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RESEARCH ARTICLE

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Selection and evaluation of phosphate-solubilizing bacteria from grapevine rhizospheres for use as biofertilizers

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Abstract

Phosphate-solubilizing bacteria (PSB) have the ability to solubilize insoluble phosphorus (P) and release soluble P. Extensive research has been performed with respect to PSB isolation from the rhizospheres of various plants, but little is known about the prevalence of PSB in the grapevine rhizosphere. In this study, we aimed to isolate and identify PSB from the grapevine rhizosphere in five vineyards of Northwest China, to characterize their plant-growth-promoting (PGP) traits, evaluate the effect of stress on their phosphate-solubilizing activity (PSA), and test their ability to stimulate the growth of *Vitis vinifera* L. cv. Cabernet Sauvignon. From the vineyard soils, 66 PSB isolates were screened, and 10 strains with high PSA were identified by 16S rRNA sequencing. Sequence analysis revealed that these 10 strains belonged to 4 genera and 5 species: *Bacillus aryabhattai, B. megaterium, Klebsiella variicola, Stenotrophomonas rhizophila,* and *Enterobacter aerogenes*. The selected PSB strains JY17 (*B. aryabhattai*) and JY22 (*B. aryabhattai)* were positive for multiple PGP traits, including nitrogen fixation and production of indole acetic acid (IAA), siderophores, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, chitinase, and protease. JY17 and JY22 showed strong PSA under stress conditions of high pH, high salt, and high temperature. Therefore, these two isolates can be used as biofertilizers in saline-alkaline soils. The inoculation with PSB significantly facilitated the growth of *V. vinifera* cv. Cabernet Sauvignon under greenhouse conditions. Use of these PSB as biofertilizers will increase the available P content in soils, minimize P-fertilizer application, reduce environmental pollution, and promote sustainable agriculture.

Additional key words: promote plant growth; inorganic phosphate; stress conditions; saline-alkaline soil; *Vitis vinifera* L. cv. Cabernet Sauvignon

Abbreviations used: ACC (1-aminocyclopropane-1-carboxylate); CAS (chrome azurol S); DF (Dworkin–Foster); GADH (gluconate dehydrogenase); IAA (indole acetic acid); NBRIP (National Botanical Research Institute's phosphate); PGP (plant-growth-promoting); PQQ-GDH (pyrroloquinoline quinone-dependent glucose dehydrogenase); PSA (phosphate-solubilizing activity); PSB (phosphate-solubilizing bacteria); TCP (tricalcium phosphate).

Authors' contributions: ML drafted the manuscript; XL made critical revision of the manuscript for important intellectual content; BSC and XLM acquired, analyzed and interpreted data; XTL coordinated the research project; XFZ performed statistical analysis; YLJ and ZM proposed revision suggestions; YLF supervised the work.

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Introduction

Phosphorus (P) is one of the major macronutrients important for the growth and development of plants, and it is involved in essential metabolic pathways, including photosynthesis, biological oxidation, nutrient uptake, and cell division (Illmer & Schinner, 1992; Gupta *et al.*, 2012). A large portion of the total P in the soil is insoluble and unavailable for plant uptake. A deficiency in soluble P in many agricultural soils is one of the major factors hampering crop production worldwide (Arcand & Schneider, 2006; Yang *et al.*, 2012).

Inorganic P as a chemical fertilizer can support crop production, but repeated use of these fertilizers is likely to have negative impacts on both the environment and the economy. Eutrophication is the main environmental problem caused by excess application of P (Park *et al.*, 2011). Therefore, it is important to explore alternative ways to improve the status of P in soils, such as the utilization of biofertilizers.

Recently, phosphate-solubilizing bacteria (PSB) have attracted the attention of agriculturists for their use as biofertilizers to improve plant growth and yield. PSB have the ability to solubilize insoluble P and release soluble P by producing various organic acids, mineral acids, siderophores, protons, humic substances, CO_2, and H_2S (Illmer & Schinner, 1995). However, the main mechanism of phosphate solubilization by PSB may involve the release of low-molecular-weight organic acids, which chelate phosphate-bound cations to convert P into soluble forms (Castagno *et al.*, 2011). Highly efficient PSB have been shown to utilize the direct oxidation glucose pathway to produce gluconic and 2-ketogluconic acids (Krishnaraj & Goldstein, 2001). Conversion of glucose to gluconic acid is facilitated by pyrroloquinoline quinone-dependent glucose dehydrogenase (PQQ-GDH). The conversion of gluconic acid to 2-ketogluconic acid involves flavin adenine dinucleotide-containing gluconate dehydrogenase (FAD-GADH) (Buch *et al.*, 2008). Both enzymes, PQQ-GDH and GADH, are localized in the outer face of the plasma membrane; therefore, acids are formed in the periplasmic space, thus also affecting the adjacent medium (Babu-Khan *et al.*, 1995). A recent study showed that *pqq* genes would be potential molecular markers of gram-negative soil PSB (Anzuay *et al.*, 2015).

Extensive research has been performed to isolate PSB from the rhizospheres of plants such as rice, pepper, sesame, spring onion (Chung *et al.*, 2005), cotton (Wu *et al.*, 2014), oil palm (Acevedo *et al.*, 2014) and peanut (Anzuay *et al.*, 2015). However, little is known about the prevalence of PSB in the rhizospheres of grapevines. Karagöz *et al.* (2012) isolated 17 PSB strains from the rhizosphere soil of grapevines in Turkey. Marasco *et al.* (2013) assessed the diversity and plant-growth-promoting (PGP) potential of the bacteria associated with the grapevine root system of different cultivars in three Mediterranean environments, and found that in all the strains isolated, including rhizobacteria and endophytic bacteria, 61% of them were able of solubilizing insoluble phosphate. But these authors did not conduct an experiment to verify whether PSB strains could improve grape growth.

Grape (*Vitis vinifera* L.) is an economically important crop in China. Because of the abundant soil resources, large temperature difference between day and night, long sunshine duration, and low rainfall, Northwest China has developed into a premium table- and wine-grape production area. Some regions of the Ningxia, Gansu, Xinjiang and Shaanxi provinces are located in the north at latitudes of 30-45 degrees, which is the golden area for growing grapes in the world (Yang & Li, 2008). However, the soil in these regions is relatively poor, and has low soluble P content. These factors adversely affect the grapevine growth. Phosphate-solubilizing microorganisms offer an alternative, eco-friendly strategy for enhancing the available P concentration in the rhizosphere soil, while the use of chemicals can be reduced.

The objectives of this study were to isolate and identify PSB from grapevine rhizospheres in five vineyards in Northwest China, to characterize their PGP traits, to evaluate the effect of stress on their phosphate-solubilizing activity (PSA), and to test their ability to stimulate the growth of *V. vinifera* cv. Cabernet Sauvignon. These objectives are consistent with the ultimate goal of using PSB as a kind of biofertilizer for saline-alkaline soils.

Material and methods

Sample collection

Soil samples were collected from vineyards of Yangling (34°27′N, 108°08′E) and Jingyang (34°26′N, 108°29′E) in Shaanxi, Yongji (34°34′N, 110°15′E) in Shanxi, Wuwei (37°23′N, 101°59′E) in Gansu, and Yinchuan (38°70′N, 106°27′E) in Ningxia during May-June, 2012. The vineyard of Yangling belongs to the College of Enology of Northwest A&F University. The other four sampling sites are commercial vineyards.

Samples were taken from ~30 cm away from the grapevine stem. Approximately 10 g of the soil adhering to the roots of each individual grape plant, considered the rhizospheric soil, was used for the bacterial isolation procedures. Plant residues and stones were removed, and the fresh soil samples were stored in sealed sterile bags at 4°C (Karagöz *et al.*, 2012).

Isolation and screening of PSB

Serially diluted soil samples were plated on the National Botanical Research Institute's phosphate (NBRIP) medium, which contained 5.0 g tricalcium phosphate (TCP) as the sole P source (Nautiyal, 1999). Each sample was plated in triplicate with suitable soil concentrations. After incubation at 28°C for 72 h, all isolates forming clear halo zones were selected as PSB.

The PSA in liquid medium was measured by following the method of Johri *et al.* (1999) with modifications. Briefly, 1 mL sample of bacterial culture (10^6 CFU/mL) was added to a 250-mL flask containing 50 mL of NBRIP. After incubation at 28°C on a rotary shaker (150 rpm) for 7 days, cultures were centrifuged at 10,000 rpm for 20 min. Supernatants were collected for pH and soluble P analyses. Phosphorus in the culture was determined by the Mo-blue method using a spectrophotometer at a wavelength of 700 nm (Watanabe & Olsen, 1965). A separate broth medium inoculated with sterile water served as the control treatment.

Identification of PSB

Bacterial genomic DNA was extracted using a bacterial genomic DNA extraction kit, and the 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCC-3') (Weisburg *et al.*, 1991). The 50-µL reaction mixture consisted of 5 µL of 10× reaction buffer, 4 µL of 200 mmol/L dNTP mixture, 2.5 µL of 10 µmol/L primer, 2.5 U of Taq DNA polymerase, and 50 ng of genomic DNA. The reaction conditions were as follows: 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 2 min, with a final extension step at 72°C for 3 min. The amplified products (nearly 1500 bp in length) were purified using an agarose gel DNA purification kit, and 16S rRNA sequencing was conducted at Beijing Huada Biological Company of China.

The top ten PSB strains, based on their PSA, were identified by 16S rRNA sequencing, and the acquired sequences were submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with the accession numbers KC776273-KC776282 (Table 1). Based on the results of the database searches, sequences were aligned with representative bacterial sequences from the GenBank database by using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) to determine their approximate phylogenetic affiliations and 16S rRNA gene sequence similarities. The phylogenetic tree was constructed using the neighbor-joining method with distance matrices and the program MEGA (v6) after bootstrap analysis of 1000 replications (Chen *et al.*, 2006).

Characterization of PGP traits

Production of indole acetic acid (IAA). The selected strains were grown in a minimal medium (50 mM KH_2PO_4, 50 mM K_2HPO_4, 5 mM $MgSO_4$, 25 mM $(NH_4)_2SO_4$, 1% glucose) with 0.05% L-tryptophan at 28°C for 48 h in a shaking incubator at 150 rpm. A separate broth medium inoculated with sterile water served as the control treatment. The culture was centrifuged at 8,000 rpm for 10 min. The supernatant (1 mL) was vigorously mixed with 4 mL of Salkowski's reagent. The mixture was incubated for 30 min at 25°C in the dark. The absorbance of the resulting solution was measured at 530 nm (Glickmann & Dessaux, 1995).

Production of ACC deaminase. The selected strains were inoculated on the Dworkin–Foster (DF) salts minimal medium containing 1-aminocyclopropane-1-carboxylate (ACC) as the sole N source. The chemical composition of DF salts minimal medium was as follows: 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 1 mg $FeSO_4 \cdot 7H_2O$, 10 µg H_3BO_3, 10 µg $MnSO_4$, 70 µg $ZnSO_4$, 50 µg $CuSO_4$, 10 µg MoO_3, 2 g glucose, 2 g gluconic acid, 2 g citric acid, and 15 g agar in 1 L distilled water. The amount of ACC added to DF salts minimal medium was 0.3033 g/L. The isolates that grew on this medium were selected for further studies.

Table 1. Phosphate-solubilizing bacteria (PSB) isolates and their closest phylogenetic relatives based on 16S rRNA gene sequencing.

Isolate	Accession No.	Closest identified phylogenetic relatives[1]	Similarity %[2]
JY2	KC776273	*Bacillus aryabhattai* B8W22 (NR_115953)	99.78
JY3	KC776274	*Klebsiella variicola* At-22 (NR_074729)	99.86
JY5	KC776275	*Bacillus megaterium* IAM 13418 (NR_043401)	99.70
JY8	KC776276	*Klebsiella variicola* At-22 (NR_074729)	99.86
JY10	KC776277	*Stenotrophomonas rhizophila* e-p10 (NR_028930)	99.58
JY11	KC776278	*Enterobacter aerogenes* KCTC 2190 (NR_102493)	99.57
JY15	KC776279	*Stenotrophomonas rhizophila* e-p10 (NR_028930)	99.51
JY17	KC776280	*Bacillus aryabhattai* B8W22 (NR_115953)	99.57
JY22	KC776281	*Bacillus aryabhattai* B8W22 (NR_115953)	99.76
JY26	KC776282	*Bacillus aryabhattai* B8W22 (NR_115953)	99.58

[1] Accession number of the representative strain is shown in parentheses. [2] Similarity value calculated by using ClustalW2.

Quantitative measurement of the ACC-deaminase activity of the selected isolates was performed by measuring the amount of α-ketobutyrate produced when the enzyme ACC deaminase cleaved ACC. The concentration of α-ketobutyrate (μmol) was determined by measuring the absorbance of samples at 540 nm (Penrose & Glick, 2003; Shahzad *et al.*, 2013).

Production of siderophores. Qualitative measurement of the siderophore-producing capacity of the selected PSB strains was performed using the universal chrome azurol S (CAS) agar plate assay. On CAS agar plates, siderophore-producing (Sid+) bacteria form colonies with an orange halo because iron is removed from the original blue CAS–Fe (III) complex during siderophore production. Inoculated plates were evaluated for the formation of siderophore halos after incubation at 28°C for 7 days (Schwyn & Neilands, 1987).

Nitrogen-fixing ability. We made some modifications based on the methods available (Lin *et al.*, 2012). The PSB strains were inoculated on modified nitrogen-deficient Ashby's agar medium (10 g sucrose, 0.2 g NaCl, 0.2 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaSO_4 \cdot 2H_2O$, 5 g $CaCO_3$, and 15 g agar in 1 L distilled water; pH 7.0). The strains that grew on this medium after incubation at 28°C for 7 days were considered to possess the ability to fix nitrogen.

Production of HCN. HCN production was detected according to the method developed by Bakker & Schippers (1987); all isolates were streaked on 10% tryptic soya agar with 4.4 g/L glycine. After 24 h of growth at 28°C, the plates were inverted, and an autoclaved filter paper soaked with picric acid (0.5%) and Na_2CO_3 (2%) solution was placed on each cover. The plates were sealed and incubated at 28°C for 48 h. HCN production is indicated by a change in coloration from orange to red. A strain of *Pseudomonas* sp. was used as a positive control (Bakker & Schippers, 1987).

Production of chitinase. Chitinase activity was evaluated by measuring the width of the edge of the translucent haloes surrounding the colonies grown on the plates after 48 h of incubation (Rojas-Avelizapa *et al.,* 1999). The medium was prepared with 13-14% (wet weight) colloidal chitin and 2.3% agar dissolved in Castaneda medium (0.625 g diammonium citrate, 0.250 g NaCl, 0.375 g KH_2PO_4, 0.125 g $MgSO_4 \cdot 7H_2O$, 0.375 g Na_2CO_3, 6.5 mL glycerol, in 1 L distilled water; pH 6.5-7.0).

Production of protease. The strains were inoculated on a medium composed of 1% casein and 2.3% agar dissolved in Castaneda medium; the pH was adjusted to 6.5-7.0 to avoid casein precipitation. Casein hydrolysis was detected by the formation of a whitish, opaque halo (coagulated casein) around a translucent area (totally hydrolyzed casein) surrounding the colony (Rojas-Avelizapa *et al*., 1999).

Effect of stress on PSA

In a 100-mL flask containing 50 mL of NBRIP, 0.5 mL of the bacterial culture (concentration, 10^8 CFU/mL) was added. The samples were incubated for 3 days at 150 rpm under various conditions of pH (7.0, 9.0, and 11.0), NaCl concentration (0%, 2.5%, and 5.0%) and temperature (15°C, 28°C, and 40°C). A separate broth medium inoculated with sterile water served as the control treatment. Soluble P content was detected under all these conditions. All treatments were performed in triplicate, and the data are presented using the means of these triplicates (Banerjee *et al.*, 2010).

Pot experiments

Experiments were conducted in the greenhouse (average air temperature between 18°C and 30°C; average relative humidity between 50% and 60%) of the College of Enology, Northwest A&F University, Yangling, Shaanxi, China (34°27′N, 108°08′E) during February-July, 2013.

The soil samples used in this experiment were a mixture of dark loessial soil and river sand (1:1, v/v), high-temperature sterilized at 121°C for 20 min. The basic soil properties were 0.84 g/kg total N, 0.63 g/kg total P, 25.46 g/kg total K, 67 mg/kg available N, 14 mg/kg available P, 111 mg/kg available K, 11.4 g/kg organic matter and pH 8.08 (Bao, 2000).

Cuttings of *V. vinifera* cv. Cabernet Sauvignon (from the vineyard of the College of Enology, Northwest A&F University) were soaked in 0.1% $HgCl_2$ for 5 min, repeatedly washed with sterilized water, soaked in 25 mg/kg 1-naphthaleneacetic acid solution for 12 h, and then planted in nutritive bowls. After 3-4 leaves had grown on the seedlings, they were transplanted to ethanol-disinfected plastic pots (inner diameter, 23 cm; height, 17 cm).

Two strains of *Bacillus aryabhattai,* JY17 and JY22, were grown separately in the nutrient broth at 28°C in a shaker (150 rpm) for 24 h. Then, 50 mL of the bacterial suspensions (10^8 CFU/mL) of two strains were separately inoculated into the middle part of the seedling roots. Sterilized nutrient broth (30 mL) was applied as the control treatment. Experiments were performed in a completely randomized block design. Three treatments (*B. aryabhattai* JY17, *B. aryabhattai* JY22 and control) were performed with 5 independent replications, 10 pots per replication. The PGP effects of bacterial treatment were assessed by measuring plant height, root and shoot dry weight (Yu *et al.*, 2012) and stem thickness after the grapevine was grown for 30 days.

Statistical analysis

A statistical analysis was conducted using an analysis of variance (ANOVA) in the Statistical Package for Social Sciences (SPSS), version 21, followed by a comparison of multiple treatment levels with the control. Duncan's multiple range test was used for multiple mean comparisons at $p < 0.05$.

Results

Isolation and identification of PSB

Sixty-six PSB strains were obtained from the rhizospheres of grapevine plants and screened based on halo formation in NBRIP medium. Among them, 23 strains were isolated from Jingyang, 14 from Wuwei, 11 from Yangling, 11 from Yinchuan, and 7 from Yongji. These PSB showed different abilities in TCP solubilization and soluble P concentrations varying from 19.41-673.99 mg/L, significantly higher than the control (11.80 mg/L). JY22 showed the highest P solubilization, followed by JY17 with a soluble P concentration of 671.64 mg/L. The ten strains with soluble P concentrations above 500 mg/L were all isolated from Jingyang.

The aforementioned ten PSB strains screened by the authors belonged to 4 genera and 5 species: *Bacillus aryabhattai, Bacillus megaterium, Klebsiella variicola, Stenotrophomonas rhizophila,* and *Enterobacter aerogenes* (Table 1). The strains JY3 and JY8 exhibited a sequence identity of up to 99% with *K. variicola*, whereas the sequences of JY10 and JY15 exhibited a strong similarity to the 16S rRNA sequence of *S. rhizophila*. The sequences of JY2, JY17, JY22, and JY26 corresponded to those of *B. aryabhattai*, and the closest identified phylogenetic relatives of JY5 and JY11 were *B. megaterium* and *E. aerogenes*, respectively. The phylogenetic tree expressing their relationships is shown in Fig. 1.

PGP traits of PSB

The selected bacterial isolates were tested for their PGP traits (Table 2). All isolated strains had the ability to produce IAA and siderophores. Production of IAA by the isolates ranged from 2.23 to 31.03 mg/L. JY22 showed the highest IAA production, followed by JY10, JY2, and JY17 (27.54 mg/L, 23.24 mg/L and 21.21 mg/L, respectively). Except for JY10, the other isolates produced ACC deaminase, with activity varying from 4.84 to 245.89 nmol/mg·h, and JY2 had the highest activity. The isolates JY2, JY3, JY5, JY8, JY17, JY22, and JY26 were able to grow on the N-free Ashby agar medium, signifying that they possessed nitrogen-fixing abilities. The PSB isolates producing HCN included JY3, JY8, JY11, and JY22. The chitinase activity could be detected in seven strains, and all the ten isolates could secrete protease.

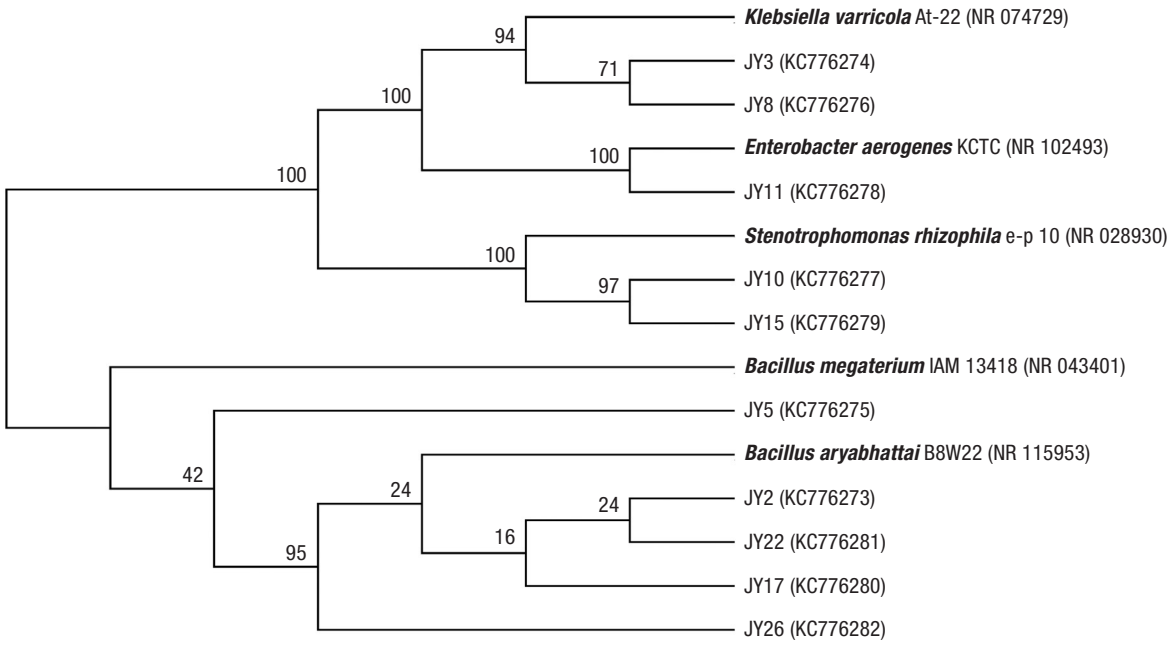

Figure 1. Phylogenetic tree showing the relationships between the phosphate-solubilizing bacteria (PSB) isolates in this study and their closest phylogenetic relatives based on 16S rRNA gene sequencing (accession numbers are given in parentheses).

Table 2. Characterization of the plant-growth-promoting traits of PSB isolates.

Isolate	Soluble-P content (mg/L)	pH of medium	IAA content (mg/L)	ACC deaminase activity (nmol/mg·h)	Siderophore production	Nitrogen-fixing ability	HCN production	Chitinase production	Protease production
JY22	673.99±4.51a	3.41±0.02e	31.03±3.18a	51.75±3.78e	+	+	+	+	+
JY17	671.64±5.98a	3.43±0.02e	21.21±2.41cd	98.52±2.75c	+	+	-	+	+
JY11	662.20±5.74ab	3.51±0.03d	2.41±0.90f	137.25±6.66b	+	-	+	+	+
JY15	661.41±13.58ab	3.53±0.03d	11.59±0.15e	4.84±1.35f	+	-	-	-	+
JY2	648.04±4.35b	3.57±0.02d	23.24±3.02c	245.89±14.97a	+	+	-	+	+
JY10	605.57±10.93c	3.55±0.02d	27.54±2.17b	-	+	-	-	-	+
JY8	594.56±6.49cd	3.76±0.04c	18.30±2.22d	79.11±5.36d	+	+	+	+	+
JY3	576.13±7.13de	4.02±0.07b	12.92±2.10e	104.83±14.81c	+	+	+	+	+
JY5	569.80±11.61e	3.99±0.02b	3.79±1.13f	108.96±7.11c	+	+	-	-	+
JY26	517.69±12.46f	4.23±0.05a	2.23±0.69f	49.33±5.24e	+	+	-	+	+

Values (means ± SD) with different letters in the same column denote significant differences at $p < 0.05$ (Duncan's multiple range test). +, tested positive; -, tested negative.

Effects of stressful environments on PSA

Under alkali stress conditions, the soluble P content in NBRIP broth significantly decreased with increasing pH, indicating that the PSA of PSB isolates was inhibited by the high initial pH of the broth medium (Fig. 2A). However, the soluble P concentration at all pH values was >200 mg/L. Under non-stress conditions, there were no significant differences between the soluble P concentrations of JY17 and JY22, but when the initial pH was 9.0, P solubilization by JY22 was significantly lower than that by JY17, showing that JY17 had better tolerance to alkali stress. When the initial pH was 11.0, the soluble P content of JY17 and JY22 was reduced by 37.6% and 40.1%, respectively, which was lower than that during the non-stress condition.

These two strains showed efficient phosphate solubilization at a NaCl concentration of 5% with the amount of solubilized phosphate exceeding 200 mg/L (Fig. 2B). Soluble P levels in JY17 and JY22 at a NaCl concentration of 2.5% were even higher than those under non-stress conditions; this indicates that moderate salt may stimulate the P-solubilizing abilities of certain PSB strains.

The soluble P levels of JY17 and JY22 increased with elevated temperature, and low temperature (15°C) clearly inhibited their PSA (Fig. 2C). However, high temperature (40°C) had no negative impacts on their PSA. The amount of soluble P at 40°C was significantly higher than that at 28°C and 15°C (increased by 33.39% and 38.23%, respectively). The PSA of JY22 at 40°C was the highest, reaching 462.30 mg/L.

Effects of PSB on plant growth

PSB treatment increased the biometric parameters of *V. vinifera* cv. Cabernet Sauvignon (plant height, stem thickness, root and shoot dry weight) over those of the control plants (Table 3). The degree of stimulation varied with respect to the growth parameter. PSB treatment had the maximum stimulatory effect on shoot dry weight, whereas the plant height was only slightly influenced compared with other parameters. The root/shoot ratio of PSB-treated plants was significantly smaller than that of the control plants.

Discussion

P-solubilizing activity is related to the microbial production of organic acids, which chelate the cation bound to phosphate, thereby converting it to a soluble form (Sagoe *et al.*, 1998; Rashid *et al.*, 2004; Lugtenberg & Kamilova, 2009). In the present study, a significant negative relationship was observed between the amount of soluble P and pH in the culture medium (R^2=0.953). This result reaffirms that phosphate solubilization by PSB is involved in the production of organic acids (Halder *et al.*, 1990; Goldstein, 1995; Kim *et al.*, 1998; Rashid *et al*., 2004). The recent research demonstrated that citric acid secreted by PSB strains can improve their P-solubilizing activity. Researchers incorporated artificial citrate operon, containing NADH insensitive citrate synthase (gltA1) and citrate transporter (citC) genes, into the genomes of *Pseudomonas fluorescens* (Adhikary *et al.*, 2014) and *Enterobacter hormaechei* strains (Yadav *et al.*, 2014), founding that the transformants secreted more citric acids than the native strains, and released more soluble P in the mediums. Further studies are required for confirming which organic acids reduce the pH of the medium.

Phosphate-solubilizing rhizobacteria are always confronted with various environmental stresses. The ability to withstand adverse environmental conditions

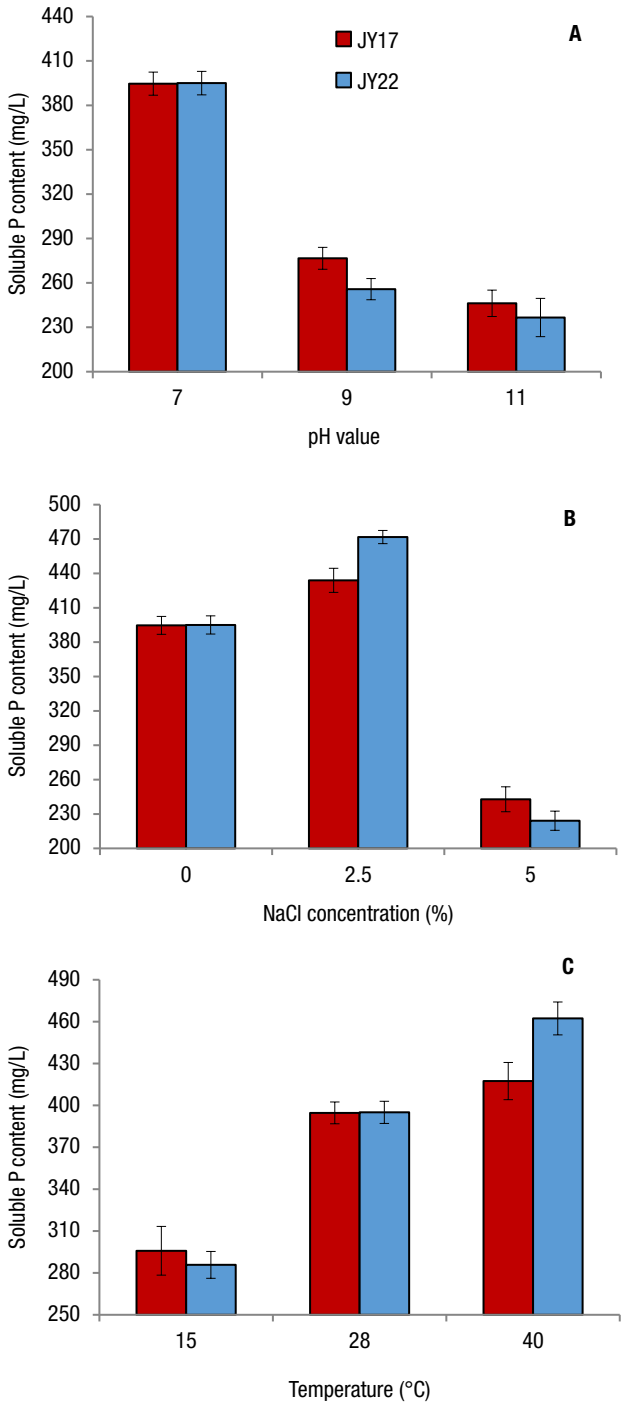

Figure 2. Effects of pH (A), NaCl concentration (B) and temperature (C) on the phosphate-solubilizing activity (PSA) of different strains. Each value represents the mean of three replicates with the standard deviation shown by error bars.

is significant not only for rhizobacterial survival in agricultural soils, but also for their use as biofertilizers (Banerjee *et al*., 2010). It this study, we isolated and selected two PSB strains, JY17 and JY22, which had high PSA even in pH of 9.0, salt concentration of 5.0% or 40 °C. They had bright application prospects as biofertilizers in saline-alkaline soils. Northwest China is located in the arid and semi-arid areas. Due to drought and strong evaporation, the processes of soil leaching and desalination are extremely weak, and the salt accumulation process is dominant. So there is a large area of saline-alkali soils in Northwest. Salt concentration of soils here may be as high as 3.25%, and pH as high as 10.3 (Sun *et al.*, 2014). It is vital to solubilize P efficiently whether PSB isolates can survive in conditions of high pH, salt, and temperature. There was a general trend that PSA decreases as NaCl concentration rises (Srividya *et al.*, 2009; Marasco *et al.*, 2013). This may result from reductions in cell growth and proliferation under saline stresses, or too many chloride ions may chelate and neutralize proton ions or acid in the medium, thereby resulting in lower solubilization efficiency (Srividya *et al*., 2009). Meanwhile, global warming is predicted to affect microbial communities, and hamper their physiology and growth (Sheik *et al.*, 2011). It has been reported that the production of food and forage in arid and semi-arid regions of the world can be increased by the application of PSB capable of withstanding such abiotic stresses (Banerjee *et al*., 2010).

A large number of researches demonstrated that PSB strains could improve plant growth, such as *Brassica napus* L. (Freitas *et al.*, 1997), *Zea mays* L. (Nadeem *et al.*, 2007), *Brassica juncea* (Kumar *et al.*, 2009), *Stevia rebaudiana* (Mamta *et al.*, 2010), sugarcane (Beneduzi *et al.*, 2013), and cotton (Wu *et al*., 2014). In this study, PSB treatment increased the plant height, stem thickness, root and shoot dry weight of *V. vinifera* cv. Cabernet Sauvignon. It was probable that PSB enhanced the amount of soluble P in soils, and consequently increasing P uptake of grapevine. The soluble P content in soils and P uptake of plants should be detected furtherly to explain this phenomenon. Gupta *et al.* (2012) found that the treatment of plants with individual PSB or mixture of them increased soil available P and P uptake in leaves of *Aloe barbadensis,* and consequently elevated all parameters of *A. barbadensis,* including leaf length, root length, total number of leaves, total gel volume, dry gel weight, and dry rind weight. However, some researchers had different results. Freitas *et al.* (1997) proved that PSB isolates had active effect on canola growth and significantly increased plant height, but did not increase P uptake of canola. Fernández *et al.* (2007) obtained a similar result. They found that aerial height of soybean shoots were increased by inoculating with one PSB strain, *Burkholderia* sp. Per2F, but there were no significant differences in shoot P content between inoculated and uninoculated soybeans.

Another reason why PSB isolates improved grape growth in this study may be that these strains produced

Table 3. Effects of PSB treatment on the biometric parameters of *V. vinifera* cv. Cabernet Sauvignon.

Treatment	Plant height (cm)	Stem thickness (cm)	Root dry weight (g)	Shoot dry weight (g)	Root/Shoot ratio
Bacillus aryabhattai JY22	31.42±0.99a	0.50±0.01a	5.76±0.07a	7.73±0.10a	0.745±0.004a
Bacillus aryabhattai JY17	30.99±1.02a	0.47±0.02a	5.63±0.07a	7.61±0.09a	0.739±0.009a
Control[1]	27.57±0.88b	0.39±0.02b	4.83±0.10b	5.62±0.08b	0.859±0.003b

[1] Uninoculated control. Values (means ± SD) with different letters in the same column denote significant differences at $p < 0.05$ (Duncan's multiple range test).

some metabolites facilitating plant growth. The strains of JY17 and JY22 could provide plants with IAA, siderophores, and ACC deaminase. IAA secreted by rhizobacteria may directly promote root growth by stimulating plant cell elongation or division, resulting in greater root surface area, which enables the plant to access more nutrients from soils. In vitro studies showed that for some PSB strains, the genes necessary for PSA and IAA production were coexpressed (Dey *et al.*, 2004). The siderophores produced by rhizobacteria may contribute to the increased mobility of Fe in the soil and rhizosphere, making it more available for the plant (Principe *et al.*, 2007). Bacteria capable of producing ACC deaminase can regulate endogenous ethylene (C_2H_4) levels in developing seedlings. C_2H_4 has been known to stimulate germination and break seed dormancy in many different plants, but root elongation is inhibited if the C_2H_4 concentration at germination is too high (Kucera *et al.*, 2005; Finch-Savage & Leubner-Metzger, 2006; Gianinetti *et al.*, 2007). Several reports showed that ACC deaminase-producing rhizobacteria increased root elongation and plant growth by reducing ethylene stress (Mayak *et al.*, 2004; Saravanakumar & Samiyappan, 2007; Shahzad *et al.*, 2013). Besides, the strains of JY17 and JY22 also secreted chitinase and protease, both of which could indirectly stimulate plant growth and development by preventing the growth of phytopathogenic microorganisms. These two characteristics are necessary for an efficient biofertilizer in the field.

In conclusion, the selected PSB strains JY17 and JY22 were positive for multiple PGP traits, including nitrogen fixation and production of IAA, siderophores, ACC deaminase, chitinase, and protease, and they revealed strong PSA even under conditions involving high pH levels, high salt concentrations, and high temperatures. Therefore, they can be used as biofertilizers in saline-alkaline soils. The present study clearly demonstrated that the inoculation of PSB significantly facilitated the growth of *V. vinifera* cv. Cabernet Sauvignon under greenhouse conditions. Use of these PSB as biofertilizers will increase the available P content in soils, minimize P fertilizer application, reduce environmental pollution, and promote sustainable agriculture. However, further research is needed to understand the specific mechanisms of phosphate solubilization by PSB and to verify these results in the field.

References

Acevedo E, Galindo-Castañeda T, Prada F, Navia M, Romero HM, 2014. Phosphate-solubilizing microorganisms associated with the rhizosphere of oil palm (*Elaeis guineensis* Jacq.) in Colombia. Appl Soil Ecol 80(8): 26-33. https:/doi.org/10.1016/j.apsoil.2014.03.011.

Adhikary H, Sanghavi PB, Macwan SR, Archana G, Kumar GN, 2014. Artificial citrate operon confers mineral phosphate solubilization ability to diverse *Fluorescent pseudomonads*. Plos One 9 (9): 1-12. https:/doi.org/10.1371/journal.pone.0107554.

Anzuay MS, Frola O, Angelini JG, Ludueña LM, Ibañez F, Fabra A, Taurian T, 2015. Effect of pesticides application on peanut (*Arachis hypogaea* L.) associated phosphate solubilizing soil bacteria. Appl Soil Ecol 95: 31-37. https:/doi.org/10.1016/j.apsoil.2015.05.003.

Arcand MM, Schneider KD, 2006. Plant- and microbial-based mechanisms to improve the agronomic effectiveness of phosphate rock: A review. Anais Da Academia Brasileira De Ciências 78 (4): 791-807. https:/doi.org/10.1590/S0001-37652006000400013.

Babu-Khan S, Yeo TC, Martin WL, Duron MR, Rogers RD, Goldstein AH, 1995. Cloning of a mineral phosphate-solubilizing gene from *Pseudomonas Cepacia*. Appl Environ Microbiol 61 (3): 972-978.

Bakker AW, Schippers B, 1987. Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp-mediated plant growth-stimulation. Soil Biol Biochem 19 (4): 451-457. https:/doi.org/10.1016/0038-0717(87)90037-X.

Banerjee S, Palit R, Sengupta C, Standing D, 2010. Stress induced phosphate solubilization by *Arthrobacter* sp. and *Bacillus* sp. isolated from tomato rhizoshpere. Aust J Crop Sci 4 (6): 378-383.

Bao S, 2000. Soil agricultural chemistry analysis. Agriculture Press, Beijing, China.

Beneduzi A, Moreira F, Costa PB, Vargas LK, Lisboa BB, Favreto R, Baldani JI, Passaglia LMP, 2013. Diversity and plant growth promoting evaluation abilities of bacteria isolated from sugarcane cultivated in the South of Brazil. Appl Soil Ecol 63: 94-104. https:/doi.org/10.1016/j.apsoil.2012.08.010.

Buch A, Archana G, Naresh Kumar G, 2008. Metabolic channeling of glucose towards gluconate in phosphate-solubilizing *Pseudomonas aeruginosa* P4 under phosphorus deficiency. Res Microbiol 159 (9-10): 635-642. https:/doi.org/10.1016/j.resmic.2008.09.012.

Castagno LN, Estrella MJ, Sannazzaro AI, Grassano AE, Ruiz OA, 2011. Phosphate-solubilization mechanism and *in vitro* plant growth promotion activity mediated by *Pantoea eucalypti* isolated from *Lotus tenuis* rhizosphere in the Salado River Basin (Argentina). J Appl Microbiol 110 (5): 1151-1165. https:/doi.org/10.1111/j.1365-2672.2011.04968.x.

Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC, 2006. Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl Soil Ecol 34 (1): 33-41. https:/doi.org/10.1016/j.apsoil.2005.12.002.

Chung H, Park M, Madhaiyan M, Seshadri S, Song J, Cho H, Sa T, 2005. Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biol Biochem 37 (10): 1970-1974. https:/doi.org/10.1016/j.soilbio.2005.02.025.

Dey R, Pal KK, Bhatt DM, Chauhan SM, 2004. Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. Microbiol Res 159 (4): 371-394. https:/doi.org/10.1016/j.micres.2004.08.004.

Fernández LA, Zalba P, Gómez MA, Sagardoy MA, 2007. Phosphate-solubilization activity of bacterial strains in soil and their effect on soybean growth under greenhouse conditions. Biol Fertil Soils 43 (6): 805-809. https:/doi.org/10.1007/s00374-007-0172-3.

Finch-Savage WE, Leubner-Metzger G, 2006. Seed dormancy and the control of germination. New Phytologist 171 (3): 501-523. https:/doi.org/10.1111/j.1469-8137.2006.01787.x.

Freitas JRD, Banerjee MR, Germida JJ, 1997. Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). Biol Fertil Soils 24 (24): 358-364. https:/doi.org/10.1007/s003740050258.

Gianinetti A, Laarhoven LJJ, Persijn ST, Harren FJM, Petruzzelli L, 2007. Ethylene production is associated with germination but not seed dormancy in red rice. Ann Bot 99(4): 735-745. https:/doi.org/10.1093/aob/mcm008.

Glickmann E, Dessaux Y, 1995. A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Appl Environ Microbiol 61 (2): 793-796.

Goldstein AH, 1995. Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. Biol Agr Hortic 12 (2): 185-193. https:/doi.org/10.1080/01448765.1995.9754736.

Gupta M, Kiran S, Gulati A, Singh B, Tewari R, 2012. Isolation and identification of phosphate solubilizing bacteria able to enhance the growth and aloin-a biosynthesis of Aloe barbadensis Miller. Microbiol Res 167 (6): 358-363. https:/doi.org/10.1016/j.micres.2012.02.004.

Halder AK, Mishra AK, Bhattacharyya P, Chakrabartty PK, 1990. Solubilization of rock phosphate by Rhizobium and Bradyrhizobium. J Gen Appl Microbiol 36 (2): 81-92. https:/doi.org/10.2323/jgam.36.81.

Illmer P, Schinner F, 1992. Solubilization of inorganic phosphates by microorganisms isolated from forest soils. Soil Biol Biochem 24 (4): 389-395. https:/doi.org/10.1016/0038-0717(92)90199-8.

Illmer P, Schinner F, 1995. Solubilization of inorganic calcium phosphates-Solubilization mechanisms. Soil Biol Biochem 27 (3): 257-263. https:/doi.org/10.1016/0038-0717(94)00190-C.

Johri JK, Surange S, Nautiyal CS, 1999. Occurrence of salt, pH, and temperature-tolerant, phosphate-solubilizing bacteria in alkaline soils. Curr Microbiol 39 (2): 89-93. https:/doi.org/10.1007/s002849900424.

Karagöz K, Ateş F, Karagöz H, Kotan R, Çakmakçı R, 2012. Characterization of plant growth-promoting traits of bacteria isolated from the rhizosphere of grapevine grown in alkaline and acidic soils. Eur J Soil Biol 50: 144-150. https:/doi.org/10.1016/j.ejsobi.2012.01.007.

Kim KY, Jordan D, Mcdonald GA, 1998. Enterobacter agglomerans, phosphate solubilizing bacteria, and microbial activity in soil: Effect of carbon sources. Soil Biol Biochem 30 (8-9): 995-1003. https:/doi.org/10.1016/S0038-0717(98)00007-8.

Krishnaraj PU, Goldstein AH, 2001. Cloning of a Serratia marcescens DNA fragment that induces quinoprotein glucose dehydrogenase-mediated gluconic acid production in Escherichia coli in the presence of stationary phase Serratia marcescens. FEMS Microbiol Lett 205 (2): 215-220. https:/doi.org/10.1111/j.1574-6968.2001.tb10950.x.

Kucera B, Cohn MA, Leubner-Metzger G, 2005. Plant hormone interactions during seed dormancy release and germination. Seed Sci Res 15 (4): 281-307. https:/doi.org/10.1079/SSR2005218.

Kumar KV, Srivastava S, Singh N, Behl HM, 2009. Role of metal resistant plant growth promoting bacteria in ameliorating fly ash to the growth of Brassica juncea. J Hazard Mater 170 (1): 51-57. https:/doi.org/10.1016/j.jhazmat.2009.04.132.

Lin L, Li Z, Hu C, Zhang X, Chang S, Yang L, Li Y, An Q, 2012. Plant growth-promoting nitrogen-fixing enterobacteria are in association with sugarcane plants growing in Guangxi, China. Microb Environ 27 (4): 391-398. https:/doi.org/10.1264/jsme2.ME11275.

Lugtenberg B, Kamilova F, 2009. Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63 (63): 541-556. https:/doi.org/10.1146/annurev.micro.62.081307.162918.

Mamta, Rahi P, Pathania V, Gulati A, Singh B, Bhanwra RK, Tewari R, 2010. Stimulatory effect of phosphate-solubilizing bacteria on plant growth, Stevioside and Rebaudioside-a contents of Stevia rebaudiana Bertoni. Appl Soil Ecol 46 (2): 222-229. https:/doi.org/10.1016/j.apsoil.2010.08.008.

Marasco R, Rolli E, Fusi M, Cherif A, Abou-Hadid A, El-Bahairy U, Borin S, Sorlini C, Daffonchio D, 2013. Plant growth promotion potential is equally represented in diverse grapevine root-associated bacterial communities from different biopedoclimatic environments. Biomed Res Int 2013 (3): 247-261. https:/doi.org/10.1155/2013/491091.

Mayak S, Tirosh T, Glick BR, 2004. Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiol Biochem 42 (6): 565-572. https:/doi.org/10.1016/j.plaphy.2004.05.009.

Nadeem SM, Zahir ZA, Naveed M, Arshad M, 2007. Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. Can J Microbiol 53 (10): 1141-1149. https:/doi.org/10.1139/W07-081.

Nautiyal CS, 1999. An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170 (1): 265-270. https:/doi.org/10.1111/j.1574-6968.1999.tb13383.x.

Park JH, Bolan N, Megharaj M, Naidu R, 2011. Isolation of phosphate solubilizing bacteria and their potential for lead immobilization in soil. J Hazard Mater 185 (2-3): 829-836. https:/doi.org/10.1016/j.jhazmat.2010.09.095.

Penrose DM, Glick BR, 2003. Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plantarum 118: 10-15. https:/doi.org/10.1034/j.1399-3054.2003.00086.x.

Principe A, Alvarez F, Castro MG, Zachi L, Fischer SE, Mori GB, Jofre E, 2007. Biocontrol and Pgpr features in native strains isolated from saline soils of Argentina. Curr Microbiol 55 (4): 314-322. https:/doi.org/10.1007/s00284-006-0654-9.

Rashid M, Khalil S, Ayub N, Alam S, 2004. Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms (Psm) under in vitro conditions. Pak J Biol Sci 7 (2): 187-196. https:/doi.org/10.3923/pjbs.2004.187.196.

Rojas-Avelizapa L, Cruz-Camarillo R, Guerrero M, Rodriguez-Vazquez R, Ibarra J, 1999. Selection and characterization of a proteo-chitinolytic strain of *Bacillus thuringiensis*, able to grow in shrimp waste media. World J Microb Biot 15: 299-308. https:/doi.org/10.1023/A:1008947029713.

Sagoe CI, Ando T, Kouno K, Nagaoka T, 1998. Relative importance of protons and solution calcium concentration in phosphate rock dissolution by organic acids. Soil Sci Plant Nutr 44 (4): 617-625. https:/doi.org/10.1080/00380768.1998.10414485.

Saravanakumar D, Samiyappan R, 2007. Acc deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (Arachis hypogea) plants. J Appl Microbiol 102 (5): 1283-1292. https:/doi.org/10.1111/j.1365-2672.2006.03179.x.

Schwyn B, Neilands JB, 1987. Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160 (1): 47-56. https:/doi.org/10.1016/0003-2697(87)90612-9.

Shahzad SM, Arif MS, Riaz M, Iqbal Z, Ashraf M, 2013. Pgpr with varied ACC-deaminase activity induced different growth and yield response in maize (*Zea mays* L.) under fertilized conditions. Eur J Soil Biol 57: 27-34. https:/doi.org/10.1016/j.ejsobi.2013.04.002.

Sheik CS, Beasley WH, Elshahed MS, Zhou X, Luo Y, Krumholz LR, 2011. Effect of warming and drought on grassland microbial communities. Isme Journal 5 (10): 1692-1700. https:/doi.org/10.1038/ismej.2011.32.

Srividya S, Soumya S, Pooja K, 2009. Influence of environmental factors and salinity on phosphate solubilization by a newly isolated *Aspergillus niger* F7 from agricultural soil. Afr J Biotechnol 8 (9): 1864-1870.

Sun J, Zhang R, Wang H, Fan W, Luo L, Han J, Ma Z, 2014. Stpatial variation of soil salt content and pH of salinized soil in Dingbian County, Shaanxi Province. Acta Agriculturae Boreali-Occidentalis Sinica 23 (9): 114-119.

Watanabe FS, Olsen SR, 1965. Test of an ascorbic acid method for determining P in water and $NaHCO_3$ extracts from soil. Soil Sci Soc Am Proc 29: 677-678. https:/doi.org/10.2136/sssaj1965.03615995002900060025x.

Weisburg WG, Barns SM, Pelletier DA, Lane DJ, 1991. 16s ribosomal DNA amplification for phylogenetic study. J Bacteriol 173 (2): 697-703. https:/doi.org/10.1128/jb.173.2.697-703.1991.

Wu Z, Peng Y, Guo L, Li C, 2014. Root colonization of encapsulated Klebsiella oxytoca Rs-5 on cotton plants and its promoting growth performance under salinity stress. Eur J Soil Biol 60: 81-87. https:/doi.org/10.1016/j.ejsobi.2013.11.008.

Yadav K, Kumar C, Archana G, Kumar GN, 2014. Artificial citrate operon and Vitreoscilla hemoglobin gene enhanced mineral phosphate solubilizing ability of Enterobacter hormaechei Dhrss. Appl Microbiol Biotechnol 98 (19): 8327-8336. https:/doi.org/10.1007/s00253-014-5912-3.

Yang H, Li Q, 2008. Studies on path for transiting from resources advantage to competition superiority of grape wine industry in Northwest China. Chin J Agr Resour Reg Plan 5: 54-57.

Yang PX, Ma L, Chen MH, Xi JQ, He F, Duan CQ, Mo MH, Fang DH, Duan YQ, Yang FX, 2012. Phosphate solubilizing ability and phylogenetic diversity of bacteria from P-rich soils around Dianchi Lake drainage area of China. Pedosphere 22 (5): 707-716. https:/doi.org/10.1016/S1002-0160(12)60056-3.

Yu X, Liu X, Zhu TH, Liu GH, Mao C, 2012. Co-inoculation with phosphate-solubilzing and nitrogen-fixing bacteria on solubilization of rock phosphate and their effect on growth promotion and nutrient uptake by walnut. Eur J Soil Biol 50: 112-117. https:/doi.org/10.1016/j.ejsobi.2012.01.004.