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CHARACTERIZATION OF RHIZOBIA ISOLATED FROM SOME TREE LEGUMES GROWING AT THE KARACHI UNIVERSITY CAMPUS

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Abstract

Rhizobial isolates from nodules of *Albizia lebbeck* (L.) Benth., and *Samanea saman* (Jacq.) Merr., were alkali-producing, slow growing and with monotrichous flagella, whereas, those from *Pithecellobium dulce* (Roxb.) Benth., and *Dalbergia sissoo* Roxb., were acid-producing and fast growers. Isolates from *P. dulce* showed amphitrichous flagella, while lophotrichous flagella were found in the isolates of *D. sissoo*. Rhizobial isolates from *D. sissoo* and *P. dulce* utilized all the 11 sugars used as carbon source, whereas isolates from *A. lebbeck* utilized 8, and those of *S. saman* utilized 7 out of 11 sugars. All isolates belonging to Mimosoideae were susceptible to Gentamycin, Neomycin and Tetracycline, but were resistant to Cephalexin, whereas reaction against other antibiotics was variable. Isolates of *D. sissoo* were resistant against Amoxicillin, Ampicillin, Cloxacillin, Erythromycin, Neomycin and Sulphamethoxazole Trimethoprim and susceptible against Gentamycin and Tetracycline.

Introduction

Members of Leguminosae are known to form nodules as a response to infection by rhizobia and fix atmospheric nitrogen (Allen & Allen, 1981). The root nodule bacteria have recently been classified into four genera viz., *Rhizobium, Bradyrhizobium, Azorhizobium* and *Synorhizobium* (Elkan, 1992), but collectively these are called rhizobia (Gaur, 1993; Michiels & Vanderleyden, 1994). The rhizobia associated with tree legumes have generally been described as slow-growing '*Bradyrhizobium*' or as 'the cowpea miscellary' (Lange, 1961; Habish & Khairi, 1970; Basak & Goyal, 1975, 1980a, 1980b). Fast- and Slow- growing rhizobia, which normally nodulate temperate legumes show a number of differences (Tan & Broughtan, 1981) but the easiest way to distinguish between the two groups is by their reaction on yeast-mannitol-agar (YMA) medium. Fast- growing isolates acidify YMA medium while the slow-growing make it alkaline (Norris, 1965; Tan & Broughton, 1981). Besides, mean generation time, carbohydrate nutrition, metabolic pathways, flagellation, symbiotic gene location, and intrinsic antibiotic resistance also vary in fast- and slow- growing rhizobia (Elkan, 1992). Although, some characteristics of rhizobia isolated from nodules of *A. lebbeck, P. dulce* (Mimosoideae) and *D. sissoo* (Papilionoideae) have been reported (Javid & Fisher, 1989; Iqbal & Mahmood, 1992), studies have been lacking on the flagellar characteristics of these tree legumes. In the present study, micro-symbionts of nodulated trees viz., *A. lebbeck* (L.) Benth., *P. dulce* (Roxb.) Benth., *S. saman* (Jack.) Merill. (Mimosoideae) and *Dalbergia sissoo* Roxb. (Papilionoideae) have been compared for their growth rates on YMA medium, colony characteristics, types of flagella, carbohydrate utilization and the antibiotic sensitivity.

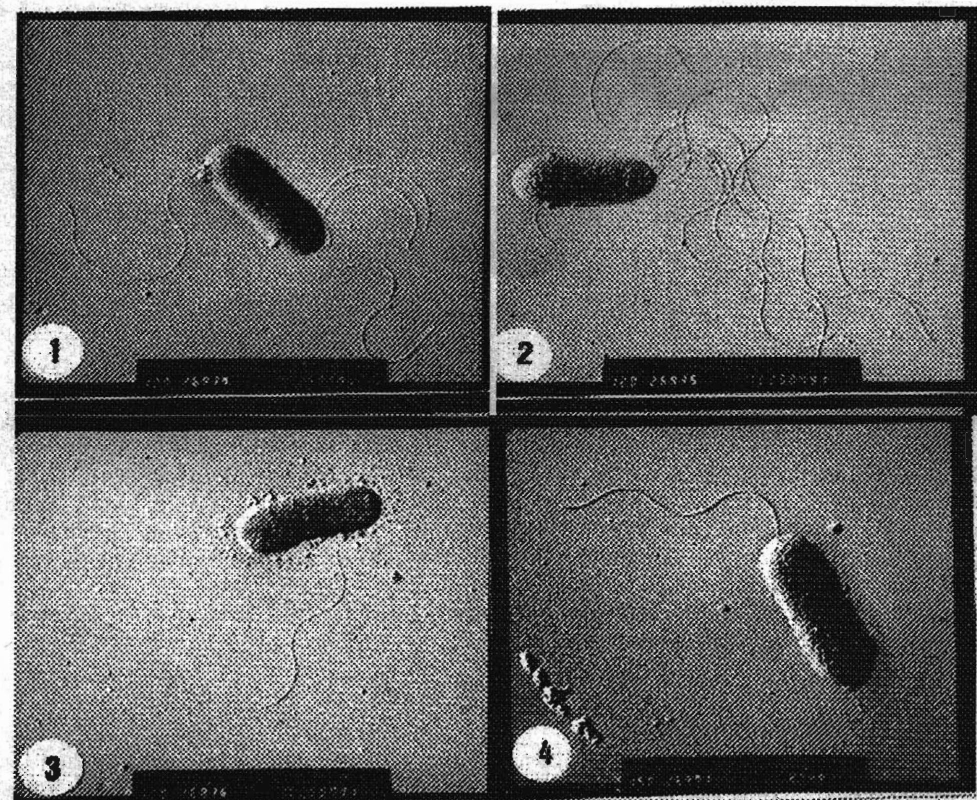

Fig. 1. Amphitrichous arrangement of flagella in the rhizobial isolates of *P. dulce*. ×13161.
Fig. 2. Rhizobial isolate from *D. sissoo* with lophotrichous flagella. ×13241.
Fig. 3. Rhizobial isolates of *A. lebbeck* with single lateral flagellum. ×13241.
Fig. 4. Monotrichous condition present in the rhizobial isolates of *S. saman*. ×1625071.

Material and Methods

Smears were prepared from surface sterilized crushed nodules of *A. lebbeck, P. dulce, S. saman* and *D. sissoo*. The smears were fixed and stained by Gram's method. Isolations of rhizobia from nodules were carried out on YMA medium as described by Vincent (1970). Cultures of rhizobia were maintained on slants according to Vincent (1970) and Somasegran & Hoben (1985). Isolates were characterized on the basis of (1) colony characteristics on different media viz., YMA, YMA-Congo red, and YMA-Bromothymol blue, (2) biochemical reactions such as carbohydrate utilization and antibiotic sensitivity, and (3) flagellar characters. For observing flagella, young cultures of rhizobia on YMA were suspended in 1-ml of sterilized distilled water in a Petri dish. After the heavier aggregates had settled, a carbon-coated grid was placed on the surface of the suspension. After 5 min., it was removed, dried at room temperature, shadowed with carbon-paladium at an angle of 40° in Edward's vacuum coating unit (model E 306A) and examined in a transmission electron microscope (Hitachi H-800) at 75 KV (Tan & Broughton, 1981).

Results

The rhizobial isolates from *P. dulce* and *D. sissoo* showed growth after 24 h of incubation at 28-30°C, whereas those from *S. saman* and *A. lebbeck* showed variable growth after 48 h. After 5-day incubation at 28°C, cultures of *A. lebbeck* and *S. saman* showed colonies, which were rounded, translucent, gummy, and 1-2 mm in diameter. On the other hand, cultures of *P. dulce* and *D. sissoo* showed colonies 2-5 mm in diameter after 3-day incubation; the colonies were rounded dome-shaped, translucent and gummy. Congo red medium was used to differentiate *Rhizobium* from *Agrobacterium*. All isolates were Gram-negative bacilli, non-sporing, and motile. Rhizobial isolates of all the four tree species did not absorb the color of congo red. Isolates from *A. lebbeck* and *S. saman* gave alkaline reaction on bromothymol blue containing YMA medium, while isolates of *P. dulce* and *D. sissoo* showed acidic reaction (Table 1). Monotrichous flagella were observed in *A. lebbeck* (Fig. 3) and *S. saman* (Fig. 4), amphitrichous in *P. dulce* (Fig. 1) and lophotrichous in *D. sissoo* (Fig. 2). Position of flagella showed different orientation in all the four isolates. These were polar in *D. sissoo* and *S. saman*, lateral in *A. lebbeck* and subpolar in *P. dulce* (Figs. 1-4).

Discussion

Rhizobia isolated freshly from nodules or the legume rhizosphere commonly show an initial vigorous growth that followed a progressive decline. Thus a slow-growing *R. japonicum* may initially grow profusely within 24 h of isolation, which within a few transfers attenuates, taking 5-7 d to achieve reasonable growth (Hubbell, 1985). In the present investigation, we found *A. lebbeck* showed variable growth after 48 h of incubation, whereas isolates from *S. saman* took 48h. Javid & Fisher (1989), Mahmood (1985, 1995), Iqbal & Mahmood (1992) reported that *Rhizobium* associated with *D. sissoo* nodules was slow-growing and alkali-producing and belonged to *Bradyrhizobium* sp. Our results on *P. dulce* and *D. sissoo* are in agreement to those of earlier studies (Mahmood, 1985, 1995). Iqbal & Mahmood (1992) reported isolates of *A. lebbeck* and *P. dulce* as slow-growing *Bradyrhizobium* types but according to our observations, isolates from *A. lebbeck* and *P. dulce* were fast-growing rhizobia. Growth rate of isolates from nodules of tropical tree legumes varies both within and between host genera (Allen & Allen, 1981; Trinick, 1982; Barnet *et al.*, 1985; Lieberman *et al.*, 1985; Sprent, 1986). Barnet & Catt (1991) included two extra growth categories viz., intermediate and very slow growing *Rhizobium* to accommodate isolates from Australian *Acacia* spp., in addition to the fast- and slow growers that did not conform to the traditional fast- and slow-growing types. Moreira *et al.*, (1993) also categorized rhizobial isolates obtained from the nodules of leguminous plants growing in Amazon region and Atlantic forest of Brazil as intermediate and very slow-growing types. On the basis of colony size after 5 days of growth, Roughley (1987) classified root nodule bacteria from *Acacia* spp., as fast-growing *Rhizobium*, and slow-growing *Bradyrhizobium*.. Oodee *et al.*, (1997) have categorized the very fast, fast and intermediate acid producing types to *Rhizobium* and the very slow, slow and intermediate alkali producing to *Bradyrhizobium*. However, this categorization may not apply to all cases, since there have been reports of alkali-producing *Rhizobium* strains (Hernandez & Focht, 1984) and acid-producing

Bradyrhizobium strains (Padmanabhan *et al.*, 1990; Moreira *et al.*, 1993). Moreover, isolates from the root nodules of *A. lebbeck* and *S. saman* were slow-growing and alkali-producing on YMA bromothymol blue medium, whereas those of *P. dulce* and *D. sissoo* were fast-growing but produced both acid and alkali in tropical habitat (Lieberman *et al.*, 1985). Tropical soils are typically acidic, and hence the ability to produce alkali would provide a selective advantage to the rhizobia (Stowers & Elkan, 1984).

A distinguishing characteristic of fast- and slow-growers is the arrangement of flagella. The slow growers have polar and sub-polar flagella while fast growers have peritrichous flagella (Elkan, 1992; Gaur, 1993). However, there are exceptions. For example, isolates from stem nodules of *Sesbania rostrata* are fast growing but had a single lateral flagellum (Dreyfus *et al.*, 1988; Elkan, 1992). Fast-growing strains from temperate regions showed more than one flagellum aggregated at the sub-polar end and tropical fast-growing isolates possess only one subpolar flagellum (Tan & Broughton, 1981). Isolate from *A. lebbeck* showed lateral flagellum while those from *S. saman* showed monotrichous polar flagellum although they were slow-growers. On the other hand isolate from *P. dulce* nodules showed sub-polar and those from *D. sissoo* polar flagella (Figs. 1-4). In agreement with some earlier reports (Tan & Broughton, 1981; Dreyfus *et al.*, 1988; Elkan, 1992), we also observed monotrichous polar flagellum in isolate from *S. saman* though they were slow-grower whereas isolate from *P. dulce* nodules were fast-growing with sub-polar flagella.

Utilization of carbohydrates by *Rhizobium* has been a subject of extensive studies in the past (Baldwin & Fred, 1927; Georgi & Ettinger, 1941; Graham, 1964). According to Hafeez *et. al.*, (1993), Moawad & Bahlool, (1993) ; Monza *et. al.*, (1992), utilization of different carbon sources is an effective tool for the classification of isolates. Fast-growing rhizobia utilize a wider range of sugars than slow-growing rhizobia, as the latter are more specialized in their sugar requirements (Graham & Parker, 1964; Vincent, 1974; Graham, 1976; Trinick, 1980; Elkan, 1992; Gaur, 1993; Irisarri *et al.*, 1996; Surange *et al.*,1997). Cowpea rhizobia behave uniformly on carbon substrate i.e., either all, or none of the strains grew on a carbon substrate though all strains showed limited growth response with maltose, lactose, arabitol and 2-ketogluconate (Stowers & Elkan, 1984). The rhizobial isolates from nodules of *A. lebbeck, P. dulce, S. saman* and *D. sissoo* tested in the present study showed a consistent carbon utilization pattern (Table 1) and conform earlier reports that the fast-growers utilize a wide range of sugar as carbon sources.

Antibiotic sensitivity tests have been widely used in identifying rhizobia (Golebiowska & Kaszubiak, 1965; Pattison & Skinner, 1974; Lim & Ng, 1979; Oodee *et al.*, 1997) and we checked the sensitivity of our rhizobial isolates against 9 antibiotics (Table 1). Tetracycline was generally most effective against fast-growing *R. trifolii, R. leguminosarum* and *R. melilotii* and a large collection of *R. japonicum* (Vintika & Vintikova, 1958; Skerdlela, 1965; Golebiowska & Kaszubiak, 1965; Pattison & Skinner, 1974; Lim & Ng, 1979). *Rhizobium* strains were more tolerant to antibiotics than *Bradyrhizobium* strains (Oodee *et al.*, 1997). In the present investigation we found that the fast-growing rhizobial isolate from Papilionoid tree (*D. sissoo*) were more resistant to antibiotics than the fast-growing isolate from Mimosoid tree (*P. dulce*). Whereas all the isolates (fast- and slow-grower) from Papilionoid and Mimosoid tree were found susceptible to Gentamycin and Tetracyclin, all of them were resistant against Cephalexin but showed varied reactions against other antibiotics (Table 1).

Table 1. Characteristics of rhizobia isolated from tree legumes growing at Karachi University Campus

S. No.	Characters	*A. lebbeck*	*S. saman*	*P. dulce*	*D. sissoo*
1.	**Growth rate on YMA**				
	After 24 h	—	—	+	+
	After 48 h	+	+	—	—
2.	**Reaction on YMA**				
	with Bromothymol blue	Alkaline	Alkaline	Acidic	Acidic
3.	**Carbon source used**				
	Arabinose	+	—	+	+
	Fructose	—	—	+	+
	Galactose	+	+	+	+
	Glucose	+	+	+	+
	Lactose	+	+	+	+
	Maltose	+	+	+	+
	Mannitol	+	+	+	+
	Mannose	+	+	+	+
	Salicin	—	+	+	+
	Sucrose	—	—	+	+
	Xylose	+	—	+	+
4.	**Antibiotic sensitivity test**				
	Amoxicillin (25 mg)*	S	R	S	R
	Ampicillin (5 mg)*	S	R	R	R
	Coxacilin (5 mg)*	S	R	R	R
	Cephalixin (30 mg)*	R	R	R	R
	Erythromycin (10 mg)*	R	R	S	R
	Gentamycin (10 mg)	S	S	S	S
	Neomycin (30 mg)*	S	S	S	R
	Sulphamethoxazole trimethoprim (25mg)*	R	S	S	R
	Tetracyclin (10 mg)*	S	S	S	S

* = Oxide sensitivity disc were used.

S = Susceptibility of isolates to antibiotic.

R = Resistance of isolate to antibiotic.

\+ = Growth was observed.

— = No growth was observed.

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