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Genotypic Characterization of Indigenous *Rhizobium* Strain from Cultivated Cowpea (*Vigna unguiculata* L.) in Bangladesh

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A B S T R A C T

A gram negative strain of *Rhizobium* sp. SOY7 has been isolated from root nodules of cow pea (*Vigna unguiculata* L.) which was later identified on the basis of biochemical tests and 16S rDNA sequencing. The optimum culture condition of *Rhizobium* sp. SOY7 was determined at pH 7.0 and 28˚C temperature. The strain was resistant to most of the antibiotics used in this study except Tetracycline, Ceftazidime, Pefloxacin, Cotrimoxazole and Mecillinam. Moreover, the isolate was sensitive to the higher concentration of NaCl (>1%). The strain is capable to produce nodules when induced rhizobia as inoculants and resulted insignificant changes in plant growth characteristics. The most abundant nodulation and improved growth of the legumes were determined in plants inoculated with *Rhizobium* sp. SOY7 was statistically significant ($p < 0.05$) compared with uninoculated controls.

Introduction

Rhizobia are gram negative bacteria that exist in a symbiotic relationship with several grain legumes as a host plants (Ngakou *et al.*, 2009). In this association, the host plant provides the bacteria symbiont with sugars and a protected environment, while the bacteria fix nitrogen from the air and make it available to the plant in the form of ammonia (Kiers *et al.*, 2002).Legumes are an inexpensive food, commonly found in diets all over the world and that can provide proteins for human beings. In Bangladesh where the population diet is mostly based on legumes, there is need to improve the production of common legumes through inoculation technology, if full benefit from these crops is to be achieved in terms of maximum yield and soil fertility. However, the yield of cowpea is very poor in Bangladesh which could be increased by using industrially produced nitrogen fertilizers. But, use of these fertilizers has led to worldwide ecological problems as well as affects the human health (Vitousek, 1997).

The symbiotic nitrogen fixation resulting from the rhizobia-legume interaction can act as a renewable and environmentally

sustainable source of nitrogen and can complement or replace fertilizer inputs (Peoples *et al.*, 1995). Hence, only rhizobia that are specifically compatible with a particular species of legume can stimulate the formation of root nodules which in turn increase nitrogen fixation for better production of legume. Legumes encompass a variety of foods in pods including cowpea, beans, groundnut and lentils. They all contain protein as well as fiber, complex carbohydrates, healthy fats, potassium, iron and magnesium. In general, the world at present is confronted with the serious problems of food and nutrition deficiencies. Increased demand of proteins has led the research workers to search for getting overproduction of crops by exploiting better colonization of their root and rhizosphere through *Rhizobium* bacteria, which can reduce nitrogenous fertilizer use and can protect environment. However rhizobial inoculants which are produced commercially in have to be specific to legume species as well as to the region. Science native rhizobia are believed to be better adapted to local environmental conditions than commercial inoculants, we conducted a screening to select for the most efficient and competitive strain among indigenous rhizobia. Therefore, isolation and characterization of new *Rhizobium* strains are necessary for production of quality inoculant for using in cultivation of cowpea in Rajshahi, Bangladesh. In this study, indigenous *Rhizobium* strains were isolated from cowpea and then the isolates were characterized to uncover their suitability as a quality inoculant for cowpea cultivation at Rajshahi, Bangladesh.

Materials and Methods

Isolation of *Rhizobium* strains

The cowpea plants were collected from the Rajshahi, Bangladesh. Healthy, unbroken nodules of cowpea were used for isolation of root nodule bacteria (*Rhizobium*) with a method as described by Saha and Haque (2005). For the identification of the isolated bacterium, morphological characterization, microscopic observations, growth characteristics, biochemical tests and antibiotic sensitivity tests were performed with method described by Aneja (2003).

Identification of *Rhizobium* strains by 16S rDNA gene sequence

Genomic DNA was extracted from the bacterial cells using TIANamp Bacteria DNA kit (Tiangen, China) and purified according to the manufacture′s instruction. The amplification products were separated by electrophoresis of 10 µl. (7 µl PCR product 3 µl loading dye, Bromothymol blue) of the reaction product in 1.0% agarose gel (wv^{-1}) in Tris- instruction. The amplification products were separated by electrophoresis of 10 µl. (7 µl PCR product 3 µl loading dye, Bromothymol blue) of the reaction product in 1.0% agarose gel (wv^{-1}) in Tris Borate buffer (0.089M Tris, O.O89M boric acid, and 0.002M EDTA, pH 8), stained with ethidium bromide (1.6 mg/ml). The gel electrophoresis was carried out at 70 V at room temperature for ~ 1.0 hour in electrophoresis unit (Bio-Rad, USA) and DNA bands were visualized using UV transilluminator in gel documentation system. A 1 kb DNA ladder was used as molecular weight markers. The PCR products were purified using TIANquick Midi purification kit (Tiangen, China) according to the manufacture′s protocol. The total DNA yield and quality were determined spectrophotometrically by Nano Drop 2000 (Thermo Scientific, USA). Sanger sequencing work flow using dye terminator technology was followed for the present study sequencing analysis was performed on a~ 800 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The 16S rRNA genes in

the Gene Bank by using the NCBI Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nih.gov/BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees were formed using Weighbor (Weighted Neighbor Joining: A likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000.The 16S rRNA gene sequences were deposited to Genbank using BankIt submission (Saitou and Nei, 1987).

Effects of temperature and pH on bacterial growth

Temperature and pH influence bacterial growth. For effect of pH, culture medium was adjusted to pH 5.0, 7.0, and 9.0. Incubation temperature was varied at, 20, 28 and 37 ^{0}C.For determination of effect of salinity, inoculated media were incubated at 1%, 2%, 3% and 4% of NaCl. Bacterial cell density of liquid cultures was determined by measuring optical density at 660 nm with photoelectric colorimeter (AE-11 M, Erma Inc., Tokyo) (Mohanta *et al.*, 2012).

Seed and soil inoculation with *Rhizobium* and its effect on growth parameters in Cowpea

Seed inoculation was done by slurry method using adhesive (Saha and Haque, 2005). Then, inoculated seeds of Cowpea were sown in in pots. For soil inoculation, liquid culture of *Rhizobium* was sprayed thoroughly in inner part (1-1.5 inches below the surface) of soil in pots. Then, fresh and dry seeds of Cowpea were sown in pots. During the experiment the soils in pots were kept moistened.

Statistical analysis

Unless indicated otherwise, all experiments were independently conducted three times and data were pooled for presentation as mean ± SEM. All data were analyzed with Prism software (GraphPad, La Jolla, CA, USA) using two-tailed unpaired Student's t-tests. P-values <0.05 were considered significant.

Results and Discussion

Bacteria were isolated by plating onto an agar solidified YEMA medium with Congored. The plates were incubated at 28^{0}C for 2 days and bacterial colonies were found to grow on the medium. Results of microscopic analysis of bacterial cells and their growth characteristics are presented in Table 1 while the biochemical and antibiotics sensitivity tests of the bacterium are presented in Table 2, 3 respectively. Isolated bacterial strain was identified by both morphological and biochemical tests and this was further confirmed by 16S rRNA gene sequence analysis. The strain showed 96% (ID KF008235.1) homology with *Rhizobium* sp. SOY7.

Many previous studies reported isolation of Rhizobia of different strains from cultivated legumes, such as, *Rhizobium leguminosarum* stranis S17/2 & S 21/6 (Pohajda *et al.*, 2016), *R. tropici* (Pinto *et al.*, 2007), *R. gallicum* strain 8a3 (Mnasri *et al.*, 2007). The soil bacterium *Rhizobium* sp. SOY7 that form nitrogen-fixing nodules on the roots of cowpea plant is being reported for the first time through this study.

In this study it was found that the colonies were circular, light pink, concave, entire and opaque. It was also observed that the bacterium was gram negative, rod shaped and motile. The isolates were showed hazy appearance in the motility media and also were positive for Catalase, Citrate utilization test, Urea hydrolysis, Congored test, Nitrification test, Oxidase test, Triple sugar iron test, MacConkey agar test and, Motility

tests. This result is supported by the finding of Lupwayi and Haque (1994) and Allito (2015). The isolates were found negative for Methyl Red (MR), Voges-Proskauer (VP), Indole, Starch hydrolysis test, Hydrogen-sulfide production and Hofer's alkaline test. These findings are in close agreement with Elsheikh and wood (1986).

Utilization of different carbon sources is an effective tool to characterize the isolates (Mirza *et al.,* 2007; Erum and Bano, 2008). In the present study sucrose, fructose, galactose, maltose and mannitol (25 mg Hi-media, India) and 20% solution of glucose, lactose, arabinose and xylose were utilized for this test. Isolates could utilize all the nine sugar. Similar results have been reported by some other paper (Stowers, 1983; Sadowsky *et al.,* 1983).

Resistance patterns of the isolates to thirteen antibiotics were studied. Screening for antibiotic resistance in our study revealed that most of the isolates were resistance to Ampicilin, Erythromycin, Gentamicin, Amoxycillin, Penicillin, Streptomycin and Nalidixic acid. But, the isolates were sensitive to Mecillinam, Ciprofloxacin, Cotrimoxazole, Pefloxacin, Ceftazidime and Tetracycline which is agreed with the results of Jordan (1984) for the genus *Rhizobium*.

Table.1 Culture characteristics and microscopic observations of the isolated bacterial strain

Agar plates	Characters	Results
YEMA slant	Abundance of growth	Moderate
	Colony	Circular, concave, entire, opaque
	Colour	Light pink
Microscopic observation	Gram staining	Gram-negative
	Motility	Motile
	Shaped	Rod shaped

Table.2 Biochemical test results for the isolated bacterial strain (*Rhizobium* sp. SOY7)

Biochemical test	Reaction	Sugar utilization	Reaction
Catalase	+	Glucose	+
Citrate utilization	+	Sucrose	+
Urea hydrolysis	+	Fructose	+
Congored	+	Mannitol	+
Nitrification	+		
Oxidase	+		
Triple sugar iron	+		
MacConkey	+		
Methyl red	-		
Voges-proskaure	-		
Indole	-		
Starch hydrolysis	-		
Hydrogen sulfide	-		
Hofer's alkaline	-		

(+ = microbial growth, - = no growth)

Table.3 Antibiotic sensitivity tests

Antibiotics	Disc distance (mm)	R	S
Mecillinam	22	-	S
Ampicilin	5	R	-
Ciprofloxacin	30	-	S
Erythromycin	5	R	-
Gentamicin	6	R	-
Cotrimoxazole	26	-	S
Amoxycillin	6	R	-
Penicillin	5	R	-
Pefloxacin	21	-	S
Ceftazidime	23	-	S
Streptomycin	5	R	-
Nalidixic acid	5	R	-
Tetracycline	20	-	S

Table.4 Effect of *Rhizobium* inoculation on various growth parameters in Cow pea

	Length (cms)	**50 pod wt. (gms)**	**100 seed wt. (gms)**	**No. of Nodules/ Plant**	**Fresh wt. of nodules (gms)**	**Dry wt. of nodules (gms)**
Inoculated	61.00 ±1.30	5.348 ±0.05	7.376±0.13	36.60 ± 0.92	0.1820±0.009	0.0700±0.004
Control	53.60 ±0.67	4.570±0.02	6.096±0.05	30.80 ± 0.73	0.1260±0.006	0.0440±0.008
Degrees of freedom	08	08	08	08	08	08
Calculated value. P=0.05	0.0010**	<0.0001***	<0.0001***	0.0012**	0.0009***	0.0231*

Table.5 Properties of Cow pea soil types before and after inoculation with *Rhizobium* strain

Parameters	Units	Before use	Control	Inoculated
pH		8.2	8.3	8.3
Organic mater	%	1.22	1.40	1.63
Potassium	Cmol/kg	0.18	0.28	0.23
Total Nitrogen	%	0.07	0.08	0.10
Phosphorous	ppm	11.5	25.5	25.9
Sulfur	ppm	15.7	28.7	41.1
Zinc	ppm	2.47	4.94	5.20

Fig.1 Effects of pH (a), temperature (b) and salinity (c) on bacterial growth

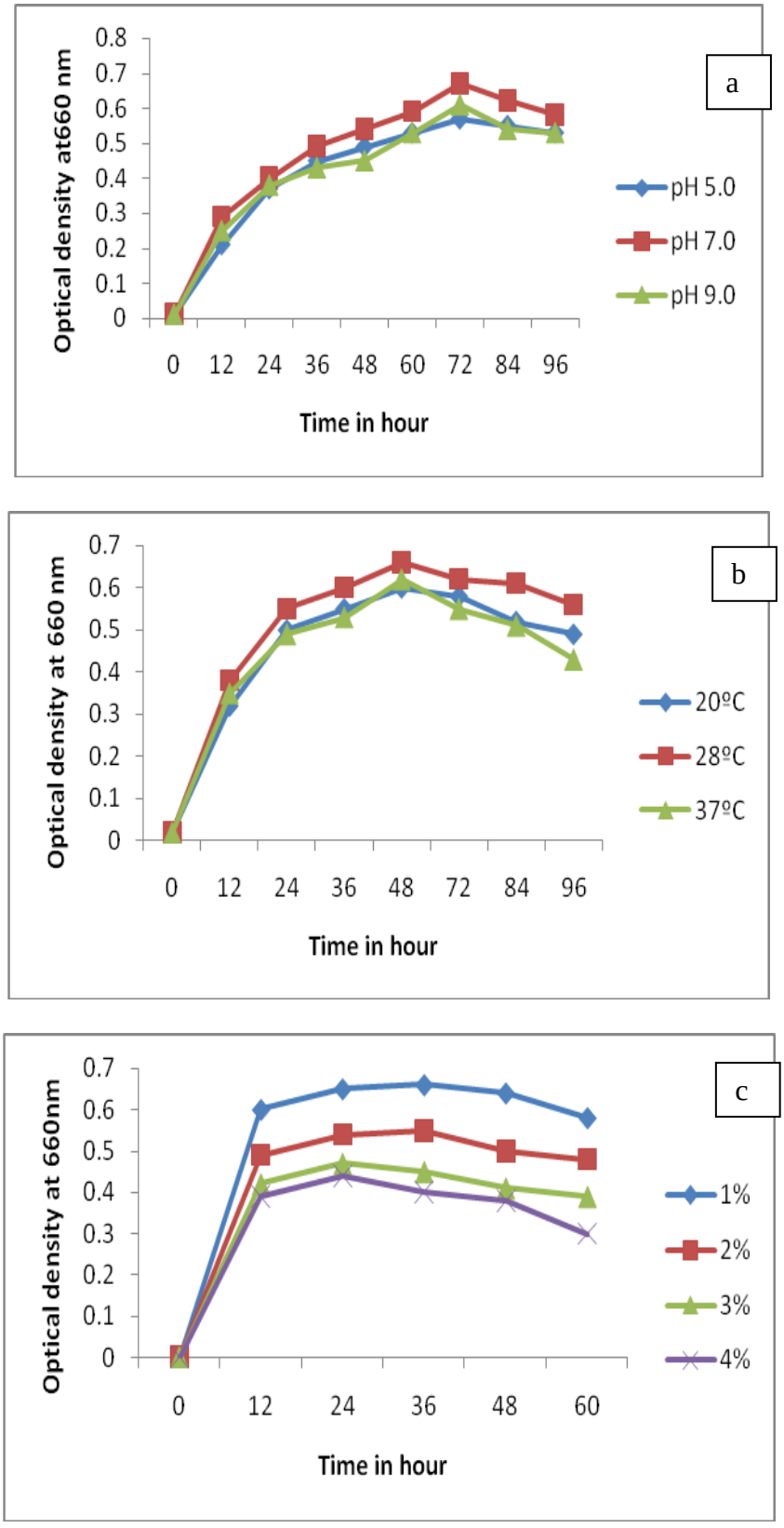
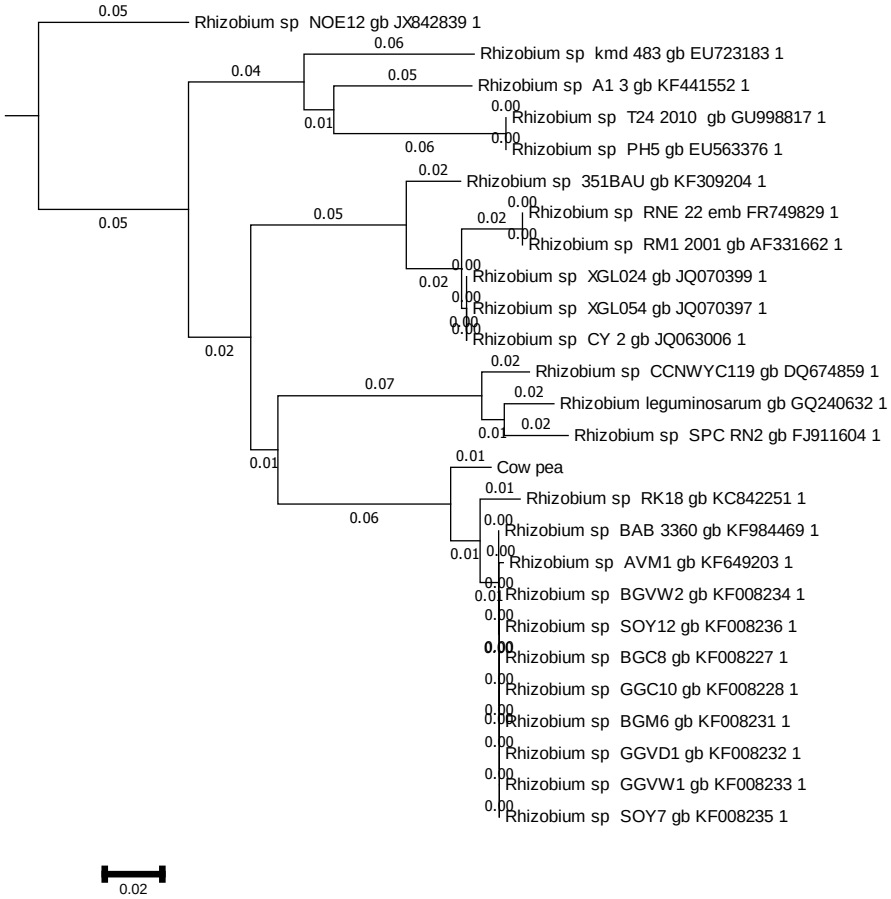


Fig.2 Unrooted phylogenetic tree showing the genetic relationship among the cultivated bacteria Cow pea and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.02 = 2% difference among nucleotide sequences



To verity the effects of temperature and pH of growth medium on the growth rate of the bacterium, a series of investigations were carried out which are presented in Fig. 1. The highest growth was observed at 28ºC (Fig. 1b). The organisms were found to be temperature sensitive as at higher and lower temperatures, a low growth was observed that might be due to a hindrance in the metabolic activity. Similarly, the best growth of *Rhizobium* was found at pH 7 (Fig. 1a). Soil characteristics, such as pH and temperature, may compromise symbiotic efficiency and plant development. pH values below 5.0 are reported to be deleterious for nodulation and nitrogen fixation (Appunu and Dhar, 2006; Mukherjee and Asanuma, 1998). Barberi *et al.*, (2004) reported that the four strains of *Bradyrhizobium* isolated from *Glycine max* (BR29, SEMIA587), *Vignaunguicuata* (INPA3-11B) and *Enterolobiumc ontortisiliquum* (BR4406), all isolates were grown satisfactorily in pH values of 5.0, 6.0 and 6.8.The experiments also showed that the cells were able to grow at 1% NaCl but unable to grow at higher concentration of NaCl, showing that the isolate was sensitive to the salt concentration (Fig. 1c). Similar findings have been reported by Kucuk *et al.*, (2006). In addition, Hashemandtheir colleagues in 1998 had proposed that salt stress may decrease the efficiency of the

Rhizobium-legume symbiosis by reducing plant growth and photosynthesis.

In this study, the isolated bacteria were identified through 16S rDNA gene sequencing. The 16S rDNA sequence revealed that the isolated strain was homologous to bacterial strain *Rhizobium* sp. SOY7. The phylogenetic distances shown in Figure 2 indicate that the relationships between this group and the *Agrobacterium* species. Moreover, this phylogenetic tree clearly showed that the isolates were belonged to the genus *Rhizobium.*

The results obtained in this study show some interesting aspects on the growth effects of *Rhizobium* inoculation on Cowpea, which was grown in pots under controlled environment. For all studied parameter of Cowpea growth *viz*. number, fresh weight and dry weight of nodules, plant height, pod weight and seed weight, the result showed significant differences between inoculation and control plants (Table 4). These findings can be supported by other studies as reported that Rhizobial inoculation induced significant changes in plant growth characteristics (Sharma and Tilak, 1974; Kapur *et al.*, 1975; Dev and Tilak, 1976).

Plant growth and microorganism activity depends upon soil reaction and on possible condition of the soil. Hence, soil properties of pots in which the Cowpea were cultivated were tested. It was found that Rhizobium inoculation can increase amount of Sulfur remarkably in soil (Table 5). Conversely, the amount of Potassium was decreased significantly in soil after *Rhizobium* inoculation. However, no remarkably changes were observed in other parameters of soil after *Rhizobium* inoculation (Table 5). Likewise, it was reported that *Rhizobium* inoculation in Cowpea enhanced the plant soil properties than control in line with Tabatabai (1994).

The indigenous strain *Rhizobium* sp. SOY7 which was isolated from cowpea of Rajshahi, Bangladesh possesses some unique characteristics of carbohydrate utilization, antibiotic resistance, salt tolerance and optimal growth condition. Altogether it can be concluded from this study the isolated indigenous *Rhizobium* sp. SOY7 strain could be an efficient candidate for production of biofertilizer for using in Rajshahi, Bangladesh as it shows some positive aspect on the growth of cowpea in this region.

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