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Screening of free living rhizobacteria associated with wheat rhizosphere for plant growth promoting traits

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The use of plant growth promoting rhizobacteria (PGPR) for the benefits of agriculture is gaining worldwide importance and acceptance and appears to be the trend for the future. PGPR are bio-resources which may be viewed as a novel and potential tool for providing substantial benefits to the agriculture. Plant growth promoting rhizobacteria (PGPR) are known to influence plant growth by various direct or indirect mechanisms. In search of efficient PGPR strains with multiple activities, a total of 58 isolates belonging to *Pseudomonas, Azotobacter* and *Bacillus* were isolated from wheat rhizospheric soils collected from various districts of Uttar Pradesh. These rhizospheric isolates were biochemically characterized and screened for their plant growth promoting traits like production of indole acetic acid (IAA), ammonia production, siderophore production, phosphate solubilization, salt tolerance and antibiotic sensitivity test activity. The isolates of *Pseudomonas* (86.36%), and *Azotobacter* (76.13%) produced IAA, whereas only 38.09% of *Bacillus* isolates were able to produce IAA. Ammonia production was most common trait of *Pseudomonas* (90.89%), and *Azotobacter* (66.43) and *Bacillus* (76.19%). Phosphorus solubilization was detected in the isolates of *Azotobacter* (66.23%), *Pseudomonas* (45.35%), and *Bacillus* (23.80%). Siderophore production was exhibited by 9.61 to 20.17% of isolates. On the basis of multiple plant growth promoting activities eighteen isolates (nine *Azotobacter*, six *Pseudomonas* and three *Bacillus*) were evaluated for quantitative IAA production, antibacterial and salt tolerance. All the *Azotobacter* isolates were shown to produce higher range (95.60 to 175.20 µg/ml) of IAA, while *Pseudomonas* produced (44.40 to 95.60 µg/ml) IAA. The isolate Bc2 also showed potential of producing high amount of IAA. The isolate Azt5, Azt9, Ps2, Bc2 and Bc3 were found resistant even at 20 µg/ml concentration of tetracycline in the medium. Salt tolerance even at 7% NaCl concentration was observed in Azt5, Bc1 and Bc3 isolates. This study has pointed out that few isolates could exhibit PGP traits, which may promote plant growth directly and indirectly.

Key words: Plant growth promoting rhizobacteria (PGPR), wheat, indole acetic acid, ammonia, siderophore, P solubilization, salt tolerance, antibacterial activity.

INTRODUCTION

Cereals such as rice, wheat and maize, are the major grains that sustain humanity. Wheat grows in temperate climates and it is staple food for 35% of the world's population. On the other hand, it provides more calories and proteins in the diet than any other crop (Laegreid et al., 1999). Wheat is one of the major crops cultivated in India and all over the world. Climatic conditions and modern agriculture have been severally modifying and

polluting the natural environment. The increasing demand for a steady and healthy food supply by a burgeoning human population will require efficient management practices along with controlling disease that reduce crop yield. During last few decades, agricultural production has increased due to the use of high yielding varieties and enhanced consumption of chemicals, which are used both as fertilizers to provide nutrition and as protection agents to control the damage caused by phyto-pathogens. Excessive use of chemicals and change in traditional cultivation practices has resulted in the deterioration of physical, chemical and biological health of the cultivable soil. Microbial diversity in soil is considered important for maintaining the sustainability of agriculture production systems (Stark et al., 2007). The variability in the performance of Plant Growth Promoting Rhizobacteria may be due to various environmental factors that may affect their growth and exert their effects on plants. The environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil. To achieve the maximum growth promoting interaction between PGPR and crop plants, it is important to discover how the rhizobacteria exerting their effects on plant and weather, the effects are altered by various environmental factors including the presence of other microorganisms (Bent et al., 2001).

The functions of soil biota are central to decomposition processes and nutrient cycling. Soil is considered a storehouse of microbial activity, though the space occupied by living microorganisms is estimated to be less than 5% of the total space. Therefore, major microbial activity is confined to the 'hot-spot', that is, rhizosphere. The growth of many microorganisms in the rhizospheric region depends upon the root exudates released by the plants (Bais et al., 2006). Interactions between plant and microbes are intensely studied and especially those that benefit plant growth. PGPRs may benefit the host by causing plant growth promotion or biological disease control. PGPR activity has been reported in strains belonging to several genera such as *Azotobacter, Pseudomonas, Azospirillum, Acetobacter, Burkhalderia* and *Bacillus* (Kloepper et al., 1989; Glick 1995; Glick and Bashan, 1997; Rangrajan et al., 2002; Ahmad et al., 2005; Fischer et al., 2007; Joseph et al., 2007; Sachdeva et al., 2009; Agrawal et al., 2011). PGPR can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of growth regulators (auxin, gibberellins, ethylene etc), siderophore, ammonia, Phosphorus solubilization, HCN and antibiotics(Wahyudi et al., 2011; Etesami et al., 2009; Ahmad et al., 2008; Ahmad et al., 2005; Dilfuza, 2005; Valverde et al., 2003). Plant diseases are responsible for annual crop losses at a total value of more than 200 billion (Agrios, 2005). Resistant plants and chemicals are often used to control plant diseases. Resistance does not exist against all diseases and the breeding of resistant plants takes many years. The use of microbes to control diseases, which is a form of biological control, is an environment-friendly approach. The microbe is a natural enemy of the pathogen, and if it produces secondary metabolites, it does so only locally, on or near the plant surface, that is, the site where it should act. In contrast, the majority of molecules of agro-chemicals do not reach the plant at all (Flores et al., 2006). An effective plant growth promoting and biological control strains isolated from one region may not perform in the same way in other soil and climatic conditions (Duffy et al., 1997; Johnson et al., 1998). Isolating of native strain adapted to the environment and their study may contribute to the formulation of inoculants to be used in region crops. The different stages of life cycle of wheat consist of elongation, flowering, fruiting and ripening stages. It is found that rate of roots exudates released by the root of the wheat at flowering stage is higher as compared to other stages, hence greater microbial biota and activity is expected during this stage (Huddedar et al., 2000). Thus, the present study aims to investigate native PGPR free living bacteria, associated with rhizosphere of wheat during flowering stage to evaluate their ability to enhance the growth and yield of wheat under the ecological condition of Uttar Pradesh.

MATERIALS AND METHODS

Soil sample collection

Soil samples were collected from the rhizosphere of different wheat growing areas of Uttar Pradesh during the flowering stage. The wheat plants were uprooted from the field and rhizosphere soil was pooled and filled in sterile polythene bags.

Isolation and characterization of plant growth promoting rhizobacteria

All the strains of *Pseudomonas, Azotobacter* and *Bacillus* were isolated from the rhizosphere of wheat grown in various locations in Uttar Pradesh, India. The *Pseudomonas* strains were isolated on Kings B medium containing (Protease peptone 20 g, Glycerol 10 ml, K_2HPO_4 1.5 g $MgSO_4.7H_2O$ 1.5 g, Agar 18 g, pH 7.2) per litre of distilled water; whereas *Azotobacter* on Jensen's medium containing (Sucrose 20 g, K_2HPO_4 1 g, $MgSO_4$ 0.5 g ,NaCl 0.5, Na_2MoO_4 0.001 g, $FeSO_4$ 0.01 g, $CaCO_3$ 2 g, Agar 18 g, pH 7.0) per litre of distilled water, and *Bacillus* on the nutrient agar containing 5 g peptone, 3 g beef extract and 18 g Agar per liter of distilled water. The plates were incubated at 30°C for 24 h. After incubation, plates were observed for different isolates based on morphological

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features. Morphologically variable colonies picked up and purified on respective media plates. The pure cultures of isolates were made and preserved on the respective media slants. The total fifty eight rhizobacterial isolates were isolated (Ahmad et al., 2008). The rhizobacterial isolates were characterized on the basis of cultural, morphological and biochemical characteristics (Cappuccino and Sherman, 1992).

Screening of soil bacterial strains for plant growth promoting activities

Indole acetic acid production

The IAA production was assayed by the modified method as described by Loper and Scroth (1986). Bacterial cultures were grown for 48 h (*Pseudomonas, Bacillus*), and 72 h (*Azotobacter)* on their respective media at 28°C on rotary shaker. Fully grown cultures were centrifuged at 10000 rpm for 15 min. The 2 ml of supernatant was mixed with 2 to 3 drops of O-phosphoric acid and 4 ml of salkouski reagent solution (1 ml of $FeCl_3$ 0.5 M mixed in 50 ml of 35% $HClO_4$). The samples were incubated for 25 min at room temperature. The development of pink color was observed and optical density was taken at 530 nm with help of spectrophotometer. The concentration of IAA produced by cultures was measured with the help of standard graph of IAA obtained in the range of 20 to 200 µg ml^{-1}.

Ammonia production

The isolates were grown in peptone water in tubes incubated at 30°C for four days. One ml of Nessler's reagent (0.09 mol/L solution of potassium tetra-iodo-mercurate (II) in 2.5 mol potassium hydroxide was added in each test tube. The observed presence of faint yellow color indicates small amount of ammonia and deep yellow to brownish color indicates maximum production of ammonia (Cappuccino and Sherman, 1992).

Phosphorus solubilization

All the isolates were spot inoculated on Pikovskaya agar medium (Yeast extract 0.5 g Dextrose 10 g, Calcium phosphate 5 g , Ammonium sulphate 0.5 g KCl 0.2 g Mg SO4 0.1 g $MnSO_4$0.0001 g, FeSO4 0.001 g , Agar 15 g and 1 L distilled water) with phosphorus source and incubated at 28°C for 4 days. Phosphorus activity was determined by development of clearing zone around the culture spot (Agrawal et al., 2011; Wahyudi et al., 2011).

Siderophore production

Bacterial isolates were tested for siderophore production on chome azurol (60.5 mg CAS,1 Mm FeCl3.6H2O, 10 M HCl, 72.9 mg HDTMA, 1 M Sucrose (3 ml), 1 M $CaCl_2$ (0.4 ml), 1 M $MgSO_4.7H_2O$ (0.8 ml), 2% K_2HPO_4 (10 ml, NaCl (0.2 g), $NaMoO_4$ (0.005 g), PIPES (30.24 g), Difco agar (15 g), 10% Casamino acid (30 ml))) s agar (CAS) medium described by Schwyn and Neilands (1987). Each isolate was streaked on surface of CAS agar medium and incubated at room temperature for 1 to 2 days. The development of orange halo around the growth has been considered as positive for siderophore production.

Antibiotic sensitivity

The isolates were tested against the tetracycline by agar dilution method as described by Ahmad et al. (2004). The stock solution (5 mg/ml) of antibiotic, that is, tetracycline has prepared and used four different concentrations 1, 5, 10 and 20 µg/ml for antibiotic sensitivity test. The tetracycline was dissolved in 70% ethanol and sterilized with membrane filler (Axiva Scihem biotech). The nutrient agar medium was prepared in 4 L flasks of 500 ml and allowed to cool to 50°C. The diluted tetracycline of different concentrations were mixed in cool molten agar medium and poured in petri plates. The isolates were spot inoculated on solidified agar plate and incubated at 30°C for 48 h. After incubation, the plates were examined for the presence or absence of growth on the spotted area. The strains which were sensitive against tetracycline did not grow on the plate and resistant strains shows the growth on the plates against tetracycline.

Salt tolerance

The pure cultures of all isolates were streaked on nutrient agar medium, containing 3 to 7% NaCl concentration. Control plates with NaCl amendment were also kept for observation for all strains. All plates were incubated at 30°C for 48 h and observed for the presence or absence of the growth.

RESULTS AND DISCUSSION

Isolation and biochemical characterization

On the basis of cultural, morphological and biochemical characteristics a total of 58 bacterial isolates were identified as *Azotobacter, Pseudomonas* and *Bacillus* as described in Bergeys manual of determinative bacteriology (Holt et al., 1994). The *Azotobacter, Pseudomonas* and *Bacillus* strains from rhizosphere of different crops were isolated and extensively studied by Kole and Hajra (1997), Gaind and Gaur (1999), Ahmad et al. (2005), Joseph et al. (2007), Fischer et al. (2007), Ahmad et al. (2008), and Wahyudi et al. (2011). The general characteristics of the isolates were illustrated (Table 1).

Screening of rhizobacteria for plant growth promoting traits

In the present study a total of 58 bacterial strains (22 isolates of *Pseudomonas* and 18 of each *Azotobacter* and *Bacillus*) were tested for IAA, ammonia production, phosphorus solubilization and siderophore production (Figure 1). IAA production was shown in most of the *Pseudomonas* isolates (86.36%) followed by *Azotobacter* (76.19%) and *Bacillus* (38.09%). Ammonia production was detected in 90.89% of *Pseudomonas* followed by *Bacillus* (76.19%) *and Azotobacter* (66.43). The *Azotobacter* (66.23%), *Pseudomonas* (45.35%) and *Bacillus* (23.80%) strains were found able to solubilize phosphate. Very few strains of *Azotobacter* (20.17%), *Pseudomonas* (13.46%) and *Bacillus* (9.61%) exhibited siderophore production. Similar to our findings of plant growth promoting activities among Rhizobacteria strains have also been reported by some other workers

Table 1. Morphological and cultural characteristic of rhizobacterial test isolates.

Biochemical characters	*Azotobacter* spp.	*Pseudomonas* spp	*Bacillus* spp
Number of isolates	18	22	18
Grams reaction	-ve	-ve	+ve
Shape	rods	rods	rod
Pigment	Transparent to light milky most isolates become light brown to black after 10 days of incubation	Cream , light to green	Cream
Colony morphology	Watery mucilaginous with smooth margins	Smooth margin, flat to raised	Circular, lobate to serrated margin
Sucrose	+	+	+
Dextrose	+	+	+
Mannitol	+	-	+
H_2S production	-	-	-
Indole	-	-	-
Methyl red	-	-	-
Vogues Prokauer	-	-	-
Citrate Utilization	+	+	+
Starch	+	+	+
Gelatin hydrolysis	-	+	-
Catalase test	+	+	+
Nitrate reduction	-	+	-
Lipid hydrolysis	+	+	+
Casein hydrolysis	+	+	+

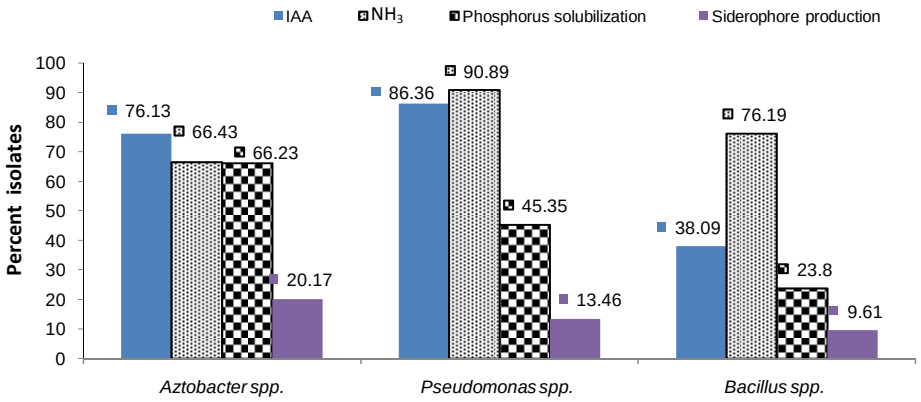

Figure 1. Plant growth promoting activities of rhizobacterial test isolate.

(Corsa and Walsh, 2002; Huddedaret al., 2002; Ahmad et al., 2004; Pedraza et al., 2004; Ahmad et al., 2008; Sachdev et al., 2009; Joshi and Bhatt, 2011; Rawat and Asrar, 2011).

Quantitative screening for IAA production by selected rhizobacterial isolates

Total of 18 selected rhizobacterial isolates of *Pseudomonas* (nine), *Azotobacter* (six) and *Bacillus* (three) were tested for quantitative IAA production. The production of IAA was recorded highest in isolates of *Azotobacter,* followed by *Pseudomonas* and *Bacillus* respectively. Among *Azotobacter* isolates, Azt-4 and Azt-7 produced highest amount (175.20 µg/ml) of IAA followed by Azt-1>Azt3 >Azt-6. However, *Pseudomonas* rhizobacteria isolates produced IAA in the range of 44.40 to 95.60 µg/ml in the broth culture medium (Tables 2 and 3). Wahyudi et al. (2011) reported that *Bacillus* spp. Cr4

Table 2. Production of Indole Acetic acid (IAA) by selected rhizobacterial isolates grown in respective medium.

S/N	Isolate	IAA Production µg/ml
1	Azt1	130.15
2	Azt2	114.66
3	Azt3	114.66
4	Azt4	175.20
5	Azt5	95.60
6	Azt6	114.60
7	Azt7	175.20
8	Azt8	114.60
9	Azt9	114.60
10	Ps1	95.60
11	Ps2	79.60
12	Ps3	44.40
13	Ps4	66.20
14	Ps5	79.60
15	Ps6	79.60
16	Bc1	70.00
17	Bc2	72.40
18	Bc3	64.00
	CD	22.08
	SEm	7.68

Table 3. Antibiotic sensitivity and salt tolerance of selected rhizobacterial test isolates.

S/N	Isolate	Antibiotic concentration (µg/ml)				NaCl concentration (%)				
		1	5	10	20	3	4	5	6	7
1	Azt1	++	++	-	-	++	-	-	-	-
2	Azt2	+++	-	-	-	++	-	-	-	-
3	Azt3	++	+	-	-	+	-	-	-	-
4	Azt4	+++	-	-	-	-	-	-	-	-
5	Azt5	+++	+++	++	-	+++	++	+	+	-
6	Azt6	-	-	-	-	++	-	-	-	-
7	Azt7	++	++	-	-	++	+	-	-	-
8	Azt8	+++	-	-	-	++	+	-	-	-
9	Azt9	+++	+++	++	++	+	+	+	-	-
10	Ps1	+++	-	-	-	-	-	-	-	-
11	Ps2	+++	+++	+++	++	+	+	-	-	-
12	Ps3	++	++	-	-	+	+	-	-	-
13	Ps4	+++	-	-	-	+	+	-	-	-
14	Ps5	+++	+++	-	-	+	-	-	-	-
15	Ps6	++	++	++	-	+	-	-	-	-
16	Bc1	+++	++	-	-	+++	+++	+++	+++	+
17	Bc2	+++	+++	+++	+++	+++	+	-	-	-
18	Bc3	+++	+++	++	-	+++	+++	++	++	+

Azt = *Azotobacter*, Ps = *Pseudomonas*, Bc = *Bacillus*, Incubation period 36 h; +++ = maximum growth, ++ = medium growth, + poor growth, - = no growth.

produced 86.82 mg/L IAA in culture medium supplemented with L Tryptophan while 32.80 µg/ml IAA production was reported by Ahmad et al. (2004). The findings of present investigation are outstanding in

reference to earlier reports.

Salt tolerance and antibiotic sensitivity test

The present study showed that out of 18 selected strains, Azt-5, Bc-2 and bc-3 tolerated even 7% NaCl concentration. All the rhizobacterial strains were found tolerant at 3% NaCl concentration except Azt4 and Ps-1 (Table 3). Rangarajan et al. (2002) screened *Pseudomonas* strains for salt tolerance; out of 256 strains, only 36 strains could grow at 4.5% NaCl concentration and no strain was able to grow at 6% NaCl concentration. Similarly, the selected strains were also tested against the tetracycline. The isolate Azt-9Ps-2 and Bc2 were found resistant even at the concentration of 20 µg/ml of antibiotic while Azt6 showed very high sensitivity against the test antibiotic and could not tolerate even 1 µg/ml concentration.

Conflict of Interest

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

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