

Relationships between rhizobial diversity and host legume nodulation and nitrogen fixation in tropical ecosystems

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Abstract

With recent advances in rhizobial phylogeny, questions are being asked as to how an ecological framework can be developed so that rhizobial classification and diversity could have greater practical applications in enhancing agricultural productivity in tropical ecosystems. Using the results of studies on tropical rhizobia which nodulate agroforestry tree legumes, three ecological aspects of rhizobial biodiversity were used to illustrate how its potential can be exploited. The results showed that legumes nodulate with diverse rhizobial types, thus contributing to the success of legumes in colonising a wide range of environments. There was an apparent shift in the relative dominance of rhizobia populations by different rhizobial types as soil pH changed. The *Rhizobium tropici*-type rhizobia were predominant under acidic conditions, *Mesorhizobium* spp. at intermediate pH and *Sinorhizobium* spp. under alkaline conditions. The *R. tropici*-type rhizobia were the most effective symbiotic group on all the host legumes. However, strains of *Sinorhizobium* spp. were as effective as the *R. tropici* types in N₂-fixation on *Gliricidia sepium*, *Calliandra calothyrsus* and *Leucaena leucocephala*; while *Mesorhizobium* strains were equally as effective as the *R. tropici* types on *Sesbania sesban*. Classification of rhizobia based on phenotypic properties showed a broad correlation with groupings based on 16S rRNA sequence analysis, although a few variant strains nested with the dominant groups in most of the clusters. Some of the phenotypic characters that differentiated different rhizobial groups are highlighted and a case is made for the need to standardise this method.

Introduction

The family Leguminosae is one of the most successful families of angiosperms, with about 650 genera and 20,000 species (Sprent 1995) and a cosmopolitan distribution (Raven and Polhill 1981). The success of legumes can in part be attributed to their ability to colonise environments with low soil nitrogen because of their symbiotic association with N₂-fixing rhizobia (Sprent 1994).

The current rhizobial taxonomy has 6 genera and 29 species, most of which were described in the last decade using rhizobia isolated from tropical legume species (Giller 2001). In spite of this relatively high turnover of rhizobial groups, it is likely that we are still orders of magnitude away from a true assessment of the diversity of tropical rhizobia (Giller 2001). This has led to questions being asked as to how this can be explored to enhance agricultural productivity in the tropics. This requires

an ecological approach, which can help us understand the relative environmental tolerances of the different rhizobial types and thus allow for predicting their ecology (Andrade et al. 2002). Such an approach is pertinent in view of the fact that the success of rhizobial inoculation, for instance, depends on inoculant strain competitiveness and persistence, which are both linked to the saprophytic competence of the strain.

Although the description of rhizobial genera and species is now essentially based on sequence analysis of small subunit ribosomal DNA, phenotypic characterisation still remains an essential ingredient of rhizobial classification (Graham et al. 1991). Since the capacity of many laboratories in developing countries is limited to the use of phenotypic characterisation, there is a need to standardise this method as a simple, low-level technology protocol for routine assessment of rhizobial diversity.

The aim of this paper is to look at rhizobial diversity from an ecological perspective, in terms of the relationship between rhizobial types and (i) nodulation (host range), (ii) N₂-fixation (effectiveness) and (iii) adaptation to soil acidity as functional attributes of rhizobial types in soil populations. Soil acidity was chosen for this study because it was found to be a major determinant of rhizobia diversity (Bala et al. 2003a). The other objective is to determine some of the phenotypic characters that can be used in differentiating groups of rhizobia. The results used are based on studies of rhizobia isolated from tropical soils of Africa, Asia and Central and Southern America using four legume tree species that are commonly used in agroforestry. These were *Calliandra calothyrsus*, *Gliricidia sepium*, *Leucaena leucocephala* and *Sesbania sesban*.

Materials and methods

Soil sampling and analysis

Soils were sampled from 13 sites that had no history of inoculation in tropical areas of Africa, Asia and Latin America. The sites ranged from cultivated fields to secondary forests, which were located in sub-humid to humid tropical climates (Table 1). None of the legume host plants used for the study was growing at any of the sites at the

time of soil sampling. Soils were sampled during the rainy season or at the beginning of the dry season. At each site, soil cores were sampled at 0–15 cm depth at several points and were bulked and thoroughly mixed to get composite samples. Precaution was taken to avoid cross-contamination of soils from different sites. Moist soil samples that were not going to be used immediately were stored in loosely tied plastic bags and stored at 4 °C. None of the soils was stored for more than 11 days after sampling before use. Routine soil analysis was carried out according to the TSBF manual (Anderson and Ingram 1993). Soil rhizobia populations were estimated using the most probable number (MPN) method (Vincent 1970). An automatic C/N Roboprep analyser coupled to a 20–20 mass spectrometer (Europa Scientific, Crewe, UK) was used for the measurement of total soil C and N in soils, and was also used to measure total N in plants as an estimate of N₂ fixed.

Seed sources and treatment

Seeds of *G. sepium* (provenance Retalhuleu) were obtained from the International Centre for Research on Agroforestry (ICRAF), Kenya, and those of *L. leucocephala* (provenance Gede) and *C. calothyrsus* (provenance Ex Maseno) from the Kenya Forestry Seed Centre. *S. sesban* seeds were obtained from Centrale de Graines Forestieres, Rwanda.

Seeds were immersed in concentrated H₂SO₄ for periods of 25–30 min for scarification and surface sterilisation, followed by thorough rinsing with sterile distilled water. Scarified seeds were germinated on 1% water agar at a temperature of 28 °C.

Nodule sampling and rhizobial isolation

Two-day old seedlings were aseptically transferred to growth pouches containing N-free solution and inoculated with serial dilutions of each soil. Seedlings were also transplanted into 6 cm diameter plastic pots containing 100 g of each soil and an equal weight of acid (0.1 M HCl) washed sand. The potted plants were irrigated with N-free nutrient solution. The seedlings were maintained in growth chamber as described by Bala et al. (2003b) for a period of 12 weeks.

Table 1. Selected site characteristics and physico-chemical properties of the soils sampled in various tropical countries.

Site and country	Vegetation and site history	Mean annual rainfall (mm)	Mean annual temp (°C)	pH in H ₂ O (1:2.5)	Organic Carbon (%)	Total N (%)	Available P (mg kg ⁻¹)	Exch. bases (cmol _c kg ⁻¹)	Exch. acidity (cmol _c kg ⁻¹)	Sand (%)	Silt (%)	Clay (%)
<i>Brazil</i>												
Itabela	Atlantic forest	1100	34	4.41	1.17	0.10	0.55	2.00	0.63	82	2	16
<i>Costa Rica</i>												
San Isidro	Moist deciduous Forest	2000	34	4.31	3.27	0.39	0.55	7.65	6.73	44	38	18
<i>Indonesia</i>												
Bromo-crater	Montane forest	2750	32	4.15	1.14	0.18	6.95	1.05	2.71	81	17	2
Lampung	Rainforest	2500	32	4.54	2.08	0.16	1.95	3.01	0.93	66	9	25
<i>Kenya</i>												
Maseno	Maize field	2500	22	5.46	1.46	0.16	1.60	530	0.49	33	20	47
<i>Malawi</i>												
Chitala	Maize field	750	28	5.75	1.02	0.07	0.45	7.49	0.09	65	9	26
River	Maize field	1000	25	5.14	2.31	0.15	0.55	12.03	0.07	51	21	28
Chitedze	Miombo woodland	800	28	5.89	0.98	0.07	19.00	7.95	0.44	71	9	20
Salima												
<i>Mexico</i>												
Yucatan	Secondary forest	1000	26	7.48	4.44	1.57	0.55	120.36	0.12	29	34	37
<i>Nigeria</i>												
Onne	Secondary rainforest	2300	32	4.18	1.01	0.11	12.95	0.85	1.81	75	8	17
<i>Zambia</i>												
Banda	Maize field	750	27	5.15	0.51	0.09	18.5	1.08	0.50	80	10	10
Fisi village	Maize field	800	27	5.07	1.42	0.11	13.5	8.37	0.51	72	10	18
Katete FTC	Maize field	800	27	5.05	0.56	0.05	11.5	3.29	0.43	78	8	14

At harvest, root nodules were randomly sampled from roots of potted plants and those inoculated with serial soil dilutions to ensure the sampling of less common rhizobia (Bala et al. 2001). Rhizobia were isolated from root nodules on yeast extract mannitol agar (YMA) containing Congo Red (Vincent 1970).

Rhizobia genetic diversity

Genetic diversity of rhizobia was determined using PCR-restriction fragment length polymorphism (RFLP) of the 16S rRNA gene and the internally transcribed spacer (ITS) region between the 16S and 23S rRNA as earlier described (Bala et al. 2002). Restriction fragment length polymorphism of 16S rRNA was used to assign the isolates into ribosomal DNA groups, representatives of which were subjected to an almost full-length DNA sequencing (Bala et al. 2003b). Variations in the RFLP patterns of ITS fragments were used to differentiate isolates as “strains”.

Phenotypic characterisation of strains and numerical analysis

Ninety six rhizobia representing different ITS RFLP groups and eight reference strains of the genera *Rhizobium*, *Mesorhizobium* and *Sinorhizobium* were used for phenotypic characterisation. Differentiation using colony characteristics was as described previously (Mpeperekki et al. 1997; Odee et al. 1997). The ability of isolates to utilise various carbon and nitrogen substrates was assessed using the method of Amarger et al. (1997).

A total of 40 variables, including colony and symbiotic characteristics of isolates and substrate utilisation, were used for numerical analysis. A cluster analysis of the phenotypic traits was based on matrix and Euclidean distance using the SAS programme.

Results

Distribution of rhizobia types and host nodulation

The rhizobial types that nodulated *C. calothyrsus*, *G. sepium* and *L. leucocephala* in four soils are

shown in Figure 1. Apart from *G. sepium* in the Chitala soil, the legumes were nodulated by rhizobia in all four soils. *S. sesban* failed to nodulate in any of the soils hence its exclusion from Figure 1. Host legumes were nodulated by at least four groups of rhizobia in the Yucatan soil, three in the Itabela and Chitala soils and two in the Lampung soil.

The *R. tropici* group was isolated in all the soils; the mesorhizobia were isolated in three soils and the *R. etli* group in two soils. The *Sinorhizobium* and *Agrobacterium* groups were limited to the Yucatan soil. All the rhizobial groups were isolated from all the hosts apart from the *R. etli* group that was not sampled from *C. calothyrsus*. The host legumes were also nodulated to various degrees by one or more of the rhizobia groups in the remaining nine soils (data not shown).

Effect of soil acidity on rhizobia groups

Changes in soil pH appeared to be associated with shifts in the dominant rhizobial groups within soil populations (Figure 2a). Below a pH of 4.2, *Rhizobium* spp., made up of members of the *R. etli*, *R. giardinii* and *R. gallicum* groups, dominated the populations, while the *R. tropici* group was dominant within a pH range of 4.2–5.0. The *Mesorhizobium* spp. were the most dominant at pH range of 5.0–6.5, while the *Sinorhizobium* spp. formed the bulk of the populations above a pH of 6.5.

There was also an apparent shift in population dominance as the exchangeable acidity changed, with the *Mesorhizobium* spp. being dominant when exchangeable acidity was below 2 cmol kg⁻¹ (Figure 2b). Above this value, the *R. tropici* group dominated the populations. The *Rhizobium* spp. and *Sinorhizobium* spp. appeared not to be dominant at any exchange acidity range.

Symbiotic effectiveness of rhizobia groups

Figure 3 shows the distribution of rhizobia types among the groups of strains that were characterised in an earlier study (Bala and Giller 2001) as being effective or very effective when inoculated on their hosts. Out of the 20 strains inoculated on *C.*

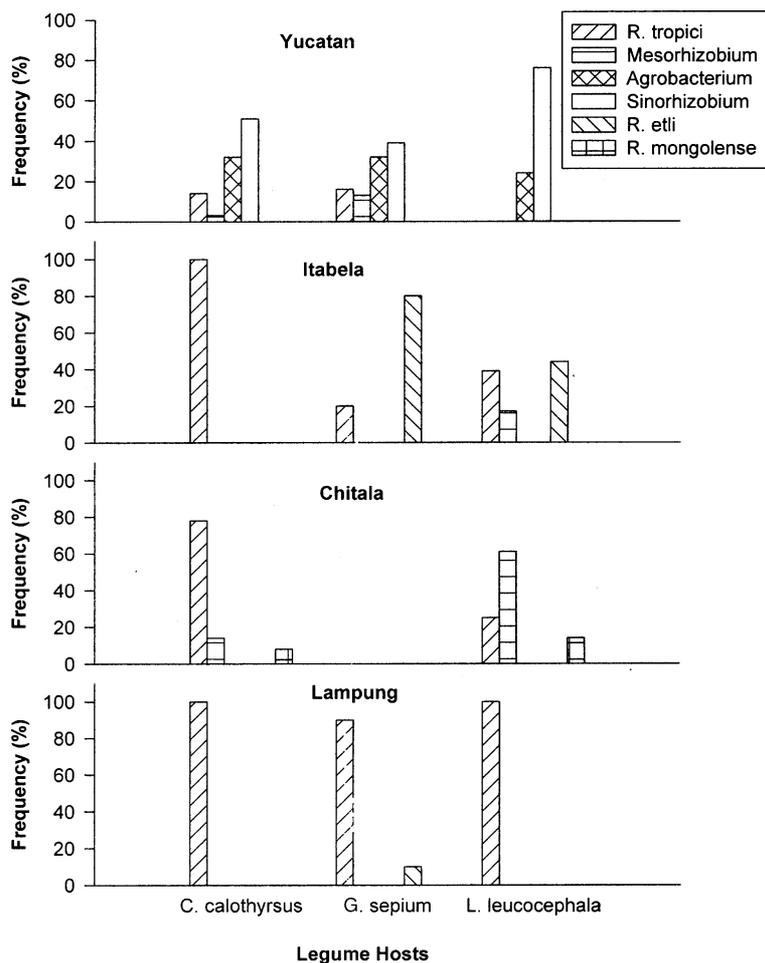


Figure 1. Distribution of rhizobial types that nodulated *C. calothyrsus*, *G. sepium* and *L. leucocephala* in different soils (Yucatan, Mexico; Itabela, Brazil; Chitala, Malawi; Lampung, Indonesia).

calothyrsus, only five were effective or very effective, with four being members of the *R. tropici* group. This group fixed an average of 5.9 mg N per plant compared with 4 mg N per plant fixed by the only *Sinorhizobium* spp. strain (GYB2-7). Nine of the thirty strains inoculated on *G. sepium* were effective or very effective with seven of these belonging to the *R. tropici* group and fixing an average of 19.5 mg N per plant. This was the same amount of N fixed on *G. sepium* by the only *Sinorhizobium* strain CYB3-5, but not as much as those of the best three strains of the *R. tropici* group. Eleven of the twenty five strains inoculated on *L. leucocephala* were effective or very effective, six of which were of the *R. tropici* group and four of *Sinorhizobium* spp. The most effective strain on *L. leucocephala* was GYB4-A7, a *Sinorhizobium*

strain, fixing about 13 mg N per plant, with a group average of 9.2 mg N per plant. The *R. tropici* group fixed an average of 8.5 mg N per plant. The most effective strain on *S. sesban* was the *R. tropici* strain GCT2, fixing about 12.2 mg N per plant. On the average, however, the *Mesorhizobium* spp. fixed 10.4 mg N per plant compared with 9.4 mg N per plant fixed by members of the *R. tropici* group.

Characterisation of rhizobia based on phenotype

The grouping of rhizobia using 40 phenotypic characters yielded 12 clusters (Figure 4 and Table 2). Clusters I, II, V, VIII, X and XII consisted of homogenous rhizobia types, while

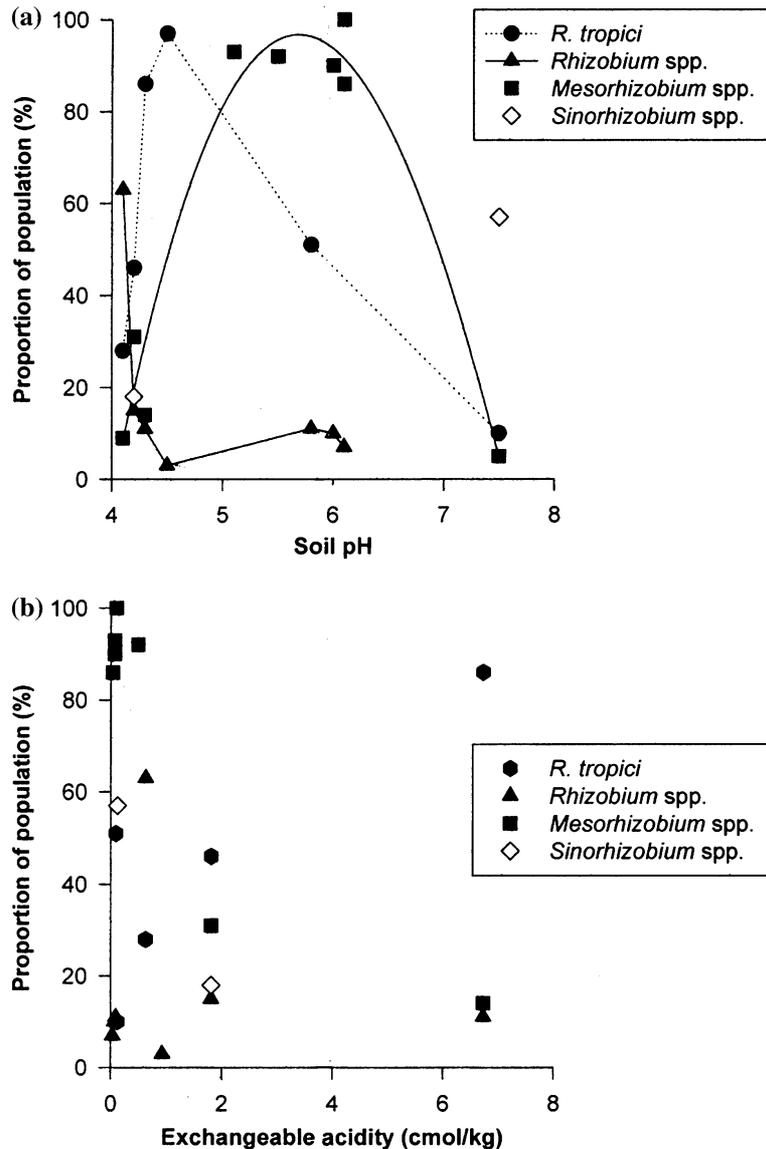


Figure 2. Relationships between the relative dominance of rhizobial types and (a) soil pH and (b) exchangeable acidity.

Clusters VI and VII were made up of single isolates. The four remaining clusters were comprised of mixtures of rhizobia sub-groups. Clusters I and II consisted of five and four isolates, respectively, which were closely related to *R. tropici*–*R. leguminosarum* lineage. Cluster III had 25 isolates, 22 of which were of the *R. tropici*–*R. leguminosarum* sub-types. Two other strains, including the type strain H152, were of the *R. giardinii* lineage, while the other was a reference strain for the *Sinorhizobium* branch. Cluster IV had five isolates of which two each were of the *R. tropici*–*R. leguminosarum*

and *R. mongolense*–*R. gallicum* branches, with the fifth isolate being of *Agrobacterium* lineage. All the five isolates in Cluster V and the six in Cluster VIII were of the *Mesorhizobium* branch. Clusters VI and VII had single isolates of *R. mongolense*–*R. gallicum* and *Mesorhizobium* lineages, respectively. Cluster IX was made up of *Mesorhizobium* and *Sinorhizobium* strains, while Clusters X and XII had *Mesorhizobium* and *Agrobacterium* types, respectively. Cluster XI was the most heterogeneous, consisting of five sub-groups of *Rhizobium* and *Sinorhizobium*. The *Rhizobium* spp. sub-group

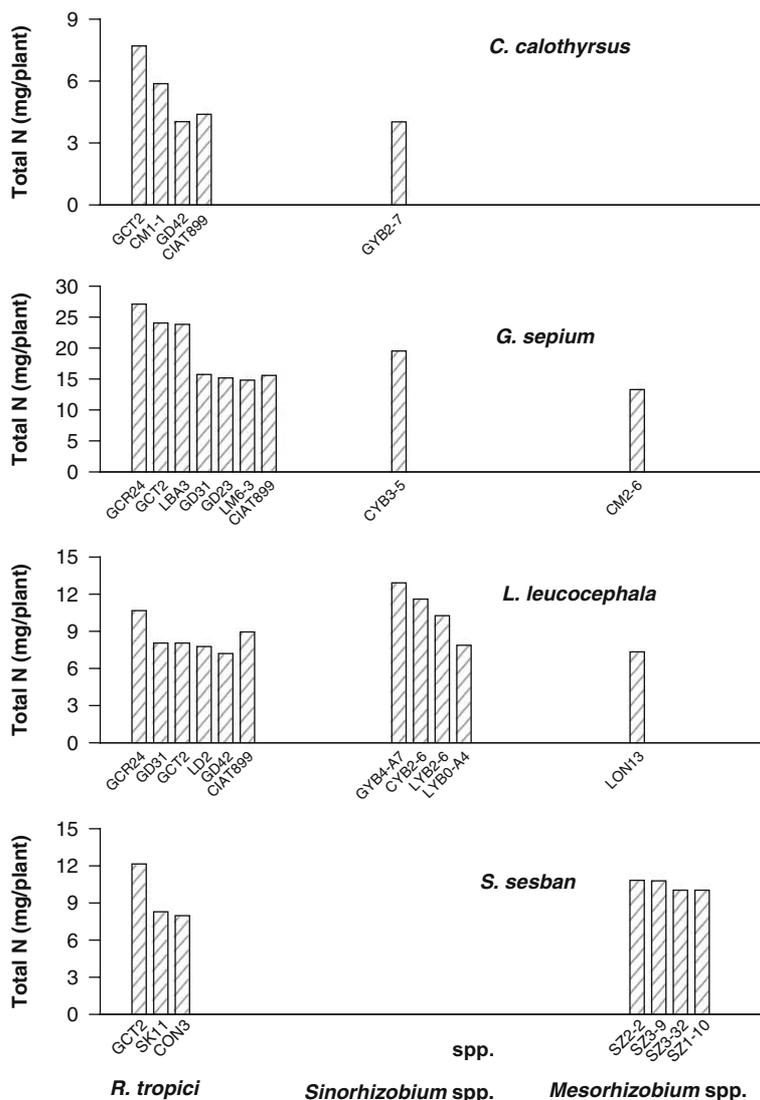


Figure 3. N_2 fixing effectiveness of different rhizobial types on host legumes.

in this cluster consisted of the reference strains for *M. loti*, *R. etli*, *R. gallicum* and *R. leguminosarum* bv. *phaseoli* and two isolates of the *R. tropici*–*R. leguminosarum* branch.

Seventeen carbon and one nitrogen compounds showed different degrees of discrimination in differentiating the various groups of rhizobia (Table 2). The *R. tropici*-type strains in the various clusters were differentiated by the ability, or otherwise, to grow on sucrose, dulcitol, maltose, PEG, cyclodextrin, tartarate, oxalate, acetate, starch and tyrosine. The *Mesorhizobium* sub-types differed in their utilisation of all the 17 substrates other than dulcitol, on which they all failed to

grow, and tartarate, which they all utilised. The differentiating compounds among the *Sinorhizobium* sub-groups were arabinose, fucose, PEG, tartarate, citrate, acetate and starch. The *Sinorhizobium* all failed to utilise dulcitol, cyclodextrin and oxalate. The two *R. mongolense*–*R. gallicum* sub-types in Clusters VI and XI differed in their ability to grow on sucrose, lactose, arabinose, PEG and starch. Both groups failed to utilise fucose, succinate, cyclodextrin, tartarate, oxalate and acetate. The *Agrobacterium*-like strains in Cluster XII were only able to utilise dulcitol, arabinose, fucose, succinate, maltose and tyrosine.

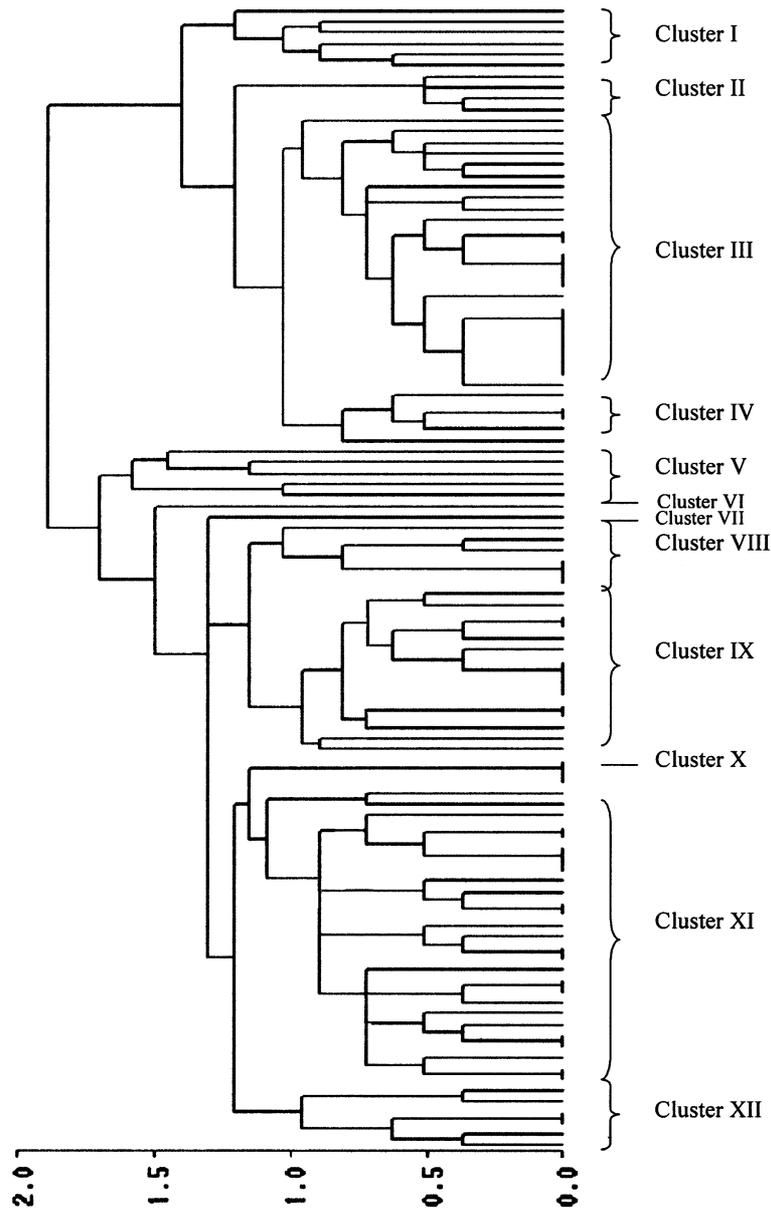


Figure 4. Differentiation into phenetic clusters of tree rhizobia isolated from thirteen soils across three continents in the tropics. The dendrogram was created from cluster analysis based on matrix and Euclidean distance using forty phenotypic traits.

Discussion

Legume nodulation in relation to diversity of rhizobia

Nodulation and N_2 -fixation by legumes occur only in the presence of compatible rhizobia. The complete dominance of the *R. tropici*-type rhizobia in

the Lampung soil suggests that the presence of just one compatible rhizobial type is sufficient for this function to be carried out. However, diversity in rhizobial types within a population could ensure the resilience of the population to environmental stress or disturbance as appears to be the case for other organisms (Giller et al. 1997). Therefore, the rhizobial population in the Yucatan soil, which

had greater rhizobial diversity, could be expected to be more resilient when faced with an environmental stress or disturbance.

Rhizobia are highly competent, heterotrophic organisms that can survive as large populations for decades in the absence of host legumes (Giller 2001), but the presence of a compatible host legume confers protection to the microsymbionts against environmental stresses (Andrade et al. 2002). On the other hand, a greater diversity of rhizobia in soil populations broadens the range of legume hosts that can be nodulated in such soils. There is thus a mutual benefit between above-ground (legume) and belowground (rhizobia) biodiversities.

There are indications that the rhizobia infecting legumes in areas outside the hosts' centre of diversity may be symbionts of local legumes, which can also infect the introduced species (Martínez-Romero and Caballero-Mellado 1996; Bala et al. 2003b). Thus promiscuity in host range appears to be the norm for tropical legumes and rhizobia (Giller 2001). This attribute seems to be driven by the huge diversity of rhizobial types in tropical soils. Contrary to the concept of a homogenous and promiscuous group of rhizobia of the 'cowpea miscellany' in tropical soils (Singleton et al. 1992), recent studies have shown that tropical rhizobia are diverse with sub-groups of varied symbiotic specificity and effectiveness (Thies et al. 1991; Mpepereki et al. 1996). This was further supported by our result, which showed rhizobia of the same phylogenetic grouping nodulating *C. calothyrsus*, *G. sepium* and *L. leucocephala* in some soils, but failing to nodulate at least one of the hosts in other soils (Figure 1), thus suggesting that rhizobial phylogeny and host range (infectiveness) are only weakly linked.

Symbiotic effectiveness in relation to rhizobial biodiversity

In spite of the relatively large numbers of rhizobial strains that nodulated host legumes and the high degree of genetic diversity amongst these strains (Bala et al. 2003b), only a small fraction was symbiotically effective on their hosts (Figure 3). Thus the relative permissiveness of the hosts may not guarantee effectiveness in N₂-fixation and may actually lead to the formation of ineffective nod-

ules when infected by some competitive strains, which are not highly evolved to fix N₂ with these legumes (Andrade et al. 2002). A balance is, therefore, necessary between the need for diversity of gene pools and the presence of effective microsymbionts for any given legume host.

The predominance of *R. tropici*-type rhizobia among the most effective strains on the host legumes was no surprise since *R. tropici* was reported as having a broad host range (Martínez-Romero et al. 1991) and the *R. tropici* strain CIAT899 is often used as an inoculant strain for these legumes. In spite of such dominance, other rhizobial types were at least as effective as the *R. tropici*-types in N₂-fixation. The fact that sinorhizobia, rather than the *R. tropici* types were the most effective on *L. leucocephala* appeared to be a reflection of the long-term co-existence of the sinorhizobia and the host in the Yucatan soil (originally from Mexico) from which the sinorhizobia strains were all isolated. Wang et al. (1999) found that *L. leucocephala* is predominantly nodulated by sinorhizobia in soils of Central Mexico. Thus environmental influences, rather than rhizobial phylogeny, may be more important in determining symbiotic effectiveness. It is pertinent that many of the isolates tested on the hosts were more effective than CIAT899, suggesting that strains with better performance than CIAT899 readily may be found.

Ecology of rhizobial types

There appeared to be an apparent effect of soil pH and exchangeable acidity on the relative dominance of rhizobial types. The dominance of *R. tropici*-type rhizobia at low pH agrees with previous reports that showed this species to be the best adapted to acidic conditions (Graham et al. 1994). Earlier studies of rhizobial populations from two Kenyan soils indicated a dominance of *R. tropici* strains in an acid soil with high aluminium-saturation, whereas *R. etli* strains were dominant in a soil with near-neutral pH (Anyango et al. 1995). *R. tropici* was also the most competitive for nodulation in acid soil conditions while *R. leguminosarum* competed better in alkaline soil (Anyango et al. 1998). In a recent study of rhizobial populations in Brazil, however, *R. leguminosarum* strains dominated in

Table 2. Differentiation of rhizobial genetic groups based on substrate utilisation.

	Glu ^a	Suc	Lac	Dul	D-Arab	Fuc	Sut	Mal	PEG	Man	Cyc	Cel	Tar	Oxa	Cit	Ace	Sta	Tyr
Cluster I																		
<i>R. tropici</i> (6) ^b	+ ^c	-	1	3	+	+	+	-	+	+	+	+	+	4	+	+	+	-
Cluster II																		
<i>R. tropici</i> (4)	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cluster III																		
<i>R. tropici</i> (25)	+	+	+	+	+	+	+	+	+	+	+	+	+	24	+	11	+	+
Cluster IV																		
<i>Rhizobium</i> spp. (5)	+	+	+	4	+	4	+	+	+	+	+	+	+	1	+	+	+	+
Cluster V																		
<i>Mesorhizobium</i> spp. (5)	-	-	1	-	-	-	2	-	-	3	-	-	+	-	-	1	-	2
Cluster VI																		
<i>R. mongolense</i> (1)	+	-	-	+	+	-	-	+	-	-	+	+	+	+	+	-	+	+
Cluster VII																		
<i>Mesorhizobium</i> spp. (1)	+	+	-	-	+	+	-	+	-	-	+	-	+	+	+	-	+	+
Cluster VIII																		
<i>Mesorhizobium</i> spp. (6)	+	+	-	-	+	+	+	+	-	+	1	+	+	-	-	+	-	4
Cluster IX																		
<i>Mesorhizobium</i> spp. (12)	+	+	-	-	7	+	+	+	+	+	+	+	+	8	+	+	10	9
<i>Sinorhizobium</i> spp. (3)	+	+	2	-	-	2	+	+	+	+	-	+	+	-	+	+	+	+
Cluster X																		
<i>Mesorhizobium</i> spp. (3)	+	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-	-
Cluster XI																		
<i>R. mongolense</i> (2)	+	+	+	+	-	-	-	+	+	+	-	+	-	-	+	-	+	+
<i>Rhizobium</i> spp. II (6)	+	+	+	+	+	+	+	+	-	+	-	+	3	-	+	-	-	+
<i>R. tropici</i> I (4)	+	+	+	-	+	+	+	+	+	+	+	+	+	-	-	-	2	+
<i>Sinorhizobium</i> spp. I (4)	+	+	3	-	+	-	+	+	-	+	-	+	-	-	-	-	-	+
<i>R. tropici</i> II (8)	+	+	+	4	+	+	+	+	-	+	-	+	-	-	+	+	-	+
<i>Sinorhizobium</i> spp. II (3)	+	+	+	-	-	+	+	+	-	+	-	+	-	-	-	+	-	+
Cluster XII																		
<i>Agrobacterium</i> spp. (6)	-	-	-	+	+	+	+	+	-	-	-	+	-	-	-	-	-	+

Number in parentheses represents the number of isolates for each rhizobial type.

^a Glu, Glucose; Suc, Sucrose; Lac, Lactose; Dul, Dulcitol; D-Arab, Arabinose (D-); Fuc, Fucose; Sut, Succinate; Mal, Maltose; PEG, Polyethylene glycol; Man, Mannose; Cyc, Cyclodextrin; Cel, Cellobiose; Tar, Tartarate; Oxa, Oxalate; Cit, Citrate; Ace, Acetate; Sta, Starch; Tyr, Tyrosine.

^b Genetic affiliation closest to *R. tropici* and *R. leguminosarum*.

^c +, all isolates were positive; -, all isolates failed to grow; Numbers are those of isolates that were positive.

the most acid soils with the highest aluminium saturation, whereas the abundance of *R. tropici* strains decreased with increasing aluminium stress (Andrade et al. 2002). Vargas and Graham (1989) also found no significant differences between the numbers of *R. tropici* strain CIAT899 and *R. leguminosarum* strain CIAT632 (supposedly acid-tolerant and acid-sensitive strains, respectively) in the rhizosphere of *Phaseolus vulgaris* at pH 4.5. These findings may explain the rather surprising result in our study, which showed that *Rhizobium* spp., consisting of close relatives of *R. etli*, and *R. giardinii*, were predominant over *R. tropici* types at soil pH below 4.2. Alternatively, the result could be attributed

to the large proportions of *R. etli* types in the Itabela soil, which was sampled from the Bahia region of Brazil. Although this soil had low pH, it also had relatively low exchange acidity, which could have enabled the *R. etli* types survive and dominate the population. *R. etli* is frequently adapted to acid soils, especially in South America where it forms effective symbiosis with several legumes including *Leucaena* species (Martínez-Romero et al. 1991).

The relative dominance of *Mesorhizobium* and *Sinorhizobium* species at intermediate and high pHs provides an interesting result that needs to be confirmed in further studies. The results, however, appear to be a reflection of the adaptation of these

rhizobia as most of the species in both genera were described using strains that originated from soils of intermediate to high pHs. The establishment of a relationship between rhizobial types and soil conditions, especially acidity, could have a significant impact on our ability to predict the adaptability of inoculant strains to specific soil types and conditions.

Phenotypic characterisation of rhizobia

The major phenotypic clusters formed were broadly consistent with genetic groupings based on 16S rRNA sequences, although variant strains were nested within some of the clusters. A comparison of substrate utilisation of strains provides some insight into the diversity of rhizobial types (Table 2). Reports show that *R. tropici* does not grow in dulcitol, in contrast to *R. leguminosarum*, *R. etli* and *R. gallicum* strains (Amarger et al. 1997). The ability of the two *R. mongolense*–*R. gallicum* sub-groups to grow on dulcitol was consistent with this result. However, only the *R. tropici* types in Cluster II and the *R. tropici* in Cluster XI failed to utilise dulcitol, indicating that the other *R. tropici*-like sub-groups may be *R. leguminosarum* or some other closely related strains. Among the *Mesorhizobium* species, *M. huakuii* does not use fucose (Jarvis et al. 1997); a similar characteristic was shown by the mesorhizobia in Clusters V and X. The phenotypic method of characterising rhizobia appears to have a potential to clearly differentiate rhizobia into homogenous groups; what is required is to use a particular number of differentiating characters that will achieve that objective. This, therefore, underlines the need to standardise the methodology and establish the set of characters required to unambiguously differentiate different rhizobial types.

Conclusions

Understanding the ecology of different rhizobial groups will further enhance our knowledge of rhizobia. This is especially poignant considering that the success of inoculation, particularly if inoculant strains are to establish in the soils, depends on the saprophytic competence of the

inoculant strain. Thus our ability to predict the environmental responses of rhizobial groups will bring a more practical meaning to rhizobial classification and diversity. The results of this study only showed a weak link between rhizobial phylogeny and function (in terms of nodulation and N₂-fixation). A better correlation appeared to exist between phylogeny and adaptation to soil acidity, although contrasting results from other studies tend to suggest that this link is tenuous. It would appear that diversity is more likely to be of major importance in stability of function by providing a diverse gene pool that imparts greater resilience to soil stresses. The broad correlation between rhizobial groupings based on chromosomal genes and those derived from phenotype suggests that a consistent and coherent rhizobial classification based on phenotypic characters may be possible with rigorous selection and evaluation of phenotypic characters. However, given the tenuous relationship between phylogeny on one hand, and nodulation and N₂-fixation on the other, it appears unlikely that the latter may eventually be part of the established set of phenotypes to be used for such a purpose. These results are largely empirical and will need confirmation through more research in this area.

References

- Amarger N., Macheret V. and Laguerre G. 1997. *Rhizobium gallicum* sp. nov. and *Rhizobium giardinii* sp. nov., from *Phaseolus vulgaris* nodules. Int. J. Syst. Bacteriol. 47: 996–1006.
- Anderson J.M. and Ingram J.S.I. 1993. Tropical Soil Biology and Fertility: A Handbook of Methods. CAB International, Wallingford, UK, 221pp.
- Andrade D.S., Murphy P.J. and Giller K.E. 2002. The diversity of *Phaseolus*-nodulating rhizobial populations is altered by liming of acid soils planted with *Phaseolus vulgaris* L. in Brazil. Appl. Environ. Microbiol. 68: 4025–4034.
- Anyango B., Wilson K.J., Beynon J.L. and Giller K.E. 1995. Diversity of rhizobia nodulating *Phaseolus vulgaris* L. in two Kenyan soils of contrasting pHs. Appl. Env. Microbiol. 61: 4016–4021.
- Anyango B., Wilson K.J. and Giller K.E. 1998. Competition in Kenyan soils between *Rhizobium leguminosarum* bv. *phaseoli* strain Kim5 and *R. tropici* strain CIAT899 using the *gusA* marker gene. Plant Soil 204: 69–78.
- Bala A. and Giller K.E. 2001. Symbiotic specificity of tropical tree rhizobia for host legumes. New Phytol. 149: 495–507.
- Bala A., Murphy P. and Giller K.E. 2001. Genetic diversity of rhizobia from natural populations varies with the soil dilution sampled. Soil Biol. Biochem. 33: 841–843.

- Bala A., Murphy P. and Giller K.E. 2002. Occurrence and genetic diversity of rhizobia nodulating *Sesbania sesban* in African soils. *Soil Biol. Biochem.* 34: 1759–1768.
- Bala A., Murphy P.J., Osunde A.O. and Giller K.E. 2003a. Nodulation of tree legumes and the ecology of their native rhizobial populations in tropical soils. *Appl. Soil Ecol.* 22: 211–223.
- Bala A., Murphy P.J. and Giller K.E. 2003b. Distribution and diversity of rhizobia nodulating agroforestry legumes in soils from three continents in the tropics. *Mol. Ecol.* 12: 917–930.
- Giller K.E. 2001. Nitrogen Fixation in Tropical Cropping Systems. 2nd edn. CAB International, Wallingford, UK, 423pp.
- Giller K.E., Beare M.H., Lavelle P., Izac A.-M.N. and Swift M.J. 1997. Agricultural intensification, soil biodiversity and ecosystem function. *Appl. Soil Ecol.* 6: 3–16.
- Graham P.H., Sadowsky M.J., Keyser H.H., Barnett M., Bradley R.S., Cooper J.E., De Ley D.J., Jarvis B.D.W., Roslycky E.B., Strijdom B.W. and Young J.P.W. 1991. Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. *Int. J. Syst. Bacteriol.* 41: 582–587.
- Graham P.H., Draeger K.J., Ferrey M.L., Conroy M.J., Hammer B.E., Martínez E., Aarons S.R. and Quinto C. 1994. Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. *Can. J. Microbiol.* 40: 198–207.
- Jarvis B.D.W., Van Berkum P., Chen W.X., Nour S.M., Fernandez M.P., Cleyet-Marel J.C. and Gillis M. 1997. Transfer of *Rhizobium loti*, *Rhizobium huakuii*, *Rhizobium ciceri*, *Rhizobium mediterraneum*, and *Rhizobium tianshanense* to *Mesorhizobium* gen. nov. *Int. J. Syst. Bacteriol.* 47: 895–898.
- Martinez-Romero E., Segovia L., Mercante F.M., Franco A.A., Graham P. and Pardo M.A. 1991. *Rhizobium tropici*: a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int. J. Syst. Bacteriol.* 41: 417–426.
- Martinez-Romero E. and Caballero-Mellado J. 1996. *Rhizobium* phylogenies and bacterial genetic diversity. *Crit. Rev. Plant Sci.* 15: 113–140.
- Mpeperek S., Wollum A.G. and Makonese F. 1996. Diversity in symbiotic specificity of cowpea rhizobia indigenous to Zimbabwean soils. *Plant Soil.* 186: 167–171.
- Mpeperek S., Makonese S. and Wollum A.G. 1997. Physiological characterization of indigenous rhizobia nodulating *Vigna unguiculata* in Zimbabwean soils. *Symbiosis* 22: 275–292.
- Odee D.W., Sutherland J.M., Makatiani E.T., McInroy S.G. and Sprent J.I. 1997. Phenotypic characteristics and composition of rhizobia associated with woody legumes growing in diverse Kenyan conditions. *Plant Soil* 188: 65–75.
- Raven P.H. and Polhill R.M. 1981. Biogeography of the Leguminosae. In: Polhill R.M. and Raven P.H. (eds), in *Advances in Legume Systematics, Part 6*. Royal Botanic Garden, Kew, England.
- Singleton P.W., Bohlool B.B. and Nakao P.L. 1992. Legume rhizobia inoculation in the tropics: myths and realities. In: *Myths and Science of Soils in the Tropics*. ASA, SSSA Special Publication No. 29, Madison, WI, USA, pp. 135–155.
- Sprent J.I. 1994. Evolution and diversity in the legume-rhizobium symbiosis: chaos theory? *Plant Soil* 161: 1–10.
- Sprent J.I. 1995. Legume trees and shrubs in the tropics: N₂ fixation in perspective. *Soil Biol. Biochem.* 27: 401–407.
- Thies J.E., Singleton P.W. and Bohlool B.B. 1991. Sub-groups of the cowpea miscellany: symbiotic specificity within *Bradyrhizobium* spp. for *Vigna unguiculata*, *Phaseolus lunatus*, *Arachis hypogaea*, and *Macroptilium atropurpureum*. *Appl. Environ. Microbiol.* 57: 1540–1545.
- Vargas A.A.T. and Graham P.H. 1989. Cultivar and pH effects on competition for nodule sites between isolates of *Rhizobium* in beans. *Plant Soil* 117: 195–200.
- Vincent J.M. 1970. *A Manual for the Practical Study of Root-nodule Bacteria*. Blackwell, Oxford, UK, pp. 164
- Wang E.T., Martinez-Romero J. and Martinez-Romero E. 1999. Genetic diversity of rhizobia from *Leucaena leucocephala* nodules in Mexican soils. *Mol. Ecol.* 8: 711–724.