JOURNAL OF PURE AND APPLIED MICROBIOLOGY, August 2014. Vol. **8**(4), p. 2613-2618

Plant Growth Promoting Activities of Phosphate-Solubilizing Bacteria *Acinetobacter calcoaceticus* YC-5a and *Enterobacter agglomerans* KMC-7

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(Received: 09 May 2014; accepted: 26 June 2014)

Two phosphate-solubilizing bacterial strains were isolated and identified as Acinetobacter calcoaceticus YC-5a and Enterobacter agglomerans KMC-7 based on the 16S rRNA gene sequence analysis. A.calcoaceticus YC-5a is less well known as phosphate solubilizing plant-associated bacteria. In this study, both A.calcoaceticus YC-5a and E. agglomerans KMC-7 produced multiple organic acids followed by a decrease in the pH of the culture medium there by solubilizing the insoluble tricalcium phosphate. The soluble phosphate production of these two strains in NBRIP medium were 518.2±17.3 mg l-1 and 435.4±15.6 mg l-1, respectively. Inoculation with these two isolates were observed to significantly ($p<0.05$) increase the stem diameter and shoot dry weight compared to the uninoculated control. And A.calcoaceticus YC-5a showed better than E. agglomerans KMC-7 in phosphate solubilizing and plant growth promoting, though E. agglomerans KMC-7 was found to produce indole acetic acid and siderophore. The results of the study revealed that the inoculation of PSB promotes plant growth mainly by phosphate solubilizing activity, and indirectly by plant growth regulators.

Key words: Phosphate-solubilizing bacteria; Plant growth promotion; Organic acids; Plant growth regulators; Corn stover.

Phosphorus (P), next to nitrogen, is the second important macronutrient required for plant growth. Soluble P is often the limiting mineral nutrient for biomass production in natural ecosystems as well. Usually the soils are supplemented with inorganic P in the form of chemical fertilizers. A large proportion of the applied P gets fixed in the soil as phosphates of iron, aluminum and calcium(Altomare *et al*. 1999). This leads to the need of frequent application of phosphate fertilizers, but its use on a regular basis has become a costly affair and also environmentally undesirable(Reddy *et al*. 2002).

Phosphate-solubilizing bacteria (PSB) are well known to promote plant growth because of their ability to convert insoluble form of P to soluble form that can be readily taken up by the plant roots. In addition to P-solubilization, PSB may also improve the plant productivity by producing other secondary metabolites(Rahi *et al*. 2010). Several evidence related to plant growth promotion by PSB through the production of indole acetic acid(IAA)(Patten and Glick 2002, Shahab *et al*. 2009) and siderophore(Koo and Cho 2009) make the PSB more suitable as biofertilizers. In this study, two PSB, *A. calcoaceticus* YC-5a and *E. agglomerans* KMC-7, which showed a strong

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phosphate solubilizing activity were used to find out the relationship between P-solibilization and other plant growth regulators(PGRs). The genera *Enterobacter* is known to be involved in the solubilization of insoluble phosphate(Alexander 1977). However, very few studies have reported strains of *Acinetobacter* as phosphate solubilizing plant-associated bacteria(Kuklinsky-Sobral *et al*. 2004).

The main objectives of the current study were to (i) examine the abilities of these two isolates to solubilize tricalcium phosphate (TCP) *in vitro* and to produce organic acids as well as PGRs; (ii) assess their potential as plant inoculants and examine their effect on maize growth and P uptake by maize in glasshouse condition; and (iii) discuss the mechanisms of plant growth promotion by PSB.

MATERIALS AND METHODS

Microorganisms

Strains *A. calcoaceticus* YC-5a and *E. agglomerans* KMC-7 (from our lab) were isolated using conventional methodology and identified using 16S rRNA gene sequence analysis by the Beijing Sunbiotech Co., Ltd, Beijing, China. These two strains were the best P-solubilizing bacteria selected for the present work from approximately 42 strains tested with TCP. They were maintained at -80! in peptone water medium containing 20% glycerol.

Phosphorus solubilization

Solubilization of P by above two PSB were estimated using insoluble $Ca_3(PO_4)_2$ in National Botanical Research Institute's Phosphate (NBRIP) broth medium(Nautiyal 1999). The composition of the medium was (g l^{-1}): glucose, 10; $Ca_3(PO_4)_2$, 5; $MgCl_2 \cdot 6H_2O$, 5; $MgSO_4 \cdot 7H_2O$, 0.25 ; KCl, 0.2; $(NH_4)_2SO_4$, 0.1. One mL bacterial suspension was transferred to a 250 mL Erlenmeyer flask containing 80 mL of the NBRIP broth medium and incubated for 7 days. A separate NBRIP broth medium inoculated with sterile Milli-Q water served as the control treatment. The cultures were harvested by centrifugation at 10174 g for 10 min and soluble-P content of culture supernatant was estimated by the phosphomolybdate method(Murphy and Riley 1962). Cell growth was estimated by the measurement of the absorbance at 600nm. Samples from NBRIP medium were previously diluted 1:1 (v/v) using 1N HCl to dissolve the residual insoluble phosphate and measured against a blank identically treated(Gonzalez and Selman 2000).The pH was determined with pH meter. Organic acids in the broth were analyzed by high-performance liquid chromatography (HPLC) using a Shimadzu Shim-pack C18 reverse phase column, VP-ODS (250mm ×4.6mm, 5µm), and Shimadzu Shim-pack G guard column (C18, 10mm×4mm, 5µm).

Antibiotic sensitivity testing

Antibiotic resistance testing was carried out using Kirby Bauer disk diffusion test. It was performed using commercially available antibiotics discs. The two strains were tested against penicillin (10 IU), ampicillin (10µg), cefazolin (30µg), amikacin (30 µg), chloramphenicol (30µg), erythromycin (15µg), sulfamethoxazole- trimethoprim (SMZ-TMP) (1.25/23.75µg), norfloxacin (10µg), gentamicin (10µg), ciprofloxacin (5µg). Growth inhibition zone diameters were measured manually.

IAA production

Strains *A. calcoaceticus* YC-5a and *E. agglomerans* KMC-7 based on their ability to solubilize P were analyzed for IAA production(Bric *et al*. 1991). Both strains were grown in Luria-Bertani's (LB) medium supplemented with tryptophan (500 µg ml^{-1}) at 28! for 48h in a shaking incubator at 150 rpm. A separate broth medium inoculated with sterile Milli-Q water served as the control treatment. One mL of aliquot of the supernatant was mixed vigorously with 2mL of Salkowski's reagent. The absorbance of pink color developed after 30 min incubation was read at 530 nm. The concentration of IAA in the culture medium was determined using a standard curve prepared with various concentration of analytical grade IAA.

Siderophore assay

Bacterial siderophore production was detected by using blue agar plates containing the dye Chrome azurol S (CAS)(Schwyn and Neilands 1987).Orange halos around the colonies on blue agar were indicative of siderophore excretion.

Pot experiments

To prepare inoculants, 60g dry corn stover powder added with 60mL LB liquid medium was packed in high density polyethylene (HDPE) bags and sterilized by autoclaving at 121 ! for 30 min. Thirty mL of bacterial cells harvested from mid-log phase culture grown in LB broth were injected

aseptically into an individual pack and covered with a label at the injecting point. Inoculated packets were thoroughly kneaded to ensure uniform adsorption of the bacterial cells into the carrier material and incubated at 28! for a period of 7days. The density of bacterial cells was approximately 109 CFU per gram of mixture. Non-inoculated stover powder was used as control. Later on, inoculated packets were preserved at cold room (4°C) for further use.

The chemical attributes of the soil were: pH 7.8, total N 810 ppm, available P 42.7 ppm, available K 136 ppm and organic matter 17700 ppm. For pot experiments, the soil was thoroughly mixed and passed through 2mm sieve to remove large particulate matter and kept under sunlight for 7 days, and filled (8kg) 26 cm-diameter pots with 20g corn stover powder based inoculums. Each pot was planted with 5 maize seeds which were surface-sterilized with a mixture of ethanol and 30% H_2O_2 (1:1) for 20 min and washed with extensive sterile water. Seeds were coated with inoculums using 1% carboxymethylcellulose (CMC) as adhesive(Hameeda *et al*. 2008), dried in air and the cell count was 107-108 CFU per seed.

After 8 weeks, whole plants were carefully removed from the pots. Growth parameters such as shoot length, fresh weight and dry weight of the plants were measured. Leaves were excised from the plant for P determination. The P content was measured using the phosphomolybdate method.

Statistical analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) IBM SPSS software, version 19.0. Comparisons of means were performed by the LSD test at $P \leq 0.05$.

RESULTS AND DISCUSSION

P solubilization

The details of the cell densities, pH, organic acids and amounts of soluble-P in the medium after 7days of incubation are showed in Table 1. The solubilization of TCP in liquid medium by A. calcoaceticus YC-5a and E. agglomerans KMC-7 was accompanied by a significant drop in pH (to 3.92 and 4.13) from an initial pH of 6.8-7.0 after 7 days. The soluble-P concentration in the broth were 518.2 mg $l^{-1}$and 435.4 mg l^{-1} respectively. In the blank treatment no soluble-P was detected as well no drop in pH was observed. For *A. calcoaceticus* YC-5a, pH and cell density were lower, meanwhile, the level of soluble phosphorus

Table 1. P solubilization in NBRIP broth medium of *A. calcoaceticus* YC-5a and *E. agglomerans* KMC-7

Bacterial strains	Growth A_{600}	pH	Solubilized P (mgl^{-1})	Organic acids*
A.calcoaceticus YC-5a	0.164±0.010	3.92±0.02	518.2±17.3	O + M + L + T
E. agglomerans KMC-7	0.256±0.013	4.13±0.01	435.4±15.6	O + L + C + S

All the values are mean of three replicates ± SD.

*O, oxalic acid; M, malic acid; L, lactic acid; T, tartaric acid; C, citric acid; S, succinic acid

Table 2. Some key traits of *A. calcoaceticus* YC-5a and *E. agglomerans* KMC-7

Strain	IAA synthesis	siderophore	antibiotic traits*									
	($mg\ l^{-1}$)	production	PEN	AMP	CFZ	AK	CL	EM	SMZco	NOR	GM	CIP
A.calcoaceticus YC-5a	13.8±1.7	-	R	I	R	S	R	I	I	I	S	S
E.agglomerans KMC-7	64.7±5.2	+	R	I	R	S	S	I	S	S	S	S

All the values are mean of three replicates ± SD.

+, Positive;-, negative;

*PEN, penicillin; AMP, ampicillin; CFZ, cefazolin; AK, amikacin; CL, chloramphenicol; EM, erythromycin; SMZco, SMZ-TMP; NOR, norfloxacin; GM, gentamicin; CIP, ciprofloxacin; S, R and I mean susceptibility, resistance or intermediate resistance, respectively.

Table 3. Plant growth of maize inoculated with *A.calcoaceticus* YC-5a and *E. agglomerans* KMC-7

Treatment	Shoot length cm	Stem diameter cm	Shoot fresh weight g	Shoot dry weight g	P uptake in leaves mg kg^{-1}
A.calcoaceticus YC-5a	149.1±7.5	1.413±0.035*	380.2±6.1	50.7±2.6*	13.9±1.09*
E.agglomerans KMC-7	152.9±3.8	1.383±0.020*	382.8±7.8	49.5±2.0*	12.2±0.80
Uninoculated control	152.6±4.3	1.329±0.016	366.9±6.8	43.4±3.5	10.5±0.89

All the values are mean of three replicates ± SD. An asterisk (*) denotes a value significantly greater than the control value ($p<0.05$).

in the culture medium was higher compared to *E. agglomerans* KMC-7. According to the experiments for screening phosphate solubilizing isolates, a higher soluble phosphate production always combined with a lower pH (data not shown). A inverse relationship between pH and soluble phosphate has been reported before(Illmer and Schinner 1995, Hwangbo *et al*. 2003). It indicated that phosphate solubility was directly correlated with the acids produced. There were reports that PSB released many kinds of organic acids, such as oxialic, citric, butyric, malonic, lactic, succinic, malic, gluconic, acetic, glyconic, fumaric, adipic, and 2-ketogluconic acids(Pecina *et al*. 1984, Illmer and Schinner 1992). HPLC analysis showed the presence of organic acids in the culture mediums of these two strains. Four different organic acids each were produced. The details are given in Table 1 and Fig.1. For *A. calcoaceticus* YC-5a, major acid oxalic in combination with other acids (malic, lactic, tartaric) was successful in decreasing pH and induced higher phosphate solubilizing activity. As for *E. agglomerans* KMC-7, was also found to produce mixtures of oxalic acid, lactic, citric and succinic acid. Production of organic acids results in acidification of the microbial cell and its surroundings. Consequently, P may be released from a mineral phosphate by proton substitution for Ca^{2+}.

Both strains *A. calcoaceticus* YC-5a and *E. agglomerans* KMC-7 were assayed for a number of other traits thought to be important for plant growth-promoting activity (Table 2). *E. agglomerans* KMC-7 had a high level of IAA, whereas *A. calcoaceticus* YC-5a exhibited only a low level of production. IAA is known to stimulate both rapid (e.g., increases in cell elongation) and long term responses in plants. IAA as the phytohormone offers great promise for sustaining the increased crop productivity by altering the plant hormonal balance. Furthermore, *E. agglomerans* KMC-7 had the capacity to produce

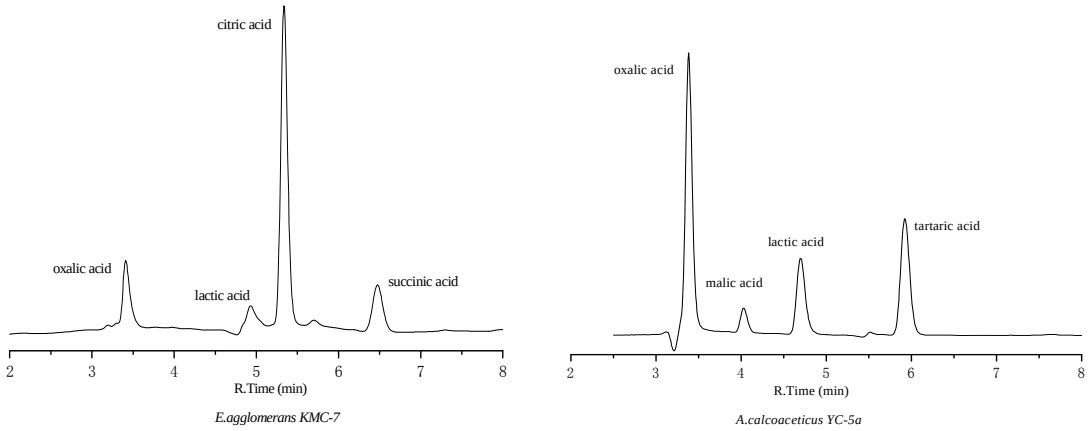

Fig. 1. HPLC analysis of the organic acids detected in the culture medium by *A.calcoaceticus* YC-5a and *E.agglomerans* KMC-7

siderophore. Siderophores are low-molecular mass iron chelators with high association constants for complexing iron(Neilands 1983, Miethke and Marahiel 2007). Thus, siderophores act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation. It was also observed that a wide antibiotic resistance range was found in both two isolates.

Pot experiments

The effects of two PSB on the growth of maize are shown in Table 3. Significant increase of stem diameter and shoot dry weight were observed when the soil was inoculated with strains *A.calcoaceticus* YC-5a and *E. agglomerans* KMC-7, compared to the uninoculated control. In case of *A.calcoaceticus* YC-5a treatment, stem diameter and shoot dry weight increased by 6.3% and 16.8% ($p<0.05$), respectively, and in case of *E. agglomerans* KMC-7 treatment, the stem diameter and shoot dry weight increased by 4.1% and 14.1% ($p<0.05$), respectively. There was no significant difference in shoot length and shoot fresh weight of maize grown in inoculated and uninoculated soils ($p>0.05$). Several studies have shown increase in plant growth due to the addition of PSB. The PSB have been considered as plant growth promoting rhizobacteria(Peix *et al*. 2001), the inoculation of soil with PSB similar to those obtained by addition of soluble phosphorus. Furthermore, the uptake of P in maize leaves was also influenced by PSB inoculation. Inoculation with *A.calcoaceticus* YC-5a and *E. agglomerans* KMC-7 showed an increase in P content of leaves(32.4% and 16.2, respectively) in comparison to control. This might be due to better utilization of P from the pool of soil nutrients by the action of PSB.

Other authors think that the inoculation of soil with PSB can increase the crops yield by other mechanisms, such as the growth factor production including phytohormones, antibiotics and siderophores etc(Kloepper *et al*. 1989). According to the results obtained, in addition to P solubilizing ability, *E. agglomerans* KMC-7, which is known to be involved in the solubilization of insoluble phosphate, showed various plant growth promoting traits(Table 2). However, the effects of promoting the maize growth and P uptake in leaves were not as good as *A.calcoaceticus* YC-5a, though *A.calcoaceticus* YC-5a could not produce high level of IAA and siderophore. Results of comparation tests have shown that mean differences of stem diameter and shoot dry weight between these two strains were significant ($p<0.05$). It indicated *A.calcoaceticus* YC-5a is more effective on the growth of maize compared *E. agglomerans* KMC-7. *A.calcoaceticus* YC-5a is less well known as phosphate solubilizing plant-associated bacteria. As the results shown in Table 1, *A.calcoaceticus* YC-5a possessed a higher phosphate solubilizing ability and could make more P released from a mineral phosphate. And P is the main nutrient elements and it may play a more important role in plant growth than other regulators. It is concluded from the present study that the mechanism of maize growth promotion by both *A.calcoaceticus* YC-5a and *E. agglomerans* KMC-7 mainly is the ability of phosphate solubilization. Phosphorus is one of the major plant nutrients limiting plant growth. Kinds of organic acids produced by both strains and they may make mineral released nutrients from surroundings of rhizosphere of plant. In combination with the plant growth promotion regulators like IAA, siderophore and antibiotics produced by PSB improved maize growth indirectly.

ACKNOWLEDGEMENTS

The work was financially supported by National Natural Science Foundation of China (No. 31200083), Shaanxi science and technology plan projects (No. 2011JY015), Hui min program of National ministry science and technology(No. 2012GS610203-2), The joint research research fund of Yulin city and Shaanxi province,Science and technology bureau of Yulin city integration of production and research projects, Education department of Shaanxi provincial government (No. 08JK453) and National innovation experiment program for university students. (No. 201210697005 and 201310697025) .

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