ISOLATION OF RHIZOBIA FROM THE ROOT NODULES OF COW PEA AND BLACK GRAM CULTIVATED IN KYAUNGKONE, MYANMAR

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Abstract

Isolation of nitrogen fixing rhizobia was made from the root nodules of Cow Pea and Black Gram during July to November 2018. Root nodules were collected from the cultivated field of Anauksu Village, Kyaungkone Township, Ayeyarwady Region, Myanmar. The aim of this research was to isolate nitrogen fixing *Rhizobium* as starter culture of biofertilizer for peas. Isolation of Rhizobium was made by using Yeast Extract Mannitol agar with Congo red and Bromothymol blue. Identification was based on colony morphology, cell morphology, basic staining reactions and motility. One strain of *Rhizobium* from Cow Pea and one strain of *Bradyrhizobium* from Black Gram were isolated. These bacteria could be used as starter culture for the production of rhizobia biofertilizers.

Keywords: *Rhizobium*, nitrogen fixing, root nodules, Cow Pea, Black Gram, biofertilizer

Introduction

Microbes are essential regulating agents for soil fertility. Microorganisms are indispensable in decay processes and in the transformation of organic substances and humus formation in soil. Bacteria are by far the most numerically abundant soil microorganisms. Among these bacteria, some are symbiotic bacteria in the root of some leguminous plants and some were associated in soil near the root region of some plants and grasses. Bacteria associated with these regions are collectively known as rhizobacteria and some have the ability to fix atmospheric nitrogen.

The rhizobia live freely in the soil and as soon as they come in contact with suitable host plant, they start the process of infection. After infection of appropriate legume, they can cause formation of nodules and participate in the nitrogen fixation. In general they are Gram negative, rod-shaped but varieties of morphological shapes are observed when isolated from the root nodule (Jadhav, 2013). The bacteria belonging to the genera *Rhizobium, Bradyrhizobium, Allorhizobium, Rinorhizobium* and *Mesorhizobium* are able to form nodules on their host plants inside of which they fix-nitrogen (Abo-Aba *et al.,* 2015).

Members of the genus *Rhizobium* have been isolated from nodules of many leguminous plants. *Rhizobium* symbiosis with legumes species is of special importance, producing 50% of 175 million tonnes of total biological N-fixation annually worldwide (Ogutcu *et al.,* 2008).

Allito (2015) studied the population and phenotypic characterization of soybean (*Glycin max*) and haric bean (*Phaseolus vulgaris*) nodulating rhizobia by using yeast extract mannitol agar (YMA). Rhizobia are unique in that they are the only nitrogen fixing bacteria living in a symbiotic relationship with legume and they can be used as biofertilizers that would reduce the need for chemical fertilizers and decrease adverse environmental effects.

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Therefore, the aim of the present work was to isolate the rhizobia bacteria from the rhizosphere of Cow Pea and Black Gram for the production of biofertilizer.

Materials and Methods

Study Period

This research was conducted at the laboratory of Zoology Department, Pathein University during July to November, 2018.

Collection of Samples

Plants and roots of Cow Pea (*Vigna catjang* Walp) and Black Gram (*Phaseolus mungo* L.) were collected from Anauksu Village, Kyaungkone Township, Ayeyarwady Region (Plate 1).

A. A plantation of Cow Pea

B. A sample plant of Cow Pea

C. A plantation of Black Gram

D. A sample plant of Black Gram

Plate 1: Plants of Cow Pea and Black Gram from which rhizobia were isolated

Preparation of Yeast Extract Mannitol Agar (Somesegaram and Hoben, 1985)

The chemical ingredients (Himedia, India) of yeast extract mannitol agar (YMA) were accurately weighed by digital balance. The ingredients were mixed in 1000 mL of distilled water. The YMA medium was added with Bromothymol blue to obtain the BTBYMA medium and similarly the YMA medium was added with Congo red to obtain the CRYMA medium. Then the chemical ingredients were sterilized by autoclaving with pressure of 1.05 kg per cm^2 (15 lb per in^2), temperature of 121ºC and duration of 15 minutes.

Culture of Bacteria

The portions of roots were taken from 5 to 15 cm below the stem base. The root segments were washed in tap water for three times to remove adhering soil particles. From each sample, two to three nodules were picked up and washed thouroughly with sterile distilled water. After washing, nodules were surface sterilized in 95% alcohol for 30 to 40 seconds to remove wax coating and subsequently immersed in 4% sodium hypochlorite for 3 to 4 minutes. Then nodules were immediately washed 5 to 6 times with sterile distilled water to remove traces of sodium hypochlorite. The surface-sterilized nodules (0.5 g) were crushed with the help of a sterile glass rod and 0.5 mL sterile distilled water was added and mixed. The milky suspension was streaked on to BTBYMA medium and incubated under aerobic condition at 30 ± 0.5ºC in the incubator until growth appeared. Single unique colonies were picked up and were streaked on to CRYMA medium. Isolation of pure cultures is made by using streak plate technique of Gillies and Dodds (1968).

Differential Staining Techniques

Gram's staining, capsule staining, acid-fast staining and endospore staining (Bradshaw, 1992) were used to identify the bacteria species.

Detection of Motility by Cultivation in Semi-solid YMA

Motility of the isolated bacteria can be detected in semi-solid agar medium using the method of Gillies and Dodds (1968). Ten milliliter of semi-solid YMA was dispensed in test tubes and they were left to set in the vertical position. A straight wire was inoculated and a single stab down was made in the centre of the tube to about half the depth of the medium. Whether the isolated bacteria were motile or not could be seen easily.

Identification of Bacteria and Plants

Bacterial species identification was followed after Breed *et al.,* (1957) Buchanan, Gibbons (1974) and Holt *et al*. (1994). The sample plants were identified at Botany Department in Pathein University.

Results

All species of Rhizobia were isolated with Yeast Extract Manitol Agar (YMA) containing Bromothymol blue (BTB) and YMA containing Congo red (CR) media. After two to five days inoculations and incubation of nodule suspension on YMA medium with BTB, colonies of bacteria were observed. This is the growth of rhizobia. Two species of rhizobia were isolated, one species of *Rhizobium* from Cow Pea, one species of *Bradyrhizobium* from Black Gram (Table 1).

Rhizobium species from Cow Pea

One species of *Rhizobium* was isolated from root nodules of Cow Pea. After two days of inoculation on YMA media containing BTB, colonies of *Rhizobium* were observed and the media turned into yellow colour. These colonies do not absorb the red colour when incubated on YMA media containing CR. The colony feature of *Rhizobium* from Cow Pea is pale yellow on

BTBYMA and white on CRYMA, convex and circular with entire edge. The diameter of colony on both media is 1.3 to 2.7 mm. Cells are ovoid with 1 to 2 µm width. They are Gram negative, capsulated, not acid-fast and non endospore. They were motile in semi-solid medium. The temperature for culture of this species is 30 ± 0.5ºC (Table 1, Plate 2. A and Plate 3).

Bradyrhizobium species from Black Gram

One species of *Bradyrhizobium* was isolated from root nodules of Black Gram. After five days of inoculation on YMA media containing BTB, colonies of *Bradyrhizobium* were observed and the medium was not changed into yellow colour. These colonies do not absorb the red colour when incubated on YMA medium containing CR. The colony feature of *Bradyrhizobium* from Black Gram is white on both BTBYMA and CRYMA, convex and circular with entire edge. The diameter of colony on both media is 1.2 to 3 mm. Cells are ovoid with 1 to 2 µm width. They are Gram negative, capsulated, not acid-fast and non endospore. They were motile in semi-solid medium. The temperature for culture of this species is 30 ± 0.5ºC (Table 1, Plate 2. B and Plate 4).

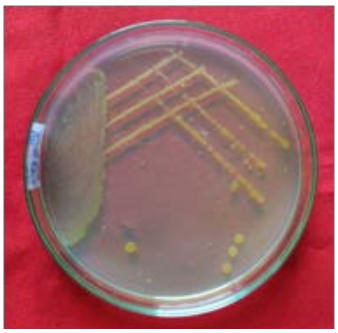
Table 1 Bacterial species isolated from root nodules of Cow Pea and Black Gram

Characteristic features	***Rhizobium* from Cow Pea**	***Bradyrhizobium* from Black Gram**
Colony morphology	Convex, circular, entire edge	Convex, circular, entire edge
Colony size	1.3-2.7 mm	1.2-3 mm
Colony colour	Pale yellow on BTBYMA and white on CRYMA	White on BTBYMA and white on CRYMA
Cell morphology	ovoid	ovoid
Cell size	1-2 µm	1-2 µm
Arrangement	Singly, pair	Singly, pair
Respiration	aerobic	aerobic
Incubated Temperature	30±0.5ºC	30±0.5ºC
Motility	Motile	Motile

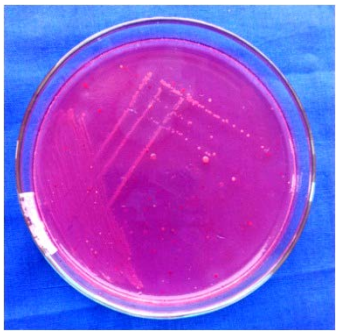
A. Motility of *Rhizobium* from Cow Pea

B. Motility of *Bradyrhizobium* from Black Gram

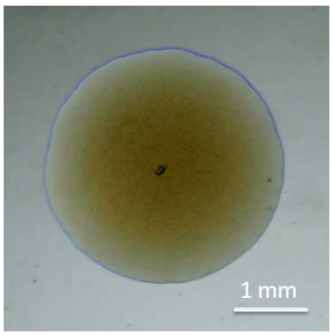
Plate 2: Motility test of isolated bacteria species in semi-solid medium



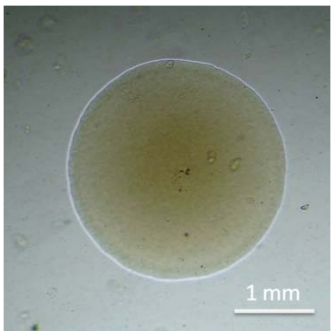
A. *Rhizobium* colonies of Cow Pea growth on BTBYMA medium



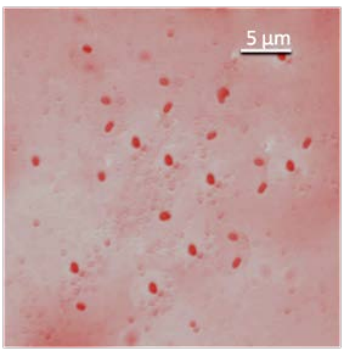
B. *Rhizobium* colonies of Cow Pea growth on CRYMA medium



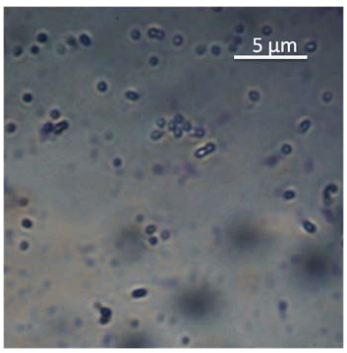
C. Single colony of *Rhizobium* on BTB medium



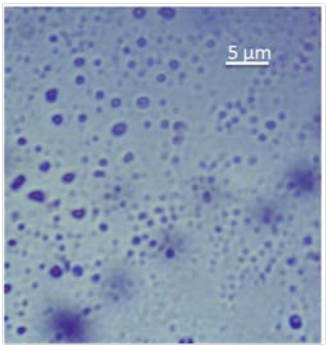
D. Single colony of *Rhizobium* on CRYMA medium



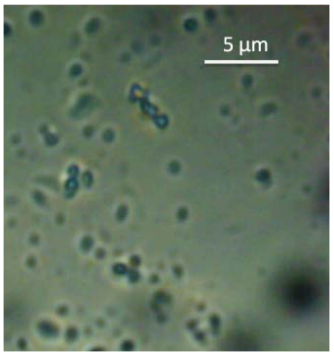
E. Gram staining of *Rhizobium*



F. Capsule staining of *Rhizobium*



G. Acid-fast staining of *Rhizobium*

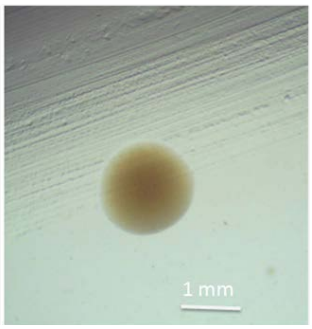


H. Endospore staining of *Rhizobium*

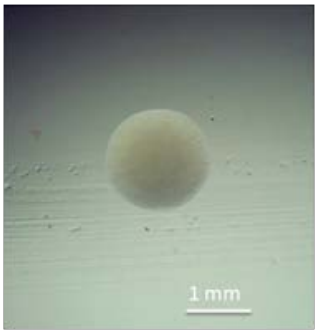
Plate 3: Colony morphologies and staining reaction of *Rhizobium* species from Cow Pea

A. *Bradyrhizobium* colonies of Black Gram growth on BTBYMA medium

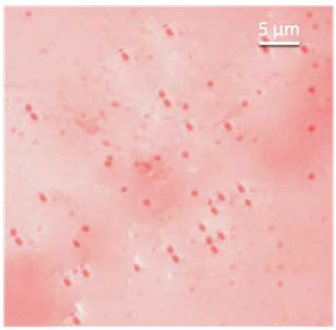
B. *Bradyrhizobium* colonies of Black Gram growth on CRYMA medium



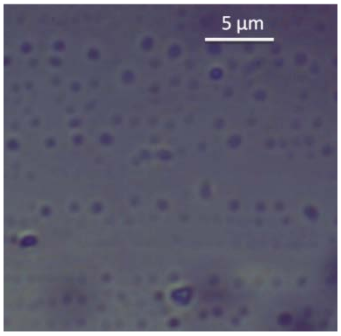
C. Single colony of *Bradyrhizobium* on BTB medium



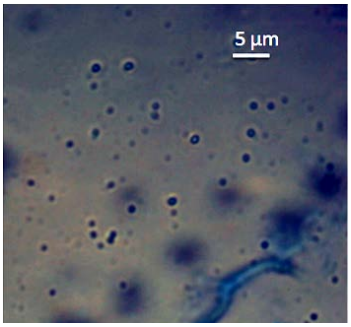
D. Single colony of *Bradyrhizobium* on Congo Red medium



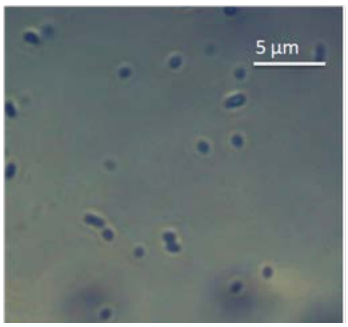
E. Gram staining of *Bradyrhizobium*



F. Capsule staining of *Bradyrhizobium*



G. Acid-fast staining of *Bradyrhizobium*



H. Endospore staining of *Bradyrhizobium*

Plate 4: Colony morphologies and staining reaction of *Bradyrhizobium* species from Black Gram

Discussion

To find out the rhizobia species (N_2 fixing bacteria) from inside root nodules of Cow Pea and Black Gram, yeast extract mannitol agar (YMA) medium with bromothymol blue or Congo red was used. Yeast extract mannitol agar (YMA) is the special medium used to grow *Rhizobium* (Neeraj *et al.,* 2009). Initial characterization of rhizobia involves observation of colonies growing in YMA containing Congo Red and bromothymol blue indicators (Bala *et al.,* 2011)

Sharma *et al.* (2010), Kaur *et al.* (2012) and Ahmed and Abdelmageed (2015) also used YMA with Congo red and YMA with bromothymol blue media to isolate and distinguish *Rhizobium* and *Bradyrhizobium* from pea and bean.

Therefore, these media were used in this work to isolate and characterize *Rhizobium.* Identification of the isolated bacteria were made based on cell and colony morphologies, basic staining reactions, motility and growing temperature. Two species of rhizobia from Cow Pea and Black Gram were isolated.

In this investigation, one of the isolated species from root nodules of Cow Pea changed the medium colour to yellow after two days incubated on YMA medium containing bromothymol blue. After five days incubation on YMA containing BTB, isolated bacteria from Black Gram nodules did not changed the medium colour into yellow.

Distinct colonies of fast-growing rhizobia begin to appear within 3-5 days, while those of slow-growers require 7-10 days to appear. Colonies of slow-growing rhizobia are characterized by a blue colouration, which indicates alkaline reaction on BTB. A yellow colour, indicating acid reaction, is produced by fast-growing rhizobia (Bala *et al*., 2011). Similarly, Sharma *et al*. (2010) classified the rhizobia as fast (medium turn yellow) and slow growers (medium turn blue) based on the yeast extract mannitol agar with bromothymol blue.

The characteristic of fast growing rhizobia is changing the YMA-BTB medium to yellow colour due to acid production (Ahmed and Abdelmageed, 2015). Therefore, in this study, isolated rhizobia from Cow Pea are fast grower, and the isolated species from Black Gram are slow grower. Distinct colonies of fast-growing rhizobia begin to appear within 3-5 days, while those of slow-growers require 7-10 days to appear.

In this work, the colonies of all isolated species do not absorb the red colour when inoculated on CRYMA medium. Typical Rhizobia colonies should show little or no Congo red absorption (Bala *et al*., 2011).

Pseudo-nodule forming bacteria *Agrobacterium* utilized Congo red but *Rhizobium* strains didn't utilize Congo Red. This test is essential to differentiate *Rhizobium* and *Agrobacterium* (Deshwal and Chaubey, 2014).

Therefore, the isolated rhizobia were not *Agrobacterium* and they may be *Rhizobium* or *Bradyrhizobium*.

In this research, colonies of all isolated strains are 1.2-3 mm in diameter and cells are gram negative, non endospore, not acid-fast and capsulated. They were motile in semi-solid medium. *Rhizobium* colonies were large (2-4 mm in diameter) mucilaginous, circular, convex with smooth edges, glistening translucent or white in YMA medium (Holt *et al*., 1994).

Rhizobium from the root of fresh rice plants are Gram negative, aerobic and rod-shape bacteria (Zhang *et al*., 2011).

The *Rhizobium* colonies were entire, opaque with regular margin, milky white, translucent, circular in shape, shiny, raised (convex), sticky consistency and 2-4 mm in diameter. They were also aerobic, non spore forming, gram negative rods and motile (Pawar *et al.*, 2014).

The coloration of colonies was milky-white translucent with a circular shape, with regular borders, shiny and raised after 3 to 5 day of growth on YMA medium at 28ºC. Colony diameter ranging from 2 to 5 mm and cells were motile, gram negative and rod shaped bacteria. All these features are characteristics of *Rhizobium* strains (Ahmed and Abdelmageed, 2015).

Therefore, *Rhizobium* species isolated from Cow Pea in this work was similar with the above reports and other standard characteristics of the isolates indicated that the isolated microorganisms were *Rhizobium* species.

The *Rhizobium japonicum* (Syn. *Bradyrhizobium japonicum*) was Gram negative, aerobic, non-spore forming and motile rods. In general, the colonies were 3.1 mm in diameter, circular, convex, whitish pink and glistening with entire margin (Gachande and Khansole, 2011).

Colony morphology of isolated bacteria from Black Gram was white on both BTBYMA and CRYMA; convex, circular, entire edge, 1.2-3mm in diameter and cells are gram negative, non endospore, not acid-fast, capsulated and motile. Therefore, the characteristics of isolated bacteria from Black Gram indicated that they were *Bradyrhizobium* species.

Conclusion

In this work, isolated bacteria from Cow Pea and Black Gram were nearly the same with the some characters of *Rhizobium* and *Bradyrhizobium*. Therefore, this research work could be expected to provide some basic information for the preparation and production of *Rhizobium* biofertilizers.

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