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Effects of media N content and rhizobial strain on N_2 fixation and partitioning in *Leucaena* seedlings

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Abstract

Research was conducted to examine the effect of *Rhizobial* race and N fertilization on N_2 fixation and N partitioning in seedlings of the genus *Leucaena*. Seedlings of *Leucaena leucocephala* (Lam) Dewit variety K-8 and *L. retusa* Gray variety Yellow Puff were grown in slender tubes filled with fritted clay and watered with nutrient solutions containing 0, 2, or 8 mM nitrate N labeled with 0.1% N^{15}. The seedlings were inoculated with *Rhizobia loti* race 94A3 or *R. loti* race 9408. After 12 weeks the plants were harvested and the effects of N fertilization and *Rhizobial* race on nodulation N_2 fixation, and N partitioning were examined. Using the N^{15}-dilution method total N fixed by dinitrogen fixation was determined as well as distribution of fixed versus fertilizer N within the plant. Both *Rhizobial* races infected both *Leucaena* species resulting in an effective symbiosis. However, the 2 *Leucaena* species responded quite differently to N fertilization. The addition of 2 mM N to the nutrient solution effectively eliminated nodulation in *L. retusa* yet increased both nodulation and N_2 fixation in *L. leucocephala*. The 8 mM N rate eliminated nodulation in both species. Due to the elimination of nodulation and N_2 fixation by N fertilization in *L. retusa*, the effect of N fertilization on partitioning of fixed N was only examined in *L. leucocephala*. Increasing the N content of the nutrient solution increased the proportion of total N in the shoot and reduced the proportion in nodules. A larger proportion of the fixed N was retained in the root and nodules than fertilizer N. Although the 2 mM N treatment increased the amount of N_2 fixed in *L. leucocephala* compared to the 0 mM treatment, the proportion of fixed N_2 was reduced to less than one third of the total N in the plant.

Key Words: *Leucaena leucocephala, Leucaena retusa,* nitrogen fixation, nitrogen partitioning

In recent years the genus *Leucaena* has received much attention as a forage and timber crop. The most common species planted in the tropics is *L. leucocephala*; however, *L. retusa* is of special interest for arid and semiarid regions. *Leucaena leucocephala* is reported to be late-nodulating under field conditions (Egara and Jones 1977, Bushby 1982, Pathak et al. 1983). Due to late nodulation, nitrogen (N) fertilization has been recommended to provide N to the seedlings after seed reserves have been depleted and before N_2 fixation is initiated (Jones 1985). *Leucaena retusa* has not nodulated well using rhizobia selected for use with *L. leucocephala*. In a pot study, the addition of the equivalent of 100 kg N/ha increased both the nodule number and nodule mass of *Leucaena leucocephala* seedlings (Jones et al. 1983). Conversely, N fertilization did not affect nodule mass or number in a subsequent field study (Jones 1985).

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Soil N and symbiotically fixed N may be partitioned differently in the plant. In alfalfa (*Medicago sativa* L.), a larger proportion of biologically fixed N remained in roots than in shoots (Henson and Heichel 1984). Due to their perennial growth habit, *Leucaena* species may behave like alfalfa, partitioning a larger proportion of fixed N to the root than to the shoot.

Further definition of the species-specific reaction of nodulation and N_2 fixation to N fertilization and rhizobial strain, and the effect of soil N on resource partitioning is needed. The objective of this experiment was to determine the effect of N fertilization and rhizobial strain on N partitioning, nodulation, and N_2 fixation in *Leucaena leucocephala* and *Leucaena retusa*.

Material and Methods

Research was conducted at College Station, Texas. Seedlings of *L. leucocephala* variety K-8 and *L. retusa* variety Yellow Puff were grown in PVC tubes similar to those described by Hickey and Engelke (1983). The PVC tubes were 120-cm long and 4.5 cm in diameter. Plastic liners of 3-mil polyethylene tubing of slightly smaller diameter were inserted in the PVC tubes. Prior to insertion, the polyethylene liners were fused and perforated at the lower end. After the liners were inserted into the tubes, they were filled with fritted clay that had been screened to remove fine particles, washed, and leached with distilled water. The fritted clay contained only trace amounts of combined N. Nutrients were supplied by a modified nutrient solution as described by Silsbury (1984). Three concentrations of N were used in the nutrient solution: 0, 2, and 8 mM. The relative level of N available to the plants in each N treatment was previously evaluated using perennial ryegrass (*Lolium perenne L.*) as a test plant. The 2 mM solution produced slow-growing, chlorotic plants and is roughly equivalent to nitrate concentrations expected in a soil with very low N levels (Barber 1986). The 8 mM solution produced healthy plants and higher N concentrations did not increase ryegrass production.

The N provided in the nutrient solutions as KNO_3 was enriched with 0.1% $^{15}NO_3$ to enable the quantification of N in the plant derived from the nutrient solution. The percent N in the plant from the rooting media, percent N from N_2 fixation, and mg N from N_2 fixation in each plant part were determined using the following equations:

$$\%\ ^{15}N \text{ excess} = \%\ ^{15}N \text{ in sample} - \%\ ^{15}N \text{ atmosphere}$$

$$\%\ N \text{ from fertilizer} = \frac{\%\ ^{15}N \text{ excess in plant part}}{\%\ ^{15}N \text{ excess in fertilizer}} \times 100$$

$$\%\ N \text{ from } N_2 \text{ fixation} = 100 - \%\ N \text{ from fertilizer}$$

$$\text{mg N from } N_2 \text{ fixation} = \%\ N \text{ from fixation} \times \text{mg N in part}$$

Seeds were pregerminated by immersion in distilled water for 12 hours and contact between moist germination paper for another 24 hours. Seeds

Table 1. Plant weight, nitrogen concentration, total nitrogen, and total fixed nitrogen of 12 week old *Leucaena leucocephala* and *L. retusa* seedlings as affected by nitrogen fertilization.

Species	Nitrogen	Plant weight	Nitrogen concentration	Total N	Total fixed N
	(mM)	(gm/plant)	(mg/gm)	(mg/plant)	(mg/plant)
L. leucocephala	0	3.5 A[1]	23 A[1]	77 C[1]	77 B[1]
	2	13.0 B	25 B	364 B	116 A
	8	23.1 C	27 C	739 A	26 C
	Avg.	13.2	25	393	73
L. retusa	0	0.4 A	22 A	9 D	9 D
	2	1.7 B	28 B	42 C	0 E
	8	2.3 B	32 C	62 C	0 E
	Avg.	1.5	27	38	3

[1]Means in the same column within a species followed by the same letter are not significantly different at the 0.05 level of probability.

were inoculated with 1 ml of inoculant broth containing either 6.5 $\times 10^7$ rhizobia of *R. loti* strain 94A3 or 2×10^8 rhizobia of strain 94A8 (both obtained from the Nitrogen Fixing Tree Association). Tubes were placed in a greenhouse with average high and low temperatures of 32.8° and 20.2° C, respectively. Photosynthetic photon flux density (PPFD) was 1215 μmol m^{-2} sec^{-1} in the greenhouse when PPFD outside was 1812 μmol m^{-2} sec^{-1}. Average day length during the study was 12.1 hours. No artificial light was used to augment light intensity or duration.

The N_2 fixation rate was estimated 3 months after planting using acetylene reduction assay (Weaver and Fredrick 1982). Acetylene was generated by placing carbide in a plastic bag, evacuating and sealing the bag, and injecting water into the bag through a previously installed septum. The plants were washed free of the rooting media and placed in 2 containers. The containers were sealed and 220 ml of acetylene was injected into the container. Ten ml air samples were removed 30 min after injecting the acetylene, stored in evacuated tubes, and analyzed using a gas chromatograph. Following the assay, plants were destructively harvested. A root branching index was determined by a visual estimation of root branching in which any secondary root arising from the primary root was defined as a root branch site. An index of 1 was assigned for 0–10 root branch sites in a 10-cm section of root 10 cm below the crown, 2 for 11–20 root branch sites, 3 for 21–30 root branch sites, and so on. Root and shoot dry weight, nodule number, nodule dry weight, and leaf area were determined. Leaf area was determined using a Li-Cor LI-3100 area meter. Leaves were separated into individual leaflets and fed through the area meter. The plant shoot, root, and nodule fractions were analyzed for total N using the digestion and steam distillation method described by Nelson and Sommers (1980). The percentage of ^{15}N in each fraction was determined using an isotope-ratio mass spectrometer according to the method described by Porter and O'Dean (1977). To determine differential partitioning of symbiotically fixed and nitrate N, the proportion of symbiotically fixed N in the nodule, root, stem, and leaf were compared to the proportion of symbiotically fixed N in the whole plant.

The study was arranged as a 3 way factorial experiment was a randomized complete block design and 6 replications. The factors included in the trial were rhizobial strain, species, and N concentration in the nutrient solution as described previously. Data were analyed by analysis of variance procedures. Unless otherwise stated, statistical significance was tested at the 0.05 level of significance. Mean separations were performed by a LSD procedure.

Results and Discussion

Rhizobial strain did not affect any parameter measured and there were no interactions with other factors. Therefore, all data presented is an average of the 2 inoculation treatments. There was frequently an interaction of legume species with N concentration. In the following discussion, individual species responses to N concentration are included in tables and figures where there was a significant species by N concentration interaction. Where no interaction occurred, averages of the 2 species were included in tables and figures.

Seedling Growth

Dry matter production was up to 10 times greater in K-8 than in Yellow Puff. Seedling dry weight of both species increased with increasing N levels in the nutrient solution. Dry matter production of K-8 was more responsive to increasing N fertilization than was Yellow Puff (Table 1).

Nodulation and Root Branching

Both strains of rhizobia formed nodules on both *L. leucocephala* and *L. retusa.* However, nodule number of *L. retusa* was more adversely affected by increasing N levels in the rooting medium than was *L. leucocephala.* No nodules were found in either the 2 or 8 mM N treatments of *L. retusa.* In contrast, the number of nodules per *L. leucocephala* seedling increased from an average of 76 at 0 mM N to 84 at 2 mM N. The highest N treatment significantly reduced nodule number in both species. This agrees with responses of alfalfa nodulation to nitrate concentration where nitrate N concentrations above 310 ppm (5 mM) reduced the number of nodules in alfalfa seedlings; however, nitrate levels of 20 to 125 ppm (0.3–2 mM) had higher nodule numbers than O N controls (MacDowall 1982, and Fishbeck and Phillips 1981). The effect of nitrate N on nodule mass was similar to that seen with nodule number. Nodule mass per plant of *L. leucocephala* increased with the addition of 2 mM nitrate N but was eliminated with the addition of 8 mM nitrate N (Fig. 1). Again this agrees with research in other species that indicates that some soil N is needed for maximum nodule formation in legumes (Rawsthorne et al. 1985, Englesham et al. 1983, Das 1982).

The greater nodule weight of *L. leucocephala* in the 2 mM N treatment compared to the 0 mM treatment is believed to be a result of increased plant vigor due to the alleviation of "N hunger". Low levels of N fertilization may increase nodulation by increasing the number of sites available for nodule formation. The infection site for nodulation in *Leucaena,* like many tropical legumes, is thought to be at root branch sites (Jones and Bray 1982). Increasing N levels in the nutrient solution increased root branching (Fig. 2). At moderate N levels (i.e., 1 mM), increased root branching could result in more sites for rhizobial infection. As a result, nodule formation of *L. leucocephala,* which was less sensitive than *L. retusa* N. fertilization, could be enhanced at low levels of N fertilization in N-deficient soils.

Dinitrogen Fixation

Nitrogenase activity (μ mole hr^{-1} $plant^{-1}$) at harvest, was significantly greater in *L. leucocephala* than in *L. retusa (Fig. 3). Nitro-*

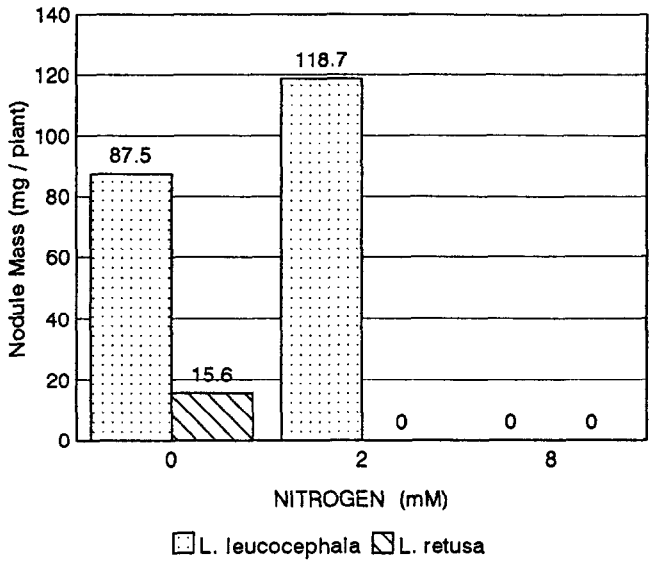

Fig. 1. Nodule Mass (mg plant^{01}) of 12-week old *Leucaena* seedlings as affected by species and nitrogen fertilization.

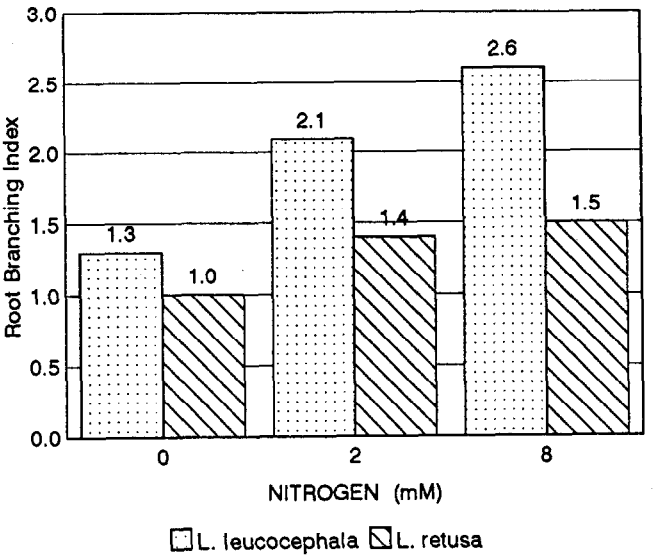

Fig. 2. Root Branching Index of 12-week old *Leucaena* seedlings as affected by fertilizer nitrogen and species.

gen fertilization affected nitrogenase activity more adversely in *L. retusa* than in *L. leucocephala* (PR = 0.057), reflecting the differing effects of combined N on nodule mass in the 2 species. A similar trend was seen in total N_2 fixation, expressed as mg fixed N per plant. Even low levels of N fertilization (2 mM) eliminated N_2 fixation in *L. retusa*. In *L. leucocephala* the 2 mM treatment actually increased the amount of N_2 fixed compared to the 0 mM N treatment (Table 1). Although the 2 mM N treatment increased total N_2 fixation in *L. leucocephala*, it reduced the proportion of fixed N_2 to less than one third of the total N in the plant (Table 2). This indicates that the plant was more actively extracting N from the nutrient solution than it was fixing N_2, even though the nitrogen status of the rooting medium was low.

The differing response of the 2 species to N fertilization may reflect differences in adaptation. *Leucaena retusa* is a native of the

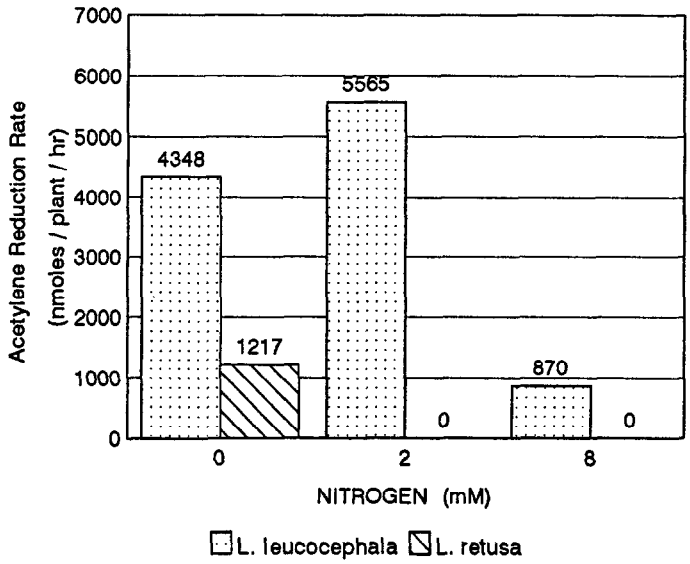

Fig. 3. Acetylene reduction rates (nmoles plant^{-1} hr^{-1}) of 12-week old *Leucaena* seedlings as affected by fertilizer nitrogen and species.

Trans-Pecos region of Texas, where the average annual rainfall is 200 to 300 mm. The cultivar Yellow Puff was selected in Uvalde, Texas, where a similar rainfall pattern prevails. In contrast, *L. leucocephala* is a native of northern Mexico, where the average annual rainfall is higher than that of the Trans-Pecos area. The cultivar K-8, which was developed in Hawaii, is not recommended for use in areas receiving less than 1,000 mm of precipitation annually. In the xeric environment to which Yellow Puff is adapted, soil N is not as limiting as soil water, while in the region of adaptation of K-8, soil N may be more limiting than soil moisture.

Table 2. The effect of nitrogen and species on the proportion of nitrogen in *Leucaena* seedlings from dinitrogen fixation (%).

Species	Nitrogen (mM)	N from Dinitrogen fixation (%)
L. leucocephala	0	100 A[1]
	2	32 B
	8	3 C
L. retusa	0	100 A
	2	0 C
	8	0 C

[1]Means followed by the same letter are not significantly different at the 0.05 level of probability.

Thus, symbiotic N_2 fixation may be far more important to the competitive ability of legumes in the zone of adaptation of K-8 compared to that of Yellow Puff.

Nitrogen Partitioning

Increasing N fertilization resulted in partitioning of a greater percentage of N to leaves and stems, accompanied by a reduction in the N percentage in nodules (Table 3). The variation of partitioning reflects changes in biomass of plant parts, not a change in the relative N content of plant parts. Nitrogen partitioning, expressed as the percentage of total plant N in a specific plant part, differed significantly between species (Table 4). The proportion of total plant N in roots was greater and the proportion in leaves was less for *L. retusa* than for *L. leucocephala*.

Table 3. The effect of nitrogen fertilization on nitrogen partitioning in *Leucaena* seedlings as the percent total plant nitrogen in the various plant parts.

	Total plant N			
Nitrogen	Leaf	Stem	Root	Nodule
	---------- (%) ----------			
0 mM	39.5	11.5	36.2	12.8
2 mM	48.2	13.4	34.5	3.9
8 mM	48.7	13.2	39.0	0.0
LSD = 4.2				

Table 4. The effect of species on nitrogen partitioning in *Leucaena* as expressed by percent total plant nitrogen in the various plant parts.

	Total plant N[1]			
Nitrogen	Leaf	Stem	Root	Nodule
	---------- (%) ----------			
L. leucocephala	49.6 A	15.1 A	36.2 B	8.4 A
L. retusa	45.3 B	12.3 A	41.0 A	9.1 A

[1]Means within a column followed by a different letter are significantly different at the 0.05 level of probability.

Differential Partitioning of Fixed and Soil Nitrogen

Differential partitioning of fixed and soil N was only examined in *L. leucocephala*. The proportion of symbioically fixed N was much higher in nodules (77%) than in other plant parts (18% or less). The percentage of symbiotically fixed N in nodules is lower than values reported by Van Kessel and Nakao (1986); under a range of N fertilization, the proportion of N in *Leucaena* nodules from N_2 fixation did not drop below 95%. In contrast to the present study, Van Kessel and Nakao applied N fertilizer to pots, at rates up to 25 mg $(NH_4)_2SO_4$ per pot. Since this level of N fertilization did not increase dry matter production or plant N accumulation, the actual concentration of N in solution was probably far lower in the study of Van Kessel and Nakao than in the 8 mM N treatment in this study.

The proportion of N from N_2 fixation was significantly higher in roots (18%) than in the stem (14%), leaf (15%), or whole plant (16%) of *L. leucocephala*. This agrees with experiments examining the differential partitioning of fixed and soil N in alfalfa (Henson and Heichel 1984). The response of leucaena and alfalfa differs from that of grain legumes in which symbiotically fixed N is preferentially partitioned to developing pods and shoot meristems (Yoneyama and Ishizuka 1982, Henson an Heichel 1984).

Summary and Conclusions

The addition of 2 mM N to the nutrient solution effectively eliminated nodulation and N_2 fixation in *L. retusa*. In contrast, 2 mM N in the nutrient solution increased nodulation and N_2 fixation in *L. leucocephala* compared with the control. These findings agree with other researchers who have found that low levels of N may actually increase nodulation and N_2 fixation in nitrogen deficient rooting media.

The large reduction in the proportion of N from N_2 fixation in *L. leucocephala* at the 2 mM level of fertilization indicates that substantial amounts of N may be taken up by this species even in soils with low to moderate levels of N. This raises serious questions on the accuracy of estimates for N_2 fixation in this species that are based on N accumualation in the shoot. Increasing the N content of the nutrient solution increased the proportion of N in the shoot and reduced the amount of N in *L. leucocephala* nodules. Differential partitioning of symbiotically fixed N and fertilizer N was similar to that of alfalfa, in which a larger proportion of symbiotically fixed N was retained in the roots than in the shoot.

Nodulation occurred much earlier in all experiments in this study than has been reported in field experiments. The optimal conditions of this study (adequate nutrients and water, moderate soil temperatures, and the lack of antagonistic or competing microbes in the rooting media) cannot be easily duplicated in the field. Thus, later nodulation should be expected in the field. This would lengthen the period between the depletion of seed N reserves and the initiation of N_2 fixation. The application of starter nitrogen to relieve "N hunger" during this period would facilitate establishment of *L. leucocephala*. This is especially true considering that low levels of fertilizer N actually increased nodulation and N_2 fixation of *L. leucocephala*. Whether starter N would aid in field establishment of *L. retusa* is less clear due to the inhibition of nodulation by low levels of fertilizer N.

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