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Competition Among *Rhizobium* spp. for Nodulation of *Leucaena leucocephala* in Two Tropical Soils†

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The successful nodulation of legumes by a *Rhizobium* strain is determined by the competitive ability of that strain against the mixture of other native and inoculant rhizobia. Competition among six *Leucaena* rhizobial strains in single and multistrain inoculants were studied. Field inoculation trials were conducted in an oxisol and a mollisol soil, both of which contained indigenous *Leucaena*-nodulating rhizobia. Strain-specific fluorescent antibodies were used for the identification of the strains in *Leucaena* nodules. Mixtures of three recommended inoculum strains for *Leucaena* spp. (TAL82, TAL582, and TAL1145) were used in peat-based inocula either alone or with one of the three other strains isolated from the sites, B213, B214, and B215. Each of these latter three strains was also used as single-strain inocula to study their competition with the native rhizobia in the two soil systems. In the oxisol soil, strains B213 and B215, when used as single-strain inocula, outcompeted the native rhizobia and formed 92 and 62% of the nodules, respectively. Strain B214 was the least competitive in oxisol soil, where it formed 30% of the nodules, and the best in mollisol soil, where it formed 70% of the nodules. The most successful competitor for nodulation in multistrain inocula was strain TAL1145, which outcompeted native and other inoculum *Leucaena* rhizobia in both soils. None of the strains in single or multistrain inoculants was capable of completely overcoming the resident rhizobia, which formed 4 to 70% of the total nodules in oxisol soil and 12 to 72% in mollisol soil. No strong relationship was detected between the size of the rhizosphere population of a strain and its successful occupation of nodules.

Numerous studies on competition among *Rhizobium* strains for nodulation of their legume hosts have emphasized that it is a major practical limitation to the establishment of superior nitrogen-fixing inoculant strains in the nodules (1, 5, 6, 10, 19, 24). In the past, the nitrogen fixation capacity of *Rhizobium* spp. has been used as the most important criterion in selection of strains for inoculum production. For the desired inoculum strain to have an impact on crop yield, however, it must be able to survive in the soil and compete with resident strains for nodulation of the host. Inoculum strains superior in nitrogen fixation have been shown to fail to compete successfully with indigenous rhizobia (5, 6, 10, 24). Poor competitiveness can sometimes be overcome by applying high numbers of the inoculum strain (5, 24), but the high numbers that are needed to overcome the resident strains are difficult to achieve for practical legume inoculation. It is therefore important to identify highly effective strains that are also competitive in situ.

The nitrogen-fixing tree legume *Leucaena leucocephala* has been identified as one of the most promising of all tropical trees (7, 14). This legume can grow successfully in a wide range of environments with large differences in salinity, rainfall, sunlight, and land terrain. It offers a wide assortment of uses, including high-quality forage, firewood, timber, and green manure (14). The main traits that make this plant attractive from a practical point of view are its ability to grow rapidly under a variety of environmental extremes and its highly efficient N_2-fixing capacity when the appropriate rhizobia are present. Although many studies have addressed the botanical, agronomical, and economical aspects of this important tree legume, only a few studies have dealt with its N_2-fixing microsymbionts, the *Rhizobium* spp. (9, 15, 22, 23).

The objective of this investigation was to evaluate the competitive ability of different strains of *Leucaena* rhizobia as single and multistrain inoculants in two different tropical soils.

MATERIALS AND METHODS

Rhizobia. *Rhizobium* strains used in these experiments were recommended by the Nitrogen Fixation by Tropical Agricultural Legumes (NifTAL) project for field trials (TAL82, TAL582, TAL1145), and four other strains, B213, B214, B215, and B217, were isolated from the sites. All strains were effective on *L. leucocephala*, and their nitrogenase activities (C_2H_2 reduction) on their hosts were not significantly different (data not shown). Strain B213 was isolated from *L. leucocephala* nodules growing in Hawaiian oxisol soil from the site used in the field plots (Poamoho Experiment Station, University of Hawaii, Honolulu). Strains B214, B215, and B217 were isolated from *L. lanceolate*, *L. collinsii*, and *L. leucocephala* nodules, respectively, growing in Hawaiian mollisol soil (Waimanalo Experiment Station, University of Hawaii, Honolulu). All strains were maintained on yeast-extract mannitol agar containing the following (concentrations in grams per liter): mannitol (10), yeast extract (Difco Laboratories) (1.0), K_2HPO_4 (0.5), $MgSO_4 \cdot 7H_2O$ (0.2), NaCl (0.1), and agar (15) with the pH adjusted to 7.0 to 7.2 with 0.1 N NaOH.

Seeds. *L. leucocephala* var. K8 was obtained from the *Leucaena* germ plasm through the courtesy of J. Brewbaker, Nitrogen Fixing Tree Association, University of Hawaii, Honolulu.

Inoculum preparation. Inocula were prepared from 1- to 2-day-old cultures of *Leucaena* rhizobia grown in yeast extract-mannitol. Each strain was grown separately. The den-

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TABLE 1. Competition between single-strain inocula and indigenous rhizobia for nodulation of *L. leucocephala* planted in oxisol and mollisol soils

Soil type and strain in inoculum	Nodule occupancy (%) of[a]:							
	Inoculum strain						Indigenous strain	
	B213		B214		B215		B217 (T)	Other (T)
	S	D	S	D	S	D		
Oxisol								
B213	58	4					0	38
B214			26	4			0	70
B215					90	2	0	8
Mollisol								
B213	36	0					32	42
B214			70	0			10	20
B215					54	1	16	29

[a] Nodule occupancy is expressed as percentage of the total number of nodules tested. S, Nodules containing only the designated strain; D, nodules containing the designated strain plus either one of the other inoculum strains or an indigenous strain; T, S + D. Figures include doubly infected nodules, which accounts for sums greater than 100%.

sity of the cells was determined with a Petroff-Hausser counting chamber. Equal numbers of cells were used in the suspensions to formulate the mixtures of inoculum strains. Forty-milliliter amounts of the suspensions were injected into polyethylene bags each containing 50 g of gamma-irradiated, dry-milled, neutralized peat. The bags were kept for 2 days before they were used for inoculation of seeds. Membrane filter count with fluorescent antibody (FA) was done for all the strains to ensure the presence of all the strains in the peat in high numbers (10^9 cells per g).

To 50 g of seeds (ca. 15 seeds per g) in polyethylene bags was added 2.0 ml of 40% gum arabic solution (wt/vol in water). The bags were swirled to coat all the seeds thoroughly with the gum. The peat-based inoculum (10 g) with the desired strain mixture was added to the seeds. The seeds were swirled until they became coated with a layer of the peat and appeared uniformly black. Precipitated calcium carbonate (5 g) was added to each bag, and seeds were shaken gently to coat the inoculated seeds with $CaCO_3$. The seeds were placed on absorbent paper to dry and planted on the same day.

Field experiments. The experiments were carried out in two locations. One experiment was done in an oxisol soil (Tropeptic Eutrustox) at the Poamoho Experiment Station, University of Hawaii, Wahiawa, Oahu, the site of a previous study (12), and the other experiment was done in a mollisol soil (Vertic Haplustol) at the Waimanalo Experiment Station, Waimanalo, Oahu, Hawaii. Ten rows spaced equally apart were arranged in each of two adjacent plots (7 by 7 m). Precoated seeds of *L. leucocephala* var. K8 were planted in the rows 30 cm apart. Uninoculated seeds were planted as controls. The three strains used for single-strain inocula were B213, B214, and B215. The following five multistrain inoculum treatments were used: (i) NifTAL-recommended *Leucaena* rhizobia strains TAL82, TAL582, and TAL1145; (ii) a mixture of the TAL strains plus B213; (iii) a mixture of the TAL strains plus B214; (iv) a mixture of the TAL strains plus B215; and (v) B213, B214, and B215. A randomized complete block design was used and each treatment was replicated once. The experiment in the oxisol soil was irrigated several times owing to the scarcity of rain, but the other experiment received water only from rain.

Immunofluorescence. Strain-specific antibodies were made against the somatic antigens of the *Leucaena* rhizobia by the method of Schmidt et al. (20). Globulin separation and FA preparations were prepared as described previously by Belser and Schmidt (2). FAs were diluted in glycerol (1:1) and stored at −20°C. Before use, the FAs were diluted in saline (0.85% NaCl) to the highest dilution that still gave maximum brightness with their homologous strains. All the strains were highly specific, as indicated by the lack of cross-reactions with each other or with any of the 78 isolates of *Leucaena* rhizobia in our collection (data not shown).

Nodule sampling was done 10 weeks after seeding. Five plants were removed from each treatment in each plot. Care was taken to harvest the roots without losing nodules. The roots were shaken to remove soil. Five nodules from each plant from each replicate plot were selected at random. Each treatment was represented by 50 nodules. Nodules were cleaned by shaking for 10 min in 10 ml of water containing 0.1 ml of Tween 80 and then rinsed with water and individually crushed in 1 ml of water. Duplicate smears from each nodule were stained (20) with the appropriate FAs, and gelatin-rhodamine isothiocyanate conjugate was used to suppress nonspecific adsorption of FA (4). Several sets of the same nodule smears were prepared for staining with the different FAs.

Rhizosphere populations of the inoculum strains were estimated by a modification of the procedure of Kingsley and Bohlool (11): at designated times, roots were carefully removed from the soil and the loosely adhering soil was gently shaken off the roots. Two plants from each replicate were analyzed separately. All nodules were removed, and each root was placed in a screw-capped bottle containing 100 ml of partially hydrolyzed gelatin (11) and shaken for 30 min on a wrist-action shaker. The soil suspension was centrifuged gently (700 × *g*, 5 min), and 1-ml aliquots were filtered through Irgalan black-treated membrane filters (Nuclepore Corp.). Immunofluorescence counts were determined as described previously (11). The rest of the soil suspension was dried down to determine the dry weight of rhizosphere soil. The mean amounts of soil (± standard deviation) adhering to the roots from harvests 1 through 4 were 196 ± 32, 221 ± 75, 337 ± 67, and 302 ± 44 mg of dry soil per root, respectively. The mean recovery (± standard deviation) of strains from the soil (11) was 67 ± 11%.

RESULTS

The results of competition of a single strain with the native *Leucaena* rhizobia in two soil types are shown in Table 1. The results show that the competitive abilities of the inoculum strains varied in the two soils. Strain B215 was the best competitor in oxisol soil, where it outcompeted most of the native rhizobia and formed 92% of the nodules. The same strain occupied 60% of the nodules on plants growing in mollisol soil. The least competitive strain in oxisol soil, B214, proved to be the most competitive against the native rhizobia of mollisol soil (70% of the nodules). Strain B213, which was originally isolated from oxisol soil, performed better in oxisol than in mollisol soil, giving 62 and 36% of the nodules in the two soils, respectively. None of the three strains could completely overwhelm the indigenous *Leucaena* rhizobia, which formed 8 to 70% of the nodules, depending on the strain used in the inoculum. In mollisol soil, one of the native strains was isolated from nodules of control plots. The serological characterization proved that this strain, B217, represents a distinct serogroup of *Leucaena* rhizobia. This resident strain B217 played an impor-

TABLE 2. Competition among *Leucaena* rhizobial serogroups of multistrain inoculants in oxisol soil

Strains in inoculum	Strain in nodule (%)[a]												
	TAL82		TAL582		TAL1145		B213		B214		B215		Indigenous (T)
	S	D	S	D	S	D	S	D	S	D	S	D	
Three TAL strains[b]	2	0	0	0	64	0							34
Three TAL strains + B213	24	8	0	0	46	2	8	0					12
Three TAL strains + B214	4	0	2	2	66	0			2	0			24
Three TAL strains + B215	44	4	2	0	28	6					10	2	4
B213 + B214 + B215							12	12	2	2	50	6	16

[a] See Table 1, footnote *a*.
[b] NifTAL-recommended *Leucaena* rhizobial strains (TAL82, TAL582, and TAL1145).

tant role in the competition against the inoculum strains in mollisol soil, where it occupied 33 to 41% of the total nodules formed by native rhizobia. Nodules containing more than one strain occurred in both soils but did not make up more than 5% of the total nodules.

Results of the competition studies in which multistrain inoculum treatments were used in oxisol and mollisol soils are shown in Tables 2 and 3. Inoculum strain TAL1145 was the most successful competitor in both soils. The only exception was the inoculum mixture containing the three TAL strains (TAL82, TAL582, and TAL1145) plus strain B215 in oxisol soil, where TAL1145 was second in nodule occupancy next to TAL82. Strain TAL582 was the least successful competitor, being recovered in not more than 4% of the nodules in any of the inoculum treatments. Although the three other strains used, B213, B214, and B215, were isolated from Hawaiian oxisol or mollisol soil, they did not compete successfully with the NifTAL-recommended strains. The inoculum mixture of the three Hawaiian strains resulted in equal strain recovery in mollisol soil (Table 3), whereas in oxisol soil, strain B215 was 14 times more competitive than B214 and 2.5 times more competitive than B213 (Table 2). Under uninoculated conditions, most of the nodules were due to indigenous strains that did not react with any of the FAs used. In multistrain inoculum treatments, the unidentified indigenous *Leucaena* rhizobia occupied 4 to 34% of the nodules in oxisol soil (Table 2) and 6 to 20% of the nodules in mollisol soil (Table 3). Strain B217 was dominant among the native rhizobia nodulating the plants in mollisol soil. Relatively small percentages of doubly infected nodules (nodules having two strains) were found on plants grown in both soils (Tables 2 and 3).

The population of each inoculum strain in the rhizosphere of the oxisol-grown plants were measured at 10, 20, 30, and 48 days by membrane filter immunofluorescence (Table 4). The rhizospheres of uninoculated plants all harbored bacteria that were cross-reactive with the six FAs used. In all cases, however, inoculation caused an increase in the rhizosphere numbers of the strains used in the inoculum (Table 4).

DISCUSSION

Immunofluorescence with strain-specific FAs was applied to study competition among *Leucaena* rhizobia in two soils and their population dynamics in the rhizosphere of plants growing in the field.

Strains isolated from the same or nearby fields were used in single-strain inocula to determine whether their nodule occupancy could be enhanced by applying them to the seeds at sowing. They were also used in mixtures with recommended inoculum strains to establish their relative competitiveness. The advantages and disadvantages of multistrain inocula have not been experimentally established (for discussion, see reference 21). In this study we used the three-strain mixture recommended by the NifTAL project for inoculation of *L. leucocephala* in tropical soils.

The six strains of *Rhizobium* used in this study differed in their competitive abilities for nodulation of *L. leucocephala*. Strain TAL1145 was the most successful competitor against other strains in different inoculant mixtures as well as against indigenous rhizobia in both the oxisol and the mollisol fields. Strain TAL1145 has been reported to form highly effective nitrogen-fixing nodules on *Leucaena* spp. (8). The coincidence in efficient N_2-fixing capacity and the competitive ability of this strain makes it an ideal choice for use in inoculum preparations. Contrary to the stable competitive ability of strain TAL1145, the other five strains showed variable performances in different inoculant mixtures. Furthermore, the soil type was found to influence the competition patterns of some strains. For example, strain TAL82 was the best competitor in one of the inoculant mixtures in oxisol soil (Table 2) but performed poorly in the same mixture in mollisol soil (Table 3). Roughley et al. (19) have

TABLE 3. Competition among *Leucaena* rhizobial serogroups of multistrain inoculants in mollisol soil

Strains in inoculum	Strains in nodules (%)[a] from inoculum													
	TAL82		TAL582		TAL1145		B213		B214		B215		Indigenous	
	S	D	S	D	S	D	S	D	S	D	S	D	B217 (T)	Other (T)
Three TAL strains[b]	18	6	0	0	40	4							26	6
Three TAL strains + B213	22	10	0	0	44	10	2	0					6	6
Three TAL strains + B214	12	2	0	0	34	6			10	4			20	12
Three TAL strains + B215	16	4	2	0	44	10					2	2	14	6
B213 + B214 + B215							12	0	14	2	14	2	36	20

[a] See Table 1, footnote *a*.
[b] NifTAL-recommended *Leucaena* rhizobial strains (TAL82, TAL582, and TAL1145).

TABLE 4. Immunofluorescence counts of inoculum strains of *Rhizobium* spp. (*Leucaena* spp.) in the rhizosphere of *L. leucocephala* grown in Hawaiian oxisol soil

No. of days after seeding and inoculum strain	Immunofluorescence counts (no. per g [dry wt] of rhizosphere soil)						LSD[a]
	TAL82	TAL582	TAL1145	B213	B214	B215	
10							
None	4.8×10^5	3.1×10^6	1.9×10^5	2.5×10^5	2.9×10^5	4.3×10^5	3.7×10^5
TAL82, TAL582, TAL1145	2.7×10^7	3.2×10^7	6.8×10^7				3.3×10^6
B213, B214, B215				3.5×10^6	1.0×10^6	3.2×10^7	6.2×10^7
20							
None	1.5×10^5	3.9×10^7	1.2×10^5	9.2×10^6	2.2×10^7	7.7×10^4	5.2×10^6
TAL82, TAL582, TAL1145	1.1×10^8	5.6×10^7	1.9×10^8				6.8×10^7
B213, B214, B215				5.1×10^7	2.8×10^7	1.4×10^7	1.3×10^7
30							
None	1.4×10^5	8.8×10^6	1.7×10^5	3.2×10^6	8.4×10^6	3.4×10^4	3.5×10^5
TAL82, TAL582, TAL1145	6.4×10^7	2.8×10^7	1.3×10^8				8.1×10^6
B213, B214, B215				3.1×10^7	3.6×10^7	1.9×10^7	1.5×10^7
40							
None	6.5×10^5	3.3×10^6	1.1×10^5	2.8×10^6	5.8×10^6	4.3×10^4	4.6×10^5
TAL82, TAL582, TAL1145	1.4×10^7	8.3×10^6	9.2×10^7				1.8×10^7
B213, B214, B215				1.2×10^7	1.8×10^7	4.5×10^6	4.2×10^6

[a] LSD, Least significant difference ($P = 0.05$).

also found that the soil type can modify the competition pattern of different *Rhizobium* strains. Although two of the Hawaiian isolates in single-strain inocula were good competitors against indigenous *Leucaena* rhizobia in both soils, they failed to compete successfully with the mixture of TAL strains in the same soils. Nevertheless, the persistence of the strains in the soil and their adaptability to the prevailing soil conditions must play a critical role in the successful establishment of inoculum strains for nodulation of perennial legumes. The resident *Leucaena* rhizobia in both soils were able to compete against the high densities of the strains in the inoculum (Tables 1, 2, and 3). The difficulty of replacing the indigenous soil rhizobia with inoculant strains, even at high rates of seed inoculation, has been documented in nodulation studies of soybeans and clover (5, 6, 10, 19, 24).

All the strains in the inoculum were able to become established in the rhizosphere and attain populations ranging from ca. 1×10^6 to 2×10^8/g of dry soil. Although the rhizosphere soil of uninoculated plants contained bacteria that were cross-reactive with the six FAs used, these bacteria did not form any nodules on uninoculated plants. In all cases, inoculation caused an increase in rhizosphere populations of the strains used in the inoculum. Although the rhizosphere numbers of the most competitive strain, TAL1145, were generally higher than those of the other strains from the inoculum, there did not seem to be a strong relationship, in general, between numbers of a strain in the rhizosphere and its successful occupation of nodules. Similar conclusions about the lack of relationship between rhizosphere numbers and nodule occupancy of strains have been reached in experiments with soybeans (3, 13, 16, 17), peas (3), and beans (18).

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