Available online at www.ijpab.com

ISSN: 2320 – 7051
Int. J. Pure App. Biosci. **3 (1):** 58-63 (2015)

Research Article

INTERNATIONAL JOURNAL OF PURE & APPLIED BIOSCIENCE

Isolation and characterization of root nodule bacteria associated with *Cassia alata* of Southern parts of Assam, India

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ABSTRACT

Rhizobium plays an important role in agriculture by including nitrogen fixing nodules on the roots of legume plants. The present study describes the characterization of Rhizobium strain isolated from root nodules of Cassia alata. The rhizobium strains were rod, gram negative and acid producing in nature. They utilized glucose and starch as sole carbon source and showed sensitivity against different antibiotics such as chloramphenicol, kanamycin and streptomycin. The growth of the isolates in glucose peptone agar medium confirmed the isolates to be Rhizobium.

Keywords*: Rhizobium, Cassia alata, antibiotic sensitivity.*

INTRODUCTION

Legumes include important grain, pasture and agro-forestry species. They are harvested as crops for human and animal consumption as well as used as pulp for paper production, fuel-woods, timber, oil production, sources of chemicals and medicines, and are also cultivated as ornamental, used as living fences and firebreaks among others[11]. “Legumes should be planted in light soils, not so much for their own crop as for the good they do to subsequent crops” [7], recognizing the importance of multiple cropping and intercropping production. Legumes form nodules with rhizobia; they have high levels of nitrogen available to them. Their abundance of nitrogen is beneficial not only to the legumes themselves, but also to the plants around them. In return for the fixed nitrogen that they provide, the rhizobia are provided shelter inside of the plant's nodules and some of the carbon substrates and micronutrients that they need to generate energy and key metabolites for the cellular processes that sustain life[16]. Leguminous plants are important not only ecologically but also agriculturally because they are dependable for the key conversion of nitrogen from atmospheric N_2 to ammonia. Throughout the world about 44-46 million tons of N_2 are fixed per annum, of which approximately half of the nitrogen is used in agriculture and this is mostly carried out by the symbiotic nitrogen fixers such as *Rhizobium*[6].

Rhizobia in association with leguminous plants have the ability to form species-specific N_2-fixing symbiotic associations. Use of nitrogenous fertilizer during the growth of leguminous crops can be relieved because of this symbiosis. Many Rhizobia can form nodules with several wild or crop legumes, and can also be a source for genetic information to improve symbiotic characters of other Rhizobia[18]. The interaction that occurs between the plant and the rhizobia during nodule formation and maintenance constitutes a novel prospect to study signal transduction in a plant system. The expression of "nodulation" genes in the bacteria is activated by flavonoids from plant roots and as a result the bacteria synthesise signals to enter this meristem via a plant-made infection thread. The consequence of Rhizobia to legumes are not constrained to their symbiotic nitrogen fixation activity, but some strains of Rhizobia also produce plant hormones, antimicrobial compounds etc.

In this study, we have characterized bacterial isolates from the roots of wild leguminous plant i.e. *Cassia alata*. The common name of *C. alata* is candle-bush or candlestick senna. It is the fourth largest genus of the family Leguminosae, the largest genus of the sub-family Caesalpinaceae. It is an erect tropical, annual herb with leathery compounded leaves. The shrub stands 3–4 m tall, with leaves 50–80 cm long. The inflorescence looks like a yellow candle. This perennial herb has an erect, waxy yellow spike that seems to be fat candles. The large leaves are bilateral-symmetrical opposed and fold together at night. The fruit is a pod, which is up to 25cm long. Its seed are distributed by water or animals. It is a type of ornamental plant. *C. alata* is a very important herbal medicinal plant throughout the world. This plant is used both internally and externally in the treatment of a variety of skin and respiratory disease. Chatterjee *et al*.,[3] in their study reported that fresh or dried leaflet of *C. alata* had been used as medicines and different parts of the plant were used in ayurvedic medicine and home remedies for regular ailments in the cure of constipation, stomach pain, ring worm and skin disease. Along with its medicinal use *C. alata* also proved to possess antibacterial property and found to be effective against some clinical isolates of Gram-positive and Gram-negative bacteria as well as against a few fungi which are mostly dermatophytes causing skin infection in human beings. Being a legume, it has a relevant role in sustainable agriculture. They can be easily planted in gardens for medicinal uses, ornamental purpose and to enhance soil fertility. The main objective of the study is to isolate and categorize diazotrophs with respect to their morphological and biochemical nature.

MATERIALS AND METHODS

Isolation of rhizobium from *Cassia alata* roots

For isolation of rhizobium, fleshy root nodules of *Cassia alata* were collected from the plants grown in five different places of Barak Valley region. The collected nodules were first washed with normal tap water and then washed with 0.1% $HgCl_2$ solution for about 5 minutes. These were then rinsed in sterile distilled water several times in order to remove the chemicals. Several drops of 70% (v/v) industrial methylated spirit was transferred to a sterile petri plate using a pipette and the wash portion of root were dipped into the alcohol and leaved immersed for 1-2 min. Sufficient amount of sterile water was pipetted into another sterile petriplate using a sterile pipette. Using a sterile forcep the portion of the roots were transferred to the sterile water in the petri plate to rinse off the alcohol. This operation was repeated at least twice more with fresh sterile water. *Rhizobium* strain were obtained by streaking the crushed root nodules on YEM (yeast extract mannitol, pH 7.0) agar plates and incubated at 29.4°C [1]. After 2 days of incubation, *Rhizobium* colonies were obtained. Streaking, spreading and visual description of colony morphology helped in isolation of pure cultures of *Rhizobium*. Further analysis and biochemical tests were performed in triplicates using pure isolates.

Morphological characterization of recovered isolates

Morphological characteristics of recovered isolates viz., colony size, form, colour, margin, elevation were taken into account for preliminary characterization of the isolates by examining the growth of the isolates on solid media in petri plates. To study the cell motility and shape, single colony from the agar plates was transferred to a drop of sterile water on glass slide and observed under light microscope (Olympus, India) at 100x magnification.

NaCl, pH and temperature variation assay

Tolerance to sodium chloride was determined on YEM agar plates containing *Rhizobium* culture having different concentrations of NaCl ranging from 1 to 6% (w/v). Tests were performed in triplicate. Growth was observed by measuring the optical density at 600 nm after 48h of inoculation. The effect of pH extremes on the growth of the organism was determined in YEM agar by adjusting the pH to 3.5, 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. All the plates were incubated at 28°C for 72 hours and YEM medium plates were used as controls. Difference in the range of growth temperature were investigated by incubation of bacterial cultures in YEM agar at 32°C, 34°C, 36°C, 38°C and 40°C. Control plates were incubated at 28°C. Strains were considered salt tolerant, resistant to acidity and temperature resistant when growth was similar to the growth in the control plates.

Biochemical characterization

Gram Staining

Gram staining is an empirical method of distinguishing bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls. For observation under light microscope, slides of isolated and purified bacterial cultures were prepared for gram staining by the Vincent's method[17]. A loopful of bacterial culture is smeared on a glass slide and allowed to air dry. The smear was then heat fixed with the help of spirit lamp, stained with crystal violet for one minute and slightly washed with distilled water. Gram's iodine solution was then added and waited for about one minute. The smear was then decolorized with 95% ethanol for one minute. This can be done in a steady stream, or a series of washes. The sample was washed with distilled water and counterstained with safranine for 2-3 minutes. Then the slide was washed with distilled water, air dried and observed under light microscope (Olympus, India) at 40 x and 100x magnification using oil immersion.

Glucose peptone agar (GPA) and lactose assay

To determine the ability of the microorganism to utilize glucose as the sole carbon source for its growth GPA assay was performed. GPA medium (40 g/L glucose, 5 g/L peptone, 15 g/L agar, pH 7.0) was inoculated with *Rhizobium* culture. After incubation growth was observed. In the same way lactose test was done to check the capability of the micro-organism to utilize lactose present in medium (10 g/L lactose, 5 g/L peptone, 3 g/L beef extract, 15 g/L agar, pH 7.0) as the sole carbon source for its growth. Presence of growth was observed after 48 hours according to Vincent[17].

Gelatin hydrolysis

The test was performed to determine the ability of microorganisms to produce gelatinase enzyme and use gelatin as media source. Degradation of gelatin indicates the presence of gelatinase enzyme[1]. The actively grown cultures were inoculated in nutrient gelatin medium (5 g/L peptone, 3 g/L beef extract, 12 g/L gelatin) and grown for 48 h. On subjecting the growing culture to low temperature treatment at 4°C for 30 min, the cultures which produce gelatinase remains liquefied while others due to presence of gelatin becomes solid.

Catalase Test

This test was performed to study the presence of catalase enzyme, which converts hydrogen peroxide to water and oxygen in bacterial colonies. Bacterial colonies were taken on glass slides and one drop of H_2O_2 (30%) was added. Appearance of gas bubble indicated the presence of catalase enzyme[14].

Starch Hydrolysis test

Starch ($C_6H_{10}O_5$) is an insoluble polymer of glucose which acts as a source of carbon and nitrogen for microorganisms, which have an ability to degrade them. To determine capability of microorganism to use starch as carbon source[4] this test was performed. Starch agar plates (5 g/L peptone, 2 g/L potato starch, 3 g/L beef extract, 15 g/L agar and pH 7.0) were prepared and streaked with suitable culture. The microbes were incubated at 37ºC for 48 hrs. Production of extracellular enzymes occurs in the presence of starch indicating the potential of the organism to use starch as carbon source. Iodine test was used to determine capability of microorganisms to use starch. Iodine solution was poured on 48h cultures grown on petri-plates. Formation of blue color indicated non-utilization of starch and vice versa.

Nitrate reduction test

Some bacteria use nitrate (NO_3^-) in place of oxygen as an exterior terminal electron acceptor. At the beginning, nitrate can be reduced to nitrite. In case of aerobic bacteria, oxygen is first used to avoid nitrate reduction and then make use of nitrite. The nitrite may promote nitrogen, ammonia; nitrogen oxide (N_2O).The enzyme reaction is catalyzed by nitrate reductase. Nitrate Broth comprising of 1g/L potassium nitrate (KNO_3), 5g/L peptone, 3g/L beef extract and pH 7.0 was prepared in test tubes. After inoculation and incubation at 37ºC for 48 hours, 3 drops of nitrate test reagent (20g/L zinc chloride, 4g/L starch, 2g/L potassium iodide) was taken in a porcelain plate and 1 drop of sulphuric acid was added to it.

To the mixture, 1 drop of bacterial culture was then added. Appearance of blue colour indicated that nitrite was produced. If no blue colour developed, then there might be two possible reason-

- there was no occurrence of nitrate to nitrite reduction or
- all the nitrite so formed must have been converted further to other products.

In such situation, zinc dust was added to each spot that appears to be negative for blue colour. Still if there was no blue colour, it means bacteria reduced nitrate and nitrite was formed. Another possibility was to get blue colour and it means that the bacteria did not reduce the nitrate.

Urease test

Urea is a waste nitrogenous material and is excreted out by animals. Some bacteria degrade urea into ammonia and CO_2. Urease is a hydrolytic enzyme that attacks the nitrogen and carbon bond in amide compounds such as urea and form alkaline end product as ammonia. Due to production of ammonia, the urease production can simply be confirmed. Urea Broth Medium comprising of 20g/L urea, 0.1g/L yeast extract, 9g/L potassium diphosphate (KH_2PO_4), 9.5g/L dipotassium hydrogen phosphate (K_2HPO_4), 0.01g/L phenol red was prepared and pH adjusted to 6.8. Bacterial culture were inoculated and incubated, which were observed for the production of color according to Lindstrom and Lehtomaki[12]. As the substrate urea is split into its product, the presence of ammonia creates an alkaline environment that causes the phenol red to turn to a deep pink colour. This is a positive reaction for the presence of urease.

Antibiotic sensitivity test

The susceptibility or resistance of rhizobia to an antibiotic was assayed with the help of antibiotic disc test. Some antibiotics kill the bacteria whereas others prevented the bacteria from multiplying so that the host's immune system could overcome them. Muller Hinton Agar media plates were prepared and antibiotic discs were placed equidistantly using sterile forcep. The plates were incubated at 37°C for 48 hrs. Resistance to an antibiotic was detected by the inhibition zone formed around the discs using Disk Diffusion method[15]. Five antibiotic discs were used viz. erythromycin, chloramphenicol, ampicillin, kanamycin and streptomycin.

RESULTS AND DISCUSSION

A total of 50 bacteria were recovered from root nodules of *Cassia alata*, collected from different agro climatic regions of Barak Valley, Assam. General microscopic observation of the isolates appeared to be non-sporing rod cells and gram negative in nature. All isolates showed the same colony characteristics, after 48 hours of incubation. The colonies were milky white, translucent, circular in shape, shiny, raised and 2-4 mm in diameter. The pH of the medium and broth during growth of isolates was changed from 7.0 to 6.0, thus showing the production of acid which is the characteristic of *Rhizobium* to produce acid during growth[2].

Nitrogen fixation and nodulation in legumes is significantly reduced by salt stress. Salt stress may decrease the competence of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis, and consequently nitrogen demand, by decreasing survival and proliferation of rhizobia in the soil and rhizosphere, or by inhibiting chemotaxis and root hair colonization, thus directly interfering with root nodule function, Hashem *et al*.[8]. Growth at 1% NaCl were indicated in our experiment but were incapable to grow on higher concentrations, thus screening that the isolate was sensitive to the salt. pH range for rhizobia was observed within 5.5 to 7.5. No growth was observed in the medium with pH 3.5. At pH 4, 50% of the isolates exhibited an acid tolerant character. Similar results were observed by Gao *et al*.,[5]; Kucuk *et al*.,[10]; Baoling *et al*.,[2] . The maximum temperature where more than 85% of the isolates grew was 36°C. No growth was seen at temperature 40°C and 45°C.

Rhizobial cells were able to grow on the GPA media showing the utilization of glucose as the carbon source by the *Rhizobium*. It is a positive test for *Rhizobium* and these are able to utilize glucose as carbon source[10]. Positive results were obtained from the starch hydrolysis assay. Applying iodine to inoculated plates, clear zones around the colonies were seen and the colonies turned yellow in appearance, whereas no growth areas appeared blue in colour. This implied that the isolates had the potential to hydrolyze starch present in the medium.

Rhizobium strains can utilize starch obtained from different sources was also observed by De Oliveira *et al*.[4]. It was observed that the rhizobial strains do not produce gelatinase enzymes as medium containing gelatin solidified when kept at 4°C for 30 and 60 minutes. Negative gelatinase activity is also a feature of *Rhizobium*[9]. In this study, all isolates were catalase and urease positive. The isolates showed evolution of bubbles hence they enhance catalase activity which complements the finding of Lupwayi and Hague[13]. In the nitrate reduction test, all isolates from *Cassia alata* resulted to be positive on addition of zinc dust. Nitrate is reduced to nitrite producing ammonia.

Antibiotics erythromycin and ampicillin resistant proved resistance towards the *Rhizobium* isolates at the amount of antibiotics under observation. However, *Rhizobium* was susceptible to chloramphenicol, kanamycin and streptomycin. 3.5, 2.5 and 2.0 cm were the diameter zone of inhibition against chloramphenicol, kanamycin and streptomycin, respectively.

Acknowledgement

The authors wish to extend their grateful thanks to Department of Biotechnology (DBT), Government of India, New Delhi for the establishment of Institutional Level Biotech Hub and Bioinformatics Centre in Gurucharan College, Silchar, India.

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