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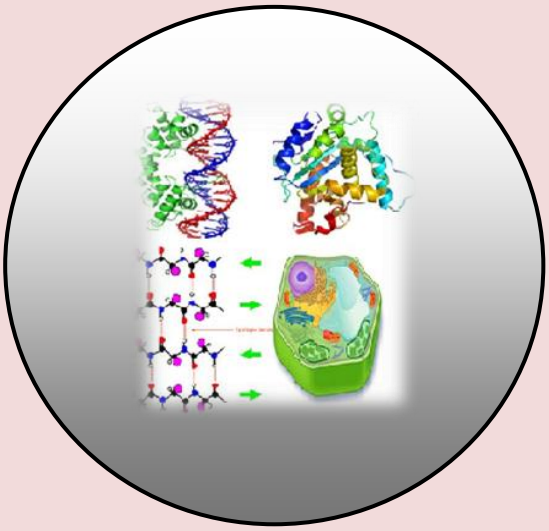

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RESEARCH PAPER

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Characterization and Antibacterial Activity of *Rhizobium spp.* from Root Nodules of Edible Crops of Fabaceae Family, Kerala

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ABSTRACT

Rhizobium spp. are formed in Nitrogen fixing nodules of leguminous plant roots. The present study describes isolation and characterization of Rhizobium species from root nodules of three leguminous plants such as (Cajanuscajan, Lablab purpureus and Vigna unguiculat) grown in Kerala agriculture field. The Rhizobium strains were rod shapes gram negative bacteria. The biochemical tests performed on the isolates showed that most were positive for citrate, catalase and Oxidase. While Voges – Proskauer, indole tests and gelatin hydrolysis were negative. It utilizes Glucose as a sole carbon source. Rhizobium strains were unable to hydrolysis the gelatin. Rhizobiumisolates showed antibacterial activity against Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Escherichia coli, Salmonella paratyphi, Klebsiella pneumonia, and Pseudomonas aeruginosa.

In conclusion, these Rhizobium strains can be used as biocontrol agents against human and plant pathogens or in bio-fertilizer.

Key words: Characterization, Antibacterial Activity, Rhizobiumsp., Root nodules and Legume plants.

INTRODUCTION

Leguminous plants have root nodules, in which bacteria live. The plant root supply minerals and the substances produced to the bacteria. These bacteria inside nodules undertake nitrogen fixation and plant absorb then in the from ammonia. This mechanism in the most important factor utilized by the agricultural biologists towards crop improvements and higher yield. Rhizobia are soil bacteria found in the leguminous plants, encourage the root hairs to taint and formation of root nodules followed by nitrogen fixation. Hence, the root nodule bacteria legume symbiosis has been studied as a mutualistic association and as beneficial in the field of agriculture. Rhizobia are nowadays considered the best biofertilizer. *Rhizobium* sp. are well known group of bacteria that act as a primary symbiotic fixer of nitrogen. These bacteria infect the root nodules of leguminous plants, leading to the formation of lumps and nodules where the nitrogen fixation is takes place. Bacteria isolated from root-nodules of *C. cajan, L. purpureus* and *V. Unguiculata* grown in different Kerala soils were diverse and could be affiliated to much genera, including *R. leguminosarum*, *Bacillus sp*. and other *Rhizobium spp*. (Darsha *et al.*,2018). *Rhizobium* plays a very important role in agriculture by inducing nitrogen fixing nodules on the roots of legumes such as peas, beans and clover.

In the 19th century the scientific demonstration of this cooperation was started and established that bacteria are present in legume root nodules which are responsible for fixing atmospheric nitrogen (Zsbrau, 1999). The *Rhizobium* species live inside the root nodules of host legumes so they are beneficial for the growth of the plants (Oblisami, 1995). They easily colonize in the plant root and promote solubilizing activity, nitrogen fixation and biocontrol activity (Deshwal *et al*., 2011). Root nodule bacteria generally grow under the following conditions 25-30°C (optimum) in the pH range of 6-7 (Somasegaran and Hoben, 1994). *Rhizobium* growth normally happens under aerobic situations. However, when fixing nitrogen, low levels of oxygen are essential to keep the enzyme nitrogenase (Goldberg *et al.,* 1987) and hence, *Rhizobium* are able to grow in microaerophilic conditions (Somasegaran and Hoben, 1994). Rhizobial nod genes are significant in the determination of host specificity, infection and nodulation and are involved in the exchange of signals between the plant and bacteria (Denarie *et al.,* 1992).Interestingly, some plants (legumes) own a exceptional capability to create symbiotic association with nitrogen-fixing bacteria of the family Rhizobiaceae. *Rhizobium* inoculants significantly advances yield in many leguminous crops and can diminish the usage of artificial fertilizer which is rather expensive and declines soil properties (Laurette*et al*., 2015). Their capabilitytofix nitrogen in symbiosis makes them excellent colonizers of low nitrogen environment and economically friendly crop grassland (Jensen and Hauggaard, 2002). In addition, nitrogen from legume fixation is principally "free" for use by both the host plant and allied or following crops (Kiers *et al*., 2002). A well-established practice for preserving soil fertility has been the agriculture of leguminous plants which refill atmospheric nitrogen through symbiosis with *Rhizobium* species in turning with non-leguminous plants (Dekaand Azad, 2006). Few endophytic bacteria isolated from young radishes can be used as biocontrol agent against human and plant pathogen (Seo *et al.,* 2010). Genetic variableness in salt tolerance happens within *Rhizobium*, and may significantly affect crop act. There is also a varied difference among chickpea *Rhizobium* strains in their ability to cultivate and live under saline condition (Zurayk *et al.,* 1998). Symbiotic *Rhizobium* species of naturally growing legumes are more tolerant to some ecological condition (salt, severe drought, raised temperature etc.) than *Rhizobium* from cultivated legumes (Zahran *et al.,* 2012). In the present study, strains of *Rhizobium* were isolated from the root nodules of three legumes plants such as *C. cajan, L. purpureus* and *V. unguiculata* and investigated its characterization and antibacterial activity against some pathogens bacteria.

MATERIAL AND METHODS

Collection and extraction of nodulated roots of legumes

Three plants included in Fabacea family such as *Cajanuscajan, Lablab purpureus* and *Vigna unguiculata*. The nodulated roots of *C. cajan* and *V. unguiculata* (Fig. 1) were collected from the paddy fields of Thiruvalathur region, Palakkad, Kerala, India. 10.74° N, 76.69° E. These plants were cultivated as mixed cropping. *L. purpureus*, cultivated as domesticated plant from the same area. The roots were first washed thoroughly with sterile distilled water and nodules were surface sterilized by washing with 95% ethanol for 10 seconds and again washed in sterile distilled water for about 5 times. Roots were mashed with pestle mortar to obtained nodules and milky white substances of bacteroids by dipping in phosphate buffer solution.

Serial dilutions of the extracted root nodules

After the extraction of bacteroid solution from the legumes root nodules, serial dilution was made. 2 ml of sterilized root nodule bacteroids solution was taken in 90 ml sterilized distilled water and serially diluted up to 10^{-6} dilution. For identification of the colonies, 10^{-4} to 10^{-6} dilution of nodule extract were plated on Yeast extract mannitol agar (YEMA). The petri plates were then kept in the incubator at 25.0 ± 1.0^0C for 5 - 7 days. After the plates were taken out of the incubator colony morphology and identification was carried out. All bacteriological isolation and the entire process of biochemical tests were carried in the laminar airflow to maintain the sterility.

Characterization of Isolates

The morphological and cultural as well as physiological or bio-chemical characteristics of the authenticated isolates were studied following the procedure given by (Rajendran *et al*., 2008).

Morphological and cultural characteristics

A) Cell characteristics:

The size, shape, motility and Gram stain reaction of the nodule bacterial cells were observed under microscope using standard procedure.

B) Colony characteristics:

The configuration, margin, elevation and color of the colonies of the test isolates grown on standard YEMA plates were observed according to (Rajendran *et al.*, 2008)

Biochemical characteristics

Biochemical characteristics of the *Rhizobium* isolates were studied using different tests like Indole production test, methyl red test, Voges Proskauer test, Citrate utilization test, urease test, oxidase test, catalase test, gelatin liquefaction test and sugars fermented tests.

Antibacterial activity assay

Antibacterial activity of *Rhizobium* isolates was tested by disc diffusion method against pathogens bacteria such as *Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Escherichia coli, Salmonella paratyphi, Klebsiella pneumonia,* and *Pseudomonas aeruginosa* isolated from different sources.

RESULTS AND DISCUSSION

In this present study, strains of root nodulating bacteria were isolated from the root nodules of *Cajanuscajan, Lablab purpureus*, and *Vigna unguiculate* (Figure 1). YEMA media was used for the isolation of *Rhizobium* bacteria. Colonies were observed on YEMA media after incubation at 25.0 ± 1.0 ^{0}C for 5 - 7 days. The colonies were circular, creamy white, raised and smooth (Figure 2). In biochemical testing, all the tests except Voges-Proskauer (VP), Indol production and gelatin hydrolysis were positive (Table 1, Figure 3 and4). Medium containing gelatin gets solidified when kept at 40^{0}C for 30 as well as 60 minutes as *Rhizobium* species was unable to hydrolyze the gelatin, therefore *Rhizobium* do not produce gelatinase enzyme (Figure 3 c.). Rhizobial cells were able to utilize glucose as a carbon source (Fig. 4.a). The colonies were large (2-5 mm in diameter) mucilaginous, circular, convex with smooth edges, glistening translucent or white (Kingchan and Chidkamon, 2014). Microscopic examination revealed that the isolates were rod shaped and gram negative in nature (Agah *et al.,* 2016). Singh *et al.*, 2008 characterized *Rhizobium* strain from the roots of groundnut bacterial species. These findings corroborate with the results of (Singh *et al.* 2008), and (Erum and Bano, 2008) who also reported these sugar tests positive during isolation and characterization of *Rhizobium meliloti* on most of leguminous plant roots. The biochemical tests performed on the isolates showed that most were positive for citrate, catalase and Oxidase. While Voges – Proskauer, indole tests and gelatin hydrolysis were negative. These findings are in close agreement with (Javed and Asghari, 2008) who have previously characterized the *Rhizobium* from soil and root nodules of groundnut with same positive biochemical tests. Similarly, (Oblisami, 1995) studied the nodulation pattern in legume plants by screening through the same tests and reported similar results. A study for Agah et al., 2016 carried out on root nodules of *Arachis hypogaea L.* and *Telfairia occidentalis,* their study revealed ten isolates of *Rhizobium* species. This shows that *Arachis hypogaea L.* and *Telfairia occidentalis* plants contain nitrogen-fixing bacteria, making them capable of self-nitrogen fertilization and valuable in croprotations. The *Rhizobium* isolates showed antibacterial activity against *Staphylococcus aureus, Micrococcus luteus, Bacillus subtilis, Escherichia coli, Salmonella paratyphi, Klebsiella pneumonia,* and *Pseudomonas aeruginosa* that activity was less than the control the antibiotic Gentamicin. The diameters of zone of inhibition were found to be 8.0 – 11.0 mm. (Table 2). A study for (Deora and Singhal, 2010) revealed the *Rhizobium sp.* had an antibacterial activity against *Streptococcus sp*., *E. coil* and *Pseudomonas sp*. the *Rhizobium* can be further easily immobilized into carrier like charcoal power which can be applied as biofertilizers. *Rhizobium* isolates were showed antibacterial activity against *Enterobacter* whereas it did not show antibacterial activity against *Pseudomonas* and *Staphylococcus*. Sensitivity of *Rhizobium* against Gentamycin, Streptomycin, Ampicillin and Chloramphenicol were studied. *Rhizobium* isolates showed sensitivity against Gentamycin, Streptomycin and Ampicillin antibiotics and resistance against Chloramphenicol. There are three determinants of bacterial permeability to an antibiotic: hydrophobicity, electrical charge, and amount of the antibiotic and the *Rhizobium* that showed a high level of resistance did not take up the antibiotics (Kumari *et al*, 2017; Hungaria *et al.,* 2000).

Table 1. Morphological and Biochemical Characteristics of *Rhizobium* species from Root Nodules of *Cajanuscajan, Lablab purpureus*, and *Vigna unguiculate.*

Sample	Colonial characteristic on YEMA		S	Sugars Fermentation		Motility	Catalase	Oxidase	Citrate test	VP	Indol	gelatin hydrolysis	GR
				Glucose	Lactose								
Cc	Creamy white	circ ular	R	+	+	+	+	+	+	-	-	-	-
Lp	Creamy white	Circ ular	R	+	+	+	+	+	+	-	-	-	-
Vu	Creamy white	Circ ular	R	+	+	+	+	+	+	-	-	-	-

***Key: Cc=Cajanuscajan, Lp= Lablab purpureus, Vu= Vigna unguiculat* YEMA= Yeast extract mannitol agar S= shape, R= Rod shape, VP= Voges-Proskauer, GR= Gram Reaction, + positive result, - Negative result**

Table 2. Inhibitory zone of Rhizobium species from Root Nodules of *C. cajan, L. purpureus*, and *V. unguiculat*.

Sample	Measurement of Zone of Inhibition (mm)± SD						
	S. aureus	*M. luteus*	*B. subtilis*	*E. coli*	*S. paratyphi*	*K. pneumonia*	*P. aeruginosa*
Cc	9.0 ± 0.45	9.0 ± 0.23	11.0 ± 0.27	10.0 ± 0.12	10.0 ± 0.37	8.0 ± 0.08	11.0 ± 0.25
Lp	9.0 ± 0.37	9.0 ± 0.36	10.0 ± 0.50	9.0 ± 0.20	8.0 ± 0.30	8.0 ± 0.11	9.0 ± 0.18
Vu	10.0 ± 0.22	9.0 ± 0.17	11.0 ± 0.38	10.0 ± 0.26	10.0 ± 0.26	8.0 ± 0.17	11.0 ± 0.24
St	19.0 ± 0.23	22.0 ± 0.26	20.0 ± 0.62	21.0 ± 0.17	25.0 ± 0.27	25.0 ± 0.14	16.0 ± 0.35

***Key: Cc=Cajanuscajan, Lp= Lablab purpureus, Vu= Vigna unguiculat*, St= Ciprofloxacin (10 µg disc)**

Figure 1. Legumes plants, a: *Cajanus cajan* (Pigeon Pea), *b: Lablab purpureus* (Hycinth Bean) *c: Vigna unguiculat* (Cow Pea).

Figure 2. *Rhizobium* Colonies on YEMA media a. from *C. cajan*, b. from *V. unguiculat*, c. from *L. purpureus*.

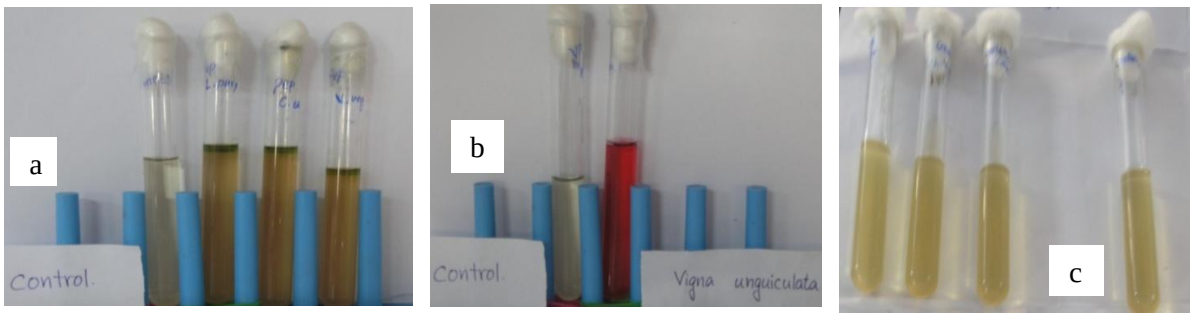

Figure 3. (a). Indol test, (b).VP test, (c). Gelatin test.

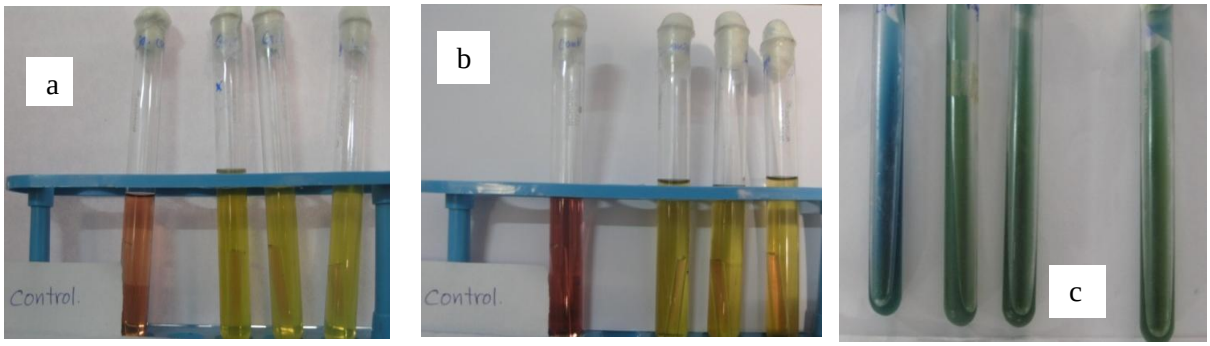

Figure 4. (a). Glucose test, (b). Lactose test, (c). Citrate test.

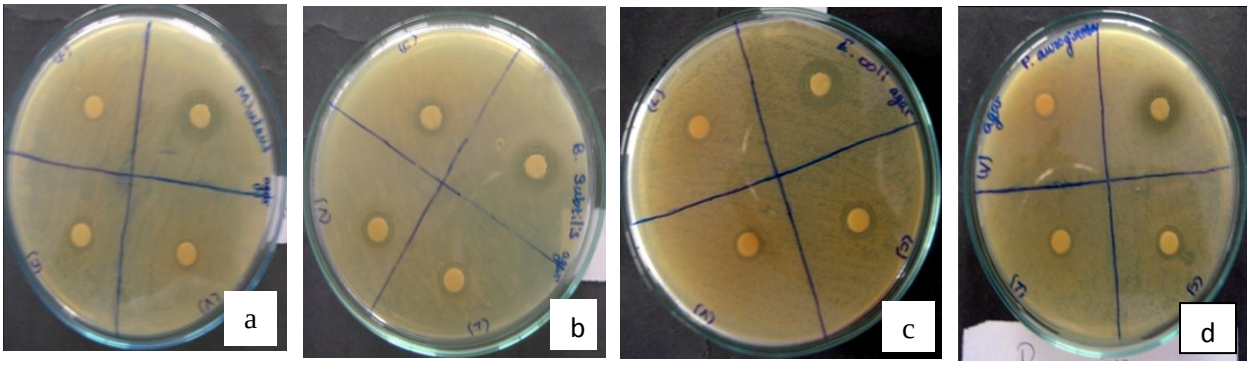

Figure 5. Antibacterial activity of *Rhizobium* isolates against: (a). *M. lutues*, (b). *B.subtilis*, (c). *E. coli*, (d). *P. aeruginosa*.

CONCLUSION

Rhizobium is an important microorganism for the environment because of its nitrogen-fixing ability when in symbiotic relationship with plants (mainly legumes). This study confirmed that the root nodules of *Cajanuscajan, Lablab purpureus*, and *Vigna unguiculate* plants harbour the nitrogen-fixing bacterium- *Rhizobium.* The present investigation seems to be promising approach to consider the optimum method for the isolation of rhizobia from legumes plant that act as a potential candidate to be used in nitrogen fixation and lab-based experiments. These findings allow us a new scope for extensive research in Agricultural Biotechnology.

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