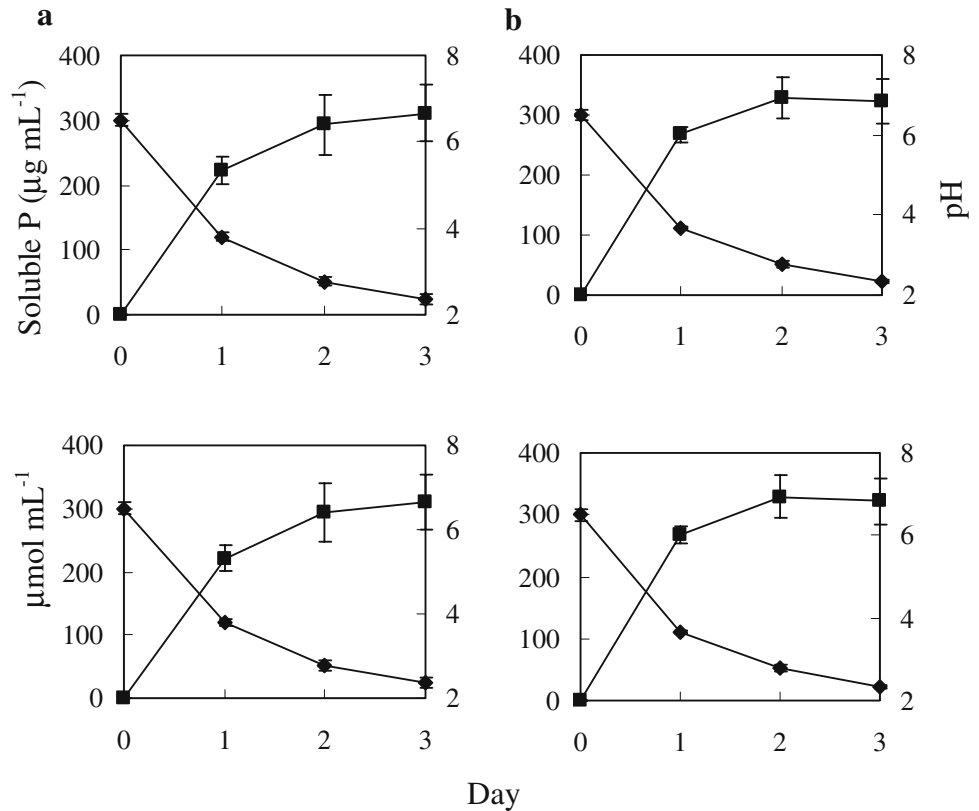

was significantly better when NH_4^+ was used as the sole N source as compared to NO_3^-, whereas with $FePO_4$ as the P source, higher solubilization was observed with NO_3^- as the sole N source. Clearly, the hypothesis that P solubilization is linked to acidification caused by NH_4^+ assimilation does not hold true for all microorganisms.

Fig. 2 Relationships between pH, organic acid production, and solubilization of iron phosphate by *Aspergillis* spp. **a** *Aspergillus* sp. 1B. **b** *Aspergillus* sp. 6A. Phosphate concentration (-■-), pH (-♦-), gluconic acid (-△-), malic acid (-◊-), oxalic acid (-□-), citric acid (-○-). Linear regression analyses are done using data connected by the *bold line*
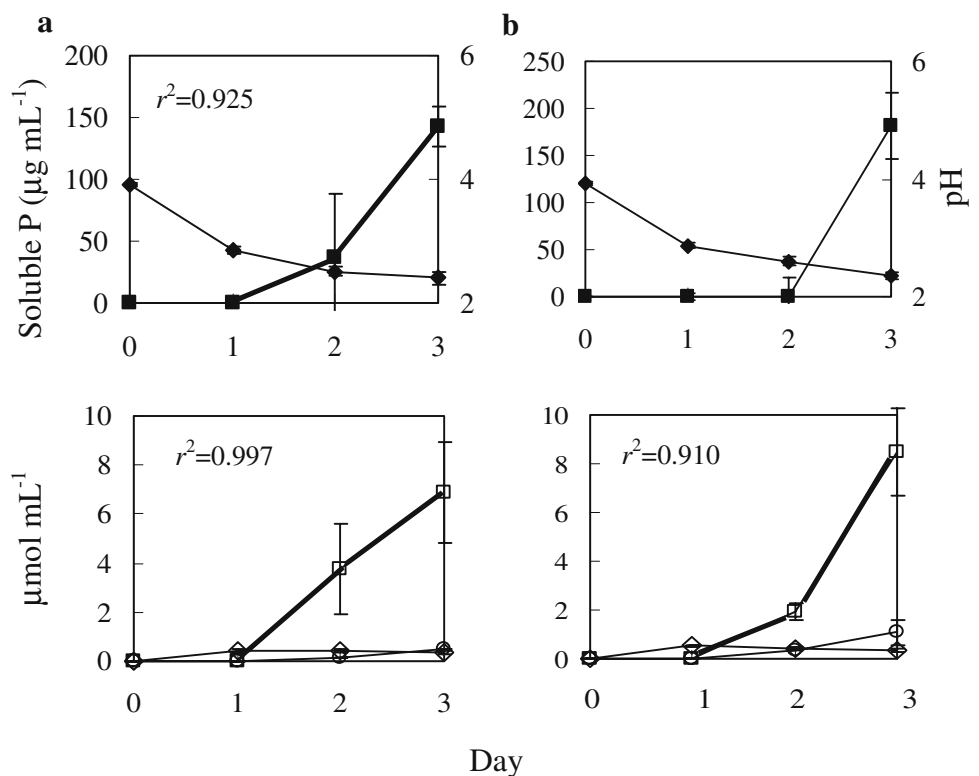
Fig. 3 Relationships between pH, organic acid production, and solubilization of aluminum phosphate by *Aspergillis* spp. **a** *Aspergillus* sp. 1B. **b** *Aspergillus* sp. 6A. Phosphate concentration (-■-), pH (-♦-), gluconic acid (-△-), malic acid (-◇-), oxalic acid (-□-), citric acid (-○-). Linear regression analyses are done using data connected by the *bold line*
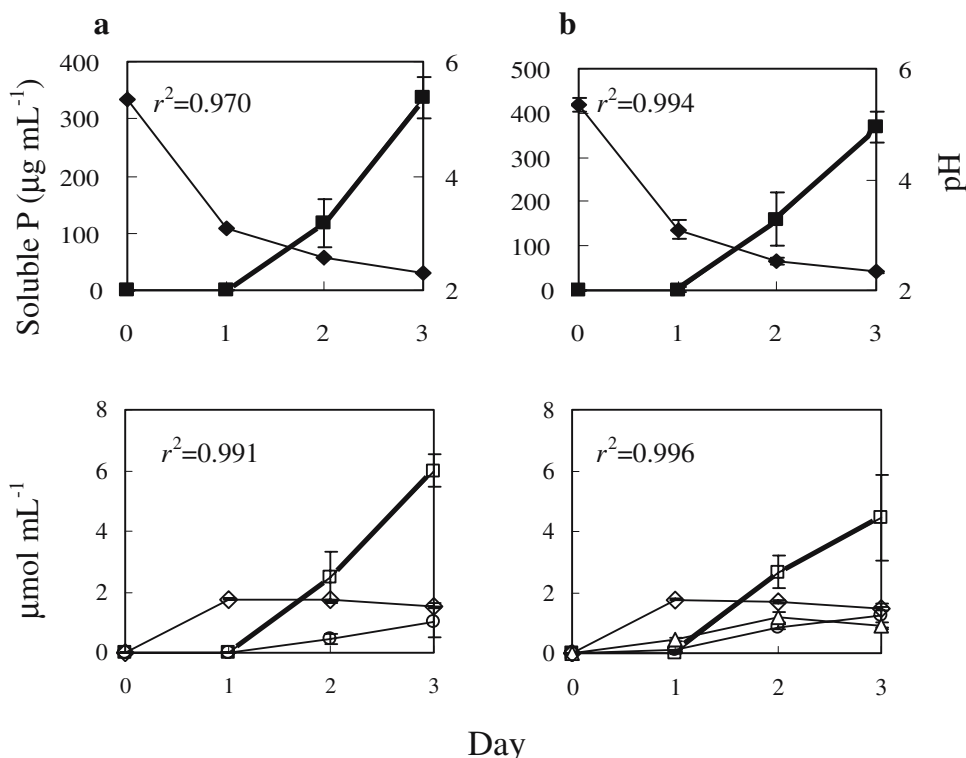
Fungal solubilization of inorganic P is associated with the production of organic acids, most frequently citrate, gluconate, oxalate, and succinate acids (Cunningham and Kuiack 1992; Gharieb 2000; Reyes et al. 2001; Vassilev et al. 2001; Vazquez et al. 2000). Organic acids may solubilize inorganic P through the release of acidic protons (Reyes et al. 1999; Whitelaw et al. 1999) and their abilities to chelate Ca^{+2}, Fe^{+3}, and Al^{+3} (Kpomblekou and Tabatabai 1994; Whitelaw et al. 1999). In the present study, using Ca-P as the P source, both *A. niger* isolates displayed trends of soluble P accumulation that closely matched the substantial increases in gluconic acid concentration (Fig. 1). When

Fe-P or Al-P comprised the P source, P accumulation corresponded to the increases in oxalic acid concentration (Figs. 2 and 3). Similarly, Reyes et al. (2001) reported that when using Utah variscite (which contains higher concentrations of Al and Fe) as the P source, the major organic acid produced by the P-solubilizing *P. rugulosum* is citric acid, whereas gluconic acid is produced in larger concentration when Florida apatite and Monte Fresco phosphate rock (which contain higher Ca concentrations) were used. It has been shown that glucose oxidase, which converts glucose to gluconic acid, is inactivated when the pH falls below 3.5 (Mischak et al. 1985). In this study, the initial pH

Table 4 Abiotic leaching of various inorganic phosphates by organic acids and HCl

Organic acid ($\mu mol\ mL^{-1}$)	Soluble phosphate ($\mu g\ mL^{-1}$)		
	$Ca_3(PO_4)_2$	$FePO_4$	$AlPO_4$
Oxalic acid (1.1)	46.9±0.3 (5.29±0.02)[a]	4.9±1.4 (3.27±0.02)	Trace (3.15±0.01)
Oxalic acid (6.6)	60.2±0.9 (2.60±0.01)	21.6±2.2 (2.45±0.01)	Trace (2.40±0.01)
Malic acid (1.5)	55.8±0.4 (5.17±0.08)	ND[b] (3.26±0.02)[c]	ND (3.25±0.01)
Gluconic acid (8.5)	146.0±1.0 (2.94±0.02)		
Citric acid (1.0)	57.8±0.5 (5.15±0.02)	ND (3.22±0.01)	ND (3.22±0.01)
Gluconic acid (6.1)+malic acid (1.5)	62.3±3.2 (3.22±0.06)		
Oxalic acid (1.1)+malic acid (1.5)	61.8±1.1 (4.74±0.03)	6.1±3.8 (3.01±0.02)	Trace (2.93±0.01)
1 M HCl	65.3±0.4 (2.50±0.01)	ND (2.50±0.01)	Trace (2.50±0.01)

[a] Final pH value of the medium. The initial pH for media containing $Ca_3(PO_4)_2$, $FePO_4$, and $AlPO_4$ were 6.50±0.15, 3.91±0.03, and 5.35±0.13, respectively.

[b] *ND* none detected

[c] –, not done

Table 5 Effect of ammonium and nitrate on the solubilization of AlPO$_4$ by *Aspergillus niger*

Treatment	Isolate 1B		Isolate 6A	
	P (μg mL^{-1})	pH	P (μg mL^{-1})	pH
NH$_4$Cl	141.4±13.1	1.8±0.1	237.0±38.2	1.8±0.1
KNO$_3$	67.5±15.2	2.1±0.2	157.3±28.5	1.9±0.1

value of the growth medium was higher (5.35±0.13) with Al-P than with Fe-P (3.91±0.03). However, in both cases, the pH rapidly fell below 3.5.

Accumulation of gluconic acid was detected in medium supplemented with Al-P, whereas none was detected in medium using Fe-P as P source. We believed that it was because higher initial pH value of the Al-P-amended medium favored the activity of glucose oxidase. Besides pH, the nature and amount of organic acids excreted by fungi are influenced by various other abiotic factors, including the presence of certain metals (Clausen and Green 2003; Gadd 1999; Sayer and Gadd 2001). Both plants (Jones 1998) and fungi (Jarosz-Wilkolazka and Gadd 2003) release organic acids (including oxalate and citrate) when subjected to metal stress (e.g., Al and Fe), and in this way, metal-organic acid complexes are formed with decrease in metal toxicity. In the present study, the importance of organic acids in inorganic P solubilization was assessed by adding organic acids, either alone or in combination, to the medium containing inorganic P at a concentration similar to the level detected in the medium after fungal growth. However, this strategy failed to produce an accumulation of soluble P in the broth, which was comparable to the accumulation observed in the presence of *Aspergillus* (Table 4). For example, in the Ca-P-amended SB supplemented with gluconic acid (final concentration, 8.5 μmol mL^{-1}), the pH decreased to 2.9, and the amount of solubilized P was 146.0±1.0 μg mL^{-1}.

In the case of oxalic acid (final concentration, 6.6 μmol mL^{-1}), soluble P detected in media containing Ca-P and Fe-P was 60.2±0.9 and 21.6±2.1 μg mL^{-1}, respectively, whereas in medium containing Al-P, only trace amount of solubilized P was detected. Statistical analysis (*t* test) indicated that the amount of P solubilized by the addition of organic acid(s) is significantly lower than the amount of P released in the presence of fungal activity (data not shown). These observations are consistent with other reports demonstrating that the mere presence of organic acid does not account for all the soluble P that is solubilized by the microorganisms (Illmer et al. 1995; Illmer and Schinner 1995). Clearly, other factor(s) plays a role in the solubilization of inorganic P. Metabolite(s) in *Pseudomonas cepacia* Al-74 culture supernatant with molecular weight between 500 and 1,000 Da are responsible for 43.5% of the P released, whereas organic acids with molecular weight lower than 500 Da (including citric acid, oxalic acid, and gluconic acid) are only responsible for 36.2% of the P released (Young et al. 2000). Similarly, in the culture supernatants of media inoculated with *A. niger* isolates 1B and 6A, metabolite(s) with a molecular weight larger than 500 Da is responsible for 48±18% and 33±17% of the solubilized phosphate, respectively.

When *A. niger* 6A was used as the soil inoculant, significant increases in dry weight and N content of *B. chinensis* were observed in all three pot trials, whereas increases in P contents were observed in two out of three trials (Table 6). Therefore, the isolate 6A has the ability to solubilize the native soil P, which is not available to plant. Several studies have also shown that the addition of P-solubilizing fungi to soil amended with rock P significantly increases the available P in soil (Salih et al. 1989; Wahid and Mehana 2000) and reduces soil pH (Wahid and Mehana 2000). However, in the present study, the addition of rock P and P-solubilizing fungi caused neither a decrease in soil pH value nor an increase in available P in the soil

Table 6 Plant dry weight and the relative N and P content

Chemical P-fertilizer treatment	Dry weight (g/plant)		N content (mg/plant)		P content (mg/plant)	
	Control	6A	Control	6A	Control	6A
Trial I						
Rock phosphate	4.0	5.8*	121	206**	19	27**
Trial II						
None added	3.4	6.6*	140	272*	15	24**
Calcium superphosphate	4.8	8.7*	157	372*	22	43*
Rock phosphate	3.4	5.7*	117	226*	12	25*
Trial III						
None added	4.6	5.9**	298	378**	24	24
Rock phosphate	5.2	6.0**	328	385**	25	25

*Value is significantly different from control at 1% level by Student's *t* test.

**Value is significantly different from control at 5% level by Student's *t* test.

(Table 1). Probably, the organic acids are rapidly degraded by soil microbiota or neutralized by soil-buffering capacity; in addition, solubilized P can diffuse from the hyphae and be reprecipitated.

In summary, the present in vitro studies have demonstrated that the accumulation of soluble P is associated with the decrease in pH. Organic acid production and H^+ excretion through NH_4^+ uptake have been shown to decrease pH, but this mechanism is not the only one responsible for P solubilization. Therefore, we have an idea that the other mechanisms are responsible for P solubilization by *A. niger*. Unknown P-solubilizing compound (s) (MW>500 Da) reported by Young et al. (2000) and found in the present study can cause a partial P solubilization when acting alone. However, further study is needed to identify these unknown compounds and to confirm their role in P solubilization.

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