

Subgroups of the Cowpea Miscellany: Symbiotic Specificity within *Bradyrhizobium* spp. for *Vigna unguiculata*, *Phaseolus lunatus*, *Arachis hypogaea*, and *Macroptilium atropurpureum*

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Rhizobia classified as *Bradyrhizobium* spp. comprise a highly heterogeneous group of bacteria that exhibit differential symbiotic characteristics on hosts in the cowpea miscellany cross-inoculation group. To delineate the degree of specificity exhibited by four legumes in the cowpea miscellany, we tested the symbiotic characteristics of indigenous cowpea bradyrhizobia on cowpea (*Vigna unguiculata*), siratro (*Macroptilium atropurpureum*), lima bean (*Phaseolus lunatus*), and peanut (*Arachis hypogaea*). The most-probable-number counts of indigenous bradyrhizobia at three sites on Maui, Hawaii, were substantially different on the four hosts: highest on siratro, intermediate on cowpea, and significantly lower on both lima bean and peanut. Bradyrhizobia from single cowpea nodules from the most-probable-number assays were inoculated onto the four hosts. Effectiveness patterns of these rhizobia on cowpea followed a normal distribution but were strikingly different on the other legumes. The effectiveness profiles on siratro and cowpea were similar but not identical. The indigenous cowpea-derived bradyrhizobia were of only moderate effectiveness on siratro and were in all cases lower than the inoculant-quality reference strain. Between 5 and 51 % of the bradyrhizobia, depending on site, failed to nodulate peanut, whereas 0 to 32 % failed to nodulate lima bean. No significant correlation was observed between the relative effectiveness of the bradyrhizobia on cowpea and their corresponding effectiveness on either lima bean or peanut. At all sites, bradyrhizobia that were ineffective on cowpea but that effectively nodulated lima bean, peanut, or both were found. Eighteen percent or fewer of the bradyrhizobia were as effective on lima bean as the reference inoculant strain; 44% or fewer were as effective on peanut as the reference strain. Only 18% of all cowpea-derived bradyrhizobia tested were able to form N-fixing nodules on both lima bean and peanut. These results indicate the need to measure indigenous bradyrhizobial population characteristics directly with the crop of interest to obtain an accurate assessment of the need to inoculate.

Rhizobial classification is based first and foremost on host specificity. In the genus *Bradyrhizobium*, the bacteria that nodulate soybean are classified as *Bradyrhizobium japonicum* and all others are assigned to the *Bradyrhizobium* spp., previously referred to as cowpea rhizobia or more loosely as tropical rhizobia. This has been the source of misconceptions about the promiscuity of tropical legumes and the ubiquity of tropical bradyrhizobia (10) and has led to false recommendations regarding the inoculation requirements of tropical legumes. For example, cowpea (*Vigna unguiculata*), lima bean (*Phaseolus lunatus*), peanut (*Arachis hypogaea*), and siratro (*Macroptilium atropurpureum*) are all thought to nodulate with the cowpea rhizobia, now classified as *Bradyrhizobium* spp. But individual bradyrhizobial isolates may not be equally infective or effective on these four host legumes. Observations of differential effectiveness led Burton (2) to separate these hosts into effectiveness groupings within the cowpea miscellany. Cowpea and siratro were included in one group, while lima bean and peanut composed separate groupings. Under this system, it is assumed that legume hosts within a given effectiveness grouping have similar bradyrhizobial requirements. While these divisions may be useful for making strain recommendations and formulating an inoculant, they fail to indicate the degree of

specificity on the part of these hosts or whether overlap exists between the various groups.

Size and effectiveness of indigenous rhizobial populations are primary factors that determine the incidence and magnitude of legume inoculation response (11, 14). Singleton and Tavares (11) isolated indigenous bradyrhizobia from cowpea, lima bean, and peanut growing in four Hawaiian soil samples and tested the effectiveness of the bradyrhizobia on the legumes from which they were isolated. They found that, within a soil sample, the range of effectiveness of indigenous rhizobial isolates for these hosts differed. Their results indicated that considerable diversity in the relative effectiveness of indigenous *Bradyrhizobium* spp. populations on these legumes exists. Such population diversity is also reflected in measured differences in the sizes of indigenous bradyrhizobial populations capable of nodulating these legumes (11, 14), differences in the ability of bradyrhizobia to compete with inoculant strains for nodulation of the different hosts (14), and differences in the incidence and magnitude of inoculation responses obtained on these hosts in the same soil samples (11, 14).

In their studies, Singleton and Tavares (11) did not characterize the effectiveness of indigenous bradyrhizobial isolates from any one of the hosts on the others. Hence, the nature of observed differences in the range of effectiveness of these isolates could not be determined. In this study, we examined the nature of these differences by assessing the degrees of specificity, in terms of both nodulation and effectiveness, exhibited by siratro, lima bean, and peanut

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TABLE 1. Location and characteristics of three sites on the island of Maui, Hawaii^a

Site no. and name	Elevation (m)	Soil subgroup ^c	Median annual rainfall ^b (mm/year)	pH	Legume genera present at site
1, Hashimoto Farm	37	Torroxic	322	6.8	<i>Leucaena</i> , <i>Prosopis</i>
2, Kula Agricultural Park	366	haplustoll	375	7.5	<i>Leucaena</i> , <i>Indigofera</i> , <i>Macroptilium</i> , <i>Prosopis</i>
3, Haleakala Station	660	Humoxic tropohumult	1,800	5.3	<i>Desmodium</i> , <i>Trifolium</i> , <i>Acacia</i> , <i>Crotalaria</i>

^a For more detailed descriptions of these sites, see reference 18.^b From reference 10.^c From reference 3.

when inoculated with bradyrhizobia from cowpea nodules. Our results further substantiate division of the cowpea miscellany into effectiveness subgroups, particularly as they relate to inoculation response, and illustrate that measurement of indigenous *Bradyrhizobium* spp. population characteristics should be made directly with the host of interest to obtain an accurate assessment of the symbiotic capability of the population.

MATERIALS AND METHODS

Soil sampling and enumeration of indigenous *Bradyrhizobium* spp. populations. Soil samples were collected from fallow areas at three field sites on the island of Maui, Hawaii (Table 1). After the top 1 cm of soil was removed, 20 to 25 2.54-cm-diameter soil cores to a depth of 25 cm were taken in a grid pattern around each field area. Soil cores were pooled, mixed, subsampled for determination of moisture content, and stored at 4°C. The most-probable-number (MPN) of indigenous bradyrhizobia in each soil sample was determined (for four test hosts: *V. unguiculata* cv. Knuckle Purplehull, *P. lunatus* cv. Henderson's Baby, *A. hypogaea* cv. Burpee Florunner, and *M. atropurpureum* cv. Siratro. Serial 1:2, 1:5, or 1:10 soil dilutions were prepared and inoculated onto four replicate test plants per dilution growing in either plastic growth pouches (cowpea, lima bean, and peanut) or test tubes (siratro) (13). Plants in pouches were kept supplied with an adequate volume of N-free nutrient solution (9), except that micronutrients were supplied by adding a commercial micronutrient mix (0.25 ml liter⁻¹; Hawaiian Horticultural). Plants were scored for nodulation 21 to 28 days after inoculation. The MPN counts were determined by using the Most-Probable-Number Enumeration System (17).

Assay for the effectiveness of indigenous *Bradyrhizobium* spp. A representative sample of nodules was taken from each of the MPN assays performed on cowpea. Nodules were selected from all dilutions when present. Three or more replicate nodules per test host formed by inoculant-quality reference strains TAL 644 (CIAT 257) and TAL 658 (CIAT 71) were used as positive controls. Nodules were surface sterilized by immersion in 70% ethanol for 1 min, which was followed by several rinses in sterile water. Individual nodules were crushed in 0.1 ml of yeast extract-mannitol broth (YMB) (16), nodule remnants were removed, and 4 ml of YMB was added. After 2 days of incubation at room temperature, 1 ml of YMB containing bradyrhizobia from individual cowpea nodules was inoculated onto one plant each of cowpea, lima bean, peanut, and siratro growing in plastic growth pouches. No fewer than seven uninoculated and four uninoculated control plants were maintained for

each test host. Nodulation characteristics were recorded, and leaf chlorophyll content (chlorophyll *a* plus chlorophyll *b*) on six leaf disks (diameter, 0.635 cm) per plant (7), taken from the most recently fully expanded trifoliate leaf 32 days after inoculation for cowpea and lima bean and 41 DAI for peanut and siratro, was determined.

Data analysis. Effectiveness of individual nodule bradyrhizobia in symbiosis with the test hosts was divided into four categories: highly effective, effective, moderately effective, and ineffective. The bradyrhizobia were considered ineffective if the chlorophyll content of host plant leaf disks was within the 95% confidence interval for the chlorophyll content of uninoculated (nonnodulated) control plants; moderately effective if host leaf disk chlorophyll content was higher than the upper confidence limit for uninoculated control plants but less than the lower confidence limit for chlorophyll content of plants nodulated by the reference strains TAL 658 for cowpea, peanut, and siratro and TAL 644 for lima bean; effective if leaf disk chlorophyll content was within the 95% confidence interval; and highly effective if leaf chlorophyll content was higher than the upper confidence limit for chlorophyll content of plants inoculated with the known reference strains listed above. Kendall tau b rank correlation analysis (8) of host leaf chlorophyll content was used to assess the degree of relatedness between the effectiveness of nodule occupants on cowpea and their corresponding effectiveness on siratro, peanut, and lima bean.

RESULTS

At all sites, MPN counts of indigenous *Bradyrhizobium* spp. were highest on siratro (Table 2). Counts of indigenous bradyrhizobia were 2.4- to 18.6-fold lower when cowpea was used as the trap host. Population counts on peanut and lima bean were from 6- to more than 1,000-fold lower than those obtained on siratro and from 2.3- to 62-fold lower than those obtained on cowpea.

TABLE 2. MPN counts^a of indigenous, homologous rhizobia for various legumes at three sites on Maui, Hawaii

Host legume	Log ₁₀ rhizobia g of soil ^{-1b}		
	Site 1	Site 2	Site 3
<i>M. atropurpureum</i>	1.70 a	1.64 a	5.56 a
<i>V. unguiculata</i>	1.08 b	1.26 a,b	4.29 b
<i>A. hypogaea</i>	0.72 b	0.87 b	2.49 c
<i>P. lunatus</i>	-0.57 c	0.68 b	2.95 c

^a Calculated by the Most-Probable-Number Enumeration System (17).^b Numbers within each column followed by the same letter are not significantly different at *P* < 0.05, on the basis of overlap of their 95% confidence intervals.

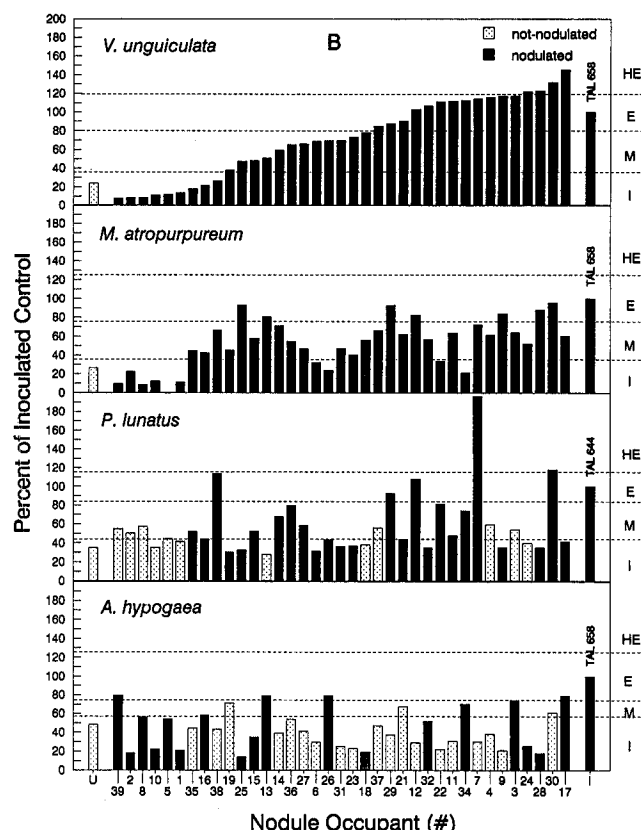
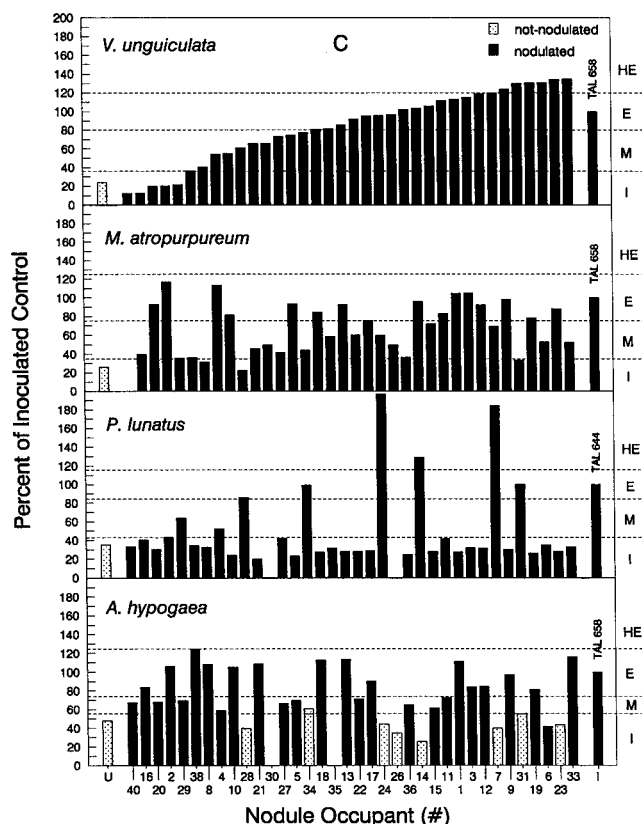
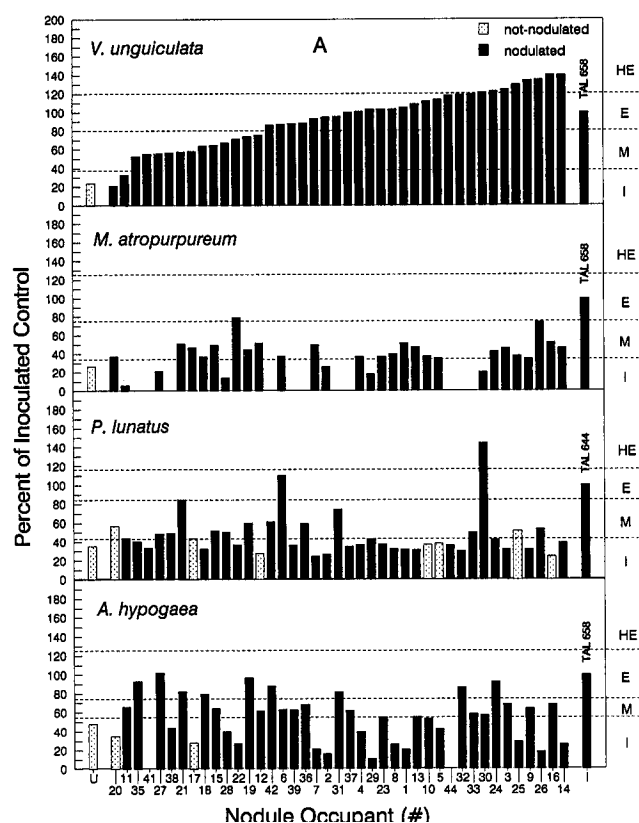


FIG. 1. Nodulation patterns and effectiveness profiles of indigenous cowpea rhizobia from site 1 (A), site 2 (B), and site 3 (C) on cowpea, siratro, lima bean, and peanut. Chlorophyll content of control plants inoculated with known strains was set at 100%. Chlorophyll content of plants inoculated with indigenous rhizobia is expressed as a percentage of the control. Areas between dotted lines represent the confidence intervals for ineffective (I), moderately effective (ME), effective (E), and highly effective

All bradyrhizobia from cowpea nodules used in the effectiveness test nodulated both cowpea and siratro. When tested on cowpea, the effectiveness of cowpea-derived nodule bradyrhizobia from the three sites was roughly normally distributed, with approximately two-thirds or more of the bradyrhizobia forming moderately effective to effective symbioses, and the remainder of the bacteria were divided between forming highly effective or ineffective symbioses (Fig. 1A to C). Two of the distributions were slightly skewed; a greater proportion of the bradyrhizobia from site 1 (Fig. 1A) formed effective to highly effective symbioses on cowpea compared with those at the other sites, whereas site 2 (Fig. 1B) yielded a larger proportion of ineffective bradyrhizobia. Across sites, 43% or more of the bradyrhizobia tested on cowpea were as effective as, or more effective than, the reference strain, TAL 658, and at least 76% formed symbioses of moderate effectiveness or better. At all sites, bradyrhizobia that were more effective on cowpea than the reference strain were present. Effectiveness and infectiveness profiles of these bradyrhizobia were significantly different on the other legumes.

Whereas all of the cowpea-derived bradyrhizobia were able to nodulate siratro, 45 to 77% of the rhizobia across sites formed symbioses of only moderate effectiveness. The

TABLE 3. Kendall tau b correlation coefficients^a between chlorophyll content of various legumes inoculated with the same rhizobia from three sites on Maui, Hawaii

Legume species	Correlation								
	<i>P. lunatus</i>			<i>A. hypogaea</i>			<i>M. atropurpureum</i>		
	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
<i>V. unguiculata</i>	-0.16	0.09	-0.02	-0.16	-0.04	-0.11	0.12	0.38***	0.15
<i>M. atropurpureum</i>	-0.16	0.13	-0.18	-0.10	-0.09	0.11			
<i>A. hypogaea</i>	0.15	0.07	-0.36**						

^a Significance of correlation coefficients: **, $P < 0.01$; ***, $P < 0.001$.

largest percentage of bradyrhizobia forming ineffective nodules on siratro was observed at site 2 (Fig. 1B), whereas the fewest ineffective and largest proportion of effective bradyrhizobia were obtained from site 3 soil (Fig. 1C). A highly significant positive correlation between the effectiveness of the indigenous bradyrhizobial population on cowpea at site 2 and their corresponding effectiveness on siratro (Table 3) was obtained. In general, the best agreement between effectiveness profiles on the four legumes was obtained between cowpea and siratro.

On lima bean, 56 to 70% of the bradyrhizobia either failed to nodulate or formed ineffective nodules, 12 to 32% formed symbioses of moderate effectiveness, and only 7 to 18% were as effective as the reference strain, TAL 644 (Fig. 1A to C). With peanut, 30 to 81% failed to nodulate or formed ineffective nodules, 5 to 31% were moderately effective, and 14 to 44% were as effective as the reference strain, TAL 658. Those bradyrhizobia forming effective symbioses on lima bean and peanut did not necessarily coincide with those forming effective symbioses on cowpea and coincided with each other only infrequently (Fig. 1A to C). Overall effectiveness of indigenous bradyrhizobia on cowpea was not significantly correlated with their effectiveness on either peanut or lima bean at any of the sites (Table 3). In all but one instance inverse relationships were obtained. The inability of some bradyrhizobia to nodulate either lima bean or peanut was not related to their effectiveness on cowpea (Fig. 1A to C).

The widest divergence in effectiveness profiles was observed between lima bean and peanut. Of the bradyrhizobia exhibiting moderate effectiveness or better on lima bean, 25, 81, and 60% from site 1, 2, and 3 soil samples, respectively, were ineffective or failed to nodulate peanut (Fig. 1A to C). Of those that were ineffective or failed to nodulate lima bean, 39, 19, and 88% from site 1, 2, and 3 soils, respectively, were effective on peanut. A highly significant inverse correlation between effectiveness of the bradyrhizobia from site 3 on lima bean and their corresponding effectiveness on peanut (Table 3) was observed. No correlation for the other sites between effectiveness on these two hosts was observed. In general, these legumes shared in common a much larger proportion of bradyrhizobia with cowpea and siratro than with each other.

DISCUSSION

Division of the cowpea miscellany into effectiveness groupings has been proposed previously (2). Yet, little is known about the comparative specificities of members in these different groupings and how the groupings might affect evaluation of the symbiotic capacity of indigenous bradyrhizobial populations. We investigated the degree of compatibility of indigenous cowpea *Bradyrhizobium spp.*

when in symbiosis with cowpea, lima bean, peanut, and siratro in order to interpret large differences in MPN counts obtained for these species from the same soil samples and to account for differential inoculation responses observed with these hosts at the same sites (11, 14).

Siratro is considered one of the more promiscuous hosts in the cowpea miscellany (5, 16) and has recently been shown to nodulate with some fast-growing rhizobial isolates (6). The consistently higher population counts we obtained on this species compared with the others (Table 2) support this conclusion. However, the frequency of effectiveness was much lower on this species compared with cowpea. This indicates that the bradyrhizobial requirement for these two hosts may differ more than previously suggested (2).

Lima bean was the most specific of the hosts examined, with greater than one-half of the bradyrhizobia at each site either failing to nodulate or forming ineffective symbioses. Peanut displayed a similar, though somewhat lower, degree of specificity. These results demonstrate that screening of strains for effectiveness should be performed with the host of interest, since results are clearly not transferable from host to host within the cowpea miscellany.

The large variation in numbers of indigenous rhizobia counted on the four legumes could be due to the differential detection limits of the plant infection assay with different host legumes. However, in a separate study (data not shown), we obtained satisfactory agreement between population counts of liquid pure cultures of rhizobia on petri plates and those obtained in plant infection assays with these hosts. Therefore, the observed variability in population counts on the four legumes is more likely due to the differential specificities of these hosts. The observation that many of the cowpea-derived bradyrhizobia tested failed to nodulate peanut and lima bean supports this view. Size of the indigenous, homologous rhizobial population is one of the primary determinants of legume inoculation response (11, 14, 15). The large differences in MPN population counts obtained for the four legumes indicate that use of the appropriate legume host in the MPN procedure may be critical for the accurate prediction of the impact of the use of legume inoculants.

Analysis of the extent to which effective indigenous bradyrhizobia were shared by cowpea, lima bean, and peanut (Fig. 2) reveals that the highest frequency of effectiveness was always observed on cowpea. Since all bradyrhizobia originated from nodules formed on cowpea, this is not surprising. Bradyrhizobia effective on peanut and lima bean appear to occupy subgroups that are not completely distinct from each other but are mostly within the group effective on cowpea. At all sites, a small percentage of bradyrhizobia effectively nodulated peanut, lima bean, or both species but were ineffective on cowpea. Hence, while

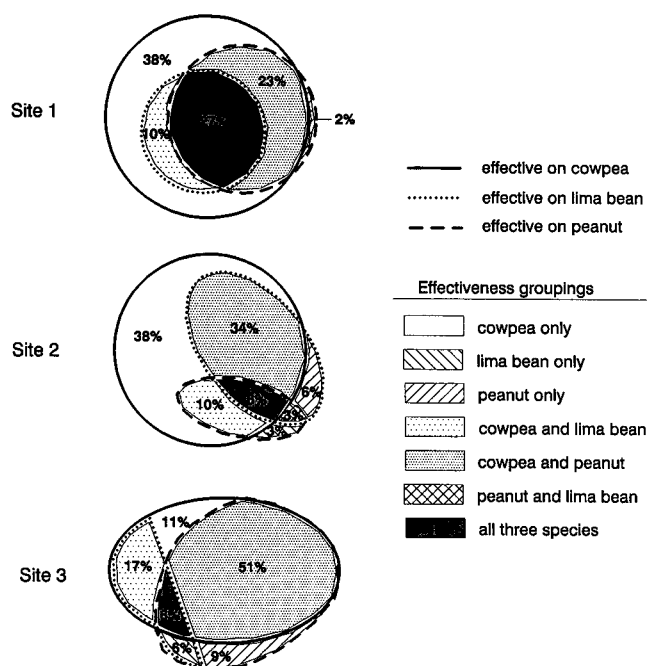


FIG. 2. Degree of relatedness of cowpea rhizobia: extent to which indigenous cowpea rhizobia of moderate effectiveness or better were shared by cowpea, lima bean, and peanut. Numbers are percentages of the total that effectively nodulate the legume indicated.

the subgroups overlap considerably, in terms of effectiveness, none was completely contained within any of the others. A close relationship was observed between the effectiveness of bradyrhizobia on lima bean and their ability to nodulate peanut, since 79% of those effective on lima bean failed to nodulate peanut. This again indicates a very high degree of specificity on the part of these two hosts and strongly supports their separation into different effectiveness groupings.

Lima bean has been shown previously to exhibit a high degree of specificity compared with other tropical legumes (1). In their cross-inoculation studies, Allen and Allen (1) showed that bradyrhizobia isolated from nodules on lima bean grown in tropical soil either failed to nodulate or formed ineffective symbioses on all of 28 tropical host legumes examined, including *Vigna sinensis* (now *V. unguiculata*), the legume host used for reference. In our study a large proportion of the bradyrhizobia tested were able to nodulate lima bean and all soil samples contained bradyrhizobia that were effective on both lima bean and cowpea, indicating that lima bean is not as exclusive as Allen and Allen (1) found it to be. Less agreement in effectiveness profiles and more clearly separated subgroups might have been observed, however, if nodules from lima bean or peanut had been used as a source of rhizobia.

Doku (4) used bradyrhizobia from a number of crushed nodules formed on lima bean, peanut, soybean, bambara groundnut, and cowpea to examine the cross-infection patterns between these hosts. He found that crushed-nodule mixtures from either cowpea or lima bean that contained effective bradyrhizobia for these two hosts failed to nodulate peanut. He also reported that lima bean nodulated freely with effective bradyrhizobia from peanut, soybean, cowpea, and bambara groundnut nodules. We used bradyrhizobia

from single nodules as inoculants in this study and found that lima bean was considerably more specific and peanut was less specific than he reported.

Effectiveness patterns within each population differed markedly among the three sites and were likely dependent on the types of indigenous legumes present at each site (Table 1), which, in part, may dictate the types of rhizobia and bradyrhizobia present (18). These patterns did not appear to be related to population size at a particular site.

In this study, effectiveness of occupants of single nodules formed by indigenous bradyrhizobia was assessed by using a chlorophyll assay (7). The results were used to develop an effectiveness index for each site by which indigenous bradyrhizobial populations for a particular legume species could be compared between sites. For example, the bradyrhizobial population estimates (by MPN) for cowpea (Table 2) can be multiplied by the percentage of each effectiveness grouping at a site to yield estimates of the size of the effective portion of the cowpea population at each site. This effectiveness index (the adjusted population size estimate) could then be used to calculate more precisely the potential yield response of legumes to inoculation by using the response models proposed by Thies et al. (15). Similar percentages and estimates of effective indigenous population size can be generated for other host legumes in the cowpea miscellany by testing in MPN assays the effectiveness of nodules formed on the host of interest.

The fact that a few nonnodulated lima bean and peanut plants had chlorophyll contents that placed them in the moderately effective grouping points out a limitation in the use of this assay. This result indicates that division between ineffective and moderately (or weakly) effective rhizobia may not be clear-cut when large-seeded legumes growing in growth pouches are used.

In summary, we have presented evidence supporting Burton's (2) separation of members of the cowpea miscellany into different effectiveness groupings. In this regard, cowpea and siratro had the most similar profiles in terms of both invasiveness and effectiveness but did not show complete homology. Lima bean and peanut showed greater specificity for both effectiveness and nodulation than either cowpea or siratro but did share some bradyrhizobia in common with these hosts. Peanut and lima bean shared in common only one nodule occupant that was as effective on both species as their respective reference strains. This demonstrates that a relatively clear division into separate effectiveness subgroups can be made between these two species but that separations from other groupings are not as clear-cut as Burton (2) suggests. However, from the standpoint of response to inoculation and determining the need to inoculate, all of these hosts could be considered to occupy effectiveness groups separate from each other. A significant proportion of the bradyrhizobia failed to form nodules on peanut and lima bean. These results explain large differences in indigenous bradyrhizobial population counts obtained from the same soil samples and from legumes which are currently classified in the same cross-inoculation group.

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