



MORPHOLOGICAL, BIOCHEMICAL AND PLANT GROWTH PROMOTING CHARACTERIZATION OF *RHIZOBIA* ISOLATED FROM ROOT NODULE OF *CAJANUS CAJAN* L.

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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 8 bacterial strains were isolated from the root nodules of *Cajanus cajan* on selective Yeast Extract Mannitol Agar (YEMA) medium (pH 7) at 28°C. All the isolated bacterial strains were subjected to morphological, biochemical characterization; confirmatory tests; screened for multiple plant growth promoting traits and antagonistic activities against *Rhizoctonia solani* were evaluated using dual culture technique. Under microscopic examination, all the bacterial strains appeared as gram negative rod shaped. On the basis of morphological properties most of the isolates were round, mucilaginous, white with raised elevation and smooth surface indicating rhizobia. All the isolates except ISSA-1 were identified as rhizobia on the basis of confirmatory test. Out of 8 rhizobial isolates, 6 isolates showed amplification with nifH primers indicating the presence of N₂ fixing genes in these bacteria. All the eight strains were positive for biochemical tests such as catalase test, oxidase test, nitrate reduction test and urease test. In this study, 63% of the isolates showed IAA production, 38% rhizobial isolates showed phosphate solubilization and 100% for ammonia, 50% for siderophores and 75% isolates showed for HCN production. Out of eight isolates, only 2 isolates exhibited inhibition potential against two soil borne plant phytopathogen viz., *Rhizoctonia solani* and *Sclerotium rolfsii* under in vitro conditions.

Keywords: *Cajanus cajan*, Nodules, Rhizobium, nifH, PGPR.

Introduction

Nitrogen is an essential element for the functioning of all the living organisms. Though, nitrogen is available in environment at an approximate level of 78%, but this form is not accessible to plants. It is worth noting that nitrogen is also one of most potent plant –growth limiting nutrients (Greenwood, 1982). Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers. The extensive use of chemical fertilizers to get high yield is not only expensive but also a threat to the environmental balance and contributes to global warming (Vitousek, 1997). Biological nitrogen fixation (BNF) is the cheapest, environment friendly and fulfills plants' requirements of nitrogen by converting atmospheric nitrogen into usable form. Rhizobial strains are able to form symbiosis with leguminous plants and to convert atmospheric N₂ into ammonium (NH₄) in the process of nitrogen fixation, which takes place in special plant organs called root or stem nodules (Franche *et al.*, 2009). The Rhizobia-legume symbiosis benefits not only the host crop but also the subsequent crops in that field. Pigeon pea (*Cajanus cajan*) 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*). Rhizobacteria that benefit plant growth by producing plant growth regulators, enhancing the nutrient(s) availability, inducing root exudation and controlling phytopathogens are termed as PGP bacteria (Kloepper and Schroth, 1980). PGP bacteria actively colonize plant roots and increase plant growth and yield. In addition to symbiotic

nitrogen fixation, rhizobia are important members of plant-growth-promoting rhizobacteria (PGPR) that exert the positive effects on plant growth via direct and indirect mechanisms. Plant growth promoting activities such as, production of phytohormones (Zahir *et al.*, 2010), siderophore production (Meyer, 2000) and increased availability of insoluble phosphorus (Fatima *et al.*, 2006; Pandey & Maheshwari, 2007) have been proposed by which Rhizobia can stimulate the growth of non-legumes directly and indirectly via suppressing or eliminating deleterious microbes by producing antibiotics (Antoun & Prevost, 2000) HCN (Antoun *et al.*, 1978). Rhizobia have a good potential to be used as biofertilizers as well as biological control agents against plant pathogens. In last few decades, there has been a growing level of interest in environmental friendly sustainable agricultural practices, thus increasing the role of biofertilizers such as Rhizobia which can decrease the need for chemical fertilizers and reduce adverse environmental effects. Keeping in view the importance of Rhizobia in legume plants as well as in non-legume plants, the present study was undertaken to shed some light on different morphological and biochemical properties of Rhizobial strain and to screen some efficient rhizobial strains based on their plant growth promoting activities, from the root nodules of pigeon pea plants.

Materials and Methods

Sample collection and isolation of root nodule bacteria

The *Rhizobium* strains were isolated from the root nodules of *Cajanus cajan* (Pigeon pea) collected from the agricultural field's sites of Mahabubnagar Telangana India. The plants were uprooted and loosely adhering soil was removed by gentle shaking. The mature nodules along with

roots were washed in running water until the removal of adhering soil particles. Large sized healthy, undamaged and pinkish nodules were selected for isolation. The surface sterilization was done with 70% ethanol for 30 sec, to break surface tension and to remove air bubbles from nodule tissues, followed by 30% hydrogen peroxide (H₂O₂) for 2 min and then rinsed thoroughly with sterile distilled water (six times) in order to remove the chemicals. Each surface sterilized nodule from different legumes was aseptically crushed with a sterile glass rod in a test tube containing 1 ml distilled water with 0.5 % NaCl. One loopful of the nodule suspension was streaked on petri plates containing yeast extract mannitol agar (YEMA) (Vincent, 1970) containing 0.0025% (w/v) Congo red as an indicator and incubated at 28°C for 4 days. At the incubation period ending, the rhizobial colonies appeared white, translucent and elevated. They were picked out using a sterile inoculating loop and were further purified by streak plate method. The most prominent isolates were maintained on YEMA slants at 4 °C in refrigerator for further studies.

Morphological characterization

The colony morphology of the bacteria (*rhizobium*) isolated from the root nodules was examined on YEM agar plates after incubation of 72 hours at 28°C, individual colony was characterized on the basis of colony- form, margin, elevation, colour, mucosity optical density (Aneja, 2003) and Gram stain reaction as per the method described by (Somasegaran and Hoben, 1994).

Confirmatory Tests of Rhizobium

Five different confirmatory tests (Growth on YEMA with Congo red, Growth on Glucose- peptone agar, Keto-lactose Test, Hoffer's alkaline Test and Bromothymol Blue Test) were performed to confirm the isolates as Rhizobia and to differentiate them from other contaminating microbes. *Growth on YEMA with Congo red:* The purity of the rhizobial isolates was detected by adding Congo red in YMA media .In general, Rhizobia absorb the dye weakly and produce white colonies, whereas many other bacteria including Agrobacteria, take up the dye strongly (Somasegaran *et al.*, 1994). *Growth on Glucose- peptone agar:* Glucose peptone agar medium containing Bromocresol purple indicator dye (glucose 40 g/L, peptone 5 g/L, agar 15 g/L, Bromocresol purple 100 mg/L pH 7.0) is used to differentiate rhizobia, which usually shows no growth or very poor growth on the media without altering the pH of the media, contaminants like Agrobacteria, shows massive growth on the media with a distinct change in pH (Singh *et al.*, 2008). *Keto-lactose Test:* Keto-lactose test widely used to differentiate Rhizobia from other contaminating bacteria (Bernaert and Daley, 1963). Keto-lactose agar medium (Lactose 10 g/L, Yeast extract 1 g/L, Agar 15 g/L, pH adjusted to 6.8) was poured into the sterile petri dishes and allowed to solidify. The isolates were streaked on the Keto-lactose agar medium and incubated for 2-3 days. The plates were flooded with Benedict's reagent and kept at room temperature for 1-2 hours. The development of bright yellow colour around the rhizobium colonies confirms the presence of *Agrobacterium*. *Hoffer's alkaline Test:* This test is based on the fact that *Agrobacterium* grows at higher pH level whereas *Rhizobium* unable to do so. A medium i.e. Hoffer's alkaline having high pH of 11.0 was used to screen isolated nodulated bacteria for this purpose. Bacterial suspension was

inoculated in broth and incubated for 24-48 hours at 28±2°C (Hofer, 1935). *Bromothymol Blue Test:* The YEMA medium incorporated with bromothymol blue was streaked with isolated strains, incubated at 28°±2°C for 3-4 days and was observed either for yellow colour due to production of acids or blue colour due to production of alkali (Norris, 1965).

Nitrogen fixing ability of the isolated bacteria

Nitrogen fixation is carried out by the nitrogenase enzyme whose multiple subunits are encoded by the genes *nifH*, *nifD*, and *nifK*. Of the three, *nifH* (encoding the nitrogenase reductase subunit) is the most widely sequenced marker gene used to identify nitrogen-fixing bacteria and archaea. For this, the pure cultures of the isolated diazotrophic bacteria were grown in yeast extract mannitol medium up to log phase and genomic DNA was isolated by using GSure Bacterial Genomic DNA Isolation Kit according to manufacturer's (GCC Biotech). One hundred nanograms of genomic DNA of the diazotrophic bacteria was used as template in PCR for the amplification of *nifH* gene using primers: *polF* (5'-TGC GAY CCS AAR GCB GAC TC-3') and *polR* (5'-ATS GCC ATC ATY TCR CCG GA -3') (Poly *et al.*, 2001). The PCR reaction mixture and conditions were followed as per the protocols mentioned by poly *et al.* (2000).

Biochemical characterization of Rhizobium

The isolates were also investigated for different biochemical characteristics namely catalase test, oxidase test, starch hydrolysis test, citrate utilization test, nitrate reduction test, urease test, and gelatin liquefaction test following standard procedure (Somasegaran and Hoben, 1994; Aneja, 2003; Cappuccino and Sherman, 2005).

In vitro Screening of Multiple Plant Growth Promoting Activities of Rhizobium spp.

The isolated bacteria were characterized for their PGP traits including indole acetic acid (IAA), siderophore production, hydrocyanic acid (HCN), ammonia production, phosphate solubilization (TCP) under *in vitro* conditions. The isolated bacteria were assayed qualitatively for indole acetic acid production by spot inoculating on nutrient agar medium amended with 5 mM L- tryptophan and after incubation for 24-48 h; the inoculated points were overlaid with 10mm-diameter nitrocellulose membrane (NCM) disk. After incubation, the NCM saturated with few drops of Salkowski reagent (1mL 0.5M FeCl₃, 50mL H₂SO₄) (Gordon and Weber, 1950). After two minutes, appearance of pink color was observed which was indicator of IAA production (Myron and Williams, 1989). Siderophore production of the isolates was carried out by spot inoculating test organism (5 µL inoculum, 1×10⁸ CFU mL⁻¹) on chrome Azurol S agar plates and incubated at 30 °C for 2-3 days in dark. Appearance of yellow to orange halo around the bacterial colonies was considered as positive for siderophore production (Schwyn and Neiland, 1987). Phosphate solubilizing ability was carried out by spot inoculating the isolated bacterial cultures on NBRIP media (Glucose 10g, Ca₃ (PO₄)₂ 5g, MgCl·6H₂O 5g, KCl 0.2g, (NH₄)₂SO₄ 0.1g, MgSO₄·7H₂O 0.25 g, Agar 15g, distilled water 1lt). The formation of halo zones around the colonies was considered as positive for phosphate solubilization (Mehta and Nautiyal, 2001). Bacterial isolates were tested for the production of ammonia in peptone water broth as per (Joseph *et al.*, 2007).

Peptone broth tubes were inoculated with freshly grown cultures and incubated for 4 days at 30 °C and 120 rpm in an incubator shaker. After incubation, few drops of Nessler's reagent were added to each tube. Development of deep yellow to brown color is a positive test for ammonia. For qualitative estimation of HCN, all the isolates were streaked on nutrient agar plate supplemented with 4.4 % glycine. A whatman filter paper no. 1 soaked in a solution of 2% Na₂CO₃ in 0.5% picric acid was placed between base and lid of petri plate and incubated at 28 ± 2 °C in inverted position for 96 h and observed for color change from yellow to orange brown as described by Bakker and Schipper (1987).

In vitro* antagonistic activity against *Rhizoctonia solani* and *Sclerotium rolfsii

Antagonistic activity against *Rhizoctonia solani* and *Sclerotium rolfsii* was detected by the dual culture technique method. Soil borne plant pathogenic fungi *Rhizoctonia solani* and *Sclerotium rolfsii* was grown on potato dextrose agar (PDA) media. A 5 mm diameter plug of fungal mycelium was cut from an actively growing fungal culture and placed on the center of the Petri plate containing potato dextrose agar. A loopful of exponentially grown culture of each isolates were streaked in a straight line on one edge of a 90 mm diameter petri plate and the distance between the fungus and the test culture was kept at 2 cm, and the plates were incubated at 28°C for 4-7 days (Rabindran *et al.*, 1996). Inhibition radial growth of test fungus was observed daily. Culture plates with the test fungus served as control. In each case three (3) replicates were taken. The diameters of the colonies were measured after five days and average values compared with control were taken as a measure of fungitoxicity. Growth inhibition (%) of test fungus was

determined by using the formula quoted by Pani and Patra (1997).

$$\text{Growth Inhibition Percentage} = \frac{\text{Control} - \text{Test}}{\text{Control}} \times 100$$

Results and Discussion

Isolation and Identification of Bacteria

A total of eight rhizobacterial strains were successfully isolated from the root nodules of *Cajanus cajan* L. (Pigeon pea). Rhizobium isolated from the root nodules showed a well-marked growth on YEMA medium at pH 7.0 after incubation for 48-72 h at 28°C. It was observed that most of the isolates showed similar colony morphology and produce white or creamy white, raised or convex, round, opaque, some translucent and gummy colonies when grown on YEMA plates. Microscopic examination revealed that the isolates were gram negative and rod in shape except (ISSA-1). The results of the morphological characteristics of the bacterial isolates are represented in Table 1 and Fig 1A, 1B. The present study revealed that that morphological and microscopic features of most of the isolates are very much similar with the *Rhizobium* spp. Similar results were also reported by various workers (Deora and Singhal, 2010; Gauri *et al.*, 2011; Deshwal and Chaubey, 2014; Gachande and Khansole, 2011). The isolates ISSA-1 produce red color colonies on YEMA media, rhizobia usually produces white or creamy white colonies on YEMA media, and microscopic examination revealed that the isolate is gram positive and rod in shape, So the isolates ISSA-1 may not be considered as a probable rhizobial isolate.

Table 1: Morphological Characterization of root nodule bacteria grown on YEMA at 28°C.

Bacterial Isolates	Colony Form	Colony Margin	Colony Elevation	Colony Colour	Colony Texture	Optical density	Cell shape	Gram staining	Suspect Organism
ISSA-1	circular	entire	flat	red	smooth	opaque	Rod	+ve	Bacillus
ISSA-2	circular	entire	raised	creamy	mucoid	translucent	Rod	-ve	Rhizobium
ISSA-3	circular	entire	convex	creamy	mucoid	translucent	Rod	-ve	Rhizobium
ISSA-4	circular	entire	raised	Milky white	mucoid	opaque	Rod	-ve	Rhizobium
ISSA-5	circular	entire	raised	Milky white	mucoid	opaque	Rod	-ve	Rhizobium
ISSA-6	circular	entire	raised	Milky white	mucoid	opaque	Rod	-ve	Rhizobium
ISSA-7	circular	entire	convex	creamy	mucoid	opaque	Rod	-ve	Rhizobium
ISSA-8	circular	entire	convex	creamy	mucoid	translucent	Rod	-ve	Rhizobium

Confirmatory Tests of Rhizobium

For confirming the isolates as rhizobia, all the 8 rhizobial spp. were screened for different confirmatory tests viz. congo red test, bromothymol blue test, growth in Hofer's alkaline broth, ketolactose medium and growth on glucose peptone agar (GPA) medium (Table 2). All the isolates did not absorb the Congo red color when streaked on YEMA-CR media except (ISSA-1), and such nature differentiates Rhizobium from Agrobacterium and other bacterial contaminants according to Deshwal and Chaubey, 2014; Trinick *et al.*, 1982. In GPA test 7 isolates showed no growth on GPA media except (ISSA-1) indicating the features of rhizobia. Regarding the growth in glucose peptone agar Vincent *et al.* reported that rhizobia showed either no growth or grow very poorly on GPA media. All the Rhizobium

strains showed the negative results on Hofer's alkaline medium except (ISSA-1), showed mild growth in this medium. Normally Rhizobium cannot grow in Hofer's medium. The results are supported by Deka and Azad, (2006). In Keto-lactose test, no yellow zone was observed around the colonies after adding Benedict's reagent which is the characteristic of Rhizobium and the same results were observed by Deshwal and Chaubey, 2014. Out of the 8 isolates, 7 isolates turned BTB indicator from deep green to yellow when streaked on YEMA enriched with bromothymol except (ISSA-1), indicating that the isolates were acid producers and fast growers which is the characteristic of rhizobium (Fig 1C). The same results were observed by many workers De-Vries *et al.*, 1980; Singh *et al.*, 2008.

Table 2: Conformity tests for differentiation of *Rhizobium* from *Agrobacterium* and other contaminants.

Bacterial Isolates	Growth on glucose peptone agar	Congo red absorption	Production of ketolactose test	Growth on Hoffer's alkaline medium	YEMA+BTB
ISSA-1	+	A	-	+	Blue
ISSA-2	-	Na	-	-	Yellow
ISSA-3	-	Na	-	-	Yellow
ISSA-4	-	Na	-	-	Yellow
ISSA-5	-	Na	-	-	Yellow
ISSA-6	-	Na	-	-	Yellow
ISSA-7	-	Na	-	-	Yellow
ISSA-8	-	Na	-	-	Yellow

Here, Na, non-absorbing; A, absorbing; YEMA+ BTB: Yeast extract mannitol agar supplemented with bromothymol blue.

Nitrogen fixing ability of the isolated bacteria

Nitrogen fixation is carried out by the nitrogenase enzyme whose multiple subunits are encoded by the genes *nifH*, *nifD*, and *nifK*. Of the three, *nifH* (encoding the nitrogenase reductase subunit) is the most widely sequenced marker gene used to identify nitrogen-fixing bacteria and archaea. Amplification of *nifH* gene segment resulted in the product of expected size (about 360 bp) from the DNA template. Out of 8 rhizobial isolates, 6 isolates showed amplification with *nifH* primers indicating the presence of N₂ fixing genes in these bacteria. The amplification of *nifH* gene of the bacterial isolates is represented in Fig 2.

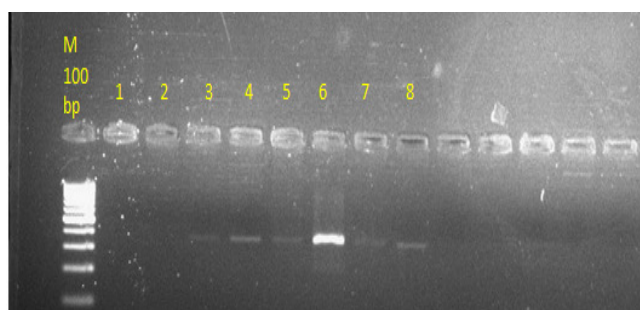


Fig. 2 : Electrophoretic profile of *nifH* primer run on 2% agarose gel with amplicon size of 360bp

1. ISSA-1, 2. ISSA-2, 3. ISSA-3, 4. ISSA-4, 5. ISSA-5, 6. ISSA-6, 7. ISSA-7, 8. ISSA-8

Biochemical characterization of *Rhizobium*

The results of the biochemical characteristics of the bacterial isolates are represented in Table 3. Most of the isolates showed positive results for Catalase test, Oxidase test, Nitrate reductase test (Fig. 1D) and Urease test. Similar results were also reported by Lupwayi and Hague, (1994). Most of the isolates showed positive results for starch hydrolysis except (ISSA-5 and ISSA-6). The same results match with those of De Oliveira *et al.* (2007), who observed that *Rhizobium* strains have capability to use starch (Fig. 1E). Most of the isolates showed negative results for citrate utilization test and gelatin liquefaction test except (ISSA-1). Negative gelatinase activity is a feature of *Rhizobium*. Negative gelatinase activity of *Rhizobium* was also observed by Hunter *et al.* (2007). The isolates ISSA-1 showed positive result for gelatinase, so the isolates may not be considered as a probable rhizobial isolate.

Table 3: Biochemical Characteristics of the root nodule bacteria

Bacterial Isolates	Cat	Oxid	Star	Cit	NR	Gel	Urease
ISSA-1	+	-	+	-	+	+	+
ISSA-2	+	+	+	-	+	-	+
ISSA-3	+	+	+	-	+	-	+
ISSA-4	+	+	+	-	+	-	+
ISSA-5	+	+	-	-	+	-	+
ISSA-6	+	+	-	-	+	-	+
ISSA-7	+	+	+	-	+	-	+
ISSA-8	+	+	+	-	+	-	+

Key: (+) Positive, (-) Negative, Cat: Catalase, Oxid: Oxidase, Star: Starch hydrolysis, Cit: Citrate utilization, NR: Nitrate reduction, Gel: Gelatinase.

Plant Growth Promoting (PGP) Traits of the Test Isolates

The bacterial isolates were screened for multiple plant growth promoting activities which are represented in the table 4. The test isolates 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). Out of 8 *Rhizobial* isolates, 5 were able to produce IAA in this

analysis. ISSA-2 and ISSA-4 showed high intensity (+++) of pink colour. ISSA-3, ISSA-6, and ISSA-8 showed moderate (++) intensity of pink colour (Fig. 1 F). Microorganisms also enhance plant growth by scavenging available iron (Fe³⁺), which involves secretion of high affinity, low molecular weight iron chelating ligands called siderophores (Anitha and Kumudini, 2014). Siderophores also play an important role in the bio control of some soil-borne plant diseases caused by several pathogens. Because siderophores sequester the

limited supply of iron in the rhizosphere, they limit its availability to pathogens and ultimately suppress their growth (Schroth *et al.*, 1984). Out of the 8 rhizobial isolates, 4 isolates were able to produce siderophores. Further, out of 4 isolates ISSA-2 and ISSA-4 exhibited strong (+++) siderophore production, and ISSA-3 showed moderate activity (++) whereas the isolate ISSA-5 showed slight activity (+) for siderophore production (Fig. 1G). All the isolates were able to produce ammonia. Further, out of 8 isolates, ISSA-2, ISSA-3, ISSA-5, ISSA-6, ISSA-7 and ISSA-8 exhibited strong (+++) ammonia production and ISSA-4 produced moderately (++) whereas the remaining 1 isolates viz., ISSA-1, showed slight activity (+) for ammonia production (Fig. 1J). Hydrocyanic acid (HCN) synthesized by some rhizobacteria inhibits diseases in plant and thereby increasing the bio control mechanism (Schippers, 1990). Out of the 8 rhizobial isolates, 6 isolates were able to produce

HCN. Further, out of 6 isolates ISSA-2, ISSA-3 and ISSA-4 exhibited strong (+++) HCN production, and ISSA-5, ISSA-6, and ISSA-8 showed moderate activity (++) whereas the remaining 2 isolates namely ISSA-1 and ISSA-7 showed no activity (-) for HCN production (Fig. 1I). After nitrogen, phosphorus (P) is the most limiting nutrient for plant growth. Rhizobia, including *R. leguminosarum*, *R. meliloti*, *M. mediterraneum*, *Bradyrhizobium* sp. and *B. japonicum* (Afzal and Bano 2008; Egamberdiyeva *et al.*, 2004; Rodrigues *et al.*, 2006; Vessey, 2003) are the potential P solubilizers. These bacteria synthesize low molecular organic acids which acts on inorganic phosphorous. Out of the 8 rhizobial isolates, 3 isolates were able to solubilize phosphate on NBRIP media containing Tri calcium phosphate. Further out of 3 Rhizobial isolates ISSA-2 and ISSA-5 recorded the highest solubilization zone (+++) and ISSA-6 showed moderate (++) solubilization zone (Fig. 1H).

Table 4: *In vitro* screening of root nodule bacterial for PGPR traits

Bacterial Isolates	IAA	SID	TCP	AMM	HCN
ISSA-1	-	-	-	+	-
ISSA-2	+++	+++	+++	+++	+++
ISSA-3	++	++	-	+++	+++
ISSA-4	+++	+++	-	++	+++
ISSA-5	-	+	+++	+++	++
ISSA-6	++	-	++	+++	++
ISSA-7	-	-	-	+++	-
ISSA-8	++	-	-	+++	++

Legend (+++; Good activity, ++; average activity, +; slight activity, -; no activity) IAA: Indole acetic acid, AMM: Ammonia production, TCP: Tri calcium phosphate solubilization, HCN: hydrocyanic acid, SID: Siderophore.

In vitro antagonistic activity against *Rhizoctonia solani* and *Sclerotium rolfsii*

None of the Rhizobia showed complete growth inhibition of the test fungi but exhibited significant growth reduction. Out of 8 Rhizobium isolates 3 isolates showed inhibition potential against *Rhizoctonia solani*, viz. ISSA-2 (55.40%), ISSA-3 (38.80%) and ISSA-4 (50.70%). Two out of 8 isolates were inhibitory to *Sclerotium rolfsii*, viz. ISSA-2 (52.40%), ISSA-3 (48.70%). Out of 8 Rhizobium isolates 2 isolates viz., ISSA-2 and ISSA-3 showed inhibition potential against both *Rhizoctonia solani* and *Sclerotium rolfsii*. Hence it can be inferred that the Rhizobium isolates ISSA-2, ISSA-3

and ISSA-4 could be considered for their bio control activity (Fig. 1K and Fig. 1L). The inhibition of fungal growth of the test fungi *in vitro* by certain of the rhizobia and formation of inhibition zones were presumably due to the metabolites released by the bacteria into the culture medium. These metabolites may include antibiotics and/or cell-wall degrading enzymes. Different studies have implicated antifungal secondary metabolites produced by Rhizobium spp. in the control of plant diseases caused by pathogenic fungi (Ehteshamul- Haque and Ghaffar, 1993; Siddiqui *et al.*, 2000).

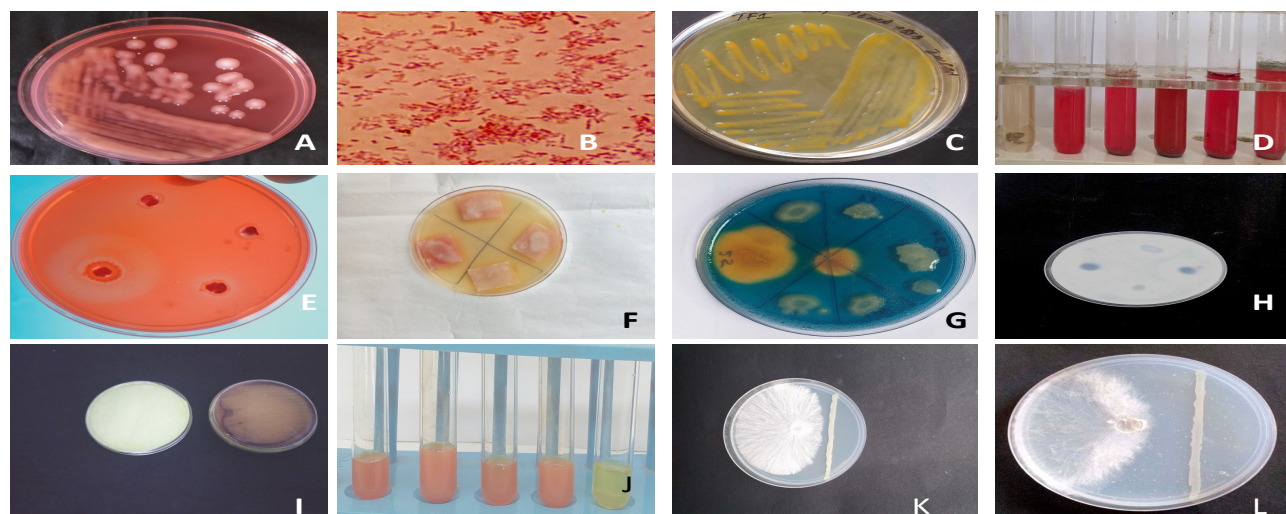


Fig. 1 : A. Rhizobium colonies on YEMA media; B. Gram staining; C. Yellow colour produced on YEMA media supplemented with bromothymol blue; D. Nitrate reduction; E. Cellulase production; F. IAA production; G. Siderophore production; H. TCP solubilization; I. Hydrocyanic acid production; J. Ammonia production; K. Antifungal activity of isolates against *Sclerotium rolfsii*; L. Antifungal activity of isolates against *Rhizoctonia solani*.

Conclusion

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 biofertilizers in preferences to chemical fertilizer is always welcome taking into consideration the suitability of agriculture. 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. In the present study the isolates were characterized based on their morphological and biochemical features, also the isolates were screened for different plant growth promoting activities. In future suitable PCR based genotypic techniques can be employed for confirming the identity of the isolates at strain level and for predicting the phylogenetic relationship of the isolates with other known isolates.

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