A Study of Phosphate Solubilizing Capacity by *Penicillium Aurantiogriseum* under Different Carbon and Nitrogen Resources

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Abstract. Phosphate-solubilizing fungi have been successfully applied to the release of phosphorus from insoluble tricalcium phosphate. A phosphate-solubilizing fungi Penicillium aurantiogriseum was isolated and investigated the phosphorus solubilizing capacity under different carbon and nitrogen resources. The highest released phosphorus content reached 1000 mg/L in ammonium and sucrose conditions. Carbon resources did not limit the release of phosphorus by Penicillium aurantiogriseum. However, nitrate and urea significantly reduced the phosphorus release, which had a low phosphorus content (~780 mg/L). Glucose and ammonium were more efficient for fungal growth and organic acid secretion. Oxalic acid secreted by Penicillium aurantiogriseum dominated the release of phosphorus. The formed calcium oxalate promoted the dissolution of tricalcium phosphate. This study indicated that the effective utilization of Penicillium aurantiogriseum to dissolve tricalcium phosphate need to support more ammonium nitrogen.

1 MIntroduction

Phosphorus (P) is a necessary element for crop growth and function in chlorophyll and proteinsynthesis[1]. However, the P uptake by crops is limited due to the low mobility of P in soil[2]. Most P in the soil exists in the form of insoluble inorganic phosphate, such as calcium, ferric and aluminium phosphate[3]. These phosphates usually have a low K*sp* value (10^{-19}~10^{-33}), which is difficult to be directly absorbed and utilized by plants[4-6]. Although chemical phosphate fertilizer supply can significantly increase the crop yield, more than 85% P was adsorbed or precipitated in soil via the formation of insoluble phosphate[7]. The amount of P stored in the soil would be continuously used by crops for 50 years[8]. In addition, the increased input of chemical phosphate fertilizer would also accelerate the consumption of phosphate ores. As a non-renewable resource, the global phosphate ores would be depleted in the next 50-100 years[9]. Therefore, improving the utilization of soil insoluble phosphate is an effective way to reduce the consumption of phosphate ore and sustainable agricultural development.

The acidic conditions can significantly promote the release of P from insoluble phosphate in the soil[10]. In nature, the release of P is mainly driven by plant roots and microorganisms[10]. On the one hand, the CO_2 produced by roots and microorganisms via respiration can form a lower pH micro-environment, which promotes the release of phosphorus[10]. On the other hand, the roots and microorganisms can also secrete organic acid to promote the release of P via acidity and chelation[10, 11]. Compared with chemical acid, the organic acid is more efficient in the dissolution of insoluble phosphate. For example, the average P released from rock phosphate by oxalic acid was 21 mg, while sulfuric acid only solubilized 14 mg[12].

Phosphate solubilizing fungi (PSF) are able to produce lots of organic acids, especially for oxalic acid. Compared with bacteria, PSF has a stronger ability of organic acid secretion. The solubilized P by PSF is ~10 times compared with bacteria[13]. Cations such as calcium and iron promote the release of phosphorus in phosphate minerals[14]. More importantly, PSF also has a cell wall and mycelium structure, which is not only improving environmental resistance, but also easy to capture more P via the extension of hyphae [13, 15]. In addition, PSF can also maintain the phosphate-solubilizing ability after continuous incubation[13]. Therefore, the PSF is usually considered as the primary candidate for the dissolution of insoluble phosphate in the soil.

The oxalic acid produced by PSF usually dominates the release of P from insoluble phosphate. Compared with other organics (e.g., citric, malic, and tartaric acids), oxalic acid has the highest acidity constant (pK_{a1} = 1.25 and pK_{a2} = 4.27)[14]. The oxalic acid secreted by PSF can significantly reduce the pH to ~2 after seven days of incubation[13]. In addition, oxalic acid also shows a high

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ability to chelate metal cations such as Ca^{2+}, Fe^{3+}, Al^{3+}, etc.

However, the secretion of oxalic acid by PSF are usually influenced by different environmental factors, including pH, carbon and nitrogen resources, metal cation, etc[14]. In the cause of pH, the optimal oxalic acid secreted by PSF occurred at pH 5-6 and was inhibited at pH<3[14]. A metal cation like Mn^{2+} can promote oxalic acid production[14]. The existence of nitrate promotes the secretion of oxalic acid by PSF, while ammonium inhibits, and then affecting the phosphate solubilizing capacity[14]. Hence, choosing a suitable environment is important for the secretion of oxalic acid by PSF, especially for the solubilization of P from insoluble phosphate.

This study aims to investigate the P solubilizing capacity by PSF-*Penicillium aurantiogriseum* (*P. aurantiogriseum*) under different carbon and nitrogen resources. The organic acid secreted by *P. aurantiogriseum* was also investigated in this research. The organic acid was analysed by high-performance liquid chromatography (HPLC). The soluble P content was analysed by inductively coupled plasma-optical emission spectrometry (ICP-OES). The mineralogical of precipitation was analysed by X-ray powder diffraction (XRD).

2 Materials and methods

2.1 Isolation and identification of fungi

2.1.1 Screening phosphate solubilizing fungi

The phosphate-solubilizing fungus was screened from the wheat rhizosphere soil located at Suzhou city, Anhui province, China. The soil sample was collected and prepared for a suspension with sterile water at a 1:9 ratio. After shaking for 30 min at 25 °C, the suspension was diluted to 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and then coated on Pikovskaya (PVK) solid medium with 0.1mL, respectively. After five days of incubation (28 ^{o}C), the fungi with obvious phosphate-solubilizing circles were collected and purified. Then, the purified fungi were punched and inoculated to PVK solid medium for the preliminary identification of P solubilizing capacity. The size of colony diameter (d, cm) and phosphate-solubilizing circle (D, cm) were observed and recorded.

2.1.2 Molecular biology identification

Genomic DNA of phosphate-solubilizing fungi with high P solubilizing capacity was extracted using a fungal DNA kit (GBCBIO Technologies). The semi-nested PCR protocol was performed to amplify the internal transcribed spacer (ITS) region. Universal primers ITS1 (5′ - TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′ - TCCTCCGCTTATTGATATGC-3′) was applied for the amplification round. The ITS rRNA gene was sequenced and searched in GenBank. All the selected sequences were aligned using the Clustal W. The neighbour-joining phylogenetic tree was constructed by the MEGA 7.0.

2.2 Carbon and Nitrogen preparation

Glucose, sucrose and starch were used as the three different carbon sources in the experiment. Ammonium-nitrogen ($(NH_4)_2SO_4$), nitrate-nitrogen (KNO_3) and urea were selected as the three different nitrogen sources. The carbon source and nitrogen source reagents used in this experiment were supplied by China Pharmaceutical Group Chemical Reagent Co., Ltd. (Shanghai, China).

2.3 Fungal incubation under different carbon and nitrogen

The P solubilizing capacity by *P. aurantiogriseum* was performed with six treatments, i.e., glucose, sucrose, starch, ammonium, nitrate and urea. Tricalcium phosphate ($Ca_3(PO_4)_2$) was used as the initial insoluble phosphate. The initial PVK liquid medium contains 10 g glucose, 0.5 g $(NH_4)_2SO_4$, 0.2 g NaCl, 0.2 g KCl, 0.25 g $MgSO_4 \cdot 7H_2O$, 0.03 g $MnSO_4 \cdot 4H_2O$, and 0.03 g $FeSO_4 \cdot 7H_2O$ in 1 L deionized water. The reagents in this study were supplied by China Pharmaceutical Group Chemical Reagent Co., Ltd, Shanghai, China. Before incubation, the PVK liquid medium with different carbon and nitrogen conditions were sterilized (121 °C, 20 min). Then, 0.5 g $Ca_3(PO_4)_2$ and 1 mL fungal spores (10^7 CFU/mL) were added into a 250 mL Erlenmeyer flask with 100 mL PVK medium. Finally, the flask was sealed with a sealed membrane (BS-QM-003, Biosharp) containing 0.22 μm air-permeable membrane. All the tests were conducted under sterile conditions. After seven days of incubation, the PVK medium with different carbon and nitrogen was filtered through a 0.45 μm polyethersulfone (PES) membrane. The filtrate was collected for the analysis of organic acids and soluble P. The filter was collected for the analysis of biomass and mineralogical. All the treatment was performed with three replicates.

2.4 Statistical analysis

The P solubilizing experiments under different carbon and nitrogen by PSF were performed with triplicates. Both the means and standard deviations were calculated and presented for each treatment. The Tukey's honestly significant difference test ($p < 0.05$) via one-way ANOVA was identified in each treatment by using SPSS 16.0.

2.5 Instrumentation

The pH value in the medium was measured by the FE20 pH meter (Mettler Toledo, Int. Inc.). The soluble P concentration in the medium was analyzed by ICP-OES (PerkinElmer Avio 200). The P calibration curves (1, 5, 10, 20, 50, 100 mg/L) were prepared before the test, which had a 0.999 R square of the external standard curve.

The organic acid concentration was analyzed by HPLC (Agilent 1200). The temperature of the chromatographic column was 30 °C. The standard curves of citric and oxalic acids were performed to 50, 100, 200, 500, 1000, and 2000 mg/L. The R square value of the standard curve was 0.999. The methanol (CH_3OH) and 2.5 wt‰ potassium phosphate monobasic (KH_2PO_4) consisted of the mobile phase with a ratio of 1:99. The mobile phase pH was adjusted to 2.8 by using phosphoric acid [16]. The flow rate of the mobile phase was 1 mL/min.

The precipitate was analyzed by X-ray diffraction (D/Max-2500, Rigaku Corporation) (Cu-Kα; 36 kV; 20 mA; scanned from 5° to 60° at a speed of 4°/sec). The XRD pattern was identified by MDI Jade 6.5 software.

3 Results & Discussion

3.1 Identification and preliminary analysis of phosphate solubilizing ability

A phosphate-solubilizing fungi strain of AHF-4 was screened from wheat rhizosphere soil in Wanbei area Anhui province. After purification, the strain was cultured on a glucose potato agar medium (PDA) to observe the colony morphology and microscopic characteristics. The formed flat colony had a regular edge and was distributed with white-yellow and blue-white circles (Fig. 1A). The slightly flocculent occurred in the centre of the colony (Fig. 1A). Microscopic observation showed that the strain has broom-shaped conidia (Fig. 1A). Combined with colony characteristics, The strain was preliminarily identified as *Penicillium*. After homologous sequences blast in NCBI (Fig. 1C). The ITS sequence of AHF-4 is highly similar to *Penicillium aurantiogriseum* in Genbank (Fig. 1C). The results of the phylogenetic tree showed that the strain of AHF-4 belongs to *Penicillium aurantiogriseum* (Fig. 1C). After one, three, five and seven days of incubation, the phosphate-solubilizing circle by *Penicillium aurantiogriseum* increased to 2.13, 2.72, 3.05 and 3.74 cm, respectively (Fig. 2).

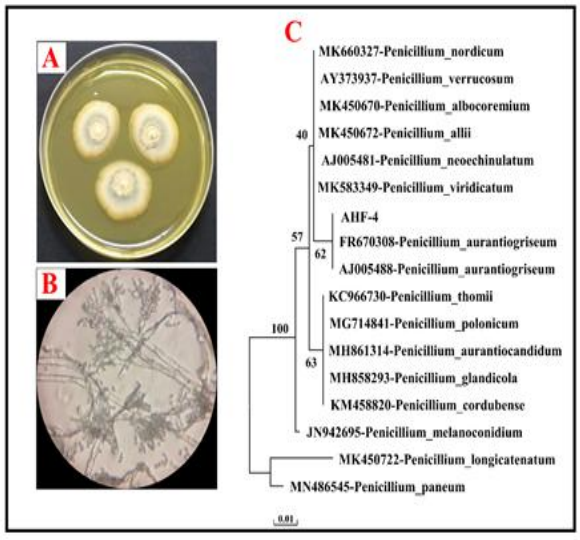

Fig. 1. The colony image (A), microscopic image (B) and phylogenetic tree analysis (C) of *Penicillium aurantiogriseum.*

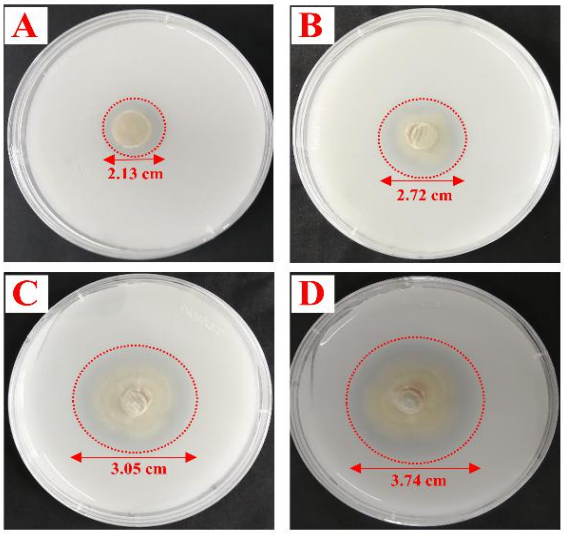

Fig. 2. The formed phosphate-solubilizing circles by *Penicillium aurantiogriseum* on the PVK solid medium after one (A), three (B), five (C) and seven (D) days of incubation.

3.2 The pH value in the medium under different C and N resources by *P. aurantiogriseum*

The initial pH of the PVK medium was 6.5. The pH values in glucose, sucrose and starch treatments decreased to 2.6, 3.3 and 2.7 after seven days of incubation, respectively (Fig. 3). Under different nitrogen resources, the medium pH decreased to 2.6, 3.7 and 3.5 in ammonium, nitrate and urea after seven days of incubation, respectively (Fig. 3). These results indicated that glucose, starch and ammonium nitrogen was more efficient for the secretion of organic acids by P. *aurantiogriseum*.

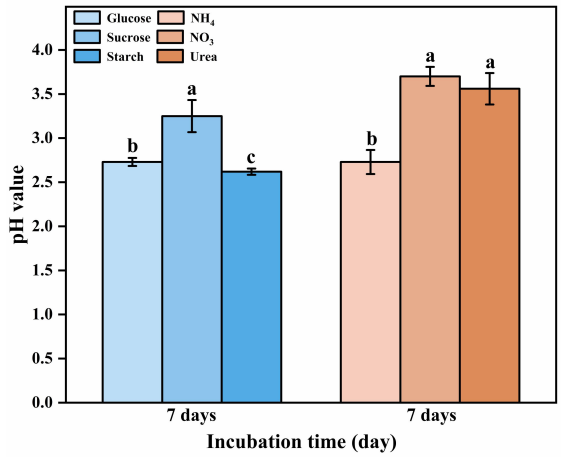

Fig. 3. The pH values in the PVK medium after seven days of incubation under different carbon and nitrogen resources. The error bars in each column represent the standard deviations of three replicates.

3.3 The dry biomass of fungi under different carbon and nitrogen resources

The dry biomass of fungi has no significant difference among the three types of carbon resources, which ranged

from 0.31 to 0.32 g (Fig. 4). However, the urea limited the growth of fungi. The dry biomass was 0.28 g, significantly lower than ammonium and nitrate, i.e., 0.32 and 0.34 g (Fig. 4). The growth of fungi did not influence by the types of carbon, but rather by nitrogen. Unlike other PSF, *P. aurantiogriseum* was favourable in both utilization of ammonium and nitrate. However, the existence of carbonate would limit the growth of PSF[17]. The adsorption of urea by fungi would release the CO_2, hence the formed carbonate would limit the growth of *P. aurantiogriseum.*

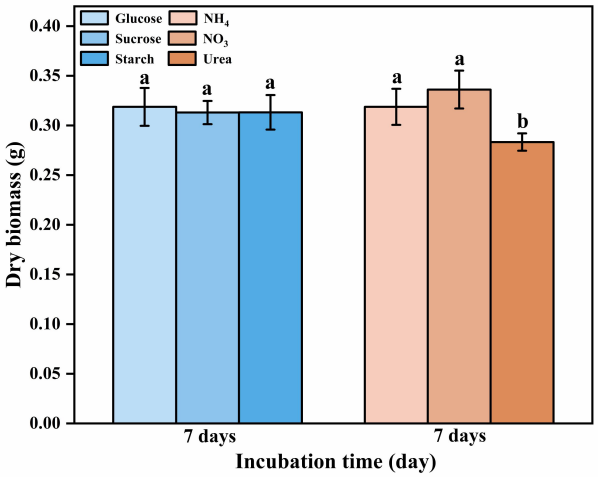

Fig. 4. The dry biomass values after seven days of incubation under different carbon and nitrogen resources. The error bars in each column represent the standard deviations of three replicates.

3.4 The release of P under different carbon and nitrogen resources

The P content in three types of carbons had no significant difference after seven days of incubation, more than 950 mg/L P was released from tricalcium phosphate (Fig. 5). However, the P content in nitrate and urea was significantly lower than ammonium after seven days of incubation, i.e., 779.5 vs. 950 mg/L (Fig. 5). This result indicated that *P. aurantiogriseum* can promote the dissolution of insoluble phosphate under different carbon and nitrogen resources (Fig. 5). Compared with other PSF(e.g., *Aspergillus niger* and *Penicillium oxalicum*), *P. aurantiogriseum* showed a higher P solubilizing capacity in calcium phosphate[13, 18, 19].

The dissolution of calcium phosphate would be more efficient in the lower pH value. However, the pH in nitrate and urea almost reached 4, much higher than 2.5 in ammonium (Fig. 3). In addition, the H^+ produced by the assimilation process of ammonium would also contribute to the decrease of pH value[14]. Hence the release of P from calcium phosphate by *P. aurantiogriseum* in ammonium was much higher than other nitrogen. In other words, the utilization of *P. aurantiogriseum* to dissolve insoluble phosphate in soil should increase the input of ammonium nitrogen.

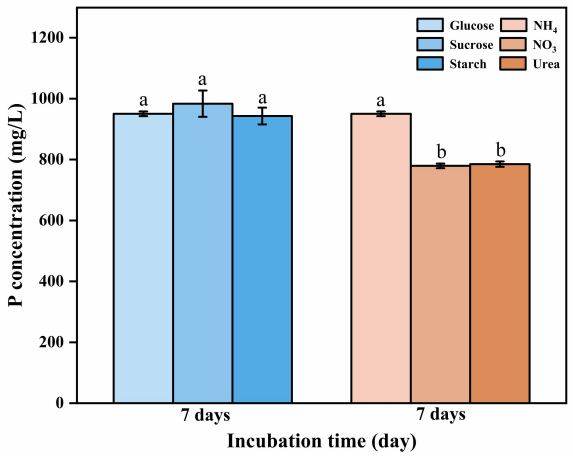

Fig. 5. The P content after seven days of incubation under different carbon and nitrogen resources. The error bars in each column represent the standard deviations of three replicates.

3.5 The organic acid secretion by fungi under different carbon and nitrogen resources

Oxalic and citric acids were the primary organic acids secreted by *P. aurantiogriseum* (Fig. 6). However, both the oxalic and citric acids content were lower than 300 mg/L. The highest oxalic acid secretion occurred in glucose and ammonium resources (Fig. 6A). While sucrose and urea can stimulate *P. aurantiogriseum to* secrete more citric acid (Fig. 6B). The primary organic acid (oxalic and citric acid) secreted by *P. aurantiogriseum* was similar to other PSF but had a lower content[18, 19]. Especially for oxalic acid, the secretion content was much lower than *Aspergillus niger* and *Penicillium oxalicum*, i.e., 205 mg/L vs. ~1000 mg/L[18, 19]. However, *P. aurantiogriseum* showed lower biomass compared with the above two PSF (0.3 vs. 0.6 g). That is to say, *P. aurantiogriseum* was more efficient in the release of P from calcium phosphate. In addition, oxalic acid also had a strong combination ability with calcium cations. The formed calcium oxalate would also contribute to the low content of oxalic acid.

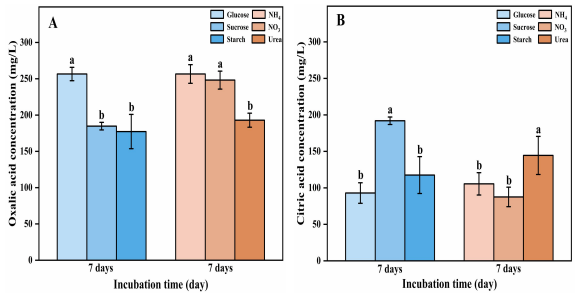

Fig. 6. The oxalic acid (A) and citric acid (B) concentration in the medium after seven days of incubation under different carbon and nitrogen resources. The error bars in each column represent the standard deviations of three replicates.

3.6 XRD analysis

The peaks at 12.1°, 25.8°, 29.5° and 32.2° stand for the mineral of calcium phosphate ($Ca_3(PO_4)_2$) (Fig. 7)[3]. The newly formed mineral of calcium oxalate (CaC_2O_4) located at 13.7° and 23.7° occurred in each treatment

(Fig. 7)[3]. This result also indicated that the oxalic acid secreted by *P. aurantiogriseum* dominated the release of P. The different carbon and nitrogen did not influence the mineralization in the process of P dissolution. This XRD result was also confirmed by the other PSF.

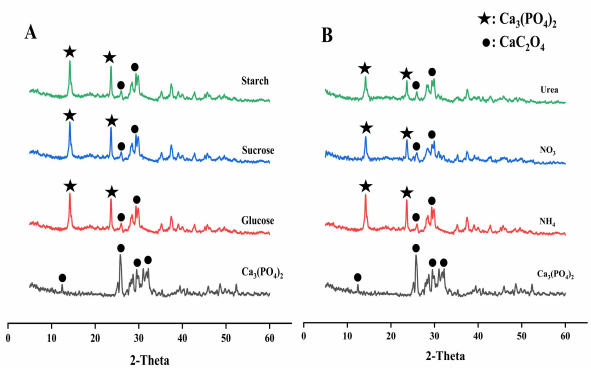

Fig. 7. The XRD patterns of precipitation in carbon (A) and nitrogen (B) resources.

4 Conclusion

The screened PSF *P. aurantiogriseum* shows a high P solubilizing capacity under different carbon and nitrogen resources. Compared with carbon, the types of nitrogen significantly influenced the release of P by fungi. Ammonium nitrogen is more efficient for fungal growth and organic acid secretion, especially for oxalic acid. Both the carbon and nitrogen did not influence the formation of calcium oxalate. The utilization of *P. aurantiogriseum* to dissolve insoluble phosphate need to increase ammonium nitrogen input.

Acknowledgements

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