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RESEARCH ARTICLE

ISOLATION, IDENTIFICATION AND CHARACTERIZATION OF *RHIZOBIUM* SPECIES FROM SOIL OF *CICER ARIETINUM* FIELD OF FARIDPUR IN BANGLADESH

Rabiul Islam[1*], Md. Abu Sayeed Imran[3], Md. Golam Mosaib[2] , Md. Ekhlas Uddin[2], Shahriar Mahmud[3], Shaharuq Nahid Sohana[3] and Md. Firoz Alam[3]

[1]Divisional DNA Screening Laboratory, Faridpur Medical College Hospital, Ministry of Women & Children Affairs, Faridpur-7800, Bangladesh
[2]Dept. of Biochemistry and Molecular Biology, Gono Bishwabidyalay, Dhaka-1344, Bangladesh
[3]Dept. of Biotechnology & Genetic Engineering, Islamic University, Kushtia-7003, Bangladesh

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ABSTRACT

Nutrient deficiency in the soil poses a big challenge to food production globally. The use of artificial nitrogen fertilizer to aid crop yield is a common farming practice, despite its undesirable effects and hazard to the environment and human population. This research work aimed at isolation, identification & characterization of *Rhizobium* species from chickpea rhizospheric soil samples collected of the southern region of faridpur district in Bangladesh. Isolation of *Rhizobium* species was culture on Yeast Extract Mannitol Agar (YEMA) medium incubated 3 days at 32^0C. A total of 10 *Rhizobium* species isolates were isolated from rhizospheric soil samples. They are also found to be gram-negative, rod-shaped morphology, fast grower, indole producers and positive for catalase test. All isolates were found with bare absorption of Congo red dye & no growth on YEMA with 2% NaCl. Out of 10 only 3 isolates (FSRS-3, FSRS-7, and FSRS-9) were identified as *Rhizobium* species on the basis of the authentication test (nodulation check with (*Cicer arietinum*). These three rhizobial isolates may be useful to increase the symbiotic biological nitrogen fixation in legume plant chickpea (*Cicer arietinum*) and can be used as potential biofertilizer owing to their plant growth-promoting characters.

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INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are a very low portion (about 2–5%) of the total rhizobacteria community. Such PGPR use one or more straight or indirect tackle to raise the growth and health of plants. These mechanisms can be effective together or independently at different stages of plant growth. Among these are phosphate solubilization, biological nitrogen fixation, development of other plant nutrients hoisted, and phyto hormone generation (like indole-3-acetic acid) are some of the moderator that profoundly impact plant growth (Geetha *et al*., 2012). Biofertilizers are actually the ordinary mini- fertilizers which are liable for providing safer plant nutrition and rising the soil fertility through natural processes (Debojyoti *et al.,* 2015). Biological nitrogen fixation is steered out by either symbiotic or free living prokaryotic, it is well documented that biological nitrogen fixation mediate by nitrogennase enzymes is a process significant to the biological activity of soil. Soil microorganisms that have retention of fixing nitrogen have repeatedly been reported as plant growth promoters. A number of microbes such as *Rhizobium*, Blue-green algae (*Cyanobacteria*), *Azotobacter*, *Azospirillum* and *Clostridium* can work important role in agriculture as Nitrogen fixing microorganisms (Temam *et al*., 2017). Nitrogen is a fundamental plant nutrient being an ingredient of amino acids, nucleic acids, nucleotides, chlorophyll, enzymes and hormones. Anyway, almost all soils are inferior of nitrogen; hence nitrogen is deliberated a limiting ingredient. Although nitrogen hold about 78% of the atmospheric air, it is not readily approach able to plant unless in the form of soil nitrate (Agah *et al*., 2016). Biological Nitrogen Fixation is a ordinary process where particular bacteria and leguminous plants with nodules in their root method are capable to convert the nitrogen gas into a form that is usable for plant life. The capability to fix atmospheric nitrogen into a form that can be used for plant growth is restricted to bacteria and cyan bacteria.

Corresponding author: **Rabiul Islam,**
Divisional DNA Screening Laboratory, Faridpur Medical College Hospital, Ministry of Women & Children Affairs, Faridpur-7800, Bangladesh.

Plants fix nitrogen only by virtue of associations with these simple organisms. The well-known associations are the symbiosis of *Rhizobium* bacteria with legumes. Nitrogen fixing leguminous plants not only supports plant growth free of mineral nitrogen in the soil but also promote soil nitrogen status for associated crops by the residues of these plants. Bacteria connected in symbiotic nitrogen fixation belong to genera *Rhizobium* and *Bradyrhizobium*. Rhizobia forms tumor like swellings called nodules on the root face of host plant. Rhizobia inside the nodules absorb air from the soil and alter gaseous nitrogen into ammonia. This association between the host plant and Rhizobia is reciprocally beneficial (Meenakshi *et al*., 2019).

MATERIALS AND METHODS

Sample Collection: Rhizospheric soil samples were collected from root zone of chickpea field at Saltha upozilla, Faridpur district in Bangladesh. Samples were kept in clean sterile bottles sealed and transferred to the Microbiology and Biochemistry laboratory at Gono Bishwabidyalay, Savar, Dhaka, Bangladesh and stored at 4^0C.

Preparation of Sample: Sample kept at room temperature before analysis. There are different ways to carry out physical fermentation process: Ten grams Rhizospheric soil samples were absorb in 90 ml of normal saline solution (8.5 g NaCl/L), homogenized for 20 min, appropriately mixed in normal saline. From every sample, serial dilutions were formed by following the method of Islam (Islam *et* al., 2020).

Isolation of Nitrogen Fixing Bacteria (*Rhizobium*): The soil samples were suspended in water by forceful vortexing and serial dilutions were formed up to 10^{-6} in barren distilled water. After appropriate dilution were added to petri plate on YEM Agar plate with the right calibration of pH (6.8-7) and cover for 72 hrs at 32^0C. Bacterial culture was repeated for three times by single colony streaking on YEMA medium. The cultures were subsequently sub-cultured and used regularly.

Morphological and biochemical Characterization

Gram Staining: The colony feature (i.e. form, guise, color, mound, margin of the bacterial colony and their growth rate) were destined by observing the colonies on YEMA plates of the overnight grown microorganisms at 32°C. Microscopic watching of the isolates was done using Gram staining fetch.

Catalase Activity: Isolates of 48 hours old culture were flooded with hydrogen peroxide to look on the release of bubbles of oxygen around the bacterial colonies.

IAA Test: YEMA Culture media add tryptophan (0.1%) then all isolates inoculated with this media. Then it kept Shaking incubator (32ºC, 100 rpm) for 48 hours.

Characterization and plant nodulation check test

Growth on 2% NaCl: To the basal medium of YEMA, 2% NaCl was added to check the growth of isolates. As 2% NaCl is inhibitory for some rhizobial isolates it may can be able to serve as tools for identification of isolates.

Congo red test: The integrity of the rhizobial isolates was discovering by adding Congo red in YEMA media. Most rhizobia bury the dye only poorly whereas contaminants including Agrobacteria, will absorb highly.

Plant nodulation check test: The different isolates were tested for their capability to nodulate *Cicer arietinum* plants grown in plastic pots. Seeds of *Cicer arietinum* were inoculated with 10 *Rhizobium* isolates by soaking seeds. Plants were gingerly uprooted after 15 days, 26 days and 40 days respectively and observed for nodulation.

Inoculum preparation: *Rhizobium* inoculant was prepared by using broth culture. Yeast mannitol broth was prepared on Erlenmeyer flask and was sterilizes at 121^0C for 15 minutes. Then the liquid medium was kept for cooling. After cooling down, a small amount of *Rhizobium* species was transferred aseptically from the agar medium to liquid medium with the aid of a sterile inoculating needle.

The flask containing broth and isolates was then placed on the shaker at 28^0C under 120 rpm for three days to accelerate the growth of *Rhizobium*. After three days, growth was observed on the flask and it was taken out from the shaker for inoculation process.

Seed preparation: Before placing, seeds were washed with tap water then it sterilized by 98% alcohol for 5 minutes afterward washed up with distilled water for 5 minutes and again with sterile distilled water for 2 minutes. Seeds were kept at laminar air flow for few minutes to become dry.

Inoculation process: Broth culture poured onto the few amount of three times autoclave sterile soils and mixed with hands by using sterile hand gloves. Sterile soils mixed with *Rhizobium* isolates were placed on one pot whereas another pot contained only sterile soil but no *Rhizobium* isolates. Seeds were then placed in the pots by maintaining a certain amount of distance between them. Every pot contained four seeds and was kept in a place full of sun light for proper growth.

RESULTS

All the isolates morphological characteristics and biochemical test result are given in (Table 1). *Rhizobium* isolate (FSRS-3) on YEMA media showed in (Figure 1). Microscopic observation of *Rhizobium* isolate (FSRS-7) showed in (Figure 2). Cultural growth on different media and nodulation check result of *Rhizobium* species isolates are given in (Table 2).Observation of nodulation check of *Rhizobium* isolate (FSRS-9) with *Cicer arietinum* plant after 26 days showed in (Figure 3).

DISCUSSION

Nitrogen is necessary in plant cells for implication of enzymes, proteins, chlorophyll, DNA and RNA, thus important for plant growth and production of food and feed. Nitrogen (N) is a component of proteins, enzymes, chlorophyll, and growth moderator to plants and its scarcity causes wanted growth, leaf yellowing, attenuated branching and small trifoliate leaves in legumes (Simon *et al*., 2014). Nitrogen is essential component for plant growth and development which is supplied by mutual symbiosis of rhizobia in cultivated legume plants. Biological nitrogen fixation could aid to enrich agricultural productivity and assure food security.

Table 1. Morphological Characteristics and Biochemical test result of *Rhizobium* species isolates

Name of the Isolates	Gram stain	Shape	Catalase test	IAA test
FSRS-1	Negative	Small rod	Positive	Negative
FSRS-2	Negative	Small rod	Negative	Positive
FSRS-3	Negative	Small rod	Positive	Positive
FSRS-4	Negative	Medium rod	Positive	Negative
FSRS-5	Negative	Small rod	Positive	Positive
FSRS-6	Negative	Small rod	Negative	Positive
FSRS-7	Negative	Medium rod	Positive	Positive
FSRS-8	Negative	Medium rod	Positive	Positive
FSRS-9	Negative	Small rod	Positive	Positive
FSRS-10	Negative	Medium rod	Positive	Positive

Table 2. Cultural growth on different media and nodulation check result of *Rhizobium* species isolates

Name of the Isolates	Congo red dye	YEMA with 2% NaCl	Nodulation check with *Cicer arietinum* plant
FSRS-1	Positive	Absent growth	Absent
FSRS-2	Positive	Absent growth	Absent
FSRS-3	Positive	Absent growth	Nodule present
FSRS-4	Positive	Absent growth	Absent
FSRS-5	Positive	Absent growth	Absent
FSRS-6	Positive	Absent growth	Absent
FSRS-7	Positive	Absent growth	Nodule present
FSRS-8	Positive	Absent growth	Absent
FSRS-9	Positive	Absent growth	Nodule present
FSRS-10	Positive	Absent growth	Absent

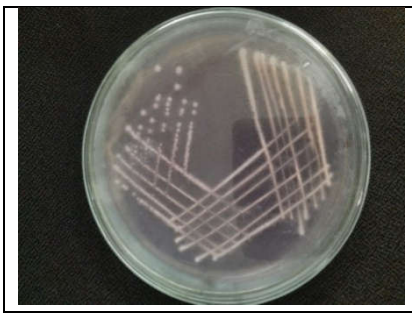

Fig 1. *Rhizobium* isolate (FSRS-3) on YEMA media

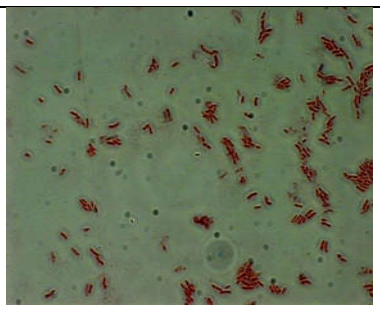

Fig 2. Microscopic observation of *Rhizobium* isolate (FSRS-7)

Fig 3. Nodulation check of *Rhizobium* isolate (FSRS-9) with *Cicer arietinum* plant after 26 days

Plant growth promoting Rhizobacteria (PGPR) are soil resident that are capable to colonize plant roots, incite plant growth, and augment crop yields (Kasa *et al*., 2020). *Rhizobium* is an significant microorganism for the environment reason of its nitrogen-fixing capability when in symbiotic relevance with plants (mainly legumes). This lesson confirmed that the root nodules of chickpea plants asylum the nitrogen-fixing bacterium- *Rhizobium* (Zeenat *et al*., 2017). The degree of specificity between leguminous plants and rhizobia is highly variable (Dhaoui *et al*., 2016). It also proved that these plants (*Cicer arietinum*) when inoculated with *Rhizobium* isolates create nodule after 26 days that are authentically identified that three isolates (FSRS-3, FSRS-7 and FSRS-9) are *rhizobia* species. After 50 days when nodule section that are showed red color that are conformed active rhizobia species. These three potential rhizobia isolates are sequencing for next time and identify their genus. This isolates will comprehensively extend agricultural production, if they are often used to inoculate legume plants basically *Cicer aritinum,* thereby abatement the environmental intimidation of artificial nitrogen fertilizers.

CONCLUSION

Symbiotic process for biological nitrogen fixation (BNF) in agriculture are most hopeful. Accordingly, the current study was directed for the isolation, identification, and Characterization of nitrogen fixing bacterial isolates of *Rhizobium* characterization of nitrogen-fixing bacterial isolates of *Rhizobium* from the legume plant of chickpea which grows extensively in almost every area of Bangladesh. In this experiment three potential (FSRS-3, FSRS-7, and FSRS-9) *Rhizobium* isolates were identification by morphological characteristics, biochemical test and plant nodulation check test. In future this isolates are experiment carried out in field experiment and prepare *Rhizobium* inoculum are use as biofertilizer which can help agricultural sector in Bangladesh.

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