

DIVERSITY OF ROOT NODULE BACTERIA FROM LEGUMINOUS CROPS

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Abstract: In the present study, a total of 353 nodule-associated bacteria were isolated from 220 legume plant samples belonging to *Cicer arietinum* (85), *Glycine max* (74), *Vigna radiata* (21) and *Cajanus cajan* (40). A total of 224 bacteria were identified as fast-growing *Rhizobium* spp. on the basis of differential staining (Gram staining and carbol fuchsin staining) and biochemical tests. All the isolates were tested for indole acetic acid production (IAA), phosphate solubilization and siderophore production on plate assay. To examine the effect of volatile organic metabolites (VOM) and water soluble soil components (WSSC) on nodule bacteria, culture conditions were optimized by observing the effects of various parameters such as pH, salt content and temperatures on the growth of bacteria. Selected rhizobia were subjected to random amplified polymorphic DNA (RAPD) and amplified ribosomal DNA restriction analysis (ARDRA) analysis to identify their species. On the basis of RAPD and ARDRA, 10 isolates were identified as *Rhizobium meliloti*. In this study, *Rhizobium* GO4, G16, G20, G77, S43, S81, M07, M37, A15 and A55 were observed as the best candidates among the tested bacteria and can be further used as potent bioinoculants.

Key words: legumes; *Rhizobium*; RAPD; ARDRA; rhizobacteria

INTRODUCTION

Rhizobium is a well-known symbiotic nitrogen fixer and hence the species belonging to this genus are widely used as biofertilizers and bioinoculants. *Rhizobium* species are also reported as a source of various biologically active compounds such as poly- β -hydroxybutyrate, indole acetic acid and siderophore (Ilic et al., 2007). Antifungal and antibacterial compounds including other plant growth promoting substances have also been developed from *Rhizobium* species for sustainable agricultural uses (Ilic et al., 2007). In recent years, researchers are focusing on co-inoculation of PGPR and *Rhizobium* and it is becoming the most popular approach for improving the growth of economically important legumes. Plant growth-promoting rhizobacteria (PGPR) may increase the efficiency of *Rhizobium* inoculation in legumes through the production of antibiotics, siderophore

and certain essential enzymes (Iqbal et al., 2012). Bioinoculants possessing certain advanced features besides nitrogen fixation are of special significance in the field of agriculture. In this respect, certain biochemical properties such as production of siderophores, indole compounds, quantification of phosphatase solubilization potential and their capability to show enhanced growth activities in the presence of volatile organic metabolites (VOM) and water soluble soil components (WSSC) of soil are of special significance in selecting strains for the development of bioinoculants. Biofertilization of legume crops with plant-growth promoting microorganisms is currently considered as a healthy alternative to chemical fertilization (García-Fraile et al., 2012). Single variable optimization methods are tedious and lead to misinterpretation of results, as the interaction between different factors are overlooked (Abdel et al., 2002). In order to overcome this prob-

lem, statistical experimental designs have been suggested for an optimization strategy by other researchers (Kim et al., 2005; Lee and Gilmore, 2005).

In the present study, we isolated, identified and characterized various isolates of *Rhizobium* spp. In order to ensure the survival of soil microorganisms, a number of soluble components including organic metabolites are produced, which may affect soil microbial populations by inhibiting or stimulating the growth of other microorganisms. Therefore, an attempt has also been made to evaluate the effect of water soluble organic compounds of soil on the survival and growth of selected isolates of nodule bacteria.

MATERIALS AND METHODS

Selection and collection of crop plants

Four legume crop plants were selected for the isolation of rhizobia from their root nodules: *Cicer arietinum* (Chick pea), *Glycine max* (Soybean), *Vigna radiata* (Moong) and *Cajanus cajan* (Arhar), collected from six different sites in the Sagar district (Madhya Pradesh), India. A total of 85, 74, 21 and 40 samples of *Cicer arietinum*, *Glycine max*, *Vigna radiata*, *Cajanus cajan*, respectively, were collected for the isolation of rhizobia.

Surface sterilization

The surface of root nodules was sterilized with 1% HgCl₂ for about 5 min and the root nodules were washed with sterile distilled water and placed in 70% ethanol for 3 min followed by washing with sterile distilled water. Preparations of root nodule suspensions followed according to the modified methods referred to previously (Subba Rao, 1995; Agrawal and Jain, 2008; Agrawal and Jain, 2009).

Isolation of bacteria

For the isolation of root-nodulating bacteria, the root nodule suspension was serially diluted to a concentration of 10⁻⁴ in sterile distilled water and streaked on yeast extract mannitol agar (YEMA) plates (Vincent,

1970). In order to reduce the risk of isolating contaminants, Congo red was added to the YEMA plates. Plates were incubated at 30°C for 3 days. Well grown colonies were taken and purified by streaking on fresh slants containing YEMA (Agrawal and Jain, 2009).

Characterization of bacterial isolates

The morphological, biochemical and physiological characteristics of the authenticated isolates were previously studied by our research group (Agrawal, 2012). Gram-staining reaction was carried out using a loop-full of pure culture grown on YEM broth and stained as per the standard Gram procedure. The purified isolates were characterized based on the shape of bacterial cells, i.e., rod shapes in single, paired chains and dense clusters that stained Gram-negative were considered rhizobia. Further, carbol fuchsin staining was carried out and the cells were characterized by their shape and color, and dark purple rounded bodies were considered as rhizobia.

Biochemical examination

Ketolactose agar test

Lactose utilization by test bacteria was tested using YEMA medium. For this test, mannitol was replaced by lactose (10g/L) in the composition of the YEM agar (Subbarao, 1993). Similarly, the ketolactose test was also performed on the YEMA plates and overlaid with Benedict's reagent to distinguish *Agrobacterium* and *Rhizobium*. The absence of a yellow zone after using Benedict's reagent was considered for the presence of rhizobium. Other physiological and biochemical tests such as the catalase production test, oxidase test, citrate utilization, nitrate reduction, motility test, growth on different salt concentrations and various pH conditions were also performed according to the previously described method (Agrawal et al., 2011).

Phosphate solubilization

Qualitative and quantitative estimations of PO₄ were carried out by the method of Pikovskaya (1948). Re-

sults were previously discussed and reported by our research group (Agrawal et al., 2011).

Determination of siderophore production

Siderophore production by test rhizobia was determined by chrome azurol sulfonate (CAS) assay as described by Schwyn and Neiland (1987). The test strains were then streaked over CAS agar medium and incubated for 3 days at 30°C. After incubation, the formation of a yellow-orange halo zone around the colony was observed as an indication of siderophore production.

Determination of indole acetic acid (IAA) production

To determine the indole acetic acid (IAA) production by test bacteria, a mineral salt broth containing 0.5% glucose and 500 µg/mL tryptophan was prepared (Agrawal et al., 2011). The growth and culture of bacteria were maintained on Luria Bertani (LB) broth, and incubated at 28°C for 24 h at 120 rpm. To obtain the cell-free supernatant, the culture from the exponential phase was centrifuged at 10000 g for 15 min at 4°C. For confirmation of IAA production by bacterial isolates, 2 drops of *o*-phosphoric acid were added to the supernatant of each bacterial isolate to generate a pink color as confirmation of IAA production.

Effect of volatile organic metabolites (VOM) of soil on the growth of rhizobia

To study the effect of volatile organic metabolites (VOM) of the soil on the growth of rhizobacteria, the test organisms were exposed to volatile emanations of different soils. The experiments were conducted using a set of 250-mL Erlenmeyer flasks with a side connecting tube. A set of two flasks was used for each test organism. In one flask, a soil sample (200 g) was taken and moistened with small amount of sterile water to activate microbial activity. These flasks were made air-tight and allowed to stand for 24 h at room temperature before connecting to culture flasks. The flasks were run as soil-filled volatile chambers. On the

other hand, the flasks of the second set were used for the cultivation of test rhizobacteria in culture broth. An aliquot (50 mL) of the broth of YEMA culture medium was taken in a culture flask and both openings cotton-plugged. Flasks were then autoclaved and inoculated with 0.2 mL of cell suspension of the test rhizobacteria. Inoculated flasks were then connected to the soil-filled volatile chambers and incubated for 48 h. A set of flasks was also run as control for each test organism. After 48 h at 35°C of incubation, the growth of bacteria in each test flask and control was noted in terms of increase or decrease in turbidity by the nephelometric method utilizing 1-100 NTU (nephelometric turbidity units) and 400 NTU standard suspensions.

Effect of water soluble soil components (WSSC) on the growth of rhizobia

The effect of water soluble soil components (WSSC) was studied using soil extract in the growth medium. Briefly, 100 g of test soil sample was suspended in 300 mL of double-distilled water in a 500-mL flask and the flask was kept for 24 h. After 24 h, when the soil particles settled to the bottom of the flask, approximately 50 mL of water was removed and centrifuged at 10000 rpm for 10 min. The supernatant was collected and filter-sterilized using a syringe filter. An amount of 2.5 mL filter sterilized soil solution was dispensed in each test flask containing 50 mL of YEMA broth. The flasks were then inoculated with 1 mL cell suspension of test rhizobacteria and incubated at 30°C for 48 h. A set of flasks was also run as a control with only YEMA broth for each test organism. The growth of each test organism in the control and test flasks was then determined by turbidimetric methods as described earlier.

Optimization of conditions for the growth of rhizobium

Optimum culture conditions such as incubation time, pH and temperature for the growth of *Rhizobium* sp. G16 were determined using the following methods (Agrawal and Jain, 2012).

Random amplified polymorphic DNA (RAPD)

RAPD finger printing was done followed by Sajjad et al. (2008) to assess diversity among root nodule rhizobia isolated from four different crop plants.

Genomic DNA and template DNA preparation

The total genomic DNA and template DNA of *Rhizobium* strains were isolated following the method of Ivanova et al. (2000), with slight modifications. The rhizobia cultures were grown in yeast extract mannitol broth on a rotatory shaker at 125 rpm and 37°C for 48 h. About 5 mL of actively grown rhizobial cultures were pelleted by centrifugation at 13000 rpm for 10 min at 4°C to harvest the cells. The cells were suspended in 500 µL of TEN buffer (50 mM Tris-HCl, 20 mM disodium EDTA, 50 mM NaCl, pH 8.0) and 0.5 mL of 1-butanol was mixed well. The contents were centrifuged at 6000 rpm for 5 min at 4°C and the supernatant was discarded. The cell pellets were then resuspended in 2 mL of TE buffer and again centrifuged at 6000 rpm for 5 min at 4°C to remove traces of butanol. The cell pellets covered with 1 mL of TE buffer and 100 µL lysozyme solution (10 mg/mL freshly prepared) and incubated at room temperature for 5 min. One hundred µL of 10% SDS and 25 µL of 100 µg/mL proteinase K were added, mixed well and incubated at 37°C for 1 h. After incubation, the cell lysates were combined with 200 µL of 5 M NaCl, 150 µL of CTAB (10% stock) and incubated at 65°C for 10 min. The cell lysate was deproteinized with 1 mL of phenol:chloroform mixture (24:1 v/v) and centrifuged at 6000 rpm for 10 min at 4°C. The aqueous layer was transferred carefully to new 2.0-mL microcentrifuge tubes. To this, 1/10 volume of sodium acetate and 600 µL of ice-cold isopropanol were added and incubated at -20°C overnight. The precipitated DNA was sedimented at 12000 rpm for 15 min at 4°C. After discarding the supernatant, the pellet was allowed to dry for 30 min at room temperature and resuspended in 100 µL of TE buffer (pH 8.0). One µL of DNase free RNase (10 mg/mL stock) was mixed, incubated at 37°C and stored at -20°C for further use.

PCR amplification

PCR amplification of isolated template DNA was carried out with two oligonucleotides primers, GLA11-(5'CAATCGCCGT3') and GLC19-(5'GTTGCCAGCC 3'), following the method as described by Sajjad et al. (2008). The amplification reactions were performed in an automated thermal cycler (Corbett, Germany), in 25 µL of reaction mixture containing 2.5 µL of 10 X buffer (pH 8.4), 3 µL of MgCl₂ (25 mM), 1 µL of dNTPs mix (2.5 mM each), 2 µL primer (15 ng/µL), 2.5 µL of genomic DNA (15 ng/µL), 0.2 µL of (1U) of *Taq* DNA polymerase and 13.8 µL of deionized water. A negative control was maintained containing all components except template DNA. The reaction mixture was overlaid with two drops of mineral oil, incubated for 5 min at 95°C for initial denaturation, and then amplified for 35 cycles consisting of 1 min at 94°C, 1 min at 36°C and 2 min at 72°C followed by a final extension of 10 min at 72°C.

Electrophoresis and pattern analysis

Ten mL of amplified product were loaded in 0.8% agarose gel with a 100-bp DNA ladder as a marker. After electrophoresis, the gel was stained with 0.5 µL/mL of ethidium bromide and photographed under UV light in a Transilluminator (Bangalore Genie, India). A culture of *Rhizobium leguminosarum* (MTCC-99) was used as a standard strain. The data are presented in binary code i.e., 0 for absence of band and 1 for presence of band, and analyzed using NTSYSpc 2.02i package.

Amplified rDNA restriction analysis (ARDRA)

Amplified ribosomal DNA restriction analyses was performed as follows:

PCR amplification of 16s rDNA genes

One mL of template DNA (10 ng DNA) was added to the PCR mixture containing 5 µL of 10xPCR buffer, 1 µL each of forward and reverse primer from a 10 M stock (i.e. 12.5 pmol of each primer), 2.5 µL of dNTPs

mix (2.5 mM each) and 0.5 μL of *Taq* DNA polymerase (3 U/ μL). The final volume was brought to 50 μL by adding sterilized distilled water. The temperature program for amplification was as follows: after initial denaturation at 94°C for 3 min, the reaction mixture was run through 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min and with a final extension at 72°C for 10 min. Primers were derived from the conserved region present at the edges of the 16S rDNA (Weisburg et al., 1991). The primers were f D1 (5'-AGAGTTTGATCCTGGCTCAG-3') and r D1 (5'-AAGGAGGTGATC-CAGCC-3'). An aliquot of 5 μL of each PCR product was electrophoresed on 1% agarose gel in 1 X TAE buffer for 1 h at 90 V/cm and stained with ethidium bromide to confirm the amplification of DNA.

Restriction endonuclease digestion

Restriction digestion was carried out using three restriction endonucleases – *Hinf III*, *Hae II* and *Rsa I*, at 37°C for 3 h. Briefly, a 20- μL volume of reaction mixture was prepared with 10 μL 16 S rRNA gene product (amplified/ PCR product), 2 μL 10 X incubation buffer, 1 μL enzyme (10 U/ μL) restriction endonuclease, and 7 μL distilled water, and used for further steps.

Electrophoresis and pattern analysis

Restriction fragment patterns were analyzed by gel electrophoresis at 95 V/cm for 3 h in 2.5% agarose gel in 1 X TAE buffer. The gels were stained with 0.5 $\mu\text{L}/\text{mL}$ of ethidium bromide. Gels were photographed using a digital camera system (Nikon, cool pi x 995, Japan) and the patterns were compared for the size of restriction fragments with a 100-bp DNA molecular marker (Bangalore Genei, India).

Cluster analysis

Multivariate analysis was conducted to generate a similarity matrix using NTSYSpc software version 2.02, based on unweighted pair-group method using arithmetic means (UPGMA) to estimate genetic distance and relatedness of rhizobial strains.

RESULTS

Isolation, characterization and biochemical examination of rhizobia

In the present study, a total of 353 nodule-associated bacteria were isolated from 220 plant samples including 85 plant samples of *Cicer arietinum*, 74 samples of *Glycine max*, 21 samples of *Vigna radiata* and 40 samples of *Cajanus cajan*. These samples were collected from different localities of the Sagar district, MP, India following the standard protocols. A total of 353 nodule bacteria were isolated, which included 125 isolates from *Cicer arietinum*, 106 from *Glycine max*, 58 from *Vigna radiata* and 64 from *Cajanus cajan*. Identification of rhizobia was done based on their Gram-staining reaction, presence of poly- β -hydroxy butyrate granules, motility, lactose agar test, growth on N_2 free medium, oxidase and catalase test, and citrate utilization. The following characters were considered typical for rhizobia and accordingly isolated bacteria were identified.

Phosphate solubilization

In all, 224 *rhizobium* isolates including 71 from *Cicer arietinum*, 70 from *Glycine max*, 43 from *Vigna radiata* and 40 from *Cajanus cajan* were used as test organisms. Phosphate solubilization activity was noted in 59 rhizobacteria of *Cicer arietinum*, 55 of *Glycine max*, 30 of *Vigna radiata* and 28 of *Cajanus cajan*. Phosphate solubilizing activity was found to be widely distributed in the strains of nodule rhizobia of all the test plants. Although some test strains including all the rhizobia isolated from *C. arietinum* and *V. radiata* and some isolates of *G. max* and *C. cajan* showed phosphate solubilization activity with the production of acid, 11 test rhizobia from *C. cajan* and 7 of *G. max* showed phosphate solubilization with the production of an alkaline reaction during the acid production test using bromothymol blue as indicator in YEMA.

Siderophore and IAA production

The ideal bioinoculant must possess certain additional properties that can promote the growth of

crop plants either directly or indirectly. The production of siderophore, phosphate solubilizing efficiency and plant growth hormones by inoculants and their growth stimulation by volatile organic metabolites (VOM) and water soluble soil components (WSSC) can be of special significance for crop productivity. The abovementioned properties of the rhizobia, selected on the basis of screening results of phosphate solubilizing potential, were studied in the present work. In all 10 *Rhizobium* strains, including 4 isolates from *C. arietinum* (strains G04, G16, G20 and G77), 2 from *G. max* (S43 and S81), 2 from *V. radiata* (M07 and M37) and 2 of *C. cajan* (A15 and A55) were studied for abovementioned properties. Test *Rhizobium* sp. A15 showed maximum phosphate solubilization activity. All the other test strains showed 10-44 mg/mL $\text{Ca}_3(\text{PO}_4)_2$ solubilization. All these strains were found to produce siderophore when tested using CAS assay medium. The test strain *Rhizobium* sp. MO7 showed an excellent production of indole acetic acid (6.5916 $\mu\text{g/mL}$) in its culture grown on mineral salt broth containing 0.5% glucose and 500 $\mu\text{g/mL}$ tryptophan (Table 1).

Table 1. Siderophore and IAA production by 10 root nodule isolates.

No.	Strain number	Yellow orange zone around the colony (mm)	Indole acetic acid ($\mu\text{g/mL}$)
1	<i>Rhizobium</i> sp. G04	1.82	3.594
2	<i>Rhizobium</i> sp. G16	2.6	5.4340
3	<i>Rhizobium</i> sp. G20	2.22	1.0200
4	<i>Rhizobium</i> sp. G29	1.0	0.196
5	<i>Rhizobium</i> sp. G30	-	0.120
6	<i>Rhizobium</i> sp. G45	-	1.011
7	<i>Rhizobium</i> sp. G75	1.12	-
8	<i>Rhizobium</i> sp. G77	2	2.869
9	<i>Rhizobium</i> sp. G88	-	-
10	<i>Rhizobium</i> sp. G98	-	-

Effect of VOM and WSSC of different crop field soil on the growth of isolated rhizobacteria

To study the effect of VOM and water soluble components of soils, three different soil samples collected from different localities of the Sagar district were used. The VOM of two test soils (i.e., soil A and soil B) stimulated the growth of almost all test *Rhizobium* species.

Table 2. Effect of Volatile organic metabolites (VOM) of different crop field soil on the growth of selected rhizobacteria isolated from root nodules of 4 crops.

Test strains	Growth of rhizobacteria (NTU)									
	Control	Soil - A			Soil - B			Soil - C		
		Growth of bacteria in test flask	Stimulation in growth (NTU)	Stimulation/ inhibition (%)	Growth of bacteria in test flask	Stimulation in growth (NTU)	Stimulation/ inhibition (%)	Growth of bacteria in test flask	Stimulation in growth (NTU)	Stimulation/ inhibition (%)
<i>Rhizobium</i> sp. G04	504	616	112	22.22	600	96	19.04	424	-80	-15.87
<i>Rhizobium</i> sp. G16	488	592	104	21.31	504	16	3.97	488	NE	NE
<i>Rhizobium</i> sp. G20	416	552	136	32.69	520	104	25.0	456	40	09.61
<i>Rhizobium</i> sp. G77	352	488	136	38.63	472	20	34.09	312	-40	-11.36
<i>Rhizobium</i> sp. S43	280	352	72	25.71	336	56	20.00	488	208	74.28
<i>Rhizobium</i> sp. S81	328	440	112	34.14	400	72	21.95	376	48	14.63
<i>Rhizobium</i> sp. M07	480	480	NE	NE	512	32	6.66	560	80	16.66
<i>Rhizobium</i> sp. M37	512	648	136	26.56	616	104	20.31	328	-184	-35.93
<i>Rhizobium</i> sp. A15	568	656	88	15.49	624	56	9.85	566	-02	-3.52
<i>Rhizobium</i> sp. A55	352	427	120	21.30	448	136	38.63	320	-32	-9.09

+ Stimulation in growth, - inhibition in growth, NE – no effect on growth.

Soil A – crop field soil of Jaat Pathariya, Soil B – crop field soil of Makroniya, Soil C – crop field soil of Damoh road.

Table 3. Effect of Water soluble soil components (WSSC) of different crop field soil on the growth of selected rhizobacteria isolated from root nodules of 4 crops.

Test Strains	Growth of Rhizobacteria (NTU)									
	Soil – A			Soil – B			Soil – C			
	Control	Growth of bacteria in test flask	Stimulation in growth (NTU)	Stimulation/Inhibition (%)	Growth of bacteria in test flask	Stimulation in growth (NTU)	Stimulation/Inhibition (%)	Growth of bacteria in test flask	Stimulation in growth (NTU)	Stimulation/Inhibition (%)
<i>Rhizobium</i> sp.G04	208	232	24	11.53	248	40	19.23	240	32	15.38
<i>Rhizobium</i> sp.G16	360	456	96	26.66	472	112	31.11	448	88	24.44
<i>Rhizobium</i> sp.G20	208	248	40	19.23	256	48	23.07	272	64	30.76
<i>Rhizobium</i> sp.G77	208	292	12	05.76	400	108	51.92	408	200	96.15
<i>Rhizobium</i> sp.S43	208	272	64	30.76	288	80	38.46	312	104	50.00
<i>Rhizobium</i> sp.S81	376	520	144	38.29	536	160	42.55	576	200	53.19
<i>Rhizobium</i> sp.M07	184	216	32	17.39	232	48	26.03	248	64	39.02
<i>Rhizobium</i> sp.M37	240	304	64	26.66	360	120	50.00	344	104	43.33
<i>Rhizobium</i> sp.A15	400	496	96	24.00	488	88	22.00	498	98	24.5
<i>Rhizobium</i> sp.A55	216	328	112	51.85	360	144	66.66	392	176	81.48

+ Stimulation in growth, - inhibition in growth, NE – no effect on growth.

Soil A – crop field soil of Jaat Pathariya, Soil B – crop field soil of Makroniya, Soil C – crop field soil of Damoh road.

On the other hand, the VOM of soil C caused both stimulation and/or inhibition in the growth of test rhizobia. It is interesting that the WSSC of all the soil samples caused growth stimulation in all the tested rhizobacteria (Table 2 and 3).

Random amplified polymorphic DNA (RAPD)

Analysis of the 10 isolates revealed that the rhizobacteria produced several RAPD band patterns by using two oligonucleotides primers, GLA-11-5'CAATCGCCGT3' and GLC-19-5'GTTGCCAGCC 3'. PCR amplification with these primers indicated that each primer template yielded distinct, easily detectable bands of variable intensities (data not shown). Ten isolates generated 4 RAPD profiles when using the GLA-11 primer. The dendrogram obtained using NTSYSpc software showed total relatedness at 1.75 (Fig. 1a). Similarly, all the isolates generated 5 RAPD profiles when using the GLC-19 primer. In this case, the isolates showed total relatedness at 2.51 (Fig. 1b).

Amplified ribosomal DNA restriction analysis (ARDRA)

Test isolates of rhizobacteria were subjected to PCR-RFLP with 16S rDNA genes using three restriction enzymes, *Hinf I*, *Hae III* and *Rsa I*. This resulted in separate restriction patterns for each enzyme (data not shown). The ARDRA produced fragments in the range of 100-900 bp. The number of bands detected was higher when the rDNA regions of test rhizobia were digested with *Hae III* (53 bands) compared to *Hinf I* (45 bands) and *Rsa I* (46 bands). A total of 144 bands were found in ARDRA. The relationship among test *rhizobium* isolates based on ARDRA is depicted in the dendrogram (Fig 2a, 2b and 2c). The UPGMA cluster analysis shows 100% similarity at a coefficient of 3.15, 2.05 and 2.93 when *Hinf I*, *Hae III* and *Rsa I* restriction enzymes were used, respectively. *Rhizobium* species showed band patterns similar to the standard culture of rhizobacteria when digested with *Hinf I*, ranging from 100-700 bp (data not shown). 16S rDNA digested with *Hae III* showed bands of 100-250 base pairs (data not

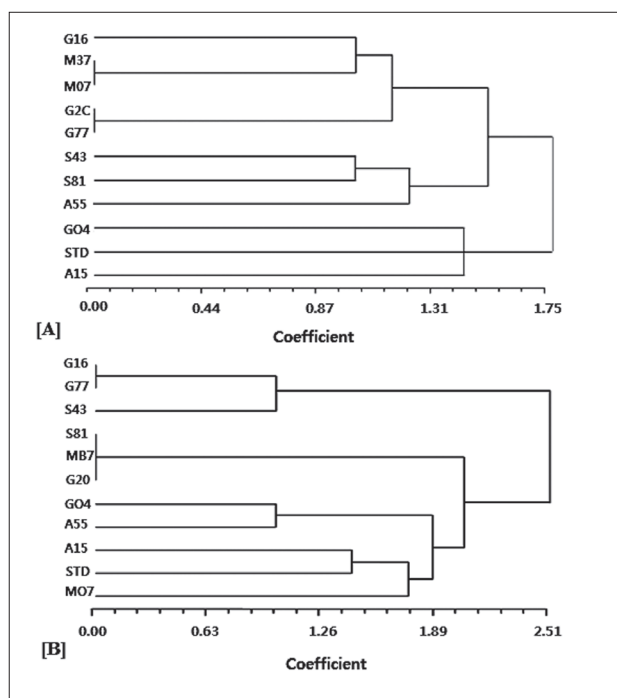


Fig. 1. UPGMA-based dendrogram showing cluster analysis of the *Rhizobium* isolates on the basis of Random Amplified Polymorphic DNA using [A] GLA 11; and [B] GLC 19.

shown). When digested with *Rsa I*, bands ranging from 100-900 base pairs were obtained (data not shown).

The UPGMA-based dendrogram obtained by ARDRA with *Hinf I* indicated three strains: M37, G04 and G20 in one cluster and 4 strains, A15, M07, G77 and A55 in another cluster, while other species showed varied band patterns. Using the restriction enzyme *Hae III* 6, test rhizobia fell in one cluster (i.e., isolates S81, G20, G77, A55, M07 and A55). Cluster analysis of the rhizobium isolates on the basis of ARDRA with *Rsa I* indicated 100% similarity at a coefficient of 1.46 with two distinct clusters, i.e., cluster I (G77, A55 and M07) and cluster II (G16 and G20), which showed 100% similarity with cluster III (S43, S81 and A15) at a coefficient of nearly 1.95 (Fig. 1 and 2).

Optimized conditions for the growth of rhizobia

Rhizobium sp. G16 showed potential to be developed as a bioinoculant and was thus selected for studies related to optimization of the best culture conditions in regard

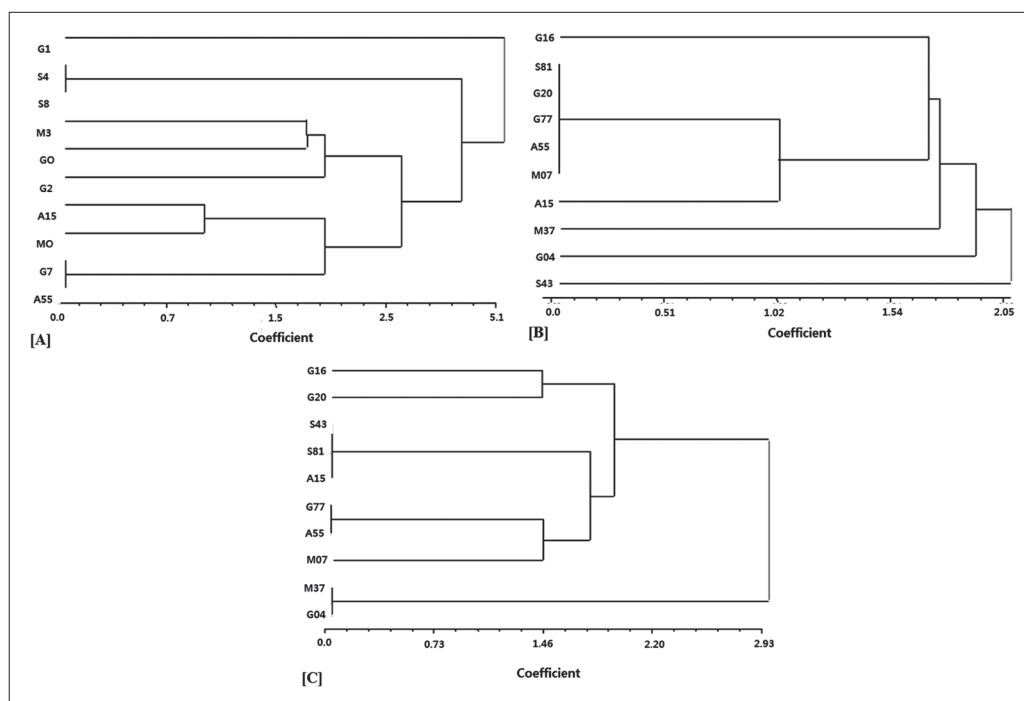


Fig. 2. UPGMA-based dendrogram showing cluster analysis of the *Rhizobium* isolates on the basis of random amplified ribosomal DNA restriction analysis (ARDRA) with [A], *Hinf I*; [B], *Hae III*; and [C], *Rsa I*.

to incubation period, pH of the medium and temperature for growth. The best incubation period was found to be 3 days for this organism. The best temperature was found to be 37°C and the organism was found to achieve its maximum growth, i.e. equivalent to NTU 497.6, in 3 days. The obtained data indicated growth of organisms at acidic pH (<7.0 pH) and at alkaline pH (>7.0 pH) while the optimum pH was found to be 7.0 in the present study (data not shown). The organism achieved an increased growth under pH 7.0, temperature 37°C and a 3-day incubation period. Under these culture conditions, the test strain *Rhizobium* sp. G16, an isolate of the root nodules of *G. max*, achieved growth equivalent to NTU 501.6. The results obtained during our investigation were of great fundamental and applied value. A number of rhizobial strains were collected which showed value-added properties such as the production of IAA and siderophore as well as the ability to solubilize phosphates.

DISCUSSION

In the present study, we studied the phosphate-solubilizing efficiency of root-nodulating bacteria isolated from four different legume crop plants. This property in *Rhizobium* inoculants may provide additional advantages to crop plants. Strains from the genera *Pseudomonas*, *Bacillus* and *Rhizobium* are considered the most powerful phosphate solubilizers and efforts should be made to find N₂-fixing bioinoculants with phosphate-solubilizing properties (Tambekar et al., 2009). In the present study, on the root nodule rhizobia of four legume crops properties such as acid production and phosphate solubilization were considered basic desirable characteristics. Depending on the extent of phosphate-solubilizing capacity, 10 strains of *Rhizobium* were selected during screening studies and further studied to quantify this ability. Wani et al. (2007) reported that among phosphate-solubilizing (PS) bacteria, *Bacillus* PSB1 and *Bacillus* PSB10 produced a clear halo zone 4 and 5 mm in size, respectively, on solid Pikovskaya medium.

Iron is another vital element required by virtually all living organisms including bacteria with

the exception of a few taxa (Posey and Gherardini, 2000). Microbial siderophores generally stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots. Marschner and Romheld (1994) reported that plants may also utilize siderophores synthesized by microorganisms present in the rhizosphere as they can be a source of soluble iron for the host plant. Strain-specific production of siderophore in *Rhizobium* species was first reported by Schwyn and Neilands (1987). Gaonkar et al. (2012) studied siderophore producing *Bacillus* sp. and *Pseudomonas aeruginosa* produced a yellowish fluorescent siderophore identified as pyoverdine. According to Guerinot et al. (1990), variation in the production of siderophores by different strains of rhizobia may be a strain-specific characteristic. Although siderophore production is not essential for both nodule formation and nitrogen fixation, it may significantly increase the efficiency of nitrogen fixation. Siderophore-mediated competition for iron appears to be a widespread phenomenon in microbial systems. Arora et al. (2001) reported siderophore production by 2 out of 12 rhizobial isolates, i.e., RMP₃ and RMP₅, using CAS agar medium. Guerinot et al. (1990) first reported citric acid release in response to iron stress by a rhizosphere bacterium. The siderophore produced by the test strain 61A152 was found to be citric acid (Guerinot et al., 1990). Moreover, strain-specific production of siderophore has been observed by a number of other researchers (Ames-Gottfred et al., 1989).

It was reported that the production of plant hormones, such as auxins, cytokinins and gibberellins may allow certain bacteria and fungi to direct the physiology of plants towards their own advantage (Lambrechet et al., 2000). Ravi Kumar and Ram (2012) examined the maximum production of IAA at 72 h of incubation in a medium containing 1 mg/mL concentration of L-tryptophan, whereas Sudha et al., (2012) reported the production and optimization of IAA by indigenous microflora using agro-waste substrate. *Rhizobium* spp. are also reported to produce IAA from tryptophan in culture (Costacurta and Vanderleyden, 1995). The physiological effect of IAA from microorganisms on plant growth depends ultimately on the amount of hormone that is avail-

able to the plant; however, it depends greatly on the interactions between the plant and the microorganisms (Patten and Glick, 1992) and also on the interactions among the microorganisms. Datta and Basu, (2000) reported the production of 28.0 µg IAA/mL in a 72-h-old culture of *Rhizobium* strain 13 isolated from *Cajanus cajan*. They also reported maximum production of IAA in a glucose-containing medium by a rhizobium isolate. In addition, Qureshi et al. (2012) observed the improved yield of *Vigna mungo* by the co-inoculation of phosphate-solubilizing bacteria and *Rhizobium* in the presence of L-tryptophan, whereas Iqbal et al. (2012) found an improved yield of lentil by integrated use of *Rhizobium* and PGPR.

The past decades have witnessed an indiscriminate use of chemical fertilizers to improve crop productivity; however, it has been realized that chemicals not only affect soil flora and fauna but also impose the threat of pollution to soil and water bodies, eventually leading to ecological imbalance. Therefore, microbial biofertilizers offer a suitable alternative to agro-chemicals and chemical fertilizers. *Rhizobium* is one of the main symbiotic bioinoculants used. Many rhizobial species have been tested for enhanced crop productivity and promising strains have been applied in field studies. However, the failure of available biofertilizers to substantially increase productivity is posing a severe problem with regard to their practicability in agriculture. This study will lead to industrial and regional economic growth through the development of region-specific native strains of *Rhizobium* for their potential use as biofertilizers, which may enhance the productivity of different leguminous crops.

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