ISOLATION AND CHARACTERIZATION OF *RHIZOBIUM* FROM THE ROOT NODULES OF *VIGNA MUNGO* L. (BLACK GRAM)

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Abstract

The present study was conducted to isolate and characterize the rhizobia from the root nodules of *Vigna mungo* L. (Black gram). Plant sample and rood nodules sample were collected from the Hinthada Township, Ayeyarwaddy Region. The experiment was performed at the Microbiology Laboratory, Department of Botany, University of Yangon in 2022. The two bacterial isolates (PBR1 and PBR2) were identified as *Rhizobium* based on the results of authentication tests (Congo red test, Keto-lactose test, Bromothymol blue test**,** Glucose- peptone agar test and Hoffer's alkaline test). On morphological properties, *Rhizobium* colonies on yeast extract mannitol agar (YEMA) medium were circular, mucoid, glistering, and white with convex elevation and smooth surface. Under microscopic examination, both isolates appeared as Gram-negative, and rod shaped. Biochemical characterization of two *Rhizobium* isolates showed positive reactions with catalase, methyl red (MR), nitrate reduction and urease while both strains indicated negative reaction with citrate utilization, gelatinase enzyme, indole, Triple Sugar Iron Agar and Vogas Proskauer (VP) test.

Keywords: *Rhizobium*, root nodules, Black gram

Introduction

Nitrogen is an essential element for the functioning of all the living organisms and is available in environment at an approximate level of 78%. It is the limiting nutrients for plant growth, but in this form is not accessible to plants. (Greenwood, 1982). The application of synthetic nitrogen fertilizers is not only expensive for the farmers but can also cause environmental damages such as pollution of water resources, also inhibit the natural flora of soil which support the growth of plant as well as also decrease the fertility of soil, and even contribute to the release of greenhouse gases (Rigby and Caceres, 2001). Therefore, there is an urgent need to find alternative strategies, that not only enhance the fertility of soil, but can also increase the production of crops without the uses of chemical fertilizers (Lee and Song, 2007).

Biological Nitrogen Fixation (BNF) is the cheapest and most environmentally friendly procedure in which nitrogen fixing micro-organisms interact with leguminous plants, fix aerobic nitrogen into the soil and are able to convert the nitrogen gas into a form that is usable for plant life (Franche *et al*., 2009). *Rhizobium* spp. are a well- known group of bacteria that act as the primary symbiotic fixer of nitrogen. Bacteria of family Rhizobiaceae are symbiotic and effectively convert atmospheric nitrogen which is utilized by the host, and which contains six genera namely *Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium and Bradyrhizobium* (Okazaki *et al*., 2004). These rhizobia are characterized into two groups on the basis of growth rate. The first group is fast growing rhizobia and the second is slow growing rhizobia (Lohis and Hansen, 1921).

The best-known associations are the symbioses of *Rhizobium* bacteria with legumes. Nitrogen fixing leguminous plants not only support plant growth independent of mineral nitrogen in the soil but also improve soil nitrogen status for associated crops through the residues of these plants. Black gram (*Vigna mungo* L.) is the most important legume and an annual pulse crop, widely cultivated in both tropical and sub-tropical countries (Gour, 1993). From a global perspective, India is the largest producer and consumer of black gram followed by Myanmar and Thailand. As an excellent source of plant protein black gram is highly responsive to nitrogen and rhizobia was believed to be the only nitrogen fixing symbiont of black gram nodules.

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Therefore, screening and selection of proper rhizobial strains is important for biological nitrogen fixation. There is a huge amount of literature available, reporting rhizobia from different pulses (Hou *et al*., 2009; Wadhwa *et al*. 2011; Riah *et al*. 2014) but limited studies are there about the authentication test and biochemical characterization of rhizobia inhabiting black gram. For this reason, this study was aimed to isolate *Rhizobium* strains from the root nodules of black gram (*Vigna mungo* L.) to conduct the authentication test to confirm the isolate as *Rhizobium* differentiated from other contaminating microbes and to identify the *Rhizobium* using morphological characters and some biochemical tests for better agriculture growth.

Materials and Methods

Sample collection and Identification of plant samples

The plant and root nodules of Black Gram (*Vigna mungo L.*) were collected from the Hinthada Township, Ayeyarwaddy Region. The collected plants were subjected to identify with the help of literature (Hooker 1879; Backer 1964; Dassanayake 1981).

Isolation of bacteria from root nodules and morphological characterization

Collected root nodules were washed in tap water to remove the adhering soil particles. The healthy, undamaged and pinkish nodules were detached from the roots and were selected for isolation of bacteria. The surface sterilization was done by washing the nodules with distilled water and, sterilizing with 70% ethanol for 30 seconds and discarding ethanol. Then, add 1% of sodium hypochlorite solution and let stand for about 2-3 minutes. To remove the sodium hypochlorite, the nodules were washed again with distilled water for 3- 5 times. The sterilized nodules put in 2 ml of microcentrifuge tube containing 0.5 ml of N- saline (0.85% NaCl) and crush them with the help of sterile glass rod or forceps. Then, one loopful of the nodule suspension was streaked on petri plates that contained yeast extract mannitol agar (YEMA) medium supplemented with 0.0025% (w/v) congo red as an indicator. The plates were incubated at room temperature for 2- 3 days. The most prominent isolates were maintained on YEMA slants at 4°C in a refrigerator for further characterization (Singh *et al*., 2008, Vincent, 1970). The morphological characteristics of isolates including colony color, form, elevation, margin, mucosity, opacity and gram's reaction, were determined by observing the colonies on YEMA plates (Somasegaran and Hoben, 1994; Aneja, 2003).

Authentication Tests of *Rhizobium*

Five different authentication (confirmatory) tests such as growth on YEMA with Congo red, Keto-lactose test, Bromothymol blue test, growth on Glucose- peptone agar and Hoffer's alkaline test were performed to confirm the isolate as rhizobia and to differentiate from other contaminating microbes.

(i) Growth on YEMA with Congo red

The purity of the rhizobial isolates was detected by addition of Congo red in YEMA medium. In general, rhizobia absorb the dye weakly and produce white colonies, whereas many other bacteria including agrobacteria, take up the dye strongly (Somasegaran *et al*., 1994).

(ii) Keto-lactose Test

The Keto-lactose test is widely used to differentiate rhizobia from other contaminating bacteria. Mannitol in YEM agar is replaced with lactose. The rhizobial isolates were streaked on the Keto-lactose agar medium and incubated for 2-3 days. The plates were flooded with Benedict's reagent and kept at room temperature for 1-2 hours. The absence of yellowish zones around the *Rhizobium* colonies indicated the purity of the isolates (Bernaertz and Daley, 1963).

(iii) Bromothymol blue Test

This assay was performed to differentiate between fast and slow growers of *Rhizobium* species. The YEMA medium containing bromothymol blue was streaked with isolated strains and observed either for green to yellow colour due to production of acids or blue colour due to production of alkali (Vincent,1970).

(iv) Growth on Glucose Peptone Agar (GPA)

GPA test was performed to determine the capability of the *Rhizobium* strains to utilize glucose as the sole carbon source for its growth medium. Isolates were streaked upon the (GPA) medium, and the plates were incubated for 18-24 hours. Rhizobia cannot or poorly utilize peptone whereas *Agrobacterium* can utilize and grow very fast on this medium (Singh *et al*., 2008).

(v) Hoffer's alkaline Test

This test is based on the fact that *Agrobacterium* grows at a higher pH level whereas *Rhizobium* is unable to do so. Hoffer's alkaline medium having a high pH of 11.0 was used to screen isolated bacteria for this purpose. Bacteria were inoculated in the above mentioned broth and incubated for 24 - 48 hours (Hofer, 1935).

Biochemical characterization of *Rhizobium*

(i) Catalase Test

The catalase test of the isolates was performed to test the the presence of catalase enzyme in bacterial colonies. *Rhizobium* colonies (24 h old) were taken on glass slides and one drop of hydrogen peroxide H_2O_2 (30%) was added. Appearance of gas bubble indicated the presence of catalase enzyme and absence of bubbles indicated a negative test (MacFaddin 2000).

(ii) Citrate utilization test

The ability to use citrate was determined by replacing mannitol in YEM agar with an equal amount of sodium citrate and bromothymol blue (25mg/l). To inoculate the slant, a loopfull of culture of bacteria was inoculated following the stab and streak method and finally observed after an incubation period of 24 - 48 hours (Koser, 1923).

(iii) Gelatin Hydrolysis Test

The test was performed to determine the capability of *Rhizobium* to produce gelatinase enzyme and use gelatin as a media source. The actively grown cultures were inoculated into nutrient gelatin medium and grown for 24 - 48 hours. On subjecting the growing culture to a low temperature at 4°C for 15- 30 minutes. The cultures which produce gelatinase remain liquefied while others due to the presence of gelatin become solid (Sadowsky *et al*., 1983).

(iv) Indole Test

A tryptone broth medium was prepared and poured into the test tubes. The bacterial isolates were incubated for 2 days. The uninoculated broth was maintained as a acontrol. After incubation, 1 mL of Kovac's reagent was added to each test tube, including the control. The formation of a red coloured ring indicates the positive results, whereas a yellow coloured or cloudy ring indicates negative results (MacFaddin, 2000).

(v) Methyl red (MR) test

The MR-Voges Proskauer (VP) broth was prepared. 5 ml of the broth was poured into sterile test tubes. The rhizobial isolates were inoculated separately into the tubes and incubated for 2 days. After the incubation period, 5 ml of MR indicator was added to each tube. Red

coloration of the broth indicates a positive result while the turning of MR to yellow is a negative result. (Dinesh *et al*. 2015).

(vi) Voges-Proskauer (VP) test

The *Rhizobium* isolates were inoculated separately into test tubes containing methyl red-Voges Proskauer broth and incubated for 2 days. After incubation, Barritt's reagents A and B were added and allowed to stand in the test tubes for 30 minutes. The red coloration on top of the culture showed the positive, whereas the negative has a yellowish color (McDevitt, 2009).

(vii) Nitrate reduction test

Nitrate broth was prepared with one loop full of culture of rhizobial isolates were inoculated separately and incubated for 2- 4 days. After the period of incubation, three drops of alpha naphthylamine and three drops of sulfanilic acid reagent were added. The cherry red color indicates the positive results (El Idrissi *et al*., 1996)

(viii) Triple Sugar Iron Agar Test

The test was performed to determine the capability of isolates to use various carbohydrates sources (sucrose, glucose, lactose) as media for growth. After inoculation and incubation color was observed on the butt and the slant (Kligler, 1918).

(ix) Urease Test

The test was performed to determine the capability of *Rhizobium* strain to produce urease enzyme or not. The rhizobial isolates were inoculated separately into the test tubes and incubated for 2 days. The appearance of deep pink color indicates positive results (MacFaddin, 2000).

Results

Identification of *Vigna mungo* L.

Scientific name -*Vigna mungo* L. Hepper
Common name - Black Gram
Myanmar name - Mat-pe
Family - Fabaceae

Outstanding Characters

Black gram is an erect, fast-growing annual, herbaceous legume reaching 30-100 cm in height. It has a well-developed taproot, and its stems are diffusely branched from the base. Occasionally it has a twining habit, and it is generally pubescent. The leaves are trifoliate with ovate leaflets, 4-10 cm long and 2-7 cm wide. The inflorescence is borne at the extremity of a long (up to 18 cm) peduncle and bears yellow, small, papilionaceous flowers. The fruit is a cylindrical, erect pod, 4-7 cm long x 0.5 cm broad. The pod is hairy and has a short, hooked beak. It contains 4-10 ellipsoid black or mottled seeds.

Fig 1. The plant of *Vigna mungo* L.

Morphological characteristics of isolated bacterial strains

Two *Rhizobium* strains (designated name as 'PBR1' and 'PBR2') were isolated from the root nodules of Black Gram. Phenotypically, both isolates grew quickly within 2-3 days of incubation and failed to absorb Congo red in YEMA medium. Colonies of both isolates were circular, mucoid, glistering, convex elevation, white, translucent with a smooth surface (Figure 2A) and gram-negative and rod-shaped characters were observed under microscopical examination (Figure 2B).

Authentication tests of *Rhizobium*

The results of five confirmatory tests were (growth on YEMA with Congo red, Keto-lactose test, Bromothymol blue test, growth on Glucose- peptone agar and Hoffer's alkaline test) shown in (Table 1) and (Figure 2).

Table. 1 Authentication tests of *Rhizobium*

Confirmatory Tests	***Rhizobium* isolates**	
	PBR1	**PBR2**
Growth on Congo red medium	White	White
Production of ketolactose test	Blue/ No color change	Blue/ No color change
Bromothymol Blue test	Yellow/ Fast	Yellow/ Fast
Growth glucose peptone agar (GPA)	No growth	Poor growth
Growth on Hoffer's alkaline medium	No growth	No growth

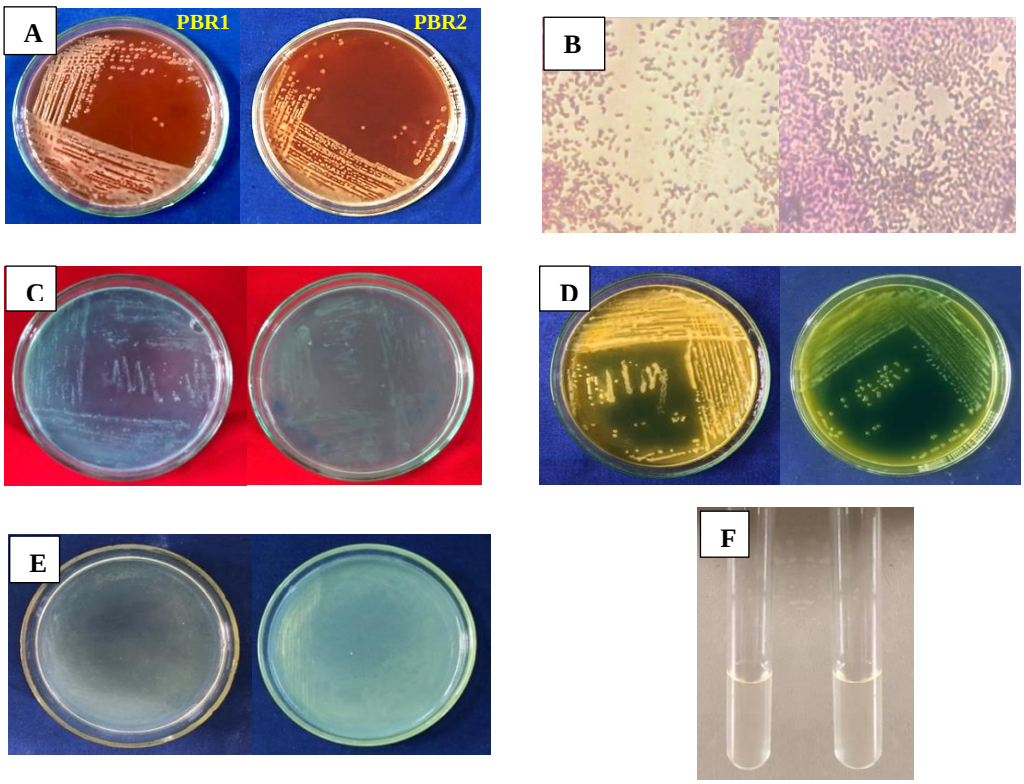

Figure. 2 (A) *Rhizobium* colonies on CR-YEMA; (B) Gram staining of isolated *Rhizobium*; (C) Keto-lactose test; (D) Bromothymol Blue test; (E) Glucose Peptone Agar (GPA) test; (F) Hoffer's alkaline Test

Table. 2 Biochemical characterization of *Rhizobium*

Biochemical Tests	***Rhizobium* isolates**	
	PBR1	**PBR2**
Catalase test	+	+
Citrate utilization test	-	-
Gelatin hydrolysis test	-	-
Indole test	-	-
Methyl red test	+	+
Vogas Proskauer test	-	-
Nitrate reduction test	+	+
Triple Sugar Iron (TSI) test	-	-
Urea Test	+	+

+ (positive test), - (negative test)

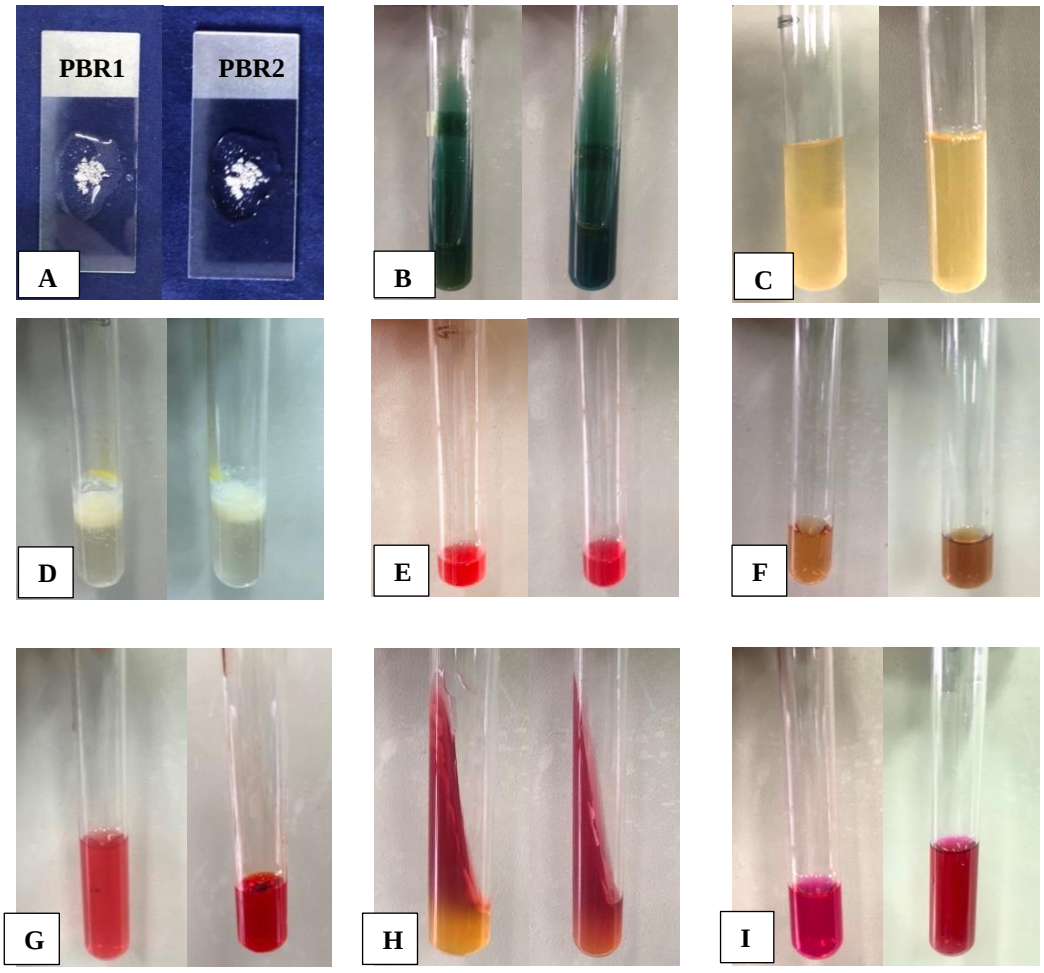

Figure. 3 Biochemical tests of two *Rhizobium* strains (A) Catalase test; (B) Citrate utilization test; (C) Gelatinase hydrolysis test; (D) Indole test; (E) Methyl red (MR) test; (F) Vogas Proskauer (VP) test; (G) Nitrate reduction test; (H) Triple sugar iron (TSI) test; (I) Urease test

Discussion and Conclusion

The Rhizobia-legume symbiosis benefits not only the host crop but also the subsequent crops in that field. *Rhizobium* bacteria stimulate the growth of leguminous plants, and they are able to fix atmospheric nitrogen into the soil by interacting symbiotically with leguminous plants (Kiers *et al*., 2003). In this research, the strains of root- nodulating bacteria; *Rhizobium* were isolated from the root nodules of Black gram (*Vigna mungo* L.). The two *Rhizobium* strains ('PBR1' and 'PBR2') were isolated and both strains were gram negative and did not absorb red colour when cultured in YEMA medium containing Congo red. In CR-YEMA (Congo red yeast extract mannitol agar) medium, *Rhizobium* colonies were white, circular, translucent, glistening, elevated convex with smooth edges, and raised colonies were smaller than stained colonies of other non-*Rhizobium* isolates. These morphological characteristics resembles the studies carried out by different researchers and also seem to approach close to the characteristics of the *Rhizobium* type as described (Jordan *et al*., 1974 and Rivas *et al*., 2009). Microscopic examination revealed that the isolates were rod shaped and gram negative in nature (Keyser, 1982 and Singh *et al*., 2008).

The confirmatory analysis of *Rhizobium* from other contaminating bacteria indicated that the colonies did not absorb the Congo red color on CR-YEMA media and that such a nature differentiates *Rhizobium* from *Agrobacterium* and other bacterial contaminants (Trinick *et al*., 1982). The two *Rhizobium* isolates showed the negative results on Hofer's alkaline medium because they normally cannot grow in Hofer's medium and are similar to Deka and Azad, (2006). In the Keto-lactose test, no yellow zone was observed around the colonies after the addition of Benedict's reagent which is the characteristic of *rhizobium*. The same results are agreed upon by Deshwal and Chaubey (2014).

In the glucose peptone agar (GPA) test, both isolates showed either poor or no growth on GPA medium after one day indicating the features of rhizobia. Similar results are reported by Vincent *et al*., (1970). On the bromothymol blue (BTB) agar plates, both fast and slow growing rhizobia formed circular, convex, colonies and were classified tentatively as fast (medium turn yellow) and slow growers (medium turn blue). The use of YEMA-BTB medium for categorizing the fast and slow growing rhizobia based on acid/alkali production was also carried out. The present investigation indicated that both strains were found to produce yellow colonies due to acid production on the medium with a high amount of mucus after 24 hours of incubation. Similar results reported by Datta *et al*., (2015) showed the fast- growing rhizobial strains isolated from *Vigna mungo*. In 2009, Alemayehu stated the same results which indicated that all strains were fast growers and acid producers. From the above observations, one could conclude that the bacterial isolates were *Rhizobium* spp.

MacFaddin (2000) stated that organisms containing the catalase enzyme will form oxygen bubbles when hydrogen peroxide (H_2O_2) exposed to it. In the present work, bubbles were appeared to show positive catalase for both strains. Some researchers also observed bubble formation around bacterial colonies (Mahana *et al*., 2000, Datta *et al*., 2015). The positive results of methyl red agreed with the studies of Raju *et al*., (2017). For nitrate reduction test, a red color change on the addition of sulphanilic acid and α-naphthylamine indicates a positive test and the similar result was agreed with Kumari *et al*. (2010). In the current study, both isolates showed a positive test for urease. Similar observations were reported by Gauri *et al*., (2011). It means that experimenting microbes are able to use urea as a source of carbon and energy for growth.

The result of citrate utilization showed a negative result, which means *Rhizobium* isolates are incapable of utilizing citrate as a carbon source and this agrees with Lupwayi and Hague (1994). The *Rhizobium* strains tested here were found negative for the production of gelatinase enzyme, similar to the findings of Hunter *et al*., (2007). Negative gelatinase activity is a feature

of *Rhizobium*. The negative indole result indicated the remaining yellow or being slightly cloudy within seconds of adding the reagent. The samples were found negative for citrate utilization, gelatinases, and indole and these findings are in close agreement with (Shahzad *et al*. 2012). In the TSI slant, and the acid produced is oxidized and alkali is formed. Alkaline (red) slant and acid (yellow) butt showed that only glucose fermentation had taken place. In the butt, acid reaction is maintained because of less oxygen and the slow growth of organisms. Our Rhizobium strains were found positive for the utilization of glucose as carbon source; similar results were reported by Kucuk *et al*., (2006). The negative result of Voges-proskauer (VP) in current study agreed with Elsheikh and Wood (1986).

From this study, it can be concluded that the screening of the effective isolates on biochemical tests is important for further investigation into black gram. The authentication test is essential to differentiate *Rhizobium* and *Agrobacterium*, it can be said that both isolates were fast growing *Rhizobium* species. In the future, the isolates can be screened for different plant growth promoting traits and nitrogen fixing ability. These two *Rhizobium* isolates might be useful to increase the symbiotic nitrogen fixation in legumes and can be used as potential biofertilizers owing to their plant growth promoting characteristics.

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