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Evaluation for Rock Phosphate Solubilization in Fermentation and Soil–Plant System Using A Stress-Tolerant Phosphate-Solubilizing *Aspergillus niger* WHAK1

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Abstract A strain WHAK1, identified as *Aspergillus niger*, was isolated from Yichang phosphate mines in Hubei province of China. The fungus developed a phosphate solubilization zone on modified National Botanical Research Institute's phosphate growth (NBRIP) agar medium, supplemented with tricalcium phosphate. The fungus was applied in a repeated-batch fermentation process in order to test its effect on solubilization of rock phosphate (RP). The results showed that *A. niger* WHAK1 could effectively solubilize RP in NBRIP liquid medium and released soluble phosphate in the broth, which can be illustrated by the observation of scanning electron microscope, energy-dispersive X-ray microanalysis, and Fourier transform infrared spectroscopy. Acidification of the broth seemed to be the major mechanism for RP solubilization by the fungus. Indeed, multiple organic acids (mainly gluconic acid) were detected in the broth by high-performance liquid chromatography analysis. These organic acids caused a significant drop of pH and an obvious rise of titratable acidity in the broth. The fungus also exhibited high levels of tolerance against temperature, pH, salinity, and desiccation stresses, although a significant decline in the fungal growth and release of soluble phosphate was marked under increasing intensity of stress parameters. Further, the fungus was introduced into the soil supplemented with RP to analyze its effect on plant growth and phosphate uptake of wheat plants. The result revealed that inoculation of *A. niger* WHAK1 significantly increased the growth and phosphate uptake of wheat plants in the RP-amended soil compared to the control soil.

Keywords Rock phosphate · Solubilization · *Aspergillus niger* · Stress · Soluble phosphate · Wheat

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Introduction

Phosphorus (as phosphate) is one of the most important nutrients that limit plant production. Phosphate in soil mostly exists in insoluble (bound) forms and thus the content of soluble phosphate in soil is very low [1]. Therefore, chemical fertilizers containing soluble phosphate are often applied to achieve maximum plant productivity.

Natural phosphate-bearing materials, such as rock phosphate (RP) have been recognized as less costly alternatives for phosphate fertilizer [2]. However, although RP is abundantly found and easily mined, their direct application is not always effective without chemical treatment, particularly for non-acidic soils. Considerable researches have been conducted in recent years on developing novel, environmentally sound methods for RP solubilization, different from the traditional processing with mineral acids.

Microorganisms play a critical role in natural phosphorus cycle, and recently, microbially based approach has been proposed to improve the agronomic value of RP [3–5]. This approach not only compensates for higher cost of manufacturing phosphate fertilizer in industry but also reduces environment pollution caused by traditional chemical process [6]. The development of commercial bioinoculants and the large-scale bioprocessing of RP through the action of phosphate-solubilizing microorganisms have resulted in the highly efficient, low-cost, and successful commercial technologies now used by the agroindustry worldwide [7].

Filamentous fungus *Aspergillus niger*, the fungus species under current report, belongs to microorganisms of extreme biotechnological importance since it is used for production of various primary metabolites (mainly organic acids) and enzymes [8]. This fungus is widely used in the fermentation industry because of its ease of culturing and lack of pathogenicity to humans and animals. The fungus was found to be very efficient in bioleaching of several heavy metals by producing organic acids [9]. Bioremediation process was also developed using *A. niger* to produce a variety of organic acids for the leaching of heavy metals from contaminated soil [10].

Solubilization of RP by *A. niger* and its use in agriculture is receiving greater attention recently. Several strains of *A. niger* have been isolated from different soils and an increase in phosphorus availability to plants through the inoculation of this fungus has been reported [11–14]. Considering the widespread use of *A. niger* in industry and agriculture, reports indicating the utilization of *A. niger* as biofertilizer are scarce. Moreover, the isolation of native *A. niger* in phosphate mines, which have more adaptability to RP solubilization has seldom been carried out. The objective of this work was to verify the potential application of a filamentous fungus, *A. niger* WHAK1, which was isolated from Yichang phosphate mines in Hubei province of China, to solubilize RP in vitro condition. It was also introduced into the RP-amended soil in order to evaluate its effect on the plant growth and phosphate uptake of wheat.

Materials and Methods

Isolation and Identification of *A. niger* WHAK1

Ten gram of RP sample from Yichang phosphate mines (Hubei, China) was added in 250-ml flask with 100 ml sterile saline (0.5 % NaCl), and mixed on the magnetic blender for 20 min to separate microorganisms from the samples completely. The serially diluted samples solution was planted on modified National Botanical Research Institute's phosphate growth (NBRIP) agar medium (pH 7), which contained (per liter): 10 g glucose, 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.5 g $MgCl_2{\cdot}6H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$, and 20 g agar [15]. Tricalcium phosphate (5 $g l^{-1}$) was added to the medium as sole phosphorus source for selectively

screening microorganisms which have phosphate-solubilizing capabilities. The isolates were incubated at 30 °C for 3–5 days until the colonies appeared. Un-inoculated plates served as control. A single colony of *A. niger* with the biggest clear zone around fungal colonies was then picked out for next inoculation. The transfer was repeated until the pure culture was obtained. Pure culture of the strain of *A. niger* named WHAK1 were maintained on potato dextrose agar slants and kept at 4 °C until required for further studies. The identity of the isolate was confirmed based on the gene sequencing of the internal transcribed spacer (ITS) regions of the ribosomal DNA. The ITS 1, 5.8S rRNA gene, and ITS 2 were amplified by using polymerase chain reaction according to White et al. [16]. Amplified products were sequenced and analyzed using the BLAST searching program at the National Center for Biotechnology Information website: http://www.ncbi.nlm.nih.gov/BLAST/.

Fermentation Experiment for RP Solubilization by *A. niger* WHAK1

The RP sample used in this experiment was obtained from Yichang phosphate mines (Hubei, China). X-ray diffraction analysis showed that the sample was mainly composed of hydroxyapatite and a small quantity of quartz and montmorillonite. The sample was ground to a particle size of 100–200 mesh. Fermentation experiments were carried out in shake flasks with 50 ml modified NBRIP liquid medium (without agar) containing 0.5 g RP as sole phosphorus source. The initial pH of the medium was adjusted to 7. Mycelial disks (10 mm) of *A. niger* WHAK1 from actively growing colonies after 3 days on modified NBRIP agar medium were added as inoculum. Flasks were shaken under 160 rpm at 30 °C for 5 days. Autoclaved, un-inoculated medium served as control. Flask was taken daily and the broth was filtered. 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. The filter residue was washed, dried, and finally examined using scanning electron microscope (JEOL JSM-5510LV), elemental composition verified by energy-dispersive X-ray microanalysis (FALCON), and Fourier transform spectrometer (Nicolet Nexus 670) with diffuse reflectance attachment. All experiments were performed in triplicate.

Solubilization of RP by *A. niger* WHAK1 Under Stress Conditions

The effects of temperature, pH, salinity, and desiccation on the solubilization of RP by *A. niger* WHAK1 were carried out by the method described above unless specified otherwise. The effect of temperature was studied at 10, 15, 20, 25, 30, 35, and 40 °C, respectively. The influence of pH was studied by adjusting the initial pH of the medium at 3, 4, 5, 6, 7, 8, and 9, respectively. Subsequently, the fungus was inoculated into the medium with different concentrations of NaCl (0, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 %, respectively) to study the effect of salinity. The tolerance of the fungus to desiccation was studied in the medium added with different concentrations of PEG 10000 (0, 5, 10, 15, 20, 25, and 30 %, respectively). One variable was changed at a time while maintaining the other factors as constant. After shaking at 160 rpm and 30 °C for 5 days, flasks were taken and assessed for the content of soluble phosphate and fungal population in the broth. All experiments were performed in triplicate.

Soil–Plant Experiment

Experiments were conducted in plastic pots using field soil (pH7.1; total phosphate, 0.41 $g\,kg^{-1}$; available phosphate, 7.13 $mg\,kg^{-1}$; and organic matter, 1.27 %). Soil was thoroughly mixed and passed through a 2-mm sieve to remove large particulate matter. The test soil was autoclaved

three times for 2 h at 121 °C and placed in 5 kg lots in ethanol-disinfected plastic pots. Ten grams RP per kilogram soil was added in the soil. The treatments consisted of: soil; soil+RP; soil+*A. niger* WHAK1 and soil+RP+*A. niger* WHAK. Wheat seeds were surface sterilized by immersing the seeds in 0.1 % of sodium hypochlorite solution for 10 min and then washed three times with distilled water before sowing. The soil from 2 cm depth was removed from pots and nine wheat seeds were placed at equal distance. At the time of seeding, the spores of *A. niger* WHAK1 from 10 mm mycelial disks actively growing 3 days on NBRIP agar medium were scraped and mixed with sterile vermiculite (2–4 mm). The vermiculite was applied on the seeds uniformly and then these were covered with a uniformly spread 2 cm thick soil layer. Water was added periodically to maintain soil moisture. Plants were harvested after 2 months of sowing and their plant height and dry weight of the shoot and root were recorded. The plants were oven-dried and then determined the phosphate content in the plants. After harvesting, soil in pot was sampled and analyzed for available phosphate and pH. Each treatment was performed in triplicate.

Analytical Methods

Content of soluble phosphate was determined by using the vanadium–ammonium molybdate colorimetric method with a UV–vis 8500 spectrophotometer at 490 nm [17]. The pH was recorded with a pH meter equipped with glass electrode. Organic acids in the broth were determined by high-pressure liquid chromatography (HPLC; Agilent 1100) analysis using C18 columns (Thermo Electron Corporation). The mobile phase consisted of a phosphate buffer (50 $\mathrm{mmol\,l^{-1}}$ KH_2PO_4, pH2.0) and acetonitrile (2.0 %, *v*/*v*). The organic acids were detected at 214 nm with a flow rate of 1.0 $\mathrm{ml\,min^{-1}}$ for 20 min. Organic acids standards included citric, oxalic, gluconic, formic, α-ketoglutaric, fumaric, lactic, pyruvic, succinic, tartaric, and ascorbic acids. For the determining the fungal population, the standard pour plate method was used in NBRIP agar medium for counting the colony-forming units (CFU) on agar plates. Dry weight of shoot and root were determined after drying at 75 °C for 48 h. The phosphate content in the plants was determined by using method described by John [18]. Soil available phosphate was extracted by the bicarbonate method and analyzed by the molybdate blue method [19]. Soil pH was measured in a 2:1 water/soil suspension by a pH meter. Values were given as mean±standard deviation for triplicate samples.

Results

Isolation and Identification of Strain WHAK1

The viable microbial population present in Yichang phosphate mines detected using isolation method described on NBRIP agar plate ranges from 0.23 to $1.16\times10^4\,\mathrm{CFU\,g^{-1}}$. A fungal strain WHAK1, which had a marked phosphate-solubilizing activity on NBRIP agar as visualized by clear zone developed around the colony (Fig. 1), was selected and later investigated for RP solubilization in NBRIP liquid medium. The isolate was rod-shaped ascomycetous fungus that produces microscopic spores inside sacs. It could growth at a wide temperature range between 5 and 45 °C, with the optimal temperature for growth at 25–30 °C. The optimum pH for growth was 6–7 and growth also occurred between wide range of pH between 2 and 11. The sequence of 603 bp ITS region of the fungus showed 99 % identity with the *A. niger* strain WM10.74 (GenBank accession number: HQ014696) ITS 1, 5.8S rRNA gene, and ITS 2 complete sequence. The strain WHAK1 was finally identified as *A. niger* based upon the results of phenotypic characterization and ITS sequences analysis. The

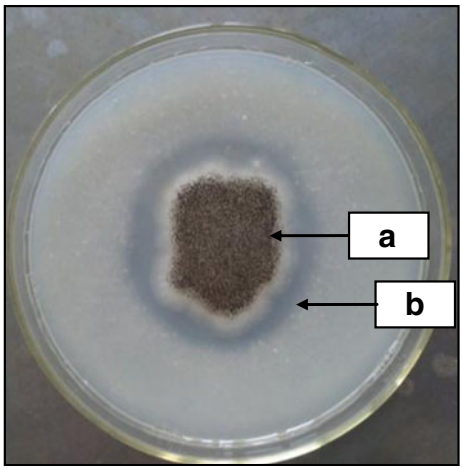

Fig. 1 Strain of *A. niger* WHAK1 (*a*) showing clear zone (*b*) of phosphate solubilization on NBRIP agar medium

sequence was deposited in the GenBank nucleotide sequence data library under the accession number JQ929761.

RP Solubilization by *A. niger* WHAK1

The results of 5 days of RP-solubilizing experiments by *A. niger* WHAK1 are presented in Fig. 2. The fungus could effectively solubilize RP in the broth and the content of soluble phosphate released increased significantly during the experiments, although a slight decrease was observed at the fourth day. As a result of growth, the pH of the broth fell from an initial value of 7–4.5 at the third day, and was maintained between 4.5 and 4.7 thereafter. Correspondingly, the titratable acidity was also detected in the broth during the experiments.

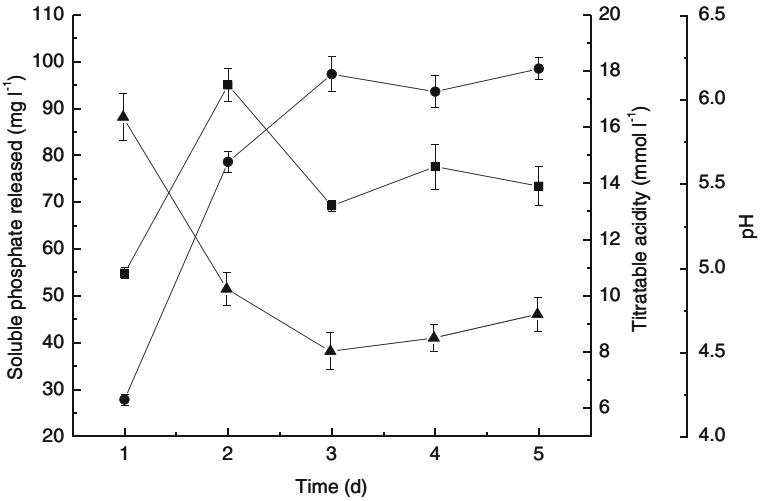

Fig. 2 Changes in content of soluble phosphate released (*filled circle*), pH (*filled triangle*), and quantity of titratable acidity (*filled square*) during 5 days of RP-solubilizing processes by *A. niger* WHAK1. Results represent the mean of three replicates±standard deviation

The major mechanism for the solubilization of RP by *A. niger* is reported to be the excretion of low-molecular weight organic acids [20–22]. Results in this study were also in agreement with this finding. In this study, HPLC analysis detected various organic acids in the broth inoculated with *A. niger* WHAK1, except α-ketoglutaric, fumaric, and tartaric acid among the organic acids standards (Table 1). Presumably, these organic acids played a vital role in the acidification of the broth, which could be illustrated by the decrease of pH and increase of titratable acidity, and thus further facilitated the solubilization of RP. Results also show that gluconic acid was predominantly produced by the fungus during the RP-solubilizing processes. Similar result in the solubilization of tricalcium phosphate by *A. niger* was reported by Chuang et al [23].

Scanning electron microscope observations show that the RP surface solubilized by *A. niger* WHAK1 after 5 days inoculation is very different from the control (Fig. 3). The fungus corroded the RP surface due to the proton attack, and thus made it scraggly and formed many chasms. In contrast, the RP surface was approximately smooth in the control.

Energy-dispersive X-ray microanalyses show the peaks of P, Ca, and other elements in the RP residues after 5 days solubilizing experiments (Fig. 4). Results exhibited a significant decrease of the amount of P and Ca in the RP residues solubilized by *A. niger* WHAK1 compared to the control. It indicated that the main composition of RP, namely insoluble tricalcium phosphate, had undergone an obvious transformation, and it was solubilized successfully by the fungus.

Figure 5 shows the Fourier transform infrared spectroscopy spectra of RP residues surface (control and the fungal treatment). As compared to the control, the trough observed at 3,496 cm^{-1}, which seems to be indicative of both amine and bonded OH groups, is slightly broad in the RP residues solubilized by *A. niger* WHAK1. The trough observed at 1,457 cm^{-1} in the fungal-solubilized RP residues was significantly reduced in intensity and broadened compared to the control, suggesting significant changes in the phosphate groups. At wave number 1,702 cm^{-1}, a shoulder is observed, which may be due to assigned to –CO–NH– group, also suggesting the action of the fungus on RP.

Table 1 Organic acids detected and their concentration (milligrams per liter) in the broth inoculated with *A. niger* WHAK1 during 5 days of RP-solubilizing experiments

	Day 1	Day 2	Day 3	Day 4	Day 5
Citric acid	–[a]	Trace	6.10±0.12	Trace	–
Oxalic acid	22.14±1.10	37.59±1.86	32.73±2.01	27.68±1.91	24.16±1.87
Gluconic acid	70.07±4.35	195.47±10.55	215.82±18.73	169.58±12.14	58.65±3.19
Formic acid	trace	13.25±0.91	14.75±1.01	13.59±0.98	12.43±1.08
α-Ketoglutaric acid	–	–	–	–	–
Fumaric acid	–	–	–	–	–
Lactic acid	1.92±0.08	2.65±0.20	2.88±0.16	4.78±0.31	8.61±0.70
Pyruvic acid	2.29±0.17	1.59±0.09	2.17±0.10	1.61±0.07	2.27±0.11
Succinic acid	4.49±0.15	7.86±0.38	5.01±0.35	3.91±0.28	2.68±0.18
Tartaric acid	–	–	–	–	–
Ascorbic acid	–	–	2.70±0.20	2.34±0.17	trace

Results represent the mean of three replicates±standard deviation

[a] Not detected

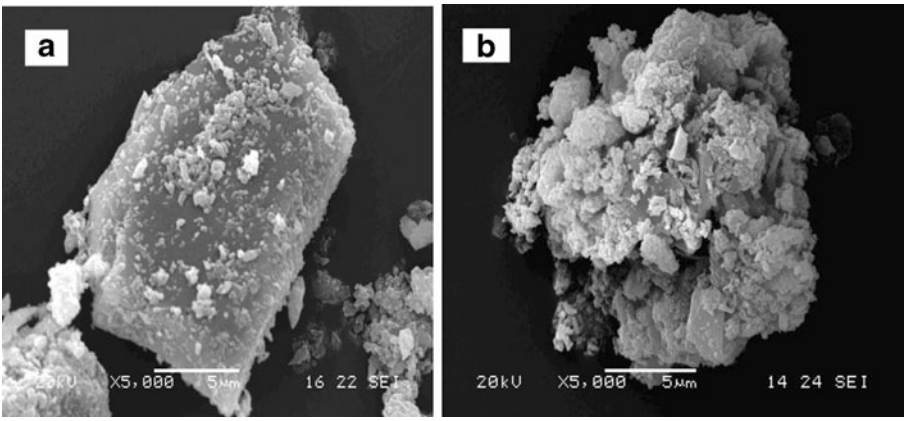

Fig. 3 Scanning electron microscope of RP residues surface of control (**a**) and solubilized by *A. niger* WHAK1 (**b**), respectively, after 5 days of RP-solubilizing experiment

Effects of Different Stresses on RP Solubilization by *A. niger* WHAK1

Effects of different stresses on RP solubilization by *A. niger* WHAK1 are given in Fig. 6. The increase in incubation temperature influenced the solubilization of RP by the fungus in the broth, with the minimum and maximum release of soluble phosphate at 10 and 30 °C, respectively (Fig. 6a). A significant increase in the fungal growth was recorded in the broths incubated at various temperature intervals through 10–30 °C. Maximum growth occurred at 30 °C, which is at par with 25 °C. Strain WHAK1 was able to grow at all the pH tested from 3 to 9, and the maximum growth was achieved at pH6–7 (Fig. 6b). The fungus was also able to solubilize RP at pH range of 3–9 and the highest release of soluble phosphate occurred at pH7. However, there was no significant difference in the release of soluble phosphate at pH5, 6, and 7. Moreover, the fungal growth and RP-solubilizing activity were significantly higher in the broth with acidic pHs than those with alkaline pHs. This is expected because fungi naturally grow better under acidic than alkaline pH conditions. Additionally, because phosphate

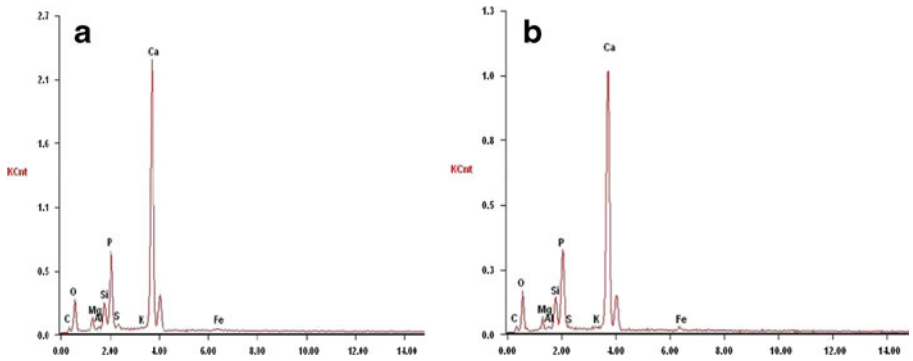

Fig. 4 Typical spectra obtained by energy-dispersive X-ray micro-analysis of RP residues surface of control (**a**) and solubilized by *A. niger* WHAK1 (**b**), respectively, after 5 days of RP-solubilizing experiment

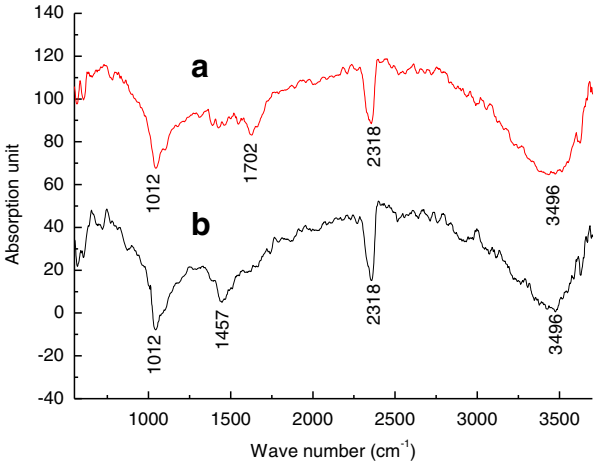

Fig. 5 Fourier transform infrared spectroscopy of RP residues surface of control (*a*) and solubilized by *A. niger* WHAK1 (*b*), respectively, after 5 days of RP-solubilizing experiment

solubilization is associated with production of acids, alkaline medium will tend to impair this process by neutralization of acidity [24].

To determine the effect of salinity and desiccation on the fungal growth and RP solubilization by the strain WHAK1, different concentrations of NaCl and PEG 10000 were added to the broth to form a saline and desiccated condition, respectively. The ability of the solubilization of RP by the fungus was enhanced in the presence of low concentrations of NaCl but was inhibited at higher concentrations (Fig. 6c). The fungus showed maximum growth in the presence of 0.5 % NaCl, which also corresponded to a maximum release of soluble phosphate in the broth. At higher NaCl concentrations than 0.5 %, however, adverse osmotic conditions impair growth and consequently, RP solubilization. The presence of PEG 10000 caused a reduction in RP solubilization, with a significantly higher reduction at higher concentrations of PEG 10000 in the broth (Fig. 6d). However, the fungus was still able to solubilize RP in the presence of 30 % PEG 10000. The fungal growth seemed to be lower with the increasing concentration of PEG 10000, and it was accompanied by an obvious decrease in the content of soluble phosphate in the broth.

Effects of Inoculation of RP-Amended Soil with *A. niger* WHAK1 on Wheat Plants Growth

Inoculation of RP-amended soil with *A. niger* WHAK1 improved the growth of wheat plants under pot culture condition (Table 2). The height of wheat plants in the RP-amended soil inoculated with the fungus was obviously higher than that of other treatments and after harvesting, 36.8 % increased were observed compared to the control. The addition of RP to soil with fungal inoculation significantly increased 24.9 and 28.3 % of shoot and root dry weight of wheat plants, respectively, compared to control plants. The application of RP, as well as inoculation of *A. niger* WHAK1 significantly increased the phosphate uptake by wheat plants, and 40.9 % increased of the phosphate accumulation in wheat plants was observed compared to control plants. Inoculation of *A. niger* WHAK1 significantly increased the available phosphate in both RP-amended and non-amended, but maximum increase of 73.2 % than that of control was recorded in soil added with RP and inoculated with the fungus. In addition, the inoculation of *A. niger* WHAK1 also caused an obvious reduction of soil pH compared to non-inoculated treatments.

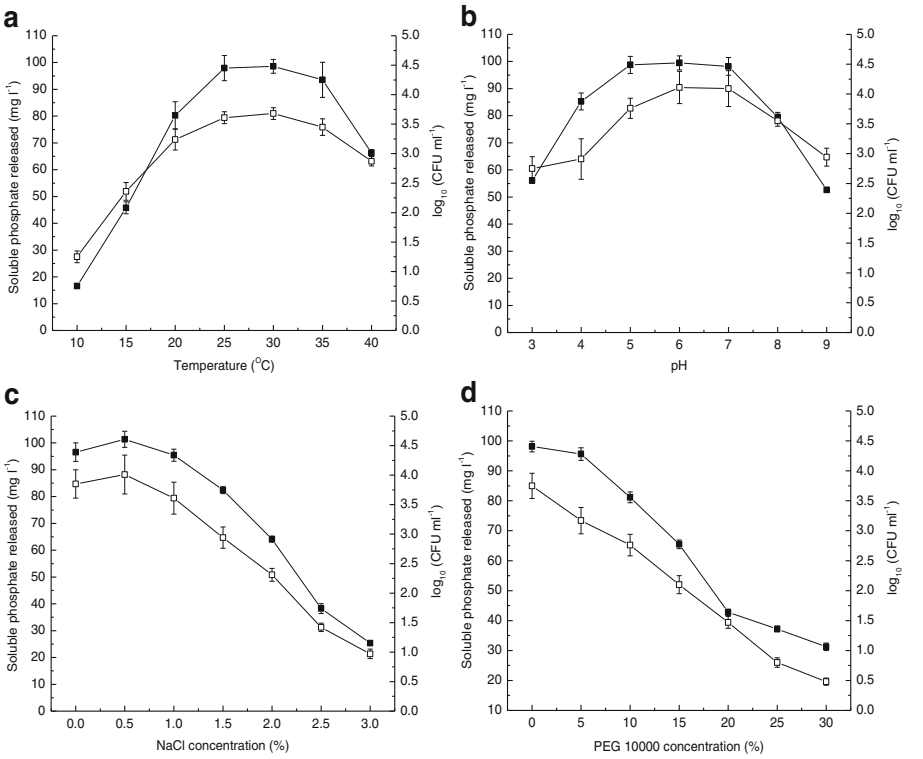

Fig. 6 Effect of different stresses (**a** temperature, **b** pH, **c** salinity, **d** desiccation) on fungal growth (*open squares*) and content of soluble phosphorus (*filled squares*) released by *A. niger* WHAK1 after 5 days of RP-solubilizing experiment. Results represent the mean of three replicates±standard deviation

Discussion

Natural RP is considered as a good and cheaper alternative to imported phosphate fertilizer. China has large deposits of natural RP, but most of them are low-grade and, therefore, have no direct use and are often rejected. There has been a growing interest in the application of phosphate-solubilizing microorganisms, including fungi and bacteria, as inoculants for

Table 2 Effects of inoculation *A. niger* WHAK1 on growth and phosphate uptake of wheat plants in RP-amended soil under pot culture condition

Treatments	Plant height (cm)	Shoot dry weight (g pot^{-1})	Root dry weight (g pot^{-1})	Phosphate uptake (mg pot^{-1})	Soil available phosphate (mgkg^{-1})	pH
Control	38.3±1.7	5.3±0.2	3.2±0.1	26.4±1.0	11.2±0.6	7.1±0.1
Soil+RP	40.7±1.4	5.6±0.3	3.3±0.2	26.9±1.2	11.8±0.7	7.2±0.3
Soil+WHAK1	45.5±2.3	5.9±0.4	3.7±0.3	32.1±1.5	18.7±1.0	6.4±0.1
Soil+RP+WHAK1	52.4±3.1	6.6±0.5	4.1±0.3	37.2±1.3	19.4±1.3	6.5±0.2

Results represent the mean of three replicates±standard deviation

solubilization of the insoluble RP [12, 25, 26]. In the present study, a strain WHAK1 of *A. niger* was isolated from Yichang phosphate mines (Hubei, China), and tested for its efficiency to solubilize RP in fermentation medium. Results showed high release of soluble phosphate from insoluble RP by *A. niger* WHAK1 in the present study. This fungus may have a better potential to serve as an agent to convert insoluble RP into soluble forms.

Although several mechanisms for phosphate solubilization may be involved, the main one in the present study is through the production of organic acids, which bound to phosphate converting them into the soluble forms [27]. A significant reduction in the pH along with increase in the titratable acidity of the broth containing RP also suggested secretion of organic acids by the fungus. In order to confirm this, the organic acids were analyzed by HPLC in the cultural filtrates. As expected, HPLC analysis showed that *A. niger* WHAK1 produced multiple organic acids, mainly gluconic acid, in the broth containing glucose as the sole carbon source (Table 1). However, the solubilization of RP have been reported to depend on their structural complexity and particle size as well as the nature and quantity of organic acids secreted by the microorganisms [28, 29]. Therefore, it needs further studies to understand the mechanisms for the solubilization of different RPs used by different microorganisms.

The performance of microorganisms is often limited by a stressful environment which affects their establishment, multiplication, and spread through the soil [30]. However in the present study, the ability of *A. niger* WHAK1 to solubilize RP over a wide range of temperature, pH, salinity, and desiccation was observed, although a decline was registered in RP solubilization under stress conditions for the fungal growth (Fig. 6). The results indicated that the fungus with potential to solubilize RP under high stress conditions is well adapted to the phosphate mines environment from which the fungus had been isolated. The stress-tolerant fungus could serve as a suitable candidate for developing microbial formulations for use in the phosphate mines for which further studies are required.

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. The soil–plant experiment showed highly significant interaction between RP amendment and inoculation with *A. niger* WHAK1. The growth and phosphate uptake of wheat plants were higher in RP-fertilized soil inoculated with the fungus, compared to control soil. The results also showed the advantageous effect of the fungus on plant growth and phosphate uptake in the absence of RP. This could be attributed to the ability of this fungus to solubilize organic and inorganic phosphate already present in the soil.

The inoculation of *A. niger* has been reported to increase soil available phosphate content [31]. Similarly, our comparison of un-inoculated soil to *A. niger* WHAK1-treated soil showed significant increase in available phosphate after harvest of wheat plants. Wheat growth promotion in the treatments with *A. niger* WHAK1 may be related to the increase in available phosphate in soil. This also suggests that it is helpful for next sowing of the crop too as more amount of available phosphate will be present in soil, which increases its fertility [32]. An obvious reduction in the soil pH was observed in inoculated treatments as compared to non-inoculated ones, which may be attributed to ability of such microorganisms to excrete organic acids, thereby decrease the pH and increase the available phosphate in soil by mechanisms involving chelation and exchange reactions [33].

Many reports are available on phosphate solubilization by phosphate-solubilizing microorganisms in fermentation and (or) pot culture conditions. In the present study, the results clearly showed that the *A. niger* WHAK1 along with RP can substitute the chemical fertilizer in soil and help in improving the crop production.

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