

Original Research Article

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Evaluation of Bacteria Isolated from Wheat Rhizosphere for Plant Growth Promoting Attributes and Antagonistic Activity

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ABSTRACT

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Plant growth promoting rhizobacteria are the soil bacteria present around the rhizosphere and are involved in promoting plant growth and development via different mechanisms. In order to promote sustainable agriculture, the present investigations were undertaken to screen the PGPR isolates from the plant rhizosphere. A total of eight rhizobacteria were isolated from the wheat plant rhizosphere. The biochemical tests such as Catalase test, oxidase test, nitrate reduction, Citrate Utilization, Urease test, Gelatinase test and carbohydrate utilization carried out for phenotypic identification of isolates. Based on functional characteristics, few isolates were found positive for siderophore, production, Phosphate solubilization potential and indole production. Subsequently, these isolates also showed *in vitro* antagonistic activity against *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Fusarium sp.* Therefore, the present study suggested that isolated bacteria showed PGPR potential and biocontrol ability which can be used for biofertilizers as well as biocontrol agents. Consequently, these isolates can be further evaluated under *in situ* soil microcosm experiments.

Introduction

The rhizosphere is a dynamic environment, was first described by Hiltner (1904) as the volume of soil surrounding plant roots, which harbours diverse range of microorganisms and the bacteria colonizing this habitat are called rhizobacteria (Schroth and Hancock, 1982). Plant-associated bacteria can be classified into beneficial, deleterious and neutral groups on

the basis of their effects on plant growth (Dobbelaere *et al.*, 2003). Among the different groups, Plant growth promoting rhizobacteria (PGPR) are a group of beneficial bacteria that colonize the rhizosphere and contribute to increased growth and yield of crop plants (Kloepper and Schroth, 1978). PGPR support the growth of host plants and can stimulate plant growth, increase yield, reduce pathogen infection, as

well as reduce biotic or abiotic plant stress, without conferring pathogenicity (Lugtenberg and Kamilova, 2009). Bacteria of diverse genera have been identified as PGPR, such as *Azoarcus*, *Azospirillum*, *Azotobacter*, *Arthrobacter*, *Bacillus*, *Clostridium*, *Enterobacter*, *Gluconacetobacter*, *Pseudomonas* and *Serratia*, however species of *Pseudomonas* and *Bacillus* are the most extensively studied (Kumar *et al.*, 2011). PGPR can promote the plant growth by various direct and indirect mechanism such as phosphate solubilisation, nitrogen fixation, Indole-3-acetic acid (IAA) production, siderophore production and repression of soil borne pathogens by production of hydrogen cyanide and antibiotics (Glick, 1995 and Ahmed and Kibret, 2014).

Wheat represents a major renewable resource for food, feed, and industrial raw material and it is the most widely grown worldwide crop. (Reynolds *et al.*, 2009; Montano *et al.*, 2014) In the realm of food crops in the world, wheat occupies a prime position in the world. In India it is second most important food crop and contributes approx. 25 % of total grain production of the country (Amrawat *et al.*, 2013). In 2010, world production of wheat was 651 million tons, making it the third most produced cereal after maize (844 million tons) and rice (672 million tons) (Reynolds *et al.*, 2009). As it is been mentioned that crops supplemented with PGPR resulted significant increase in grain yield and protein content with lower fertilizer doses as compared to conventionally applied crops (Rosas *et al.*, 2009). Additionally, significant increase was observed in the wheat yield after supplement of rhizobacterial inoculants at locations with continuous low yields (Mader *et al.*, 2011).

Thus, wheat (*Triticum aestivum*) plant has been selected as a source of rhizobacteria for the present study because among the different crops undergoing cultivation in Madhya

Pradesh, it is one of the major cash crop of this region. On account of that, the present investigation has been undertaken to isolate and characterize the rhizobacteria for plant growth promoting parameters which could be useful in preparation of bioinoculants for wheat crop.

Materials and Methods

Sampling site

The rhizospheric sample was taken from the field of Badwa village located near Karond, Bhopal. Wheat (*Triticum aestivum* L.) was selected as the source of rhizobacteria. Wheat Seed of variety Lok 1 were regularly used for sowing by the farmers. The geographical location of wheat field supplemented was longitude: 23°09'02.1", latitude: 077°27'04.3" and altitude: 1590 ft.

Collection of soil sample

In order to determine the indigenous bacterial population, samples were collected in a randomized manner. A quadrant of 1 m² area (1 m x 1 m) was selected at five different places of the experimental site. Samples were collected from all four corners and centre of the quadrant. This was repeated for all quadrants made in the field. Sampling was done at maturation stage of the plant. Rhizosphere soil was collected in polythene bags, closed and brought to the laboratory, and used within 6 hours for further experiment.

Isolation of Rhizobacteria

10 gm of soil of rhizospheric soil was weighed and mixed with 90 ml sterile distilled water in 250 ml conical flask. Flask was kept in shaker incubator for vigorous shaking for 5 to 10 min to form homogenous suspension. The soil solution was then than allowed to

settle for 10-15 min before further processing. The isolation was carried out using serial dilution technique (Saini *et al.*, 2016; Patel *et al.*, 2015).

The bacterial colonies were recovered on Nutrient agar medium and were observed on the basis of colony colour, shape, margin, elevation and texture. Pure culture of these morphotypes was obtained by following streak plate method.

Biochemical Characterization of the isolated rhizobacteria

The potential isolates were further characterized on the basis of their staining characteristics and further investigated in terms of biochemical properties like indole, catalase, urease, citrate, ammonia, nitrate producing abilities which helped in identifying the bacteria up to genus level (Cappuccino and Sherman., 1996).

Characterization of PGPR

Screening for Siderophore Production

Siderophore production was observed on Chromeazurool 'S' agar medium (Schwyn and Neilands, 1987). For this, the test organism was spot inoculated on the Chromazurool S (CAS) agar plates which were incubated at 28°C. Formation of an orange halo around the bacterial colony indicated siderophore production.

Phosphate solubilization

Pikovskaya's agar medium was used to demonstrate phosphate solubilization by bacteria. Bacterial isolates were spot inoculated on plates and incubated for 2-3 days at 28 ± 2°C. Clear zone formation after incubation around bacterial colony indicated positive result (Pikovskaya, 1948).

Indole production

Bacterial culture was grown in tryptone broth followed by incubation at 28±2°C for 72 h at 120 rpm. After incubation 1 ml of Kovac's reagent was added in tubes, shaken vigorously and allowed to stand for some time. Appearance of cherry red layer at the top indicated positive result.

Extracellular enzymes production

The bacterial isolates were evaluated for following extracellular enzymatic activity following standard protocols.

Protease production

Bacterial isolates were streaked on skim milk agar plates to screen for protease production. The plates were incubated at 28 ± 2 °C for 2-3 days. Formation of clear zone around the bacterial colony indicated a positive result (Cappuccino and Sherman, 1996)

Pectinase production

Bacterial isolates were spot inoculated on M₉ minimal medium supplemented with 0.5% pectin. The plates were incubated at 28 ± 2 °C for 2-3 days. After incubation plates were overlaid with 2% CTAB. After 30 min CTAB solution was poured off, the plates were then washed with 1M NaCl. A zone around bacterial growth indicated positive result (Aneja, 2001).

Cellulase production

Pure cultures of bacteria were spot inoculated on M₉ minimal salt medium supplemented with 0.5% carboxymethyl cellulose (CMC). Plates were incubated at 28 ± 2 °C for three days. After three days the plates were overlaid with 0.1% congo red solution and washed with 1M NaCl. Cellulase positive isolates

showed clear zone around the bacterial colonies (Amore *et al.*, 2013).

Xylanase production

Bacterial isolates were spot inoculated on M₉ Minimal medium supplemented with 0.5% larch wood xylan as carbon source. The plates were incubated at 28 ± 2 °C for 2-3 days. After three days the plates were flooded with congo red solution for 30 min. Zone of clearance around the bacterial colonies was considered as positive result.

Chitinase production

Bacterial isolates were spot inoculated on M₉ minimal medium supplemented with 0.5% chitin for the assay of chitinase production. The plates were incubated at 28 ± 2 °C for 2-3 days. After three days the plates were observed. Formation of clear Zone around the bacterial growth indicated positive result (Amore *et al.*, 2015)

Amylase production

Bacterial cultures were spotted on starch agar medium containing 0.5% starch. Plates were incubated at 28°C for 3days. The plates were over laid with freshly prepared Lugol's iodine solution. A clear zone against blue-background confirms starch hydrolysis (Aneja, 2001).

Antifungal assay

In vitro antagonistic activity of isolated bacteria was tested against fungal pathogens *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Fusarium sp.* by employing dual plate assay (Huang and Hoes, 1976). The culture of fungal pathogens was procured from Indian Institute of Soybean Research, Indore. Fungal cultures

were grown and maintained on potato dextrose agar (PDA) medium (HiMedia). The radii of the fungal colony towards and away from the bacterial colony were noted. The percentage growth inhibition was calculated using the following calculation:

$$\% \text{ Inhibition} = [(R - r)/R \times 100]$$

Where, r is the radius of the fungal colony opposite the bacterial colony and R is the maximum radius of the fungal colony away from the bacterial colony (Fatima *et al.*, 2009).

Results and Discussion

Isolation and morphological characteristics

Total 8 bacterial morphotypes were obtained based on colony characteristics from the wheat rhizosphere soil samples. Based on Gram staining result total 5 isolates were found to be Gram Negative and 3 were Gram Positive. A KOH result also confirms the discrimination of the bacteria into Gram positive and Gram Negative group (Table 1). The isolates WRS-2, WRS-3, WRS-4 were found to be endospore positive.

Biochemical characterization and Identification

The biochemical tests such as Catalase test, oxidase test, nitrate reduction, Citrate Utilization, Urease test, Gelatinase test and carbohydrate utilization carried out for phenotypic identification of isolates (Holt *et al.*, 1994).

All the isolates were positive for maximum biochemical activities. (Table3). Based on Biochemical results all the 8 eight isolates were positive for the catalase, Citrate and gelatinase activity.

Table.1 Morphological characteristics of the rhizobacteria isolated from wheat rhizosphere

S. No.	Isolate No.	Gram Reaction	Endospore	KOH test	Colour of the colony	Cell Shape	Margin	Texture	Elevation
1	WRS-1	-	-	KOH+	Offwhite	Circular	Entire	Smooth	Raised
2	WRS-2	+	+	KOH-	Creamish white	Circular	Entire	Smooth	Flat
3	WRS-3	+	+	KOH-	offwhite	Circular	Entire	Smooth	Flat
4	WRS-4	+	+	KOH-	Creamish white	Irregular	Undulate	Rough	Raised
5	WRS-5	-	-	KOH+	Yellow	Circular	Entire	Smooth	Flat
6	WRS-6	-	-	KOH+	offWhite	Circular	Entire	Smooth	Flat
7	WRS-7	-	-	KOH+	Creamish Brown	Circular	Entire	Smooth	Flat
8	WRS-8	-	-	KOH+	Red	Circular	Entire	Smooth	Raised

Table.2 Biochemical characteristics of the rhizobacteria isolated from wheat rhizosphere

S. No.	Isolate No.	Catalase	Oxidase	Nitrate reduction	Citrate Utilization	Urease	Gelatinase
1	WRS-1	+	+	-	+	-	+
2	WRS-2	+	+	+	+	-	+
3	WRS-3	+	-	+	+	-	+
4	WRS-4	+	+	+	+	-	+
5	WRS-5	+	+	+	+	-	+
6	WRS-6	+	+	-	+	-	+
7	WRS-7	+	+	-	+	-	+
8	WRS-8	+	-	+	+	+	+

“- = No production; += production”

Table.3 Functional characteristics of the rhizobacteria isolated from wheat rhizosphere

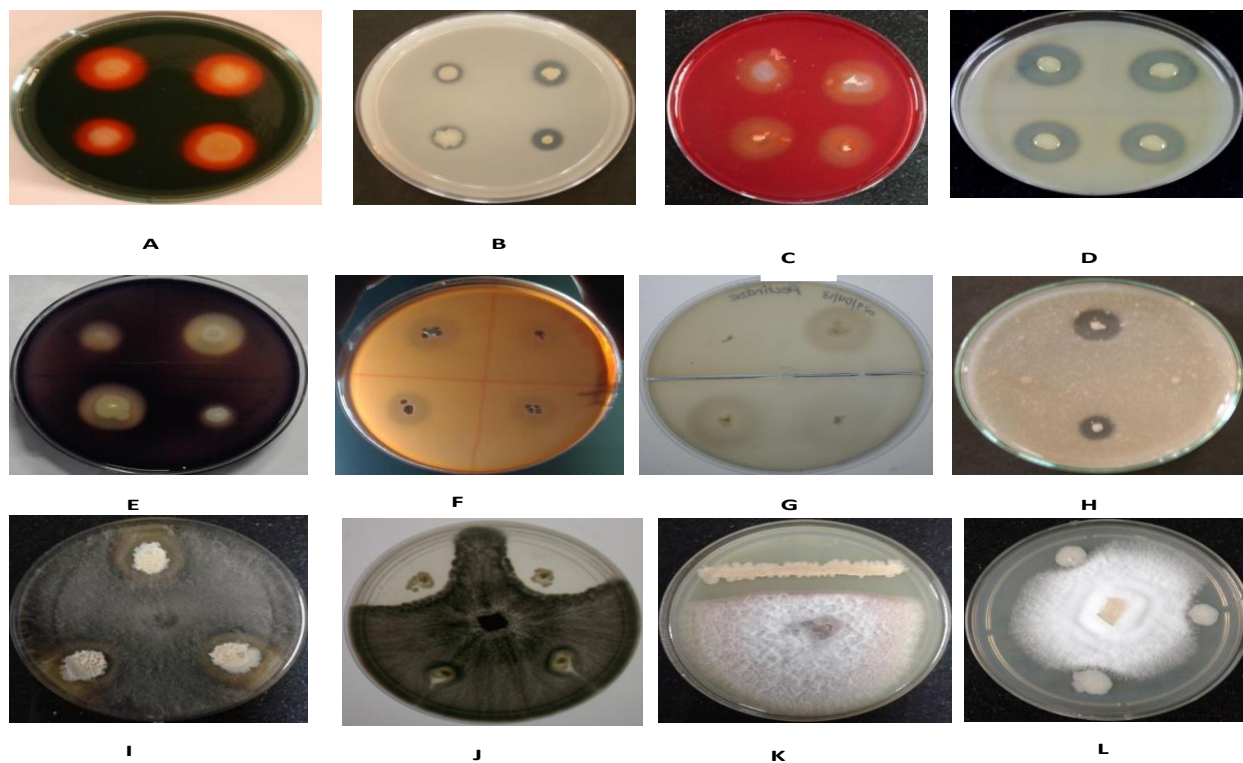
S. No.	Isolate No.	Siderophore	Phosphate	Indole	Protease	Pectinase	Cellulase	Xylanase	Chitinase	Amylase
1	WRS-1	-	-	-	+	-	-	-	-	-
2	WRS-2	-	+	-	+	-	-	-	-	+
3	WRS-3	-	+	-	++	+	+	-	-	+
4	WRS-4	++	+	-	+++	+	++	+	-	++
5	WRS-5	+	-	-	-	+	-	-	-	-
6	WRS-6	+++	+	+	++	-	+++	+	-	-
7	WRS-7	++	-	-	+	-	-	-	-	-
8	WRS-8	-	-	-	+	-	-	-	++	-

- = No production; + = Weak production; ++ = medium production; +++ = high production

Table.4 Antagonistic activity against *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, *Fusarium sp.* and *Macrophomina phaseolina*

S. No.	Isolate No.	% Inhibition			
		<i>Macrophomina phaseolina</i>	<i>Sclerotinia sclerotiorum</i>	<i>Fusarium Sp.</i>	<i>Sclerotium rolfsii</i>
1	WRS-1	—	—	—	—
2	WRS-2	—	—	—	—
3	WRS-3	—	—	—	—
4	WRS-4	65%	75%	79%	55%
5	WRS-5	—	—	—	—
6	WRS-6	45%	56%	55%	54%
7	WRS-7	55%	79%	60%	60%
8	WRS-8	—	—	—	—

Fig.1 Functional Characterization of the isolates: A) Siderophore production B) Phosphate production, Enzymatic activities: (C) Cellulase activity (D) Protease activity (E) Amylase activity (F) Xylanase activity (G) Pectinase activity (H) Chitinase activity. Antagonistic activity of the isolate WRS-4 against (I) *Macrophomina phaseolina* (J) *Sclerotinia sclerotiorum* (K) *Fusarium sp.* (L) *Sclerotium rolfsii*



Functional characterization

Plant growth-promoting rhizobacteria (PGPR) are known to promote plant growth by improving plant nutrition, modifying root growth architecture and plant responses to external stress factors simultaneously (Salem *et al.*, 2007; Glick, 1995). Several bacterial genera like *Bacillus*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bradyrhizobium*, *Rhizobium*, have been determined as suitable candidate for plant growth promotion. In present study the morphological characteristics and biochemical activities (Table-2) reveals that the isolated bacteria have properties similar to the genus *Enterobacter*, *Bacillus*, *Pseudomonas*, *Serratia marscecens* etc. Three isolates WRS-2, WRS-3, WRS-4 were positive for endospore formation which is common characteristics of the genus *Bacillus* (Clauss and Berkeley, 1986). Among the eight isolates, four were siderophore, three showed Phosphate solubilization potential and one was positive for indole production (Fig. 1). Isolate WRS-6 which is positive for siderophore production also showed positive activity for phosphate solubilization and indole production. It is well documented that rhizospheric bacteria harbors multiple plant growth promoting traits (Upadhyay and Srivastava, 2010; Kumar *et al.*, 2012; Saini *et al.*, 2016). There are different mechanisms by which rhizobacteria promote plant growth directly by hormone production and phosphate solubilization or indirectly by siderophore production (Lugtenberg *et al.*, 2009). It is also well documented that rhizobacteria isolated from wheat rhizosphere showed multiple plant growth promoting traits (Rana *et al.*, 2011).

Results of the present work revealed that out of 8, 7 isolates were positive for one or more multiple plant growth promoting traits and are thus can be used as significant PGPRs (Table 3). Bacterial extracellular enzymes production

plays an important role in the management of plant pathogens as well as holds enormous economic potential (Geetha *et al.*, 2014). Soil enzymes like cellulase, chitinase, protease, phosphatase play a critical role in maintaining soil ecology, fertility and health (Sinsabaugh *et al.*, 1991). Due to potential industrial applications of extracellular enzymes all the 8 PGPR isolates selected were tested for their extracellular enzyme production like Cellulase, Xylanase, Pectinase, Protease, amylase, chitinase, activity. In our study all the isolates WRS-1, WRS-2, WRS-3, WRS-4, WRS-5, WRS-6, WRS-7, WRS-8 were positive for one or more extracellular enzymatic activity. (Table.3). Only one isolate WRS-8 was found to be positive for Chitinase activity. Intriguingly, isolate WRS-4, WRS-6 and WRS-8 showed *in vitro* antagonistic activity against fungal pathogens *Macrophomina phaseolina*, *Sclerotinia sclerotiorum*, *Sclerotium rolfsii*, *Fusarium sp* (Table-4). These results were supported by Fatima *et al.*, 2009, Walia *et al.*, 2009, who isolated the PGPRs from wheat rhizosphere and showed *in vitro* antagonism against soil borne plant pathogen. Thus, it can be concluded that the isolates evaluated for multiple traits can be used as a potential candidate in plant growth promotion and biocontrol.

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