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# Molecular identification of root nodule bacteria from edible crops of Fabaceae family, Kerala

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## Abstract

The root nodule bacteria were isolated from three different leguminous plants belonging to the Fabaceae family such as *Cajanus cajan, Lablab purpureus* and *Vigna unguiculata* using YEMA medium and identified. The isolates were submitted to 16S rRNA and PCR–RFLP typing. A representative sample was further submitted to sequence analysis of 16S rRNA. Isolates were assigned to two genera and were related to *Rhizobium spp.* (75%) and *Bacillus spp*. (25%) respectively. This study opens the doors to researchers to do specific studies on this root nodule bacteria which are symbiotic with these legume plants in this area for their antibacterial activity, medicine and food application, and sustainable agriculture. *Rhizobium* and other beneficial microbes may be used as biofertilizers as a substitute for chemical fertilizers.

**Keywords:** Fabaceae, Isolation, Leguminous, PCR-RFLP, Root nodule bacteria, 16S rRNA

## INTRODUCTION

In some plants, especially legumes, the bacteria live in small outgrowths on the roots called nodules. The plant roots supply vital minerals and newly produced substances to the bacteria (Dariel and John, 2003). Inside these nodules, the bacteria do nitrogen fixation and the plant absorbs it in the form of ammonia. The soluble form of nitrite and nitrate can be adjusted by plant's roots and used in creating proteins and nucleic acids. This form of nitrogen is usually transformed to ammonia by plants, animals and microorganisms (Robertson, and Groffman, 2015 ). *Rhizobium* and *Bradyrhizobium* are designated as soil bacteria that have ability to taint root hair of leguminous plants and it to encourage nodule formation with following fixation ofnitrogen. Nitrogen fixing food and silage legumes lenient of environmental pressures denote an important strategy to recover agricultural productivity. The biological nitrogen fixation, because of root nodule bacteria, legume symbiosis benefits not only the host crop but also the subsequent crops in that field. It may also act as a non-symbiotic PGPB as in the case of certain non-legume crops such as rice or wheat, which are the best-studied examples that benefit from root nodule bacteria as endophytes (Biswas *et al.,* 2000). For all these reasons, the root nodule bacteria -legume symbiosis has been widely studied as a model of mutualistic associations and as a beneficial association for sustainable agriculture. With increasing use of *Rhizobium* and other beneficial microbes as bio fertilizers, reduction in the need for chemical fertilizers can be observed. Therefore, bio fertilization has great importance in decreasing environmental pollution and deterioration of nature (Vessey, 2006; Erman *et al.,* 2011). The inoculation of seeds with Rhizobium is known to increase nodulation, N uptake, growth and yield parameters of legume crops (Erman *et al.,* 2011). Other study outlined the potential participation of legume nodules in increasing risks related to human pathogens (Muresu, 2010). In the present study, the root nodule bacteria from three leguminous plants such as *Cajanus cajan, Lablab purpureus* and *Vigna unguiculata* of the family Fabaceae, were isolated and identified.

## MATERIALS AND METHODS

**Collection and extraction of nodulated roots of legumes:** The nodulated roots of *C. cajan* and *V. unguiculata* 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 and *L. purpureus* was 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 obtain nodules and milky white substances of bacteroids by dipping in phosphate buffer solution.

**PCR–RFLP and sequencing analysis:** PCR–RFLP of the 16S rRNA genes was conducted for the 51 isolates using the restriction endonucleases *Msp*I and *Nde*II according to (Mhamdi et al., 2002).Representative isolates of the different ribotypes were selected for nearly full-length sequencing of the 16S rRNA gene according to (Saıdi *et at*., 2011). The sequence obtained from isolates were matched with the nucleotide database available at the Gene Bank, using BLAST program.

## RESULTS

In the present study, strains of root nodulating bacteria are isolated from the root nodules of *C. cajan, L. purpureus* and *V. unguiculata* plants growing in Thiruvalathur Region, Palakkad, Kerala, India (Fig. 1).

A collection of 51 strains isolated from surface-sterilized root-nodules of *C. cajan*, *L. purpureus*, and *V. unguiculata* plants nodules were first investigated with PCR– RFLP of 16S rRNA genes. All the Isolates belonged to the two genera *Rhizobium spp.,* and *Bacillus spp.,* having similarity of 75 % and 25 % respectively (Table 1). The three representative isolates of the dominant ribotype A (49 % of total isolates) showed (99.70 %) sequence identity with the type strain of *Rhizobium leguminosarum* (USDA 2370). When re-examined for nodulation, all members of ribotype A induced nodules on *C. cajan*, *L. purpureus*, and *V. unguiculata* plants. Non-symbiotic rhizobia lacking symbiotic genes (ribotypes B, C, D, and E) were also frequently isolated in 13 strains, their closest respective relatives, such as *R. grahamii*, *R. huautlense*, *R. albertimagni* (formerly *Agrobacterium albertimagni*), *R. nepotum*, and *R. pusense* (formerly *Agrobacterium tumefaciens* genomovar), then *Bacillus* (ribotypes F, G, and H) were also frequently isolated in 13 strains approximately 25% of total isolates their closness to *B. megaterium*, *B. muralis,* and *B. subtilis*. (Table 1).

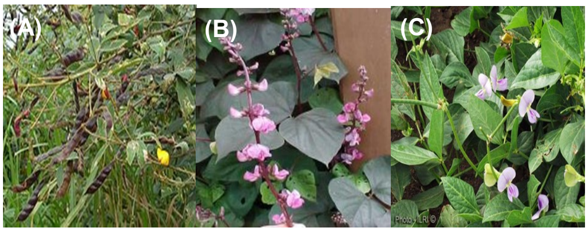

**Fig. 1.** *Legumes plants, A: Cajanuscajan (Pigeon Pea), B: Lablab purpureus (HycinthBean) C: Vigna unguiculate (Cow Pea).*

## DISCUSSION

*C. cajan* and *L. purpureus* are grown as a pulse crop (crop harvested for dry seed) or eaten green as a vegetable. The grain is popularly consumed in India, Asia, and Africa. India is the largest importer and producer (Phatak et al., 1993; Maass et al., 2010). *V. unguiculata* is commonly cultivated as a nutritious and highly palatable food source in the southern United States, Middle East, Africa, Asia, and throughout the tropics and subtropics. The seed is reported to contain 24% crude protein, 53% carbohydrates and 2% fat (FAO,2012). The leaves and flowers can also be consumed. These crops are usually well nodulated in Indian soils; however, there are much studies about the diversity and the effectiveness of the nodulating rhizobia. Hence, special interest has been focused on microbial diversity of three plants. A unique isolate

**Table 1.** Identification of the bacteria using 16S rDNA gene of the bacterial isolates present in the nodules of the plants *C. cajan, L. purpureus* and *V. unguiculate.*

| Ribotype[a] (RFLP) | Strain Accession | Accession number | Closest relative species | Similarity (%) | Nodulation ability | Amplification of *nif*H |
|---|---|---|---|---|---|---|
| **A (25)** | FB1006 | JN558659 | *Rhizobium leguminosarum* USDA2370 | 99.70 | + | + |
| | FB14022 | JN558660 | *R.leguminosarum* USDA2370 | 99.70 | + | + |
| | FB14022 | JN558660 | *R.leguminosarum* USDA2370 | 99.70 | + | + |
| **B (1)** | FB803 | JX943843 | *R. grahamii* CCGE 502 | 100 | - | - |
| **C (3)** | FB2501 | JX943844 | *R. huautlense* SO2 | 100 | - | - |
| | FB11041 | JX943845 | *R. albertimagni* AOL15 | 99.80 | - | - |
| **D (7)** | FB1802 | HM194623 | *R. nepotum* LMG26435 | 100 | - | - |
| | FB19011 | HQ007644 | *R. nepotum* LMG26435 | 99.90 | - | - |
| **E (2)** | FB17031 | HQ007645 | *R. pusense* NRCPB10 | 99.90 | - | - |
| **F (8)** | FB1502 | JX943846 | *Bacillus megaterium* IAM 13418 | 98.50 | - | - |
| **G (1)** | FB705 | JX943855 | *B. muralis* LMG 20238 | 98.50 | - | - |
| **H (4)** | 1FB2005 | JX943862 | *B. subtilis* subsp. subtilis DSM10 | 97.90 | - | - |

[a]Total number of isolates in each ribotype is indicated between parentheses.

representing the major colony-morphology phenotype was retained for each nodule analyzed. Only 49 % of isolates were able to re-nodulate their original host. They were almost closely related to *R. leguminosarum*. However, a high percentage of isolates (55 %) could not nodulate *V. faba* and either *L. culinaris*. The most frequent were non-symbiotic rhizobia, mainly taxa formerly known as agrobacteria (25 %), and taxa associated to *Pseudomonas* (16 %) and Enterobacter (16 %), which have been commonly isolated from root-nodules of several legumes (Lajudie *et al.,* 1999; Ibanez *et al.,* 2009; Ramezanpour, 2011). These results agree with the studies carried out in India (Rajeswari et al., 2017; Wadhwa et al., 2017). The results found from the examination of nodulation displayed that all the strains have been able to nodule their host plants. Some authors have studied the symbiotic parameters of diverse strains nodulating the *Lablab* for the collection of couples of rhizobia – *Lablab* of high symbiotic act (Kurlovick *et al.,* 1997). The selection of performant symbiotic couples of *Lablab* and their microsymbiotes can rise the profit of the symbiosis (Younes *et al.,* 2005).

## Conclusion

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* and other *Rhizobium spp*. However, other endophytes included taxa identified as human pathogens, rising the concern of the hazards posed by pathogens. These crops are usually well nodulated in Indian soils. In this study, a special interest has been engrossed on microbial diversity of three legumes plants that would help as biofertilizers for the crop of the area.

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