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Physiochemical characterization of PGPR isolates of *Rhizobial* strains from Shekhawati region in India

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Soils contain natural reserves of plant nutrients, but these reserves are largely in forms unavailable to plants, and only a minor portion is released each year through biological activity or chemical processes. For optimum plant growth, nutrients must be available in sufficient and balanced quantities. Nitrogen being an essential component of proteins and nucleic acids plays an important role in improving the crop production. A study on physiochemical characterization *viz.*, physiological, biochemical characteristics and antibiotic resistance signifies that rhizobial strains were sensitive to pH and antibiotic like rifampicin and ciproflaxin. It was also concluded that these rhizobial bacterial strains utilize glucose, arabinose and sorbitol as sole carbon source.

Key words : *Rhizobium*, Biochemical, Antibiotic, Leguminous plants.

INTRODUCTION

Leguminous plants are known as a dietary protein source. These plants show symbiotic nitrogen fixation with the association of *Rhizobium* present in its root nodules. Soil bacteria are one of the major groups of microbes which are abundant in rhizosphere soil ranging between 106 to 108 colony-forming units (cfu) per gram (Clark, 1967) and some of them have shown great potential as biocontrol agents of nematodes (Zavaleta-Mejia and VanGundy, 1982). Plant growth promoting rhizobacteria (PGPR) are free-living bacteria and some of them colonies the tissues of living plants and cause unapparent and symptomatic infections when applied to seeds or crops, enhance the growth of the plant or reduce the damage from soil borne plant pathogens (Kloepper *et al.*, 1980). Rhizobacteria that exert beneficial effects on plant growth and development are referred to as plant growth-promoting rhizobacteria (PGPR). In last few decades a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus* and *Serratia* have reported to enhance plant growth (Okon and Labandera-Gonzalez, 1994; Glick, 1995). In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil (Cattelan *et al.*, 1999). The use of PGPR to promote plant growth has increased in various parts of the world. PGPR can affect plant growth by producing and releasing secondary metabolites and facilitate the availability and uptake of certain nutrients from the root environment (Zahir *et al.*, 2003). Unfortunately, the interaction between associative PGPR and plants can be unstable. The good results obtained *in vitro* cannot always be dependably reproduced under field conditions (Chanway and Holl, 1993; Zhender *et al.*, 1999). Therefore, it is necessary to develop efficient strains in field conditions. One possible approach is to explore physiochemical characterization for PGPR having combination of PGP activities and well adapted to particular soil environment. So keeping in view the above constrains, the present study was designed to screen certain rhizospheric bacterial isolates belonging to the genera *Rhizobium* from different sites of Shekhawati region of India for their multiple plant growth promoting activities.

MATERIALS AND METHODS

Isolation of *Rhizobium* strains:

The rhizospheric soil samples (six) were collected from fields growing different leguminous plants were collected from different areas of Shekhawati region of India. *Rhizobium* bacterial strains were isolated on isolated on yeast extract mannitol agar (Vincent, 1970) containing (per liter) 0.5 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$,

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0.1 g NaCl, 10 g mannitol, 1 g yeast extract, and 1.5% (w/v) agar, with congo red as an indicator (Nelson and Child, 1981). The YEMA plates were then allowed to grow at 28+2^{0}C in an incubator. After 36-48 hours incubation period, Rhizobial colonies were appeared on YEMA plates. Single colonies that appeared on YEMA plates were picked and re-streaked on fresh YEMA medium to obtain pure cultures.

Characterization of rhizobacteria for PGP traits:

Production of Indole acetic acid:

Indole acetic acid (IAA) production was detected as described by Brick *et al.* (1991).Bacterial cultures were grown for 72 h (*Azotobacter* and *Rhizobium*) and 48 h (*Pseudomonas* and *Bacillus*) on their respective media at 36±2^{0}C. Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was mixed with two drops of orthophosphoric acid and 4 ml of the Salkowski reagent (50 ml, 35% of perchloric acid, 1 ml 0.5 M $FeCl_3$ solution). Development of pink colour indicated IAA production.

Production of ammonia:

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72 h at 36±2^{0}C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman, 1992).

Production of HCN and catalase:

All the isolates were screened for the production of hydrogen cyanide by adapting the method of Lorck (1948). Briefly, nutrient broth was amended with 4.4 g glycine/l and bacteria were streaked on modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 36±2^{0}C for 4 days. Development of orange to red colour indicated HCN reduction. Bacterial cultures were grown in a nutrient agar medium for 18-24 h at 36±2^{0}C. The cultures were mixed with appropriate amount of H_2O_2 on a glass slide to observe the evolution of oxygen.

Siderophore production:

Siderophore production was detected by the universal method of Schwyn and Neilands (1987) using blue agar plates containing the dye chrom azurol S (CAS). Orange halos around the colonies on blue were indicative for siderophore production.

Biochemical characterization of rhizobacteria:

Each bacterial strain was then identified on the basis of its growth rate, color, shape and gum production. For gram's strains reaction, the slides of isolates and purified *Rhizobial* strains were prepared (Vincent, 1970) and observed under light microscope. The pH tolerance of the *Rhizobium* isolates was tested by inoculating them in 50 ml YEMA broth with pH adjusted to 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0. (Graham *et al.*, 1991).

Rhizobial isolates were further also examined for biochemical characteristics such as utilization of citrate, lysine, ornithine, nitrate reduction, H_2S Production, hydrolysis of urea, phenylalanine deamination and utilization of different carbon sources using KB002 HiAssorted™ Biochemical Test Kit (Genei, Bangalore, India). This kit was a standardized colorimetric identification system utilizing seven conventional biochemical tests and five carbohydrate tests.

Antibiotic resistance test:

The susceptibility or resistance of rhizobia to an antibiotic was assayed according to Singh *et al.* (2008) with the help of antibiotic disc test. The following antibiotics were used for analysis- Vancomycin, Kanamycin and Nalidixic Acid (30 mcg/ disc), Ciproflaxin (5 mcg/ disc) and Rifampcin (2 mcg/ disc).

RESULTS AND DISCUSSION

Plant rhizosphere is known to be preferred ecological niche for soil microorganisms due to rich nutrient availability. Reports are available on *Azotobacter* spp. isolated from different sources showed IAA production (Gonzalez-Lopez *et al.*, 1986; Jagnow, 1987; Nieto and Frankenberger, 1989). In the present investigation 5 isolates belonging to *Rhizobium* spp., were screened for *in vitro* PGP activities. Screening results of PGP traits are depicted in Fig. 1. IAA production was shown in all

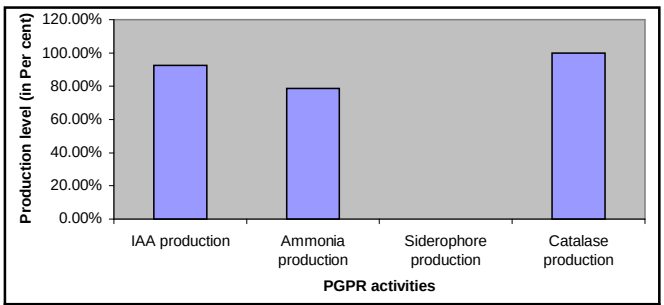

Fig. 1 : Plant growth promoting characteristics of rhizobcterial isolates

the isolates of *Rhizobium* at a level of 92.5%; ammonia production was detected in 78.8% of isolates of *Rhizobium*. None of the isolates of *Rhizobium* spp. produced siderophore. Production of catalase was exhibited by all the isolates of rhizobacteria. However, production of HCN was not detected in rhizobacterial isolates under study (data not shown). Catalase activity was detected in all the *Rhizobium* strains that may be potentially very advantageous. IAA production in rhizobial isolates are in agreement with earlier reports. The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant. Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities (Arshad and Frankenberger, 1993; Glick, 1995). Production of IAA by bacterial strains is a general characteristic of our test isolates. Higher level of IAA production by rhizobial was recorded by other workers (Xie *et al.*, 1996). Another important trait of PGPR, that may indirectly influence the plant growth, is the production of ammonia. Mostly all the isolates were able to produce ammonia. However, ammonia production was observed less frequently. Reliable identification of specific Rhizobial strains is necessary for the study of their symbiotic association with plants (Sajjad *et al.*, 2001). The characterization of new prospective isolates for application requires the development of easy, rapid and reliable methods for their identification (Selenska- Pobell *et al.*, 1996). Siderophores chelates iron and other metals contribute to disease suppression by conferring a competitive advantage to biocontrol agents for the limited supply of essential trace minerals in natural habitats (Hofte *et al.*, 1992; Loper and Henkels, 1997). All five rhizobial isolates were obtained from the root nodules of *Cicer aerietinum*, *Trigonellla foenumgraecum*, and Mungbean and they showed sufficient growth and more as well as less gum production having a mean generation time of 24- 48 hrs. The colonies obtained on YEMA containing congo red were cream colored, gummy, mucilaginous, translucent and circular with entire or smooth margins. All the strains were found gram negative and rod shaped during gram's staining. All isolates showed growth in YEMA broth with pH values of 4 to 10, but they showed weak growth at pH 4 and pH 10 (Fig. 2). All isolates were grown at highly acidic as well as highly basic condition. Differences between isolates were verified by antibiotic resistance. All the *Rhizobium* isolates were resistant to rifampcin and sensitive to ciproflaxin (Table 1). Siderophores may directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria. Antibiotic and siderophores may further function as stress factors or singles including local and systematic host resistance. It has been assumed that inoculation with the tested

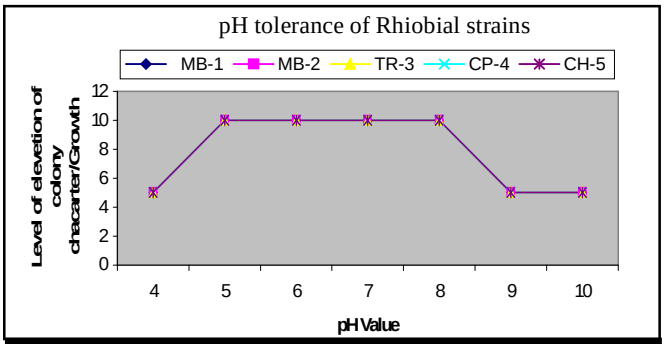

Fig. 2 : Showing pH tolerance results of all isolates at different pH values
0-5 - weak growth, 6-10 high growth

Table 1: Morphological and cultural characteristics of rhizobacteria

Sr. No.	Physiological character	Stability level
1.	Grams reaction	G -ve
2.	Shape	rods
3.	Pigments	+/-
4.	Lactose	Less
5.	Dextrose	-
6.	Sucrose	-
7.	Mannitol	+
8.	Oxidase	+
9.	OF test	-
10.	H_2S production	+
11.	Indole	+
12.	Methyl red	+
13.	Vogues Proskauer	+
14.	Citrate utilization	-
15.	Nitrate reduction	+
16.	Starch hydrolysis	+
17.	Gelatin hydrolysis	-
18.	Sorbitol	+
19.	Arabinose	+
20.	Adonitol	+
21.	H_2S production	+
22.	Citrate utilization	+
23.	Nitrate reduction	+
24.	Lysine utilization	+
25.	Ornithine utilization	+
26.	Urease	-
27.	Adonitol	-

Table 2: Results of antibiotic resistance test

Isolates	Antibiotic discs				
	Ciproflaxin	Vancomycin	Kanamycin	Nalidixic acid	Rifampcin
MB-1	+	–	+	+	–
MB-2	+	–	+	–	–
TR-3	+	–	–	–	–
CP-4	+	–	–	–	–
CH-5	+	+	–	–	–

Absence of growth(-) and presence of growth (+)

bacterial strains of rhizobacteria may enhance the plant growth as a result of their ability to fix nitrogen. All the bacterial isolates in the present study were able to produce catalase. Rhizobial strains showing catalase activity must be highly resistant to environmental, mechanical, and chemical stress. Similar to our findings of multiple PGP activities among PGPR have been reported by some other workers while such findings on indigenous isolates of India are less commonly explored (Gupta *et al.*, 1998). Biochemical tests were based on the principle of pH change and substrate utilization. On incubation organisms undergo metabolic changes which were indicated by a color change in the media that could be either interpreted visually or after addition of the reagent. All the *Rhizobium* isolates are negative to adonitol and urease test. All five isolates had given 17 positive and 7 negative biochemical tests (Table 2). Any microbial utilization in agriculture requires an evaluation of the environmental risks associated with the introduction of indigenous or non-indigenous microorganisms into the plant rhizosphere (Jackman *et al.*, 1992) as well as an assessment of the most suitable conditions for effective and successful establishment of the PGPR inoculation in the rhizosphere of the host plant (De Leij *et al.*, 1995). Furthermore, it is known that some PGPR strains are able to express multiple beneficial functions (Kloepper and Schrot, 1978). In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil. Unfortunately, the interaction between associative PGPR and plants can be unstable. The good results obtained *in vitro* cannot always be dependably reproduced under field conditions. It is expected that inoculation with rhizobacteria containing PGP characteristics consequently promote root and shoot growth as well as nodulation. Further evaluation of the isolates exhibiting multiple plant growth promoting (PGP) traits on soil–plant system is needed to uncover their efficacy as effective PGPR.

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