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Soundarya K R
Department of Microbiology, School of Life Sciences, JSS Academy of Higher Education and Research (Deemed to be University), SS Nagar, Mysuru, India.

Bhavana D
Department of Microbiology, School of Life Sciences, JSS Academy of Higher Education and Research (Deemed to be University), SS Nagar, Mysuru, India.

Harsha T S
Department of Environmental Science, Karnataka State Open University, Mukthagangotri, Mysuru, India.

Nagalambika Prasad
Department of Microbiology, School of Life Sciences, JSS Academy of Higher Education and Research (Deemed to be University), SS Nagar, Mysuru, India.

Correspondence:
Nagalambika Prasad
Department of Microbiology, School of Life Sciences, JSS Academy of Higher Education and Research (Deemed to be University), SS Nagar, Mysuru, India.

Isolation, Identification and Biochemical Characterization of *Rhizobium* spp. from *Mimosa pudica*

Soundarya K R, Bhavana D, Harsha T S, Nagalambika Prasad

Abstract
The Rhizobium bacteria is a symbiont, associated with the roots of the leguminous plant and forms root nodule. Plants affords the nutrients which are needed for the metabolism of the bacteria and in turn provides the atmospheric nitrogen needed for the plants. These organisms convert the atmospheric nitrogen into accessible forms and makes this able to utilize by the plants for their development. Nitrogen is one of the essential elements needed for the growth and development for the plants which mainly acts as a building block of the protein which are responsible for the main metabolism in the plant body. The bacteria are isolated and identified on the YEMA media and confirmed by the morphological and most appropriate biochemical tests.

Keywords: Rhizobium, Root nodule, Symbiotic association, Mimosa pudica.

Introduction
The Rhizobium species are soil bacteria and basically found in soil. These are non-spore forming, Gram-negative rod-shaped bacteria possess flagella at its polar ends. It is well known for the symbiotic association with various leguminous plants (Chaintreuil et al., 2000). Martinus Beijerinck was the first person to isolate and cultivate Rhizobium from the nodules of legumes in 1888. These are classified as follows, it belongs to kingdom - Bacteria, lacks membrane bound organelles. Phylum - Proteobacteria, responsible for fixing nitrogen fixation. Class - Alpha proteobacteria, composed of Gram negative varying in characters. Order- Rhizobiales, forms a symbiotic relationship with plant roots. Family - Rhizobiaceae, includes species showing positive and negative development on plants. Species - includes different types of species, namely Rhizobium leguminosarum has an ability to fix the free nitrogen from the air. Rhizobium binae enhances growth of lentil peas. Some other species are Rhizobium alamii, Rhizobium lentis, Rhizobium japonium, Rhizobium smilacinae etc (Xingyan Tan et al., 2019).
The plant Mimosa pudica is commonly known as touch me not, live and die, shame plant and humble plant, touch me not, shame weed, shame bush, sensitive plant, sensitive weed, shame lady plant (Fig 1). It is a perennial herb usually found in the tropical America, Australia and also in tropical and subtropical parts of India. There are about 4000 species of Mimosa. The periodic movements of closing and opening of leaves are due to the presence of derivatives of 4-O-gallic acid (Genest et al., 2008).
These plants are usually propagated by the seeds and vegetative methods. This plant majorly possesses antibacterial, antivenom, antifertility, anticonvulsant, antidepressant, aphrodisiac and other pharmacological activities (Ueda and Yamamura, 1999). These herbs are in use since ages, for the treatment of urinogenital disorders, piles, dysentery, sinus and can also be applied on wounds (Damaris et al., 2017).

Scientific classification
Kingdom: Plantae
Division: Magnoliophyta

Class: Magnoliopsida
Order: Fabales
Family: Fabaceae
Genus: Mimosa
Species: Mimosa pudica

The extracts of the plants are very effective in treating reproductive disorders, diarrhea and dysentery. The decoction of the root extract is useful to treat renal stones, urinary complications, bleeding piles, relieve vaginal prolapse and anal prolapse. The powder of the seeds is used for increasing low sperm count. The leaf extracts are effectively used in treatment of skin diseases, menorrhagia, nasal bleeding, asthma and chronic respiratory disorders and fractures of the bone. This study is directed to investigate the presence root nodule in the plant root and also isolated the *rhizo* bacteria from the root nodules.

Materials And Methods

Isolation

The uprooted plant samples are brought into the laboratory, tagged properly. The roots were handled carefully during washing so that the nodules are not separated from the root surface. Pink and healthy root nodules were picked with sterile forceps. These nodules are then surface sterilized with the help of 1% sodium hypochlorite, followed by washing with alcohol and finally with distilled water (Damaris *et al.*, 2017). Then the nodules are placed on a clean glass slide and crushed smoothly with another slide, so to obtain a creamy juice liquid containing the bacteria. This liquid substance is used as the inoculum for the isolation of Rhizobium. The isolation is done on selective medium for Rhizobium i.e., YEMA (Yeast Extract Mannitol Agar) media by streak plate inoculation method. The streaked plates were incubated at 35ºC for 24 hours and are observed for the appearance of growth. White mucoid colonies appear on streaked Petri plates than it proved the presence of *Rhizobium*. These white and mucoid colonies are picked up separately and inoculated on nutrient agar slants in two replicates and incubated at 35ºC for 24 hours (Baby joseph *et al.,* 2013). After incubation, if proper growth occurred, then the agar slants were preserved at 4ºC in refrigerator for further biochemical characterization.

Gram staining

Bacterial smear was prepared separately and were heat fixed by passing over a Bunsen burner flame and then using a basic stain crystal violet (primary stain) for one minute. Then, it was washed with distilled water and immersed in Gram's iodine for one minute. Then washed with distilled water and blot dried smear was flooded with 95% ethyl alcohol (decolorize) for 30 sec. It was again washed with water and then it was counter stained with safranin (secondary stain), again washed with distilled water and finally dried and examined under 10X, 45X and finally with oil immersion objective (100X) microscope. Gram-negative rod-shaped bacteria retain the pink/red colour while Gram-positive bacteria retain the crystal-violet (Aneja KR. 2003.)

Biochemical characterization

Biochemical characterization of different isolates was done for the identification of Rhizobium on the basis of different biochemical tests viz., Indole test, MR VP test, oxidase test, Sugar fermentation test, Citrate utilization test, Urease test, Catalase test, Starch utilization test and Nitrate reduction test (Aneja KR. 2003).

Indole test

Tryptone broth medium was prepared by dissolving 0.25 grams of tryptophan in 25 ml distilled water. The medium was poured into the test tubes. The culture was inoculated and incubated at 30ºc for 2days. The uninoculated broth is maintained as control. After the period of incubation about 1ml of kovac's reagent is added including control tube and allow it for some 10 minutes and observe the formation of red coloured ring formation indicates the positive results whereas the presence of yellow ring indicates the negative result (Akbar Hossain *et al*., 2019).

Methyl red test

The broth is prepared and for about 5ml of broth was prepared and transferred into test tubes. Tubes are incubated for about 24 to 48 hours (Agarwal *et al.,* 2013). Then methyl red indicator is added to the tubes and the development of red colour in the broth indicates the positive result and yellow colour indicates negative result.

Citrate utilization test

In this medium citrate is the only carbon source available to the bacteria; however, the *Rhizobium* cannot grow on the citrate and therefore, no change in colour occurs. To inoculate the slant, a loop full of culture of *Rhizobium* was used; the slant was inoculated following stab and streak method and finally observed after incubation period of 24 h at 37°C.

Catalase test

This test was performed to study the presence of enzyme Catalase which hydrolyzes H_2O_2 into H_2O and O_2 in bacterial strains20. Rhizobial colonies (2-3 days old) were taken on glass slide and one drop of H_2O_2 (30%) was added.

Starch Hydrolysis:

This test was performed to determine the capability of Rhizobium to use starch as a carbon source[19].Starch Agar Medium was inoculated with Rhizobium and analyzed for starch utilization. Iodine Test was used to determine the capability of microbes to use starch. A drop of iodine (0.1N) was spread on 24-hour old culture and clear zone of inhibition were formed (De Oliveira et al., 2007).

Oxidase test

A clean glass slide is taken and the disc is placed on the slide and loopful of culture is added on to that the disc turns blue colour within a minute after adding loopful culture this indicates the positive result.

Sugar fermentation test

Sugar fermentation broth is prepared and for about 0.25grams of glucose and lactose is added to the medium separately along with the durham's tube and a loopful of culture is inoculated into the broth containing the sugars and incubated at 30c for about 24 to 48hours. The change in the colour of media indicates the utilization of sugars and the gas bubble in the durham's tube indicates that the organism is capable of producing the gas.

Hydrogen sulphide production test

The hydrogen agar medium is prepared and the loopful of culture is inoculated and incubated for about 2 to 3 days at 30^0c and then results are recorded. The formation of black precipitate on the medium indicates the positive result. No blackening of the medium indicates the negative result.

Nitrate reduction test

Nitrate broth prepared and distributed into the tubes and loopful culture is inoculated and incubated for about 2 to 4 days. After the complete growth for about 2 to 3 drops of reagent A sulphanalic acid is added and 2 to 3 drops of reagent B α - naphthalamine is added for the appearance of red colour indicates the positive result.

Results And Discussion

Root nodule samples collected from leguminous plant Mimosa pudica (Fig. 2) of Bangalore region were used for the isolation and identification of Rhizobium bacteria. The sample were found positive for the presence of Rhizobium on the basis of white mucoid growth on YEMA medium when incubated for 24 h at 35^0C (Datta et al., 2015; Mahana et al., 2000) (Fig. 3). All the samples that showed the presence of Rhizobium when subjected to Gram staining and all the isolates were found Gram-negative as the cells appeared pink (Fig. 4). After Gram staining, all the isolates are preserved on nutrient agar slants for further characterization.

Characterization of isolate was done on the basis of different biochemical tests viz. Citrate utilization test, Triple sugar iron test, Glucose peptone agar utilization test, Gelatin liquefaction test, Starch utilization test and Catalase test gave positive result and the isolate did not ferment the lactose (Figs. 5 - 14) (Ishihara et al. 2002; Küpper et al. 2011; Dinesh et al. 2015; McDevitt 2009; Chhetri et al., 2019; Tyagi et al., 2019). The results of different biochemical tests are represented in Table 1. All these tests confirmed that findings of the present study have been isolated Rhizobium species and identified on the basis of morphological and biochemical characters.

Fig. 1: Flower of Mimosa pudica.

Fig. 2: root nodules of *Mimosa pudica.*

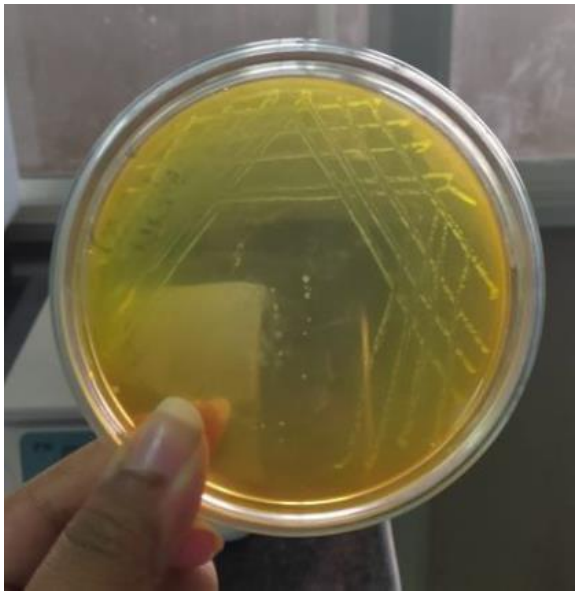

Fig. 3: Pure culture of *Rhizobium.*

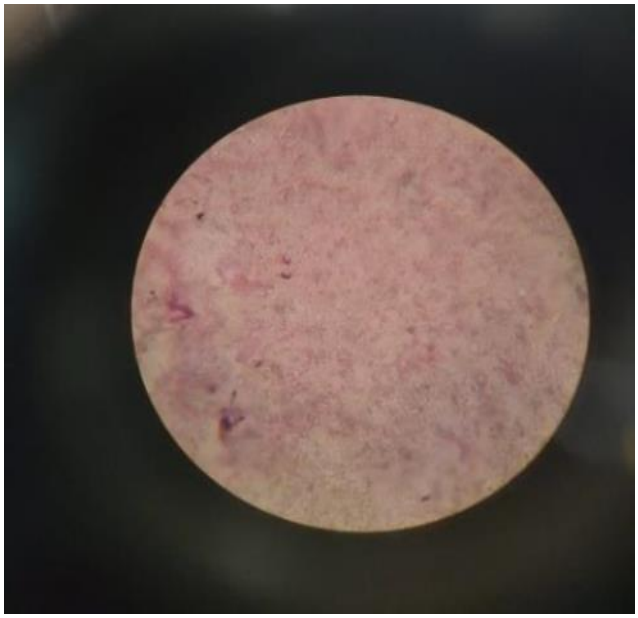

Fig. 4: microscopic view of *Rhizobium.*

Fig. 5: Carbohydrate.

Fig. 6: carbohydrate test (+) Fermentation test (-).

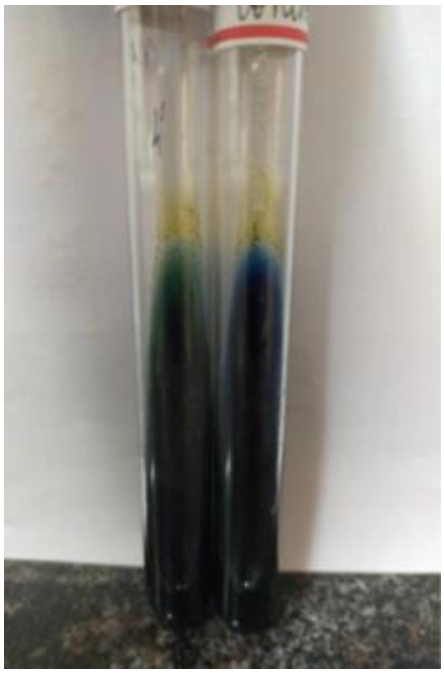

Fig. 7: citrate utilization.

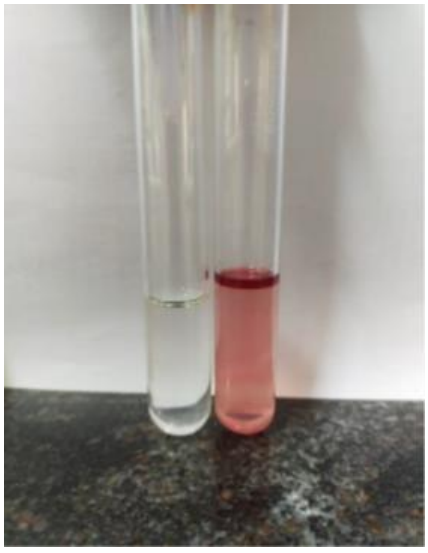

Fig. 8: Indole test (+).

Fig. 9: sulfide production test (-).

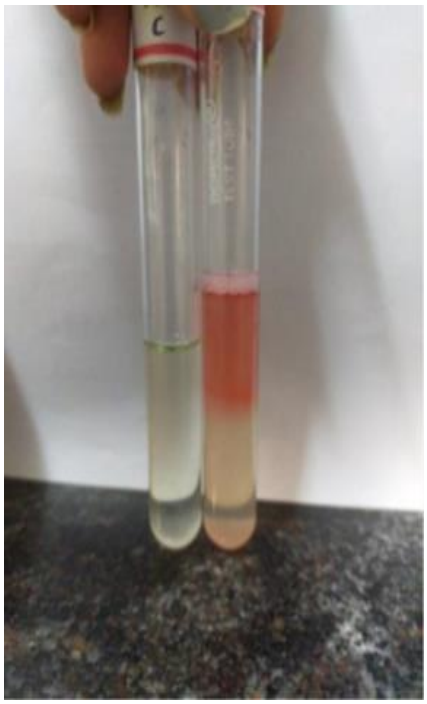

Fig. 10: MR Test.

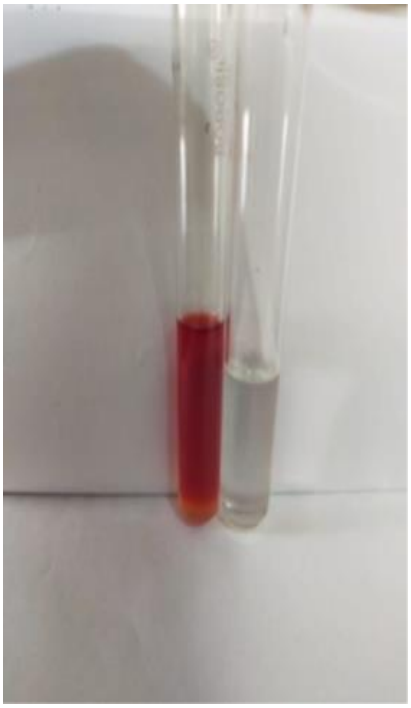

Fig. 11: VP Test.

Fig. 12: Oxidase test (+).

Fig.13: Catalase (+).

Fig 14: Starch hydrolysis test (+).

Table 1: Results of Biochemical characterization.

Sl no	Biochemical tests	Result
1.	Indole production test	+
2.	Methyl red test	+
3.	Voges Proskauer test	+
4.	Citrate utilization test	+
5.	Hydrogen sulfide production test	+
6.	Sugar fermentation test	
a	Glucose	+
b	lactose	-
7.	Starch hydrolysis test	+
8.	Catalase test	+
9.	Oxidase test	+

Conclusion

The conclusion of the present study, the rhizobial isolates of Mimosa pudica to enhance the plant growth and nodulation of bean plant in natural environmental conditions. The symbiosis among Rhizobium and legumes is an inexpensive and usually effective for ensuring tolerable supply of nitrogen for legume-based crop and pasture fabrication than the application of organic fertilizers.

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