Isolation and characterization of *Rhizobium* spp. and determination of their potency for growth factor production

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Abstract

The most important step of this study was to isolate *Rhizobium* species and determination of their potency for growth factor production. We isolated 260 type bacteria on PCA (Plate Count Agar) media from adjacent soil samples of ten leguminous plants, two non-leguminous plants and control soil (without plant). Then pure cultures of 53 nitrogen fixing bacterial strains were isolated on selective Yeast Extract Mannitol Agar (YEMA) medium. Five isolates (Ma-G1, Ch-H2, Lo-F1, Sh-J1and Ra-I2) were selected as *Rhizobium hainanense* and better result was regarded in their assessment for production potency of various growth factors. All *Rhizobium* spp. were able to fix nitrogen in media. Among these isolates, indole acetic acid (IAA) was produced by Ma-G1 and Ra-I2. The exopolysaccharide production rate of Ma-G1 was enhanced expectedly (1.25-fold increase) by treating with IAA. Unfortunately, these five strains were unable to separate soluble phosphorus content from insoluble tri-calcium phosphate (TCP). High salt tolerance was also observed by all strains in media supplemented with IAA containing 5-10% KH_2PO_4.Therefore, this *Rhizobium* spp. would be the beneficial bacteria to interplay with the growth promotion of leguminous plants along with biological nitrogen fixation.

Keywords: Leguminous plants, *Rhizobium hainanense,* growth factor production, biological nitrogen fixation.

INTRODUCTION

Nitrogen is an essential nutrient for plant growth and development. Plants usually depend upon combined, or fixed, forms of nitrogen, such as ammonia and nitrate because it is unavailable in its most prevalent form as atmospheric nitrogen. Much of this nitrogen is provided to cropping systems in the form of industrially produced nitrogen fertilizers. Use of these fertilizers has led to worldwide ecological problems as well as affects the human health (Vitousek, 1997). Biological nitrogen fixation (BNF) is the cheapest and environment friendly procedure in which nitrogen fixing micro-organisms, interacting with leguminous plants, fix aerobic nitrogen into soil (Franche et al., 2009).

Rhizobium is the most well known species of a group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria can infect the roots of leguminous plants, leading to the formation of lumps or nodules where the nitrogen fixation takes place. The bacterium's enzyme system supplies a constant source of reduced nitrogen to the host plant and the plant furnishes nutrients and energy for the activities of the bacterium. *Rhizibium* bacteria stimulate the growth of leguminous plants and they are able to fix atmospheric nitrogen into soil by interacting symbiotically with leguminous plants, using the nitrogenase enzyme complex (Kiers et al., 2003). The legume-rhizobium interaction is the result of specific recognition of the host legume by *Rhizobium*. Various signal molecules that are produced by both *Rhizobia* and the legume confer the specificity (Phillips, 1991). Exopolysaccharide (EPS) produced by *Rhizobium* is one such signal for host specificity during the early stage of root hair infection (Olivares et al., 1984). It also protects the cell from

Table 1. List of the leguminous plants and non-leguminous plants species

Plant Type	Local Name	English Name	Scientific Name
Leguminous	Sim	Field Beans	*Lablab purpureus* (L.) Sweet
	Masoor	Lentil	*Lens culinaris* Medic
	Cholai	Bengal Gram	*Cicer arietinum*
	Lojjaboti	Sensitive Plant	*Mimosa pudica* Linn.
	Shishu	Sisso/Indian Rosewood	*Dalbergia sissooo* Roxb.
	Babla	Indian Gum-Arabic Tree	*Acacia Nilotica* (L.)
	Sojne	Drumstick Tree	*Moringa olefeira*
	Tetul	Tamarind	*Tamarindus indica* Linn.
	Radhachura	Copper Pod Tree	*Peltophorum pterocarpum*
	Krisnachura	Peacock Flower	*Caesalpinia pulcherrima*
Non-leguminous	Shorisha	Black Mustard	*Brassica Nigra*
	Tal	Asian palm	*Borassus flabellifer*

desiccation and predation and helps in nitrogen fixation by preventing high oxygen tension (Jarman et al., 1978). In addition, *Rhizobium* strains secrete growth hormones like indole acetic acid (IAA), which shows positive influence on plant growth and also plays an important role in the formation and development of root nodules (Nutman,1977). Hence, the production of EPS and IAA are considered as important traits of plant growth-promoting *Rhizobacteria*. Moreover, *Rhizobium* species also have other various enzymatic activities. These benefits of this species were indentified and measured biochemically by various researchers (Kumari et al, 2009).

Screening an effective *Rhizobium* strain and enhancement of their quality is very much important for sufficient nitrogen fixation. The main purpose of this study was to isolate more efficient strains of *Rhizobia* those can compete with less effective strains for effective nodulation of leguminous plants. Throughout our investigation we isolated five effective strains of *Rhizobia* those had a substantial contribution in biological nitrogen fixation along with capability for various growth factor production. Further depth research is required for their use in nodulation of legume plants in future.

METHODS AND MATERIALS

Sample Collection, Plant Selection And Experimental Site

Soil samples were collected from the adjoining soils of plant roots. Ten leguminous plants and two non-leguminous plants species were selected for soil sample collection (Table 1). Experimental site was at Jhenidah district in Bangladesh that is located at 23^0-15'-00"N to 23^0-45'-00"N and 88^0-45'-00"E to 89^0-45'-00"E (LGED, 2012).

Isolation And Morphological Characterization Of Root-Associated Bacteria

Fresh soil was diluted for isolation and morphological characterization of root-associated bacteria from thirteen soil samples and diluted solutions were spread on the PCA (Plate Count Agar) media in the petri dish. Bacterial colonies were counted and about 20 colonies were observed for their morphological characters from each sample. In this way total 260 colonies were isolated and the cultures were streaked on another PCA media in the second step.

Isolation Of *Rhizobium* Bacteria From Adjoining Soil Of Leguminous Plants

Nitrogen fixing bacteria were identified by streaking 260 selected colonies on the selective YEM (Yeast Extract Mannitol) media (composition for 1L media: mannitol 10.0g; K_2HPO_4 0.5gm; $MgSO_4$ -$7H_2O$ 0.2gm; NaCl 0.1gm; yeast extract 1.0g; agar 20g; 10ml of stock solution of 250mg bromothiol blue in 100ml water; pH was adjusted to 7.0 with 1 M NaOH or HCl before autoclaving at 121^0C for 15 min). Various biochemical tests were performed to screen the particular species of *Rhizobium* (Wang et al., 1998).

Assessment of the production potency of *Rhizobium* strains for growth factors

For the estimation of EPS production, *Rhizobium* strains were inoculated into conical flask containing 20 ml of YEM broth supplemented with 1% of carbon source. The inoculated flasks were incubated at 30°C in a shaker for 72 h. After incubation, the culture broth was centrifuged 3500 × g and the supernatant was mixed with two

Table 2. CFU (colony forming unit) of various samples.

Plant Types	Sources of Soil Samples	CFU
Leguminous	Beans	1.19 x 10^7
	Rosewood	1.77x 10^7
	Lentil	4.23x 10^6
	Sensitive Plant	8.46x 10^6
	Bengal Gram	1.79x 10^7
	Gum-Arabic Tree	8.3x 10^7
	Drumstick Tree	2.37x 10^7
	Tamarind	9.96x 10^5
	Copper Pod Tree	2.98x 10^6
	Peacock Flower	2.02x 10^7
Non-Leguminous	Asian palm	1.07x 10^6
	Black Mustard	7.47x 10^5
Without Plant Interaction	Control Soil	8.30x 10^6

volumes of chilled acetone (Merck KGaA, Germany). The crude polysaccharide developed was collected by centrifugation at 3500 × g for 30 min. The EPS was weighed after half an hour of drying at 105°C (Damery and Alexander, 1969). A 50µl IAA solution (5%) was added to the 20ml YEM media of *rhizobium* strains to enhance the production rate. In later stage the increased amount of production was measured. Acid production was studied by measurement of pH, using a pH meter (Hanna instrument, Mauritius) and bacterial growth was observed visually for the change in color from green to yellow. Salt tolerance was detected on YEM media containing 5%- 10% KH_2PO_4. A 150µl IAA solution (5%) was spread over on YEM media containing 5% - 10% KH_2PO_4. *Rhizobium* strains were inoculated on both YEM media. The isolates were observed after 72 h.

Phosphate solubilization test was made according to the method of Goldstein (1986). The appearance of clearing zone around bacterial colonies after 96h of growth at 30°C was used as indicator for positive Phosphorus solubilization. Spot test for indole was performed on growing *Rhizobium* strains on Trypton Soya Agar medium amended by adding glucose (10g), K_2HPO_4 (0.5g); $MgSO_4.7H_2O$ (0.2gm); NaCl (0.1g); yeast extract (1.0g) for 1L medium. A Whattman Filter paper was placed on the bacterial growth on YEM medium. Then the filter papers were saturated with few drops of Salkawski reagent (1mL 0.5M $FeCL_3$, 50mL H_2SO_4) (Gordon and Weber, 1950). After two minutes, appearance of pink color was observed which was indicator of IAA production (Myron and Williams, 1989).

Statistical Analysis

Analysis of Variance (ANOVA) was performed using SPSS version 20. The statistical significance was set at the $P<0.05$ confidence level. Test of significance of the means was by the Least Significance Difference (LSD).

RESULTS AND DISCUSSION

Isolation And Morphological Characterization Of Root-Associated Bacteria Of Leguminous And Non-Leguminous Plants

Total 43 type morphologies were found among 260 colonies in PCA media. This means that bacterial diversity exists largely in the soil microbial population. The microbial populations of leguminous plants were larger in number than that of non-leguminous. Total colonies were counted as shown in Table 2. Majority number of colonies was found in adhered soil of Drumstick Stick Tree (leguminous plant) and was 2.37x 10^7, whilst the minimum number (7.47x 10^5) was in adjacent soil of Black Mustard (non-leguminous plant).

Isolation Of Nitrogen Fixing Bacteria (*Rhizobium*) From Adjoining Soil Of Leguminous Plants

The nitrogen fixing bacteria can be isolated directly from the root nodules of the host plant or from the soil (Geniaux et al., 1993), using yeast extract mannitol selective culture media (YEM) (Handley et al., 1998; Castro et al., 2003; Kukuc et al., 2006). We isolated nitrogen fixing bacteria from adjoining soil samples of legumes. Bromthimol blue (BTB) was used as indicator in order to detect the multiplication of the nitrogen fixing bacteria (Figure 1A). The yellow halos around the colonies on blue were observed after three days (Figure 1B). Yellow color was produced due to the production of acid from nitrogen fixing bacteria. Their morphological shapes were observed under light microscope. Gram staining of these microbes was performed and was observed microscopically. Total 53 isolates were selected as nitrogen fixing bacteria from which only ten isolates, identical to *Rhizobium* in colony morphology, were chosen for further characterization (Table 3). Regarding

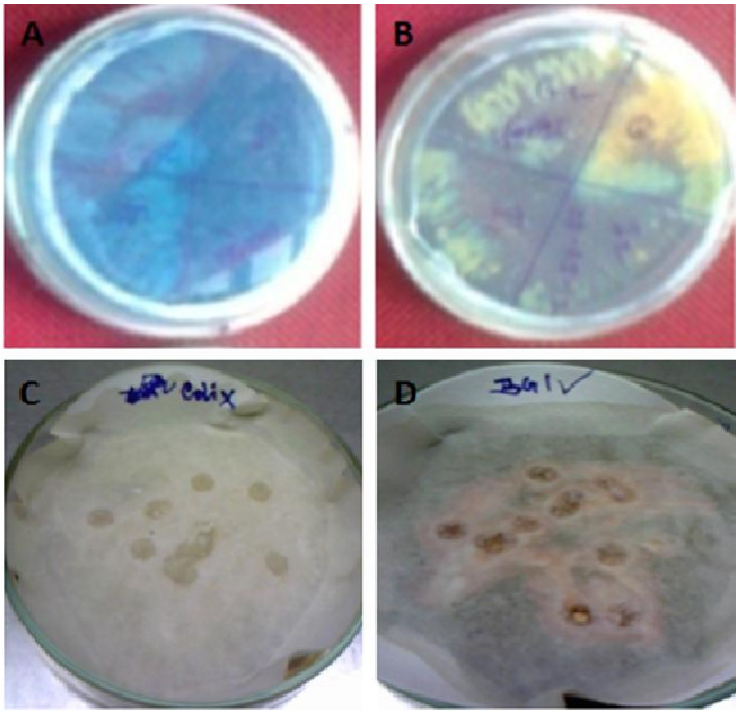

Figure 1. Isolation of nitrogen fixing bacteria and spot test for indole-3- acetic acid production. Bromthimol blue (BTB) was used as indicator in order to detect the multiplication of the nitrogen fixing bacteria (A). The yellow halos surrounding the bacterial colonies indicate nitrogen fixing bacteria (B). No color change was detected by Kr-D2 during spot test (C). Pink color indicates the production of indole-3- acetic acid by Ba-G1 (D).

Table 3. Isolation of *Rhizobium* genera from nitrogen fixing bacteria.

Samples	Growth on YEM	Color	Gram Staining	Cellular Morphology	Genus
Si-D2	Yes	Yellow	Negative	Cocci	-
Ma-G1	Yes	Yellow	Negative	Rod	*Rhizobium*
Ch-H2	Yes	Yellow	Negative	Rod	*Rhizobium*
Lo-F1	Yes	Yellow	Negative	Rod	*Rhizobium*
Sh-J1	Yes	Yellow	Negative	Rod	*Rhizobium*
Ba-G1	Yes	Yellow	Negative	Irregular, branched	-
So-A2	Yes	Yellow	Negative	Cocci	-
Te-B1	Yes	Yellow	Negative	-	-
Ra-I2	Yes	Yellow	Negative	Rod	*Rhizobium*
Kr-D2	Yes	Yellow	Negative	Irregular	-

Here, "–'' indicates not detected.

the cellular morphological analysis, 5 isolates were proved as rod shape and gram negative. These 5 strains were considered as *Rhizobium* genera (Garrity, 1982). Various biochemical tests were performed to screen the particular species of *Rhizobium*. Interestingly, the five *Rhizobium* general showed nearly same features and

Table 4. Screening of *Rhizobium* species.

Sample	Growth Factors Required:			pH	Temperatue	Salt Tolerance	Acidic Reaction	Indole	N_2	Species
	Biotin	Pantothenate	Thiamin							
Ma-G1	-	-	-	7-4.5	+	+	+	+	+	
Ch-H2	-	-	-	7-4.5	+	+	+	-	+	
Lo-F1	-	-	-	7-5	+	+	+	-	+	
Sh-J1	-	-	-	7-5	+	+	+	-	+	*Rhizobium*
Ra-I2	-	-	-	7-5	+	+	+	+	+	*hainanense*

Here,"–'' indicates negative result and "+" indicates positive result, pH indicates acidity range required for bacterial growth, temperature indicates growth in 28^0c-35^0c, salt tolerance indicates growth at 1% NaCl, acidic reaction indicates growth on YEM media,N_2 indicates N_2 fixation capacity of *Rhizobium*

Table-5. Assessment of the potency of five *Rhizobium hainanense* strains.

Samples	EPS (Per 1mg Bacteria)	EPS with IAA (Per 1mg Bacteria)	Increase of EPS (Times)	Acidity (pH)	Phosphorus Solubilization	IAA Producing Isolates
Ma-G1	0.80 mg	1 mg	1.25	4.50	-	+
Ch-H2	0.81 mg	0	-	4.58	-	-
Lo-F1	0.50 mg	0	-	4.75	-	-
Sh-J1	0	0	-	5.01	-	-
Ra-I2	0.82 mg	0	-	4.67	-	+

Here, "–'' indicates negative result and "+" indicates positive result

were identical to *Rhizobium hainanense* (Table 4). According to Wang et al (1998), these five isolates were selected as *Rhizobium hainanense*.

Assessment Of The Production Potency Of *Rhizobium Hainanense* Strains For Growth Factors

Measurement Of Acid Production

Acid production was studied by measurement of pH, using a pH meter (Elico, India) and bacterial growth was observed visually for the change in color from green to yellow. The highest acidity was created by the strain Sh-J1and it was 5.01, while the Ma-G1 strain occupied the lowest rank by producing pH4.50, shown in Table 5. According to Fred et al. (1932), the strains in the present study can be considered as fast growers as all can produce acid. Therefore, all the strains in the present study may be suitable for better nodulation.

Exopolysaccharide Production

According to the result of EPS production test, maximum amount of EPS was produced by Ra-I2 sample and it was .82mg /1mg cell, after three days. EPS was produced by four strains; but the amount was varied from strain to strain. The possible cause is that various acidic conditions were created by different strains themselves, and these acidic conditions influenced the EPS production rate. Gorret et al (2001) studied that the optimal pH for exopolysaccharide production may differ from the optimal pH for bacterial growth. Research on *Sinorhizobium meliloti* (*Rhizobium sp.)* revealed that IAA solution treated cells released more EPS into the medium than the control strain (Carmen et al. 2009). In our present study, for enhancement of EPS production, 50µl IAA solution (5%) was added to the 20ml YEM media. EPS production was increased markedly by one sample (Ma-G1) (Table 5). These EPS producing strains are beneficial to plant-microbe symbiosis (Olivares et al., 1984; Jarman et al., 1978).

Salt Tolerance

The soil was rich in phosphorus and potassium. A weak correlation co-efficient (0.43) was observed between these two parameters (Figure 2). For this reason, there was a possibility to form potassium-phosphate weak chemical bond in the soil samples. Hence the salt tolerance was detected on KH_2PO_4 wherein a feeble

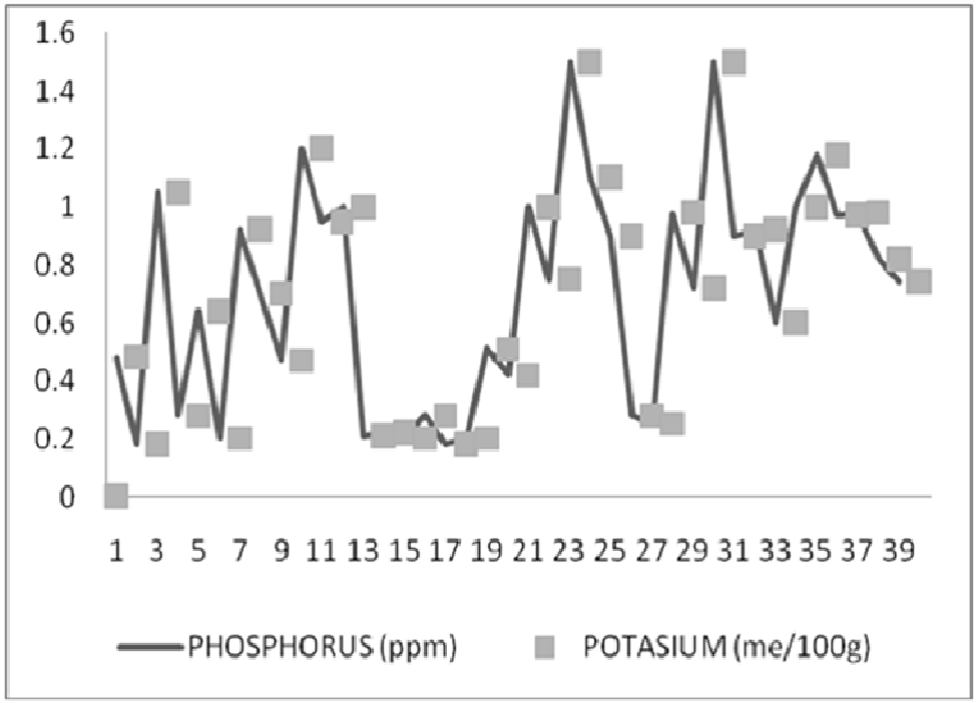

Figure 2. Correlation of potassium and phosphorus

bond has been stabilized by hydrogen. In case of salt tolerance test, five *Rhizobium hainanense* strains (Ma-G1, Ch-H2, Lo-F1, Sh-J1and Ra-I2) were grown on YEM media containing 5% KH_2PO_4 with IAA solution, whereas only one isolates (Ra-I2) was grown on the same media without IAA solution. On the other hand, three isolates (Ma-G1, Lo-F1 and Sh-J1) were grown on 10% KH_2PO_4 containing YEM media with IAA solution, whereas no bacterial colony was grown on the same media without IAA solution. It indicates that enhancement of salt tolerance of some isolates were occurred by IAA solution. Carmen et al. (2009) opined that the increased levels of trehalose, EPS and biofilm are correlated to the enhanced resistance against stress conditions observed for RD64 cells.

Phosphate Solubilization

Test for phosphate solubilization was made by following the method of Goldstein (1986). Unfortunately, no clear zone was found on PSB media. It indicates that the isolates were not phosphorus solubilizing bacteria (Table 5).

Indole Production

Amended Trypton Soya Agar Media was used for indole spot test. Minor amount of tryptophan was available in this medium from which indole was produced by *Rhizobium hainanense*. The appearance of pink color was the indicator of positive result of indole production (Figure 1C and 1D). Positive result of Ma-G1 and Ra-I2 isolates was observed in this study (Table 5). These two strains may be considered as effective plant growth-promoting bacterial strains (Nutman, 1977).

Statistical Analysis For Growth Factor Production

All five *Rhizobium hainanense* strains were almost identical in respect of cellular mass dry weight, EPS production rate and acid production activity. Since the F-statistic (.001) value was smaller than F-critical value (3.47), the null hypothesis could be accepted at a 95% confidence level. Thus, the data was not significant ($p=0.99$) and there was no significant variation amongst the five *Rhizobium hainanense* strains (Table 6). We used Analysis of variance (ANOVA) to test the homogeneity of bacterial strains, because this method is used to compare measurements to determine if the measurements are sampled from the same or different distributions (Gelman, 2005).

CONCLUSION

In the present study we isolated *Rhizobium* spp. from adjoining soil samples of leguminous plants and

Table-6. ANOVA (Single Factor analysis) for *Rhizobium hainanense* strains.

SUMMARY

Groups	Count	Sum	Average	Variance
Ma-G1	3	5.31	1.77	5.7457
Ch-H2	3	5.4	1.8	5.9563
Lo-F1	3	5.252	1.750667	6.809001
Sh-J1	3	5.012	1.670667	8.363361
Ra-I2	3	5.5	1.833333	6.199033

ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	0.045071	4	0.011268	0.001703	0.999993	3.47805
Within Groups	66.14679	10	6.614679			
Total	**66.19186**	**14**				

determined their ability for growth factor production. We found five *Rhizobium* strains those are capable for fixing higher amount of nitrogen along with different growth factors. Thus those strains might be applied in efficient nodulation of legume plants for biological nitrogen fixation. These findings allow us a new scope for extensive research.

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