Isolation and characterization of *Rhizobium* spp. from root of legume plants species

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ABSTRACT: Isolation and Characterization of *Rhizobium* sp. were collected from root nodules of legume plants in subfamily Mimosoideae, Caesalpiniodeae and Papilionoideae. One hundred thirteen strains were characterized by biochemical assay. Morphological properties of Eighty six isolates were fast growing indicated that isolated rhizobia and had color of colony in white and pink. There were produced gummy colonies on YMCA plates after 3 days of incubation at 37°C. All strains were rod shaped, gram-negative and capable of producing poly hydroxyl butyrate. All strains utilized glucose, manitol, lactose as fermentation sugar. The isolates from present study may be useful to increase the symbiotic nitrogen fixation in legume plants

Keywords: isolation, characterization, *Rhizobium*, legume plant

Introduction

Nutrient enrichment of soils by nitrogen fixing symbiotic bacteria present in legumes has been known for centuries. Scientific demonstration of this symbiosis was started in 19th century and it established the facts that bacteria present in nodules on legume roots are responsible for fixing atmospheric nitrogen (Deshwal etal., 2011). *Rhizobium* species are known as bacteria that act as the primary symbiotic fixer of nitrogen. These bacteria infect the roots of leguminous plants, leading to the formation of lumps or 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. This symbiosis reduces the requirements for nitrogenous fertilizers during the growth of leguminous crops (Zsbrau, 1999). *Rhizobium* species are symbiotically associated with several leguminous plants such as *Pisum sativam, Glycine max, Alfa alfa* etc. These *Rhizobium* are Gram negative, motile, and non-endospore forming bacteria. These bacteria are generally cultured in Yeast Mannitol Agar medium (YEMA medium) (Holt et al., 1994). Rhizobium species give colorless gummy appearance when grown on YEMA medium supplemented with congo red. The gummy appearance is because of extracellular polysaccharide production. Importantly, they are able to accumulate a high amount of poly hydroxyl butyrate (PHB) intracellular (Kumari and Dhingra, 2013). Rhizobiaceae family contains six genera namely *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium* and *Bradyrhizobium*, respectively (Okazaki et al., 2004). Biofertilizer promotes plant growth and productivity has internationally been accepted as an alternative source of chemical fertilizer. Rhizobacteria effectively colonize plant root and increase plant growth by production of

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various plant growth hormones, P-solubilizing activity, N_2 fixation and biological control activity (Deshwal et al., 2011). A well established practice for maintaining soil fertility has been the cultivation of leguminous plants which replenish atmospheric nitrogen through symbiosis with rhizobia in rotation with non leguminous plants (Shahzad et al., 2012). In the present study, *Rhizobium* spp. were isolated from root nodules. Further characterization was done by performing various biochemical tests.

Materials and Methods

Rhizobium Isolation from root nodules of legume plant

The fresh and plump root nodules of legume plants of subfamily Mimosoideae, Caesalpiniodeae and Papilionoideae were collected from different locations in Udon Thani province, Thailand. The collected nodules were surface-sterilized with 95% ethanol and 3% H_2O_2 and washed thoroughly with distilled water. *Rhizobium* strains were obtained by streaking the crushed root nodules on YMCA (10g/L manitol, 0.5g/L K_2HPO_4, 0.2g/L $MgSO_4 \cdot 7H_2O$, 0.1g/L NaCl, 4g/L $CaCO_3$,0.4g/L Yeast extract, 0.25% congo red, 15g/L agar, pH 7.0) agar plates and incubated at 37°C. After 5 days of incubation, *Rhizobium* colonies were obtained. The white, translucent, elevated and mucilaginous colonies were picked up and transferred to YMA slant for further characterization.

Microbiological assays

The morphological traits were evaluated by comprised of colony morphology, mucous production and pH changing of YMBA medium (10g/L manitol, 0.5g/L K_2HPO_4, 0.2g/L $MgSO_4 \cdot 7H_2O$, 0.1g/L NaCl, 4g/L $CaCO_3$,0.4g/L Yeast extract, 0.5% bromthymol blue, 15g/L agar, pH 7.0) during growth. Mucous morphology assay was measured on type, elasticity and appearance, while colony morphology parameters were diameter, form, transparency and color. Gram staining reaction was performed to evaluate type of strain and cell shape (Holt et al., 1994).

Sudan black B staining method

PHB producing bacteria was further confirmed using Sudan black B staining method (Schlegel et al., 1970) with some minor modifications. Sudan black B stain was prepared as 0.3% solution (w/v) in 60% ethanol. The smear of cultures was prepared on glass slides and heat fixed. The samples were stained for 10 mins. with Sudan black solution, rinsed with water and counter stained with 0.5% safranin for 5 mins. and observed at 1000X magnification.

Glucose peptone agar (GPA) and lactose assay

GPA assay was performed to determine the capability of the microorganism to utilize glucose as the carbon source for its growth. The single colony of *Rhizobium* spp. was streak on GPA medium (5g/L glucose, 10g/L peptone, 15g/L agar, pH 7.0) an inocubation in 37°C for 3-5 days . Similarly, lactose assay was performed to determine the capability of *Rhizobium* spp. to utilize lactose present in YLA medium (10g/L lactose, 0.5g/L K_2HPO_4, 0.2g/L $MgSO_4 \cdot 7H_2O$, 0.1g/L NaCl, 4g/L $CaCO_3$,0.4g/L Yeast extract, 15g/L agar, pH 7.0) an inocubation in 37°C for 3-5 days.

Litmus milk test

Litmus milk is a complex medium that can potentially distinguish among many species of bacteria. Litmus milk has several components that can be metabolized: lactose (milk sugar); casein (milk protein); and litmus (a pH indicator is purple to blue at neutral to alkaline pH and pink under acidic conditions). Litmus milk broth (100g/L Skim milk powder, 0.075g/L Litmus, pH 6.8) was inoculated with *Rhizobium* culture, incubated in 37°C for 3-5 days and growth was observed.

Results and Discussion

There were 113 isolates of *Rhizobium* spp. collected from 12 legume plants contained with *Sesbania javanica, Mimosa pigra, M. pudica, Leucaena leucocephala, Tamarindus indica, Butea monosperma, Doliches lablab, Dalbergia duoerreana, Vigna unguiculata, Pterocarpus indicus, Sesbania grundiflora,* and *Arachis hypogaea*, respectively (**Figure 1** and **Table 1**). The coloress gummy colonies were found in all isolates of *Rhizobium* sp. after streaked on YMCA plates for 5 days at 37°C with fast growing rate. Gram negative was observed in Gram's reagent and also rod shape of bacteria cell with pink color was observed under microscope (**Figure 2**). To distinguish PHB producer, they were stained with sudan black B dye. Dark black to purple granules were observed intracellularly with pink background when counterstained with safranin. This confirmed that all isolates were capable to accumulate PHB intracellularly. All *Rhizobium* isolates were able to grow on GPA medium showing the utilization of glucose as the carbon source. However, some pure *Rhizobium* isolates are unable to grow on lactose. Casein utilization and peptonization were resulted in litmus milk test by *Rhizobium* isolates. Most of the biochemical tests were giving the same results as reported for *Rhizobium* spp. in literature. Shahzad et al., (2012) reported that *Rhizobium* from root nodules of *Alfafa* (*Medico sativa*) collected equally from district Quetta Balochistan, Pakistan. After series of biochemical and sugar fermentation assay, twenty five samples were identified as *Sinorhizobium meliloti*. Singh et al. (2008) also observed that *Rhizobium* strain isolated from root nodules of fenugreek. The *Rhizobium* isolates were rod shape, gram negative, acid and mucous producing. They were temperature and pH sensitive. The *Rhizobium* species have the potential to produce industrially important enzymes; amylase and cellulase. The isolates from present study may be useful to increase the symbiotic nitrogen fixation in legume plants. This study therefore provides the basis for further research on isolation and characterization of *Rhizobium* strains nodulating the legume plants.

Figure 1 Root of *Dalbergia duperreana* showing nodules developed by symbiotic bacterium *Rhizobium* spp.

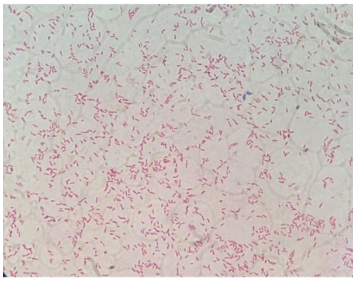

Figure 2 Gram negative rod shaped *Rhizobium* spp. cell showed at 1000X magnification

Table 1 Colony characterization and number of isolates of *Rhizobium* spp. isolated from various of legume plants

Legume plant species	Colony color on YMCA	Colony texture on YMCA	Number of isolates
Sesbania javanica	white	gummy	4
Mimosa pigra	white	gummy	14
M. pudica	white	gummy	5
Leucaena leucocephala	white	gummy	15
Tamarindus indica	white	gummy	4
Butea monosperma	white	gummy	7
Pterocarpus indicus	white	gummy	13
Sesbania grundiflora	white	gummy	22
Arachis hypogaea	pink	gummy	8
Doliches lablab	pink	gummy	7
Dalbergia duperreana	pink	gummy	12
Vigna unguiculata	pink	gummy	2
		Total	113

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References

Deshwal, V.K., K. Vig, D.M. Amisha, P. Yadav, D. Bhattacharya, and M. Verma. 2011. Synergistic effects of the inoculation with plant growth-promoting *Rhizobium* and *Pseudomonas* on the performance of Mucuna. Ann. Forestry. 19: 13-20.

Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley, and S.T. Williams. 1994. Bergey's Manual of Systematic Bacteriology. 9th ed. Williams & Wilkins, USA.

Kumari, P., and K.H. Dhingra. 2013. Isolation and characterization of PHB producing micro-organisms isolated from root nodules of leguminous plants. An international quarterly journal of life science. 8: 109-113.

Okazaki, S., N. Nukui, M. Sugawara, and K. Minamisawa. 2004. Rhizobial strategies to enhance symbiotic. Interactions: Rhizobiotoxine and 1-Aminocyclopropane-1-Carboxylate deaminase. Microb. Environ. 19: 99-111.

Schlegel, H.G., R. Lafferty, and I. Krauss. 1970. The isolation of mutants not accumulating poly-beta-hydroxybutyric acid. Arch. Microbial. 70: 283-294.

Shahzad, F., M. Shafee, F. Abbas, S. Babar, M.M. Tariq, and Z. Ahmad. 2012. Isolation and biochemical characterization of *Rhizzobium meliloti* from root nodules of Alfalfa (*Medico sativa*). J. Anim. Plant Sci. 22: 522-524.

Singh, B., R. Kaur, and K. Singh. 2008. Characterization of rhizobium strain isolated from the roots of *Trigonella foenumgraecum* (fenugreek). Afr. J. Biotechnol. 7: 3671-3676.

Zsbrau, H.H. 1999. *Rhizobium*-Legume symbiosis and nitrogen fixation under sever conditions and in arid climate. Microbiol. Mol. Biol. Rev. 63: 968-989.