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Evaluation of *Pseudomonas* isolates from wheat for some important plant growth promoting traits

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Plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms. The use of PGPR is steadily increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides and supplements. In the present study, isolation and characterization of PGPR from the rhizospheric soil of wheat was done for the enhancement of wheat growth. Rhizospheric soils samples were collected from different areas of Uttar Pradesh, India. Twenty two (22) isolates of bacteria were successfully isolated, biochemically characterized and screened for their plant growth promoting traits like production of indole acetic acid (IAA), ammonia production, siderophore production and phosphate solubilization. On the basis of multiple plant growth promoting activities among 22 isolates, 17 isolates were also evaluated for their quantitative production of IAA. The isolates Ps-AB4, Ps-BK1 and Ps-JN 2 showed highest IAA production (79.67-110.12 µg/L). Out of 22, 20 and 6 isolates produced ammonia and siderophore, respectively, while 10 isolates solubilized phosphate on the Pikovskaya's agar medium. The present study reveals that the use of *Pseudomonas* isolates Ps-AB4, Ps-BK1 and Ps-JN2 as inoculants biofertilizers might be beneficial for wheat cultivation.

Key words: Indole acetic acid, NH_3, *Pseudomonas* spp., phosphate solubilization, wheat.

INTRODUCTION

Bacteria that colonize the rhizosphere and plant roots, and enhance plant growth by any mechanism are referred to as plant growth-promoting rhizobacteria (PGPR). In the context of increasing international concern for food and environmental quality, the use of PGPR for reducing chemical inputs in agriculture is a potentially an important issue. PGPR have been applied to various crops to enhance growth, seed emergence and crop yield, and some have been commercialized (Herman et al., 2008; Sachdev et al., 2009, Dilfuza, 2010; Rawat et al., 2011; Minaxi et al., 2013). The *Pseudomonas* spp. isolated from the roots of graminaceous plants has been shown to colonize the roots of various plants, and to increase the height, flower number, fruit number and total fruit weight of tomato plants. Under salt stress, PGPR have shown positive

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effects in plants on such parameters as germination rate, tolerance to drought, weight of shoots and roots, yield and plant growth (Kloepper et al., 2004; Kokalis-Burelle et al., 2006). Another major benefit of PGPR is to produce antibacterial compounds that are effective against certain plant pathogens and pests (Herman et al., 2008; Fatima et al., 2009). PGPR mediate biological control indirectly by eliciting induced systemic resistance against a number of plant diseases (Jetiyanon and Kloepper, 2002). In addition to improvement of plant growth, PGPR are directly involved in increased uptake of nitrogen, synthesis of phytohormones, solubilization of minerals such as phosphorus, and production of side-rophore that chelate iron and make it available to the plant root (Mohite, 2013; Schoebitz et al., 2013). Wheat is the most important staple food in several developing countries, and chemical fertilizer is the most important input required for wheat cultivation. The high-yielding wheat variety has resulted in an increase in wheat production but requires large amount of chemical fertilizers, leading to health hazards and environmental pollution. In order to make wheat cultivation sustainable and less dependent on chemical fertilizers, it is important to know how to use PGPR that can biologically fix nitrogen, solubilize phosphorus and induce some sub-stances like indole acetic acid (IAA) that can contribute to the improvement of wheat growth. Recently, there is a growing interest in PGPR due to their efficacy as biological control and growth promoting agents in many crops (Thakuria et al., 2004). There is very little infor-mation regarding the use of PGPR as biofertilizers in wheat. Therefore, the present study was undertaken to screen the PGPR strains that are compatible with wheat in agro-ecological conditions of Uttar Pradesh India.

MATERIALS AND METHODS

Isolation of *Pseudomonas* from wheat rhizosphere

Soil samples were collected from the rhizosphere of wheat plants at flowering stage from Faizabad (FZ), Kanpur (KN), Barabanki (BK), Muzzaffarnagar (MZ), Ambedkarnagar (AB), Jaunpur (JN) and Lucknow (LK) districts of Uttar Pradesh, India. The wheat plants were uprooted from the agricultural fields and the rhizosphere soil was pooled together, placed in plastic bags and stored at 4°C and immediately microbiological processing was carried out. Serial dilutions were made up to 10^{-4} for all seven soil samples and 10^{-3} and 10^{-4} dilutions were taken for spread plating on Kings B medium containing per liter of distilled water: 20.0 g peptone, 10.0 g glycerol, 1.50 g K_2HPO_4, 1.50 g $MgSO_4$:$7H_2O$, 18.0 g agar and pH 7.2. The plates were incubated at 30°C for 48 h. After incubation, plates were observed for different isolates based on morphological traits. Morphologically variable colonies were picked up and purified on Kings B medium plates. Pure cultures were maintained on the respective medium slants.

Characterization of *Pseudomonas* isolates

The bacterial isolates were characterized by their morphological (shape, size, elevation, surface, margin, color, pigmentation and Gram's reaction) and biochemical (carbohydrate fermentation, oxidase test, H_2S production, starch and gelatin hydrolysis, NO_2 reduction, citrate and catalase reactions) characteristics using standard methods (Cappuccino and Sherman, 1992). Gram reaction was determined by making a thin smear of inoculums of rhiozbacterial isolates in a separate glass slide and heat fixed. The smear was covered with crystal violet for 30 s. The slide was washed with distilled water for few seconds by using wash bottle. The smear was stained with iodine solution for 60 s. The iodine solution was washed with 95% ethyl alcohol. Ethyl alcohol was added drop by drop until no more colour flows from the smear. The slides were washed with distilled water and drained. Saffranin was applied to smear for 30 s and washed with distilled water and blot dried with absorbent paper. The slide was examined under oil immersion objective. Carbohydrate fermentation pattern of isolates was determined by medium containing specific carbohydrate source (dextrose, sucrose and lactose) along with phenol red as indicator at 37°C for 48 h. The starch hydrolysis capacity of test isolates was checked on starch nutrient agar medium. Cultures were streaked and incubated at 37°C for 24 h. After incubation, plates were flooded with Gram's iodine and zone of clearing around the growth was observed on the medium plate. For gelatin hydrolysis, Stab tubes of gelatin nutrient agar were inoculated with cultures and incubated. Tubes were chilled in ice and liquefaction of gelatin was observed. To test the presence of nitrates, nitrate broth was inoculated with test cultures and incubated at 35°C for 48 h and equal parts of sulfuric acid and alphanaphthyl amine was mixed. Pink color development was observed for the presence of nitrite. Cimmons citrate agar medium slants were inoculated by isolates and incubated at 35°C for 48 h for citrate utilization. Observations were recorded for presence of growth which indicated citrate utilization. To check the catalase reaction, a loopful of culture from freshly grown slant of nutrient agar was taken out and placed on slide. A drop of H_2O_2 (3%) was added for growth. Production of bubbles indicated presence of catalase. Oxidase reagent (Kovacs) was added to colonies growing on nutrient agar medium. Development of purple color indicated presence of oxidase.

Production of indole acetic acid

Indole acetic was detected as described by Brick et al. (1991). The bacterial cultures were grown in100 mL of conical flask containing Luria-Bertani broth for 48 h on a rotary shaker. After incubation, fully grown cultures were centrifuged at 10000 rpm for 10 min at 40°C. The supernatant 2 mL was mixed with two-three drops of Ortho-phosphoric acid and 4 mL of the Salkouski reagent (50 mL, 35% of perchloric acid 1 mL 0.5 $FeCl_2$ solution). Development of pink color indicates IAA production. Optical density was taken at 530 nm with help of UV visible spectrophotometer. Concentration of IAA produced by culture was measured with the help of standard graph of IAA obtained in the range of 20-200 µg/mL.

Phosphate solubilization

The plates were prepared with Pikovskaya's medium. All the bacterial isolates streaked on the surface of Pikovskaya's agar plate and phosphate solubilizing activity was estimated after 4 days of incubation at 28°C. Phosphate solubilization activity was determined by the development of the clear zone around the bacterial colonies (Frioni, 1990).

Production of Ammonia

All the bacterial isolates were tested for the production of ammonia in the peptone water (peptone 10 g, NaCl 5 g in litre, pH 7.0). Freshly

Table 1. Morphological and cultural characteristics of test isolates.

Biochemical characters	*Pseudomonas* spp.
Number of isolates	22
Grams reaction	Negative
Shape	Short rods
Pigment	Cream , yellow to greenish
Colony morphology	Round colonies to entire margin
Arrangement	Mostly single
Endospore Position	No Spore
Sucrose	+
Dextrose	+
Mannitol	+
H_2S production	-
Citrate Utilization	+
Starch	+
Gelatin hydrolysis	+
Catalase test	+
Nitrate reduction	+
Lipid hydrolysis	+
Casein hydrolysis	+

grown culture were inoculated in 10 mL peptone water in each test tube and incubated for four days at 30°C. Nessler reagent (1 mL) was added in each test tube. Development of brown to yellow color was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Siderophore production

Bacterial isolates were assayed for siderophore production on the chome azurol agar (CAS) medium (Husen, 2003). Bacterial isolates were spot inoculated on the chrome azurol s (CAS) ager plates and incubated at 30 for 72 h. Siderophore production was indicated by orange halos around the colonies after incubation.

RESULTS AND DISCUSSION

Isolation and biochemical characterization

Twenty two bacterial strains were successfully isolated from wheat field and identified as *Pseudomonas* spp. on the basis of cultural, morphological and biochemical characteristics as described in Bergeys manual of determinative bacteriology (Holt et al., 1994). The *Pseudomonas* spp. strains from rhizosphere of different crops were isolated and extensively studied by Fischer et al. (2007), Ahmad et al. (2008), Fatima et al. (2009), Joshi et al. (2011) and Rawat et al. (2011). Sinha and Simon (2013), characterized *Pseudomonas fluorescens* on the basis of morphological (Grams stain and colony morphology), physiological and biochemical tests (gelatin hydrolysis, IAA production test, starch hydrolysis, urease, oxidase, ammonia production, HCN, Levan production from sucrose). Similarly, Kushwaha et al. (2013) also isolated *Pseudomonas* strains from cauliflower and characterized for cultural, morphological and biochemical characters while Singh et al. (2013) isolated various strains of *Pseudomonas* from wheat rhizosphere and characterized them by morphological and biochemical characteristics like production of IAA, HCN, catalase, phosphatase enzymes etc. The general characteristics of the isolates are illustrated in Table 1.

IAA production

All 22 bacterial strains were tested for quantitative estimation of IAA, most interestingly all the test *Pseudomonas* strains showed IAA production in the range of 2.22-110.12 µg/mL (Figure 1). The strain Ps-BK1 and Ps-AB4 were able to produce 95.60 and 110.00 µg /mL IAA in the broth culture medium, respectively. The produced IAA was measured with the help of standard graph of IAA obtained in the range of 20-200 µg/mL. It has been reported that IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Mirza et al., 2001). Ahmad et al. (2004) reported that *Pseudomonas* isolate Ps_1 produced 53.20 µg/mL IAA in culture medium supplemented with L Tryptophan @5 mg/mL. Deshwal and Kumar (2013) screened out qualitatively 55 *Pseudomonas aeruginosa*, 22 *Pseudomonas putida*, 26 *Pseudomonas cepacia* and 37 *Pseudomonas fluorescens* strains for IAA and most of the *Pseudomonas* strains showed encouraging results for IAA as 44 *P. aeruginosa*, 14 *P. putida* , 19 *P. cepacia* and *22 P. fluorescens* were found positive while Bhakthavatchalu et al. (2013) observed significant increase in IAA production (80 µg/mL) by *P.s aeruginosa* when L- tryptophan was supplied as precursor. The present investigation findings are outstanding with reference to earlier reports.

Ammonia production and phosphorus solubilization

Out of 22 *Pseudomonas* strains, 20 strains were found to produce NH_3. Few isolates like Ps-FZ2, Ps-BK1, Ps-JN1, Ps-JN3, Ps-AB1, Ps-AB2, Ps-AB4, Ps-AB5, Ps-MZ3 and Ps-LK3 which produced deep brown color indicated higher production of ammonia. Only two *Pseudomonas* isolates namely Ps-JN2 and Ps-MZ1 did not produce ammonia where as the rest isolates were found to be medium producer of ammonia (Table 2). Sinha and Simon (2013) isolated 13 *Pseudomonas* strains from wheat rhizosphere and were found positive for ammonia production, likewise Kaushal et al. (2013) reported ammonia producing *Pseudomonas* from rice rhizosphere. Only ten strains solubilized phosphate on Pikovskaya's agar plates at 30°C and the remaining strains did not

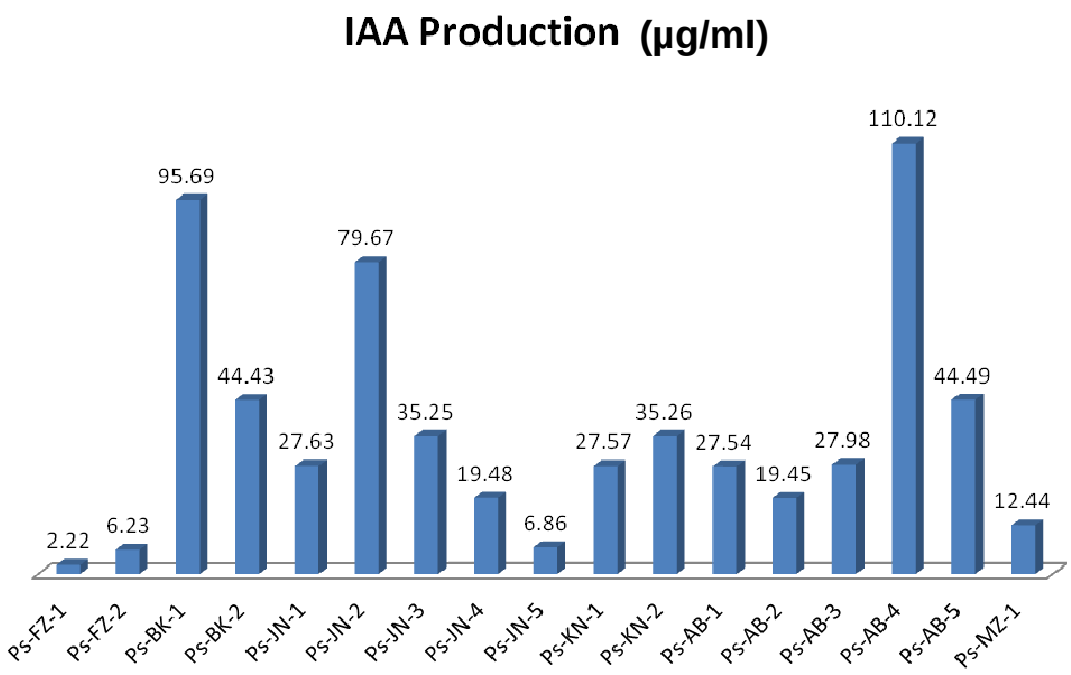

Figure 1. Indole acetic acid production by *Pseudomonas* isolates.

Table 2. Plant growth promoting characteristics of *Pseudomonas* isolates.

Isolate	Siderophore production[b]	Phosphate solubilization[c]	Ammonia production[a]
Ps-FZ-1	-	-	+
Ps-FZ-2	-	-	+++
Ps-BK-1	-	+	+++
Ps-BK-2	-	+	++
Ps-JN-1	+	++	+++
Ps-JN-2	-	-	_
Ps-JN-3	+	++	+++
Ps-JN-4	-	-	+
Ps-JN-5	-	-	++
Ps-KN-1	-	-	+
Ps-KN-2	-	-	++
Ps-AB-1	-	+++	+++
Ps-AB-2	-	-	+++
Ps-AB-3	-	++	+
Ps-AB-4	+	++	++++
Ps-AB-5	+	-	++++
Ps-MZ-1	+	-	-
Ps-MZ-2	-	-	+
Ps-MZ-3	-	++	+++
Ps-LK-1	-	-	++
Ps-LK-2	-	+++	+
Ps-LK-3	+	+++	+++

[a]; + = weak producer of NH_3, ++ = medium production of NH_3, +++ = good producer of NH_3,++++ = strong producer of NH_3, [b]; + = Siderophore Production, - = no Siderophore Production [c]; + = poor solubilization of Phosphate in Pikovskaya's medium, ++ = medium solubilization of phosphate in Pikovskaya's medium, +++ = very good solubilization of phosphate in Pikovskaya's medium, - = not solubilization of phosphate in Pikovskaya's medium.

show phosphate solubilization. PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to rice that represent a possible mechanism of plant growth promotion under field conditions (Verma et al., 2001). Several other worker like, Ahmad et al. (2008), Fischer et al. (2007), Sachdev et al. (2009), Rawat et al. (2011) and Joshi et al. (2011) observed phosphate solubilization and ammonia production by *Pseudomonas* isolated from wheat and other crops while Schoebitz et al. (2013) found that *P. fluorescens* from wheat rhizosphere solubilized phosphate in the range of 89-93 µ g/mL.

Siderophore production

Out of 22 isolates, six were found to produce siderophore. All six isolate formed orange halos surrounding the colonies on CAS medium (Table 2). This study has demonstrated that some of the isolates produced siderophore which are able to suppress the phyto-pathogenic fungi. Deshwal and Kumar (2013) also found *Pseudomonas* species to be producer of siderophore which could suppress the phyto-pathogenic fungi and Bhakthavatchalu et al. (2013) confirmed the ability of *P. aeruginosa* FP6 to produce siderophore and maximum production of siderophore (85.70 µM) was recorded after 36 h of incubation.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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