RESEARCH PAPER

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Selection of effective indigenous *Rhizobium* strains in district Sagar for chickpea bioinoculant

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An attempt has been made to isolate and characterize nitrogen fixing bacteria, *Rhizobium*, from the root nodules of chick pea collected from different fields of the district Sagar, (M.P.). A total of 26 isolates were obtained. The isolated strains were identified on the basis of colonial and morphological characteristics on Yeast mannitol agar medium by Congo red test. Out of 26 isolates, 14 were confirmed to be *Rhizobium* on the basis of the results of Gram staining, carbol fuchsin staining for the presence of Poly β hydroxybutyrate granules, motility test and lactose agar test. The isolates were further characterized biochemically by performing indole, methyl red, vogues proskauer, citrate utilization, nitrate reduction test. The isolates were also tested for its phosphate solubilising efficiency, capability to tolerate variability in salt concentration, pH and presence of amylase and catalase enzymes. Out of 14, 5 isolates showed very good phosphate solubilising zones upto 15 mm on one day incubation and 2 isolates namely CP3 and CP13 also showed siderophore production and thus were found to be the best strains which could be further characterized for their use as bioinoculant.

Key words : *Rhizobium*, Chickpea, Bioinoculant

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INTRODUCTION

Nitrogen is required by all living organism for the synthesis of proteins, nucleic acids and other nitrogen- containing compounds. *Rhizobium* is soil-inhabiting bacteria with the potential of forming specific root structures called nodules. In effective nodules the bacteria fix nitrogen gas from the atmosphere into ammonia (O'Gara and Shanmugam, 1976), which is assimilated by the plants and supports growth, particularly in nutrient deficient soils. Chickpea is one of the most important grain legumes traditionally cultivated in deprived areas and saline soils (Rao *et al*., 2002). The agronomical importance of chickpea (*Cicer arietinum* L.) is based on its high protein concentration 25.3- 28.9% (Hulse, 1991) for the human and animal diet being used more and more as an alternative protein source. Chickpea can obtain a significant proportion of its N requirement through symbiotic nitrogen fixation when grown in association with effective and compatible *Rhizobium* strains. However, indigenous population of *Rhizobium* are present in soil, they cannot establish an effective association, hence, inoculation is essential to ensure that a large and effective rhizobial population is available in the rhizosphere of the plant (Hynes *et al*., 1995).

The isolation of superior *Rhizobium* is very important because the effective rhizobial strains are used as inoculants to ensure the effective nodulation (Moxley *et al*., 1986). The competitive ability of a inoculant strain is a major factor determining the success of rhizobial inoculation.

Rhizobia like other growth promoting rhizobacteria (PGPR), stimulate the plant growth and are also able to solubilize both organic (Abd-Alla, 1994) and inorganic phosphates (Antoun *et al.*, 1998). The main advantage of rhizobia, as PSM will be their dual beneficial nutritional effect resulting both from phosphate mobilization and nitrogen fixation (Peix *et al.,* 2001).

The present study was therefore, undertaken for isolation and characterization of *Rhizobium* from the root nodules of chickpea, to study their efficiency and to bring up an efficient strain which can be used as bioinoculant for chickpea.

RESEARCH METHODOLOGY

Isolation of *Rhizobium* from chickpea:

Collection of crop plants:

Different samples of chickpea plants have been collected from different localities of district Sagar (M.P.) and were brought to the Microbiology Laboratory.

Method of Isolation:

The process of isolation of *Rhizobium* from root nodules of chick pea includes many steps which are described in the following text.

Surface sterilization of nodules:

The root nodules were surface sterilized with the help of 3% hydrogen peroxide. The nodules were then placed in 70% ethyl alcohol for 2-3 minutes and then again washed with distilled water 2-3 times. Serial dilution of crushed nodules was prepared and this suspension was then used for the isolation of bacteria using YEMA medium having Congo red.

Isolation of bacteria:

The isolation of *Rhizobium* was carried out by serial dilution of the nodule suspension followed by a streak plate method (Waksman, 1927; Vincent, 1970) on Yeast mannitol agar medium. The colonies with typical *Rhizobium* growth (small, white, translucent, glistening) were picked up on a separate medium for further examination.

Purification:

Purification of the colonies was done by adopting a streak plate technique in which a particular colony will be picked up from the streaked plates showing bacterial colonies, using a loop and then streaked onto a plate containing YEMA with congo red (Waksman, 1927). These plates were then incubated at 37^0C to obtain the pure culture or specific colonies of *Rhizobium*.

Morphological and biochemical characterization:

Morphological characterization:

Gram's staining:

The Gram stain procedure is the most widely used differential staining procedure in bacteriology. The stained slide was then examined under the oil immersion objective to determine whether the organism is Gram positive or Gram negative.

Carbol fuchsin staining:

The smear of bacteria, was flooded with dilute carbol fuchsin stain for 10-20 seconds. The slide was then washed and then examined under microscope for the presence or absence of poly-β-hydroxy butyrate granules.

Biochemical characterization:

Lactose – agar test:

This test was used to differentiate *Agrobacterium* from *Rhizobium* (Agrawal and Jain, 2008).

IMViC test:

Indole, methyl red, vogues proskauer and citrate utilization test was performed on all the isolates biochemically.

Nitrate reduction test :

For this test organisms were grown in nitrite broth The reduction of nitrate to nitrite was be detected by adding 0.5 ml 1% sulphanilic acid in 5 N acetic acid followed by 0.5 ml of 0.6% dimethyl-β-naphthylamine in 5 N acetic acid. The development of a red colour indicated a positive reaction.

Phosphate solubilisation activity (Pikovskaya et al., 1948):

This activity shows the efficiency of bacteria to solubilize phosphate. For this test organism was grown in Pikovskaya's medium. The development of clear zone at inoculation site on the culture plates indicate that phosphate has been solubilized.

Test of pH requirement:

In current investigation bacteria was grown on YEMA medium at varying pH of 4 and 10.

Test for salt tolerance:

For the test, YEMA medium with varying salt concentration as 3%, 4.5% and 5% were streaked with bacterial culture to observe the growth.

Amylase activity (Aneja, 2003):

For testing the amylase activity of the isolates, Nutrient agar medium was prepared supplemented with 0.2% starch and inoculated with bacteria and incubated. Gram's iodine solution was flooded over the plates and observed for the clear zone.

Catalase activity (Aneja, 2003):

For Catalase activity a loopful of culture of bacteria

was reacted with the 3% H_2O_2 which was used as substrate. Formation of bubbles due to release of oxygen indicated positive Catalase activity.

RESULTS AND ANALYSIS

For the isolation of *Rhizobium*, chickpea samples were collected from different fields of the district Sagar (M.P.) as given in Table 1. A total of 26 isolates were obtained from 12 different chickpea samples collected from 9 different fields and isolated by using Yeast mannitol agar medium with Congo red as differential reagent. The isolates obtained formed gummy and sticky colonies due to exopolysaccharide secretion. The colonies were white in colour and elevation found to be convex also called as pelvinate colonies.

Table 1: Isolation of *Rhizobium* from different chickpea samples collected from different fields of district Sagar (M.P.)

Sr. No.	Location	No. of fields	No. of samples	No. of isolates obtained
1.	Jaat Pathariya	4	7	13
2.	Makronia	2	3	8
3.	Bhopal road	2	2	5
	Total	9	12	26

Characterization and identification of microorganisms traditionally relies on the phenotypic and biochemical characteristics that are observed by growing them on some selective and differential media. The pattern of physiological and metabolic characteristics distinguishes one microbial species from another, forming basis for identification. In the present investigation, an attempt has been made to characterize the isolated *Rhizobium* strains for their morphological and biochemical characteristics.

The isolates were characterized primarily on the basis of Gram staining techniques and Carbol fuchsin staining for the presence of poly β hydroxybutyrate granules inside the cells and motility was observed using YEMA medium with 1% agar.

Out of 26 isolates 18 were found to be Gram negative and only 14 of them were found to be perusing PHB granules. The 14 isolates were further characterized in the basis of production of 3- ketolactose on lactose agar medium. Thus, all the 14 isolates were confirmed to be as *Rhizobium*. The results of biochemical characteristics are given in Table 2.

In the IMViC test (Indole, Methyl red, Vogues Proskauer, Citrate utilization) (Aneja, 2003), all the 14 isolates were found to be negative. For the nitrate reduction test all the 14 isolates were able to reduce nitrate to nitrite.

The ability of *Rhizobium* to solubilize inorganic phosphate was tested on Pikovskaya's media, for which different isolates showed variability in their capability to solubilize tricalcium phosphate present in the media, the halo zones of different radius were obtained. Agrawal and Jain (2009) had also reported the same. A major portion of phosphorus is fixed as insoluble forms soon after its application and becomes unavailable to plants (Rodriguez and Fraga, 1999). The work done by Abd-Alla (1994) and Antoun *et al.* (1998) showed that rhizobia are able to solubilize both organic and inorganic phosphates. The solubilization of inorganic phosphorus by phosphate solubilising microorganisms is believed to be due to the production of organic acids (Whitelaw *et al.,* 1999; Maliha

Table 2 : Biochemical characterization of the isolates isolated from the chickpea samples

Sr. No.	Strain No.	Identified as	Gram staining	Presence of PHB granules	Motility	IMViC test	Nitrate reduction	Phosphate solubilisation (mm)
1.	CP 1	*Rhizobium*	-	s	+	-	+	10
2.	CP 2	*Rhizobium*	-	+	+	-	+	No zone
3.	CP 3	*Rhizobium*	-	+	+	-	+	13
4.	CP 4	*Rhizobium*	-	+	+	-	+	5
5.	CP 5	*Rhizobium*	-	+	+	-	+	5
6.	CP 6	*Rhizobium*	-	+	+	-	+	10
7.	CP 7	*Rhizobium*	-	+	+	-	+	3
8.	CP 8	*Rhizobium*	-	+	+	-	+	12
9.	CP 9	*Rhizobium*	-	+	+	-	+	5
10.	CP 10	*Rhizobium*	-	+	+	-	+	2
11.	CP 11	*Rhizobium*	-	+	+	-	+	3
12.	CP 12	*Rhizobium*	-	+	+	-	+	3
13.	CP 13	*Rhizobium*	-	+	+	-	+	15
14.	CP 14	*Rhizobium*	-	+		-	+	8

Table 3: Physiological characterization of selected strains based on phosphate solubiling efficiency

Sr. No.	Strain no.	Identified as	Growth at					Amylase activity	Catalase activity
			pH		NaCl Concentration				
			4	10	3%	4.5%	5%		
1.	CP 01	*Rhizobium*	+++	++++	++++	++	+	_	++
2.	CP 03	*Rhizobium*	+++	++++	++++	++	++	+	+
3.	CP 06	*Rhizobium*	+++	++++	++++	+++	+	_	++
4.	CP 08	*Rhizobium*	++	++++	++++	++	+	_	++
5.	CP 13	*Rhizobium*	+++	++++	++++	+++	++	+	++

Note - Growth patterns

++++ - Excellent growth +++ - Good growth

++ - Fair growth + - Poor growth

et al., 2004) or the formation of soluble complexes with metal ions associated with insoluble phosphate (Omar, 1998).

Out of 14 isolates, 5 best phosphate solubilizing bacteria were selected and further studied for pH and salt tolerance, catalase and amylase activity.

The chickpea nodulating rhizobia tested showed a wide diversity in their pH tolerance (Table 3). Most of the isolates grew well in acidic as well as alkaline conditions of pH 4 and pH 10. Thus, they showed an acido-tolerant character since they grew without restriction at pH 4. But some isolates showed very poor growth at pH 4, all the isolates showed excellent growth at pH 10.

Chickpea rhizobia exhibited a wide diversity in their salt tolerance. The salt inhibitory concentrations varied among strains. Tolerance of sodium chloride (NaCl) was found since all the isolates continued to grow with 3% NaCl. However, at higher concentrations, the per cent of tolerant strains decreased rapidly and only 2 of the 5 isolates showed moderate growth at 4.5% and 5% NaCl concentration.

Under iron limiting conditions, many bacteria secrete ferric iron- specific ligands, generally termed siderophores, to aid in the sequestering and transport of iron. Two isolates, CP3 and CP13 were detected for the production of siderophore in the CAS assay.

For phosphate solubilization, pH and salinity, two out of five strains were able to grow on different pH and salt concentrations and able to effectively solubilize phosphate and thus could be good candidate for chickpea inoculation.

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