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Classical and Molecular Characterization of Rhizobial Isolates

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ABSTRACT

Traditionally the name Rhizobia portrays root and stem nodulating bacteria that live in N_2 fixing symbiosis mainly with leguminous plants. Understanding rhizobial diversity at classical and molecular level could have a profound implication in agriculture. Hence, the present study is aimed at isolation, identification, biochemical and molecular characterization of rhizobia from the fourteen different soil series of Thanjavur district. All the test isolates showed uniform morphological and biochemical characteristics except colony size and isolates growth on YEMA containing bromothymol blue medium, revealed that Vlm6 and Mud8 isolates were fast growers while others were slow growers. Based on morphological, cultural and biochemical characterization, all the isolates were preliminarily identified as species of Rhizobium. The soil samples from the 14 different sites showed variation not only in the number of colonies per gram of soil, but also in their physico-chemical properties. Positive correlation was noted between physicochemical parameters such as bulk density, EC, organic carbon, nutrients such as N, K, Cu and number of rhizobial colonies. Negative correlation was observed with soil pH, nutrients such as P, Fe, Zn, Mn and number of colonies. However, the relationship is not statistically significant in the present study. The isolates showed variation in their carbon utilization, fatty acid and amino acid profiles confirming the existence of diversity.

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Introduction

The complexity of the soil system is determined by the numerous and diverse interactions among its physical, chemical, and biological components, as modulated by the prevalent environmental conditions (Buscot, 2005). In particular, the varied genetic and functional activities of the extensive microbial populations have a critical impact on soil functions, based on the fact that microorganisms are driving forces for fundamental metabolic processes involving specific enzyme activities (Nannipieri *et al.,* 2003). Many microbial interactions, which are regulated by specific molecules/signals (Pace, 1997), are responsible for key environmental processes, such as the biogeochemical cycling of nutrients and matter and the maintenance of plant health and soil quality (Barea *et al.,* 2004). Soil system supports a conglomerate of microorganisms with a high degree of diversity and their interactions are extremely complex. This sometimes creates a hostile environment for the inocula. The introduced microorganisms in the soil are also subjected to abiotic stress (Barnert, 1991; Dowling and Broughton, 1986; Van Veen *et al.,* 1997).

Many studies have demonstrated that soil borne microbes interact with plant roots and soil constituents at the root-soil interface (Barea *et al.,* 2002; Bowen and Rovira, 1999; Glick, 1995; Kennedy, 1998; Linderman, 1992). The great array of root-microbe interactions results in the development of a dynamic environment known as the rhizosphere where microbial communities also interact. The differing physical, chemical, and biological properties of the root-associated soil, compared with those of the root-free bulk soil, are responsible for changes in microbial diversity and for increased numbers and activity of micro-organisms in the rhizosphere micro-environment (Kennedy, 1998).

Symbiosis between leguminous plants and soil bacteria commonly referred to as rhizobia is of considerable environmental and agricultural importance, since they are responsible for an estimated 180 × 10^6 tonnes per year of biological nitrogen fixation worldwide (Postgate, 1998). Rhizobia reduce atmospheric nitrogen to ammonia using the enzyme nitrogenase and supply this essential nutrient to the host plant cells. The symbiotic association has been reported to fix 24-584 kg N ha^{-1} annually in different legumes. In this study, effective native rhizobial isolates from different soil samples were collected from Thanjavur district. Based on the soil type, thanjavur district has already been classified into fourteen different soil series. From the 14 different soil series, the rhizobia would be isolated and characterized based on classical and molecular level. The initial identity of a bacterial organism can be inferred from morphological, cultural and biochemical studies. These classical approaches have relied on laboratory cultivation of isolates from natural environments. Though these approaches provide information on five scale diversity, it suffers from bias, resulting from the media and cultivation conditions employed (Sudhakara Reddy, 2005). The molecular approaches include fatty acid profile by GLC and amino acid profile using HPLC. The assessment of diversity within rhizobial natural populations in various regions of the world has received considerable attention. The present study is an attempt in this direction.

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Materials and methods

Collection of soil sample

Characterization of Rhizobial isolates in the soils of Thanjavur District was studied by isolating them from the soil samples representing each series. Soil survey and land use organization in its publication entitled *Soil Atlas* (1998) reported about 14 different soil series in thanjavur district of Tamil Nadu, India. They are listed in table 1 and fig.1.

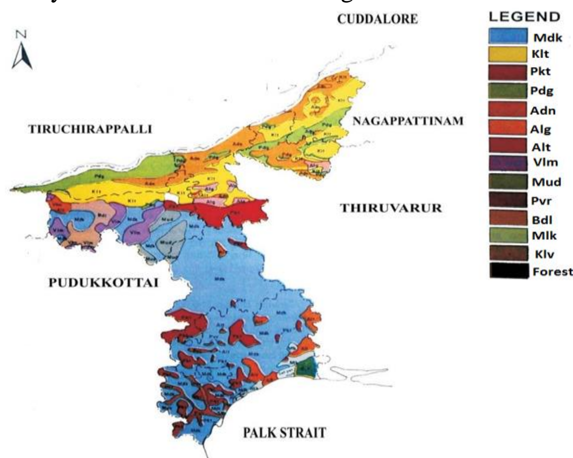


Fig. 1. Soil series map of thanjavur District

Soil samples were collected within an area of approximately 500 m^2 in each location. Before the soil samples were taken loosening top 10 cm of soil with a sterile trowel followed by removal of a core at different places and pooled together. The collected samples were placed in a cool box for transporting and stored at 4°C. About 250 g of soil was used as the test sample for each of the soil series.

Soil analysis

The physico-chemical properties of soil such as EC, pH, bulk density (g/cc), organic carbon (%), nitrogen, phosphorus, potassium, zinc, copper, manganese and iron content were analysed by the following methods of Barnes (1959) and Muthuvel and Udhayasoorian (1999).

Isolation of rhizobium (Somasegaran and Hoben, 1994; Vincent, 1970)

The soil samples were serially diluted up to 10^{-8}. For the isolation of *Rhizobium* from soil, one ml each of 10^{-6}, 10^{-7} and 10^{-8} were transferred onto sterile Petri plates containing YEMA medium. The plates were incubated at 28°C ± 2°C for 2 to 3 days. This medium allowed *Agrobacterium* and *Rhizobium* to grow and develop into colonies. The *Rhizobium* appeared as white translucent, elevated and gummy colonies on YEMA medium. They were removed and purified by repeated streaking and maintained on YEMA slants with code names. The Agrobacterium it present will appear as red colored elevated colonies.

Rhizobial isolates growth and culture maintenance

The rhizobial isolates were grown on YEM agar and cultured in YEM broth with constant shaking at 100 rpm on an orbital shaker. Mean generation time was recorded for each isolate and the isolates were gram stained after harvesting (Vincent, 1970). The morphological and growth characteristics of all rhizobial isolates were studied by light microscopy.

Morphological cultural and biochemical characterization of rhizobial isolates

The test isolates were subjected to the following microscopic and cultural tests in order to confirm their identity as *Rhizobium*. No. of colonies g^{-1} of soil, colony colour and texture, the test isolates were grown on YEMA medium. After 24h, the colony shape and size (diameter) cell shape, cell length and cell diameter were measured and recorded. Gram staining, staining of poly β-hydroxy butyrate (PHB) Brudon, (1946) capsular polysaccharide staining (Mc Kinney, 1953) motility test, congo red test (Hahn, 1966), hofer's alkaline agar test (Hofer, 1941), growth in lactose agar (Bernnerts and Deley, 1967), growth in litmus milk agar (Vincent, 1970), growth in glucose peptone agar (Subba Rao, 1971), growth on YEMA containing bromothymol blue (Chen and Lee, 2001; Cubo *et al.,* 1988) to one-liter yeast extract mannital agar medium (YEMA), 5ml of bromothymol blue stock solution (0.5 g of BTB dissolved in 100ml ethanol) was added. Plates were read for reactions after 3-5 days for fast growers and 5-7 days for slow growers. A blue colour indicates an alkaline reaction of BTB with slow growing rhizobial strains (*Bradyrhizobium* sp.) a yellow colour (acid) indicates the fast growing *Rhizobium* sp. The biochemical tests were conducted the following method of Cappuccino and Sherman (1999) Graham and Parker (1964) and Somasegaran and Hoben (1985) respectively.

Molecular characterization

Fatty acid profile by GC (Miller and Berger, 1985)

The test cultures were centrifuged at 5,000 rpm for 10 min. From the pellet 100 mg was taken in separate screw cap test tubes. To each tube l ml of saponification reagent was added and tightly sealed with Teflon-Lined screw cap. Then the tubes were vortexed for 10 seconds and kept in a boiling water bath for 5 min. Again the tubes were vortexed for 10 seconds. Then the tubes were kept in the water bath for an additional period of 20 min. After a total of 30 min of saponification, the tubes were removed from the water bath and cooled to room temperature. To each tube 2 ml of methylation reagent was added by uncapping the tubes. After vortexing for 10 seconds, the tubes were placed in a water bath set at 80°C for 10 min. Finally, 1.25 ml of the extraction solvent was added to each cooled tube. Then the test tubes were tightly closed and rotated end-over-end for 10 min. From the tubes, the lower aqueous phase was removed and discarded. To the upper phase, 3 ml of base wash was added and rotated end-over-end for 5 min. With the help of clean Pasteur pipette 2/3 of the organic extract from each tube was transferred to GC vials and kept in deep freezer by capping with Teflon-lined septum. From each vial, 2 µl of sample was analysed with Hewlett-Packard 5890 Gas Chromatograph fitted with 10 per cent DEGS column and a flame ionization detector. The carrier gas, N_2 was supplied at the rate of 30 ml min^{-1}. The detector gas flow rates were 30 ml of H_2 min^{-1} and 300 ml of air min^{-1}. The chromatograph oven was set at 180°C with injector and detector temperature at 210 and 230°C respectively. From the peak area, the amount of fatty acid was calculated using respective standards.

Amino acid profile by HPLC (Rajendra, 1987)

To each vial, containing 100 µl of amino acid sample, 500 µl of OPA-reagent was added. The vials were capped, shaken and kept for 2 min to allow derivatisation. Then, 20 µl of each sample was injected separately into HPLC for analysis. The peaks of the chromatogram were identified and quantified from the retention time (RT) and peak area of the known standard amino acids.

Results and discussion

Rhizobia are usually defined as nitrogen fixing soil bacteria capable of forming root and stem nodules on leguminous plants, fixing atmospheric nitrogen and reducing it to ammonia for the benefit of the plant. Due to their considerable agricultural and environmental significance, these legume symbionts have been extensively studied. In the recent past, the assessment of diversity within natural populations of rhizobia in different parts

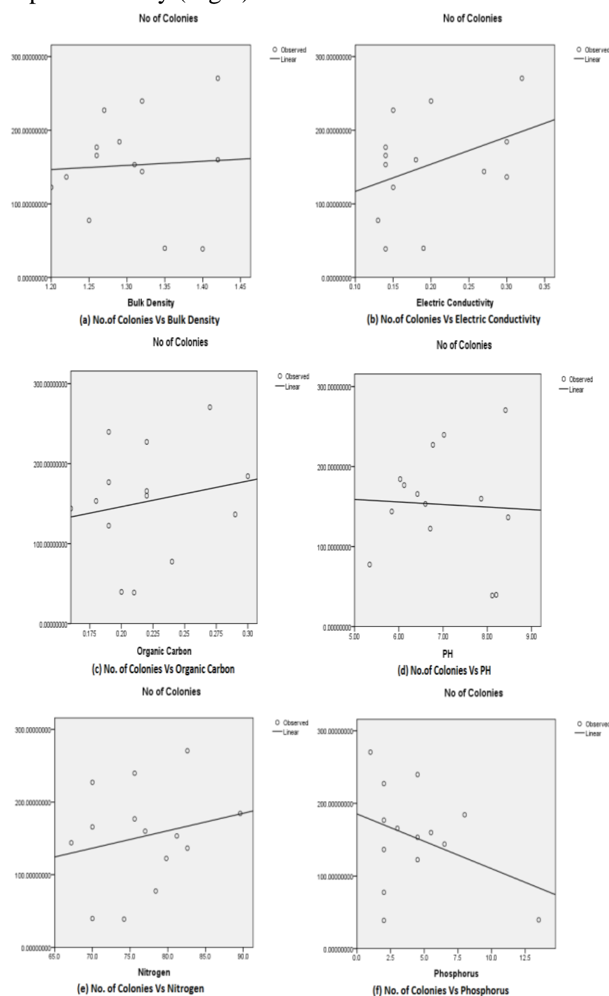
of the world have received considerable attention (Chen *et al.,* 2000; Madrzak *et al.,* 1995).

Physico-chemical properties of soil

Physico-chemical properties of collected soil samples representing 14 different series are presented in table 2. Irrespective of the series, all samples were of sandy clay loam soil type except for Alangudi (Alg10) series, which has clay loam soil. The bulk density of the soil samples ranged between 1.20 to 1.42 g/cc and EC from 0.14 to 0.32 se ds^{-1}. The lime status, was nil in 11 soil samples and middle in rest of 3 soil samples. The pH of the soil ranged between 5.34 to 8.47. The organic carbon content of the soil samples ranged from 0.16 to 0.30%. All the soil samples were low in their N, P and K content. Micronutrients such as Fe, Zn, Mn and Cu were also analysed and there were variation among the soil samples with regard to their content.

Relationship between number of rhizobial colonies and physico-chemical properties of soil

The population of rhizobia from the different soil series showed great variation with colonies ranging from 38.8 × 10^7 to 270.6 × 10^7. The soil samples from the 14 different sites showed variation not only in the number of colonies per gram of soil, but also in their physico-chemical properties. Positive correlation was noted between physicochemical parameters such as bulk density, EC, organic carbon, nutrients such as N, K, Cu and number of rhizobial colonies. Negative correlation was observed with soil pH, nutrients such as P, Fe, Zn, Mn and number of colonies. However, the relationship is not statistically significant in the present study (Fig.2).



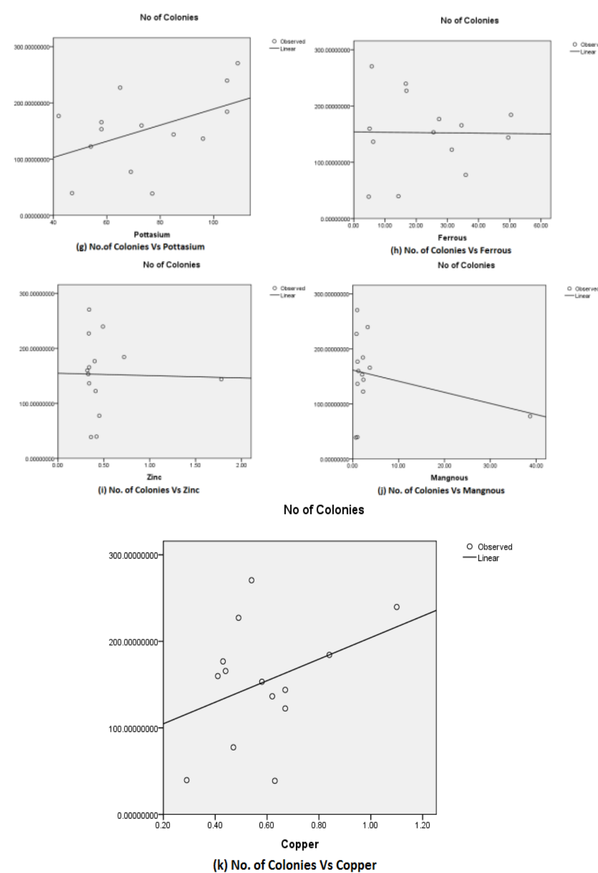


Fig 2 (a-k). Correlation between number of Rhizobial colonies and Phyco-chemical properties of soil in different study sites of Thanjavur district

Abiotic factors such as soil structure, nutritional availability, moisture, pH, salinity and temperature normally affect the population of microbes in soil (Sindhu and Dadarwal, 2000; Slattery *et al.,* 2001). Among the inorganic soil components, clay fraction is the most reactive one, which can provide protection from extreme fluctuations in the physico-chemical micro environment by forming an envelope around bacterial cells and enhance bacterial survival. This may probably the reason for the maximum number of rhizobial colonies in Alg10 series where the soil is of the clay loam type while in all others it is of sandy clay loam type. The microbial colonies that developed on YEMA medium. These colonies were further purified and subjected to morphological, biochemical and molecular characterization.

Morphological cultural and biochemical characterization

This includes results of microscopic tests and cultural test (Table 3). The colonies that developed on YEMA medium from all samples were milky white except in Adonur soil series (Adn5) where it was watery and translucent. Colony diameter ranged in size from 1.5 to 3.7 mm and it was circular in shape. All the rhizobial isolates were Gram-negative, rod shaped and motile in nature. The cell length and diameter of all rhizobial strains ranged from 0.5-1 to 1.5-3 mm and 0.5 to 1 mm respectively. The diagnostic features Graham and Parker (1964) include staining and morphology, colonial and growth characteristics, biochemical properties, utilization of carbohydrates and infective properties. Of these, staining and morphology, colonial and growth characteristics were studied as part of the cultural tests in the present study. Vincent (1970) reports explain the usefulness of the following routine cultural tests in the identification of rhizobia viz. growth on media, Grams reaction, PHB granules, congo red test, Hofer's alkaline broth test, lactose agar and growth in litmus milk agar.

Table 1. Rhizobia isolated from different soil series

S. No.	Soil series	Place of collection	Code Name of isolates
1.	Madukkur	Kattuthottam	Mdk1
2.	Kalathur	Thittai	Klt2
3.	Padugai	Mathur	Pdg3
4.	Pattukkottai	Kudikadu	Pkt4
5.	Adonur	Thandangorai	Adn5
6.	Vallam	Achampatti	Vlm6
7.	Alathur	Sorakudipatti	Alt7
8.	Mudukulam	Vilar	Mud8
9.	Peravurani	Puthu Parapanar Vayal	Pvr9
10.	Alangudi	Annappanpettai	Alg10
11.	Budalur	Budalur	Bdl11
12.	Melkadu	Parakalakottai	Mlk12
13.	Kollivayal	Nadiyam	Klv13
14.	Reserved Forest	Adirampattinam	RF14

Table 2 . Physico-chemical properties of soil at different study sites (Soil Series) of Thanjavur district

S. No	Code*	Texture#	Bulk density (g/cc)	LS	EC $_{sc}$ ds^{-1}	Organic carbon %	pH	Macronutrient (Kg/acre)**			Micronutrient (ppm)			
								N	P_2O_5	K	Fe	Zn	Mn	Cu
1.	Mdk1	SCL	1.32	Nil	0.27	0.16	5.84	67.2	6.5	85	49.52	1.78	2.33	0.67
2.	Klt2	SCL	1.22	Middle	0.30	0.29	8.47	82.6	2.0	96	6.21	0.34	1.03	0.62
3.	Pdg3	SCL	1.42	Nil	0.18	0.22	7.86	77	5.5	73	5.04	0.32	1.23	0.41
4.	Pkt4	SCL	1.26	Nil	0.14	0.19	6.12	75.6	2.0	42	27.33	0.40	1.04	0.43
5.	Adn5	SCL	1.32	Nil	0.20	0.19	7.02	75.6	4.5	105	16.67	0.49	3.26	1.10
6.	Vlm6	SCL	1.25	Nil	0.13	0.24	5.34	78.4	2.0	69	35.88	0.45	38.66	0.47
7.	Alt7	SCL	1.26	Nil	0.14	0.22	6.42	70	3.0	58	34.53	0.34	3.73	0.44
8.	Mud8	SCL	1.20	Nil	0.15	0.19	6.71	79.8	4.5	54	31.41	0.41	2.27	0.67
9.	Pvr9	SCL	1.40	Middle	0.14	0.21	8.11	74.2	2.0	77	4.77	0.36	0.68	0.36
10.	Alg10	CL	1.42	Middle	0.32	0.27	8.41	82.6	1.0	109	5.73	0.34	0.98	0.54
11.	Bdl11	SCL	1.29	Nil	0.30	0.30	6.03	89.6	8.0	105	50.39	0.72	2.22	0.84
12.	Mlk12	SCL	1.27	Nil	0.15	0.22	6.77	70	2.0	65	16.85	0.36	0.82	0.49
13.	Klv13	SCL	1.31	Nil	0.14	0.18	6.60	81.2	4.5	58	25.54	0.33	2.06	0.58
14.	RF14	SCL	1.35	Nil	0.19	0.20	8.20	70	13.5	47	14.30	0.42	1.01	0.29

*Definition as per table 1.

S – Sandy, SL – Sandy Loam, SCL – Sandy Clay Loam Soil, LS – Lime Status (Nil), C – Clay, CL – Clay Loam.

**General nutrient status of the soils (Anonymous, 1998)

	N	P_2O_5	K_2O
Low	<140	<24.2	<140.7
Medium	141-280	24.3-32.2	140.8-281.6
High	>280	>32.3	>281.6

Table 3. Cultural and morphological characteristics of rhizobial isolates

S.No.	Morphological characteristics	Rhizobial isolates													
		Mdk1	**Klt2**	**Pdg3**	**Pkt4**	**Adn5**	**Vlm6**	**Alt7**	**Mud8**	**Pvr9**	**Alg10**	**Bdl11**	**Mlk12**	**Klv13**	**RF14**
1.	No.of colonies g^{-1} of soil	143.9x10^{7}	138.5x10^{7}	159.9x10^{7}	176.8x10^{7}	239.7x10^{7}	77.5x10^{7}	165.7x10^{7}	122x10^{7}	38.8x10^{7}	270.6x10^{7}	184.3x10^{7}	227.2x10^{7}	153x10^{7}	39.6x10^{7}
2.	Colony colour and texture	Milky white	Milky white	Milky white	Milky white	Watery translucent	Milky white	Milky white	Milky white	Milky white	Milky white	Milky white	Milky white	Milky white	Milky white
3.	Colony shape	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular	Circular
4.	Colony diameter (mm)	2.7	3.5	2.3	2.4	3.7	3.4	1.5	2.4	3.5	2.3	3.5	2.4	2.7	2.3
5.	Cell shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
6.	Cell length (µm)	1 – 2.5	1.5 – 3	0.5 – 2	1 – 2.5	1.5 – 2	1.5 – 2	1 – 2.5	0.5 – 1	1 – 2.5	0.5 – 3.0	1.5 – 3	1 – 2.5	0.5 – 2	1 – 2.5
7.	Cell diameter (µm)	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1	0.5 – 1
8.	Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9.	Gram's staining	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
10.	Capsular polysaccharide staining	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
11	Growth on YEMA containing bromothymol blue	Blue	Blue	Blue	Blue	Blue	Yellow	Blue	Yellow	Blue	Blue	Blue	Blue	Blue	Blue

+ : Positive; - : Negative; Yellow: Fast growing; Blue: Slow growing.

Table 4. Carbon Utilization Tests for Rhizobial Isolates

S.No.	Carbon source	Rhizobial isolates													
		Mdk1	**Klt2**	**Pdg3**	**Pkt4**	**Adn5**	**Vlm6**	**Alt7**	**Mud8**	**Pvr9**	**Alg10**	**Bdl11**	**Mlk12**	**Klv13**	**RF14**
1.	Adonitol	-	+	+	+	+	+	+	-	+	-	+	+	+	+
2.	Galactose	+	+	+	+	+	-	+	+	+	+	+	+	+	+
3.	Glucose	+	+	+	+	+	+	+	+	-	+	+	+	+	+
4.	Inositol	+	+	-	+	+	+	+	+	+	+	+	+	+	+
5.	Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6.	Maltose	+	+	+	+	+	+	+	+	+	-	+	+	+	+
7.	Mannitol	+	+	+	+	+	+	-	+	+	+	+	+	+	+
8.	Sorbitol	+	+	+	+	+	+	+	+	+	+	-	+	+	+
9.	Sucrose	-	+	+	+	+	+	+	+	+	+	+	+	+	+
10.	Xylose	+	+	-	+	+	+	+	+	-	+	+	+	+	+

+ : Positive

- : Negative

Table 5. Fatty Acid Profile of Rhizobial Isolates (Mg/G^{-1} of Lipid)

S.No.	Fatty acid	Rhizobial isolates													
		Mdk1	Klt2	Pdg3	Pkt4	Adn5	Vlm6	Alt7	Mud8	Pvr9	Alg10	Bdl11	Mlk12	Klv13	RF14
1.	Undecanoic acid (C11:0)	-	0.64	0.43	-	-	-	13.00	2.23	-	-	14.27	13.41	35.06	8.13
2.	Tridecanoic acid (C13:0)	4.51	-	-	-	-	4.61	-	30.15	-	23.64	6.04	-	-	15.83
3.	Mystric acid (C14:0)	-	5.36	-	14.90	3.73	-	-	-	-	-	2.78	-	6.84	23.09
4.	Pentadecanoic acid (C15:0)	10.93	7.51	11.10	1.62	-	8.05	-	-	7.51	-	4.71	11.10	-	19.91
5.	Palmitic acid (C16:0)	9.59	8.80	12.23	13.67	2.26	2.26	-	-	8.80	6.74	9.38	10.96	12.23	10.64
6.	Palmitioleic acid (C16:1)	-	-	-	8.69	3.13	-	-	-	-	-	-	-	-	2.03
7.	Heptadecanoic acid (C17:0)	13.02	11.37	15.32	2.50	-	10.88	28.78	26.10	18.80	-	-	-	-	-
8.	Cis-10 Hepta decanoic acid (C17:1)	-	1.56	1.21	-	5.06	2.99	54.78	14.93	54.78	14.50	11.99	2.04	11.99	13.61
9.	Stearic acid (C18:0)	1.21	1.21	2.29	-	-	1.87	-	-	-	-	-	9.37	-	-
10.	Oleic acid (C18:1)	-	-	2.80	3.43	-	-	-	2.22	-	7.08	14.89	7.70	-	-
11.	Non decanoic acid (C19:0)	-	-	3.05	-	2.93	3.20	-	-	2.93	-	-	-	-	-
12.	Arachidic acid (C20:0)	-	7.37	-	-	14.64	-	-	-	-	-	-	-	-	-
13.	Gadoleic acid (C20:1)	-	-	-	11.29	-	-	-	-	-	-	-	-	-	-
	Total	5	8	8	7	6	7	3	5	5	4	7	5	4	7

Table 6. Amino Acid Profile of Rhizobial Isolates (μG/Mg Dry Wt.)

S.No.	Amino acid	Rhizobial isolates													
		Mdk1	Klt2	Pdg3	Pkt4	Adn5	Vlm6	Alt7	Mud8	Pvr9	Alg10	Bdl11	Mlk12	Klv13	RF14
1.	Alanine	1.47	3.51	3.39	2.65	2.65	0.70	0.18	0.21	1.37	1.53	0.42	1.20	4.37	1.28
2.	Arginine	0.23	6.03	-	1.20	-	2.26	0.17	2.40	-	4.37	0.98	0.76	2.40	1.61
3.	Cystine	3.39	4.13	1.14	1.37	1.29	0.70	0.47	1.20	2.65	0.76	1.58	3.39	0.46	9.73
4.	Glycine	4.43	2.65	1.37	-	1.58	6.03	1.58	2.10	0.23	1.73	6.03	1.47	0.97	-
5.	Glutamate	-	0.81	0.21	1.12	1.21	2.22	0.23	4.20	-	-	2.40	-	8.55	0.47
6.	Histidine	4.21	-	1.74	3.26	0.21	-	4.37	0.42	1.59	3.26	0.70	4.33	-	0.17
7.	Isoleucine	1.20	7.16	0.52	-	0.81	2.26	0.52	-	1.14	4.20	-	1.32	1.58	-
8.	Methionine	9.47	4.23	4.20	0.17	0.16	1.53	1.50	2.01	1.37	2.65	8.55	6.36	1.96	9.47
9.	Phenylalanine	1.31	9.03	0.97	0.47	0.47	1.14	1.74	1.02	1.12	1.13	7.86	4.43	1.53	9.80
10.	Serine	0.73	0.43	3.51	-	-	0.21	2.23	0.21	-	1.58	1.74	4.21	-	1.73
11.	Tryptophan	0.43	1.23	0.23	4.20	4.21	4.42	3.08	2.40	1.29	0.62	1.31	1.14	1.03	3.39
	Total	10	10	10	8	9	10	11	10	8	10	10	10	9	9

The results of the present study revealed that all the fourteen isolates were uniform in their morphological and cultural characteristics except for the colony size. Based on the cultural tests, the isolates could be preliminarily identified as *Rhizobium* in the light of earlier reports.

Conformation test (cultural characterization)

All the 14 different rhizobial isolates showed positive result to PHB (Poly β hydroxy butyrate), negative results to congored, hofers alkaline agar, lactose agar, litmus milk and growth on glucose peptone agar tests. The rhizobial isolates of Vlm6 and Mud8 produced yellow colour on YEMA containing bromothymol blue medium, indicating their fast growing nature. All other rhizobial isolates produced blue colour when grown on YEMA containing bromothymol blue medium, thereby indicating their slow growing nature (Table 3). The results suggest that all the 14 isolates belong to *Rhizobium*.

Biochemical characterization

For biochemical characterization of the 14 different test isolates following tests were done. They include indole test, methyl red test, voges-proskauer test, macconkey agar test, , urease test, citrate utilization test, H_2S production test starch hydrolysis, nitrate reduction test and catalase test. All the isolates were uniform in their response. The results obtained suggest that all these isolates belong to *Rhizobium* as they were all in conformity with the results reported in Bergey's manual for *Rhizobium*. Thus both cultural as well as biochemical tests point to the identity of these isolates as belonging to *Rhizobium*. However, in order to find out whether any difference is observed with regard to their nutritional preferences, carbon utilization tests were done.

With regard to the utilization of carbon sources, variation could be observed among the isolates. While the isolates such as klt2, pkt4, Adn5, Mlk12, Klv13 and RF14 utilized all the ten different carbon sources, the rest of the isolates could not utilize one or two carbon sources. Adonitol could not be utilized by Mdk1, Mdu8 and Alg10; galactose by Vlm6; glucose by Pvr9; inositol by Pdg3; maltose by Alg10; mannitol by Alt7; sorbital by Bdl11; sucrose by Mdk1; xylose by Pdg3 and Pvr9. Among the ten carbon sources, only lactose was utilized by all the isolates (Table 4). The results revealed variations among the rhizobial isolates of the present study. Such kind of variation in the utilization of carbon sources were reported even earlier. For example, the glucose, galactose, fructose, arabinose and xylose were reasonably well used by fast and most slow growers, whereas rhamnose, lactose, trehalose and raffinose were poorly utilized by the slow growers (Bergersen, 1971). The fast growing rhizobia used a wide range of sugars, sugar alcohols and organic acids, but the usage by slow growers is restricted. Glucose, galactose, fructose, xylose and mannitol were utilized by most of the rhizobial strains, whereas sucrose, trehalose and dulcitol were least utilized was also reported by Doudoroff and Palleroni (1974).

Molecular characterization

Fatty acid profile

The feasibility of utilizing gas chromatography as a sensitive and rapid method for the analysis of lipids as a natural basis for the classification of microorganisms was investigated by Abel *et al.* (1963). In the present study gas liquid chromatography has been used to produce the fatty acid profile of the rhizobial isolates. The diversity of rhizobia can be assessed by an array of methods. One such method is the analysis of cellular fatty acids (FAME). For many years, analysis of short chain fatty acids has been routinely used in the identification of anaerobic bacteria. In several studies involving Gram negative organisms, fatty acids between 9 and 20 carbons in length have also been used to characterize the genera and species of bacteria. Short chain hydroxyl acids often characterize the lipopolysaccharides of the Gram negative bacteria. Cellular fatty acid analysis is a useful tool for identifying unknown strain of rhizobia and establishing taxonomic relationship between the species (Tighe *et al.,* 2000).

In the present study, totally 13 fatty acids were detected from 14 rhizobial isolates. Among them, 9 were saturated and the remaining 4 unsaturated fatty acids (Table 5). None of the isolates in the present study seem to possess all the 13 fatty acids; reported in the present study. Klt2 and Pdg3 had 8 fatty acids, Pkt4, Vlm6, Abd11 and RF14 had 7; Adn5 had 6; Mdk1, Mud8, Pvr9 and Mlk12 had 5; Alg10 and Klv13 had 4 where as Alt7 had only 3 fatty acids. The number of fatty acids reported from various isolates ranged from 3 (Alt 7) to 8 (Klt2 and Pdg3). Such a kind of diverse distribution of fatty acids could be best understood from the earlier works (Graham *et al.,* 1995; Jarvis *et al.*, 1992; 1996; Jarvis and Tighe, 1994; Tighe *et al.,* 2000). The nod factor requirements for efficient stem and root nodulation of the tropical legume, *Sesbania rostrata* reported that nod factors are essential for effective nodulation in *Sesbania rostrata* and the nod factors of *Azorhizobium caulinodans* are mainly pentamers that are vaccenoylated (C18:1), polymitoylated (C16:0) and stearoylated (C18:0) Haeze *et al.* (2000). In the fatty acid profile of the present study also, palmitic acid and stearic acid that function as nod factors as reported by Haeze *et al.* (2000) were detected in several isolates. The presence of these fatty acids (Palmitic and Stearic) in many wild-legume rhizobia, suggesting that many of the isolates in the present study show affinity to wild legume rhizobia (Zahran, 2000).

Amino acid profile

Amino acid profile of rhizobial isolates was investigated by using HPLC and the results are summarized in table 6. A total number of 11 amino acids could be identified among the 14 different rhizobial isolates. Among the 14 isolates, one isolate (Alt7) had all the 11 amino acids, 8 isolates had 10 amino acids, and 3 had 9 amino acids while the remaining two isolates contained 8 amino acids. The amino acid profile of many Gram negative bacteria was studied by many (Britten and McClure, 1962; Brown, 1976; Lillich and Elkan, 1973; Measures, 1975; Tempest and Meers, 1970) workers. Amino acid profile of test isolates in the present investigation using HPLC revealed that a total 11 amino acids constituted the amino acid pool. However, only five amino acids like alanine, cystine, methionine, phenylalanine and tryptophan were found in all the isolates. It has been well established that glutamate is predominant in the intracellular aminoacid pool in Gram negative bacteria (Hua *et al.,* 1982). However, in the present study this trend could not be observed. This may be due to the fact that the metabolic activities of microorganisms vary considerably with changes in their growth environment. Environmental changes are most likely to affect the intracellular concentrations of low molecular weight pool constituents (Brown, 1976; Measures, 1975). Further, the results of the amino acid profile from the 14 different rhizobial isolates in the present study revealed the existence of diversity. Thus for example, glycine was found in twelve isolates excepting Pkt4 and RF. Arginine and histidine were present in 8 isolates while arginine was absent in Pdg3, Adn5 and Pvr9, histidine was absent in Klt2, Vlm6 and Klv3. Of the two isolates containing only 8 aminoacids, Pkt4 did not have glycine, isoleucine and serine while Pvr9 did not have arginine, glutamate and serine. In other words, the present study confirms

the existence of diversity with regard to the pattern of distribution of amino acids among the isolates studied.

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