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Solubilization of aluminum phosphate by specific *Penicillium* spp.

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Abstract: The solubilization of hardly soluble aluminum phosphate ($AlPO_4$) by specific *Penicillium* spp. isolated from wheat rhizospheric soils was investigated in Pikovskaya agar and liquid medium, respectively. Most of the *Penicillium* isolates except *P. simplicissimum* AP11 and *P. variabile* AP15 developed clear transparent zone around the colony margin in plate assays. Results of broth assays show that the *Penicillium* isolates can efficiently solubilize aluminum phosphate in Pikovskaya liquid medium, and vary in their capabilities to release soluble phosphate from aluminum phosphate. All the isolates exhibit different abilities to lower the pH and increase the titratable acidity in the broth compared to the control. HPLC analysis shows that most of the isolates except the species of *P. aurantiogriseum* can excrete different concentrations of organic acids, including gluconic acid, citric acid, oxalic acid, malic acid and tartaric acids, in the broth. The release of soluble phosphate by the isolate *P. oxalicum* AP2, which is the best solubilizer of aluminum phosphate among the isolates, is accompanied by a significant drop of pH and an obvious rise of titratable acidity during 7 d of aluminum phosphate-solubilizing experiments. The effects of temperature, initial pH, concentration of aluminum phosphate and shaking speed on aluminum phosphate solubilization by *P. oxalicum* AP2 were also investigated, and the maximum contents of soluble phosphate released are recorded at temperature 30 °C, initial pH 6, aluminum phosphate concentration 20 g/L, and shaking speed 160 r/min.

Key words: solubilization; aluminum phosphate; *Penicillium* spp.; soluble phosphate; *Penicillium oxalicum*

1 Introduction

Phosphorus is one of the major macronutrients required for plant growth. However, phosphorus is sequestered by adsorption to the soil surface and precipitation by reaction with soil cations, particularly calcium, iron and aluminum [1]. Such hardly soluble forms of phosphorus are not readily available to crops for nutrition and yields may be limited.

A number of soil microorganisms have been reported as phosphate-solubilizing microorganisms having ability to solubilize hardly soluble inorganic phosphates [2−4]. These phosphate-solubilizing microorganisms can transform the insoluble form of phosphorus to soluble form by acidification, chelation, and exchange reactions [5]. Therefore, the use of phosphate-solubilizing microorganisms in agricultural practice would not only offset the high cost of manufacturing phosphatic fertilizers, but also reducenvironment pollution caused by traditional chemical phosphatic fertilizers production [6].

Penicillium fungi are considered to be a key group of soil microflora involved in phosphorus cycling, and are of special interest to solubilize hardly soluble inorganic phosphates [7−9]. Several *Penicillium* spp. strains have been isolated from soils and the capabilities of these *Penicillium* isolates to solubilize hardly soluble inorganic phosphates have been studied [10−11]. However, most of these studies were focused on the isolation of *Penicillium* spp. able to solubilize calcium-bearing phosphates, such as tricalcium phosphate and rock phosphates, and seldom studies have demonstrated the isolation of *Penicillium* spp. having the ability to solubilize aluminum-bearing phosphates, such as aluminum phosphate ($AlPO_4$).

Since most of the phosphorus is found as aluminum phosphate rather than calcium phosphate in soils [12], the present work thus focused on isolating phosphate-solubilizing *Penicillium* spp. from wheat rhizospheric soils in Hubei province of China and examining their phosphate-solubilizing potential in Pikovskaya agar and liquid medium containing aluminum phosphate as sole phosphorus source by plate and broth assays, respectively.

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2 Experimental

2.1 Isolation of aluminum phosphate-solubilizing *Penicillium* spp.

The strains of *Penicillium* spp. were isolated from soil samples collected from 15−25 cm depth from the rhizosphere of wheat in the farm located in the suburb of Wuhan city (Hubei Province, China). For the collection of rhizospheric soils, plants were uprooted and the soils attached to roots were then suspended in sterilized water, and mixed on the magnetic blender for 20 min to separate microorganisms from the soils completely. Serially diluted soil solution was planted on Pikovskaya agar medium (pH 7), which contained (per liter): 10 g glucose, 0.5 g/L yeast extract, 0.5 g/L $(NH_4)_2SO_4$, 0.2 g/L KCl, 0.1 g/L $MgSO_4·7H_2O$, 0.000 1 g/L $MnSO_4·H_2O$, 0.00 0 1 g/L $FeSO_4·7H_2O$ and 20 g agar. 5 g/L aluminum phosphate was added to the medium for selectively screening of microorganisms capable to release soluble phosphate from it. After 3−5 d of incubation at 30 °C, isolates colonies were further purified and identified. Specific *Penicillium* isolates were selected for further analysis in this work.

2.2 Plate and broth assays for aluminum phosphate solubilization by *Penicillium* isolates

Each *Penicillium* isolate was planted on Pikovskaya agar medium containing 5 g/L aluminum phosphate in plate assays. After being incubated for 7 d at 30 °C, the sizes of phosphate-solubilizing zone were measured. Broth assays were carried out in shake flasks with 100 mL Pikovskaya liquid medium (without agar) containing 0.5 g aluminum phosphate as sole phosphorus source. The pH of the broth was initially adjusted to 7. The 10 mm mycelial discs of each isolate from actively growing for 4 d on Pikovskaya agar medium were added as inoculum. Flasks were shaken under 160 r/min at 30 °C. Autoclaved, uninoculated medium was served as control. After 7 d of inoculation, the broth was first filtered and then the filtrate was centrifuged at 11 000*g* for 20 min, and the supernatant was assessed for the soluble phosphate, pH, titratable acidity, and organic acids, respectively. All experiments were performed in triplicate.

2.3 Broth assays and optimization of aluminum phosphate solubilization by *P. oxalicum* AP2

Solubilization of aluminum phosphate by *P. oxalicum* AP2 was tested in 250 mL flasks with 100 mL Pikovskaya liquid medium containing 0.5 g aluminum phosphate. The pH of the medium was initially adjusted to 7. The 10 mm mycelial discs of each isolate from actively growing for 4 d on Pikovskaya agar medium were added as inoculum. Flasks were shaken under 160 r/min at 30 °C for 7 d, and samples were taken every day for the examination of soluble phosphate, pH, and titratable acidity, respectively. To study the optimal temperature for aluminum phosphate solubilization by *P. oxalicum* AP2, flasks were shaken at different temperatures (20, 25, 30, 35, and 40 °C, respectively). The influence of initial pH was studied by adjusting the initial pH of the medium at 5, 6, 7, 8, and 9, respectively. Subsequently, the effect of the concentration of aluminum phosphate was studied at 1, 5, 10, 20, and 30 g/L, respectively. Finally, the effect of shaking speed (100, 120, 140, 160, and 180 r/min, respectively) on aluminum phosphate solubilization was also investigated. All experiments were performed in triplicate.

2.4 Analytical methods

Content of soluble phosphate in the filtrate was determined by using the vanadium-ammonium molybdate colorimetric method with a UV-vis 8500 spectrophotometer at 490 nm [13]. The pH was recorded with a pH meter equipped with glass electrode. The titratable acidity was determined by titrating the filtrate with 85.7 mmol/L standard NaOH solutions [14]. Organic acids were detected by HPLC (Agilent 1100) at 214 nm [15]. Values were given as means ± standard deviation for triplicate samples.

3 Results and discussion

3.1 Isolation of *Penicillium* spp. and assays for solubilization of aluminum phosphate

Fungi could be easily isolated from soils, and by isolating from a diverse range of soils using different techniques, and it was anticipated that a more diverse range of *Penicillium* would be encountered [16]. In this work, a total of 121 fungi were isolated from the soil samples collected from the rhizosphere of wheat by serially transferring to Pikovskaya agar medium containing aluminum phosphate as sole phosphorus source. Out of 121, 15 fungal isolates representing 6 species of *Penicillium* were further tested for aluminum phosphate solubilization in Pikovskaya agar and liquid medium.

Most of the *Penicillium* isolates except the two isolates (*P. simplicissimum* AP11 and *P. variabile* AP15) developed clear transparent zone between 5.36 and 7.91 mm around the colony margin due to solubilization of aluminum phosphate on Pikovskaya agar medium in plate assays (Table 1). However, the remaining five isolates, i.e., *P. oxalicum* AP2 and AP3, *P. rugulosum* AP5 and AP6, and *P. radicum* AP13 developed diffused zone of aluminum phosphate solubilization.

Results of broth assays show that all the isolates

Table 1 Aluminum phosphate solubilization in Pikovskaya agar and liquid medium by *Penicillium* spp. isolated from wheat rhizospheric soils (Values given as means ± standard deviation for triplicate samples)

Strain	Isolate	Zone size/ mm	Soluble phosphate/ ($g \cdot L^{-1}$)	pH	Titratable acidity/ ($mmol \cdot L^{-1}$)	Organic acid/ ($mmol \cdot L^{-1}$)
Control (Uninoculated)		—	—	7.37 ± 0.6	—	—
P. oxalicum	AP1	6.84 ± 0.6	1.81 ± 0.2	4.26 ± 0.4	29.93 ± 3.0	Malic (10.34 ± 1.0), Oxalic (4.16 ± 0.4), Gluconic (2.75 ± 0.3)
	AP2	Diffused	2.28 ± 0.3	3.95 ± 0.3	32.54 ± 3.2	Malic (9.67 ± 0.9), Oxalic (5.33 ± 0.5), Gluconic (2.71 ± 0.2)
	AP3	Diffused	1.94 ± 0.3	3.74 ± 0.3	33.62 ± 3.2	Malic (11.46 ± 1.1), Oxalic (4.83 ± 0.5), Gluconic (3.96 ± 0.3)
	AP4	7.91 ± 0.7	1.57 ± 0.2	4.27 ± 0.4	27.45 ± 2.8	Malic (7.95 ± 0.8), Oxalic (4.16 ± 0.4), Gluconic (3.37 ± 0.3)
P. rugulosum	AP5	Diffused	1.86 ± 0.2	3.55 ± 0.3	29.53 ± 2.6	Citric (10.34 ± 0.9), Gluconic (6.76 ± 0.7)
	AP6	Diffused	1.72 ± 0.2	4.37 ± 0.4	29.57 ± 3.1	Citric (9.49 ± 1.0), Gluconic (5.37 ± 0.5)
	AP7	5.77 ± 0.5	1.57 ± 0.2	4.19 ± 0.4	28.63 ± 2.9	Citric (6.5 ± 0.6), Gluconic (4.47 ± 0.4)
P. aurantiogriseum	AP8	6.38 ± 0.5	1.37 ± 0.3	4.33 ± 0.3	30.25 ± 2.9	—
	AP9	5.49 ± 0.6	1.51 ± 0.2	4.78 ± 0.4	31.24 ± 3.1	—
P. simplicissimum	AP10	5.36 ± 0.5	1.90 ± 0.2	4.34 ± 0.4	40.25 ± 4.2	Gluconic (10.67 ± 1.1), Citric (2.93 ± 0.3)
	AP11	—	1.78 ± 0.2	4.28 ± 0.4	38.67 ± 4.1	Gluconic (9.45 ± 0.9), Citric (2.81 ± 0.2)
P. radicum	AP12	7.24 ± 0.7	1.58 ± 0.1	4.62 ± 0.4	33.24 ± 3.2	Gluconic (11.25 ± 1.2)
	AP13	Diffused	1.65 ± 0.2	4.07 ± 0.3	37.16 ± 3.8	Gluconic (10.93 ± 1.0)
P. variabile	AP14	5.62 ± 0.4	1.53 ± 0.2	4.76 ± 0.4	32.08 ± 3.4	Gluconic (7.04 ± 0.6); Tartaric (3.29 ± 0.3)
	AP15	—	1.61 ± 0.2	4.43 ± 0.4	27.65 ± 2.3	Gluconic (7.71 ± 0.7), Tartaric (1.83 ± 0.2)

could effectively release soluble phosphate from aluminum phosphate compared to the controlled one (Table 1). However, the isolates vary in their capacities to release soluble phosphate from aluminum phosphate, even though they are the same species. For example, whilst *P. oxalicum* AP2 was the most effective at aluminum phosphate solubilization among the isolates, but another isolate AP4 of the same species just ranked twelfth effective. This indicates that the phosphate-solubilizing activity is not necessarily a species-wide trait [16].

Isolates not developing clear transparent zone on solid agar plates were not relatively ineffective at solubilizing aluminum phosphate in liquid medium, and vice versa. Results show that the two isolates, namely *P. simplicissimum* AP11 and *P. variabile* AP15, which did not develop clear transparent zone, could efficiently solubilize aluminum phosphate.

An obvious drop of the pH in the broth inoculated with the *Penicillium* isolates was observed compared to the controlled one (Table 1). However, the extent of pH reduction was different with different isolates. For example, the *P. aurantiogriseum* AP8, was the least effective at aluminum phosphate solubilization but its pH

was 4.33, which ranked sixth high among the isolates. Moreover, *P. oxalicum* AP2 released the highest content of soluble phosphate yet the pH was higher than that of *P. oxalicum* AP3 and *P. rugulosum* AP5.

Significant increase of titratable acidity was detected in the broth inoculated with the *Penicillium* isolates compared to the controlled one, and the amount of titratable acidity varied with the isolates involved (Table 1).

Penicillium spp. strains are often known to produce a wide range of secondary metabolites, such as organic acids [17]. Phosphate solubilization by *Penicillium* fungi is generally attributable to the production of organic acids that can directly dissolve phosphate precipitates, or chelate phosphate precipitating cations with the concomitant release of soluble phosphate into solution [18]. In this work, from HPLC analysis multiple organic acids detected, including gluconic acid, citric acid, oxalic acid, malic acid and tartaric acids, in the broth inoculated with most of the *Penicillium* isolates (Table 1). The production of organic acids played a vital role in the acidification of the broth, which could be illustrated by the decrease of pH and the increase of titratable acidity in the broth compared to the controlled one, and thus released soluble phosphate from aluminum phosphate.

Although organic acids production appeared to be the major reason for the solubilization by *Penicillium* fungi, it was not the sole factor responsible for aluminum phosphate solubilization. In this work, the two isolates of *P. aurantiogriseum* AP8 and AP9 could not excrete any organic acid in the solubilization of aluminum phosphate. However, they could still lower the pH and increase the titratable acidity in the broth compared to the control, and thus also effectively released the soluble phosphate from aluminum phosphate. The result indicates that there must have some other factors responsible for aluminum phosphate solubilization. For example, there are some reports that the cellular H^+ exudation to balance NH_4^+ uptake may be participated to lower the pH in the broth and thus increase the solubility of hardly soluble inorganic phosphates [11].

However, phosphate solubilization by microorganisms is not a simple phenomenon, and it may be determined by many factors, such as nutritional, physiological and growth conditions of the microorganisms [19]. Therefore, it can be accomplished by a range of mechanisms, and needs further studies to understand the mechanisms of aluminum phosphate solubilization used by different *Penicillium* spp. isolated from different soils.

3.2 Broth assays for aluminum phosphate solubilization by *P. oxalicum* AP2

The species of *P. oxalicum* were reported to solubilize Ca-bearing phosphate, such as rock phosphate in the pot culture or field conditions [20−22]. In this work, *P. oxalicum* AP2 was the best solubilizer of aluminum phosphate among all the isolates of *Penicillium* spp., and the results of aluminum phosphate solubilization by this isolate in Pikovskaya liquid medium are presented in Fig. 1. Results show that the content of soluble phosphate released by *P. oxalicum* AP2 increases gradually during 7 d of aluminum phosphate-solubilizing experiments, and the increase of the soluble phosphate released in the broth is accompanied by a significant drop of pH and an obvious rise of titratable acidity. Simple regression analyses indicate that there is a significant negative correlation (r=−0.93; p<0.01) between the content of soluble phosphate released and pH, and a significant positive correlation between the content of soluble phosphate released and the titratable acidity (r=0.96; p<0.01), respectively.

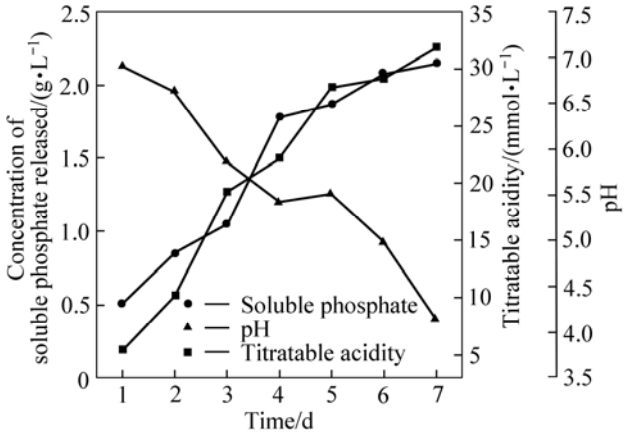

Fig. 1 Changes in concentration of soluble phosphate released, pH and titratable acidity during 7 d of aluminum phosphate-solubilizing experiments by *P. oxalicum* AP2

3.3 Optimization of temperature, pH, concentration of aluminum phosphate and shaking speed for solubilization of aluminum phosphate by *P. oxalicum* AP2

The effect of temperature on the soluble phosphate released during 7 d of aluminum phosphate-solubilizing experiments by *P. oxalicum* AP2 is shown in Fig. 2. Results show that the optimal temperature for the solubilization of aluminum phosphate by *P. oxalicum* AP2 is 30 °C, and when the temperature is higher or lower than the optimal temperature, the concentration of soluble phosphate released decreases.

It is observed from Fig. 3 that initial pH of the broth has significant effect on aluminum phosphate solubilization by *P. oxalicum* AP2, and relatively acidic environments favor the solubilization of aluminum

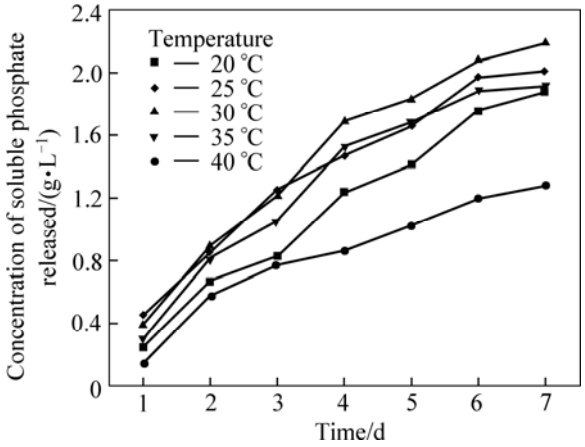

Fig. 2 Effect of temperature on soluble phosphate released by *P. oxalicum* AP2 during 7 d of aluminum phosphate-solubilizing experiments

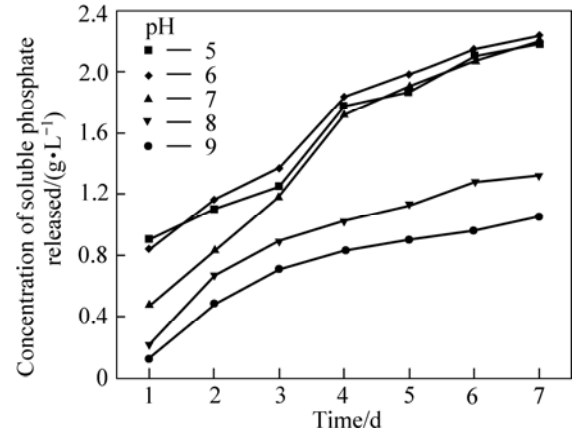

Fig. 3 Effect of initial pH on soluble phosphate released by *P. oxalicum* AP2 during 7 d of aluminum phosphate-solubilizing experiments

phosphate as compared to that under the alkaline conditions. The concentration of soluble phosphate is recorded the highest at initial pH 6, when higher or lower than this pH, the concentration of soluble phosphate obviously decreases.

Figure 4 shows that the greatest concentration of soluble phosphate released during 7 d of aluminum phosphate-solubilizing experiments by *P. oxalicum* AP2 is obtained when the concentration of aluminum phosphate is 20 g/L. Increasing concentration of aluminum phosphate promotes the solubilization of aluminum phosphate by *P. oxalicum* AP2. The results are similar to the results reported by SINGH and REDDY [21]. However, when the concentration of aluminum phosphate increases to 30 g/L, the concentration of soluble phosphate released decreases. This adverse effect could be attributed to the limited availability of nutrients and O_2 with increasing concentration of aluminum phosphate and the mechanical damage to fungal cells by solids.

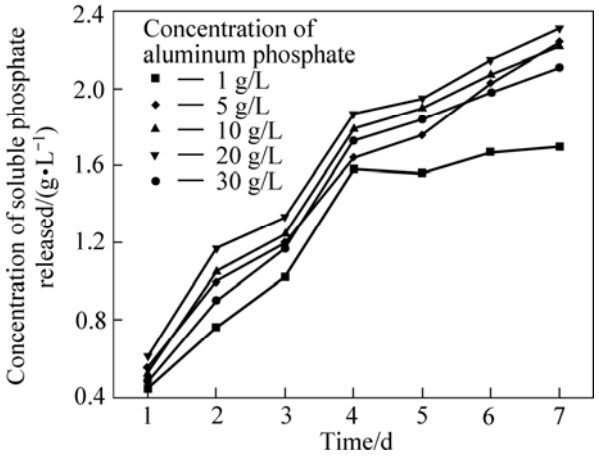

Fig. 4 Effect of concentration of aluminum phosphate on soluble phosphate released by *P. oxalicum* AP2 during 7 d of aluminum phosphate-solubilizing experiments

The data of the content of soluble phosphate released by *P. oxalicum* AP2 at different shaking speeds are shown in Fig. 5. The content of soluble phosphate increases with the increase of shaking speed from 100 to 160 r/min. However, at the excessive shaking speed of 180 r/min, the growth of the isolate is often weakened by the shear stress resulting from the strong stirring. That is why the concentration of soluble phosphate released decreases when the shaking speed increases from 160 to 180 r/min.

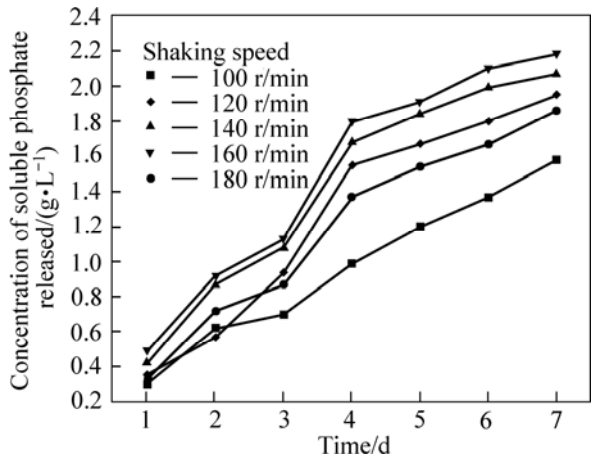

Fig. 5 Effect of shaking speed on soluble phosphate released by *P. oxalicum* AP2 during 7 d of aluminum phosphate-solubilizing experiments

4 Conclusions

1) A total of 15 fungal isolates representing 6 species of *Penicillium* are tested for the solubilization of aluminum phosphate in Pikovskaya agar and liquid medium, respectively. All the *Penicillium* species could effectively solubilize aluminum phosphate in Pikovskaya liquid medium, although the two isolates, namely *P.*

simplicissimum AP11 and *P. variabile* AP15, do not develop clear transparent zone around the colony margin in qualitative plate assays.

2) Acidification of the broth seems to be the major mechanism for aluminum phosphate solubilization by the *Penicillium* isolates, and the increase of soluble phosphate released is significantly correlated with a drop of pH and an increase of titratable acidity, which demonstrates experimentally that different concentrations of organic acids, including gluconic acid, citric acid, oxalic acid, malic acid and tartaric acids, are detected in the broth inoculated with most of the *Penicillium* isolates except the species of *P. aurantiogriseum*.

3) The isolate *P. oxalicum* AP2 leads to the most extensive soluble phosphate released at 30°C and initial pH of 6, respectively. Increasing concentration of aluminum phosphate no more than 20 g/L is shown to promote the release of soluble phosphate by *P. oxalicum* AP2. The maximum concentration of soluble phosphate released by *P. oxalicum* AP2 is recorded when shaking speed is 160 r/min, and when the shaking speed is higher or lower than this speed, the concentration of soluble phosphate released decreases.

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