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Isolation of Phosphate-Solubilizing Fungi from Phosphate Mines and Their Effect on Wheat Seedling Growth

Chunqiao Xiao · Ruan Chi · Huan He · Guanzhou Qiu · Dianzuo Wang · Wenxue Zhang

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Abstract Three phosphate-solubilizing fungi, identified as *Penicillium expansum*, *Mucor ramosissimus*, and *Candida krissii*, were isolated from phosphate mines (Hubei, People's Republic of China) and characterized. All the isolates demonstrated diverse levels of phosphate-solubilizing capability in National Botanical Research Institute's phosphate growth medium containing rock phosphate as sole phosphate source. Acidification of culture medium seemed to be the main mechanism for rock phosphate solubilization. Indeed, citric acid, oxalic acid, and gluconic acid were shown to be present in the culture medium inoculated with these isolates. Moreover, the isolates produced acid and alkaline phosphatases in culture medium, which may also be helpful for RP solubilization. A strong negative correlation between content of soluble phosphorus and pH (r=– 0.89; p<0.01) in culture medium was observed in this study. All the isolates promoted growth, soil available phosphorus, phosphorus, and nitrogen uptake of wheat seedling in field soil containing rock phosphate under pot culture conditions, thus demonstrating the capability of these isolates to convert insoluble form of phosphorus into plant available form from rock phosphate, and therefore hold great potential for development as biofertilizers to enhance soil fertility and promote plant growth.

Keywords Isolation · Phosphate-solubilizing fungi · Phosphate mines · Rock phosphate · Soluble phosphorus · Wheat

C. Xiao · H. He · G. Qiu · D. Wang
Key Laboratory of Biometallurgy of Ministry of Education,
School of Resources Processing and Bioengineering, Central South University, Changsha 410083, China

C. Xiao · R. Chi (✉)
Key Laboratory for Green Chemical Process of Ministry of Education,
Hubei Key Lab of Novel Reactor and Green Chemical Technology, Wuhan Institute of Technology,
Wuhan 430073, China
e-mail: whjhx@tom.com

W. Zhang
Yunnan Phosphate Chemical Group Co. Ltd., Jinning 650600, China

Introduction

Phosphorus is an essential nutrient element required for plants, but the low availability of phosphorus in soil has become a limiting factor for plant growth [1]. Therefore, the optimal growth of plants demands an input of high, often costly phosphate fertilizer. Recently, natural phosphate-bearing materials, such as rock phosphate (RP) have been recognized as less costly alternatives for phosphate fertilizer [2]. However, in natural conditions, most of RP in the world is not suitable for the manufacturing of commercial phosphate fertilizer because of its low phosphorus content and poor solubility [3].

Microorganisms play a critical role in natural phosphorus cycle, and recently, microbially-based approach has been proposed to improve the agronomic value of RP [4, 5]. This approach not only compensates for higher cost of manufacturing phosphate fertilizer in industry but also reduces environment pollution caused by traditional chemical process.

Considering this factor, many phosphate-solubilizing microorganisms have been isolated from different soils, and an increase in phosphorus availability to plants through the inoculation of phosphate-solubilizing microorganisms has been reported under pot and field conditions [6–8]. However, in fact, these microorganisms are only a small percentage of the total microbial population, and few of them present a high potential to solubilize RP under natural conditions, which seriously restrains the application of this microbially based technique. Moreover, a main problem, indeed, is connected with the fate and activity of introduced microorganisms [9]. Normally, many isolates present high phosphate-solubilizing capability in growth medium, but when they are inoculated to natural conditions, they often exist in a form characterized by non-growth and/or low phosphate-solubilizing capability. In addition, so far, the isolation of native phosphate-solubilizing microorganisms have seldom been carried out from phosphate mines. Therefore, it is necessary to explore this field of study.

The study was aimed at isolation and characterization of three phosphate-solubilizing fungi from phosphate mines in Hubei province (People's Republic of China), and their characteristics of RP solubilization were investigated. In addition, the potential value of these isolates as biofertilizers for wheat was assessed in pot experiments.

Materials and Methods

Isolation of Phosphate-Solubilizing Fungi

Samples for isolation were collected from phosphate mines in Hubei province (People's Republic of China). Three 10 m×10 m areas were sampled and 100 g samples which contain almost 70% of rock phosphate, 20% of clay, and 10% of silt were taken from 0 to 10 cm depth after removing 3 cm surface residues, and then homogenized by mixing, sieved (<2 mm) and placed in a sterile tightly closed polyethylene bag. The samples were stored at 4 °C and processed within 48 h. For isolation of phosphate-solubilizing microorganisms, 10 g samples were added to 100 ml sterilized water, and mixed on the magnetic blender for 20 min to separate microorganisms from the sample completely. The serially diluted sample solution was planted on National Botanical Research Institute's phosphate growth (NBRIP) agar [10] [glucose, 10.0 g; $(NH_4)_2SO_4$, 0.15 g; KCl, 0.2 g; $MgCl_2{\cdot}6H_2O$, 0.5 g; $MgSO_4{\cdot}7H_2O$, 0.25 g; agar, 20.0 g; distilled water, 1,000 ml) containing 5.0 g tricalcium phosphate as sole phosphate source for selectively screening microorganisms which have phosphate-solubilizing capabilities. After 3 days of incubation

at 30 °C, isolates colonies with clear zones were further purified by replanting on medium maintained above supplemented with tricalcium phosphate. The best three fungal isolates from the preliminary screening were selected based on the content of soluble phosphorus released in culture medium and were again assayed for RP solubilization. The isolates were designated as HB-1, HB-2, and HB-3.

Identification of the Isolates

Morphology characteristics of the isolates were determined according to the methods described by Wei et al. [11]. Physiological and biochemical characteristics were performed as per standard procedures by plate assays. The 18S rRNA genes of the isolates were amplified and sequenced according to the methods described by White et al. [12]. The sequences of 18S rRNA genes were first analyzed using the BLAST searching program at the National Center for Biotechnology Information (NCBI) website: http://www.ncbi.nlm.nih.gov/BLAST/. Related sequences were preliminarily aligned with the default setting of Clustal X (2.0) [13]. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4.0 [14].

RP Solubilization Assays by the Isolates

The RP sample used in this experiment was obtained from Yichang phosphate mines (Hubei, People's Republic of China). The main elemental composition of the sample was 41.89% O, 2.74% H, 8.35% P, 24.78% Ca, 9.85% Si, 9.66% Mg, 1.24% Na, 0.93% Al, and 0.56% Fe, respectively. The sample was ground to a particle size of 100–200 mesh. XRD analysis showed that the sample was mainly composed of hydroxyapatite and a small quantity of quartz and montmorillonite. RP solubilization assays were carried out in flasks with 50 ml of NBRIP medium (without agar) added 0.1 g RP sample as sole phosphate source. The culture medium was sterilized in an autoclave at 121 °C for 20 min. The pH of culture medium was periodically adjusted at 6.0. Ten millimeter mycelial discs of each isolate from actively growing 72 h on NBRIP medium were added as inoculum. Flasks were shaken under 140 rpm at 30 °C for 14 days. Autoclaved by the same method described above, uninoculated medium served as control. Each flask with 50 ml culture medium was taken every other day for examination for 14 days. The culture medium was centrifuged at 10,000 rpm for 20 min, and supernatant was filtered through blue ribbon filter paper. The filtrate was assessed for the content of soluble phosphorus, pH, organic acid, and acid and alkaline phosphatases activity. All experiments were performed in triplicate.

Pot Experiments to Determine the Effects of the Isolates on Wheat Seedling Growth

Wheat seeds were sterilized by soaking in 5% NaOCl solution for 10 min and then washed thrice with distilled water. Thirty sterilized seeds were placed on moist filter papers in Petri dishes and germinated in the dark at 25 °C. Five seedlings of uniform size (almost 2 cm length) were transferred into ethanol-disinfected plastic pots containing 1.0 kg sterile soil and placed in a temperature-controlled growth chamber at 25–28 °C and a 16:8 h day/night regime. The test soil was thoroughly mixed and passed through a 2-mm sieve to remove large particulate matter, and the basic properties of the soil were as follows: pH (H_2O), 6.8–7.0; available phosphorus, 15.4 mg kg^{-1}; available nitrogen, 532.4 mg kg^{-1}; total kalium, 2.4%; and organic carbon, 1.1%. Ten millimeter mycelial discs of each isolate from actively growing 72 h on NBRIP medium were suspended in distilled water as inoculum and sprayed

on the soil surface after seedling transferred. Adequate amount of distilled water was added so as to raise the moisture content of soil to field capacity. The sets included: (1) control (soil without both the isolates inoculation and RP addition); (2) soil + RP (10 g kg^{-1} soil); (3) soil + HB-1; (4) soil + HB-2; (5) soil + HB-3; (6) soil + RP (10 g kg^{-1} soil) + HB-1; (7) soil + RP (10 g kg^{-1} soil) + HB-2; and (8) soil + RP (10 g kg^{-1} soil) + HB-3. After 30 days, the plants were carefully removed from the pots, and the root surface was cleaned several times with distilled water. Growth parameters such as shoot and root length, shoot and root dry weight of the plants were measured and recorded. The plants were oven-dried, and then the phosphorus and nitrogen content were determined. After harvesting, soil in pot were sampled and analyzed for available phosphorus. Each treatment was performed in triplicate.

Analytical Methods

Content of soluble phosphorus in the filtrate was determined by using the method described by Kitson and Mellon [15]. The pH was recorded with a pH meter equipped with glass electrode. For the analysis of organic acids, 10 μl of filtrate was injected to high-performance liquid chromatography (HPLC; Agilent 1100), using C_{18} columns (Thermo electron corporation). The mobile phase consisted of a phosphate buffer (50 mmol l^{-1} KH_2PO_4, pH 2.0) and acetonitrile (2.0%, *v*/*v*). The organic acids were detected at 214 nm with a flow rate of 1.0 ml min^{-1} for 20 min. Acid and alkaline phosphatases activity were determined using a modification of the method of Tahatabai and Bremmer [16]. One milliliter filtrate was blended with 1 ml of 25 mmol l^{-1} *ρ*-nitrophenyl phosphate (ρNPP) and 4 ml modified universal buffer (pH 6.5 or pH 11 for the assay of acid or alkaline phosphatases, respectively) at 37 °C in the dark. The reaction was terminated after 1 h by adding 1 ml of 0.5 mol l^{-1} $CaCl_2$ and 4 ml of 0.5 mol l^{-1} NaOH. The mixtures were filtered, and the filtrate was measured at 410 nm. Dry weight of shoot and root were determined after drying at 75 °C for 48 h. The phosphorus content of dried plants was determined by using the method described by John [17], and the nitrogen content was determined using the colorimetric method described by Linder [18]. Available phosphorus content of soil was determined using sodium bicarbonate extractable phosphorus colorimetric method [19]. Values were given as means±SD for triplicate samples.

Results

Identification of the Isolates

The isolates presumably identified as phosphate-solubilizing fungi were rod-shaped ascomycetous fungi with circular spores. The full details of morphology, physiological, and biochemical characteristics were summarized in Table 1.

The 18S rRNA gene sequences of the isolates were determined, and a phylogenetic tree was constructed (Fig. 1). The sequences of the isolates were deposited in the GenBank nucleotide sequence data library under the following accession numbers: EU590663 (HB-1), EU590664 (HB-2), and EU590665 (HB-3). The 18S rRNA gene sequences comparison revealed that HB-1 had 97.4% similarity with *Penicillium expansum* (*P. expansum*), while HB-2 had 98.7% similarity to *Mucor ramosissimus* (*M. ramosissimus*), and HB-3 had 99.2% similarity to *Candida krissii* (*C. krissii*). Based on their 18S rRNA gene sequences and phylogenetic positions, the isolates were designated as *P. expansum* HB-1, *M. ramosissimus* HB-2, and *C. krissii* HB-3, respectively.

Table 1 Morphology, physiological and biochemical characteristics of the isolates.

Test items	HB-1	HB-2	HB-3
Origin	Huangmei	Yichang	Yichang
Colony morphology	Green, circular	White, slender	White, circular
Cell shape	Filamentous	Filamentous	Filamentous
Aerial spore mass	Dark green	White	White
Spore morphology	Rectiflexible	Spiral	Single conidia
Motility	+[a]	+	–
Gram staining	–[b]	–	–
Aerobic growth	+	+	+
Anaerobic growth	–	–	+
Optimum temperature	28–32 °C	28–30 °C	25–28 °C
Optimum pH	6.0–7.0	6.0–7.0	5.0–6.0
Nitrate reduction	–	+	+
Methyl red test	–	–	–
Voges-Proskauer	+	+	+
Indole production	–	–	–
Lysine decarboxylase	–	+	+
Catalase	+	+	+
DNase	+	+	+
Oxidase	–	–	–
Hydrolysis of			
Gelatin	–	–	+
Starch	+	+	+
H_2S production	–	–	–
Citrate utilization	+	+	–
Lipase production	+	–	–
Carbon source utilization			
Glucose	+	+	+
Maltose	+	+	+
Mannose	+	+	–
Lactose	–	–	–
Sucrose	+	+	–
Fructose	+	+	+
Galactose	–	–	–
Starch	+	+	+

[a] Positive result

[b] Negative result

Characteristics of RP Solubilization by the Isolates

Contents of soluble phosphorus in culture medium during 14 days of RP solubilizing experiments were presented in Fig. 2. Results showed that the isolates had different capabilities to release soluble phosphorus from RP. Among the three isolates, *C. krissii* HB-3 was the most efficient strain for RP solubilization releasing the highest of 108.44 mg l^{-1}

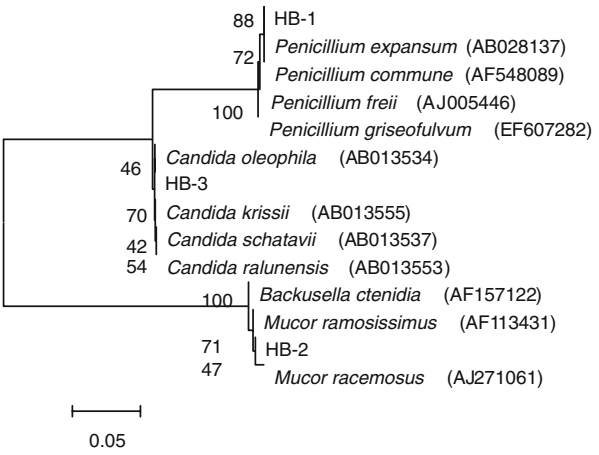

Fig. 1 18S rRNA-based phylogenetic relationship between the isolates and representatives of other related taxa (GenBank accession numbers in parentheses). The numbers at the nodes indicate the levels of bootstrap support based on data for 1,000 replicates; values inferred greater than 50% are only presented. The scale bar indicates 0.05 substitutions per nucleotide position

soluble phosphorus in culture medium, followed by *P. expansum* HB-1 and *M. ramosissimus* HB-2, in which the highest contents of soluble phosphorus were recorded of 98.67 and 89.65 mg l^{-1}, respectively (Fig. 2).

Results also showed that the content of soluble phosphorus released by the isolates in culture medium increased significantly before the tenth day, after which it began to decrease. This fact may be attributed to the availability of soluble phosphorus, which had an inhibitory effect on further RP solubilization, or the depletion of carbon source limited both the production of organic acids and microbial activity [20]. However, there was no significant change in the content of soluble phosphorus under the control, which only resulted in a slightly increase of 5.32 mg l^{-1} during 14 days.

Changes of pH in culture medium during 14 days of RP solubilizing experiments were presented in Fig. 3. In all cases, RP solubilization was concomitant with a significant pH decrease. Among the three isolates, *C. krissii* HB-3 had the largest reduction of pH in culture medium from an initial value of 6.0 to 3.95 after only 6 days, compared to that of

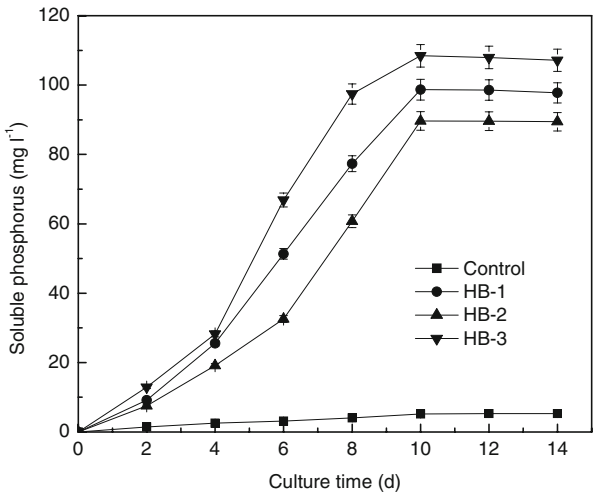

Fig. 2 Content of soluble phosphorus in culture medium during 14 days of RP solubilizing experiment in flasks with 50 ml of NBRIP medium and 0.1 g RP sample as sole phosphate source at 30 °C. Culture medium uninoculated served as control. Results represent the mean of three replicates ±SD

P. expansum HB-1 and *M. ramosissimus* HB-2, which showed a reduction to 4.22 and 4.46, respectively, after 8 days. However, no decrease in pH was found in culture medium over the last 6–8 days. This may be due to the limited supply of substrate in culture medium, which caused the growth cease of the isolates. In addition, Fig. 3 also showed that under the control, the pH remained almost constant for the duration of the experiment.

A significant increase in content of soluble phosphorus and a concurrent significant reduction in pH were shown in the culture medium inoculated with the isolates compared with the uninoculated culture medium (Figs. 2 and 3). A strong negative correlation (r=– 0.89; p<0.01) between the content of soluble phosphorus and pH was observed. It was accordance with some studies which also showed a negative correlation between soluble phosphorus released and pH in phosphate solubilization [21, 22].

HPLC analysis detected citric acid (retention times 2.338 min) in the culture medium inoculated with *P. expansum* HB-1, citric acid (retention times 2.221 min), and oxalic acid (retention times 2.438 min) in the culture medium inoculated with *M. ramosissimus* HB-2, and gluconic acid (retention times 2.393 min) in the culture medium inoculated with *C. krissii* HB-3, respectively. The time and concentration when these organic acids could be detected are shown in Table 2. Presumably, the production of these organic acids played a vital role in the acidification of culture medium and following by the decrease of pH in culture medium (Fig. 3), and thus further facilitated the solubilization of RP.

It could be seen from Figs. 4 and 5 that the activity of acid and alkaline phosphatases varied greatly among the three isolates. The activity of acid phosphatases produced by *C. krissii* HB-3 was the highest among the three isolates, followed by *P. expansum* HB-1 and *M. ramosissimus* HB-2 (Fig. 4). However, the activity of alkaline phosphatases produced by *C. krissii* HB-3 was lower than that of *P. expansum* HB-1, and very little alkaline phosphatases activity was detected in the culture medium inoculated with *M. ramosissimus* HB-2 (Fig. 5). Moreover, both the activity of acid and alkaline phosphatases produced by the isolates increased sharply after inoculation. However, they began to decrease rapidly after the sixth day, and this may be due to the soluble phosphorus released by the isolates, which inhibited the activity of acid and alkaline phosphatases.

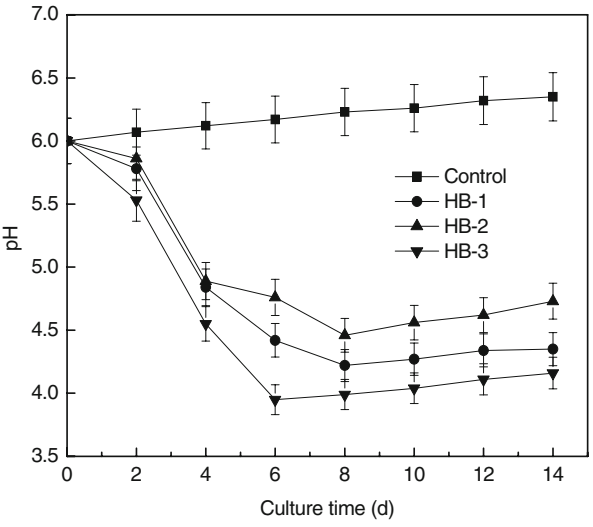

Fig. 3 Changes of pH in culture medium during 14 days of RP solubilizing experiment in flasks with 50 ml of NBRIP medium and 0.1 g RP sample as sole phosphate source at 30 °C. Culture medium uninoculated served as control. Results represent the mean of three replicates ±SD

Table 2 Organic acids and time and concentration when they could be detected in culture medium inoculated with the isolates during 14 days of RP solubilizing experiment in flasks with 50 ml of NBRIP medium and 0.1 g RP sample as sole phosphate source at 30 °C.

Isolates	Organic acids	Time and concentration (mmol l^{-1})
HB-1	Citric acid	d6 (2.2±0.2), d8 (8.5±0.6), d10 (4.7±0.4)
HB-2	Citric acid	d6 (1.5±0.2), d8 (4.3±0.4), d10 (3.1±0.3)
	Oxalic acid	d6 (2.3±0.2), d8 (1.2±0.1)
HB-3	Gluconic acid	d4 (2.4±0.2), d6 (7.5±0.8), d8 (14.2±1.1), d10 (3.9±0.6)

Results represent the mean of three replicates ±SD

Effects of the Isolates on Wheat Seedling Growth

The results of pot culture experiments were shown in Table 3. According to these results, a significant increase of most parameters measured in this study under pot culture conditions was observed in treatments both inoculated with the isolates and added with RP. However, there were no significant increases under the control, and in the treatments added with RP alone and inoculated with the isolates alone, respectively.

Under pot culture conditions, all the isolates promoted the shoot and root length of wheat seedling in pots containing RP over control (Table 3). Among the three isolates, the effect of *C. krissii* HB-3 inoculation was highest with an increase in shoot length (10.2%) and root length (15.4%), followed by *P. expansum* HB-1 inoculation showing an increase in shoot (8.3%) and root length (12.6%). However, *M. ramosissimus* HB-2 inoculation also showed an increase in shoot (6.0%) and root length (9.8%) over control, but to a lesser extent.

All the isolates promoted shoot and root length of wheat seedling, with a concomitant increase in the shoot and root dry weight of wheat seedling in pots containing RP compared to control (Table 3). Among the three isolates, inoculation of *C. krissii* HB-3 enhanced

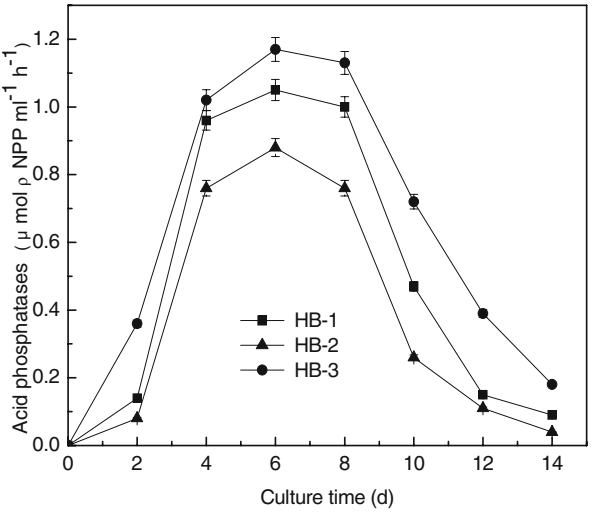

Fig. 4 Acid phosphatases activity in culture medium during 14 days of RP solubilizing experiment in flasks with 50 ml of NBRIP medium and 0.1 g RP sample as sole phosphate source at 30 °C. Culture medium uninoculated served as control. Results represent the mean of three replicates ±SD

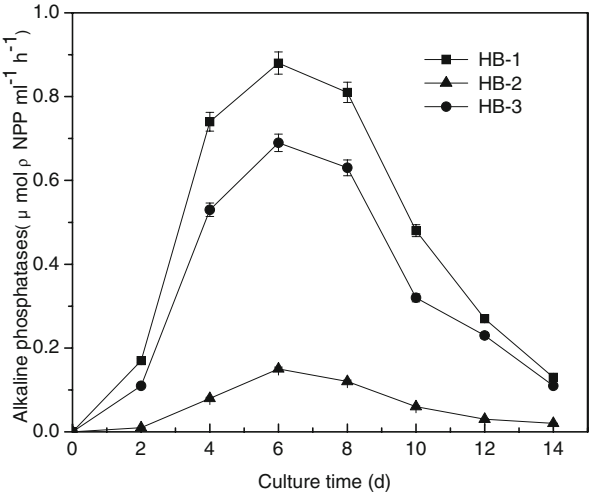

Fig. 5 Alkaline phosphatases activity in culture medium during 14 days of RP solubilizing experiment in flasks with 50 ml of NBRIP medium and 0.1 g RP sample as sole phosphate source at 30 °C. Culture medium uninoculated served as control. Results represent the mean of three replicates ±SD

shoot and root dry weight by 12.4% and 17.3%, respectively, over control, followed by *P. expansum* HB-1 (10.1% and 12.5%, respectively) and *M. ramosissimus* HB-2 (7.1% and 8.7%, respectively) inoculation.

The available phosphorus content in the soil mixed with RP and inoculated with the isolates was significantly higher compared to the control. This suggested that a subsequent plant will reap the benefits imparted by these isolates to the soil as more available phosphorus was present in soil, which increased its fertility. Among the three isolates, the available phosphorus content in the soil was in the order: *C. krissii* HB-3 inoculation (18.8% increased) > *P. expansum* HB-1 inoculation (17.5% increased) > *M. ramosissimus* HB-2 inoculation (14.3% increased; Table 3).

Table 3 also showed that inoculation of the isolates with the application of RP resulted in significantly higher phosphorus and nitrogen uptake by wheat over control. The highest phosphorus and nitrogen uptake was obtained in *C. krissii* HB-3 inoculated pot. It recorded an increase of 27.6% in phosphorus uptake and 19.3% in nitrogen uptake over the control,

Table 3 Growth, soil available phosphorus, and phosphorus and nitrogen uptake of wheat seedlings in pots.

Treatments	Shoot length (cm)	Root length (cm)	Shoot dry weight (g pot^{-1})	Root dry weight (g pot^{-1})	Soil available phosphorus (mg kg^{-1})	Phosphorus uptake (mg pot^{-1})	Nitrogen uptake (mg pot^{-1})
Control	21.5±1.3	14.3±0.8	1.69±0.5	1.04±0.3	15.4±1.1	10.5±0.7	95.4±7.7
Soil + RP	21.6±1.6	14.5±0.7	1.71±0.6	1.02±0.5	15.8±1.3	10.6±0.7	96.2±8.1
Soil + HB-1	21.8±1.4	14.6±0.7	1.74±0.7	1.06±0.5	15.7±1.3	10.6±0.6	95.9±7.4
Soil + HB-2	21.7±1.7	14.4±0.9	1.72±0.5	1.04±0.4	15.6±1.2	10.5±0.4	95.6±7.2
Soil + HB-3	22.0±1.9	14.5±1.1	1.75±0.5	1.07±0.6	15.9±1.4	10.7±0.5	96.1±7.9
Soil + RP + HB-1	23.3±2.0	16.1±1.2	1.86±0.7	1.17±0.7	18.1±1.5	12.8±0.9	112.6±9.3
Soil + RP + HB-2	22.8±2.2	15.7±1.2	1.81±0.8	1.13±0.6	17.6±1.3	12.5±0.8	107.7±9.2
Soil + RP + HB-3	23.7±2.4	16.5±1.3	1.90±0.8	1.22±0.8	18.3±1.6	13.4±1.0	113.8±9.7

Pots were placed in a temperature-controlled growth chamber at 25–28 °C and a 16:8 h day/night regime for 30 days. Results represent the mean of three replicates ±SD

followed by *P. expansum* HB-1 (21.9% and 18.0%, respectively) and *M. ramosissimus* HB-2 (14.3% and 12.9%, respectively). The significant increase of phosphorus and nitrogen uptake by wheat seedling growing in soil inoculated with the isolates proved that these isolates have not only the capability to solubilize RP in vitro but also that this phenomenon can be observed in vivo with a beneficial effect for plant growth.

Discussion

Phosphorus deficiency is limiting crop production in many agricultural soils worldwide. Therefore, chemical phosphate fertilizer is often used in agriculture to improve crop yield [23]. However, chemical phosphate fertilizer is often expensive and easy to cause extremely environment pollution. Therefore, there requires a strong reduction in agrochemical inputs and their replacement by more ecological, efficient, and cheap natural products, such as RP [24].

China has large deposits of natural RP, but most of which is low-grade and, therefore, have no direct use and are often rejected. In view of environmental concerns and current developments in sustainability, research efforts are concentrated on development of techniques to use RP as less costly alternatives for phosphate fertilizer [3]. Recently, there has been a growing interest in the application of phosphate-solubilizing microorganisms, mainly filamentous fungi and bacteria, as inoculants for solubilizing the insoluble RP to soluble phosphorus in fermentation and soil conditions [25–27]. Because the fertilizer value of insoluble RP was substantially increased by the exogenous introduction of phosphate-solubilizing microorganisms replacing the costly chemical phosphate fertilizer, the low-cost ecotechnology engineered through specific microorganisms responsible for RP solubilization is of considerable economic importance, especially in developing country like China.

For this reason, this study succeeded in the isolation and subsequent characterization of certain biological activities of three promising phosphate-solubilizing fungi (*P. expansum* HB-1, *M. ramosissimus* HB-2, and *C. krissii* HB-3) from phosphate mines that showed considerable promise for application in the solubilization of RP. They are reported as phosphate-solubilizing fungi for the first time in this paper.

The role of phosphate-solubilizing microorganisms in phosphate solubilization has been attributed mainly to their abilities to reduce the pH of the surroundings by the production of organic acids [28, 29]. The results in this study were also in agreement with this finding. Indeed, three organic acids, including citric, oxalic, and gluconic acid, were detected in the culture medium inoculated by the three isolates. Moreover, it has been well established that the role of phosphate-solubilizing microorganisms in phosphate solubilization has also been attributed to their abilities to reduce the pH of the surroundings by the production of acid or alkaline phosphatases [3]. The results in this study also confirmed it, although there was a variation in the production of acid and alkaline phosphatases by different isolates. However, phosphate solubilization is not a simple phenomenon and may be determined by many factors, such as nutritional, physiological, and growth conditions of the microorganisms [30]. Therefore, it needs further studies to understand the mechanisms of phosphate solubilization used by different phosphate-solubilizing microorganisms.

It was expected that the fertilizer value of naturally abundant, cheap, easily accessible but sparingly soluble RP could be profitably enhanced by inoculating phosphate-solubilizing microorganisms into the soil ecosystem in which RP was applied as a source of phosphate fertilizer. It was obvious that the pot trial in this study showed that these isolates were still able to benefit wheat growth through RP solubilization. Presumably, the isolates involved in RP solubilization as well as better releasing the soluble phosphorus can

enhance wheat growth by enhancing the availability of plant growth promoting substances and other trace elements.

The inoculation of phosphate-solubilizing microorganisms has been reported to increase soil available phosphorus content [31, 32]. Results in this study were also in agreement with this finding. Wheat growth promotion in the treatments with the isolates combined with RP may be related to the increase in available phosphorus in soil. In addition, the level of phosphorus uptake in wheat seedling was also improved with the inoculation of the isolates in soil containing RP. This may be due to better utilization of soluble phosphorus from the pool of available phosphorus in the soil by the actions of the isolates. The result was in agreement with previous reports which also showed an increase in the phosphorus uptake levels in treated plants following the application RP in soil inoculated with phosphate-solubilizing microorganisms [32, 33]. Moreover, it was reported that the production of organic acids by phosphate-solubilizing microorganisms possibly also help in the acquisition of nutrients other than phosphorus [34]. Our results were also in agreement with this finding. In this study, it was obvious that the nitrogen uptake of the plants was promoted by the actions of the isolates. However, the experimental results presented here could not evaluate the effect of all interacting factors that took place, but it was clear that RP previously solubilized by the isolate inoculation favored the growth of wheat seedling.

However, plant growth promotion by the action of phosphate-solubilizing microorganisms is a well-established and complex phenomenon. Colonization of soil by non-indigenous phosphate-solubilizing microorganisms depends both on their interaction with indigenous microorganisms associated with plants and their ability to utilize diverse substrates in soil [35]. It is expected that this report will prompt further screenings of phosphate-solubilizing microorganisms, as potential RP solubilizers for use in natural soil.

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