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Screening the Rhizobium from *Cajanus cajan*for Plant Growth Promoting Factors

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Abstract:
Plant growth promoting rhizobacteria (PGPR) found in the rhizosphere in association with roots are beneficial bacteria which can heighten the growth of plant directly or indirectly. In the present study root nodules were collected from Cajanus cajan cultivated field, Karimganj of Barak Valley Region, Assam (India). A total of five (5) bacterial strains were isolated and they were further subjected to biochemical characterization and screened for plant growth promoting traits. The strain KRN5 showed high IAA production and KRN1 showed the highest solubilization of phosphate among others. KRN1 and KRN5 also showed tolerance to salt at 10 % NaCl concentration, which may be effective for soil with high salinity. The IAA production and phosphate solubization showed by these isolates make them suitable for further suitable pot trials.

Keywords: *Rhizobium, PGPR, Salinity*

1. Introduction

Leguminous plants have always been significant both agriculturally and ecologically as they are the key source of biological nitrogen fixation (BNF) through root nodule formation (Sprent, 1987). Pigeon pea (*Cajanuscajan*L var. Manak), a legume is a major source of protein for most of the vegetarian population worldwide. India is a principal pigeon pea-growing country contributing nearly 90% of total world's production (Dubey*et al*., 2010). Bacteria present in root nodules of legumes are mainly species of *Rhizobium*(*Mesorhizobium, Bradyrhizobium, Azorhizobium, Allorhizobium* and *Sinorhizobium*), responsible for fixing atmospheric nitrogen through symbiosis with legumes. *Rhizobium* spp. are gram- negative bacteria having scientific and agronomic importance also maintains the soil fertility (Singh, 2013).

Bacteria isolated from the rhizosphere, which have been shown to improve plant health or increase yield, are usually referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1980). In addition to symbiotic nitrogen fixation, *Rhizobium* can also produce phytohormones, siderophores, HCN; thereby reducing the threat of pests and diseases and ultimately improve plant growth and yields of legumes. They can solubilize sparingly soluble organic and inorganic phosphates, and they can colonize the roots of many non-legume plants (Antoun*et al*., 1998). Rhizobia have a good potential to be used as biological control agents against some plant pathogens.The present investigation was undertaken to isolate and screen rhizobium based on their plant growth promoting activities, from root nodules of pigeon pea plants.

2. Material and Methods

2.1. Isolation of Root Nodule Bacteria / Rhizobium

Root nodules were collected from the roots of *Cajanus cajan* (Pigeon pea) from Karimganj district of Assam, India. Nodules present on the root were fresh, healthy and spherical of about 2-3mm size. The root samples were protected well in polythene bags and brought to the laboratory. The collected nodules were first washed with normal tap water to remove soil and then surface sterilized by dipping in 95% ethanol solution and then in 0.1% $HgCl_2$ solution for about 5 minutes. The nodules were then rinsed in sterile distilled water several times in order to remove the chemicals (Anjum *et al*., 2011). The surface sterilized nodules were crushed with sterile glass rods in a minimal volume of distilled water so as to obtain a milky suspension. A loopful of this suspension was then streaked on yeast mannitol agar plates and kept for incubation at 28^0C for 24 hrs.

2.2. Identification of Bacteria

Well isolated and distinct colonies of shiny appearance were picked up and streaked freshly to obtain pure cultures and incubated at 37^0C. The pure cultural strains were preserved on slants at 4^0C and maintained for later experimentation. The bacterial isolates

were identified on the basis of morphological and biochemical characteristics on YEMA by the standard protocol given by (Cappuccino and Sherman, 2005) and (Dubey and Maheshwari, 2012).

2.3. Morphological and Biochemical Characterization

Morphological characteristics of all isolates viz., Colony morphology (colour, shape, margin, elevation and surface) and cell morphology (size, shape and arrangement) were studied.

All the collected samples were processed through different biochemical tests viz, Catalase Test, Indole Production Test, Methyl Red Test, Voges Proskauer Test, Citrate Utilization Test, Starch hydrolysis Test, Urease test, Nitrate reduction test, Acid and H_2S production as described by (Cappuccino and Sherman, 2005).

2.4. Effect of Salinity on Bacterial Growth

Tolerance of bacterial strains to NaCl was evaluated on YEM agar medium supplemented with increasing NaCl concentrations of 0.5%, 2%, 5%, 10% and 20% and one kept as a control. The YEM agar media were poured in petriplates, bacterial strains were streaked on each plate and incubated at 37°C for 24 hrs (Dubey and Maheshwari, 2012).The influence of NaCl concentrations on degree of inhibition of bacterial growth was recorded.

2.5. Plant Growth Promoting Traits

2.5.1. IAA Production

Qualitative (Brick *et al*., 1991) and quantitative (Patten and Glick, 1996) analyses of indole acetic acid (IAA) were carried out. Development of pink colouration indicates positive result. Absorbance of supernatant mixture was measured at 535 nm and quantified using tryptophan standard.

2.5.2. Siderophore production

Isolates were qualitatively detected (Schwyn and Neilands, 1987) using chrome azurol S (CAS). After 24 hr of incubation, colonies were observed for orange halo formation.

2.5.3. HCN Production

Isolates were screened for hydrogen cyanide (Lorck, 1948) synthesis. Nutrient broth was amended with glycine (4.4 g/l). After incubation, tubes were observed for the development of orange to red color.

2.5.4. Ammonia Production

Bacterial isolates were tested for ammonia synthesis in peptone water as per (Joseph *et al*., 2007). After incubation, tubes were observed for the development of brown yellow color.

2.5.5. Phosphate Solubilization

All isolates were first screened on Pikovskaya's agar plates for phosphate solubilization as described by (Gaur, 1990).

3. Results and Discussion

3.1. Isolation and Identification of Bacteria

A total of five root nodule bacteria from roots of *Cajanus cajan* L var. Manak were isolated on the basis of cultural, morphological and biochemical characteristics as described in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). Total viable counts ranges from 5 X 10^4 (CFU/g) to 17 X 10^4 (CFU/g). It was observed that all the bacterial colonies were gram negative rod, creamish in colour and slimy nature. The biochemical characteristics of the bacterial isolates are illustrated in Table 1.

Bacterial Isolates	Gram Staining	Shapes	Indole	MR	VP	Catalase	Starch	Citrate	Oxidase	H_2S produ--ction	Suspect Organism
KRN1	-ve	rod	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Rhizobium*
KRN2	-ve	rod	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Rhizobium*
KRN3	-ve	cocci	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Rhizobium*
KRN4	-ve	rod	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Rhizobium*
KRN5	-ve	rod	-ve	+ve	+ve	+ve	+ve	-ve	+ve	+ve	*Rhizobium*

Table 1: Biochemical characteristics of the bacterial isolates
Legend: +ve -- Positive; -ve -- Negative

3.2. Effect of Salinity on Bacterial Growth

The salt concentration in an environment is the major contributor to the osmotic effect of ions on growth. Bacteria require ions that are provided by salts and typically moderate salt concentrations. In the present study, all isolates showed 2% NaCl tolerance as compared to the control, while only two strains (KRN1, KRN5) showed tolerance at 10% NaCl concentration. High level of NaCl (20%) repressed bacterial growth. Results are tabulated in Table 2.

Isolates	Control	0.5% NaCl	2% NaCl	5% NaCl	10% NaCl	20% NaCl
KRN1	+++	+++	+++	+++	++	-
KRN2	+++	+++	+++	++	-	-
KRN3	+++	+++	+++	-	-	-
KRN4	+++	+++	++	-	-	-
KRN5	+++	+++	+++	+++	++	-

Table 2: Salt Tolerance of bacterial isolates

Legend: +++ = Excellent growth; ++ = Moderate growth; + = Less growth; - = No growth

3.3. Plant Growth Promoting (PGP) Traits of the Test Isolates

The bacterial isolates were screened for multiple plant growth promoting activities which are listed in the table 3. Among the five isolates, three isolates (KRN1, KRN2 and KRN3) responded positively for siderophore (Figure1). Except KRN1, none showed ammonia production. KRN1 is best among others as it showed positive result for all the attributes. IAA production was highest by KRN5 (Figure 2).

Isolates	IAA Production		Siderophore Production		Ammonia Production	HCN Production	Phosphate Solubilization	
	Pink Colouration	Conc. At 535 nm	CAS assay (halo formation in blue agar)	Halo size (cm)			Halo formation	Diameter of Halo zone (cm)
KRN1	+	0.101	+	0.2	+	+	+	0.9
KRN2	+	0.134	+	1.00	-	+	+	0.7
KRN3	+	0.135	+	0.7	-	+	+	0.3
KRN4	+	0.171	-	-	-	+	+	0.4
KRN5	+	0.186	-	-	-	+	+	0.8

Table 3: Assessment of multiple PGP traits of the bacterial isolates

Legend: +ve -- Positive; -ve – Negative

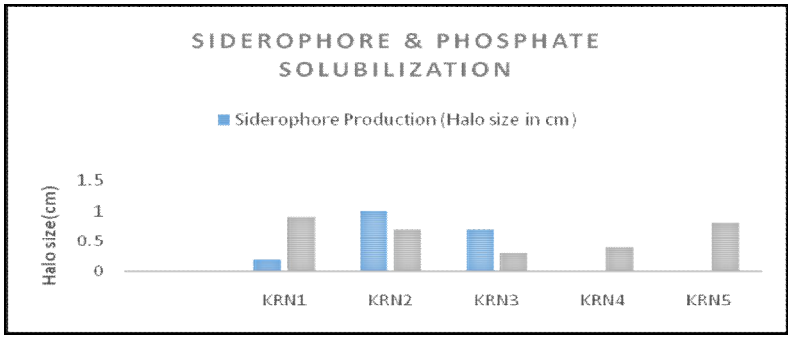

Figure 1 shows the diameter of halozone size (cm) of the isolates KRN1, KRN2, KRN3, KRN4 and KRN5 while producing siderophore and solubilizing phosphate. The highest siderophore production was shown by KRN2 of halo size measuring 1.0 cm.

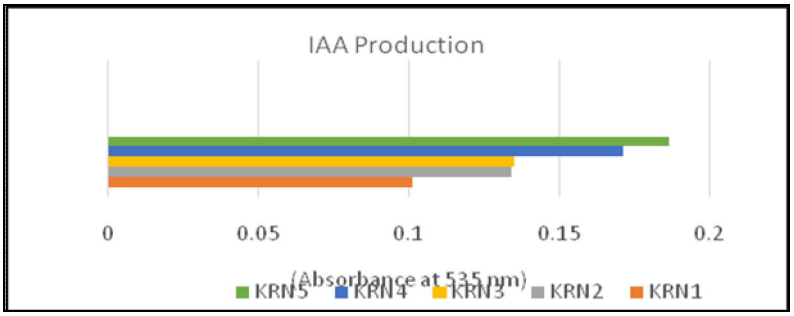

Figure 2 shows the IAA production of the 5 isolated strains and their absorbance at 535 nm. Absorbance ranged from 0 – 0.2, and the highest was of KRN5, 0.186

4. Discussion

Beneficial bacteria were isolated, in the present study from *Cajanus cajan* rhizosphere. The test were screened for plant growth promoting traits. IAA, i.e. indole-3-acetic acid is considered to be the best categorized auxin found in plants. IAA is known to enhance cell elongation, cell division and differentiation in plants (Singh*et al*., 2013). All *Rhizobium* isolates were able to produce IAA in this analysis. Mature root nodules of *Phaseolus mungo* produced higher amount of indole acetic acid compared to non-nodulated roots (Ghosh and Basu, 2006). High amounts of IAA (99.7 µg/ ml) were produced by *Rhizobium* isolates from root

nodules of *Cajanus cajan* in L-tryptophan supplemented basal medium (Datta and Basu, 2000). Besides IAA production, microorganisms also enhance plant growth by scavenging available iron (Fe^{3+}), which involves secretion of high affinity, low molecular weight iron chelating ligands called siderophores (Anitha and Kumudini, 2014). In this investigation, only 60% isolates were able to form halo zone CAS agar symbolizing siderophore production. Hydrogen cyanide (HCN) synthesized by some rhizobacteria inhibits diseases in plant and thereby increasing the biocontrol mechanism (Schippers, 1990). In the present study, all bacterial isolates were able to grow upto 5% NaCl, five isolates shows the in vitro efficiency to grow in 10% NaCl concentration. However, at 20% NaCl concentration, no growth was observed by any isolated strains even after 48 hrs of incubation.In the study of Damodaran *et al*., (2013), 5 out of 16 bacteria showed tolerance to high salt concentration (7.5 % NaCl) and among them two isolates, *B. pumilus* and *B. subtilis* had higher uptake of sodium when cultured under in-vitro conditions in 1 M NaCl solution. Upadhyay *et al*., 2009, has also furnished that bacteria isolated from saline soil are more likely to overcome saline conditions. In our study, all the 5 strains tested were positive for hydrogen cyanide (HCN) production. Production of ammonia also indirectly influences the plant growth which signifies an attribute of PGPR. Only 20% were positive for ammonia production. Nadeem *et al*., 2009 reported that Rhizobacteria containing ACC-deaminase deliberated salt tolerance in maize grown on salt-affected fields whereas in our study KRN1 solubilising phosphate and KRN5 producing high amount of IAA among others also were salt tolerant upto 10% NaCl. Use of chemical fertilizers or pesticides for high production of crops so as to fulfill the demands for food primed to environmental damage which directly or indirectly is hazardous for all organisms residing in earth. Growing awareness of this environmental damage has motivated the study of biological alternatives. The use of bio-fertilizer in preferences to chemical fertilizer is always welcome taking into consideration the suitability of agriculture. It is advantageous always in terms of soil fertility, ecological health etc. In India, the use of bio-fertilizer is clearly insufficient so far, therefore with more stress the production, consumption and also appropriate distribution are to be considered (Sayyed andPatel, 2011).For effective outcome of inoculation of microbes in agricultural soil of host plant, their assets must be suitably arbitrated as it is necessary to prevent environmental threats which may be associated while supplementing with the introduction of native and non-native microorgaisms as well as a design of the most appropiate conditions (Samuel and Muthukkaruppan, 2011).

5. Conclusion

Thus, from the present study it can be concluded that the application of beneficial microbes devouring plant growth promoting traits will reduce the use of such chemical fertilizers to some extent thereby remediating the crop soil.Further, efficiency of these isolates are needed to be measured by pot trials for sustainable use of these microorganism in agricultural purpose.

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