Classification of Tropical Tree Rhizobia Based on Phenotypic Characters Forms Nested Clusters of Phylogenetic Groups

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Abstract

Phenotypic variables were used to characterize 97 rhizobial isolates of *Calliandra calothyrsus*, *Gliricidia sepium*, *Leucaena leucocephala*, and *Sesbania sesban* sampled from several tropical soils, and had earlier been characterized using 16S rRNA analysis. Isolates were evaluated based on 40 variables, including colony morphology, growth rate, acidification of culture media, and the ability to utilize a wide range of carbon and nitrogen substrates. There was an overall agreement between phenotypic and phylogenetic classifications, with many of the phylogenetic groups nesting within different phenotypic clusters. In general, isolates did not cluster either according to host or geographical origin, suggesting that the ability to utilize certain substrates may not be an adaptive response. Although no one substrate was in itself distinctly diagnostic of any of the rhizobial groups, members of the *Agrobacterium* spp. sub-cluster exhibited poor growth on mono- and disaccharides, while the mesorhizobia and sinorhizobia failed to utilize dulcitol. All the rhizobial groups utilized fructose, arabinose, fucose, succinate, maltose, trehalose, and cellobiose as good energy sources and either grew poorly on polyethylene glycol (PEG), cyclodextrin, oxalate, and soluble starch or failed entirely to utilize them.

Introduction

In addition to symbiotic traits, a wide range of morphological and cultural properties has traditionally been used in the characterization and identification of rhizobia. The phenotypic characters that are routinely used for this purpose are growth rate, colony characteristics on YEM media, and the utilization of carbon and nitrogen substrates as sole sources of nutrition. Different laboratories also use additional methods to characterize rhizobia, such as analyses of cell lipopolysaccharides or protein banding patterns (de Lajudie *et al.*, 1994), multilocus enzyme electrophoresis (Martínez-Romero *et al.*, 1991) and tolerance to stresses such as acidity, salinity, heavy metals and high temperatures (Zhang *et al.*, 1991; Mpepereki *et al.*, 1997; Odee *et al.*, 1997). Soon after Jordan (1982) separated rhizobia into two genera, *Rhizobium* and *Bradyrhizobium* on the basis of growth rate, it became evident that the use of phenotypic characters as the primary basis for rhizobial species classification was fraught with inconsistencies.

Although molecular phylogeny has now been established as the primary basis for species classification, most laboratories in developing countries, especially in sub-Saharan Africa, do not have the tools nor the skills to undertake recombinant DNA-based studies.

In addition, the International Committee of Systematic Bacteriology recommends that sufficient phenotypic characters are examined to complement phylogenetic classification (Graham *et al.*, 1991). Use of phenotypic characters, therefore, remains a useful tool, at least in the initial characterisation of rhizobial strains. It is thus imperative that this methodology is standardised, using genetic relatedness as a basis for comparison, for routine use in laboratories.

In this study, rhizobia isolated from several tropical soils across three continents using *Calliandra calothyrsus, Gliricidia sepium, Leucaena leucocephala,* and *Sesbania sesban* as trap hosts, were characterized using phenotypic characters. The classification was evaluated to see how well it compared with classification of the isolates using 16S rDNA sequences. The isolates had earlier been characterized based on restriction fragment length polymorphism (RFLP) of PCR-amplified 16S rRNA and the internally transcribed spacer (ITS) region between 16S and 23S rRNA genes.

Materials and methods

Bacterial isolates

The rhizobia isolated from *C. calothyrsus, G. sepium,* and *L. leucocephala* were sampled from seven soils: Yucatan (from Mexico), San Isidro (Costa Rica), Itabela (Brazil), Onne (Nigeria), Chitala (Malawi), and Lampung and Bromo-crater (both from Indonesia). The Yucatan, San Isidro and Itabela sites were chosen because they lie within the centre of diversity of the three legume species. The Lampung and Bromo-crater sites lie within areas of earliest introduction of these species, while the Chitala and Onne sites represent areas of later introductions and also lie within the area of distribution of *S. sesban.* The *S. sesban* isolates were further sampled from a wide variety of soils from Eastern and Southern Africa where *S. sesban* is being promoted as improved fallow species. A total of 97 isolates was used for the study, with 7 reference strains belonging to the genera *Rhizobium, Sinorhizobium* and *Mesorhizobium* also included.

Characterisation of rhizobia based on growth rate and colony characteristics

Isolates were characterized using appearance, colour, shape and texture of the colonies growing on YEM agar and incubated at 28 °C. Other characters used to differentiate the isolates were the ability to change the pH of growth media, exopolysaccharide (EPS) production, and the size of first discrete colonies to appear. Colony diameter measured after 3, 5 and 7 days of incubation on YEM media was used to group the isolates according to the following growth rate categories described by Jordan (1984) and modified by Odee et al. (1997):

(a) Very fast–colonies ≥ 5 mm in diameter after 3 days of incubation, acid producers.

(b) Fast - colonies ≥ 2 mm in diameter after 5 days of incubation, acid producers.

(c) Intermediate - colonies 1–2 mm in diameter after 5–7 days of incubation, acid producers

Colony appearance was scored as either translucent or opaque; texture as buttery or elastic; EPS production as copious, moderate, or low; shape as flat or raised; and colour as creamy, milky, watery, or white.

Utilization of carbon and nitrogen substrates

Isolates were evaluated for their ability to utilize the following substrates as sole sources of carbon: D(+)glucose, sucrose, D-fructose, dulcitol, D-arabinose, α-L(-) fucose, succinate, L(+)arabinose, maltose, polyethylene glycol (PEG), D(+)mannose, D(+)trehalose, α-cyclodextrin (cyclohexaamylose), D-(+)cellobiose, sodium hydrogen tartarate, di-potassium oxalate, tri-sodium citrate, sodium acetate, and soluble starch.

Isolates were also tested for their ability to utilize the following nitrogen substrates: thiamine, L-isoleucine, L-arginine, L-tyrosine, L-asparagine, L-valine, L-aspartic acid, L-alanine, L-β-phenylalanine, and urea.

To test for carbon substrate utilization, the different carbon sources were added at a final concentration of 1 g L^{-1} to a minimal medium (Amarger *et al.*, 1997) containing (L^{-1}) 1 g of K_2HPO_4, 1 g of KH_2PO_4, 0.01 g of $FeCl_3 \cdot 6H_2O$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$ 1 g of $(NH_4)_2SO_4$, and 10 g of agar. Bromothymol blue was added at 0.005% as pH indicator. Substrates used as nitrogen sources were added at a concentration of 0.5 g L^{-1} to a medium with the same composition as the carbon sources, except that $(NH_4)_2SO_4$ was omitted, and mannitol was added at a concentration of 1 g L^{-1}. Stock solutions of all substrate sources were filter sterilized using 0.22 µm millipore filters and added to the minimal medium, which had been autoclaved and cooled to about 50 °C. The resulting medium was thoroughly mixed and poured onto Petri dishes and allowed to solidify under a laminar flow cabinet (Bassaire, Southhampton, UK) for at least 30 min before use.

Starter cultures were grown in Trypton Yeast (TY) broth for between 36 and 48 hours and growth on the different substrates was tested by applying the bacterial suspension onto the surface of the agar using drop plate method. Three 5 µl drops of each suspension were added to a plate and replicated on two other plates. Each isolate was also grown on Petri dishes containing YEM agar to serve as control. All plates were incubated at 28 °C and growth was recorded after 5 days. Where an isolate failed to grow on a substrate, the experiment was repeated for that treatment combination.

Numerical analysis

Mix, a discrete character programme from the phylogenetic programme PHYLIP (Felsenstein, 1993), was used to produce a dendogram based on substrate utilisation, growth rate, EPS production, colony texture and shape and final pH of culture media. Growth was scored as 2 for heavy growth, 1 for light growth and 0 for no growth. For clustering analysis, both heavy and light growths were scored as 1, and no growth as 0. Fast- or very fast- growth was scored as 1 and medium growth as 0. Moderate to copious EPS was 1 and low to no EPS was scored 0, while acid reaction was scored 1 and neutral reaction as 0. A total of forty variables were used for the clustering analysis.

Results

Characterization of isolates by growth rate and colony morphology

The 97 isolates tested could be categorised into 4 colony types: milky translucent, white opaque, watery translucent and creamy opaque (Table 1). The first 3 colony types were circular to irregular in shape, raised and elastic; and all produced moderate to copious EPS. These groups together constituted 96% of all the isolates tested. The milky translucent and white opaque colonies contained isolates with intermediate, fast and very fast growth, while the watery translucent group was solely made up of very fast- growing isolates. The fourth colony type (creamy opaque) constituted 4% of the total isolates. Members of this group were all of intermediate growth rate, had flat, circular colonies with buttery texture and produced low or no EPS. Very fast- and fast-growing isolates constituted 33% and 24% respectively of all the isolates, while the other 43% had intermediate rates of growth (Table 2).

Distribution of colony types according to hosts and sites of origin

All the isolates with creamy opaque colonies originated from the Itabela (Brazil) soil. The other colony types were isolated from virtually

TABLE 1
Growth rates and colony characteristics of rhizobial isolates grown on YEM agar.

Colony type	Shape	Texture	EPS slime production	Growth rate	Proportion of isolates (%)
Milky translucent	raised	elastic	moderate to copious	very fast, fast, intermediate	52
White opaque	raised	elastic	moderate to copious	very fast, fast, intermediate	25
Watery translucent	raised	elastic	copious	very fast	19
Creamy opaque	flat	buttery	low to none	intermediate	4

TABLE 2
Growth rate characteristics of rhizobial isolates based on their sites and hosts of isolation.

Site of isolation	Number of isolates with growth rates that are: very fast	fast	intermediate	Proportion of isolates with fast and very fast growth (%)
Yucatan	9	3	8	60
Lampung	6	13	0	100
Chitala	6	1	3	70
Itabela	5	0	7	42
San Isidro	1	2	1	75
Bromo-crater	1	1	0	100
Onne	3	2	1	83
Other soils*	1	1	22	9
Total of growth rate types	32	23	42	
Percentage of total isolates	33	24	43	

*Other soils comprising a wide range of soils from East and Southern Africa from which *S. sesban* was used to isolate the rhizobia.

all the soils, the exception being the group of African soils from which *S. sesban* was used to trap isolates. The colonies of isolates from this group of soils were all of the milky translucent types with intermediate growth rates. Isolates with fast- or very fast-growing rates were predominant in all the other soils except the Itabela soil (Table 2).

Relationships between growth rate and phylogenetic affiliation of isolates

Fast- and very fast-growing isolates were roughly of equal proportions among the various genetic groupings (Table 3). All agrobacteria and most rhizobia were fast- or very fast growers, while all mesorhizobia and most sinorhizobia had intermediate growth rates. The other isolates

TABLE 3
Relationships between growth rate characteristics and the genetic affiliations of rhizobial isolates.

Genus affiliation	Total number of isolates	Number of isolates with growth rates that are: very fast	fast	intermediate
Agrobacterium	8	5	3	0
Mesorhizobium	26	0	0	26
Rhizobium grp I*	35	19	14	2
Rhizobium grp II*	7	3	3	1
Sinorhizobium	8	1	0	7
Others	13	4	3	6

*_Rhizobium_ grp I comprised of all isolates with 16S rRNA sequence homology closely affiliated with *R. tropici* and *R. leguminosarum*; *Rhizobium* grp II isolates are closely affiliated with *R. giardinii*, *R. mongolense* or *R. gallicum*.

of unknown phylogenetic affiliation were distributed in roughly equal proportions among fast-, very fast- and intermediate-growers.

Substrate utilization performance of rhizobial isolates

The result of the cluster analysis performed on the 97 isolates based on their ability to utilize a wide range of carbon and nitrogen substrates is shown in Figure 1. Six main clusters were formed, each containing isolates with varying degrees of genetic heterogeneity. Cluster I comprised six isolates, all of which were of the *Agrobacterium* lineage. Members of this cluster were able to utilize all the substrates tested except cyclodextrin, tartarate, and oxalate (Table 4). Only one isolate was able to grow on PEG and soluble starch even though growth in both cases was light. Growth of members of this cluster on six other carbon substrates was light.

Cluster II was made up of 21 isolates, 15 of which were of the *Rhizobium* lineage including strains R602 (*R. gallicum*), CIAT632 (*R. etli*) RCR3644 (*R. leguminosarum* bv. *phaseoli*), and CFN42 (*R. etli*). The other group in Cluster II was made up of strain USDA3471 (*M. loti*), a *Mesorhizobium* isolate, and four other isolates of unknown phylogenetic affiliation. The *Rhizobium* species were able to grow on all the substrates except oxalate, although growth was light on dulcitol, cyclodextrin, and soluble starch. Members of the other group in this cluster were also capable of utilizing all the substrates except PEG and soluble starch.

Cluster III was made up of 32 isolates forming four subgroups. The first consisted of 16 isolates which belong to the *Mesorhizobium* lineage. Members of this group failed to grow on dulcitol, PEG, and soluble starch. Most of the isolates that could grow on six other substrates had light growth. The second sub-group had four isolates which belong to the *Rhizobium* lineage. These isolates failed to grow on dulcitol, PEG, cyclodextrin, tartarate, oxalate, and soluble starch. The third subgroup was made up of eight members of the *Sinorhizobium* lineage. They could grow on all substrates except dulcitol and oxalate, and only one isolate used cyclodextrin and soluble starch. The fourth subgroup contained the isolates whose phylogenetic affiliations are yet to be determined. These failed to grow on PEG, cyclodextrin, oxalate, and soluble starch.

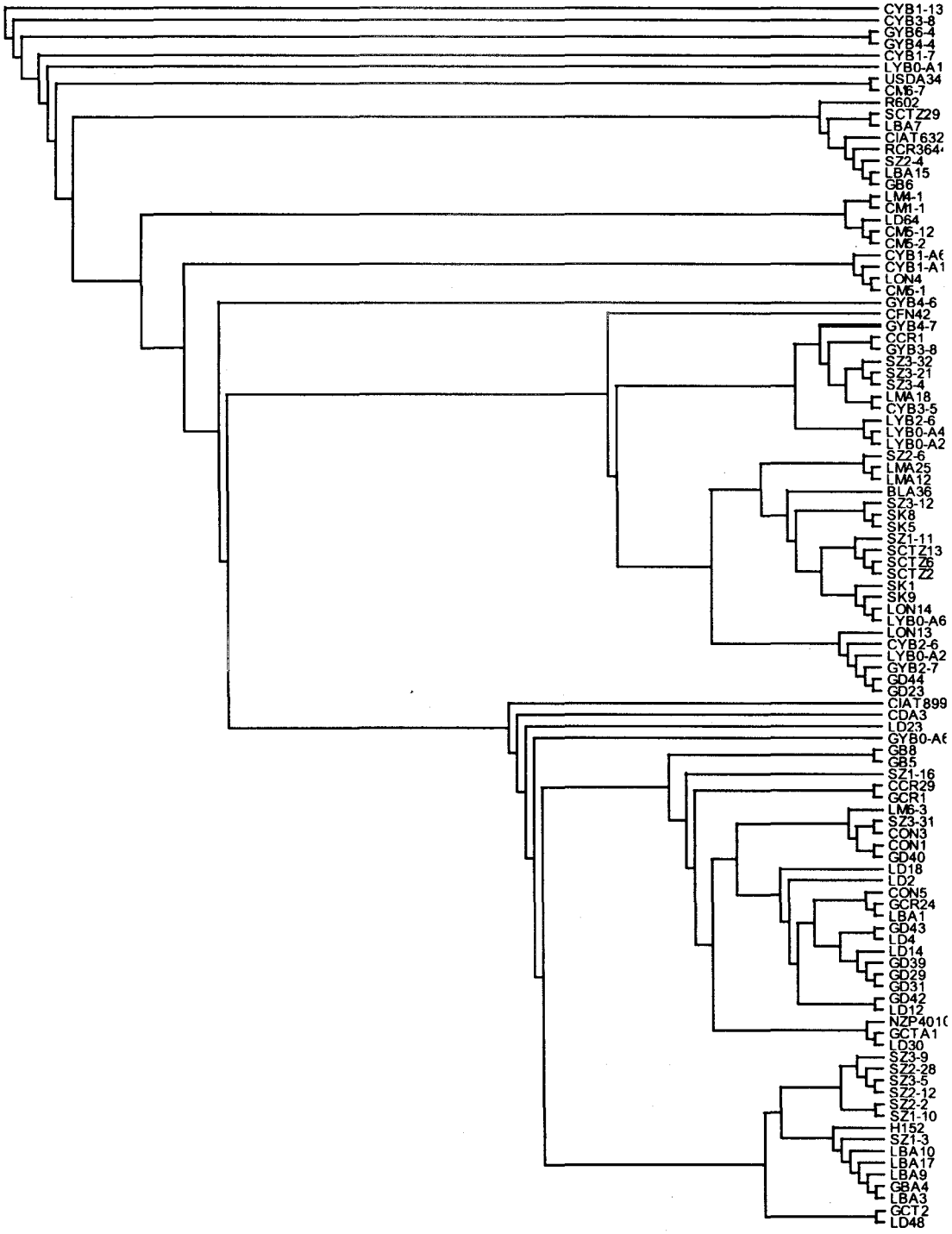

Figure 1. Dendrogram showing phenetic clusters of tropical tree rhizobia and eight reference strains representing the genera *Mesorhizobium* (USDA3471), *Rhizobium* (R602, CIAT632, RCR3644, CFN42, CIAT899 and H152) and *Sinorhizobium* (NZP4010).

TABLE 4
Results of substrate utilization tests performed with different groups of tree rhizobia and some reference strains.

Substrate	Cluster I	Cluster II		Cluster III				Cluster IV			Cluster V	Cluster VI
	Ag[a]. spp	*R.* spp	others	*M.* spp	*R.* spp	*S.* spp	others	*Ag.* spp	*R.* spp	others	*M.* spp	
	No. of isolates											
	6	15	6	16	4	8	4	2	24	4	6	9
Glucose	+(±)[b]	+	+	15	+	7	+	1	+	+	+	+
Sucrose	+(±)	+	5	+	+	+	3	+	+	+	+	+
Lactose	+(±)	+	+	6(±)	+	6	2	+	+	+(±)	+(±)	8
Fructose	+	+	+	+	+	+	+	+	+	+	+	+
Dulcitol	+	10(±)	+	–	–	–	1	–	19	+	–	2
D- Arabinose	+	+	5	15(±)	+	7	2	+	+	+	+	8
Fucose	+	+	5	12	+	5	3(±)	+	+	+	+	+
Succinate	4	14	+	+	+	+	+	+	22	+	5(±)	+
L+ Arabinose	+	13	+	15	+	+	+	+	+	+	5	+
Maltose	+	+	5	15	+	+	2	+	+	+	+	8
PEG	1(±)	2	–	–	–	7	–	+(±)	21(±)	3(±)	3(±)	+(±)
Mannose	+(±)	+	+	15	+	+	+	+	+	+	5	+
Trehalose	+	+	5	+	+	+	2	+	+	+	+	+
Cyclodextrin	–	3(±)	1	3(±)	–	1(±)	–	+(±)	20(±)	3(±)	+(±)	+
Cellobiose	+	+	+	15	+	7	+	+	23	+	+	+
Tartarate	–	4	3	4(±)	–	1	1	+(±)	23	3(±)	+(±)	+
Oxalate	–	–	1(±)	4(±)	–	–	–	–	17(±)	2	+(±)	+(±)
Citrate	+(±)	+	+	14(±)	+	6(±)	2(±)	+	+	+	+	+
Acetate	+(±)	+	+	14	+	+	+	+	+(±)	+	5	+
Soluble starch	1(±)	2(±)	–	–	–	1(±)	–	+(±)	+(±)	3(±)	4(±)	+(±)
Thiamine	+	+	+	+	+	7	+	+	+	+	+	8
Isoleucine	+	+	+	+	+	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	+	+	+	+	+	8
Tyrosine	+	+	+	8	+	+	+	+	+	+	+	8
Asparagine	+	+	+	+	+	+	+	+	23	+	+	8
Valine	+	+	+	+	+	+	+	+	+	+	+	+
Aspartic acid	+	+	+	15	+	+	+	+	+	+	+	+
Alanine	+	+	+	+	+	7	+	+	23	+	+	+
Phenyl alanine	+	+	+	+	+	+	+	+	+	+	+	8
Urea	+	+	+	15	+	+	+	+	+	+	+	+

[a] *Ag, Agrobacterium*; *R, Rhizobium*; *M, Mesorhizobium* and *S, Sinorhizobium*. Others are of unknown phylogenetic affiliations. [b] Number of isolates
+ all the isolates are positive; (±), at least 75% of the isolates had light growth; –, all the isolates failed to grow. The values are numbers of isolates that did grow.

Cluster IV contained 30 isolates, 24 of which were of the *Rhizobium* lineage, including the *R. tropici* IIB type strain, CIAT899. These utilized all the substrates tested, although their growth on five of the substrates was light. The two agrobacteria and four others were also able to utilize all the substrates tested.

Cluster V was a homogenous group consisting of six mesorhizobia. This group failed to grow on dulcitol, and grew only lightly on seven other substrates. Cluster VI contained nine isolates including three *Rhizobium* species and strain H152–the type strain for *R. giardinii*. Members of this cluster could utilize all the substrates tested.

Acid and alkali production on substrates

Growth of some isolates on each of the carbon substrates used in this study was accompanied by the acidification of the culture medium. Nearly all the isolates tested utilized each of the nitrogen sources with varying degrees of acid reactions (data not shown).

Discussion

Growth rates and colony characteristics

The variations in growth rate and colony characteristics as observed in this study are consistent with those reported for other fast-growing rhizobia (Mpepereki *et al.*, 1997; Odee *et al.*, 1997). With their characteristically dry small (< 2 mm), buttery opaque colonies which were hardly confluent, and often punctiform with flat elevations, the intermediate-growing creamy opaque colony types isolated from the Itabela (Brazil) soil rather had characteristics that bore more resemblance to bradyrhizobia than rhizobia (Eaglesham *et al.*, 1987). However, unlike bradyrhizobia, these isolates had relatively fast growth rates, with the first discrete colonies on YEM appearing within 2 to 3 days just like other fast-growing types. Given that members in this group also formed a distinct 16S rRNA-RFLP group (Bala *et al.*, 2002), it is probable that they belong to a separate and perhaps as yet undescribed species.

Acid and alkali reactions on growth media

In this study, acid production occurred to various degrees on most of the carbon and nitrogen substrates and was observed to be dependent on the isolates, rather than the carbon or nitrogen source, except for mannitol (Bala, 1999). On the contrary, other reports indicate that the final pH of culture media for both slow- and fast-growing rhizobia is dependent on the carbon and nitrogen sources (Stowers and Eaglesham, 1984; Stowers and Elkan, 1984; Ahmad and Smith, 1985). The observation by Bala (1999) may only be valid in so far as the duration of the study was concerned. Since observation for colour changes only lasted for 10 days, the possibility of acidification occurring beyond this period could not be precluded, and variations in colour changes observed within the ten-day period may be a mere reflection of the growth rates of the different isolates on individual substrates.

Colony characteristics and phylogenetic affiliations

Consistent with the description of the genus *Mesorhizobium*, which signifies intermediate growth rate (Young, 1996), all the *Mezorhizobium* strains were of intermediate growth rate irrespective of site or host of isolation. The exhibition of fast- and very fast-growth rate by the agrobacteria and rhizobia was also consistent with the characteristics of the two genera (de Lajudie *et al.*, 1994; Young, 1996). On the hand, the intermediate-growth rates observed for most of the sinorhizobia was contrary to the observed growth rates of members of the genus *Sinorhizobium* (de Lajudie *et al.*, 1994).

Substrate utilization ability of rhizobial isolates

Auxanographic analyses of rhizobial isolates revealed considerable diversity in substrate utilization, on the basis of which six phenotypic clusters were formed. All the carbon and nitrogen substrates tested were used, by some or all of the isolates, as sources of growth. Given that substrate utilization is used to characterize both functional and structural differences among microbial communities (Buyer and Drinkwater, 1997), it will be expected that the pattern of utilization of some substrates by rhizobia will be sufficiently distinctive to differentiate them from other organisms. In this study, nearly all the isolates utilized fructose, arabinose, fucose, succinate, maltose, trehalose, and cellobiose as good energy sources, and most isolates showed either light growth on dulcitol, PEG, cylodextrin, oxalate, and soluble starch or failed to utilize them. These variations in substrate utilization are consistent with the general patterns reported for other fast-growing rhizobia (Stowers and Eaglesham, 1984; Zhang *et al.*, 1991; 1992; Rome *et al.*, 1996; Amarger *et al.*, 1997; de Lajudie *et al.*, 1994; 1998).

Although no one substrate was in itself distinctly diagnostic of any of the rhizobial groups, some features were observed to be characteristics of certain groups at the genus level. For instance, most of the agrobacteria showed poor growth on monosaccharides and disaccharides such as glucose, sucrose, and mannose, while all the other groups of isolates utilized these substrates as good energy sources. Poor growth or lack of growth on monosacharides and disaccharides is a feature more associated with some types of bradyrhizobia and azorhizobia (Stowers and Elkan, 1984; Eaglesham *et al.*, 1987; de Lajudie *et al.*, 1994), and typical *Ag. tumefaciens* and *Ag. vitis* strains are known to grow well on these substrates (Lindström and Lehtomäki, 1988; de Lajudie *et al.*, 1994; Amarger *et al.*, 1997). Another instance of a distinctive feature is that of the mesorhizobia and sinorhizobia which failed to utilize dulcitol. This result is also consistent with that of de Lajudie *et al.* (1994).

In some cases, isolates belonging to the same genus fell into more than one cluster with the different groups varying in their ability to use certain substrates. For instance, while most of the *Rhizobium* isolates in Cluster II could grow only lightly on dulcitol, those in Cluster III failed to utilize it at all, while most of the cluster IV isolates grew luxuriantly on the substrate (Table 4). Although previous studies have shown that some substrates could be used as distinctive features in differentiating several species of a genus (de Lajudie *et al.*, 1994; Nour *et al.*, 1994; Rome *et al.*, 1996), it is not clear at this stage whether such sub groups constitute distinct species, but the possibility cannot be precluded.

Except for Cluster I in which all the agrobacteria were obtained from the Yucatan (Mexico) soil, isolates did not form clusters either according to host or geographical origin. Isolates obtained from widely separated areas were found in the same clusters, while others from the same areas were found in different groups. This result is inconsistent with the suggestion that substrate utilization may be affected by environmentally induced physiological changes (Buyer and Drinkwater, 1997), and may be an indication that the ability to utilize certain substrates is not an adaptive response.

The fact that each of the phenetic clusters was broadly composed of genetically similar isolates not only underlined the usefulness of phenotypic characterization as the first step in strain characterization and species description, but also highlighted the benefit of using large numbers of differentiating characters. McInroy *et al.* (1999) tested the ability of 15 reference strains representing all

the rhizobial genera (except *Allorhizobium*) to utilize 95 carbon sources on Biolog microtitre plates. They found close correlation between the groupings based on these characters and those based on 16S rRNA homologies. Other workers using API galleries have also had close homologies between groupings based on phenotypic characters and those based on genetic similarities (Nour *et al.*, 1994; Rome *et al.*, 1996; de Lajudie *et al.*, 1994; 1998).

Conclusion

The rhizobial isolates used in this study differed in their colony characteristics, and although both the intermediate and the fast (or very fast) isolates were of similar proportions, most of the soils were dominated by the latter. These produced moderate to copious slime and were characterized by a mucilaginous appearance. Growth of any of the isolates on YEM was accompanied by an acid reaction, but acidification of culture media with other C and N sources was strain dependent. All the mesorhizobia and sinorhizobia were of intermediate growth rate, while the agrobacteria and rhizobia were fast or very fast growers. Grouping the isolates based on phenotypic characters was broadly correlated with their genetic relationships, thus highlighting the usefulness and relevance of phenotypic characterization in rhizobial taxonomy. Given that most of the main phenetic clusters contained several phylogenetic groups, there may be a need to increase the number of phenotypic characters, or further fine-tune the ones used so that only characters that show distinct differences between isolates are used.

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