

Original Article

Genetic diversity of *Rhizobium* spp. isolated from soil samples of Bangladesh

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Rhizobia are Gram-negative soil-inhabiting bacteria commonly found in nodules of leguminous plants. These bacteria exclusively fix atmospheric nitrogen into ammonia, which is convertible to urea to serve natural fertilizer to the plants. However, rhizobial microbiota of Bangladesh have been studied poorly. Here, twenty isolates were identified as *Rhizobium* through observation of cultural characteristics, morphological characteristics and different biochemical tests. Isolates differed from one another in terms of their stress response characteristics like salt-stress tolerance, temperature tolerance, antibiotic susceptibility, and pH. Most of the isolates were resistant to ampicillin and erythromycin and streptomycin; and were sensitive to kanamycin and ciprofloxacin. Isolates were clustered into three genotypic groups according to the banding patterns of Amplified Ribosomal DNA Restriction Analysis (ARDRA). All the strains in ARDRA group 1 were *Rhizobium azibense*, and the others were *Rhizobium bangladeshense* and *Rhizobium qilianshanense*. Future studies would validate their capacity of nitrogen fixation and the scope of improvement of these strains to increase their efficiency of nitrogen fixation.

Keywords: *Rhizobium*, biodiversity, stress response, ARDRA.

Introduction

Nitrogen is an essential nutrient for all living organisms and necessary to produce high-yield and high-quality agricultural crops¹. Nitrogen is one of the key atoms in amino acids, nucleotides, vitamins, co-factors, and various metabolites commonly found in living systems, as well as vital component of various drugs². Although molecular nitrogen (N₂) is the most abundant gas in the atmosphere, no plant or animal can convert the atmospheric nitrogen to the metabolically active form of nitrogen (i.e. -NH₂ or -NO₃)³. Rhizobia are a genetically diverse and physiolo-

gically heterogeneous group of soil inhabiting bacteria that have the capacity to form nodules on legume roots (and occasionally on stems) and can fix atmospheric nitrogen through a process called biological nitrogen fixation to partially or fully meet the nitrogen requirements of the host plant⁴. Such process contributes approximately 16% of total nitrogen input in crop land⁵. Thereby, Rhizobia are a major contributor in nitrogen fixation, and the legume-*Rhizobium* symbiosis can fix up to 450 Kg N/ha/year^{3,4}.

Genetic and phenotypic diversity study helps to classify the variants of *Rhizobium* in different groups and to find efficient nitrogen fixing strain having desired traits⁶. The rhizobia from wild leguminous trees had been classified after extensive analysis of the phenotypic, genetic and molecular characteristics⁷. In another study, rhizobia from *Acacia senegal* and *Prosopis chilensis*, grown in Sudan, had been clustered according to the numerical analysis of phenotypic characteristics and

lipopolysaccharide (LPS) profiles^{8,9}. Despite the identification of 74 rhizobial species to date including one new species from Bangladesh, few studies have been reported on the diversity of *Rhizobium* spp. of Bangladesh^{10,11}. Since Bangladesh is a tropical country, the soil microbiota of this country is highly diversified¹². Therefore, more studies are required to define such biodiversity, especially the diversity of rhizobial microbiota those are associated with nitrogen fixation.

This study was carried out with 20 rhizobial isolates from the nodules of seven leguminous plants species. These isolates were identified through morphological characteristics, biochemical tests, and partial 16S rDNA sequencing. Phenotypic diversity of the isolates was analyzed through observation of cultural characteristics, stress response characteristics and antibiotic sensitivity tests. Genetic diversity was analyzed by Amplified Ribosomal DNA Restriction Analysis (ARDRA). Thus, this study can be considered as a preliminary study on different rhizobial strains associated with nodules of these leguminous plants.

Materials and Methods

Collection of soil samples

Soil samples adjoining roots of seven different leguminous plants were collected from ten different regions of Bangladesh using sterile bucket trowel. The samples were sealed in sterile plastic bags and were carried to the laboratory. The leguminous plants include *Caesalpinia pulcherrima*, *Delbergia sissoo*, *Lablab purpureus*, *Mimosa pudica*, *Moringa oleifera*, *Sesbania*

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bispinosa, and *Vigna unguiculata*. The locations of sampling areas include Gazipur, Manikganj, Rajshahi, Mymensingh, Faridpur, Khulna, Patuakhali, Narsingdi, Sylhet and Chittagong districts.

Isolation of rhizobial strains

The soil samples were suspended in normal saline at 1:9 (w/v) ratios. The suspension was serially diluted and spread onto Congo Red Yeast Extract Mannitol Agar (CRYEMA) medium¹³. Plates were then incubated at 30 °C for 24-48 hours. Single colonies were isolated and pure cultures were obtained in CRYEMA plates. For long term storage glycerol stocks were prepared from the pure culture of the isolates. The stocks were kept at 4°C for 24-48 hours followed by storage at “20°C.

Morphological and biochemical characterization of rhizobial isolates

Isolates were Gram stained and observed under both bright field microscope and Phase contrast microscope (Zeiss Primo Star, Germany). Biochemical tests that were performed included Catalase test, Indole production test, Methyl red test, Voges-Proskauer test, Citrate utilization test, Kligler’s iron agar (KIA) test as described by Lowe; Motility test as mentioned by Arora and ONPG test (O-Nitrophenyl-D-Galacto-Pyranoside) with the protocols as described before¹⁴. The results of biochemical tests were observed after incubation at 30°C for 24-48 hours.

Stress response characteristics of rhizobial isolates

To see the stress response characteristics, isolates were grown in Yeast Extract Mannitol Agar (YEMA) medium at different range of temperatures (30°C-45°C), pH levels (4.5-9.5) and salt (NaCl) concentrations (1%- 4% (w/v)). Following incubation at 30°C for 24-72 hours, growth of the isolates was observed. Antibiogram of the strains were performed using ampicillin (10 mg), chloramphenicol (30 mg), ciprofloxacin (5 mg), erythromycin (15 mg), kanamycin (30 mg), nalidixic acid (30 mg), rifampicin (5 mg), and streptomycin (10 mg) discs following the Kirby-Bauer disk diffusion method¹⁵. The zone diameters for individual antibiotics were translated to sensitive, intermediate and resistant categories by referring to Clinical & Laboratory Standards Institute (CLSI) guidelines¹⁶.

Molecular characterization

DNA was extracted and purified from the isolates using standard phenol-chloroform-isoamyl alcohol method⁷. Quality and quantity of isolated DNA was measured by NanoDrop™ spectrophotometer (Thermo Fisher Scientific, USA). Approximately 1.5 kb of the 16S rDNA gene of the isolates was amplified in a thermal cycler (Eppendorf Mastercycler®, Germany) by PCR using the primers Forward: 52 - AGAGTTTGATCMTGGCTCAG -32 , and Reverse: 52 - GGTTACCTTGTTACGACTT -32 . The PCR condition was: initial denaturation of 5 minutes at 95 °C followed by 30 cycles of denaturation of 45 seconds at 94°C, annealing of 45 seconds at 58 °C, extension of 2.5 minutes at 72°C with a final extension

of 10 minutes at 72°C. The PCR products were electrophoresed in a 1% agarose gel containing ethidium bromide and observed under UV transilluminator. For amplified rDNA restriction analysis (ARDRA), the PCR products were digested overnight with *AluI* (Thermo Fisher Scientific, USA) restriction enzyme. After agarose gel electrophoresis on 1.5% gel containing ethidium bromide, the resulting digested products were visualized on a UV transilluminator. For sequencing, the PCR product was sent to FirstBase Malaysia and the sequences were processed using FinchTV. The sequences were blasted at NCBI, and were also analyzed using MEGA v6.0.

Results and Discussion

The importance of rhizobial species identification particularly stands on the increasing demands of biofertilizers for organic farming. Also, the methods and typical morphological and cultural characteristics of nodulating rhizobial strains have been standardized¹⁷. It is reported that inoculation of indigenous rhizobial strains in soil increases the fertility of soil as well the agricultural production¹⁸. To understand the rhizobial diversity of soil samples of Bangladesh, we have successfully isolated 20 strains capable of producing moderate to large colonies on CRYEMA medium. Among them, 16 showed pinkish colonies and the remaining 4 showed whitish colonies. These four isolates produced copious amount of extracellular polysaccharide (EPS). All the isolates were Gram negative rod-shaped, non-spore-forming and motile bacteria (Figure 1A and 1B). They were able to utilize citrate as a source of energy, and positive for catalase and b-galactosidase activities. Also, these strains were non-fermentative, do not produce acetoin and hydrogen sulfide, and negative for typtophanase, urease, and gelatinase activities. All these findings indicated that the isolated strains were rhizobial strains¹⁹⁻²¹.

Salinity intrusion has a common effect on food crops, livestock, and fish species of Bangladesh and the response to such stress are diverse in microbial community²². In addition to that, response to other stress like temperature, pH, and antibiotics are highly variable among the rhizobial species¹⁷. Changes in these stresses are one of the limiting factors to restrict the efficiency of nitrogen-fixation in legume-rhizobia symbiotic association²³. In our study, we have observed that all the isolates were able to grow on 2% (w/v) NaCl, at 30-33 °C, and within the pH range 6.5-7.5. However, the growth declined with the increased salt concentration (Figure 2A). Only 2 isolates were able to tolerate 4% (w/v) NaCl. This kind of salt tolerance is similar to the previous studies²⁴. Growth of the isolates were also inhibited with an increase or decrease of pH from 6.5-7.5, except the same two isolates (Figure 2B). The growth of the isolates hindered when the temperature is increased, and none of the isolates could tolerate temperature above 45 °C (Figure 2C). 16 out of the 20 strains were resistant to ampicillin and erythromycin, and 15 strains showed resistance to rifampicin and streptomycin. However, all these strains were sensitive to ciprofloxacin and nalidixic acid, and 18 strains were sensitive to chloramphenicol and kanamycin (Figure 2D). All these data indicated that the

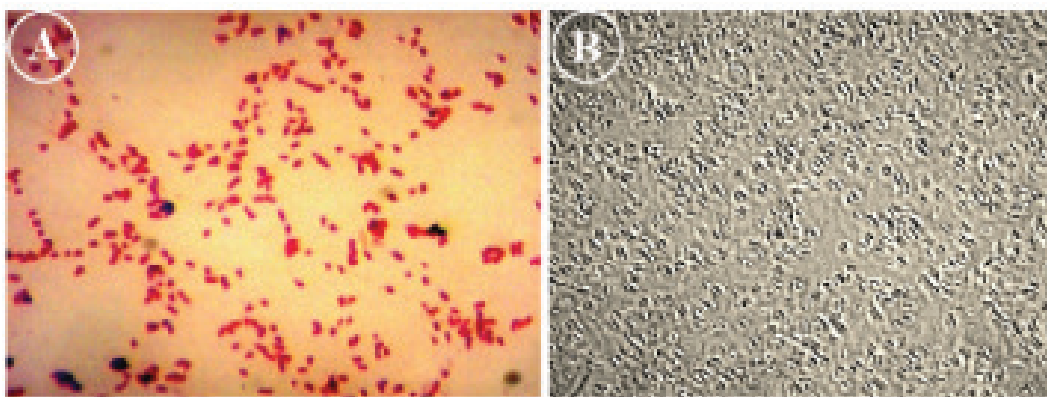


Figure 1. Representative microscopic images of the rhizobial isolates at 100X. (A) Gram staining revealed that they were Gram negative. (B) Phase contrast microscopic image of the isolates.

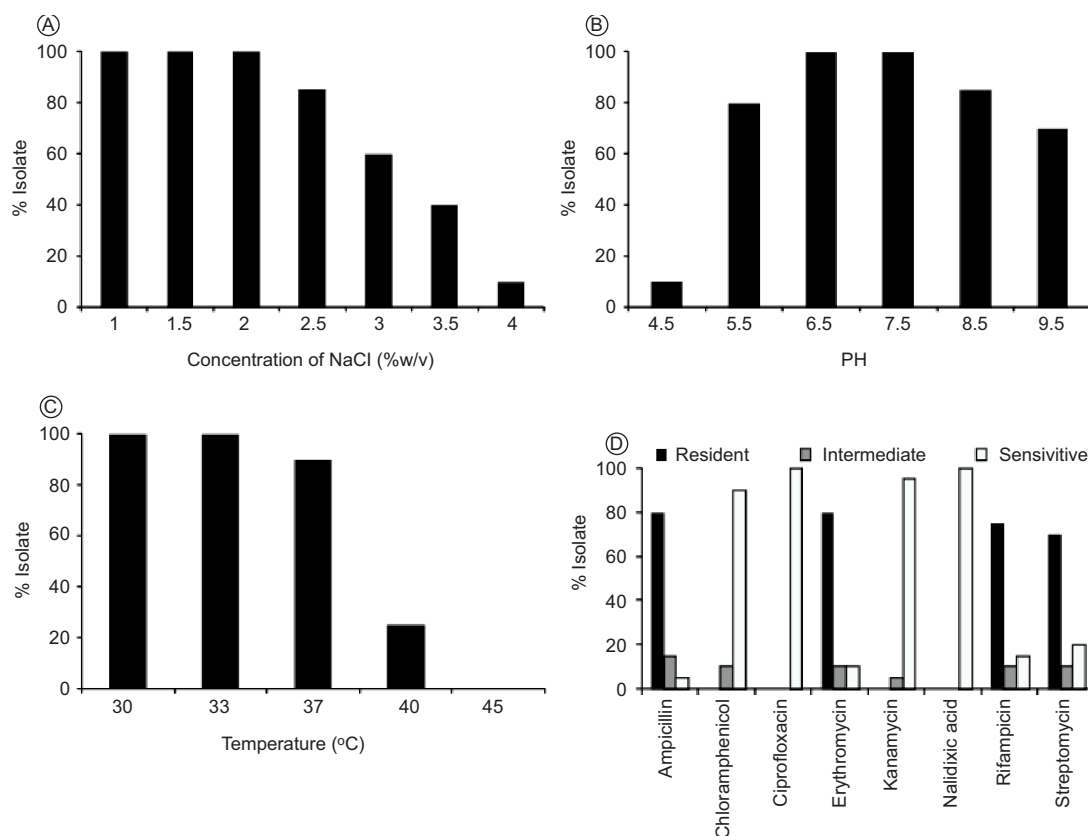


Figure 2. Stress tolerance of the isolates. Individual isolates were grown in (A) different concentrations of NaCl, (B) different pH, (C) different temperature, and (D) in presence of different antibiotics.

isolated strains were diverse in response to salt, temperature, acid, and antibiotic stresses.

16S rDNA sequencing is a powerful tool for the identification of microbial species²⁵ and we have identified our isolated rhizobial strains by 16S rDNA genotyping. For this, Universal primers (also known as primers 27F and 1492R) were used to partially amplify the 16S rDNA. The expected PCR product of around 1.5kb was observed in case of all isolates (Figure 3A). The PCR products were subjected to sequencing to identify the species, and also the amplified rDNA restriction analysis (ARDRA) to define the genetic diversity. The digestion of 16S rDNA PCR

products by *Alu1* for ARDRA indicated 3 genotypic groups. ARDRA group 1 consisted of 18 isolates. Of the two remaining isolates, one was in ARDRA Group 2 (*R. bangladeshense*) and the other one was placed in ARDRA group 3 (*R. qilianshanense*) (Figure 3B) revealed these strains were of three ARDRA groups with group 1 consisting of 18 strains. Partial 16S rDNA sequence analysis revealed that these 18 strains belong to *Rhizobium azibense*. The remaining two strains were *Rhizobium bangladeshense* and *Rhizobium qilianshanense*. These *R. bangladeshense* and *R. qilianshanense* were isolated from the soil samples adjoining the root of *Caesalpinia pulcherrima* and

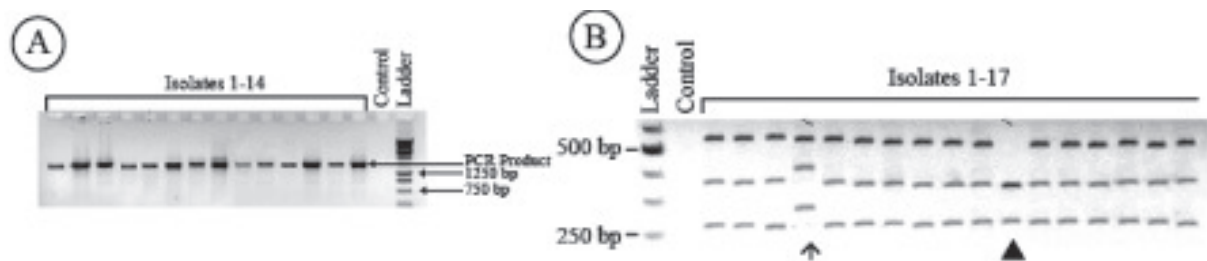


Figure 3. Amplification of partial 16s rDNA and ARDRA of the isolates. (A) Partial 16s rDNA was amplified using universal primer. (B) Three distinct ARDRA pattern was observed confirming three rhizobial species.

Lablab purpureus respectively. These observations indicated that the rhizobial species mostly associated with the nodules of different legumes is *Rhizobium azibense* and the rhizobial community in the tested soil samples were genetically less diverse.

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