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Isolation and identification of *Rhizobium* from saline soils of Bangladesh and preparation of mother culture

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Abstract: Nitrogen is an essential plant nutrient which is required for the maximum growth and yield of agriculturally important crops. Microbial inoculants may supplement N-requirement and reduce the dependency on costly synthetic N-fertilizer in respect of crop yield. The ability of soil microorganisms to fix atmospheric nitrogen is an important trait in promoting plant growth and increasing crop yield. The study was conducted for the isolation and identification of nitrogen fixing bacteria from saline soils of different locations of tidal floodplain region of Bangladesh. Three *Rhizobium* strains were isolated and purified. The isolates were preliminarily identified on the basis of their morphological and biochemical characteristics. Based on the results, it can be concluded that the isolates possess great potential to be developed as biofertilizers to enhance soil fertility and plant growth. However, their performance under green house and field conditions should be assessed before being recommended for biofertilizer production and their applications.
Key words: Nitrogen, inoculants, biofertilizer,rhizobia, saline soils.

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 on 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 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). *Rhizobium* is the soil microorganism that can survive in the soil or forms a symbiotic association with the host legume.The most convenient method of obtaining *Rhizobium* from nature is by isolation from root nodules. In contrary to popular belief, many of the bacteriods in nodule are viable. It is impractical to isolate rhizobia directly from the soil because of their fastidious growth.The primary objective of the proposed research is to develop a cheap organic nitrogen fertilizer that could supplement synthetic nitrogen fertilizer.

Materials and Methods

Sampling site: Selected sampling sites were Dumuria upazilla of Khulna AEZ 13 (Ganges Tidal Floodplain).

Collection of soil samples: For isolation of Rhizobia, soil samples were collected from selected areas. Ten surface soil samples were collected from each location. For the convenience of discussion, the 3 soils are referred to as soil -1, soil-2, and soil-3 and these soil samples were collected from Rangpur, Dumuria, Khulna; Rudroghor, Dumuria, Khulna; and Gutudia, Dumuria, Khulna, respectively.

Preparation of the soil sample: Some portions of collected soil sample were kept in refrigerator at 4^{o}c for isolation of bacteria. The rest portions of soil samples were then air dried ground to pass through a 2 mm sieve andthen mixed to from a composite sample.Then these composite sampleswere kept in clean and sterilized bottles for physical and chemical analysis.

Experimental site: The laboratory experiment was conducted at the Department of Soil Science and central laboratory, Patuakhali Science and Technology University, Dumki, Patuakhali during july, 2014 to june, 2015.

Soil analysis: Theinitial soil samples were analyzed for physical and chemical characteristics. The physical characteristics includes textural class and the chemical properties include soil pH, organic matter, electrical conductivity, total N. exchangeable K, available P and S content. Results of this analysis have been presented in Table -1.

Table. 1. Physical and chemical characteristics of collected soils

Properties of soils	Soil-1	Soil-2	Soil-3
% Sand	29.2	28.9	29.15
% Silt	60.75	60.82	60.56
% Clay	10.05	10.12	10.21
pH (H_2O)	7.77	7.67	7.65
EC	0.411	0.423	0.416
%OC	1.195	1.21	1.179
% OM	2.06	2.08	2.02
%N	0.017	0.018	0.015
P(ppm)	10.01	10.09	10.10
S(ppm)	99.33	99.45	99.57
K(meq/100g)	1.136	1.207	1.263

Culture media: Yeast mannitol agar media were used for culture of *Rhizobium.*

Method of isolation: Enrichment culture technique (in liquid medium) was used for isolation of bacteria.

Composition of Yeast extactmannitol agar: Mannitol - 10.0 g, K_2HPO_4- 0.5 g, $MgSO_4$, $7H_2O$ - 0.2 g, NaCl - 0.1 g, Yeast extract - 0.5 g, Distilled water - 1 litre, and Agar- 15 g. The medium was prepared and was autoclaved at 121 ^{0}C and 15 psi for 20 minutes. In the meantime all accessories like Petridis and pipette (1 ml) was also sterilized by autoclave.

Colony Isolation: The growth of *Rhizobium* was streaking on medium and incubated until pure growth was obtained. Finally, pure *Rhizobium* was cultured on slant medium as mother culture and stored in refrigerator. Then different biochemical test is to be done and new mother culture was done after 3 – 4 months. The colonies showing clear zones around them developed within 48 hours were transferred to agar slants of Yeast mannitol agar medium and allowed to grow at 30°±2°C for three days. The cultures were then repeatedly plated in the same agar medium till pure strains were obtained and finally 20 bacterial cultures were maintained in the Yeast mannitol agar medium.

Estimation of bacterial population: The viable cells were calculated by the following formula stated by Somasegaran and Hobben, 1985. Number of cells/ml(CFU/ml) = [{(Number of colonies) × (Dilution factor)} ÷ (Volume per drop)].

Purification of isolates: Three isolates of each *Rhizobium* were taken from respective cultured media and streaked on respective prepared plate's media. The streaked plates were incubated at 28^0C for 2-4 days. Repeated streaking was done until purification.

Preparation and preservation of mother culture: Purified isolates of *Rhizobium* were transferred into Yeast Mannitol Agar media and preserved for further study.

Identification of *Rhizobium* isolates: The isolates of *Rhizobium* obtained from soils were described according to their growth characteristics on solid and liquid Yeast Mannitol Agar media. Some morphological characters such as the shape, size, color, texture of colonies and ability to alter pH and some biochemical characters such as carbohydrate utilization and fermentation, gelatin and starch hydrolysis, Congo red dye absorption.

Results and Discussion

Estimation of Rhizobia: Bacterial populations of collected soils were determined and presented in Table 2. The results show that the highest populations of *Rhizobium* 3.4×10^6 was found in soil no. 1 (Rangpur, Dumuria, Khulna) and the lowest populations of *Rhizobium* 2.8×10^6 was found in soil no. 2 (Rudroghor, Dumuria, Khulna).

Table 2. Bacterial population of *Rhizobium* from collected soil sample

Location	*Rhizobium* ($CFUg^{-1}$)
1. Rangpur, Dumuria, Khulna	3.4×10^6
2. Rudroghor, Dumuria, Khulna	2.8×10^6
3. Gutudia, Dumuria, Khulna	2.9×10^6

Isolation of *Rhizobium* from saline soils of coastal region: Three *Rhizobium* isolates were obtained from Saline soil of coastal region. They were designated as R_1, R_2, and R_3, respectively (Table 3).

Table 3. List of isolates from saline and non saline soil areas

Sl. No.	Isolate name	Location
1.	R_1	Rangpur, Dumuria, Khulna
2.	R_2	Rudroghor, Dumuria, Khulna
3.	R_3	Gutudia, Dumuria, Khulna

Characterization of the isolates: Results of isolation as well as morphological and biochemical characteristics of isolates are presented below.

Morphological characteristics: Morphological characteristics of the isolates i.e. colony morphology have been presented in Table 4 and Table 5. The colony characteristics of isolates did not vary widely. All the isolates were found round shape, medium flat elevation, whitish colour, smooth surfaces, odour less, viscous consistency, opaque opacity with entire margin on congo red yeast extract mannitol agar (CRYFMA) plates. All the isolates were foundmedium, small, large in size.

Microscopic tests

Simple staining (shape and size of cells): The shape of the cells of rhizobia isolates are presented in Table 6. All the isolates were found short rod in shape. Vincent et al. (1980) stated that Rhizobium was rod shaped.

Motility test: All the 3 isolates under study were found motile in nature. Vincent *et al.* (1980) stated that Rhizobium was generally motile.

Gram reaction test: All the 3 isolates have shown gram negative in reaction. Vincent *et al*. (1980) stated that Rhizobium was gram negative.

Table 4. Colony characteristics of Rhizobium isolateson Yeast Mannitol Agar media

Isolate	Shape	Size	Elevation	Odor
R_1	Round	Medium	Medium flat	Odor less
R_2	Round	Small	Medium flat	Odor less
R_3	Round	Large	Medium flat	Odor less

Table 5. Colony characteristics of Rhizobiumisolates on Yeast Mannitol Agar media

Isolate	Margin	Surface	Opacity	Colour	Consistency
R_1	Entire	Smooth	Opaque	Whitish	Viscous
R_2	Entire	Smooth	Opaque	Whitish	Viscous
R_3	Entire	Smooth	Opaque	Whitish	Viscous

Table 6. Morphological (Microscopic) characteristics of Rhizobium isolates

Isolate	Shape	Gram reaction	Motility
R_1	Short rod	Gram negative	Motile
R_2	Short rod	Gram negative	Motile
R_3	Short rod	Gram negative	Motile

Biochemical tests: Results of biochemical tests are presented below-

Congo red absorption test: From the Table 7 it was observed that all the bacterial isolates did not absorb Congo red at young stage but absorbed slightly when

cultures became old. The isolates absorbed counter stain. Vincent *et al*. (1980) stated that Rhizobium was gram negative, rod shaped and generally motile. The isolates produce circular, low convex to convex, mucous and opaque white. The isolates were observed to lack the ability to absorb Congo red from yeast extract mannitol agar medium containing this dye (Plate 1). Similar result was observed by Barbar *et al*., (1983).

Table 7. Congo red absorption of different rhizobial isolates

Isolate	Congoredabsorption	
	Young culture	Old culture
R_1	Not absorbed	Weakly absorbed
R_2	Not absorbed	Weakly absorbed
R_3	Not absorbed	Weakly absorbed

Plate 1. Congo red absorption test of *Rhizobium*

BTB test: All the bacterial isolates produced acid in variable amounts on BTB-YEMA plates. The results are presented in Table 8. The growth of the all fast growers develops yellow color that results acidic in nature but the growth of all slow growers develops blue color that results alkaline in nature.

Table 8. Biochemical observation of different rhizobial isolates

Isolate	BTB test			Hoffer's alkaline test
	Growth	Observation	Result	Growth
R_1	Fast growth	Yellow colour	Acidic	No growth
R_2	Fast growth	Yellow colour	Acidic	No growth
R_3	Fast growth	Yellow colour	Acidic	No growth

Hofer's alkaline broth test: Among the nine isolates none had grown on Hofer's alkaline broth (Table 8).

Growth on Different pH: The growth responses of the Rhizobium isolates were investigated in the YEMA medium having 5 levels of pH. The pH levels 4.0, 5.0, 6.0, 7.0 and 8.0 were created adding HCl solution and 8.0 adding NaOH as required. Results in the Table 9. Show that all the isolates viz., R1, R2, and R3 were heavy growers at pH 6.0 and 7.0.But at pH 4 and 5 isolates were found slight to minimum growth. At pH 8.0 all isolates viz., R_1, R_2 and R_3 were found medium growth. Shraddha Bhatt *et al*., (2013) also found that rhizobia were grown in YEM medium with pH values of 4, 5, 7 and 9.

Table 9. Effect of different pH on *Rhizobium* isolates in yeast mannitol agar media

Isolate	Different pH				
	4	5	6	7	8
R_1	_	+	++	++	+
R_2	+	-	++	++	++
R_3	-	+	++	++	++

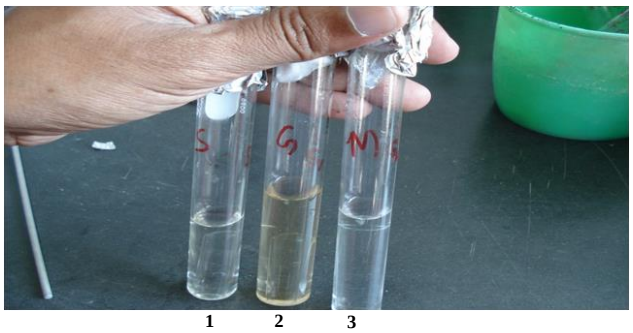
+++ =Heavy growth, ++ =Medium growth , + = Minimum growth, - = Slight growth and, - - = No growth.

Carbohydrate utilization: Results of carbohydrate utilization by the isolates are presented in Table 10. The sign of carbohydrate utilization was observed from the growth and fermentation characteristics of the isolates in a given carbohydrate medium and the variation in growth was identified by measuring the optical density of the media. It was observed that the isolates R_1 and R_2 showed heavy growth in mannitol. R_1 and R_2 showed heavy growth in sucrose. It was also observed that all the isolates showed minimum growth in glucose and produced gas in carbohydrate media used (Plate 2). Chowdhury and Knan (1968) and Podder and Habibullah (1977) working with chickpea isolates also recorded similar results. Graham (1964) reported that most of the rhizobial strains utilized glucose, xylose and arabinose but lactose and sucrose were utilized by a few slow growing rhizobia.

Table 10. Carbohydrate utilization and fermentation by the strains

Strain	Sucrose	Glucose	Manitol
R_1	+ + +	+	+ + +
R_2	+ +	+	+ + +
R_3	+ + +	+	+ +

+++ =Heavy growth, ++ =Medium growth and + = Minimum growth



1 2 3

Plate 2. Carbohydrate utilization test, 1-Sucrose, 2-Glucose, 3-Mannitol

Results in Table 11 show that all the isolates had the capacity to hydrolyse gelatin. But Podder and Habibullah (1977) reported that none of the isolates from chickpea used in his study could hydrolyse gelatin.

Table 11. Gelatin test of the *Rhizobium* isolates

Isolates	Gelatin hydrolysis
R_1	(+)
R_2	(+)
R_3	(+)

(+) = hydrolytic/, (-) = nonhydrolytic

Starch hydrolysis: R_1 and R_2 of the isolates gave positive results for starch hydrolysis (Table 12). R3 isolate gave

negative results for starch hydrolysis (Table 12). Halos developed around the bacterial colonies (Plate 3). Podder and Habibullah (1977) also noted that the isolates from chickpea failed to cause hydrolysis of starch.

Table 12. Starch hydrolysis and Catalase test

Isolate	Starch	Catalase
R_1	+	+
R_2	+	+
R_3	-	+

+++ = Rapid evolution of oxygen (O_2), ++ = Medium evolution of oxygen (O_2), + = Slow evolution of oxygen (O_2)

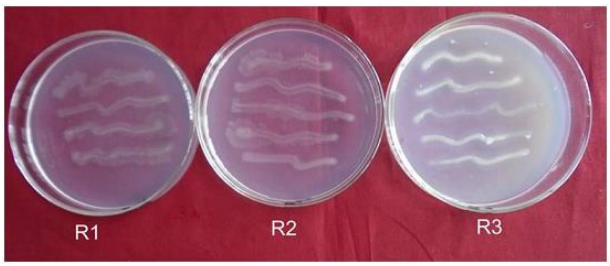


Plate 3. Growth of *Rhizobium* isolates on yeast mannitol agar media with starch

Catalase test: Results in the Table 12 show that All of the test isolates gave positive results for catalase test (Plate 4).

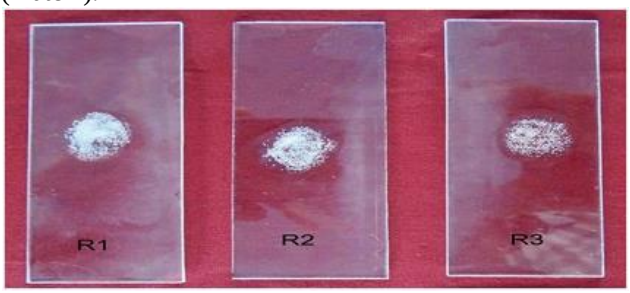


Plate 4. Catalase test of *Rhizobium* isolates

Growth at different temperature conditions: All the isolates showed good growth at temperature 28°C and 32°C (Table 13). Most of the isolates grew weakly at 14"C. At 22°C most isolates exhibited medium growth while five isolates R_l and R_2 recorded poor growth. All the isolates grew at 38°C. Only three isolate R_3 showed medium growth. Most of the isolates exhibit poor growth at 45°C.

Table 13. Growth of *Rhizobium* isolates in different temperature conditions

Isolate	Growth in different temperature condition					
	14℃	22° C	28℃	32℃	38"C	45℃
R_1	+ +	+ +	+ + + +	+ + + +	+ + + +	+
R_2	-	+ +	+ + ++	+ + ++	+ + ++	+
R_3	+ +	+ + +	+ + + +	+ + + +	+ +	-

- = No growth, + + + = Medium growth, + = Very poor growth, + + + + = Good growth, + + = Poor growth

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