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Symbiotic Characteristics and *Rhizobium* Requirements of a *Leucaena leucocephala x Leucaena diversifolia* Hybrid and Its Parental Genotypes

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In 56-day-old plants, *Leucaena leucocephala* and its hybrid with *L. diversifolia* showed 100% more total N than did *L. diversifolia.* Significant (P < 0.01) host-inoculation interaction in total N was 14.4% of the total phenotypic variation. The most effective and competitive *Rhizobium sp.* for the leucaenas was TAL 1145. Three-strain mixed inoculation was inferior to TAL 1145 alone.

Leucaena is the common name for *Leucaena leucocephala* (Lam.), which is a fast-growing, tropical, leguminous tree species. It is referred to as a miracle tree (6) because of its exceptional capacity to produce biomass and protein besides its use in agroforestry, soil improvement, and land reclamation and for wood and forage. Leucaena is nodulated by soil bacteria of the genus *Rhizobium,* and the symbiosis is highly specific (16). Though *L. leucocephala* has exceptional qualities, it is a tree species for the lowland areas below 500 m, and it continues to grow at higher elevations but without its lowland vigor (10). Other species, notably *L. diversifolia, L. esculenta,* and their crosses with *L. leucocephala* appear to have distinct potential for higher elevations (6). Hybrids of *L. leucocephala* and *L. diversifolia* have been bred and found to have high-acid soil tolerance with the desirable qualities of *L. leucocephala* still retained (6). Many other interspecific hybrids of leucaena have also been obtained (2) and field tested for their agronomic and genetic improvements (1). However, no data are available on the symbiotic relationships of the leucaenas and their hybrids with the microsymbionts. This investigation addressed the symbiotic characteristics and nitrogen-fixing potentials of an *L. leucocephala-L. diversifolia* hybrid and its parental genotypes.

Seeds of *L. leucocephala* var. K-8 and *L. diversifolia* var. K-156 were obtained from the nitrogen-fixing trees germ plasm at the NifTAL Project, University of Hawaii, Paia. *L. leucocephala-L. diversifolia* hybrid seeds (var. K-743) were obtained from J. L. Brewbaker, Department of Horticulture, University of Hawaii, Honolulu. Seeds were immersed in a minimal amount of concentrated sulfuric acid for 15 min to simultaneously scarify and surface sterilize. Treated seeds were rinsed in at least five changes of sterile water to remove acid and left to imbibe in sterile water for 4 to 6 h at room temperature. After imbibition and a further rinse, seeds were pregerminated on 0.75% (wt/vol) water agar for 2 days at room temperature. Three germinated seeds were selected and planted per modified Leonard jar (17) containing agricultural-grade vermiculite as the rooting medium. Each seed was inoculated immediately with 1 ml of a peat inoculantwater suspension containing ca 10' rhizobia per ml.

Three *Rhizobium sp.* strains, TAL 1145 (CIAT 1967; CB 3060), TAL 582 (CB 81), and TAL 82 (recommended for leucaena [4]), were obtained from the NifTAL *Rhizobium* germ plasm resource. The individual strains were cultured separately in yeast-mannitol broth (17) and later injected into gamma-irradiated peat (5). Ten-day-old peat inoculants of each strain were plate counted. Based on the viable counts, a mixed inoculant containing the three strains in a 1:1:1 ratio was prepared. For inoculating the seeds, suspensions of the individual strains or the mixture were prepared in sterile water.

The experiment was set up in a greenhouse in a randomized complete-block design with four replications. There were four inoculation treatments (TAL 1145, TAL 582, TAL 82, and a mixture of all three) and an uninoculated control without N. At 7 days, plants were thinned down to two per jar. At harvest (56 days), excised roots free of rooting

TABLE 1. Growth, nodulation, and nitrogen fixation characteristics among *L. leucocephala, L. diversifolia,* and the hybrid combined over four inoculation treatments

Leucaena species	N (mg/plant)[a]	Shoot dry wt (mg/plant)[a]	TNA[b]	SNA[c]	Nodule dry wt (mg/plant)	Nodule no./plant
L. leucocephala	14.78 (a) (0.81)[d]	701.75 (a) (94)	2.95 (b)	15.98 (b)	40 (a)	18.25 (b)
L. diversifolia	7.39 (b) (0.54)	291.06 (c) (28.3)	1.91 (c)	21.71 (a)	21.69 (b)	10.13 (c)
Hybrid	14.78 (a) (0.65)	610.31 (b) (54.5)	3.72 (a)	17.27 (b)	47.19 (a)	25.4 (a)

[a] The numbers in parentheses show data from uninoculated controls (not included in statistical analysis).
[b] Micromoles of ethylene produced per plant per hour.
[c] Micromoles of ethylene produced per gram of nodule fresh weight per hour.
[d] Means having the same letter within a column were not significantly different by Duncan's multiple-range test ($\alpha = 0.05$).

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TABLE 2. Coefficient of correlation (*r*) of the growth nodulation and nitrogen fixation traits of the three leucaenas

Trait	Total N	TNA	SNA	Nodule dry wt	Nodule no.
TNA	0.84[a]				
SNA	0.50 (NS)[b]	0.51 (NS)			
Nodule dry wt	0.84[a]	0.93[a]	0.75[c]		
Nodule no.	0.81[a]	0.88[a]	0.69[d]	0.92[a]	
Shoot dry wt	0.95[a]	0.87[a]	0.50 (NS)	0.90[a]	0.85[a]

[a] $P < 0.001$.
[b] NS, Not Significant.
[c] $P < 0.01$.
[d] $P < 0.05$.

TABLE 4. Competition for nodulation by three *Rhizobium* strains on *L. leucocephala, L. diversifolia,* an the *L. leucocephala-L. diversifolia* hybrid

Leucaena species	Nodule occupancy (% of total) by[a]: TAL 82	TAL 582	TAL 1145	TAL 82 + 1145[b]
L. leucocephala	18.3	0	78.5	3.3
L. diversifolia	10.3	0	88.0	1.7
L. leucocephala-L. diversifolia hybrid	6.8	0	91.8	1.7

[a] The three *Rhizobium* strains were equally represented in the peat-based mixed inoculum. Fifteen nodules were typed per replication for each treatment. The means of the *Rhizobium* strains were as follows: TAL 82, 11.8%; TAL 582, 0%; TAL 1145, 86.1%; TAL 82 + 1145, 2.23%.
[b] Double-strain occupancy of nodules.

medium were incubated in 10% (vol/vol) acetylene in airtight 1-liter polypropylene containers. Gas samples were analyzed for ethylene productions with a Varian aerograph 940 gas chromatograph equipped with a flame ionization detector. Nodules were picked, and fresh weight and number were determined before oven drying at 65°C for 48 h for dryweight determination. Plant tops were oven, dried similarly, weighed, and ground. Ground samples (0.25 g) were treated with 5 ml of H_20_2 and digested with concentrated sulfuric acid, and the N contents were determined by the method of Mitchell (7) with a Technicon autoanalyzer.

Oven-dried nodules from the mixed-inoculation treatment (15 nodules per replication) were analyzed (15) for strain competition by the fluorescent-antibody technique (13). Statistical analysis of the data (uninoculated controls not included) was performed by the ANOVA procedure (12). Simple correlation coefficients among the traits were computed on entry means.

The growth, nodulation, and nitrogen fixation traits of the parent leucaenas and the hybrid are shown in Table 1. Symbiotically fixed nitrogen was the only source of N for the three leucaenas. The controls were not nodulated. The hybrid and the *L. leucocephala* parent had similar nitrogen contents, but *L. diversifolia* showed 100% lower N content than *L. leucocephala* or the hybrid. Significant differences were detectable in all of the other parameters besides total N but in general, the symbiotic characters of the hybrid closely resembled those of *L. leucocephala* more than those of *L. diversifolia.* These represented significant inheritance of desirable symbiotic characters in favor of the hybrid, which has been shown to have high-acid soil tolerance and a potential for higher elevation similar to that of the *L. diversifolia* parent (6).

It was interesting (Table 1) that the specific nitrogenase activity (SNA) of *L. diversifolia* ranked highest, but its N content, shoot dry weight, total nitrogenase activity (TNA), and nodule dry weight and number were the lowest. This misleading relationship of SNA was further reflected by its generally poor correlations (Table 2) with other growth, nodulation, and nitrogen fixation traits. However, significant correlations of SNA did exist with nodule number ($r = 0.69$; $P < 0.05$) and nodule dry weight ($r = 0.75$; $P < 0.01$). Therefore, SNA should be used with caution when analyzing symbiotic effectiveness of the association in the *LeucaenaRhizobium* symbiosis. Similar misleading relationships were shown in the *Phaseolus vulgaris-Rhizobium phaseoli* symbiosis (11).

The results of this investigation indicated that, for evaluating symbiotic effectiveness in *L. leucocephala, L. diversifolia,* and the hybrid, shoot or nodule dry weight, TNA, or even nodule numbers can be used in place of total N because of the high correlations shown among these traits (Table 2).

All three strains of rhizobia were capable of nodulation and nitrogen fixation even though TAL 82 and TAL 582 were not as effective as TAL 1145. However, the SNA data for the three strains were not significantly different (Table 3). Though TAL 1145 was the most dominant strain, forming 86.1% of the nodules (Table 4), the mixed-inoculation treatment was significantly lower (20%) and ranked second in total N (Table 3) compared with the best-ranked treatment, which was TAL 1145. Our data suggested that use of single-strain inoculants of TAL 1145 would be preferred for the three leucaenas for better N fixation.

In inoculation studies with *L. leucocephala,* TAL 1145 has been found to be fully effective under acid soil conditions (3), and further, it out competed TAL 82, TAL 582, and native leucaena rhizobia for nodulation in two soils (8). These data further supported TAL 1145 as an elite strain for inoculation of *Leucaena* species.

Analysis of variance (Table 5) showed that the main effects due to the leucaena host were significant ($P < 0.001$ or <0.01) for all of the growth, nodulation, and nitrogen fixation traits. Unlike in the case of the hosts, the *Rhizobium* inoculation data for SNA and nodule number were not

TABLE 3. Growth, nodulation, and nitrogen fixation characteristics among the single- and mixed-strain inoculations combined over the three leucaenas

Rhizobium inoculation	N (mg/plant)	Shoot dry wt (mg/plant)	TNA[a]	SNA[b]	Nodule dry wt (mg/plant)	Nodule no./plant
TAL 82	7.4 (c)[c]	437.6 (b)	2.8 (ab)	16.7 (a)	37.8 (ab)	19 (a)
TAL 582	8.6 (c)	397.4 (b)	2.2 (b)	17.4 (a)	28.8 (b)	15 (a)
TAL1145	18.4 (a)	700 (a)	3.4 (a)	19.9 (a)	41 (a)	12 (a)
TAL 82 + 582 + 1145	14.8 (b)	602.5 (a)	3.1 (a)	19.4 (a)	37.6 (ab)	17 (a)

[a] Micromoles of ethylene produced per plant per hour.
[b] Micromoles of ethylene produced per gram of nodule fresh weight per hour.
[c] Means having the same letter within a column were not significantly different by Duncan's multiple-range test ($\alpha = 0.05$).

TABLE 5. Analysis of variance for growth, nodulation, and nitrogen fixation traits for the three leucaenas in response to *Rhizobium* inoculation

Sources of variation	df	Mean square of: N (mg/plant)	Shoot dry wt (mg/plant)	TNA[a]	SNA[b]	Nodule dry wt (mg/plant)	Nodule no./plant
Rhizobium sp.	3	320.9[c]	240,795.5[c]	3.4[d]	NS[e]	326.4[f]	NS
Leucaena sp.	2	291.1[c]	743,855[c]	13.4[d]	144.6[d]	2,766[c]	3,648.4[c]
Rhizobium-Leucaena interaction	6	45.4[d]	NS	NS	NS	NS	NS
Error	33	9.6	14,301.3	0.88	27.8	125.1	218.8

[a] Micromoles of ethylene produced per plant per hour.
[b] Micromoles of ethylene produced per gram of nodule fresh weight per hour.
[c] $P < 0.001$.
[d] $P < 0.01$.
[e] NS, Not significant.
[f] $P < 0.05$.

sources of variation. The host-inoculation interaction was significant ($P < 0.01$) only for total N.

Assuming a fixed-effect model (14), an analysis of variance was performed on the mean squares of variation for total N to quantify the relative contribution of the three genetic components of symbiotic variation. Results of the analysis indicated that the host-inoculation interaction component of variation accounted for only 14.4% of the total phenotypic differences. Additive effects of inoculation and the leucaena accounted for only 41.8 and 28.3% of the phenotypic variations, respectively. In the *Vicia faba-R. leguminosarum* symbiosis, *host-Rhizobium interaction* accounted for 73.8% of the phenotypic differences (9). The data in our study with the three leucaenas suggested that *host-Rhizobium interac*tion can be significant but not necessarily as important as the *Rhizobium* sp. or the host.

Hybridization with the lesser-known and low-N-fixing *L. diversifolia* yielded a hybrid with N-fixing and dry-matter production potential resembling that of the popular *L. leucocephala* parent. Also, a highly effective *Rhizobium sp.* strain (TAL 1145) was identified for inoculation of the hybrid. Further research needs to be done in long-term studies under different agroclimatic conditions to fully evaluate the hybrid for N fixation and biomass production.

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