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Biosolubilization of Rock Phosphate by Three Stress-Tolerant Fungal Strains

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Abstract Three stress-tolerant phosphate-solubilizing fungal strains identified as *Aspergillus niger*, *Aspergillus japonicus*, and *Penicillium simplicissimum* were isolated from wheat rhizospheric soil. The strains demonstrated different capabilities of phosphate solubilization in National Botanical Research Institute's phosphate medium containing rock phosphate (RP) as sole phosphorus (P) source, and the solubilization of RP by *P. simplicissimum* was the most effective among these strains, followed by *A. niger* and *A. japonicus*. All the strains exhibited high levels of stress tolerance like 10~45°C temperature, 4~11 pH, 0~3.5% NaCl, and 0~35% PEG 10000. The strains also differed in their abilities to survive and release soluble P from RP under different stresses. *A. niger* showed significantly higher tolerance to temperature and pH over the other two strains. Higher amount of spores and content of soluble P in the medium were observed in the presence of 3.5% NaCl with *P. simplicissimum*, followed by *A. niger* and *A. japonicus*. *P. simplicissimum* could not solubilize RP in the presence of 35% PEG 10000, which exhibited the lowest tolerance to desiccation stress among the three strains.

Keywords Biosolubilization · Rock phosphate (RP) · Phosphate-solubilizing fungi · Stress · Phosphorus (P)

Introduction

Phosphorus (P), next to nitrogen, is the second important macronutrient required for plant growth [1]. In order to reduce P deficiencies and ensure plant productivity, large quantities of expensive chemical phosphate fertilizer are applied worldwide every year. In recent years, the use of rock phosphate (RP) as an alternative phosphate fertilizer has received significant interest since it is a natural, inexpensive, and available fertilizer [2]. However, its

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solubilization and direct application as phosphate fertilizer are only effective in acid soil and rarely occur in neutral or alkaline soil [3]. Therefore, a proper processing is required before its application in nonacidic soil.

Microorganisms play critical roles in natural P cycle, and the use of phosphate-solubilizing microorganisms has been proposed as a low-cost and low-energy mechanism to help increase the agronomic effectiveness of RP [4, 5]. Several recent scientific reports showed that some microorganisms, including bacteria, fungi, and actinomycetes, were indeed able to promote the solubilization of RP and increase crop yields [6–9]. These phosphate-solubilizing microorganisms render insoluble phosphate into soluble form through the process of acidification, chelation, and exchange reactions [10, 11].

Recently, filamentous fungi, such as *Aspergillus* and *Penicillium* species, were of special interest to solubilize RP in fermentation systems or be inoculated directly into soil as biofertilizer since they were widely used as producers of organic acids [12, 13]. This fungal-mediated process is of physiological and biogeochemical significance but severely affected by environmental factors, especially under stressful conditions, such as alkalinity, salinity, and desiccation [14]. However, little information is available on the occurrence of these fungi to solubilize RP under stressful conditions. In this study, two *Aspergillus* species and a *Penicillium* specie were isolated from wheat rhizospheric soil, and the biosolubilization of RP by these fungal strains was investigated. Moreover, the growth and RP solubilizing activity of these strains under different stresses, such as temperature, pH, salinity, and desiccation, were also studied.

Materials and Methods

Isolation of Phosphate-Solubilizing Fungal Strains

The phosphate-solubilizing fungal strains 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, China). The soil characteristics were pH 6.8~7.2, available phosphorus (18.7 mg kg^{-1}), available nitrogen (670.5 mg kg^{-1}), total kalium (1.9%), and organic carbon (1.5%). For the collection of rhizospheric soil, plants were uprooted, and the soil attached to roots was then suspended in sterilized water and mixed on the magnetic blender for 20 min to separate microorganisms from the soil completely. Serially diluted soil solution was planted on National Botanical Research Institute's phosphate (NBRIP) agar [15] (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 P source for selectively screening microorganisms which have phosphate-solubilizing capabilities. The best three fungal strains from the preliminary screening were selected based on the content of soluble P released in the NBRIP medium (without agar). The three fungal strains were characterized as *Aspergillus niger*, *Aspergillus japonicus*, and *Penicillium simplicissimum* on the basis of phenotypic characters and 18S rRNA gene sequencing.

RP Solubilization Assays by the Strains

The RP sample used in this experiment was obtained from Yichang phosphate mines (Hubei, China). 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 shake flasks with 50 ml NBRIP medium containing 0.5 g RP sample as sole P source. The pH of culture medium was periodically adjusted at 7.0. Spore suspensions of the strains were counted by a hemocytometer to adjust the count to approximately 1.0×10^7 spores per milliliter. Each flask was inoculated with the spore suspensions at 10% (*V*/*V*). Flasks were shaken under 160 rpm at 30°C for 7 days. Autoclaved, uninoculated medium served as control. Each flask with 50 ml culture medium was taken daily for examination for 7 days. The culture medium was first filtered through blue ribbon filter paper and then filtered through a 0.22-μm Millipore filter to collect spores of the fungal strains. The spores were resuspended in autoclaved deionized water and counted. The filtrate was centrifuged at 10,000 rpm for 20 min, and the supernatant was assessed for the content of soluble P. Solid residues were washed, dried, and finally examined using scanning electron microscope (JEOL JSM-5510LV), with elemental composition verified by energy-dispersive X-ray microanalysis (EDXA, FALCON). All experiments were performed in triplicate.

Stress Tolerance of the Strains

The effects of temperature, pH, salinity, and desiccation on the growth and RP solubilization of the fungal strains were carried out in flasks containing 50 ml NBRIP medium with 10% (*V*/*V*) of 1.0×10^7 spores per milliliter of initial inoculum under different stresses. The effect of temperature was studied at 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, and 45°C, respectively. The influence of pH was studied by adjusting the initial pH of the medium at 4, 5, 6, 7, 8, 9, 10, and 11, respectively. Subsequently, the strains were inoculated into the medium with different concentrations of NaCl (0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, and 3.5%, respectively) to study the effect of salinity. The tolerance of the strains to desiccation was studied in the medium added with different concentrations of PEG 10000 (0%, 5%, 10%, 15%, 20%, 25%, 30%, and 35%, respectively).

Analytical Methods

Content of soluble P in the filtrate was determined by using the vanadium–ammonium molybdate colorimetric method with a UV–vis 8500 spectrophotometer at 490 nm. The spores of the strains were counted by a hemocytometer in a microscope. Values were given as mean±standard deviation for triplicate samples.

Results

RP Solubilization by Different Fungal Strains

Contents of soluble P in NBRIP medium during 7 days of RP-solubilizing experiments by different fungal strains are presented in Fig. 1. Results show that all the strains could effectively release soluble P from RP in the medium compared to the control, and strains varied with respect to levels of RP solubilization achieved. However, there was no significant change in the content of soluble P under the control, which only resulted in a slight increase of 5.32 mg l^{-1} during 7 days. The phosphate-solubilizing capacity of *P. simplicissimum* was slightly greater than that of *A. niger* and *A. japonicus*, and the highest soluble P of 85.43 mg l^{-1} was obtained at the seventh day, followed by *A. niger* and *A.*

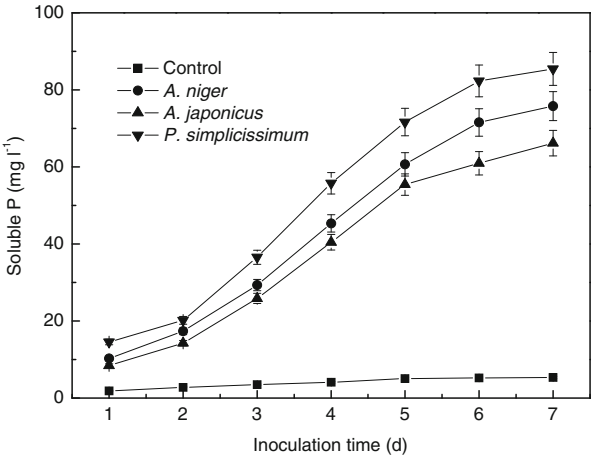

Fig. 1 Content of soluble P released by different fungal strains during 7 days of RP-solubilizing experiment. Results represent the mean of three replicates±standard deviation

japonicus, in which the highest contents of soluble P recorded were 75.79 mg l^{-1} and 66.18 mg l^{-1}, respectively, at the seventh day (Fig. 1).

Scanning electron microscope observations revealed the morphological features of RP residue surfaces solubilized by different strains after 7 days, and the typical images are shown in Fig. 2. The surface of the control was approximately smooth compared to surface in the presence of different strains, which presented scraggly and formed many chasms. In all cases, all the strains could make different extents of corrosion of the RP residue surfaces.

Energy-dispersive X-ray microanalysis of the RP residues showed peaks for P, Ca, and other elements (Fig. 3). Results exhibited a significant decrease of the amount of P and Ca in the RP residues solubilized by the strains compared to the control after 7 days. It indicated that the main composition of RP, namely insoluble tricalcium phosphate, had undergone an obvious transformation, and it was solubilized successfully by the strains.

Fungal Growth and RP Solubilization by the Strains Under Different Stresses

To determine the optimum temperature for the fungal growth and RP solubilization by the strains, shake flask cultures were carried out at different temperatures (10~45°C). As shown in Table 1, all the strains were able to grow at all the temperature tested, and the soluble P production increased gradually up to 30°C, at which the highest content of soluble P was obtained. However, the amount of spores and content of soluble P were obviously decreased when temperature was higher than 30°C. Among the three strains, *A. niger* exhibited higher growth and RP solubilizing capacity over the other two strains, suggesting a good adaptation of this fungus to the temperature variety.

The influence of pH on the fungal growth and RP solubilization by the strains was investigated in the range of pH 4 to 11. Table 2 shows that all the strains tolerated the pH of 4 to 11, and the fungal growth and RP solubilization were almost similar for the pH of 4 to 9. It indicated that these strains are acid- and alkali-tolerant fungi which can be applied to the acidic or alkaline soil. Similar to the effect of temperature on the fungal growth and RP solubilization, *A. niger* also exhibited the highest amount of spores and content of soluble P among the three strains when the initial pH in the medium changed from 4 to 11.

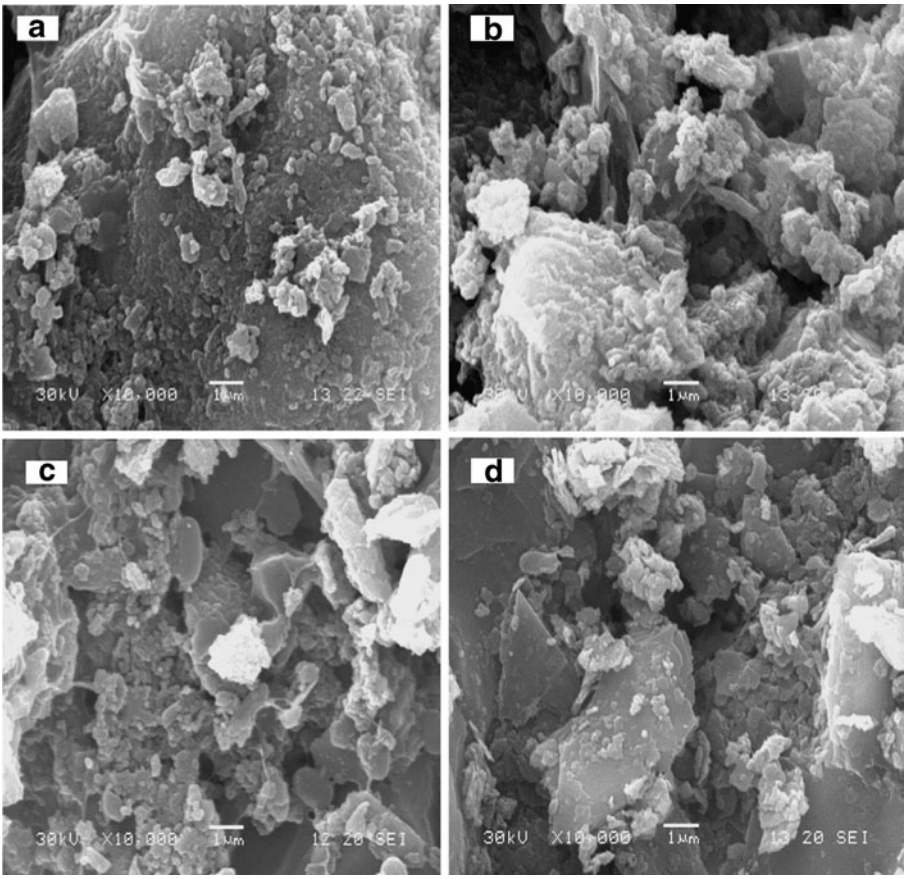

Fig. 2 Scanning electron microscope of RP residue surfaces of control (**a**) and solubilized by *A. niger* (**b**), *A. japonicus* (**c**), and *P. simplicissimum* (**d**), respectively, after 7 days of RP-solubilizing experiment

To determine the effect of salinity on the fungal growth and RP solubilization by the strains, different concentrations of NaCl from 0% to 3.5% were added in the medium, respectively. The amount of spores and content of soluble P increased when the concentration of NaCl increased from 0% to 1.0% (Table 3). However, there were gradual decreases in the amount of spores and content of soluble P at above 1.0% NaCl. Nevertheless, all the strains still survived, and the soluble P was also released at 3.5% NaCl. Among the three strains, *P. simplicissimum* was the least sensitive and the most tolerant to salinity, causing minimal decrease of the amount of spores and content of soluble P in the presence of 3.5% NaCl.

To determine the fungal growth and RP solubilization under desiccation stress, the strains were inoculated into the medium containing from 0% to 35% (*w*/*v*) PEG 10000. The results showed that a decrease in the amount of spores and content of soluble P was observed with increase in PEG 10000 concentration (Table 4). All the strains tolerated the desiccation regime of 30% PEG 10000. However, *P. simplicissimum* did not survive when the concentration of PEG 10000 was up to 35%, which exhibited the lowest tolerance to desiccation stress among the three strains.

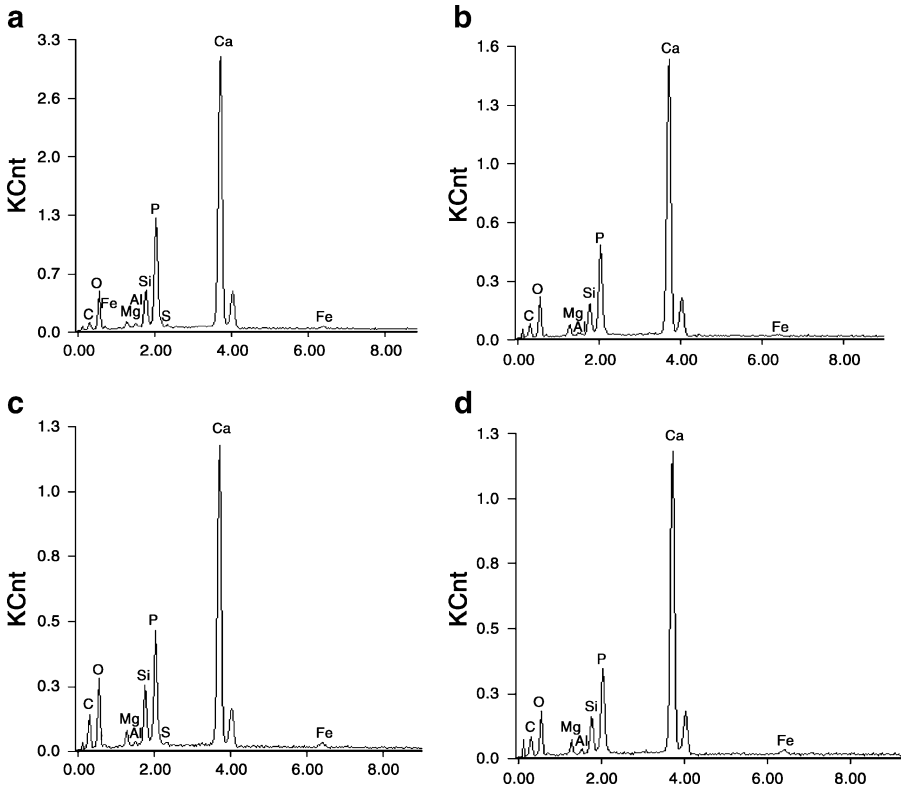

Fig. 3 Typical spectra obtained by energy-dispersive X-ray microanalysis of RP residue surfaces of control (**a**) and solubilized by *A. niger* (**b**), *A. japonicus* (**c**), and *P. simplicissimum* (**d**), respectively, after 7 days of RP-solubilizing experiment

Table 1 Effect of temperature on the fungal growth and content of soluble P released by the strains after 7 days of RP-solubilizing experiment

	A. niger		*A. japonicus*		*P. simplicissimum*	
Temperature	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})
10°C	4.03±0.38	33.15±3.37	2.96±0.25	22.54±2.31	2.07±0.23	13.58±1.31
15°C	7.98±0.79	44.72±4.55	7.34±0.69	42.15±4.48	6.68±0.59	35.21±3.52
20°C	10.43±1.06	77.53±7.84	9.16±0.94	75.76±7.25	9.13±0.92	75.71±7.71
25°C	11.27±1.07	90.84±9.23	10.92±1.02	88.93±8.11	11.34±1.03	91.93±9.15
30°C	11.54±1.11	91.55±9.17	11.13±1.10	89.47±9.04	11.77±1.12	92.76±9.47
35°C	11.17±1.03	89.96±8.56	10.45±1.13	88.32±9.08	10.62±1.15	91.38±9.28
40°C	10.45±0.99	80.17±7.92	9.21±0.97	79.03±8.02	9.12±0.98	78.27±8.05
45°C	8.62±0.87	57.68±5.85	6.23±0.62	46.61±4.58	6.07±0.60	44.95±4.20

Results represent the mean of three replicates±standard deviation

Table 2 Effect of pH on the fungal growth and content of soluble P released by the strains after 7 days of RP-solubilizing experiment

	A. niger		*A. japonicus*		*P. simplicissimum*	
pH	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})
4	10.24±0.97	90.92±9.11	9.93±0.97	85.87±8.54	9.14±0.90	82.77±8.27
5	10.93±0.99	91.75±9.24	10.18±0.99	88.86±8.11	10.71±1.07	85.46±9.10
6	11.26±1.04	92.88±9.26	11.05±0.94	89.03±8.37	11.28±1.13	90.55±9.33
7	11.18±1.24	91.27±9.77	11.22±1.11	80.64±8.75	11.37±1.10	91.48±9.25
8	10.84±1.31	90.73±9.26	10.72±1.06	89.53±8.33	10.43±1.01	90.26±9.04
9	10.15±1.23	86.41±8.27	10.08±1.03	86.15±8.10	9.74±1.05	85.86±8.19
10	8.94±0.82	75.84±7.16	8.06±0.85	77.54±7.92	7.97±0.75	72.12±7.47
11	5.91±0.58	51.54±5.77	5.85±0.60	52.76±5.10	5.32±0.58	49.15±4.56

Results represent the mean of three replicates±standard deviation

Discussion

Traditionally, RP solubilization was conducted by inorganic acids. However, it was very costly and unsuitable for commercial phosphate fertilizer production due to the low reactivity and impurities in RP. In order for RP to become an efficient phosphate fertilizer, innovative methods must be found to free the P from its strong ionic interactions with the calcium or other elements in the RP.

Filamentous fungi are capable of producing organic acids and widely used to solubilize RP in fermentation systems or be inoculated directly into soil, particularly some *Aspergillus* and *Penicilium* species [16, 17]. The advantage of using fungi for such processes includes their tolerance to high concentrations of potentially toxic metals [18], and better acid and alkali tolerance than bacteria [19], although fungi might be inferior to bacteria in their

Table 3 Effect of the concentration of NaCl on the fungal growth and content of soluble P released by the strains after 7 days of RP-solubilizing experiment

	A. niger		*A. japonicus*		*P. simplicissimum*	
NaCl concentration (%)	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})
0	11.04±1.13	90.54±8.96	9.85±0.94	88.74±8.14	11.21±1.15	91.65±9.13
0.5	11.27±1.14	90.88±9.02	9.87±1.05	88.86±8.58	11.43±1.10	91.97±9.14
1.0	11.54±1.12	91.14±9.18	9.93±0.97	88.91±8.83	11.52±1.04	91.98±9.10
1.5	10.75±0.97	88.15±8.93	9.42±0.95	85.47±8.25	11.34±0.99	90.77±8.93
2.0	9.16±0.95	77.56±7.12	8.36±0.85	76.47±7.87	10.07±0.92	83.23±8.21
2.5	5.24±0.57	51.49±5.20	5.03±0.54	50.76±5.10	7.55±0.73	64.12±6.52
3.0	3.17±0.34	37.53±3.33	2.85±0.23	35.46±3.67	4.78±0.51	43.54±4.45
3.5	1.75±0.18	18.70±1.92	1.48±0.15	17.38±1.83	3.12±0.32	24.14±2.30

Results represent the mean of three replicates±standard deviation

Table 4 Effect of the concentration of PEG 10000 on the fungal growth and content of soluble P released by the strains after 7 days of RP-solubilizing experiment

	A. niger		*A. japonicus*		*P. simplicissimum*	
PEG 10000 concentration (%)	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})	Growth (10^7 spores per milliliter)	Soluble P (mg l^{-1})
0	11.43±1.18	90.15±9.01	10.81±1.01	88.48±8.72	11.86±1.23	92.30±9.12
5	11.36±1.15	90.33±9.36	10.77±1.09	88.12±8.28	11.01±1.15	90.24±9.43
10	11.03±1.16	89.57±9.12	10.66±1.04	87.74±8.16	10.73±1.08	87.83±8.68
15	10.28±1.10	83.27±8.54	9.89±0.92	81.38±8.41	9.26±0.91	75.36±7.98
20	9.23±0.91	71.87±7.43	8.94±0.90	70.59±7.42	7.78±0.72	53.51±5.76
25	7.93±0.73	67.39±6.77	7.53±0.73	62.15±6.18	5.93±0.54	37.68±3.35
30	4.48±0.49	34.67±3.38	3.90±0.37	30.58±5.53	2.33±0.25	18.35±1.40
35	1.96±0.20	21.43±2.27	1.48±0.12	19.87±2.01	0.61±0.05	6.77±0.57

Results represent the mean of three replicates±standard deviation

ability to colonize plant root. Therefore, fungi may have a much better potential to serve as an agent to convert insoluble inorganic P into a soluble form (e.g., HPO_4^{2-}, $H_2PO_4^-$) usable by plants in low or high pH soil.

Three fungal strains, viz *A. niger*, *A. japonicus*, and *P. simplicissimum*, were isolated from wheat rhizospheric soil in this study, and these strains exhibited powerful capacity to release soluble P from RP in NBRIP medium at 30°C under pH 7.0 (Fig. 1). Moreover, the results also revealed the ability of these strains to solubilize RP over a wide range of temperature, pH, salinity, and desiccation, although a decline was registered in the fungal growth and soluble P released under stressful conditions (Tables 1, 2, 3, 4). Overall, the three strains exhibited high levels of stress tolerance, and all the strains were able to grow and release soluble P from RP at temperatures of 10°C to 45°C, pH of 4 to 11, NaCl concentration of 0% to 3.5%, and PEG 10000 concentration of 0% to 35% (except *P. simplicissimum* at 35%). It indicated that these strains could act as efficient strains to solubilize RP in NBRIP medium under different stresses. Stress-tolerant trait of these strains might be of some significance for their survival in stressful conditions and further benefits the solubilization of RP.

Tolerance to various stresses is important in the growth and survival of fungi in soil. The present studies have generated useful information about the phosphate-solubilizing fungi with tolerance to extremes in temperature, pH, salinity, and desiccation. In this study, *A. niger*, *A. japonicus*, and *P. simplicissimum* were reported as phosphate-solubilizing fungi under normal and stressful conditions in vitro. It is expected that these stress-tolerant strains could serve as suitable candidates for solubilizing RP in rigorous environments. However, since the conditions in soil are much more complex than those in vitro, further study of environmental factors affecting phosphate solubilization by these strains in soil should be of practical importance for crops.

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