



## Original Article

### Molecular characterization of rhizobial isolates from *Sesbania bispinosa*

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**ABSTRACT:** Amongst the soil bacteria one unique group, the Rhizobia have a beneficial effect on the growth of plants. The bacterium within root nodules converts atmospheric nitrogen to ammonia and provides organic nitrogenous compounds to the plants. The symbiotic association between rhizobia and leguminous plants is one of the major contributors to the total biological nitrogen fixation, which is an alternative to the use of nitrogen fertilizers that lead to unacceptable pollution levels. Of the different legumes, one well-known legume of Bangladesh, Dhaincha (*Sesbania bispinosa*) have generally been considered as an important green manure among the farmers. Thus it was important to study the rhizobial isolate of this less studied legume in order to understand its association to increase soil fertility. The test isolates were previously isolated and characterized and identified through biochemical tests. They gave the correct size amplification product for the *nifH* gene and *nodC* gene, which indicated their possession of nodulation and nitrogen fixation ability. This was further confirmed when the same isolates were found to produce nodules in plant infection test in the laboratory. Furthermore, the amplification band of *nifH* gene was observed only in plasmid, further proving that the symbiotic genes might be plasmid borne.

Lipopolysaccharide profiling showed variation in some bands indicating certain diversity prevails among the rhizobial isolates with respect to LPS expression. The isolates were observed further and found to be capable of phosphate solubilization. These characteristics made these rhizobia a suitable choice for use as in symbiotic association with *Sesbania bispinosa* to work as biofertilizer.

Biological Nitrogen fixation (BNF) technology can play an important role in substituting for commercially available N fertilizer. The test isolates from *Sesbania* sp. can be an environmental friendly solution in the face of N-fertilizer pollution. Thus it's important to further characterize these isolates in the molecular level to choose the ideal strain of rhizobia for *Sesbania bispinosa*.

**KEYWORDS:** Rhizobium, *Sesbania bispinosa*, biofertilizer, *nif* gene, *nod* gene, LPS

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## INTRODUCTION

Rhizobia are alpha-proteobacteria which lives in a symbiotic relationship with leguminous plants by forming nodules in root where these bacteria fix atmospheric nitrogen. These N<sub>2</sub> fixing microorganisms are able to enhance the nitrogen fixation performance and also may increase nutrient level in soil through the production of substances like hormones, siderophores, phosphate solubilization, water uptake etc. These microbes help to enrich soil fertility and also counteract agro environmental problems<sup>1</sup>. The most limiting nutrients for plant growth are N and P<sup>2</sup>. Although soil may contain vast amounts of either nutrient, most is not readily available for plant use. Most of N is tied into the soil organic matter. Even after fertilization, plants have to compete with soil microbes for easily available soluble N. Problems with P are different. In acidic soils, even when added in substantial quantities as fertilizer, P precipitates with iron or aluminum, whereas in alkaline soils P precipitates as calcium phosphates<sup>3</sup>. Accordingly, P limitation may be a difficult problem to

overcome through the addition of P-containing fertilizers. The extensive fertilization required to overcome N and P limitations may lead to (sub-)surface runoff, and the soluble nutrients can easily end up in surface-water bodies or groundwater. For example, the loss of P from agricultural systems is one of the main causes of eutrophication and hypoxia in lakes and estuaries in the developed world<sup>4</sup>. In Bangladesh the consumption of chemical fertilizer increased to a peak value of 4.45 billion tons in 2007 and is still increasing<sup>5</sup>.

Methods for identifying rhizobial strains include serological techniques<sup>6</sup>, introduction of antibiotic resistance markers, and analysis of total cell protein profiles<sup>7</sup>. All of these techniques have practical drawbacks. Antisera to rhizobial cells are cross-reactive with many strains within the same species, and pre-absorption<sup>8</sup>, purification of strain-specific antigens<sup>9</sup> or production of strain-specific monoclonal antibodies<sup>10</sup> is required. An antibiotic resistance marker may interfere with symbiotic functions<sup>11</sup> or be exchanged between strains<sup>12</sup>. Comparison of outer

membrane protein and/or lipopolysaccharide (LPS) profiles has been used for strain identification in other species<sup>13,14,15</sup>.

Present study was about the symbiotic association of *Rhizobium* sp. with the leguminous plant *Sesbania bispinosa*, which is a well-known green manure plant in Bangladesh. The biological nitrogen fixation (BNF) is a way to eliminate the usage of chemical fertilizer and also to prevent the damage to the agricultural ecosystem. This biological process is certainly the cheapest and the most effective tool for maintaining sustainable yields in less developed, poor countries where the consumption of N fertilizer is limited for economic reasons. To use *Rhizobium* as biofertilizer, its characteristics should be focused starting from its plasmid study, its ability of nitrogen fixation and nodulation through genomic characterization, lipopolysaccharide profiling and also growth promoting attributes. The second key molecules in legume infection are surface polysaccharides including exocellular-(EPS), capsular-(KPS) and lipopolysaccharides (LPS)<sup>16</sup>. Several lines of evidence indicate that these compounds prevent elicitation of, or protect against, plant defense reactions triggered by invading bacteria, either by contributing a structural protection (e.g. against reactive oxygen species) or a signaling function<sup>17-20</sup>.

The present research aims at attaining knowledge about the physiological, biochemical and molecular aspects of the organisms that nodulate the local *Sesbania* sp. Nitrogen fixation and plant growth enhancement by rhizosphere bacteria might be important factors for achieving a sustainable agriculture in the future.

## METHODS AND MATERIALS

### *Collection of nodules*

The host plant, *Sesbania bispinosa* was collected from four different places of Bangladesh that includes Savar, Demra, Kishorganj and Sylhet. The selected nodules were usually light brown or pinkish in color, to indicate that an active nitrogen fixation has been established between the nodule bacteria and the legume plant. Besides collection of nodule from leguminous plant, the rhizospheric soil around the plant was also collected.

### *Isolation and Identification of root nodule bacteria*

Nodules were thoroughly washed under tap water and then carefully severed from the root with sterile forceps. Intact, undamaged nodules were sterilized<sup>22</sup>. The first step of the isolation process was to crush the sterile nodules with a blunt tipped forcep in a large drop of sterile water in a petri dish. One loopful of nodule suspension was streaked on Yeast- Mannitol Agar (YMA) plates. The colony characteristics (i.e. shape, size, color, opacity, elevation, edge, margin of the bacterial colony and their growth rate) were determined by observing the colonies on YMA plates after growth at 30°C.

### *Growth promoting properties of the Isolates* *Phosphate Solubilization Test*

Ability to solubilize phosphate was observed by inoculating Pikovskaya's agar medium<sup>23</sup>. Presence of clear zone around the colony on the streaked plates was indicative of phosphate solubilization.

### *Analysis of Plasmid profile*

Plasmid profile analysis involved the direct lysis of bacteria and observed on an agarose gel prior to electrophoresis. The plasmid profile can be used as phenotypic markers to identify particular strains.<sup>24</sup> Plasmids were observed by Agarose Gel Electrophoresis.

### *Amplification of nif H gene and nodC gene from the Rhizobial strain*

Rhizobial cultures meant for genomic DNA extraction were propagated overnight at 28°C in 10 mL YEM broth. The genomic DNA of rhizobial isolates was extracted<sup>25</sup>.

Two primers, nif H1 5' CGT TTT ACG GCA AGG GCG GTA TCG GCA 3' and nif H2 5' TCCTCC AGC TCC TCC ATG GTG ATC GG 3' were used for amplification of nif H region (781 bp) from genomic DNA and plasmid<sup>26,27</sup>.

The nodC gene from DNA and plasmids of root nodule isolates was amplified using the nodC gene primers nodC1 5' GCC ATAGTG GCA ACC GTC GT 3' and nodC2 5' TCA CTC GCC GCTGCA AGT C 3'<sup>26</sup>.

### *Analysis of Lipopolysaccharide profile through SDS-PAGE analysis*

Analysis of lipopolysaccharide of rhizobia isolated from *Sesbania bispinosa* was carried out by SDS-PAGE method<sup>27</sup>.

## RESULTS AND DISCUSSION

The host plant *Sesbania bispinosa* and 36 rhizobial isolates were collected from four different places. Among these, 14 isolates were extracted from nodule and 22 were from associated rhizosphere.

### *Growth promoting Attributes of the strains:*

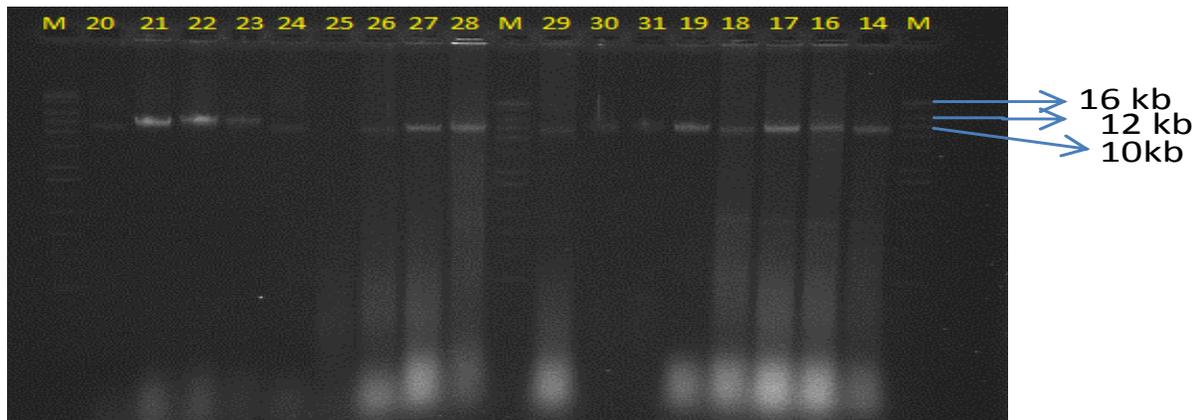
Rhizobia, like other plant growth promoting bacteria, were able to solubilize both organic and inorganic phosphates. Growth promoting characteristics was observed in 24 isolates among 36 isolates through phosphate solubilization. Nine isolates displayed a large hollow zone around the colonies by solubilizing tricalcium phosphate on the Pikovskaya's agar within 24 hrs of growth (Fig. 1). In one study, it was found that phosphate solubilization started after 24 h of incubation maximum in *Sinorhizobium* spp<sup>28</sup>.

### *Analysis of plasmid profile of the isolates*

The study revealed, all isolates contained plasmids of moderate size. The average plasmid sizes were around 10 Kb (Fig. 2). One isolate from Sylhet soil contained one large plasmid (>16 kb). Megaplasmids, from *R. meliloti* bearing important functional genes have been reported earlier<sup>29</sup>. These plasmids were not always associated with symbiosis; in some cases they also contain genes involved in overall cellular activities.



**Figure 1:** Rhizobium isolated from soil (Savar) with hollow zone around colony on Pikovskaya's Agar

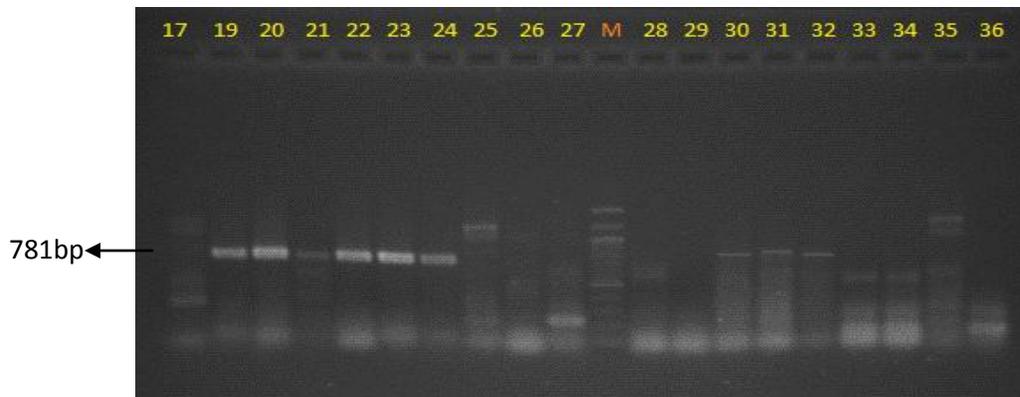


**Figure 2:** Plasmid profile of the test isolates observed in agarose gel electrophoresis. M- Marker size is mentioned here.

**PCR analysis of *nifH* gene and *nodC* gene**

The genomic DNA of rhizobial isolates were subjected to PCR-based analysis for *nifH* and *nodC* diversity. The test isolates were amplified by PCR and a product of 781 bp was observed in some isolates. Amplification was done from both DNA and plasmids. PCR using DNA from 15 isolates gave the correct size product. However only one nodule isolate showed amplified 781 bp band from plasmid. The amplification of *nifH* using plasmids gave 781 bp band and was observed in one nodule (savar) sample only.

The isolates were further investigated for the presence of *nodC* gene. Only two isolates from Sylhet nodule sample showed band at 500 bp position, when genomic DNA was amplified (Fig. 3). But no band was observed when plasmid was amplified using the same primer pair for *nodC* gene. These two isolates were similar to *Sinorhizobium* strains by amplification of *nifH* and *nodC* fragments, as *Sinorhizobium* strains contain *nifH* of 781 bp and *nodC* at 500 bp<sup>25, 26</sup>.

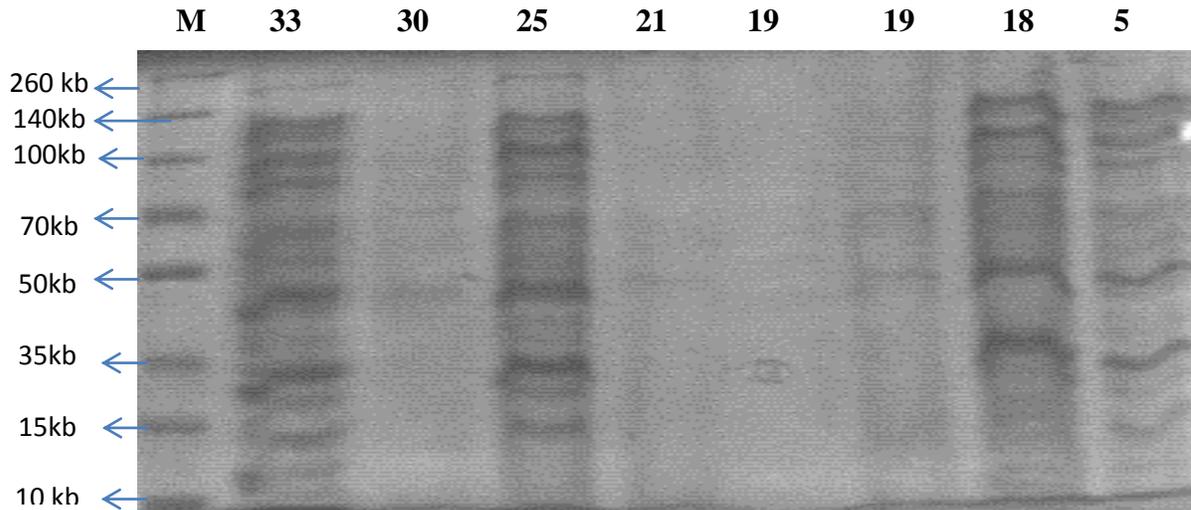


**Figure 3:** PCR profiling of *nifH* gene isolates from *Sesbania* (from DNA) [lane 19, 20, 21, 22, 23, 24 contains 781bp band]

An earlier report, suggested that the size of the amplified product of the symbiotic gene region can vary within the species. They characterized 43 strains of *Rhizobium leguminosarum* bv. *viciae*, *trifolii*, and *phaseoli* based on PCR amplification of chromosomal and symbiotic gene regions<sup>25</sup>.

#### Lipopolysaccharide profile analysis

Lipopolysaccharide profiling was done to investigate the diversity among the isolates from same plant. Four nodule isolates and three isolates from soil were randomly selected to observe the LPS profile. Some of the strong bands of different sizes were common to all isolates (Fig. 4).



**Figure 4:** Lipopolysaccharide profile analysis of isolates (lane-5, 19, 25, 30 are Nodule isolates and lane- 18, 21, 33 are soil isolates)

The SDS-PAGE analysis of LPS not only helps in identification of the rhizobial strains but was also useful in differentiating among the isolates within the same serogroup<sup>29</sup>. The profiles of purified LPS from both bacteria and bacteroids were identical and this is true for the LPS profiles of whole-cell lysates, bacteroids, or nodule squashes. This indicates that no changes occur during nodulation. It was suggested in a study that the differences in the LPS profiles were associated to the differences in host specificity or to the loss of a symbiotic plasmid<sup>30</sup>.

In the present study, the LPS profile as observed in an SDS gel showed some bands common to all isolates (ranging from 35kb-150kb) both from the nodule and soil isolates. However the thicknesses of the bands varied which could be due to the variation in the expression of the same LPS band for the different strain. This could be explained that there was certain diversity among the strains depending on the type of gene expression. At the same time some bands were observed in some strains while those were absent from the others. A single large sized band of 260 kb was observed in two of the isolates that were not present in the other lanes (Fig 4), one of the isolate was from nodule sample and the other one was from soil sample.

From Growth promoting attributes, Plasmid profile, PCR amplification and Lipopolysaccharide profile analysis, it was observed that the rhizobia isolates from same host *Sesbania* indicated diversity in their characteristics. These isolates showed high capability of solubilizing phosphate and all isolates contained plasmids. The nitrogen fixing or nodulation capability can be transferred to other bacteria

through further studies. In future, extended research is needed to be done to explore other important characteristics of these isolates to use these as a potential biofertilizer.

#### CONCLUSIONS

Increases in crop production have been made possible through the use of commercial man-made fertilizers. The use of nitrogen (N) fertilizer has increased almost ninefold and phosphorus (P) more than fourfold<sup>2</sup>. The tremendous increase of N and P fertilization, in addition to the introduction of highly productive and intensive agricultural systems, has allowed these developments to occur at relatively low costs. The increasing use of fertilizers and highly productive systems have also created environmental problems such as deterioration of soil quality, surface water, and groundwater, as well as air pollution, reduced biodiversity, and suppressed ecosystem function. In order to increase our understanding of the role of various root-associated organisms in plant growth and health as well as make use of their potential beneficial features as biofertilizers in plant production, more information is urgently needed on the interactions among plants and rhizosphere microorganisms.

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