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Serological and Antigenic Relationship of Rhizobium of Indian Rosewood (*Dalbergia latifolia* Roxb.)

G. Prasada Babu[1], Venkata Subbaiah Kotakadi[2 *], Ch. Paramageetham[3], K. Gopal[4] and T. K. K. Reddy[1]

[1]Department of Botany, Sri Venkateswara University, Tirupati - 517 502. A. P.
[2]Department of Virology, Sri Venkateswara University, Tirupati-517502, A.P
[3]Department of Microbiology, Sri Venkateswara University, Tirupati-517502, A.P.
[4]AICRP on Tropical fruits(Citrus), AP Horticultural University, Tirupati-517 502. A. P.

ABSTRACT

The production of effective inoculants for agricultural use presumes a sensitive method for detection of bacteria used as inoculants. The competitive ability of the introducing strain against indigenous rhizobia and other soil organisms, is often necessary to re-inoculate for soil management to encourage the development of an effective symbiosis? Until recently the agglutination and immunodiffusion tests (AGDDT) have been the ones most commonly used for Rhizobium strain identification. In the present studies a rapid sensitive method of enzyme-linked immunosorbent assay (ELISA) was developed for serotyping of Rhizobium isolates was used for serological identification of fast- and slow-growing Rhizobium strains belonging to different species. For the study the homologous antisera was raised against Rhizobium of Dalbergia latifolia (TKDL2) strain. ELISA was also successfully used for strain identification in mixed inoculated plants. The enzyme-linked immunosorbent assay (ELISA) technique for examining the serological diversity of fast growing rhizobia of twenty one isolates (diverse strains) from three countries were examined with homologous antisera. Some strains showed no antigenic relatedness with each other while others were closely related, and some showed a greater affinity with the Rhizobia of Dalbergia latifolia strain than with other strains. All of the strains showed antigenic homology to an isolate from USDA 4128 (Rhizobium galegae) and Leucaena leucocephala sp., these patterns of relatedness and diversity clearly demonstrated the utility of the DAC ELISA method.

***Key words**: AGDDT, DAC- ELISA, Rhizobia , Serological diversity, Antigenic homology*

INTRODUCTION

Dalbergia latifolia Roxb. (Leguminosae, sub-family Papilionoideae) is a premium quality timber species internationally known as "Indian Rosewood". *Dalbergia latifolia* is predominantly a single-stem deciduous tree with a dome shaped crown of lush green foliage. On wet sites, it may remain evergreen. The trees reach a height of 20-40 meters with a girth of 1.5-2.0 meters [13]. Farmers use the nitrogen-rich foliage of *Dalbergia latifolia* as a green manure and fodder. Tannins from the bark are used to produce medicines for the treatment of diarrhoea, worms, indigestion and leprosy. The chemical constituents of bacterial cells behave as antigens and produce antibodies in vertebrates. Flagellar antigen was found to be widely shared amongst different strains and internal non-agglutinating antigens within the slow

growing rhizobia of one type. The surface somatic antigens of the rhizobia were the most strain specific. Hence, for the strain identification of rhizobia, antiserum was raised against them [23,20]. Mature, Newzealand white rabbits were used for the production of antisera against rhizobial antigens [21, 23]. The optimum titre value of antisera ranges from 1600 [20] to 3200 [23] . Hoben *et al*.[7] used mixed - rhizobial species antigen preparation for developing polyclonal antisera for strain identification by agglutination, flouorescent bodies, immunoblot and ELISA. The root nodule bacteria of leguminous plants are serologically heterogenous. Agglutination tests revealed that different bacteria isolated from different plant species differ serologically [10]. Rhizobia of different species of *Arachis* were found to be serologically different [4]. Sadowsky *et al*. [17] reported rhizobia of one host plant can be serologically unrelated as shown in the case of *Bradyrhizobium japonicum* by using agglutination, immunodiffusion and immunofluorescent techniques. Khan *et al.* [9] studied the differentiation of *Rhizobium* sp using three different antisera by means of DAC-ELISA, agglutination tests and Dot immuno blot assay (DIBA) for identification of specific *Rhizobium* species into four different serogroups. Broughton reported that 6 strains of rhizobia from *Psophocarpus tetragonolobus* reacted positively with 62 different strains by agglutination and immunodiffusion system, produced strong precipitin bands. Khan *et al*.[9] used Dot immunoblot assay for determination of nodules produced on Soybean french bean, pigeon pea and mung bean by *Bradyrhizobium japonicum* USPA-110 ; *R.leguminosarum* bv.*phaseoli* FB-77 and N-3; *Rhizobium* sp., A-3 and U-1 respectively.

MATERIALS AND METHODS

SEROLOGICAL IDENTIFICATION OF RHIZOBIA OF *D.latifolia*

Preparation of antigens for rhizobia of *Dalbergia latifolia* Roxb.

Whole cell antigen was prepared by growing pure culture of *Rhizobium* isolated from Indian rosewood on yeast extract mannitol agar slants [21]. The culture was harvested and suspended in Phosphate buffer saline (PBS), [potassium phosphate buffer pH 7.0 with 0.85% NaCl (v/v)] and the culture suspension was centrifuged at 15,000 rpm for 30 minutes on Sorvall Rc 5c centrifuge. The cells were washed thrice with the same buffer followed by further centrifugation. The concentrated antigen was suspended in PBS and stored at 4°C before injecting in to rabbit.

Production of Polyclonal antiserum

Healthy New Zealand white rabbit was taken for Immunization. Whole cell culture of *Rhizobium* of *D.latifolia* bacteria (1 mg) was emulsified with an equal volume of incomplete Freund's adjuvant and injected into the thigh muscle of the rabbit. Five injections were given at one week interval. The final and fifth injections were given with complete adjuvant as booster injection. Seven days after the last injection, the rabbit was test bleed by making a cut on the marginal ear vein with a sharp sterile blade. Blood was allowed to clot for 3 h at 4^0C. The tubes containing the blood were centrifuged at 6,000 rpm for 15 minutes and the antiserum was collected into 1ml aliquots and was stored at -20^0C in a deep freezer.

Agarose Gel Double Diffusion Test (AGDDT)

Agarose double diffusion test was performed as described by Purcifull and Batchelor [14]. The agarose gel was prepared by using 0.8% agarose melted in PBS (0.01M potassium phosphate buffer pH 7.0 and 0.85% Sodium Chloride). The molten agarose at 50^0C was poured into 5x5 cm glass plate with the help of pipette and allowed to solidify. The wells (4mm) were cut in the solidified gel using a template with a cork borer (6 peripheral wells at a distance of 3mm from the edge of the central well). The agarose plugs were taken out with the help of a needle. The bottom of the wells was sealed with molten agarose to prevent seepage of the samples.

Enzyme Linked Immunosorbent Assay (ELISA)

The Direct Antigen Coating (DAC) form of indirect ELISA described by Hobbs *et al*., [16] was adopted to determine antiserum titer and serological relationships of *Rhizobium* of *D.latifolia*. A programme sheet was prepared according to the number of antigen samples. The samples were prepared in carbonate buffer and 200µl was added to each well of the plate (Greiner Microlon, Germany) as shown in the programmed sheet and incubated for 2 hours at 37^0C. The plate was washed 3 times with PBS-T (by keeping 3 minutes interval between each wash). Antiserum dilutions were prepared with PBS-TPO (1: 100, 1:200, 1:300, 1:400, 1:500, 1:750, 1:1000, 1: 1500, 1:2000, 1:2500) and added to the wells (200µl /well) as shown in the program sheet. The plate was incubated at 37^0C for 2 hours. After incubation the plate was washed 3 times with PBS-T and goat antirabbit antibodies labeled with ALP (Sigma) was diluted (1:1000) with PBS-TO and added to the plate. The plate was incubated at 37^0C for 2 hours and washed with PBS-T for 3 times. The substrate P-nitro phenyl phosphate (Sigma) was added to the wells and incubated at

room temperature for 30 min. The reaction was terminated by adding 50µl of 3 M NaOH solution to each well. The reactions were recorded according to the intensity of yellow colour development. The antiserum titre was detected using homologous antigen of $TKDL_2$ rhizobial isolate of *D.latifolia.*

SEROLOGICAL RELATIONSHIPS

Serological affinities of *Rhizobium* of *Dalbergia latifolia*

Serological relationship of rhizobia of Indian Rosewood was tested against the antigens of the following cultures by AGDDT and DAC-ELISA These cultures which were found positive in AGDDT were used in DAC-ELISA.

USDA cultures -*Bradyrhizobium japonicum* (USDA 6**)**, *Mesorhizobium loti* (USDA 3471), *Rhizobium galegae* (USDA 4128),*Rhizobium monogolense* (USDA 1844), *Rhizobium tropici* (USDA 9030),*Rhizobium leguminosarum*(USDA 2370).

Australian cultures -*Rhizobium leguminosarum bv.trifolii* **(**WSM409**),***Rhizobium leguminosarum bv. phaseoli* **(**CC511**)**, *Rhizobium leguminosarum bv. viciae* **(**SU303**)**, *Sinorhizobium medicae* **(**WSM688**),** *Rhizobium lupini* ***(***RRI 128***)***. *Bradyrhizobium japonicum* (CB1809), *Bradyrhizobium sp.* (WU425).

Tree legumes - *Dalbergia sissoo, Pongamia pinnata, Pterocorpus santalinus, Gliricidia sepium, Albizzia lebbeck, Leucaena leucocephala, Acacia nilotica, Acacia auriculiformis, Acacia leucophloea and Pithecolobium dulce* .

Crop legumes - *Arachis hypogaea, Cajanus cajan,Cicer arietinum, Glycine max, Phaseolus vulgaris, Pisum sativum, Vigna unguiculata,* and *Vigna radiata.*

AGDDT

Agarose plates were prepared as described earlier and template was used such that the central well was surrounded by six peripheral wells of 3mm in diameter. The central well was filled with 50µl of antiserum and peripheral wells with 50µl of USDA cultures, Australian cultures, tree legumes and crop legumes. Plate was incubated in humid chamber for 36 hours for the formation of precipitin bands.

DAC-ELISA

The rhizobial cultures of USDA cultures, Australian cultures, Tree legumes, crop legumes and rhizobial pure culture of *Dalbergia latifolia* were prepared in carbonate buffer and added at the rate of 200 µl to each well. Each culture was loaded in duplicates on the wells of the plate. The plate was incubated over night at 4^0C and washed with PBS-TO thrice at 3 min interval. Then the plates were coated with 1:5000 dilution of homologous antiserum and incubated at 37^0C for 2 hours and washed thrice at 3 minutes intervals. The plates were coated with IgG (antigen-anti rabbit) tagged with alkaline phosphatase enzyme (in PBS-TO) at 1:5000 dilution and incubated at 37^0C for 2 hours. After washing thrice at 3 minutes interval followed by developing with p-nitro phenyl phosphate (PNP, 10 mg in 20 ml) in diethanolamine substrate buffer, development of yellow colour confirmed as positive to homologous culture. A buffer control was maintained.

RESULTS

SEROLOGICAL STUDIES

Antiserum production for *Dalbergia latifolia* rhizobia

The results of rhizobial authentication studies by growth on congo red medium; YEM – Bromothymol blue broth; Glucose peptone agar medium and using test of gram staining: ketolactose test and Hofers alkaline broth test have revealed similarities between the ten isolates of *Dalbergia latifolia.*

Since, all the ten isolates have shown similar electrophoretic banding pattern profiles (Data unpublished) only one isolate, viz. $TKDL_2$ was used for further serological studies. Accordingly, antiserum was produced against the $TKDL_2$ isolate by injecting rhizobial antigen into a New Zealand white albino rabbit. The purity of the antiserum produced against rhizobia of $TKDL_2$ antigen was tested by Agarose Gel Double Diffusion Test (AGDDT) and Direct Antigen Coating Enzyme Linked Immunosorbent Assay (DAC-ELISA).

Titre of antiserum

The titre of antiserum of rhizobial isolate $TKDL_2$ was detected upto 1/64 dilution in AGDDT (Fig.1) and reacted upto 1:2500 dilution in DAC-ELISA. The produced antiserum has reacted positively with homologous antigen ($TKDL_2$) and also with the nine other rhizobial isolates of *Dalbergia latifolia.*

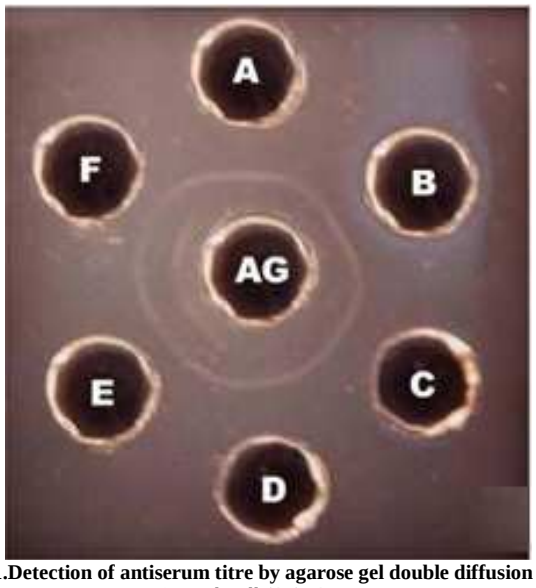

Fig.1.Detection of antiserum titre by agarose gel double diffusion test.
Central well (Ag): Antigen
Peripheral wells (A-F): Different dilutions (1/2, 1/4, 1/8, 1/16, 1/32, 1/64) of homologous antisera of TKDL2 rhizobia.

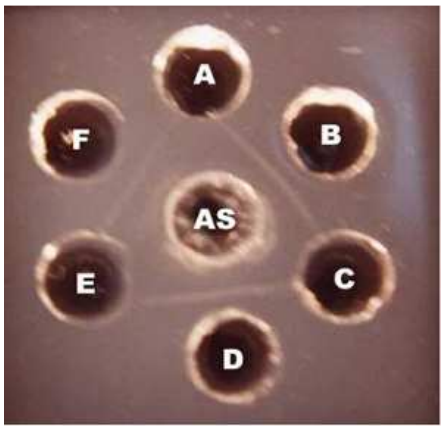

Fig.2. Serological cross reaction of TKDL2 Rhizobium with rhizobia of USDA 4128 and *Leucaena leucocephala* by agarose gel double diffusion test.
Central well (AS): TKDL2 Antiserum
Peripheral wells (A-F): A: Buffer control B: TKDL2C: Buffer control
D: USDA 4128 E:Buffer control F: Leucaena leucocphala

Serological relationship of *Rhizobium* isolate $TKDL_2$

The serological relationship of *Rhizobium* isolate $TKDL_2$ was determined by employing AGDDT and DAC-ELISA methods. The antiserum of $TKDL_2$ *Rhizobium* isolate was tested against the antigens of **USDA cultures** (*Bradyrhizobium japonicum*-USDA 6, *Mesorhizobium loti*- USDA 3471, *Rhizobium galegae* USDA 4128, *Rhizobium monogolense*-USDA 1844, *Rhizobium tropici*- USDA 9030, *Rhizobium leguminosarum*-USDA 2370, **Australian cultures** (*Rhizobium leguminosarum bv. Trifolii*-SM409**,** *Rhizobium leguminosarum bv. Phaseoli*-CC511**)**, *Rhizobium leguminosarum bv. Viciae* -SU303, *Sinorhizobium medicae*-WSM688, *Rhizobium lupini*-RRI128, *Bradyrhizobium japonicum*-CB1809, *Bradyrhizobium sp.*-WU425**), Tree legumes** (*Dalbergia sissoo, Pongamia pinnata, Pterocorpus santalinus, Gliricidia sepium, Albizzia lebbeck, Leucaena leucocephala, Acacia nilotica, Acacia auriculiformis,*

Acacia leucophloea and Pithecolobium dulce) and **crop legumes** (*Arachis hypogaea , Cajanus cajan, Cicer arietinum, Glycine max, Phaseolus vulgaris , Pisum sativum , Vigna unguiculata, Phaseolus radiatus*). Amoung all the cultures tested positive reaction was observed only with its own antigen, $TKDL_2$, USDA 4128 (*Rhizobium galegae*) and *Leucaena leucocephala* in AGDDT (Fig.2) and in DAC ELISA and the OD values of DAC ELISA were given in Table-1 and Fig.3

Table 1: Absorbance values of homologous and non-homologous reactionsof antiserum and antigens of different crop and tree rhizobial cultures* and nodulation of *Dalbergia latifolia***

S. No	Type of rhizobial antigen coating	Absorbance value (at 405 nm)	Difference over control	Nodulation on *Dalbergia latifolia*
1	Buffer control	0.130		-
2	*Brady rhizobium japonicum* (USDA 6)	0.132	0.002	-
3	*Mesorhizobium loti* (USDA 3471)	0.130	0.000	-
4	*Rhizobium galegae* (USDA 4128)	**1.654**	**1.524**	+++
5	*Rhizobium monogolense* (USDA 1845)	0.132	0.002	-
6	*Rhizobium tropici* (USDA 9030)	0.130	0.000	-
7	*Rhizobium leguminosarum bv. Trifolii* (WSM 409)	0.131	0.001	-
8	*Rhizobium leguminosarum bv, phaseoli* (CC 511)	0.132	0.002	-
9	*Rhizobium leguminosarum bv. Viciae* (SU 303)	0.132	0.002	-
10	*Sinorhizobium medicae* (WSM 688)	0.130	0.000	-
11	*Rhizobium lupini* (RRI 128)	0.130	0.000	-
12	*Rhizobium of Dalbergia sissoo* (DS 01)	0.130	0.000	-
13	*Rhizobium of Dalbergia latifolia* (TKDL 1)	**1.804**	**1.674**	+++
14	*Rhizobium of Pongamia pinnata* (PP 02)	0.130	0.000	-
15	*Rhizobium of Pterocarpus santalinus* (PS 03)	0.131	0.001	-
16	*Rhizobium of Gliricidia sepium* (GS 04)	0.132	0.002	-
17	*Rhizobium of Albizzia lebbeck* (AL 05)	0.130	0.000	-
18	*Rhizobium of leucaena leucocephala* (LL 06)	**1.564**	**1.434**	+++
19	*Rhizobium of Acacia nilotica* (AN 07)	0.131	0.001	-
20	*Rhizobium of A. auriculiformis* (AA06)	0.130	0.000	-
21	*Rhizobium of A. leucophloea* (AA 08)	0.131	0.001	-
22	*Rhizobium of Pithecolobium dulce* (PP 09)	0.130	0.000	-

**Values are mean of 4 wells; results for homologous reactions are underscored. **+++ good nodulation, + scanty nodulation, - no nodulation*

DISSCUSION

Rhizobial strains in addition to the usual criteria have been distinguished by various serological techniques. Although ELISA procedures have been used for differentiation of rhizobial strains of many legumes, no information is available on *Rhizobium* of Indian Rosewood (*Dalbergia latifolia* Roxb.). Indian Rosewood is a tropical tree legume in Cuddapah and Chittoor districts of A.P., India. Since it is of economic importance, serological relationship of its nodule bacteria with rhizobia of some other legumes was examined. The rhizobia of Indian Rosewood were further characterized serologically to determine the affinities with other rhizobia. The antiserum of Indian rosewood rhizobia had a titre of 1/64 in AGDDT and at 1 : 2500 dilution in ELISA, which was more or less similar to other reports [23,20]. In Agarose Gel Double Diffusion Test (AGDDT) and Direct Antigen Coating - Enzyme Linked Immunosorbent Assay (DAC-ELISA), all the rhizobial isolates of Indian rosewood reacted positively to the antiserum. The antiserum when tested against **USDA cultures, Australian cultures, Tree legumes** and **crop legumes** the positive reaction was obtained with *Rhizobium galegae* (USDA 4128) and *Leucaena leucocephala* only. The above serological tests showed that the rhizobia of Indian Rosewood demonstrated antigenic similarities with *Rhizobium galegae*. Similar antigenic similarities were demonstrated serologically between *Rhizobium* sp. and *Bradyrhizobium japonicum* [1,18], and *Bradyrhizobium* species and *R. meliloti* [2,15]. Strains belonging to the same species of *Rhizobium* may exhibit serological differences and serogroups were reported among strains of *Rhizobium phaseolus* [16]. Rhizobia of different plant species differ serologically [10,4,]. The antigenic reactions of rhizobia also revealed the number of internal antigens shared among fast growing rhizobia *R.trifolii, R. leguminosarum* and *R. phaseolus* [19, 5]. Rangarajan *et al* [15] reported that antigenic relationship existed in *R. meliloti* strains of tropical and temperate regions. The gel diffusion pattern of *Rhizobium* strains were reported to indicate one or more widely shared antigens[22].

The affinities of Indian Rosewood rhizobia with other tree legumes were determined with antiserum of Indian rosewood rhizobia in DAC-ELISA, against the rhizobia of tree legumes, rhizobia from soils of Indian Rosewood plantations and

Rhizobium galegae (USDA 4128). The antiserum reacted positively with the antigens of *Leucaena leucocephala*, ten rhizobial isolates of Indian Rosewood plantations and *Rhizobium galegae* only. Sadowsky *et al.*, [17] reported that rhizobia of one host plant can be serologically unrelated as shown in case of *Rhizobium japonicum* and *R. fredii*, which were related to *R. meliloti* and some fast growing rhizobia of *Leucaena*, *Lablab* and *Sesbania* sp. The strain identification by ELISA was made in *Rhizobium meliloti* [11], rhizobia of *Trifolium repens* [12] and of *Psophocarpus tetrogonolobus* [8]. Thus, the serological studies confirmed the strain identification and affinities with other rhizobia.

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