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CHARACTERIZATION OF RHIZOBIA FROM ROOT NODULE AND RHIZOSPHERE OF *LABLAB PURPUREUS* AND *VIGNA SINENSIS* IN BANGLADESH

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ABSTRACT

Nitrogen fixation resulting from mutual symbiosis of rhizobia and cultivated legume plants is therefore critical to food security as it directly affects agricultural production. Biological Nitrogen Fixation (BNF) can be an important factor in sustainable agriculture. The isolation and identification of different slow growing and fast growing rhizobial strains from the nodules of two leguminous plant species. Symbiotic nitrogen fixing Rhizobium spp. was isolated from (Lablab purpureus and Vigna sinensis). Nodules samples were collected from plants growing in different Districts of Bangladesh and the Glucose-Peptone Agar (GPA), Congo red, Yeast Mannitol Agar (YMA) containing 2% NaCl were employed to make presumptive decisions on the recognition and classification of the isolated bacterial strains. All the isolates were found with poor absorption of dye Congo red and little or no growth on the media of GPA and without altering the pH. Almost all of the isolates exhibit growth on 2% NaCl, poor growth on GPA, thus confirming the rhizobia. After biochemical tests like catalase test and citrate utilization test isolates were confirmed as Rhizobia. The presence of rhizobia on root nodules of leguminous Plant. Not only the leguminous Plant but also the rhizosphere contains rhizobia which help in soil fertilization

KEYWORDS: Root Nodules, Leguminous Plant, Rhizobia, Symbiosis, Bangladesh

INTRODUCTION

Ecologically and agriculturally leguminous plant is important and this plant possesses a unique ability to establish symbiosis with nitrogen fixing bacteria of the family Rhizobiaceae from the genera Bradyrhizobium, Rhizobium, Allorhizobium, Mesorhizobium and Sinorhizobium [1], which are collectively referred as rhizobia, helps in biological nitrogen fixation by formation of root nodules. An efficient source of nitrogen is BNF [2]. Within the root nodules, colonization of bacteria occurs and it converts atmospheric nitrogen to ammonia and provides the plants an organic nitrogenous compounds. The soluble form of nitrite and nitrate can be assimilated by plant roots and utilized in synthesizing proteins and nucleic acids. Without these bacteria, within a short time organic matter such as straw or leaves would accumulate and soil would not be fertile [3]. BNF is on expanded interest of ecology that has drawn attention to the fact that is ecologically benign and its greater occlusion can minimize the use of fossil fuels and can be subsidiary in reforestation and in restoration of misused lands to productivity [4], [5]. Nitrogenous wastes are returned by the animals to the environment as uric acid [6]. The main way of 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 the human health [7]. It has been proven that the presence of rhizobia increases plant productivity without any harm to human health.

So for maintaining soil fertility, cultivation of leguminous plants is important which replenish atmospheric nitrogen through symbiosis with rhizobia in rotation with non leguminous plants. This study is aimed to isolate and identify rhizobium species from root nodules of Lablab purpureus and Vigna sinensis from the target area of Bangladesh for better agricultural growth.

This module introduces the general role of microorganisms in the soil, and specifically the rhizobia. Rhizobia are special bacteria that can live in the soil or in nodules formed on the roots of legumes. In root nodules, they form a symbiotic association with the legume, obtaining nutrients from the plant and producing nitrogen in a process called biological nitrogen fixation, or BNF. The rhizobia are broadly classified as fast or slow growing based on their growth on laboratory media. Rhizobia are further classified according to their compatibility with particular legume species. Farmers can stimulate BNF by applying the correct rhizobia to their legume crops, a process called inoculation. The module describes the diversity of rhizobia and the selection of superior strains, as well as plant and environmental factors that affect rhizobia in the soil.

METHODS

Study Area

This study was conducted in Primeasia University and samples were collected from different district of Dhaka division in Bangladesh

Collection of Nodules

Two different legume plants (Table 1) were collected from rural areas of Bangladesh. Fresh and healthy root nodules were selected from each plant for the present study. The selected nodules were usually light brown or pinkish in color, which indicates that an active nitrogen fixation had 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.

Table 1: List of the Sample and their Host Under Investigation

Sample Designation	Host Scientific Name
S-1	Lablab purpureus
S-2	Vigna sinensis

Surface Sterilization of the Nodules

Total fifty (50) samples of root nodules from *Lablab purpureus* and *Vigna sinensis* were collected randomly from five different localities of Dhaka city. Nodules were thoroughly washed under tap water and then carefully severed from the root with sterile forceps. Intact, undamaged nodules were immersed in 95% ethanol for 5-10 second to break the surface tension and then transferred to 3% solution of H_2O_2 and soaked for 2-3 minutes. Nodules were then rinsed in five changes of sterile distilled water using sterile forceps for transferring.

Isolation of Root Nodule Bacteria

The first step of the isolation process was to crush the sterile nodules with a blunt tipped forceps in a large drop of sterile water in a petri dish. Using yeast extract mannitol selective culture media (YEM) [8], [9],[10]) the nitrogen fixing bacteria can be isolated directly from the root nodules of the host plant or from the soil [11].

Maintenance of Cultures

The isolates were sub-cultured on YMA slants. Growth was observed at 30°C and then the slants were kept at 4°C. Periodic subcultures from these stock cultures were performed at a 15 days interval. For long time storage, the isolates were streaked on YMA slants and after incubation at the same temperature; sterile glycerol was added on the media and then stored at room temperature.

Morphological Characteristics of the Isolates

Colony Characteristics

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. Microscopic Observation of the Isolates was done using Gram staining technique as described by [12].

Cultural and Metabolic Characteristics

Presumptive Tests

Strain of rhizobia can be described according to their growth on the solid and liquid media. The size, shape, color, texture of the colonies and their growth on different media and their ability to alter the pH of the media are generally stable characteristics useful in defining strains.

Congo Red Test

The purity of the rhizobial isolates was detected by adding Congo red in YMA media [13]. Most rhizobia absorb the dye only weakly whereas contaminants including Agrobacteria, will absorb strongly.

Growth on Glucose- Peptone Agar

Glucose–peptone media was used to differentiate rhizobia, which usually shows little or no growth on the media without altering the pH of the media, contaminants like Agrobacteria, shows massive growth on the media with a distinct change in pH.

Growth on 2% NaCl

To the basal medium of YMA, 2% NaCl was added to check the purity of the isolates. As 2% NaCl is inhibitory for most rhizobial isolates it can serve as an identification tool.

Differentiation between Fast and Slow Growers on the Bromothymol Blue Media

Yeast Mannitol Agar (YMA) media incorporated with bromothymol blue was used to distinguish fast-(acid producing) growing strains from slow (non acid producing or alkali producing) growing rhizobia [13]. In this medium, the fast growers require 48 hours to produce an acidic reaction by turning the color of the media yellow from green, whereas the slow growers take >96 hours to produce alkaline endpoints with or without changing the color of the media from green to blue.

Biochemical Characteristics

To confirm whether the isolates were rhizobia or not, they were incubated in different media for each

physiochemical tests and then incubated depending upon their growth rate at 30°C.

Catalase Activity Test

The presence of the enzyme catalase in the rhizobial isolates was examined by suspending one loopful of organism in a drop of 3% H_2O_2 on a glass slide. This test was performed as per standard procedure [14]. Production of bubbles indicates a positive result.

Citrate Utilization Test

Citrate utilization by the isolates was observed by the growth on slants of Simmon's Citrate Agar. A distinct change in color from green to blue refers to as a positive test.

RESULTS

The present study encompassed the isolation and identification of different slow growing and fast growing rhizobial strain from the nodule of two leguminous plants and from the associated rhizospheres. Identification and Characterization.

Staining Properties

Microscopic observations were performed to investigate the isolate such as shape, Gram reaction. All the isolates were found to be Gram negative, short, rod shaped and are non- sporeforming.

Colony Morphology

Growth rate and colony morphology of the isolates were observed. Most of the isolates were found to produce translucent colonies with deep center, entire, convex, and are mucoid.

Presumptive Tests

The presumptive test was performed for strains to differentiate between fast growing and slow growing strains. The results of presumptive tests are summarized in Table-2.

Table 2: Presumptive Tests Results

Isolates	Growth on Glucose-Peptone Agar	Congo Red Test	Growth on 2% NaCl	Bromo-Thymol Blue Test
Vigna sinensis (nodule)	No growth	Pink colour	No growth	Blue colour
Vigna sinensis (rhizosphere)	No growth	Pink colour	No growth	Yellow colour
	Little growth	Pink colour	No growth	Yellow colour
Lablab purpureus (nodule)	Little growth but no colour change	Pink colour	No growth	Blue colour
Lablab purpureus (rhizosphere)	No growth	Pink colour	No growth	Yellow colour

All the isolates from rhizosphere acted as fast grower and produced acid but isolates from nodule acted as slow grower in Bromothymol blue. Isolates of Vigna sinensis (rhizosphere) grown on GPA showed little growth or no growth and isolates of Vigna sinensis (nodule) showed no growth on GPA. Isolates of Lablab purpureus (nodule) showed little growth but no colour change and isolates from Lablab purpureus (rhizosphere) showed no growth. In 2% NaCl no growth of the isolates was also observed. In congo red all the isolates showed pink colour or absorbed weakly. All the isolates,

showed poor absorption of dye Cong red, little or no growth on the media of GPA without altering the PH. This fact give further evidence for purity of the Rhizobial isolates [13]. High salt concentrations, like 2% NaCl, act as an inhibitor for Rhizobial growth, some common bacterial contaminant e.g. Agrobacterium shows considerable growth on similar salt concentration. But almost all of the isolates exhibit growth on 2% NaCl which support the investigation carried out by [15]. Poor growth on GPA can be explained as such that, rhizobia do not prefer the peptone as a source of nitrogen, vitamin or growth factor or amino acids.

Biochemical Characteristics of the Isolates

All the isolates were tested for selective biochemical tests which are presented in Table 3. Mahana *et al.*, (2000) [16] also reported catalase activity in some isolates from nodules and rhizosphere were catalase positive and citrate negative. So in catalase test bubbles were produced and in citrate test no colour change observed and it remained green.

Table 3. Biochemical Behaviors of the Test Strains

Isolates	Catalase Test	Citrate Utilization Test
Vigna sinensis (nodule)	+	_
Vigna sinensis (rhizosphere)	+	_
Lablab purpureus (nodule)	+	_
Lablab purpureus (rhizosphere)	+	_

DISCUSSIONS

Bangladesh has made impressive progress in improving the production of rice and other crops during the past few years. However there are still much lacking in nutrition. We are mainly concerned with carbohydrate and protein intake. A vast majority of the poor in Bangladesh cannot afford animal or fish proteins, and thus have to depend on cheap and easily available vegetable protein – mainly pulses and legumes, which are known as “poor man’s beef”. Research on the rhizobia has revealed a lot about the role of biological nitrogen fixation in the field of agriculture. Rhizobia by diminishing input of the fertilizer in the field and positively influencing plant crop growth, contribute to the development of the sustainable agriculture, which is necessary for the agriculture based, under developed country like Bangladesh. The present study is expected to reveal the diversity of these Rhizobial strains native to Bangladesh, to some extent, especially with the agronomically and ecologically interesting pulse legume. This study showed the presence of rhizobia on root nodules of leguminous Plant. Not only the leguminous Plant but also the rhizosphere contains rhizobia which help in soil fertilization. Further studies are recommended to identify additional characteristics of rhizobia, and to assess biological nitrogen fixation

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CONCULSIONS

Our study confirmed that rhizobia is a common species usually associated with root nodule and rhizosphere. Results from different biochemical test revealed their presence in that specific location in very crucial for our agriculture. So for increasing soil fertilization, association of rhizobia is a key factor which could help in fulfilling our protein demand

by nitrogen fixating process. Rhizobia could be used as replacement of fertilizer in the field which will contribute to the development of the sustainable agriculture.

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