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Short Communication

SCREENING OF SOIL BACTERIA FOR PLANT GROWTH PROMOTION ACTIVITIES *IN VITRO*

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ABSTRACT

Fourteen isolates of soil bacteria, including two known plant growth promoting rhizobacteria (PGPR) strains, *Azotobacter vinelandii* Mac 259 and *Bacillus cereus* UW 85, were tested *in vitro*. Parameters assessed were indoleacetic acid (IAA) production, phosphate solubilization, dinitrogen fixation, and siderophore (Fe-III chelating agent) production. IAA production was assayed colorimetrically using ferric chloride-perchloric acid reagent. Phosphate-solubilization and siderophore production were tested qualitatively by plating the bacteria in Pikovskaya and chrome azurol S agar, respectively. The ability to fix dinitrogen was measured based on nitrogenase activity of the bacteria by gas chromatography. The results showed that twelve isolates produced IAA, ranged from 2.09 to 33.28 µmol ml^{-1}. The ability to solubilize precipitated phosphate was positively exhibited by four isolates (BS 58, BTS, TCaR 61, and BTCaRe 65). Seven isolates including Mac 259 positively produced siderophore. None of the isolates showed nitrogenase activity. Only one isolate (TS 3) did not exhibit any of the traits tested. Isolate TCeRe 60 and reference strain Mac 259 were found to have IAA- and siderophore-producing traits. Four P-solubilizing bacteria (BS 58, BTS, TCaR 61, and BTCaRe 65) were also IAA- and siderophore-producing bacteria. Potential use of these PGPR isolates needs further test in enhancing plant growth.

[*Keywords*: Soil bacteria, screening, plant growth promotion]

INTRODUCTION

The microbe-plant interaction in the rhizosphere can be beneficial, neutral, variable, or deleterious for plant growth. Rhizobacteria that exert beneficial effects on plant development are termed plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978). The term rhizobacteria is used for bacteria that aggressively colonize the rhizosphere (Subba Rao 1999). Although the mechanisms by which PGPR promote plant growth are not yet fully understood, many different traits of these bacteria are responsible for growth promotion activities (Cattelan *et al*. 1999). It includes the ability to produce or change the concentration of the plant hormones indoleacetic acid (IAA), gibberellic acid, cytokinins, and ethylene; fix dinitrogen; suppress the growth of deleterious microorganisms by production of siderophore, β-1,3-glucanase, chitinases, antibiotics, and cyanide; and dissolve phosphates and other nutrients.

Initially, *Azotobacter* and *Azospirillum* were believed to promote plant growth due to their ability to fix dinitrogen. Later, it was known that other plant growth stimulating hormones such as IAA was also involved (Kennedy 1998). The use of P-solubilizing bacteria was reported to increase plant growth in some cases, but in other cases it was not. It indicated that other mechanisms may involve in growth response (De Freitas *et al*. 1997).

Ample studies on the biological dinitrogen fixation have been documented. A number of free-living bacteria have the ability to fix dinitrogen and increase nitrogen availability for plant. IAA produced by bacteria improves plant growth by increasing the number of root hairs and lateral roots (Okon and Kapulnik 1986). Microbial biosynthesis of IAA in soil is enhanced by tryptophan from root exudates or decaying cells (Frankenberger and Arshad 1991; Benizri *et al*. 1998). P-solubilizing bacteria are potential to increase available P for plant, especially in soils with large amounts of precipitated phosphate (Goldstein 1986). These bacteria release bound phosphate by secreting a number of organic acids although it is not the only way by which P is solubilized (De Freitas *et al*. 1997; Kim *et al*. 1997). Siderophore-pruducing bacteria promote plant growth indirectly by sequestering the limited iron in the rhizosphere, especially in neutral and alkaline soils, and thereby reduce its availability for the growth of pathogen (Alexander and Zuberer 1991; Subba Rao 1999).

This study was aimed to assess the potential of twelve soil bacteria isolates in producing plant growth promotion, i.e. IAA, phosphate solubilizer, nitrogenase, and siderophore (Fe-III chelating agent).

MATERIALS AND METHODS

The study was conducted in the National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines at Los Banos, Philippines. Twelve soil bacteria isolates used (Table 1) were kindly provided by BIOTECH. These bacteria were isolated from soil, rhizosphere, and plant roots in Bohol and Tarlac, Philippines. Two known PGPR that produce IAA (Paterno 1997), *Azotobacter vinelandii* Mac 259 and *Bacillus cereus* UW 85, were included in the study as reference strains. Two out of fourteen bacteria (TCeR 68 and UW 85) are Gram-positive.

Table 1. Bacteria isolates and reference strains used in the study.

Bacteria isolates and reference strains	Sources
BS 58	Soil sample from Bohol
BTS	Soil sample from Bamban - Tarlac
TS 3	Tarlac soil sample (lahar affected)
TS 17	Tarlac soil sample (lahar affected)
TCaR 59	Rhizosphere of *Calopogonium* sp. from Tarlac (lahar affected)
TCaR 61	Rhizosphere of *Calopogonium* sp. from Tarlac (lahar affected)
TCeR 68	Rhizosphere of *Centrosema pubescens* from Tarlac
TCeRe 60	Root extract of *Centrosema pubescens* from Tarlac (lahar affected)
TCeRe 66	Root extract of *Centrosema pubescens* from Tarlac (lahar affected)
TCeRe 66/71	Root extract of *Centrosema pubescens* from Tarlac (lahar affected)
TCeRe 76	Root extract of *Centrosema pubescens* from Tarlac (lahar affected)
BTCaRe 65	Root extract of *Calopogonium* sp. from Bamban - Tarlac
Azotobacter vinelandii Mac 259	Department of Microbiology, MacDonald College of McGill University, Quebec, Canada
Bacillus cereus UW 85	Institute of Environmental Studies, University of Wisconsin, Madison, USA

IAA Production

Indoleacetic acid produced by bacteria was assayed colorimetrically using ferric chloride-perchloric acid reagent ($FeCl_3$-$HClO_4$) (Gordon and Weber 1951). This method estimated the quantities of indole compounds produced by bacteria in medium containing precursor L-tryptophan. The test was replicated two times.

The bacteria were grown in modified Nutrient Broth-M26 for 24 hours on a gyratory shaker (150 rpm) at room temperature as seed culture. The medium contained (in 1,000 ml distilled water) 5 g NaCl, 10 g peptone, and 10 g beef extract. After overnight incubation, 100 µl of culture was inoculated to 10 ml minimal salt (MS) medium amended with 5 mM L-tryptophan (Frankenberger and Poth 1988) and grown again for 48 hours on the shaker. The MS medium contained (in 1,000 ml distilled water) 1.36 g KH_2PO_4, 2.13 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, and trace elements. The pH of MS medium was adjusted to 7.0 before autoclaving. L-tryptophan solution was prepared as stock solution containing (in 100 ml distilled water) 10 g glucose, 1 g L-tryptophan, and 0.1 g yeast extract. The stock solution was filtered through a sterile 0.2 µm membrane filter (Millipore).

To measure the amount of IAA produced, 1.5 ml bacterial broth culture was centrifuged at 12,000 rpm for 5 minutes. One milliliter of the supernatant was added to 2 ml $FeCl_3$-$HClO_4$ reagent. After 25 minutes (after color density reaches its maximum), the mixture was read in UV-spectrophotometer at 530 nm absorbance. The amount of IAA produced per milliliter culture was estimated using a standard curve. The number of bacterial population in the culture expressed in colony forming unit (cfu) was estimated by the Miles and Misra drop-plate method.

P-Solubilization

Phosphate-solubilization test was conducted qualitatively by plating the bacteria in agar containing precipitated tricalcium phosphate. The medium was a modification of Pikovskaya medium (Subba Rao and Shinta 1963 *in* Subba Rao 1999), consisted of 10 g glucose, 5 g tribasic phosphate ($Ca_5HO_{13}P_3$), 0.5 g $(NH_4)_2SO_4$, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, trace of $MnSO_4$ and $FeSO_4$, 0.5 g yeast extract, and 15 g agar, in 1,000 ml distilled water. Bacterial culture was streaked on the surface of replicated agar plates. The presence of clearing zone around bacterial colonies after overnight incubation was used as indicator for positive P-solubilization.

Nitrogenase Activity

The ability of the bacteria to fix dinitrogen was measured based on their acetylene reduction activity (ARA) or the ability of nitrogenase complex produced by bacteria to reduce acetylene (C_2H_2) to ethylene (C_2H_4) (Hardy *et al*., 1968; Hardy *et al*. 1973). The

medium used was Burk's N-free medium (Subba Rao 1999). It consisted of (in 1,000 ml distilled water) 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.2 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.013 g $CaSO_4.2H_2O$, 1 ml Fe-Mo mixture (1.45 g $FeCl_3.6H_2O$, 0.253 g $Na_2MoO_4.2H_2O$, 1,000 ml distilled water), 20 g sucrose, and 4 g agar.

Bacterial culture (50 µl) was inoculated to 1 ml of the Burk's N-free medium in 6 ml vacutainer sealed with a cotton plug. After 48-hour incubation at room temperature, the cotton plug was replaced with a rubber stopper, and 0.5 cm^3 of the atmosphere (10%) in the vacutainer was replaced with acetylene by injection, then incubation was continued for 20-24 hours. Gas sample (1 ml) was removed from the vacutainer using 1 ml syringe and the ethylene concentration in the gas sample was measured by gas chromatography. The test was replicated three times.

Siderophore Production

Siderophore production was tested qualitatively using chrome azurol S (CAS) agar as described by Alexander and Zuberer (1991). The CAS agar was a mixture of four solutions that were prepared and sterilized separately before mixing. Solution-1 (Fe-CAS indicator solution) consisted of 10 ml of 1 mM $FeCl_3.6H_2O$ (in 10 mM HCl), 50 ml of an aqueous solution of CAS (1.21 mg ml^{-1}), and 40 ml of aqueous solution of hexadecyl-trimetylammonium bromide (HDTMA) (1.82 mg ml^{-1}). Solution-2 (buffer solution) was prepared by dissolving 30.24 g of PIPES (peperazine-N,N'-bis[2-ethanesulfonic acid]) in 750 ml of salt solution. The distilled water was added to bring the volume to 800 ml after adjusting the pH to 6.8 with 50% KOH. The solution was autoclaved after adding 15 g agar. Solution-3 contained 2 g glucose, 2 g mannitol, and trace elements in 70 ml distilled water. Solution-4 was 30 ml filtered-sterilized 10% (w:v) casamino acid. At 50°C after autoclaving, solutions 3 and 4 were added to the buffer solution. Indicator solution (solution-1) was added last with sufficient stirring to mix the ingredients. This mixture (Fe-CAS dye complex) yielded blue to dark green color.

The bacterial culture were spreaded on the CAS agar plates with two replications. Orange halos around the colonies after overnight incubation indicated siderophore-production.

RESULTS AND DISCUSSION

Table 2 shows the PGPR traits of all bacteria screened. Twelve bacteria produced IAA that ranged from 2.09 to 33.28 µmol ml^{-1}. Isolates TCeR 68 and TS 3 did not produce IAA. On the other hand, two isolates (TCaR 59 and TCeRe 60) produced remarkably high amount of IAA (more than 30 µmol ml^{-1}). The ability to solubilize precipitated phosphate was positively exhibited by isolates BS 58, BTS, TCaR 61, and BTCaRe 65 (Fig. 1). Seven bacteria including the reference strain Mac 259 positively produced siderophore (Fig. 2). However, none of the bacteria showed nitrogenase activity, hence their ability to fix dinitrogen was negligible.

Table 2. Indoleacetic acid (IAA) production, P-solubilization, and siderophore production of fourten soil bacteria.

Bacteria isolates and reference strains	IAA production (µmol ml^{-1})	P-solubilization	Siderophore production
BS 58	14.93	+	+
BTS	27.51	+	+
TS 3	nd[1]	-	-
TS 17	8.27	-	-
TCaR 59	33.28	-	-
TCaR 61	19.99	+	+
TCeR 68	nd[1]	-	+
TCeRe 60	31.45	-	+
TCeRe 66	6.72	-	-
TCeRe 66/71	7.23	-	-
TCeRe 76	21.44	-	-
BTCaRe 65	9.83	+	+
Azotobacter vinelandii Mac 259	2.09	-	+
Bacillus cereus UW 85	4.28	-	-

[1]Not detected, + = Positive for P-solubilization or siderophore production, - = Negative for P-solubilization or siderophore production.

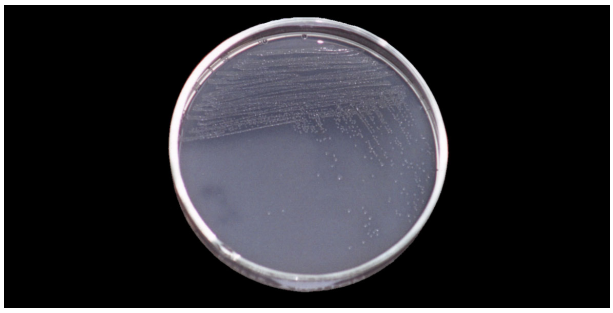

Fig. 1. Clearing zones around the colonies of TCaR 61 indicating the ability of this isolate to solubilize precipitated phosphate in Pikovskaya agar medium.

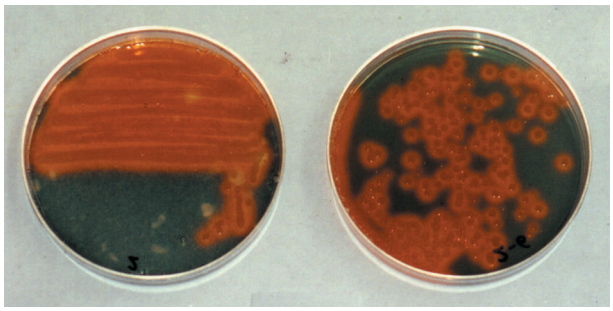

Fig. 2. Orange halos around the colonies of BS 58 indicating the ability of this isolate to excrete siderophore that removes Fe from Fe-CAS agar medium.

The overall results showed that only one isolate (TS 3) did not show any of the four PGPR traits. Isolate TCeRe 60 and strain Mac 259 shared two PGPR traits, i.e., produce IAA and siderophore. Four P-solubilizing bacteria (BS 58, BTS, TCaR 61, and BTCaRe 65) shared two more PGPR traits, i.e. IAA- and siderophore-producing traits.

The amount of IAA produced by some isolates was higher than that have been reported by De Freitas *et al*. (1997), which range from 2.31 to 9.43 µmol ml^{-1}. Further study is required to utilize potential application of the IAA high-producing bacteria.

Seven siderophore-producing bacteria found in this study including the reference strain *A. vinelandii* Mac 259 are good candidates to be used for plant growth promotion, especially in neutral to alkaline soil. Four P-solubilizing bacteria (BS 58, BTS, TCaR 61, and BTCaRe 65) have good prospects to improve plant growth, especially in soil with large amount of precipitated phosphate.

CONCLUSION

Twelve of fourteen bacteria screened produced IAA, seven of which positively produced siderophore and four of them also positively solubilized precipitated phosphate. These four bacteria (BS 58, BTS, TCaR 61, and BTCaRe 65) are potential to be examined their capability in enhancing plant growth.

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