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Study on endosymbiotic bacterial dynamics on selected legumes around Thanjure district

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Abstract
The largest and most widely distributed angiosperm is Legumes, which plays a significant role in the ecology of microbial community structure. Endophytic rhizobia have also been reported in the roots and stems of legumes and other plants. The present study aimed to isolate root nodule associated bacteria from five selected host legumes and to find out different harboring bacterial species in their root nodules. The highest bacterial diversity was observed in Mimosa pudica and Crotalaria albida. Totally 23 bacterial isolates from five different plant root nodules were isolated. Based on colony morphology and biochemical characters, isolates were identified as *Agrobacterium* sp (1), Bacillus spp (6), *Bradyrhizobium* spp (2), Pararhizobium spp (1), *Enterobacter* spp (3), Ensifer sp (1), Methylobacterium sp (1), Pseudomonas spp (3), Streptomyces sp (1), Rhizobium spp (3), Mesorhizobium sp (1). Of these 23 isolates, 13 belonged to nonrhizobial endosymbionts that come under Bacillus sp, Pseudomonas sp, Streptomyces sp, and Enterobacter sp. The remaining 10 where αProteobacteria that belong to the family of Bradyrhizobiaceae, Methylobacteriaceae, and Rhizobiaceae.

Keywords: legumes, Rhizobium sp, endosymbionts, phosphate, siderophore

Introduction
The root nodule based symbiosis between legumes belongs to the *Leguminosae* / *Fabaceae* family and partners of rhizobia is an intense microbial interaction plays a significant role in nitrogen fixation in terrestrial ecosystems. In legume plants, infection begins at the end of host-derived tubular that grows inward in the root trichoblast and form an infection thread [1]. The interaction is initiated by signals of legumes that exude a series of phenolic compounds into the rhizosphere, under low nitrogen availability [2]. These molecules are stumilate rhizobia and activating the transcriptional regulator (nodD), which in turn triggers the transcription of genes of the Nod factor [3]. In root legume symbiosis, the interaction is based on the capacity of rhizobia to convert atmospheric N_2 into chemical forms that can be incorporated into the plant metabolism. The success of this interaction depends on the recognition of the right partner by the plant within the richest microbial ecosystems on Earth, the soil. It was estimated that a gram of soil contains an average of 10^6–10^9 bacterial cells representing more than 10,000 different species [4]. Rhizobia are primarily soil bacteria that are able, when they encounter a compatible host, to induce the organogenesis of root nodules that they colonize intracellularly. Rhizobia belong to various genera of α- and β-proteobacteria [5], indicating that a large diversity of bacteria has developed the ability to enter into a nitrogen-fixing symbiosis with legumes during evolutionary times [6]. All legumes were thought to be infected by this single species of rhizobia. More studies and advanced molecular techniques have, however, revealed that there are many species of rhizobia and some strains are host-specific which only induce nodules in specific legume hosts. Recent studies on legume tree species in the tropics have described various new rhizobia species. As a result, the taxonomy of rhizobia has changed rapidly.

Traditionally, rhizobia are included in the well-known genera Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium, Azorhizobium and Allorhizobium of α Proteobacteria [7].

Materials and method
Collection of sample
The five different root nodule producing plants *Crotalaria albida*, *Aeschynomene indica*, *Vigna trilobata*, *Vigna mungo* L and *Mimosa pudica* were collected from in and around the village of Thanjavur district. The plants were identified by Dr. S. John Britto, Director, Rapinat Herbarium, Tiruchirappalli.

Preparation of root extract
The collected plant roots were washed and then dried under shade. The coarse powder of the roots (5 g) was soaked in 100 ml of water, ethyl acetate, acetone and chloroform under the cold condition for 3 days with occasional shaking. The solvent from the total extract was filtered & concentrated on water bath for 8 hrs. The remaining was used for the analysis of phytochemical test.

Phytochemical analysis of root
Test for carbohydrates
Fehling's test Equal volume of Fehling A and Fehling B reagents were mixed together and 2ml of it was added to crude extract and gently boiled. A red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

Benedict's test
Crude extract when mixed with 2 ml of Benedict's reagent and boiled, a brick red precipitate formed which indicated the presence of the carbohydrates.

Iodine test
Crude extract was mixed with 2 ml of iodine solution. A dark blue or purple coloration indicated the presence of the carbohydrate.

Test for phenols and tannins
Crude extract was mixed with 2 ml of 5% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

Test for flavonoids (Shinoda test)
One to five drops of concentrated hydrochloric acid (HCl) and few fragments of magnesium ribbon were added to little amount of ethanolic extract of the plant material. Immediate development of a pink color indicates the presence of flavonoids.

Test for saponins
Crude extract was mixed with 5 ml of distilled water in a test tube and it was shaken vigorously. The formation of stable foam was taken as an indication for the presence of saponins.

Test for glycosides
Salkowski's test Crude extract was mixed with 2 ml of chloroform. Then 2 ml of concentrated H_2SO_4 was added carefully and shaken gently. A reddish brown color indicated the presence of steroidal ring, i.e., glycone portion of the glycoside.
Keller-kiliani test Crude extract was mixed with 2ml of glacial acetic acid containing 1-2 drops of 5% solution of Fe Cl_3. The mixture was then poured into another test tube containing 2 ml of concentrated H_2SO_4. A brown ring at the interphase indicated the presence of cardiac glycosides.
Test for terpenoids Crude extract was dissolved in 2ml of chloroform and evaporated to dryness. To this, 2 ml of concentrated H_2SO_4 was added and heated for about 2 minutes. A reddish brown color indicated the presence of terpenoids.
Test for quinones A small amount of extract was treated with concentrated HCl and observed for the formation of red color precipitate
Test for alkaloids Two mL of extract was taken in a test tube and then 0.2 mL dilute HCl was included, followed by 1 mL of Mayer's reagent. Precipitation indicates alkaloids presence.

Isolation of Rhizobium from root nodules [8]**.**
The healthy nodules were taken from freshly uprooted plants. Roots of the plant were thoroughly washed under tap water to remove the mud and soil particles. Healthy and pink nodules were selected for the isolation of Rhizobium. The nodules of root were washed under running tap water and then for 30 sec in 70% ethanol solution. They were then treated with 0.1% HgCl2 for 2 min and successively washed three times with sterile distilled water under aseptic condition for 1 min each. The nodules were put in 1.5 mL microfuge tubes containing 0.5 mL N-saline. Then the nodules were crushed with the help of sterile forceps and the 100 µL contents were spread on YEMA plate containing 25 µg/ml Congo red. All the plates were incubated at 28 ± 2°C for 5 days. Colonies were picked after 5 days of incubation. The cultures were maintained on YEMA slants.
Morphological Characteristics [9]: All the plates were incubated at 28 ± 2°C for 5 days. Colonies were picked after 5 days incubation. The circular, raised with smooth edges and musky odor of the colony were observed under low power microscope

Gram's staining
Thin smear of isolates were prepared on a clean glass slide and air dried. The smear was covered by crystal violet (CV) for 1 min. and then washed. The slide was treated with grams iodine (I) for 1 min. The 70 percent ethanol was added over the slide to destain the CVI complex. After 30 sec., the slides were washed and counter stained by safranin for 1 min. Stained slide was air dried and observed under microscope.

Biochemical test
Isolates were subjected to Indole, methyl red,Vogeus praskure,citrate,oxidase and catalse test for further identification
Plant growth promoting trait test [10]**.**
Indole Acetic Acid (IAA) Production: Bacterial endophytes were tested for their ability to produce IAA under liquid culture. The bacterial cultures were inoculated in tryptone soya broth supplemented with 100 µg ml^{-1} DL-tryptophan and were incubated at 30° C for 72 h. Indole acetic acid was determined in the culture supernatant by adding Salkowski reagent. Two ml of Salkowski reagent was added to 2 ml of culture supernatant, mixed and allowed to stand for 30 min for the development of pink color.
Phosphate Solubilization Phosphate solubilization ability of the endophytes was determined by spotting of cultures on Pikovskaya's agar plates. Solubilization of organic complex phosphates by the isolates was screened by using lecithin (inositolphosphate) as phosphate source in the media. Solubilization of lecithin by the isolates was indicated by the formation of clear zone around the bacterial colony. The halo size produced by therespectivebacteriawas calculated according to the formula:
Solubilization Index= zonediameter (cm) -colony diameter (cm)/ colony diameter (cm)

Organic acid production
Bacterial cultures were grown in MM9 agar medium and observed for drop in pH using methyl red as an indicator dye which changed from yellow to pink below pH 5.0. Isolates having the ability to produce organic acid gave a pink zone around the colonies.
Ammonia production. Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48–72h at 28℃. Nessler's reagent (0.5 ml) was added in each tube. The development of color from yellow to brown was a positive test for ammonia production
Siderophore production test
The chromeazurol (CAS) agar was prepared and subsequently inoculated with bacterial strains and incubated in the dark (28°C for 5 days). Positive results were indicated by the formation of a clear halo zone around the colonies, showing a visual change in color from dark-blue to yellow.

Results and Discussion

Photochemistry of leguminous plant

Root nodule producing legume plants were collected from Thanjure district, Tamilnadu, India the herbarium accessed as SJCBOT2101 for *Crotalaria albida*, SJCBOT2102 for *Aeschynomene indica*, SJCBOT2103 for *Vigna trilobata*, SJCB8OT2105 for *Vigna mungo L* and SJCBOT2104 for *Mimosa pudica.* The nodules of collected legume plant samples varied in size and weight. The minimum mass of nodules has a mean value of 1.4 ± 0.02 mg and the larger is 80 ±0.004 mg mass with 30-70 mm diameter. The phytochemical screening of selected legumes shows diverse phytochemistry (table 1). Qualitative analysis clearly showed positive results and confirms the presence of alkaloids, flavonoids, saponins, tannins, and phenols. From phytochemical screening it was observed that the aqueous extracts gave a positive result with Benedict`s test and the Fehling`s test, which indicated the presence of mono and reducing sugars. None of the tests were negative among *Aeschynomene indica and C. albida*. The Meyers reagent failed to show the presence of alkaloids in the aqueous extract for *V. mungo* and *V. trilobata*. The frothing test confirmed the presence of saponins in the aqueous extract of all the four plants except *V. mungo*. The phenols, tannins and, glycoside test were found to be negative among the *M. pudica, V. mungo and V. trilobata*. The ferric chloride test for flavonoids gave positive results in all plant extracts. The test of cardiac glycosides gave positive results in 4 plant extracts except for M. pudica. The present investigation revealed the presence of carbohydrates, alkaloids, flavonoids and the absence of cardiac glycoside, phenols, and terpenoids in root nodule extracts of *M. pudica* and a similar observation was reported by Pratap Chandran *et al*.[11]. All of these phytochemicals tested in these legumes were compared and the results were favorable with those reported from some medicinal plants found in Nigeria [12].

The frequency of bacterial isolates among samples

Both the rhizobial and non rhizobial bacterial colonies were isolated from sterilized root nodules and the colony-forming units (CFU) is given in table 2. The tested leguminous plants are most frequently isolated with the rhizobia and less frequently by nonrhizobial bacteria. Among the five plant root nodules, *V.mungo* (PS3) showed 54X10^7 CFU followed by 43X10^7 from *Aeschynomene indica* (PS2). The CFU of *Mimosa pudica, Crotalaria albida* and, *Vigna trilobata* showed 36, 37 and 38 X 10^{7}CFU respectively on YEMA plates. Colony morphology observation reveals that many isolated colonies were elevated mucoid, smooth edged and a musky odor. Among the isolates, the frequency of rhizobia were recorded as 43 % and majority of them were fast growing formed single colonies with a diameter of 3 mm within 3 days on YEMA. Each of them was effective symbionts of their original hosts, evidenced by the formation of pink nodules. Nearly 13 colonies developed on YEMA within 48 h was considered as endophytic nonrhizobial colonies and theire frequency is 56%.

The diversity of bacterial isolates the bacterial studies on collected leguminous plants showed a total of 23 morphologically distinguished colonies on YEMA plates which were further purified and subcultured. Most of the colonies were opaque, umbonate followed by translucent and glistening surface texture. The most abundant colony morphology on agar plate was a circular form with a smooth edge creamy white in nature. Microscopic studies were performed to investigate the characteristics of PGPR isolates such as shape, gram reaction and, motility. Out of 23 isolates, 60.8 percentages were found to be Gram-negative varying thicknesses and lengths and30.4 % isolates come under Grams positive rod and 8.6 % were Gram's variable (Figure 1). The rods observed had. Among these Gram-positive isolates, four were rod-shaped, one is coccobacilli and two were pleomorphic in nature. The isolates that belonged to the Gram negative cell wall had eight Gram-negative rod, three negative cocci and one pleomorphic in nature. Only two isolates were found to be Gram's variable in nature. Figure 2 revealed that the diversity of bacterial isolates was predominant in *Crotalaria albida, Mimosa pudica* and, *Vigna mungo, Vigna trilobata* showed moderate diversity and *Aeschynomene indica* is less significantly diverse colonization. The mucoid colonies is a survival characteristic of Rhizobium promotes the adaptation in adverse conditions [13]. The existence of non-rhizobial and Gram-negative bacteria are most frequently isolated and colonized in root nodules among several geographical conditions [14, 15]. The collected leguminous plants have shown a symbiotic relationship with the subclass of proteobacteria (αrhizobia). These findings support the co-operative interaction between rhizobia and other plant root colonizing bacteria. The coexistence of Gram-positive bacteria also reported in many studies. The presence of Bacillus species as endophytes has been reported from different plants such as pigeon pea, wheat, and soybean nodules [16, 17]. They have been shown to benefit their hosts by promoting nodulation and growth of leguminous plants. Legumes, establish symbiotic relationships with bacteria nodulating legumes in addition to a great variety of endophytic bacteria, non-nodulating strains [18]

Plant growth traits

Out of tested endophytic bacterial isolates the plant growth traits characters data reveals the production of hormone IAA, siderophore, organic acid and phosphate solubilization (table 3). Figure 3 shows 59% of isolates are found to be IAA producer. Kumar *et al*. [19] reported isolates of Bradyrhizobium sp. From Vigna mungo stimulate plant growth by production of IAA. Among the 23 isolates 27% were phosphate solubliser and 18% were organic acid and siderophore producer. Production of ammonia is less frequently found among islates. These non-rhizobial nodule endophytes improved plant growth and nodulation when co-inoculated with Rhizobium, than with the inoculation of Rhizobium alone. Bacillus species comprise one of the most common soil bacteria and they are frequently isolated from the rhizospheres of plant, as well as from different plant tissues. The occurrence of non rhizobial species as nodule endophytes has an advantages on plant growth stimulation have been reported in different wild legumes [20].

Conclusion

Study concludes that the root nodules of indigenous plants have vast array of microbial community contain Rhizobium sp coexist with non rhizobial endosymbionts promote the plant growth in addition to nitrogen fixation.

Table 1: Qualitative phytochemical analysis of root extract of legume plants

S. No	Phyto chemical test	Interference	*Mimmosa indica*	*A indica L*	*V mungo L*	*V trilobata*	*C albida*
1.	Carbohydrate Benedict's test	Brick red- positive	positive	positive	positive	positive	positive
	Fehling's test	Brick red- positive	positive	positive	positive	positive	positive
	Iodine test	Purple- positive	positive	positive	positive	positive	positive
2.	Saponins test	Stable foam- positive	positive	positive	positive	Negative	positive
3.	Phenols and tannins test	Black colour- positive	Negative	positive	Negative	Negative	positive
4.	Glycoside Salkowski's test (steroid ring)	Reddish brown- positive	Negative	positive	Negative	Negative	positive
	Keller Kilani test (cardiac glycoside)	Brown ring- positive	Negative	positive	positive	positive	positive
5.	Flavonoid test- Shinoda test	Pink colour- positive	positive	positive	positive	positive	positive
6.	Quinones test	Red colour- positive	positive	positive	positive	positive	positive
7.	Terpenoids test	Red brown- positive	positive	positive	positive	positive	positive
8.	Alkaloids test- Wagners test	Precipitation- positive	positive	positive	negative	negative	positive

Table 2: CFU frequency of bacterial isolates among leguminous plants

Sample code	Plant name	Total CFU	Number of Epiphytic genera	Numbers of Endophytic genera
PS-1	*Mimmosa indica*	$36X10^{-7}$	4	2
PS2	*Aeschynomene indica*	$43X10^{-7}$	1	2
PS3	*Vigna mungo*	$54X10^{-7}$	3	1
PS4	*Vigna trilobata*	$38X10^{-7}$	3	1
PS5	*Crotalaria albida*	$37X10^{-7}$	3	3

Table 3: physiological characteristics of root nodule associated isolates

S.no	Isolates	Phosphate solublization	Siderophore production	IAA	Organic acid	Ammonia
1.	Methylobacterium sp	-	-	Positive	-	-
2.	Bacillus sp	-	-	Positive	-	-
3.	Pseudomonas sp	-	positive	Positive	-	-
4.	Mesorhizobium sp	Positive	positive	Positive		
5.	Bacillus sp	-	-	-	-	-
6.	Enterobacter sp	-	-	-	-	-
7.	R.nepotum	-	-	Positive	Positive	Positive
8.	Blastobacter sp	-	positive	-	-	positive
9.	Bacillus sp	-	-	-	-	-
10.	Agrobacterium sp	-	-	Positive	-	-
11.	Pseudomonas sp	positive	-	-	Positive	-
12.	Bacillus sp	-	-	Positive	-	-
13.	Bradyrhizobium sp		-	Positive	-	-
14.	Rhizobium sp	positive	-	Positive	-	-
15.	Pseudomonas sp	-	-	-	Positive	-
16.	Bacillus sp	positive	-	-	-	-
17.	Entrobacter sp	-	-	-	-	-
18.	Streptomyces	positive	-	Positive	Positive	-
19.	Enterobacter sp	-	-	-	-	-
20.	Methylobacterium sp	-	-	Positive	-	-
21.	Bradyrhizobium sp	-	positive	Positive	-	-
22.	Ensifer sp	-	-	Positive	-	-
23.	Bacillus sp	positive	-	-	-	-

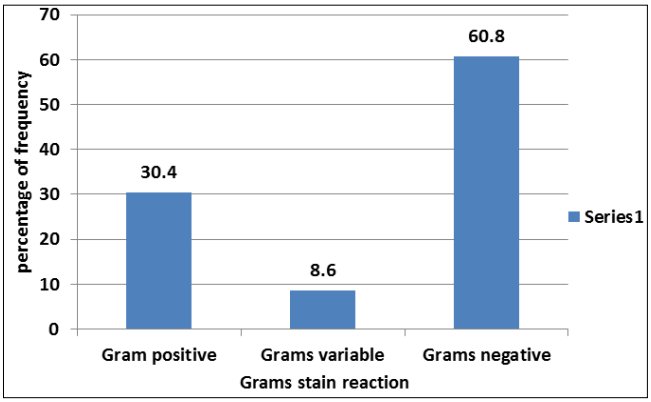

Fig 1: Frequency of isolates from root nodules

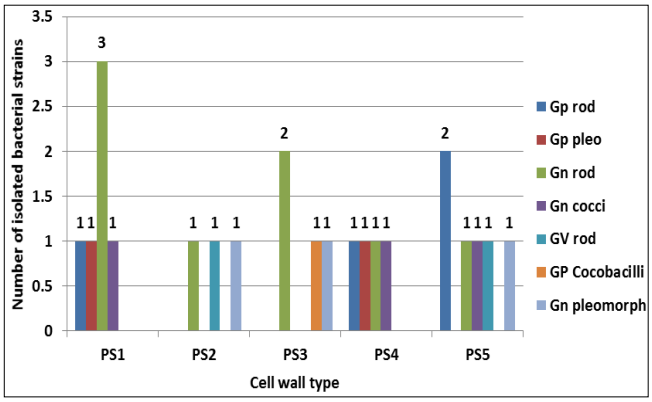

Fig 2: Diversity of Gram positive and Grams negative cell isolates

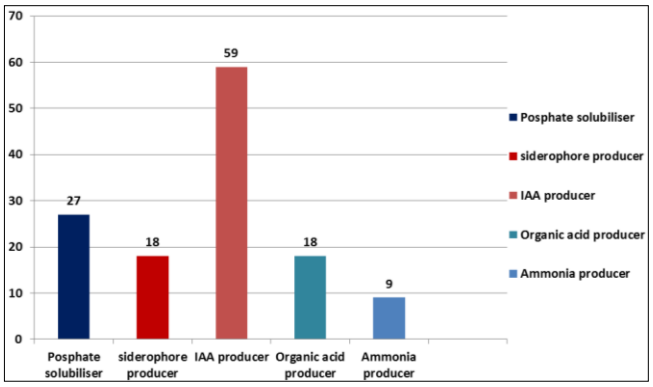

Fig 3: Percentage of bacterial population with plant growth trait

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