THE RESPONSE OF TREE LEGUMES TO INOCULATION

WITH RHIZOBIA IN RELATION TO THEIR RHIZOBIAL SPECIFICITY

AND THE DENSITY OF INDIGENOUS RHIZOBIA

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By

Robert Daniell Turk

Thesis Committee:

Paul W. Singleton, Chairman
Harold H. Keyser
Russell S. Yost
James H. Fownes

For my parents

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Chapter 1

Thesis Introduction

Nitrogen is the most limiting plant nutrient in agriculture (Singer and Munns, 1987). Despite its abundance, plants are unable to use N_2 directly. Most legumes, through symbiosis with rhizobia (in this thesis, rhizobia will be used to refer to both *Rhizobium* and *Bradyrhizobium*, unless specified differently), have the ability to reduce N_2 through biological nitrogen fixation (BNF) into a form usable for growth. Increasing demand for agricultural products combined with a need to conserve the world's limited resources make increased use of BNF in agriculture and forestry an important global objective. Fast-growing N_2 fixing leguminous trees are being widely promoted as sources of renewable energy and biologically fixed N for associated crops (Brewbaker et al., 1982; Dommergues 1987; Kang et al., 1984).

When appropriate rhizobia are absent from a soil, legume BNF does not occur. Then, inoculation of legumes with rhizobia can supply sufficient appropriate rhizobia to satisfy a plant's rhizobial requirements for maximal N_2 fixation and provide considerable return on investment in inoculant technology. However, where inoculant or needed infrastructure are not available,

the cost of inoculant can be prohibitive. For farmers
to determine if inoculation is a wise investment and
for regional planners to decide if and where to develop
needed associated infrastructure, it is important for
both to be able to predict whether or not a response to
inoculation will occur and the magnitude of the
response.

Several approaches have been advanced for
assessing the need for inoculation. Field experiments
have the advantage of directly measuring the effect of
inoculation on yield. However, results from field
experiments are site specific and their cost generally
precludes wide use.

Another approach, the focus of this thesis, is to
use factors that determine response to inoculation as
indicators of the magnitude of the response.
Relationships between these factors and response to
inoculation can be used to predict responses to
inoculation in other areas. Experiments with grain and
forage legumes have identified density of indigenous
rhizobia as the major determinant of response to
inoculation (Singleton and Tavares, 1986; Thies et al.,
1991a and 1991b). Available soil N also influences the
magnitude of response to inoculation (Thies et al.,
1991b).

The use of rhizobial density for assessing the need to inoculate involves three key components: (i) use of the most-probable-number plant-infection (MPN) assay, (ii) a knowledge of rhizobial specificity, and (iii) establishment of the relationship between rhizobial density and response to inoculation. Because the accuracy of the MPN assay has not been established using trees and little is known about the rhizobial specificity of important tree legumes, these components will be addressed in Chapters 2 and 3 respectively. Chapter 4 examines the relationship between rhizobial density and response to inoculation of six tree species and the effect of available soil N on this relationship.

Chapter 2

Comparison of Most-Probable-Number Estimates of Tree Rhizobia with Plate Counts

ABSTRACT

Enumeration of rhizobia by most probable number plant infection (MPN) assays has been conducted with only a few leguminous tree species as hosts. To see if reasonable agreement could be obtained using conventional plant infection growth systems, MPN estimates were compared with plate counts of pure cultures using seven tree species grown in both growth pouches and on agar slants in glass tubes and seven other species grown in growth pouches alone. Reasonable agreement can be obtained between plate counts and MPN estimates, with closer agreement in agar slants than growth pouches for small-seeded species. Agar slants and growth pouches should be scored for nodulation 5 and 7 weeks after inoculation, respectively.

INTRODUCTION

Although there has been significant interest in the potential of fast-growing nitrogen-fixing leguminous trees as fuelwood, fodder, construction

material, and a source of biologically-fixed N_2 for associated crops in agroforestry systems, there has been little systematic study of their associated rhizobia. The most probable number plant-infection (MPN) assay, a technique widely used to enumerate populations of indigenous rhizobia, has only rarely been used with leguminous trees (Table 2.1). Rarer still are evaluations of its accuracy when used with trees. The purpose of this paper is to evaluate the accuracy of MPN assays with a variety of tree legumes grown in traditional growth systems through comparisons of plate counts and MPN estimates of the same pure rhizobial cultures.

The MPN assay is based on the capacity of rhizobia to form nodules on appropriate host plants under suitable conditions. MPN assays use the pattern of nodulated and non-nodulated plants inoculated with a dilution series to estimate the density of the rhizobial population in the original solution. Because no selective medium has proven reliable for distinguishing rhizobia from other microorganisms, the MPN assay is widely used for enumerating rhizobia in the presence of other microorganisms (Brockwell, 1980). Enumeration of indigenous rhizobia is especially useful for predicting the need to inoculate legumes

with rhizobia (Brockwell et al., 1988; Thies et al., 1991b).

The agreement of plate counts with MPN estimates of pure rhizobial cultures provides a measure of compliance of the MPN assay with the underlying assumptions (see Scott and Porter, 1986) and a means for comparing the appropriateness of different growth systems for different legume species. An important assumption is that a single rhizobial cell causes nodule formation (Cochran, 1950). Although agreement between plate counts and MPN assays with pure cultures does not guarantee accuracy in the presence of other microorganisms, it is an essential step for establishing the reliability of the technique. While reasonable agreement has been obtained in some cases with grain and forage legumes, there is a tendency of MPN assays to underestimate plate counts (Scott and Porter, 1986). Boonkerd and Weaver (1982) reported underestimations greater than 100 fold.

It is important to keep in mind that plate count-MPN comparisons cannot be evaluated using conventional statistical approaches. The fiducial limits developed by Cochran (1950) were based solely on the dilution ratio and the number of replicate growth units per dilution level, given the assumptions of the assay.

When the assumptions are not met, the actual fiducial limits will be higher than those calculated from theory (Scott and Porter, 1986).

A variety of growth systems have been used in MPN assays, including agar slants, tubes and cups filled with sand or horticultural vermiculite, Leonard jar assemblies (Leonard, 1944), and plastic growth pouches (Gibson, 1980). Agar slants have been recommended for legumes whose seeds are smaller than those of vetch (Vincent, 1970). Growth pouches are particularly useful for larger-seeded legumes.

No studies have investigated the number of weeks growth units need to be kept prior to scoring. Published reports of MPN assays conducted with tree legumes indicate that growth units have been kept for 3 to 12 weeks after inoculation before scoring (Table 2.1). It is unclear from these reports whether 3 or 4 weeks was sufficient for nodulation to occur or whether longer periods were essential.

The objectives of the experiments presented in this paper were to (i) evaluate the accuracy of the MPN assay for a variety of tree legumes; (ii) compare the suitability of growth pouches and agar slants for seven tree legumes; and (iii) determine how long the assay should continue.

MATERIALS AND METHODS

Growth systems

Two growth systems were used in this study.
Acacia auriculiformis, *Acacia mangium*, *Acacia mearnsii*,
Leucaena diversifolia, *Paraserianthes falcataria*,
Robinia pseudoacacia, and *Sesbania grandiflora* were
grown in plastic growth pouches (Northrup King Co.) and
on agar slants in 25 mm x 250 mm glass tubes.
Additional MPN assays were conducted in pouches with
Albizia lebbeck, *Albizia saman*, *Calliandra calothyrsus*,
Enterolobium cyclocarpum, *Flemingia macrophylla*,
Gliricidia sepium, *Leucaena leucocephala*, and *Sesbania
sesban*.

Agar slants were prepared according to Somasegaran
and Hoben (1985) using 15 ml per tube of a plant
nutrient solution containing 0.482 mM P, 0.96 mM K,
0.50 mM S, 0.21 mM Mg, and 0.29 mM Ca as K_2HPO_4,
$MgSO_4 \cdot 7H_2O$, and $CaSO_4 \cdot 2H_2O$; 10 ìmFe as FeEDTA; 0.0625 ml l$^-$
^{1} of a micronutrient solution (Liquid Hawaiian
Horticultural Mix, Monterey Chemical Co.); and 15 g
agar l^{-1}. After autoclaving, the agar was slanted at an
angle of 10° from the horizontal, producing a slant
approximately 10 cm long, beginning at the base of the
tube. The slant was designed to promote maximal
contact between roots and rhizobia at the base of the

slant and at the bottom of the tube. Growth pouches
were prepared by filling each pouch with 50 ml of the
same solution used in the tubes, minus the agar. An
additional 50 ml of the same solution was added prior
to inoculation. After inoculation pouches were
provided with sterile water as needed until harvest.

Plant Culture and Selection

Seeds were scarified and surface sterilized with
appropriate treatments (Table 2.2). After allowing the
seeds to imbibe water for 5-12 hours, they were
germinated on water-agar in petri plates according to
Somasegaran and Hoben (1985). Two to 5 days later,
when the radicles had reached 0.5 to 1.5 cm in length,
the seedcoats of uniform seedlings were carefully
removed to prevent hardening of the seed coat and to
facilitate examination of tubes for nodulation. In
tubes, seedlings were placed on the surface of the
agar, with the root collar approximately 6 to 7 cm from
the bottom of the slant. One and two seedlings were
planted in tubes and pouches respectively. After
planting, racks of pouches and tubes were placed in a
growth room receiving > 300 ìEinsteins m^{-2} s^{-1} of
photosynthetically active radiation at plant height
from 1000 W high pressure sodium lamps for 16 hours day^{-1}.

Prior to inoculation growth units with poorly growing plants were eliminated, leaving enough uniform plant growth units for at least 6 dilution levels with 4 replicate growth units each. In tubes, seedlings with tap roots that penetrated the agar were eliminated in accordance with the observation of Woomer et al. (1988b) that penetration of the agar by *L. leucocephala* roots resulted in poor nodulation. Of the trees grown in tubes for these experiments, *A. mangium* roots had the greatest tendency to penetrate the agar.

Inoculation

Rhizobial strains (Table 2.3) were grown for 6-10 days in either yeast-extract mannitol broth (Vincent, 1970) or arabinose-gluconate medium (Sadowsky et al., 1987). Prior to inoculation, cultures were diluted in a solution containing the salts found in yeast-extract mannitol broth (Vincent, 1970) with 0.01 % Tween 80 (Fisher Scientific Co.) added as a surfactant. Dilution procedures followed those outlined in Somasegaran and Hoben (1985). Either four-fold or 10-fold dilutions were used.

Seedlings were inoculated 7 to 12 days after planting, when root radicles reached the bottom of the slants or had begun to differentiate into secondary roots in pouches. One ml of diluted rhizobial culture

was applied directly to the roots in pouches and to the lower portion of the roots in tubes. An average of seven uninoculated growth units were included per MPN assay as checks for contamination within the system. Uninoculated controls received 1 ml of rhizobia-free diluent.

Plate Counts

Plate counts were conducted at the time of inoculation using the Miles and Misra drop plate method as modified by Somasegaran and Hoben (1985). At least four replicate 0.03 ml aliquots were used from each of three dilution levels from the inoculation dilution series. Prior to data collection, plates were incubated at 27°C for 7 to 10 days for slow-growing strains, and 3 to 5 days for fast-growing strains.

MPN determinations

With two exceptions scored at 4 weeks, growth units of MPN assays were scored for nodulation 5 to 7 weeks after inoculation. The most probable number was determined using a computer program (Woomer et al., 1990), beginning with the highest dilution step in which all growth units nodulated.

Assessment of time to scoring

MPN assays in pouches and tubes of *A.*

auriculiformis, *A. mangium*, *A. mearnsii*, and *R.
pseudoacacia*, were scored weekly for nodulation from
the second to the seventh week after inoculation.
Weekly scores were also recorded for *S. grandiflora* and
L. diversifolia, grown in pouches and tubes
respectively.

RESULTS

The plate count was within the corresponding 95%
confidence interval of the MPN (Cochran, 1950) for 18
of 54 plate count-MPN comparisons (Appendix B
summarized in Table 2.4), indicating lack of a
significant difference between them. However, in all
but three plate count-MPN comparisons the MPN
underestimated the plate count. The rhizobial density
of the original solution ranged from 3.67 x 10^7 to 7.55
x 10^9 rhizobia ml^{-1} as measured by the plate count.
Over all MPN assays, only five of 384

uninoculated controls nodulated, with never more than
one nodulated control per assay.

In tubes, all species with seed weights less than
25 mg seed^{-1} (Table 2.1) had average plate count:MPN
estimate (PC:MPN) ratios of less than 26. *A. mearnsii*,
L. diversifolia, and *P. falcataria* in tubes and *L.*

diversifolia, *C. calothyrsus*, and *S. sesban* in pouches all had average PC:MPN ratios less or equal to 2.1. *S. grandiflora*, with a seed weight of 36 mg/seed, had an average PC:MPN ratio of 1428.9 in tubes but only 5.8 in pouches. In pouches, seven of 14 species had average PC:MPN ratios of less than 35. For 11 of 14 tree species, PC:MPN ratios of less than 35 were obtained in either tubes or pouches.

Five of 10 MPN estimates from nodulation scores taken weekly from 2 to 7 weeks after inoculation increased after the fourth week (Figure 2.1). None of the 48 uninoculated controls nodulated in these assays. For species grown in both pouches and tubes, the average number of weeks to arrive at the final MPN estimate were 6.0 in tubes compared with 4.8 in pouches. In tubes, the MPN estimates of *A. auriculiformis* and *A. mearnsii* increased during the interval from 6 to 7 weeks after inoculation whereas in pouches, the MPN estimate of only one species, *A. mangium*, increased in the interval from 5 to 6 weeks and none after 6 weeks.

DISCUSSION

The excellent agreement between plate counts and MPN estimates observed with *A. mearnsii*, *L.*

diversifolia, and *P. falcataria* in tubes and with *L.*
diversifolia, *C. calothyrsus*, and *S. sesban* in pouches,
indicates that relatively accurate measurement of
rhizobial density can be achieved with pure cultures
for a range of tree legumes. Tree legumes appear to be
no different from herbaceous legumes in this respect.
A high level of agreement between MPN estimates and
plate counts (PC:MPN ratio < 6) has been obtained with
many herbaceous legumes including clover (Brockwell,
1963; Tuzimura and Watanabe, 1961), alfalfa (Weaver and
Frederick, 1972; Scott and Porter, 1986), chick pea
(Toomsan et al., 1984), and soybean (Weaver and
Frederick, 1972; Brockwell et al., 1975).

   Growth pouches were not suitable for seven of 14
tree species and tubes not suitable for *S. grandiflora*.
 These species had PC:MPN ratios > 100, indicating that
more than 100 rhizobia were required for nodule
formation. Other authors have reported large
discrepancies between plate counts and MPN estimates.
Boonkerd and Weaver (1982) reported consistent
underestimations involving *Bradyrhizobium* strains
applied to cowpea and siratro. They obtained PC:MPN
ratios of 260.6 for cowpea in pouches (mean of 4
strains), 255.0 for siratro in pouches (mean of five
strains), 125.6 for siratro in vermiculite-filled cups

(mean of two strains), and 15.3 for siratro in agar
slants (mean of two strains). In our assays, of seven
species grown in both pouches and tubes, good agreement
of five species in only one of two growth systems
suggests that the problems in obtaining agreement are
growth-system related, rather than indicative of an
inherent inability of trees to nodulate with a single
rhizobial cell, as was suggested by Boonkerd and Weaver
(1982) for cowpea and siratro.

     Our results demonstrate the appropriateness of
agar slants for small-seeded tree species and emphasize
the need to tailor the growth system to the needs of
the plant. Vincent (1970) reported that enclosed tubes
were only appropriate for legumes with seed weight less
than that of "vetch" (approximately 25 mg seed^{-1}). *S.
grandiflora*, with a seed weight of 36.4 mg seed^{-1}, was
the only species tried that did not grow well in tubes.
 Woomer et al. (1988b) noted the importance of placing
the diluted inoculant directly on the roots of the
plants to get good nodulation and noticed that the
roots of *Macroptilium atropurpureum* tended to
accumulate at the bottom of the tube without growing
extensively on the surface of the slant where the
inoculant was applied. Observations in our experiments
confirmed that root growth of the *Acacia* spp. in

particular was not extensive, especially in tubes, with most nodulation occurring at the bottom of the tubes. Therefore, the quantity of agar we used and the angle of the slant were designed to channel both roots and rhizobia directly to the bottom of the tube to ensure maximal contact between them. We found that 15 ml of agar was sufficient to support growth of the tree species we used for the duration of the assays.

Weekly assessment of MPN estimates (Figure 2.1) indicates the importance of keeping pouches for at least 5 weeks and tubes for at least 7 weeks after inoculation. Two to 3 weeks had been recommended as adequate by Vincent (1970), and 4 weeks by Brockwell (1980), based on MPN assays with herbaceous legumes. Scoring at 3 weeks had also been recommended as adequate for cowpea and siratro in pouches (Boonkerd and Weaver, 1982).

Reports of times to assessment of MPN assays with tree legumes (Table 2.1) indicate a range from 2-3 weeks to 11 weeks after inoculation, with nodulation in four of seven reports assessed at 3 weeks or less. The agreement Davis (1982) obtained between an MPN estimate and plate counts with *L. leucocephala* (PC:MPN ratio < 1) was from plants scored for nodulation approximately 11 weeks after inoculation.

In conclusion, our results indicate that reasonable agreement between plate counts and pure rhizobial cultures can be obtained with tree legumes but that results are very species and growth system dependent. MPN estimates using agar slants agreed well with plate counts for *A. auriculiformis*, *A. mangium*, *A. mearnsii*, *L. diversifolia*, *P. falcataria*, and *R. pseudoacacia*, while growth pouches gave acceptable agreement for *C. calothyrsus*, *G. sepium*, *L. diversifolia, L. leucocephala*, *R. pseudoacacia*, *S. grandiflora*, and *S. sesban*. Because of delayed nodulation, it is recommended that tubes and pouches be scored for nodulation at least 5 and 7 weeks after inoculation respectively. These results provide a basis for selecting appropriate growth systems for MPN assays with tree legumes and suggest that accurate MPN assays can be obtained in soils.

Table 2.1

MPN assays conducted with tree legumes.

Reference	Species	Growth System	Time to Assessment[a]
Davis, 1982	*Leucaena leucocephala*	agar deeps	up to 11 weeks
Sanginga et al., 1985	*L. leucocephala*	not recorded	2-3 weeks[b]
Sanginga et al., 1987	*L. leucocephala*	not recorded	2-3 weeks[b]
Singleton and Tavares, 1986	*L. leucocephala*	growth pouches	2-3 weeks
Singleton et al., 1991a	*L. leucocephala*	not recorded	not recorded
Thies et al., 1991a	*L. leucocephala*	growth pouches	3-4 weeks
Woomer et al., 1988a	*L. leucocephala*	agar slants	7 weeks
Woomer et al., 1988b	*L. leucocephala*	agar slants	2-3 weeks
Virginia et al., 1987	*Prosopis glandulosa*	dibble tubes	6 weeks
Singleton et al., 1991a	*Sesbania sesban*	not recorded	not recorded

[a] weeks after inoculation
[b] according to the reference cited, Vincent (1970).

Table 2.2. Seed sources, weight, and scarification and surface sterilization methods.

Species	Seed Source[a]	Weight mg seed^{-1}	Scarification[b]	Surface Sterilization[c]
A. auriculiformis	AOP	24.0	A (15 min) + B	
A. mangium		9.8	A (20 min) + B	
A. mearnsii	local	16.8	A (20 min) + B	
A. lebbeck	AOP	135.2	C	D (1 min)
A. saman	AOP	221.2	C	D (1 min)
C. calothyrsus	NFTA	51.6	none	D (1 min)
F. macrophylla	IITA	17.2	C	D (1 min)
G. sepium	IITA	131.2	none	D (1 min)
L. diversifolia	local	18.4	A (15 min)	
L. leucocephala K 8	NFTA	46.8	A (15 min)	
P. falcataria	Dr. I.L. Domingo, Phillipines	21.6	A (20 min)	
R. pseudoacacia	Pakistan Forest Inst.	17.8	A (20 min) + B	
S. grandiflora	AOP	36.4	C	D (2 min)
S. sesban	NFTA	14.6	C	D (1-2 min)

[a]AOP, Agroforestry Outreach Project, Port au Prince, Haiti; IITA, International Institute of Tropical Agriculture, Ibadan, Nigeria; local, Maui, Hawaii; NFTA, Nitrogen Fixing Tree Association, Waimanalo, Hawaii.

[b]A = soaked in concentrated sulfuric acid for no. of minutes in parentheses, followed by 8-10 rinses with sterile water.

B = seeds covered with boiling water following the last rinse in treatment A and allowed to cool

C = seeds scarified mechanically, either nicked with scissors or a scalpel, in one case (*Sesbania sesban*), scarified by scratching with sand paper.

[c]D = seeds soaked in 50 % bleach for the no. of minutes in parentheses, followed by 8-10 rinses in sterile water.

Table 2.3

Strains used in MPN assays.

Host Species in MPN Assay	Strain	Rhizobial genus[*]	Original Host	Other Name
Acacia auriculiformis	TAL 569	*Bradyrhizobium*	*Desmodium uncinatum*	MAR 472
Acacia auriculiformis	TAL 651	*Bradyrhizobium*	*Calopogonium mucunoides*	UMKL 44
Acacia auriculiformis	TAL 1446	*Bradyrhizobium*	*Acacia auriculiformis*	-
Acacia mangium	TAL 1867	*Bradyrhizobium*	*Acacia mangium*	LB 5
Acacia mearnsii	TAL 940	*Bradyrhizobium*	*Acacia mearnsii*	Num 777
Acacia mearnsii	TAL 941	*Bradyrhizobium*	*Acacia mearnsii*	Num 778
Acacia mearnsii	TAL 1388	*Bradyrhizobium*	*Acacia mearnsii*	-
Albizia lebbeck	TAL 1536	*Bradyrhizobium*	*Albizia lebbeck*	-
Albizia saman	TAL 833	*Bradyrhizobium*	*Albizia saman*	UMKL 27
Calliandra calothyrsus	TAL 1455	*Bradyrhizobium*	*Calliandra surinamensis*	-
Flemingia macrophylla	TAL 1883	*Bradyrhizobium*	*Flemingia macrophylla*	Nit 52A1
Gliricidia sepium	TAL 1806	*Rhizobium*	*Gliricidia sepium*	BR 8801
Gliricidia sepium	TAL 1145	*Rhizobium*	*Leucaena leucocephala*	CIAT 1967
Leucaena leucocephala	TAL 1145	*Rhizobium*	*Leucaena leucocephala*	CIAT 1967
Paraserianthes falcataria	TAL 45	*Bradyrhizobium*	*Paraserianthes falcataria*	-
Robinia pseudoacacia	TAL 1889	*Rhizobium*	*Robinia pseudoacacia*	USDA 3436
Sesbania grandiflora	TAL 1114	*Rhizobium*	*Sesbania sp.*	IC 71
Sesbania grandiflora	TAL 1119	*Rhizobium*	*Sesbania sp.*	IC 91
Sesbania sesban	TAL 674	*Rhizobium*	*Sesbania rostrata*	-
Sesbania sesban	TAL 1042	*Rhizobium*	*Sesbania longifolia*	Nit 145B1

[*] = determined by IPTG XGal assay in conjunction with growth on sucrose and lactose (Appendix A).

Table 2.4

Comparison of MPN Estimates with Plate Counts (PC) for Tree Legumes

Species	No. of MPN Assays Performed	No. with plate count within 95% C.I. of MPN	Average PC:MPN Ratio	Standard Deviation of PC:MPN Ratios	Range of PC:MPN Ratios	
AGAR SLANTS						
Acacia auriculiformis	3	0	25.7	29.2	5.0 -	67.0
Acacia mangium	4	0	16.0	6.4	5.0 -	21.0
Acacia mearnsii	4	3	2.0	0.9	0.6 -	2.2
Leucaena diversifolia	4	2	1.6	1.6	0.3 -	4.2
Paraserianthes falcataria	2	2	1.4	0.4	1.0 -	1.9
Robinia pseudoacacia	3	1	14.7	19.9	0.2 -	42.9
Sesbania grandiflora	2	0	>1428.9	1252.7	176.2 -	>2682
GROWTH POUCHES						
Acacia auriculiformis	3	0	8057.7	9305.3	746.4 -	21189.2
Acacia mangium	3	0	2370.3	2450.7	517.3 -	5833.3
Acacia mearnsii	3	0	234.8	300.2	5.5 -	658.9
Leucaena diversifolia	2	2	1.3	0.4	1.0 -	1.7
Paraserianthes falcataria	2	0	548.7	335.6	213.1 -	884.4
Robinia pseudoacacia	3	1	5.9	6.8	0.1 -	15.4
Sesbania grandiflora	3	1	5.8	2.9	1.9 -	8.8
Albizia lebbeck	1	0	118.8			
Albizia saman	1	0	12823.5			
Calliandra calothyrsus	1	1	2.1			
Flemingia macrophylla	1	0	865.1			
Gliricidia sepium	2	1	7.1	5.9	1.1 -	13.0
Leucaena leucocephala	3	1	34.3	26.8	0.7 -	66.3
Sesbania sesban	3	3	1.2	0.4	0.9 -	1.8
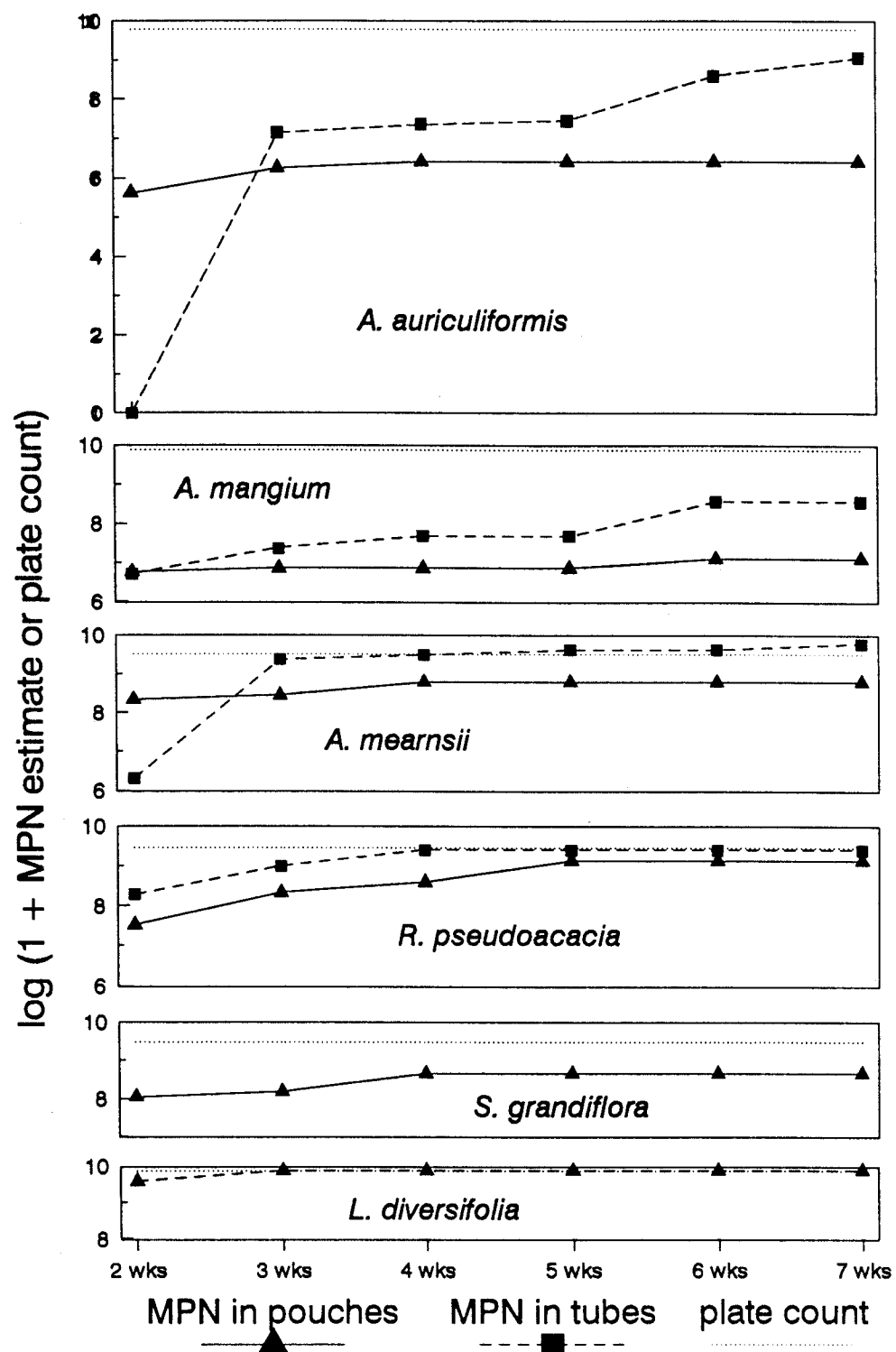
Figure 2.1. Weekly assessment of MPN estimates.

Chapter 3

Rhizobial Specificity of Fast-Growing Tree Legumes

ABSTRACT

Rhizobial specificity, defined in terms of nodulation and BNF effectiveness characteristics of a group of rhizobia on a host legume, has served as a basis for predicting the need to inoculate, selecting species for most-probable-number plant-infection assays, and preparing rhizobial inoculants suitable for a range of legume species. A series of cross-inoculation experiments were performed under growth room and greenhouse conditions to delineate rhizobial specificity of a variety of tree legumes. *Gliricidia sepium*, *Calliandra calothyrsus*, and *Leucaena leucocephala* nodulated effectively with rhizobia isolated from each of the three genera. With a few exceptions, *Sesbania grandiflora* and *Robinia pseudoacacia* nodulated effectively only with rhizobial strains isolated from each genus respectively. Considerable specificity was found among species that nodulate with *Bradyrhizobium*. While *Acacia mearnsii* nodulated with most strains but fixed N_2 effectively with relatively few, *Acacia mangium* and *Lysiloma latisiliqua* were specific for both nodulation and effectiveness.

INTRODUCTION

Nitrogen-fixing leguminous trees (NFLTs) are being planted on a wide scale in the tropics to provide fuelwood, construction materials, fodder, and nitrogen-rich biomass for improving soil fertility. Although biological nitrogen fixation is an important attribute of NFLTs, little has been done to delineate their rhizobial specificity in terms of nodulation and effectiveness.

Cross-inoculation is the reciprocal application of rhizobia isolated from different legumes to each other. A series of cross-inoculation experiments were conducted with a variety of tree legumes to delineate their rhizobial specificity in terms of nodulation and effectiveness.

Trees, in general, nodulate effectively and almost exclusively with either fast-growing rhizobia (genus *Rhizobium*) or slow growing-rhizobia (genus *Bradyrhizobium*) (Dreyfus et al., 1987). A few species nodulate effectively with some rhizobial strains from both genera such as *Acacia seyal*, *Acacia sieberana* (Dreyfus and Dommergues, 1987; Dreyfus et al., 1987), *Acacia longifolia* (Barnet et al., 1985), and *Prosopis glandulosa* (Jenkins et al., 1987). Tree species that nodulate effectively with fast-growing rhizobia are

specific for nodulation and effectiveness (Duhoux and Dommergues, 1985; Gibson et al., 1982; Trinick, 1982) whereas species that nodulate with *Bradyrhizobium* are less specific (more promiscuous) for both characteristics (Graham and Hubbell, 1975; Date, 1977 and 1982). Trees that nodulate with *Rhizobium* have not been examined extensively for specificity for effectiveness with respect to each other. Similarly, specificity for effectiveness has not been systematically evaluated in tree species that nodulate with *Bradyrhizobium*. There is evidence that some non-woody legume species and cultivars that nodulate with *Bradyrhizobium* are specific for effectiveness and/or nodulation (Date and Norris, 1979; Burton, 1952; Keyser and Cregan, 1987, Thies et al., 1991c) and that *A. mangium* is specific for effectiveness (Galiana et al., 1990).

There is considerable confusion surrounding the generic classification of rhizobia that effectively nodulate some tree species (Table 3.1). Apparent misidentification of the genus of rhizobia that effectively nodulates some trees (eg. *Calliandra* and *Gliricidia*) is misleading given the view that species that nodulate effectively with *Rhizobium* are more likely to respond to inoculation than species that

nodulate effectively with *Bradyrhizobium* (Dommergues, 1987; Dreyfus and Dommergues, 1981; Peoples et al., 1989).

The use of a small-seeded legume has been recommended for performing most-probable-number plant-infection (MPN) assays to determine the rhizobial population density of symbiotically related legumes (Vincent, 1970; Brockwell, 1980 and 1982). The only recommended substitution involving trees has been *Desmanthus virgatus* for *Leucaena leucocephala* (Brockwell, 1980 and 1982; Davis, 1982). *Macroptilium atropurpureum* has been recommended as a substitute for "cowpeas and symbiotically related species" (Brockwell, 1980 and 1982). Whether or not bradyrhizobial populations nodulating tree legumes can be enumerated accurately with *M. atropurpureum* has not been investigated.

Effectiveness groups of legumes are subsets of cross-inoculation groups that respond similarly to a set of rhizobial strains (Burton, 1979). They have served primarily as a guideline for inoculant preparation. Only four tree species have been ascribed to effectiveness groups: *Leucaena leucocephala*, *L. retusa*, *Robinia pseudoacacia*, and *Albizia julibrissin* (Burton, 1979; NiFTAL and FAO, 1984).

To delineate effectiveness groups of 14 tree legumes in terms of nodulation and effectiveness, two cross-inoculation experiments were performed in pouches. The purpose of the first experiment, termed Pouch Experiment A, was to evaluate fast-growing tree legumes using strains of both *Rhizobium* and *Bradyrhizobium*. The purpose of the second experiment, Pouch Experiment B, was to evaluate the specificity of a larger group of trees known to nodulate effectively with strains of *Bradyrhizobium*, using a larger number of these strains.

To confirm that data from the pouch experiments were not artifacts of the pouch system, effectiveness experiments were performed in pots in a greenhouse with three species, *A. auriculiformis*, *A. mangium*, and *A. mearnsii*. An additional objective of the pot experiments was to evaluate the effectiveness of a group of homologous strains (strains tested on the same species from which they were isolated) with *A. auriculiformis* and *A. mearnsii*.

MATERIALS AND METHODS

Tree species and rhizobial strains

Tree species (Table 3.2) were selected for their importance in reforestation and agroforestry in the

tropics. Cultivars and seed sources are the same as described in Chapter 2 except for *Lysiloma latisiliqua*, obtained from Agroforestry Outreach Project, Haiti; *Enterolobium cyclocarpum* obtained from trees on Maui, Hawaii; and *Vigna unguiculata* cv. knuckle purple hull.

 Pouch experiment B evaluated seven tree species inoculated with 35 rhizobial strains. In pouch experiment B eight tree species were used that were either known to nodulate with bradyrhizobial strains from written reports (Allen and Allen, 1981) or from experience at the NifTAL project. *M. atropurpureum* and *V. unguiculata* were also included in Pouch Experiment B because they are recognized as nodulating effectively with a wide variety of bradyrhizobial strains (Vincent, 1970). Eight uninoculated pouches were included for each tree species.

    Rhizobial strains (Table 3.3) were from the NiFTAL Project (1000 Holomua Ave., Paia, HI). As much as possible, in Pouch Experiment A at least three strains isolated from each tree species (or genus) were used. In Pouch Experiment B 34 *Bradyrhizobium* strains were used including NifTAL's recommended strains for cowpea (TAL 173, 209, 658), peanut (TAL 1000, 1371), and lima bean (TAL 22, 209, 658). The same set of strains was used on each species. Strains used in the pot

experiments were a subset from Pouch Experiment B, including the best strains identified from each of the three *Acacia* species. In addition, all strains isolated from *A. mearnsii* and *A. auriculiformis* in the NiFTAL collection were used to inoculate their respective homologous hosts.

Growth systems and experimental design

Pouch experiments

In the pouch experiments, trees were grown in growth pouches (Northrup King Co.), with a single plant of each of two species grown in each pouch (Table 3.2). Pouches were prepared as described in Chapter 2. Growth room conditions were the same as used in Chapter 2.

Four replicates of each treatment were included in Pouch Experiment A, with six uninoculated controls per species. Six uninoculated controls were included per species in Pouch Experiment B. In Pouch Experiment A, pouches were not randomized but received uniform light. Other experiments in the growth room had not shown a significant differences across blocks (data not shown). For Pouch Experiment B, pouches were randomized in racks (Somasegaran and Hoben, 1985) in three blocks. Every two days racks were rotated around the table to ensure uniformity of light for all racks.

Pot experiments

In the pot experiments, plants were grown in 1 l plastic pots containing moistened horticultural vermiculite (Grace and Co.). Two, three, and four plants were grown per pot for *A. mangium*, *A. mearnsii*, and *A. auriculiformis*, respectively. A microtube irrigation system supplied nutrient solution daily. The nutrient solution was the same as used to fill the pouches except for the first 6 weeks with *A. mangium* when double strength solution was used. In addition, pots with *A. mangium* and *A. mearnsii* received 3 mM starter N in the nutrient solution in the form of $CaNO_3$ for the first 3 weeks after planting. Pots were covered with sterile gravel following inoculation to help prevent rhizobial contamination.

Each of the three pot experiments was set up in a randomized complete block design with three blocks for *A. mangium* and four for *A. auriculiformis* and *A. mearnsii*.

Seedling preparation and inoculation

Seeds for all experiments were scarified and surface sterilized as described in Chapter 2. *Enterolobium cyclocarpum*, and *L. latisiliqua* were scarified in the same manner as *Albizia lebbeck*. Seeds

for the pouch experiments were germinated on water agar plates except for *Albizia saman*, *A. lebbeck*, and *E. cyclocarpum*, which were germinated in expanded vermiculite. All seedlings for the pot experiments were germinated in horticultural vermiculite (Grace and Co.) and transplanted into the pots 10 to 15 days later. For *A. mangium* and *A. mearnsii*, an extra seedling was transplanted into each pot; the least vigorous seedling was removed after 5 weeks.

Rhizobial strains were grown in yeast-extract mannitol (YEM) broth (Vincent, 1970) for 8 and 11 days respectively for Pouch Experiments A and B and for 8 days for the pot experiments. Plants were inoculated 8 to 16 days after planting in pouches, and within 24 hours after planting in pots. Pouches were inoculated by applying 1 ml of undiluted inoculant supplying approximately 10^9 rhizobia ml^{-1} in Pouch Experiment A and 1 ml of inoculant diluted 10-fold supplying approximately 10^8 rhizobia ml^{-1} in Pouch Experiment B to the roots of each plant. In pots, 1 ml of rhizobial broth culture containing approximately 10^9 rhizobia ml^{-1} was applied to each plant within 24 hours after planting and washed in with approximately 25 ml of sterile water.

Harvest and analysis

Plants were grown for various times before harvest to achieve maximal treatment differences (Table 3.2). At harvest plants in Pouch Experiment A were scored for effectiveness using a scale of 0 if no nodules formed on at least three of four replicates, "I" if plants nodulated but were not greener than uninoculated plants, "e" if at least two replicates were slightly greener than uninoculated plants, and "E" if at least two plants were greener and larger than "e" plants.

In Pouch Experiment B specificity for nodulation and effectiveness was assessed by single linkage cluster analysis of nodule numbers and shoot dry weight after standardization of values from zero to one. For shoot dry weight, uninoculated controls and treatments with values less than those of the uninoculated controls were given a value of zero. Specificity for nodulation and effectiveness was also assessed by rank correlation of nodule numbers and shoot dry weight respectively.

In the pot experiments, strains were evaluated using analysis of variance of $\log_{10}$ transformed data except for shoot dry weight of _A. auriculiformis_ where the data were not transformed because Bartlett's test showed that the variances were not significantly different at $P = 0.05$. Performance of strains used for

the same species in Pouch Experiment B and in the corresponding pot experiment were compared by Spearman rank correlation.

Correlation and cluster analyses were carried out using the SYSTAT program (Wilkinson, 1990); the SAS program (SAS Institute, 1986) was used to perform analysis of variance with Tukey's HSD test in pouch experiment B the pot experiments.

RESULTS

Pouch Experiment A

In Pouch Experiment A, tree species that nodulated effectively with *Rhizobium* formed three distinct groups based on specificity for effectiveness while species that nodulated effectively with *Bradyrhizobium* formed a single group but still exibited a range of specificity for nodulation and effectiveness (Table 3.4). *Leucaena leucocephala*, *Gliricidia sepium*, and *Calliandra calothyrsus* consistently formed effective symbioses with rhizobia isolated from members of all three genera with some effective nodulation with strains from *Sesbania* species. *Sesbania grandiflora* and *Robinia pseudoacacia* only nodulated effectively with rhizobia isolated from their respective genera except for effective and moderately effective nodulation of *S.*

grandiflora with a *Calliandra* strain (TAL 1801) and an *Acacia mangium* strain (TAL 1867) respectively and moderately effective nodulation of *R. pseudoacacia* with TAL 1145 and a strain from *Calliandra* (TAL 1455). *R. pseudoacacia* nodulated ineffectively with 19 of 35 strains, including both *Rhizobium* and *Bradyrhizobium*.

A. auriculiformis, *A. mangium*, *A. mearnsii*, *Paraserianthes falcataria*, and *Tephrosia candida* nodulated effectively only with *Bradyrhizobium* strains but revealed a range of specificity for nodulation and effectiveness. *P. falcataria* and *T. candida* formed effective symbioses with all *Bradyrhizobium* strains applied except TAL 102. At the other extreme, *A. mangium* failed to nodulate with five of 12 *Bradyrhizobium* strains and nodulated ineffectively with four of the remaining strains. All species that nodulated effectively with *Bradyrhizobium* strains nodulated ineffectively with some of the *Rhizobium* strains.

Pouch Experiment B

Distinct differences in the performance of different species were observed in Pouch Experiment B with respect to nodule numbers and shoot dry weight. *Albizia saman*, *Enterolobium cyclocarpum*, and *Vigna unguiculata* were not included in species comparisons

due to lack of significant differences ($P < 0.05$) between the shoot dry weight of the uninoculated control and that of the best strain as evaluated by Tukey's HSD test. *M. atropurpureum* (Figure 3.1), *A. auriculiformis* (Figure 3.1), and *A. lebbeck* (Figure 3.3), had a higher proportion of relatively effective strains compared with *A. mangium* and *A. mearnsii* (Figure 3.2) as evaluated by shoot dry weight. *L. latisiliqua* (Figure 3.3) and *P. falcataria* (Figure 3.4) were intermediate between these groups. *A. mangium* (Figure 3.2) and *L. latisiliqua* (Figure 3.3) did not form nodules with three and four strains respectively. All other species nodulated with all 34 strains. The three strains that produced the highest shoot dry weight on *A. mearnsii* were all homologous, as was the best strain on *A. mangium*.

Single-linkage cluster analyses were used to summarize these relationships (Figure 3.2). With respect to nodulation, there were no differences discernable between *M. atropurpureum*, *A. auriculiformis*, and *A. mearnsii*. The other species were separated from this group by regularly increasing intervals, with *A. mangium* being furthest apart. Similarly, whereas *A. mearnsii* had significant rank correlation with *M. atropurpureum* and *A. auriculiformis*

with respect to nodule numbers, nodulation of *A. mangium* was not significantly correlated with nodulation of any other species (Table 3.5).

In terms of shoot dry weight, *A. auriculiformis*, *M. atropurpureum*, *A. lebbeck*, and *P. falcataria* formed a group distinct from the other species (Figure 3.2). *A. mearnsii* was the only species whose shoot dry weights were not significantly correlated with shoot dry weights of *M. atropurpureum* by Spearman rank correlation (Table 3.4).

Pot Experiments

Nodulation and shoot dry weight response patterns observed in pouch experiment B were also evident in the pot experiments. The majority of strains were effective on *A. auriculiformis* in pots, with all but five of 27 producing significantly higher shoot dry weight than the uninoculated control (Figure 3.6). As in pouches, a large proportion of strains were relatively ineffective on both *A. mangium* and *A. mearnsii* (Figure 3.7). Correlation of shoot dry weight in the pot experiments with the dry weights from the same strains used in pouch experiment B yielded significant Spearman correlation coefficients ($P< 0.05$) for shoot dry weight and nodule numbers of *A. mangium*

and *A. mearnsii* (Table 3.6).

The 10 strains with the highest dry weights for *A. mearnsii* in pots were all homologous isolates (Figure 3.7). Homologous isolates also were among the best strains for *A. auriculiformis* (Figure 3.6) and *A. mangium* (Figure 3.7) in the pot experiments.

DISCUSSION

Effective cross nodulation of *G. sepium*, *C. calothyrsus*, and *L. leucocephala*, by rhizobia from each of the three species but failure to nodulate effectively with most rhizobia isolated from other legumes, indicates that *G. sepium*, *C. calothyrsus*, and *L. leucocephala* belong to a common effectiveness group. Previously, *L. leucocephala* had been reported to nodulate with a strain isolated from *C. calothyrsus* (Halliday and Somasegaran, 1983) but without any information on effectiveness. Other researchers have reported effective nodulation of *G. sepium* (Somasegaran et al., 1989) and *G. maculata* (Akkasaeng et al., 1986) with fast-growing rhizobial strains. Our data conflict with unsubstantiated reports that *G. sepium* (Date, 1977; Dreyfus et al., 1987; Peoples et al., 1989; Trinick, 1982) and *Calliandra* (Peoples et al., 1989) nodulate with "cowpea," "slow-growing" or

Bradyrhizobium strains.

The results from Pouch Experiment A support reports that *S. grandiflora* has highly specific rhizobial requirements (Abdel Magid et al., 1988; Johnson and Allen, 1952; Ndoye et al., 1990; Trinick, 1982). The data support reports of specificity for effectiveness of *R. pseudoacacia* (Allen and Allen, 1981; NifTAL and FAO, 1984). They indicate that *R. pseudoacacia* nodulates with a broad range of rhizobia in support of some reports (Burton, 1977; Crow et al., 1981; Wilson, 1944) but in disagreement with the conclusions of Allen and Allen (1981).

Based on the pattern of effective nodulation in Pouch Experiment A and on cluster analyses and rank correlation of shoot dry weight and nodule numbers in Pouch Experiment B, *A. auriculiformis*, *A. lebbeck*, *P. falcataria*, and *T. candida* appear to be promiscuous for both nodulation and effectiveness while *A. mearnsii* is promiscuous for nodulation but specific for effectiveness, and *A. mangium* and *L. lysiliqua* are specific for both nodulation and effectiveness. These data support the findings of Allen and Allen (1939) for *A. lebbeck* and of Galiana et al. (1990) for *A. mangium*, but are in disagreement with an unsubstantiated report that *A. lebbeck* is specific in its rhizobial

requirements (Duhoux and Dommergues, 1985).

The results suggest that species-specific
inoculants should be developed for *S. grandiflora*, *R.
pseudoacacia*, *A. mangium*, *A. mearnsii*, and *L.
latisiliqua*. A common inoculant could be developed for
L. leucocephala, *G. sepium*, and *C. calothyrsus*, an
expansion of the host range suggested by Somasegaran et
al. (1989).

Similarly, a single inoculant could be developed
for use with all species that are promiscuous for both
nodulation and effectiveness. However, because no
strains emerged as distinctly superior for all of the
species in this category used in pouch experiment B and
because a strain commonly recommended for inoculating
grain and forage legumes in the cowpea miscellany, TAL
309 (CB 756), failed to nodulate *A. auriculiformis*
effectively in pots, it would seem prudent to develop
individual inoculants for each species. Another
approach would be to develop a group inoculant
consisting of a mixture of highly effective strains for
each species rather than to rely on inoculant developed
for use with other legumes that are promiscuous for
both nodulation and effectiveness such as *M.
atropurpureum* or *Vigna unguiculata*.

The data suggest that MPN assays conducted with *L.*

leucocephala, *G. sepium* or *C. calothyrsus* should
provide a reasonable approximation of the population of
rhizobia present capable of nodulating the other two
species. Similarly, *M. atropurpureum* will likely
provide a reasonable estimate of the rhizobial
densities of *A. auriculiformis*, *A. mearnsii*, *A.
lebbeck*, *P. falcataria*, and *T. candida*, but not for *A.
mangium* and *L. latisiliqua*
due to greater specificity of the latter species for
nodulation.

In keeping with view that species that are more
specific for effectiveness are more likely to respond
to inoculation in the field than species with less
specific rhizobial requirements (Dommergues, 1987), *L.
leucocephala*, *G. sepium*, *C. calothyrsus*, *A. mearnsii*,
A. mangium, and *L. latisiliqua* should be more likely to
respond to inoculation than *A. auriculiformis*, *A.
lebbeck*, *P. falcataria*, or *T. candida*.

In summary, the trees studied in the experiments
described here can be categorized based on effective
nodulation with *Rhizobium* or *Bradyrhizobium* and
specificity for nodulation and effectiveness (Table
3.7).

Table 3.1

Conflicting reports of rhizobial affinities with tree legumes.

Genus or Species	Effective Nodulation with:	
	Bradyrhizobium	*Rhizobium*
Albizia lebbeck	Allen and Allen, 1939*	Duhoux and Dommergues, 1985
Calliandra	Peoples et al., 1989	P. Somasegaran, pers. comm.
Desmanthus	Date, 1977 and 1982 Date and Halliday, 1982 Trinick, 1982	Date, 1991* Davis, 1982*
Gliricidia	Date, 1977 Dreyfus et al., 1987 Peoples et al., 1989 Trinick, 1982	Akkasaeng et al., 1986* Somasegaran et al., 1989*
Samanea (*Albizia*)	Allen and Allen, 1939*	Gibson et al., 1982

* supporting data presented in report

Table 3.2. Plant combinations and time from inoculation to harvest for each combination.

Experiment	Plant combinations	Days to harvest
Pouch A	*Acacia auriculiformis - A. mangium*	62
Pouch A	*Acacia mearnsii*	49
Pouch A	*Calliandra calothyrsus - Paraserianthes falcataria*	47
Pouch A	*Gliricidia sepium - Lysiloma latisiliqua*	39
Pouch A	*Robinia pseudoacacia*	43
Pouch A	*Sesbania sesban - Tephrosia candida*	32
Pouch B	*Acacia auriculiformis - Albizia lebbeck*	88
Pouch B	*Acacia mangium - A. mearnsii*	74
Pouch B	*Albizia saman - Enterolobium cyclocarpum*	92
Pouch B	*L. latisiliqua - P. falcataria*	87
Pouch B	*Macroptilium atropurpureum - Vigna unguiculata*	38
Pot A	*A. auriculiformis*	101
Pot B	*A. mangium*	73
Pot C	*A. mearnsii*	59

Table 3.3

Strains used in pouch and pot experiments.

Strain (TAL No.)	Original Host	Rhizobial Genus[a]	Other Name[b]	Experiments[c]
7	*Gliricidia sepium*	*Rhizobium*	-	A
22	*Phaseolus lunatus*	*Bradyrhizobium*	-	A,B,C
33	*Calliandra calothyrsus*	*Rhizobium*	-	A
45	*Paraserianthes falcataria*	*Bradyrhizobium*	-	A,B,C
47	*Enterolobium cyclocarpum*	*Bradyrhizobium*	-	B,C
60	*Enterolobium cyclocarpum*	*Bradyrhizobium*	-	B
63	*Acacia mearnsii*	*Bradyrhizobium*	Nit 1B2	B,C
69	*Erythrina indica*	*Bradyrhizobium*	Nit 47A1	B,C
82	*Leucaena leucocephala*	*Rhizobium*	-	A
102	*Glycine max*	*Bradyrhizobium*	USDA 110	B
111	*Acacia mearnsii*	*Bradyrhizobium*	-	B,C
112	*Acacia mearnsii*	*Bradyrhizobium*	-	C
126	*Acacia mearnsii*	*Bradyrhizobium*	-	C
132	*Acacia mearnsii*	*Bradyrhizobium*	-	C
133	*Acacia mearnsii*	*Bradyrhizobium*	-	C
134	*Acacia mearnsii*	*Bradyrhizobium*	-	C
169	*Vigna unguiculata*	*Bradyrhizobium*	Nit 176A22	C
173	*Vigna unguiculata*	*Bradyrhizobium*	Nit 176A30	B,C
179	*Acacia mearnsii*	*Bradyrhizobium*	-	C
180	*Acacia mearnsii*	*Bradyrhizobium*	-	C
201	*Canavalia ensiformis*	*Bradyrhizobium*	Nit 22A4	B
209	*Vigna radiata*	*Bradyrhizobium*	-	B
309	*Macrotyloma africanum*	*Bradyrhizobium*	CB 756	C
363	*Albizia lebbeck*	*Bradyrhizobium*	-	A
569	*Desmodium uncinatum*	*Bradyrhizobium*	MAR 472	B,C
582	*Leucaena leucocephala*	*Rhizobium*	CB 81	A
583	*Leucaena leucocephala*	*Rhizobium*	NGR 8	A
644	*Phaseolus acutifolius*	*Bradyrhizobium*	CIAT 257	B,C
651	*Calopogonium mucunoides*	*Bradyrhizobium*	UMKL 44	B,C
658	*Stylosanthes sp.*	*Bradyrhizobium*	CIAT 71	B
749	*Erythrina indica*	*Bradyrhizobium*	CIAT 35	B,C
795	*Tephrosia glauca*	*Bradyrhizobium*	CIAT 496	A
833	*Samanea saman*	*Bradyrhizobium*	UMKL 27	B,C
850	*Crotalaria sp.*	*Bradyrhizobium*	UMKL 71	B,C
881	*Acacia koa*	*Bradyrhizobium*	-	B,C
940	*Acacia mearnsii*	*Bradyrhizobium*	Num 777	C
941	*Acacia mearnsii*	*Bradyrhizobium*	Num 778	C
942	*Acacia mearnsii*	*Bradyrhizobium*	Num 779	C
1000	*Arachis hypogaea*	*Bradyrhizobium*	-	B
1119	*Sesbania sp.*	*Rhizobium*	IC 91	A
1122	*Albizia stipulata*	*Bradyrhizobium*	NGR 143	B
1137	*Sesbania sp.*	*Rhizobium*	CIAT 175	A
1145	*Leucaena leucocephala*	*Rhizobium*	CIAT 1967	A
1280	*Samanea saman*	*Bradyrhizobium*	ALLEN 708	B

Table 3.3 (Continued)

Strains used in pouch and pot experiments.

1371	*Arachis hypogaea*	*Bradyrhizobium*	Nit 8A11	B
1380	*Crotalaria paulina*	*Bradyrhizobium*	Nit 32H1	A,B,C
1384	*Acacia mearnsii*	*Bradyrhizobium*	-	C
1385	*Acacia mearnsii*	*Bradyrhizobium*	-	C
1386	*Acacia mearnsii*	*Bradyrhizobium*	-	C
1387	*Acacia mearnsii*	*Bradyrhizobium*	-	C
1388	*Acacia mearnsii*	*Bradyrhizobium*	-	B,C
1389	*Acacia mearnsii*	*Bradyrhizobium*	-	C
1446	*Acacia auriculiformis*	*Bradyrhizobium*	-	A,B,C
1449	*Acacia auriculiformis*	*Bradyrhizobium*	-	C
1450	*Acacia auriculiformis*	*Bradyrhizobium*	-	C
1455	*Calliandra surinamensis*	*Rhizobium*	-	A
1457	*Acacia albida*	*Bradyrhizobium*	-	B
1521	*Acacia auriculiformis*	*Bradyrhizobium*	-	A,B
1522	*Acacia auriculiformis*	*Bradyrhizobium*	-	C
1530	*Enterolobium cyclocarpum*	*Bradyrhizobium*	-	B,C
1536	*Albizia lebbeck*	*Bradyrhizobium*	-	A,B,C
1597	*Albizia lebbeck*	*Bradyrhizobium*	-	B,C
1770	*Gliricidia sepium*	*Rhizobium*	-	A
1779	*Sesbania grandiflora*	*Rhizobium*	-	A
1788	*Gliricidia maculata*	*Rhizobium*	-	A
1801	*Calliandra calothyrsus*	*Rhizobium*	BR 4301	A
1806	*Gliricidia sepium*	*Rhizobium*	BR 8801	A
1829	*Sesbania rostrata*	*Azorhizobium*	ORS 571	A
1852	*Albizia caribaea*	*Bradyrhizobium*	-	B
1867	*Acacia mangium*	*Bradyrhizobium*	LB 5	A,B,C
1869	*Acacia mangium*	*Rhizobium*	LB 7	A
1883	*Flemingia macrophylla*	*Bradyrhizobium*	Nit 52A1	A,B
1886	*Sesbania longifolia*	*Rhizobium*	Nit 145B1	A
1887	*Leucaena leucocephala*	*Rhizobium*	MS 111	A
1908	*Glycine max*	*Bradyrhizobium*	USDA 94	B

[a] determined by IPTG XGal assay (Sambrook et al., 1989) in conjunction with growth
on sucrose and lactose (Appendix A).

[b] strains with no other name were isolated by the NiFTAL Project.

[c] A = Pouch Experiment A, B = Pouch Experiment B, C = pot experiments.
For pot experiments, homologous strains were used only on the original host except
for TAI nos. 1446, 1388, 63, and 111 which were used in all three pot experiments.
TAL 309 and TAL 850 were not used in the *A. mangium* pot experiment.

Table 3.4. Effectiveness of *Rhizobium* and *Bradyrhizobium* strains on seven tree legumes.

Rhizobial Strains		Tree Species									
TAL No.	Original Host	Ll	Gs	Cc	Sg	Rp	Aa	Am	Ame	Pf	Tc
1887	*Leucaena leucocephala*	E	E	e	O	I	O	I	I	I	O
583	*Leucaena leucocephala*	E	E	e	O	I	O	I	I	O	np
1145	*Leucaena leucocephala*	E	E	e	O	e	O	O	O	I	I
582	*Leucaena leucocephala*	e	I	I	O	I	I	I	I	O	O
82	*Leucaena leucocephala*	e	E	e	O	O	O	I	O	O	O
7	*Gliricidia sepium*	E	E	E	O	I	I	I	O	I	O
1788	*Gliricidia maculata*	E	E	E	I	I	I	O	O	I	I
1806	*Gliricidia sepium*	E	E	E	O	I	O	O	O	I	I
1770	*Gliricidia sepium*	E	E	E	O	I	O	O	O	I	O
1801	*Calliandra calothyrsus*	e	E	O	E	I	I	I	O	O	O
33	*Calliandra calothyrsus*	E	E	E	O	I	I	O	O	I	I
1455	*Calliandra surinamensis*	E	E	E	O	e	O	O	O	I	I
1779	*Sesbania grandiflora*	O	O	O	E	I	O	I	O	I	O
1886	*Sesbania longifolia*	e	E	I	E	I	O	I	O	I	O
1137	*Sesbania* sp.	e	E	E	O	O	O	I	I	I	I
1114	*Sesbania* sp.	O	O	O	E	I	O	O	O	O	np
1119	*Sesbania* sp.	O	O	O	E	O	O	O	O	O	O
1829	*Sesbania rostrata*	O	O	e	I	I	I	I	O	I	I
183	*Robinia pseudoacacia*	O	O	O	O	E	O	O	O	O	np
1889	*Robinia pseudoacacia*	O	O	O	O	E	O	O	O	O	np
1907	*Robinia pseudoacacia*	O	O	O	O	E	O	O	O	O	np
1869	*Acacia mangium*	e	I	e	I	I	I	I	e	O	O
1867	*Acacia mangium*	O	O	O	e	O	E	E	e	E	E
1446	*Acacia auriculiformis*	O	O	O	O	O	E	O	E	E	E
1521	*Acacia* sp.	O	O	O	O	I	E	O	e	E	E
45	*Paraserianthes falcataria*	O	I	O	I	I	?	E	E	E	E
363	*Albizia lebbeck*	O	O	O	O	O	O	I	I	e	E
1536	*Albizia lebbeck*	O	I	O	O	I	E	np	I	E	np
795	*Tephrosia glauca*	O	O	O	O	O	O	O	I	E	E
1883	*Flemingia macrophylla*	O	O	O	O	O	E	I	O	e	E
22	*Phaseolus lunatus*	O	O	O	O	O	E	E	e	E	np
1380	*Crotalaria paulina*	O	I	O	O	O	E	I	E	E	E
1000	*Arachis hypogaea*	O	O	O	O	O	E	O	e	e	np
1908	*Glycine max*	O	I	O	O	I	e	I	I	e	E
102	*Glycine max*	O	O	O	O	I	O	O	I	I	np

Effectiveness code: O = no nodules, e = moderately effective,
I = ineffective, E = effective, ? = inconsistent response, np = not performed.
Legume species: Ll = *Leucaena leucocephala*, Gs = *Gliricidia sepium*,
Cc = *Calliandra calothyrsus*, Sg = *Sesbania grandiflora*, Rp = *Robinia pseudoacacia*, Aa = *Acacia auriculiformis*, Am = *Acacia mangium*, Ame = *Acacia mearnsii*, Pf = *Paraserianthes falcataria*, Tc = *Tephrosia candida*

☐ = *Leucaena-Gliricidia-Calliandra Rhizobium* group
⌐ ⌐ = *Sesbania Rhizobium* group
⌐ = *Robinia Rhizobium* group
☐ = *Bradyrhizobium* group

Table 3.5. Comparison of species used in Pouch Experiment B on the basis of rank correlation of shoot dry weight (A) and nodule numbers (B) produced by individual rhizobial strains.

A. Shoot Dry Weight

	Ma[a]	Aa	Am	Ame	Al	Ll
	-Spearman rank correlation coefficients-					
Aa	0.402*[b]					
Am	0.374*	0.328*				
Ame	0.189	0.192	0.530**			
Al	0.375*	0.316*	0.230	0.366*		
Ll	0.366*	0.372*	0.314*	0.090	0.164	
Pf	0.528**	0.238	0.019	0.086	0.693**	0.296

B. Nodule Numbers

	Ma	Aa	Am	Ame	Al	Ll
	-Spearman Rank Correlation Coefficients-					
Aa	0.212					
Am	0.209	0.269				
Ame	0.304*	0.445**	0.149			
Al	0.094	0.277	0.148	0.320*		
Ll	0.300*	0.289	0.109	0.059	-0.294	
Pf	0.020	-0.159	-0.258	-0.404	0.104	0.094

[a] Legume species: Ma = *M. atropurpureum*, Aa = *A. auriculiformis*, Am = *A. mangium*, Ame = *A. mearnsii*, Al = *Albizia lebbeck*, Ll = *Lysiloma latisiliqua*, Pf = *P. falcataria*.
[b]*, ** = significant at $P < 0.05$ and $P < 0.01$, by 1 tailed test

Table 3.6. Comparison of species used in Pouch Experiment B with the same species used in the pot experiments by rank correlation of shoot dry weight and nodule numbers for strains used in both experiments.

Spearman rank correlation coefficients				
Shoot dry weight			Nodule number	
A. auriculiformis	*A. mangium*	*A. mearnsii*	*A. mangium*	*A. mearnsii*
0.25	0.72***	0.67***	0.72***	0.80***

*** = significant at *P* <0.001

Table 3.7

Rhizobial Specificity of Tree Legumes.

|	Specificity*	
Trees nodulating effectively with *Rhizobium*	Nodulation	Effectiveness
Leucaena leucocephala, Gliricidia sepium, Calliandra calothyrsus	S	S
Sesbania grandiflora	S	S
Robinia pseudoacacia	P	S
Trees nodulating effectively with *Bradyrhizobium*		
Acacia auriculiformis, Albizia lebbeck, Paraserianthes falcataria, Tephrosia candida	P	P
Acacia mearnsii	P	S
Acacia mangium	S	S
Lysiloma latisiliqua	S	S

*S = Specific, P = Promiscuous
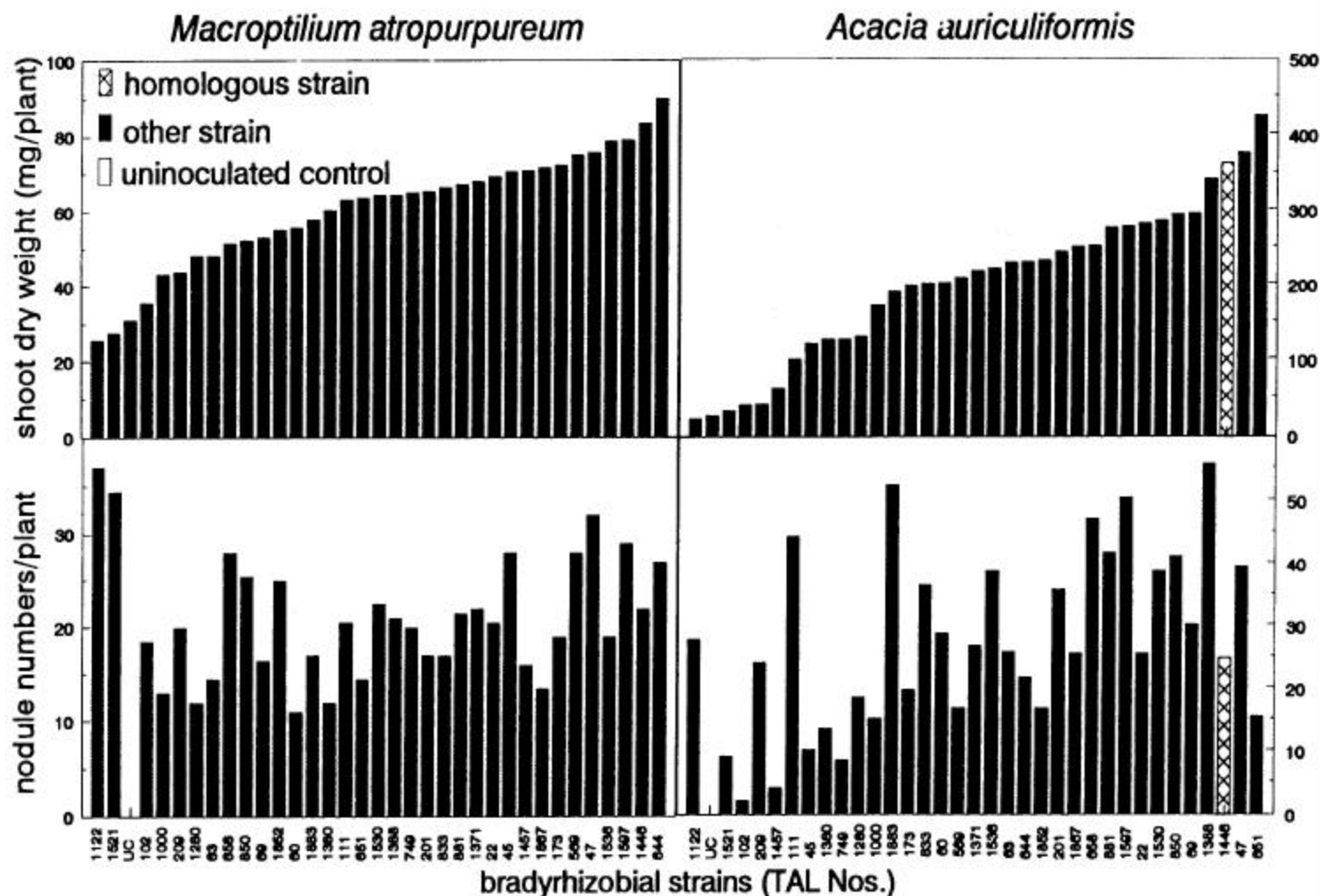
Figure 3.1. Nodule numbers and shoot dry weight of M. atropurpureum and Acacia auriculiformis inoculated with 34 bradyrhizobial strains in Pouch Experiment B.
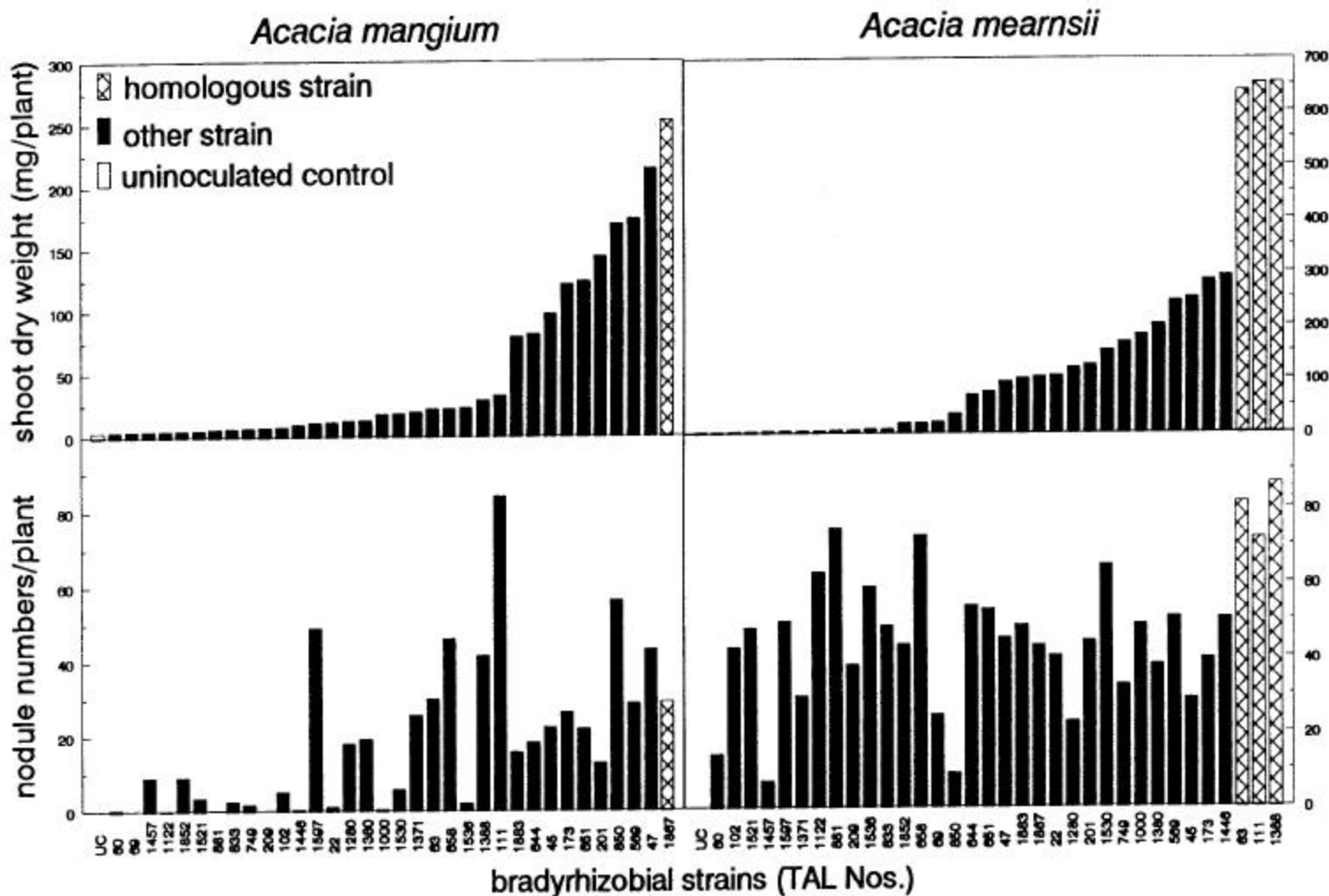
Figure 3.2. Nodule numbers and shoot dry weight of A. mangium and A. mearnsii inoculated with 34 bradyrhizobial strains in Pouch Experiment B.
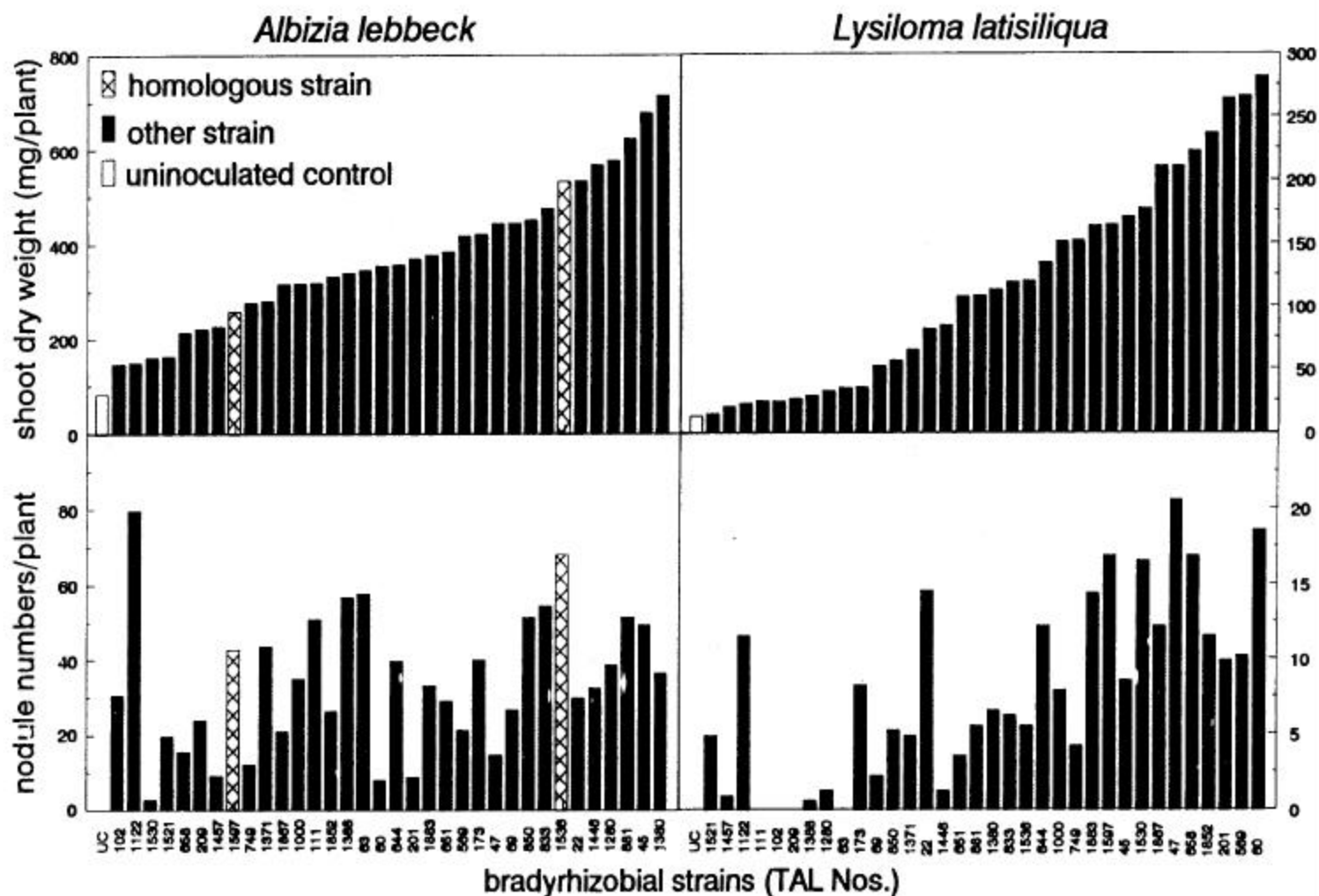
Figure 3.3. Nodule numbers and shoot dry weight of A. lebbeck and L. latisiliqua inoculated with 34 bradyrhizobial strains in Pouch Experiment B.
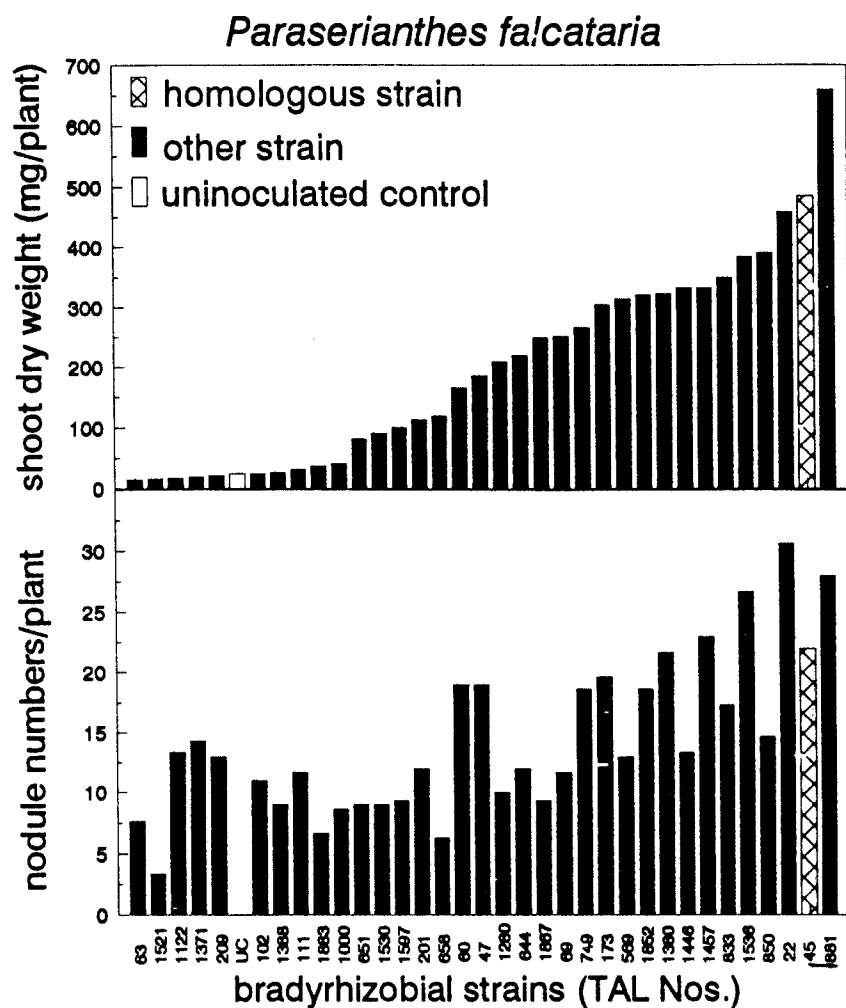
Figure 3.4. Nodule numbers and shoot dry weight
of Paraserianthes falcataria inoculated with 34
bradyrhizobial strains in Pouch Experiment B.

A

Acacia mearnsii
Acacia mangium
Lysiloma latisiliqua
Paraserianthes falcataria
Albizia lebbeck
Acacia auriculiformis
Macroptilium atropurpureum

B

Acacia mangium
Lysiloma latisiliqua
Paraserianthes falcataria
Albizia lebbeck
Acacia auriculiformis
Acacia mearnsii
Macroptilium atropurpureum

0.20   0.25   0.30   0.35   0.40
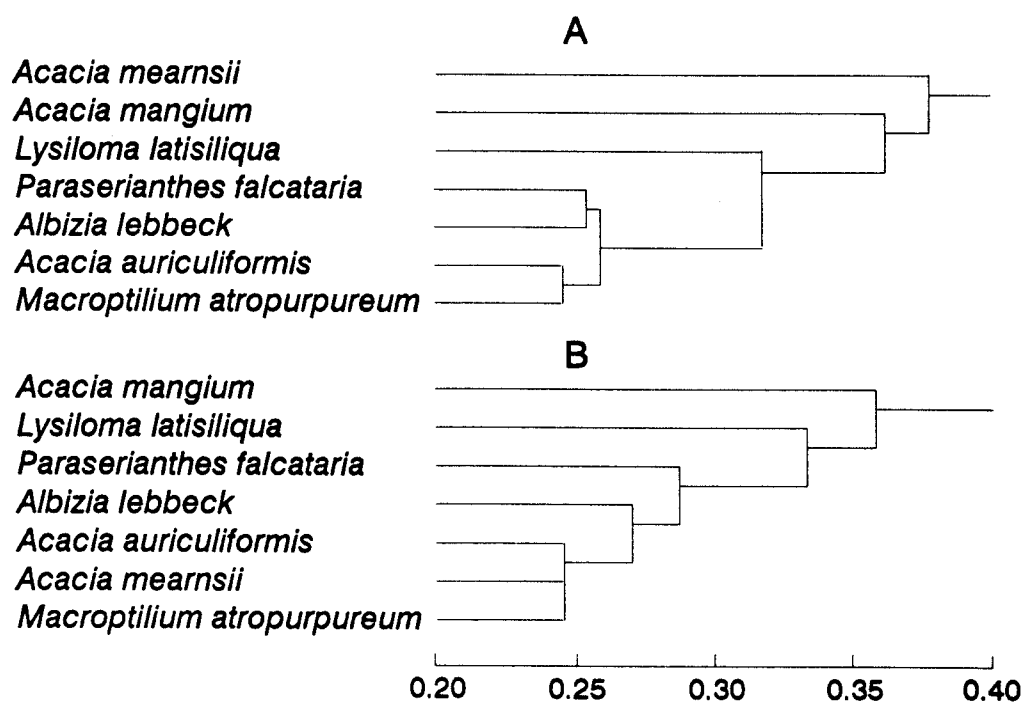
Figure 3.5. Single linkage cluster analysis by Euclidean distance of shoot dry weight (A) and nodule numbers (B) of legume species from Pouch Experiment B.
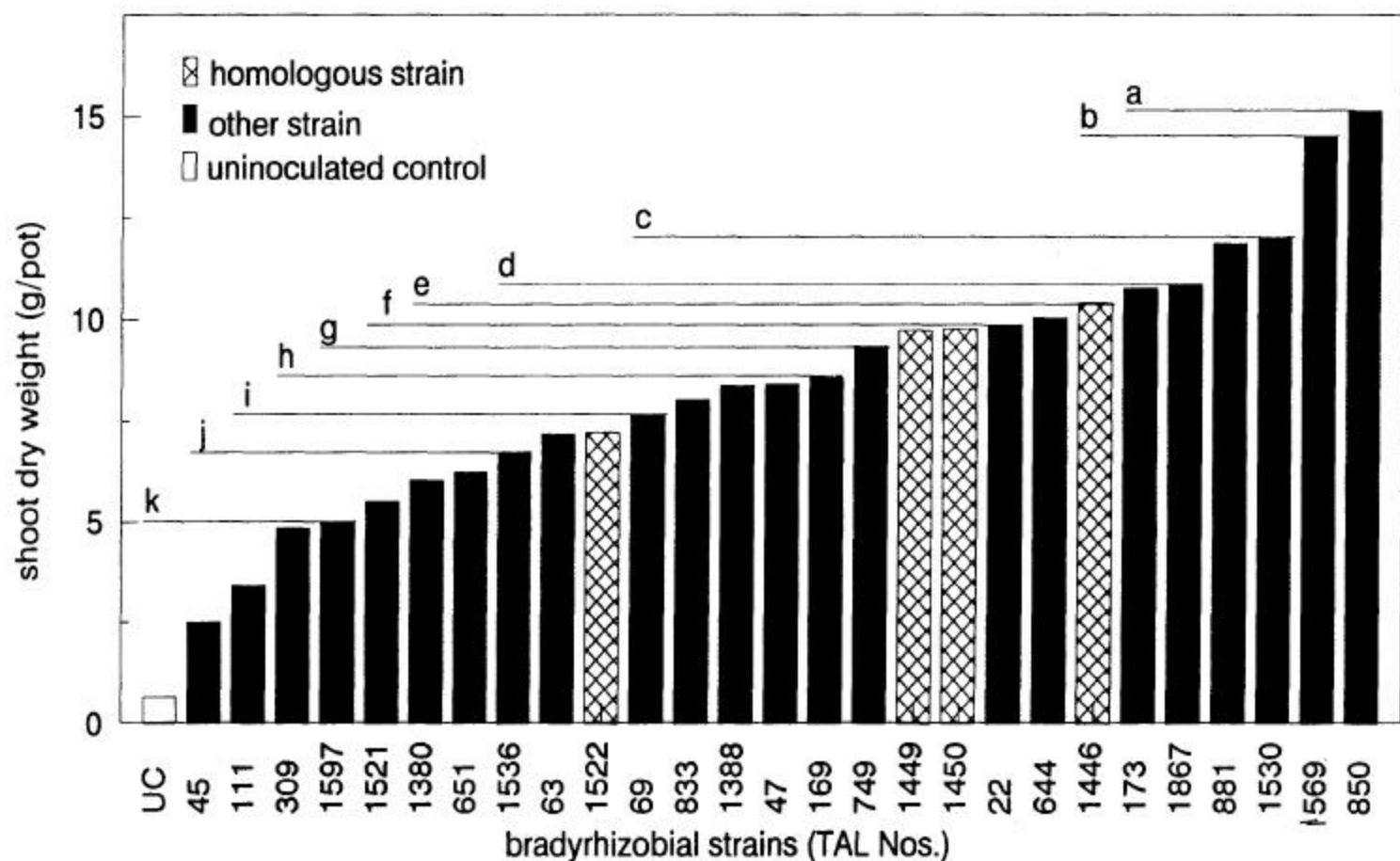
Figure 3.6. Shoot dry weight of Acacia auriculiformis inoculated with 27 bradyrhizobial strains in a pot experiment. Bars under a line are not significantly different (P < 0.05) by Tukey's HSD test.
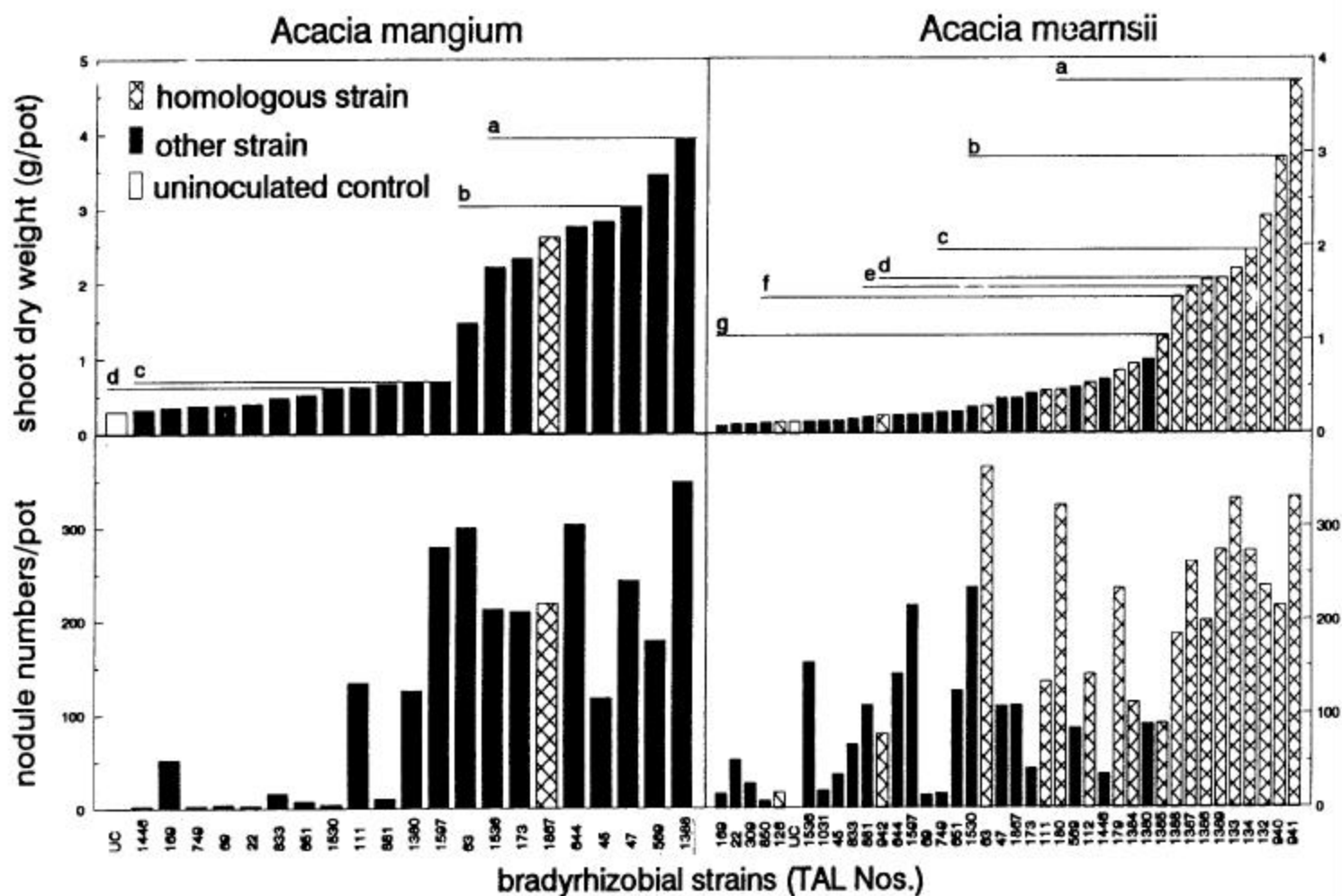
Figure 3.7. Shoot dry weight and nodule numbers of A. mangium and A. mearnsii from pot experiments. Bars under a line are not significantly different (P < 0.05) by Tukey's HSD test.

Chapter 4

Response of Tree Legumes to Rhizobial Inoculation

in Relation to the Population Density

of Indigenous Rhizobia

ABSTRACT

To determine the relationship between yield response to
inoculation and rhizobial population density for
leguminous trees, inoculation experiments were
conducted in pots containing four soils in a greenhouse
on the island of Maui, Hawaii, with *Acacia
auriculiformis*, *A. mangium*, *A. mearnsii*, *Leucaena
diversifolia*, *Robinia pseudoacacia*, and *Sesbania
grandiflora*. Densities of indigenous rhizobia were
determined by most-probable-number plant-infection
assays. Response to inoculation was calculated for
each species-soil combination as the increase in shoot
nitrogen due to inoculation. The magnitude of the
response was inversely related to the density of
rhizobia in the soil, with the greatest responses where
rhizobial densities were less than 50 rhizobia g^{-1} soil.
Tree species known to nodulate effectively with
Rhizobium had a higher proportion of significant
responses to inoculation than species known to nodulate
effectively with *Bradyrhizobium*. *R. pseudoacacia* and
A. mearnsii responded significantly ($P < 0.05$) to

inoculation in three and one soils respectively despite
the presence of more than 1000 rhizobia g^{-1} soil. A
hyperbolic model best described the relationship of the
increase in shoot dry weight due to rhizobial
inoculation to the density of indigenous rhizobia.
Incorporation of an index of available soil N into this
model resulted in models with lower residual mean
square values, indicating the important role of mineral
N in mediating responses to rhizobial inoculation.

INTRODUCTION

As natural forests are depleted and fallow periods
diminished in shifting agricultural systems of the
tropics, fast-growing nitrogen-fixing trees are
becoming more important as sources of fuelwood, fodder,
and nitrogen-rich biomass. Inoculation of tree seeds
or seedlings with rhizobia can insure that enough
highly effective rhizobia are present to satisfy the
trees' demand for biological nitrogen fixation (BNF).
Where available mineral N limits growth and appropriate
indigenous rhizobia are scarce or absent, inoculation
can increase yields and and monetary returns. However,
infrastructure and educational constraints often
prevent farmer use of rhizobial inoculant in the
tropics. Because of the expense involved in overcoming

these constraints, knowledge of if, and how much, inoculation is likely to increase yields can help farmers and regional planners make sound decisions concerning investment in rhizobial inoculant technology. In this paper, a method is evaluated for predicting the magnitude of response to inoculation for six tree legumes based on the density of indigenous rhizobia.

The quantification of factors regulating the response of legumes to inoculation has been proposed as an approach for assessing the magnitude of responses to inoculation with rhizobia without resorting to pot or field experiments (Singleton and Tavares, 1986; Brockwell et al., 1988). Major factors likely to influence response to inoculation are (i) limitations to plant growth other than N; (ii) density of indigenous rhizobia in soil; (iii) effectiveness of indigenous rhizobia; and (iv) available mineral N (Singleton et al., 1991a). A major advantage of this approach is that it permits extrapolation of results beyond the fields where soils were tested.

Models of the response to inoculation of field-grown grain and forage legumes (Thies, 1991b) indicated that when limitations other than N are removed, rhizobial density as estimated by the MPN assay is the

primary factor determining the magnitude of response to inoculation. Models that accounted for the effects of mineral N availability in addition to rhizobial density improved the agreement between observed and predicted inoculation responses. Singleton et al. (1991a) noted that measures of indigenous rhizobial population effectiveness have not proven to be good indicators of the magnitude of response to inoculation.

This study was conducted to determine the relationship of shoot N response to rhizobial inoculation of six tree legumes to an index of available mineral N and to the density of their indigenous rhizobial populations. A second purpose was to evaluate how specificity for nodulation and effectiveness of tree legumes (Chapter 3) relate to patterns of indigenous rhizobial densities and responses to inoculation.

MATERIALS AND METHODS

General Experimental Approach

Pot experiments were conducted in a greenhouse at 110 m elevation at Hamakuapoko, Maui, Hawaii. Four soils (Table 4.1) were selected, two with total indigenous rhizobial densities greater than 10 000 rhizobia g^{-1} soil, and two with rhizobial densities less

than 200 rhizobia g^{-1} soil, as determined by MPN counts
on five legumes representing the cross-inoculation
groups of the legumes growing at the sites (Woomer et
al., 1989a). *Leucaena leucocephala* was growing at one
low rhizobial density site and at one high density
site. Tree species were selected on the basis of
rhizobial specificity as determined in Chapter 3.
Three species known to nodulate with *Rhizobium*,
Leucaena diversifolia, *Robinia pseudoacacia*, and
Sesbania grandiflora, were from genera determined in
Chapter 3 to belong to distinct effectiveness groups.
The three species chosen that nodulate effectively with
Bradyrhizobium, *Acacia auriculiformis*, *A. mangium*, and
A. mearnsii, showed a range of specificity for
effectiveness and infectiveness (Chapter 3).

 Seed sources were the same as indicated in Chapter
2. *Macroptilium atropurpureum* cv. siratro, a
herbaceous forage legume which nodulates effectively
with many *Bradyrhizobium* (Vincent, 1970) was included
for comparison with the *Acacia* spp. Treatments were
(i) inoculation with rhizobia and (ii) no inoculation.
 Two species in each soil received mineral N to measure
yield potential in the growth system. The experiment
was divided into two. The trials in Pane and Makawao
soils were conducted from 7 December 1990 to 8 February

1991 and in Keahua and Waiakoa soils from 1 February

1991 to 5 April 1991. Pots were arranged in a

randomized complete block design, with four replicates.

 Results were analysed using SYSTAT, version 5.0

(Wilkinson, 1990), by dependent one-tailed t tests for

each species-soil combination.

Soil Collection and Plant Culture

At each soil collection site the soil was

excavated to a depth of 20 cm and passed through a

screen with a 5 mm opening. Five-liter black plastic

pots lined with polyethylene bags were filled with

2000, 3300, 3500, and 4520 g of soil (dry weight basis)

per pot for Pane, Makawao, Keahua, and Waiakoa soils

respectively. These weights produced approximately

equivalent volumes of each soil. The following

fertilizers were added (Kg^{-1} soil): 1.07 g K_2HPO_4, 0.25

g $MgSO_4 \cdot 7H_2O$, and 0.5 ml kg^{-1} of a liquid micronutrient

mix (Hawaiian horticultural mix, Monterey Chemical

Co.). A total of 450 mg of N Kg^{-1} soil as ammonium

nitrate was added to the mineral nitrogen controls at

three intervals: 50 mg kg^{-1} soil at the time of

inoculation, 150 mg kg^{-1} soil 3 weeks after inoculation,

and 150 mg kg^{-1} soil 6 weeks after inoculation.

Tree seeds were scarified and surface sterilized

as described in Chapter 2. *M. atropurpureum* seeds were

treated in the same manner as *L. diversifolia*, except
exposure to concentrated sulfuric acid was limited to 8
minutes. Imbibed seeds were planted in trays
containing expanded horticultural vermiculite (Grace
and Co.) 4 to 10 days prior to transplanting into the
pots. Four to eight seedlings were planted per pot and
thinned to four plants per pot after 3 weeks.
Following inoculation, the surface of each pot was
covered with 850 g of sterile washed road gravel to
guard against rhizobial contamination. Pots with *M.
atropurpureum* were provided with sterile stakes because
they have a climbing growth habit. Pots were watered
to field capacity every 3-4 days as required throughout
the course of the experiment.

Inoculation and Harvest

The inoculant consisted of a three strain mixture
of appropriate strains for each species (Table 4.2).
Each strain was grown separately for 6 to 10 days in
either yeast-extract manitol (YEM) broth (Vincent,
1970) for the first two soils or in arabinose-gluconate
medium (Sadowsky et al., 1987) for the second two
soils. Within 2 days after planting, each pot received
5 ml of a 10^{-3} dilution of the appropriate three-strain
mixture. The inoculum was applied evenly around the
roots of each seedling to give approximately 5 x 10^{6}

rhizobia per pot, an inoculation rate within recommended rates. The inoculant was washed into the soil with 50 ml of sterile water.

Plants were harvested 9 weeks after inoculation. Tops were severed at gravel level, dried at 70°C, weighed, ground, and analysed for N content using a Leco 600 C-H-N analyser. The root systems of three replicates were cleaned by washing over 1 mm mesh screens. Nodules were removed and counted, and both roots and nodules weighed after drying at 70°C.

Soil N Availability

N mineralization values for each soil were determined following the anaerobic incubation method of Keeney (1982). A standard curve was used to determine the amount of NH_4^+ present after distillation to avoid the need to standardize H_2SO_4. N mineralization values were multiplied by the dry weight of soil per pot and the number of weeks the plants were grown to yield an index of N mineralized per pot over the course of the experiment. Demand for symbiotic N was assessed by calculating the percent N derived from fixation (%NDF) of *S. grandiflora* in each soil. For each soil, %NDF was calculated as:

$$\%NDF = \frac{(N_{SI} - N_{SU})}{N_{SI}} \times 100$$

where N_{SI} = average total shoot N of *S. grandiflora* in
inoculated pots, and N_{SU} = average shoot N of *S.
grandiflora* in uninoculated pots. The few nodules
produced by *S. grandiflora* in uninoculated pots were
completely white in cross-section except for a single
plant in Pane soil. Shoot dry weight from this pot was
not used for the calculation of %NDF.

MPN assays

 MPN assays for each species-soil combination were
conducted using the best growth system identified for
each species in Chapter 2. For each of the two sets of
greenhouse experiments, plate counts and MPN assays of
pure rhizobial cultures (Table 4.2) were compared as
described in Chapter 2 to provide an evaluation of the
growth systems used, in accordance with the

recommendations of Thompson and Vincent, 1967; Scott
and Porter, 1986; and Singleton et al., 1991b.

 Soil for the MPN assays were taken just prior to
planting from extra pots used in the pot experiment.
Fifty g of soil (dry weight equivalent) was used for
the initial dilutions. Dilution ratios were 4.0 for *L.
diversifolia*, *R. pseudoacacia*, and *S. grandiflora* in
Pane and Makawao soils and 5.0 for all other species-

by-soil combinations. The amount of soil applied at
the lowest dilution level was 1 g for Pane and Makawao
soils, and 0.2 g for the other two soils. Eight
uninoculated controls were interspersed among the
growth units of each MPN assay.

Nodulation was assessed at 7 weeks after
inoculation. The most-probable-number was determined
with a computer program (Woomer et al., 1990), using as
initial dilution the highest dilution in which all four
replicates nodulated or, in the case of *A. mearnsii* in
Makawao soil where no dilution level had four nodulated
growth units, the third from last dilution level.

Models of Response to Inoculation

Models relating the response to inoculation to the
density of indigenous rhizobia were fit to the data
using a variety of functions with the 'nonlin' module
of the SYSTAT program version 5.0 (Wilkinson, 1990).
Response to
inoculation for each species-soil combination was
defined as

$$R = \frac{(N_{SI} - N_{SU})}{N_{SU}} \times 100$$

where R = response to inoculation and N_{SI} and N_{SU} are as
described for %NDF.

The indices of available mineral N and symbiotic N

demand were incorporated into a mathematical equation
to model the effect of mineral N and symbiotic N demand
on the relationship of rhizobial density to response to
inoculation. The model used was:

$$y = \frac{a}{1+MPN}$$

where y is shoot N increase due to inoculation
expressed as a percentage of the shoot N of plants in
uninoculated pots, a is the y-intercept, and x = 1 plus
the density of the indigenous rhizobial population,
expressed as the number of rhizobia g^{-1} soil on a dry
weight basis. Linear, exponential, power, and
hyperbolic functions of mineralized N and %NDF were
incorporated into this model through replacement of the
y-intercept.

Rhizobial Isolation and Effectiveness Tests

Rhizobia were isolated from at least 20 nodules of
R. pseudoacacia in both Makawao and Pane soils. These
rhizobia were selected for characterization because the
response of *R. pseudoacacia* in these two soils despite
the presence of over 20 000 rhizobia g^{-1} soil suggested
that the rhizobia were ineffective at fixing N_2. Random
nodules from extra uninoculated pots were used for
isolating the rhizobia. Rhizobia from each nodule were
isolated, authenticated, and their pH reactions on

bromothymol blue determined following methods described in Somasegaran and Hoben (1985).

Isolates from *R. pseudoacacia* from the two soils were inoculated onto agar slants containing legumes in the effectiveness groups of legume species nodulating with *Rhizobium* found growing at each soil collection site. The purpose was to determine the natural host(s) of the rhizobia that nodulated *R. pseudoacacia* from among the legumes growing at each site. The species used were: *Trifolium repens*, *Vicia* sp., and *Medicago sativa* in Pane soil and *T. repens* and *L. diversifolia* in Makawao soil.

An effectiveness test was conducted with 20 isolates from each soil. Isolates from each soil were grown for 7 days in YMB, mixed, and inoculated onto *R. pseudoacacia* in growth pouches (two plants/pouch) prepared as described in Chapter 3, using 1 ml of inoculum per pouch. Other pouches were inoculated in the same manner with a mixture of the three inoculant strains used in the pot experiments, or left uninoculated. Seven replicates were used per treatment in a completely randomized design. Plants were harvested after 8 weeks. Shoot dry weight was measured and analysed by analysis of variance with each soil analysed separately.

RESULTS

MPN Assays

   MPN results (Table 4.3) are species and soil specific. No rhizobia for *S. grandiflora* and *L. diversifolia* were detected in Pane soil where the MPN estimate for *R. pseudoacacia* was over 10^4 rhizobia g^{-1} soil. No rhizobia from *S. grandiflora* were detected in Makawao soil where the rhizobial densities of *R. pseudoacacia* and *L. diversifolia* were both greater than 10^4 rhizobia g^{-1} soil. All pure culture MPN estimates had plate count:MPN ratios less than 20.

Response to Mineral N and Inoculation with Rhizobia

   Response to mineral N was significant ($P< 0.05$) for all applicable species-soil combinations (Table 4.4).

   Response to inoculation as evaluated by total shoot nitrogen (Figure 4.1) was species and soil specific. Of 12 tree-soil combinations involving species that nodulate with *Rhizobium*, increased N accumulation was observed in 11 cases of which 9 were significant at $P < 0.05$, with another significant at $P < 0.10$. *L. diversifolia* did not respond significantly to inoculation in the two soils from sites where *L. leucocephala* occurred. Of 12 tree-soil combinations

involving species that nodulate with *Bradyrhizobium*,
increased N accumulation was observed in seven cases of
which *A. auriculiformis* in Waiakoa soil, *A. mangium* in
Keahua soil, and *A. mearnsii* in Pane and Keahua soils
were significant at $P < 0.05$. *M. atropurpureum* did not
respond significantly to inoculation in any soil.
Response to inoculation in terms of shoot dry weight
(Figure 4.2), and nodule dry weight (Figure 4.3) were
very similar to that of total shoot nitrogen: 13 and 11
total responses were significant respectively.
Increased root weight (Figure 4.2), significant in 8
cases, and nodule numbers (Figure 4.3), significant in
seven cases were not as good indicators of response to
inoculation. Correlation coefficients of total shoot
nitrogen with shoot mass, nodule mass, root mass, and
nodule number were significant ($P < 0.05$): 0.98, 0.88,
0.51, and 0.43, respectively.

Significant shoot nitrogen responses to
inoculation occurred in eight out of nine cases where
the rhizobial population was less than 50 rhizobia g^{-1}
soil. Where the population was greater than 50
rhizobia g^{-1} soil, significant responses were observed
in six out of 14 cases. Those species that responded
where the MPN estimate was greater than 50 rhizobia g^{-1}
soil were *R. pseudoacacia* in Pane, Makawao, and Waiakoa

soils, *A. mearnsii* in Pane and Keahua soils, and *A. auriculiformis* in Waiakoa soil. The mean shoot nitrogen response where the rhizobial density was less than 50 rhizobia g^{-1} soil was 375 %. Where the rhizobial density was greater than 50 rhizobia g^{-1} soil the mean response was 21.5 %.

A hyperbolic model, $y=596.341/(1+MPN)$, where y=increase in shoot N due to inoculation expressed as a percent of the shoot N of uninoculated plants, best described the relationship between rhizobial density and inoculation response (Table 4.5). The residual mean square for this model was lower than that of linear, quadratic, and power models. Models where the y-intercept of the hyperbolic model was replaced with functions of soil N availability and symbiotic N demand had even lower residual mean square values (Table 4.5).

R. pseudoacacia Effectiveness Tests and Characterization of *R. pseudoacacia* Rhizobial Isolates

All of the indigenous nodule isolates from *R. pseudoacacia* grown in Pane soil produced an acid reaction on bromothymol blue. They were distinctly different from isolates from *R. pseudoacacia* grown in Makawao soils which produced a neutral reaction. Of 23 rhizobial isolates from *R. pseudoacacia* in Pane soil,

18 nodulated both *Trifolium repens* and *Vicia* sp., and
five nodulated *T. repens* but not *Vicia* sp. *Medicago
sativa* did not form nodules with any of the strains.
Five of the strains that nodulated *T. repens* were
effective as evaluated by visual observation of greener
foliage and increased growth relative to uninoculated
and ineffectively nodulated plants. Fifteen of the
strains that nodulated *Vicia* sp. formed effective
symbioses as demonstrated by pink to red nodule color
in cross section. Of 20 *R. pseudoacacia* strains
isolated from Makawao soil, 16 nodulated *L.
diversifolia* effectively in tubes; 1 strain nodulated
T. repens ineffectively. The effectiveness test
revealed that the strains that nodulated *R.
pseudoacacia* in Pane soil were ineffective while those
from Makawao soil were at least moderately effective
(Table 4.7).

DISCUSSION

The primary goal of our experiment was to
determine the relationship between density of rhizobia
in soil and response to inoculation of six tree
legumes. The data and models generated from these
experiments indicate a precipitous decline in response
to inoculation as rhizobial density increases from 0 to

50 rhizobia g^{-1} soil. The relationship is very similar to those reported by Thies et al. (1991b) and Singleton et al. (1991a) for field-grown grain and forage legumes.

As in these experiments, Thies et al. (1991b), found that a hyperbolic model best described the relationship between rhizobial density and increase in economic yield due to inoculation. Measures of soil N availability and symbiotic N demand improved the fit of their predictive models, as they did with the model developed from our data. Our data therefore suggest that the relationship between rhizobial density and response to inoculation is fundamentally the same with trees compared with grain and forage legumes and that mineral N has a similar effect on woody and non-woody legumes.

Species differences in MPN results and response to inoculation are consistent with what is known about the effectiveness groups of the trees used in this study. The data support the general view (Dommergues, 1987; Dreyfus and Dommergues, 1981; Peoples et al., 1989) that species nodulating with *Rhizobium* respond more often to inoculation than species nodulating with *Bradyrhizobium*. The data also support the separation of *Sesbania*, *Leucaena*, and *Robinia* into distinct

effectiveness groups (Chapter 3) since both the density of indigenous rhizobia and magnitude of response to inoculation were species dependent. Effective nodulation of *R. pseudoacacia* with rhizobia capable of effective nodulation with *L. diversifolia* is consistent with observations of a moderately effective symbiosis between *R. pseudoacacia* and TAL 1145 (Chapter 3). The response of *R. pseudoacacia* to inoculation in the presence of effective strains does not support the conclusion of Singleton and Tavares (1986) that no response will occur in the presence of a large rhizobial population as long as some strains are effective.

The inoculation response of *A. mearnsii* in Pane soil despite a rhizobial population of over 10 000 rhizobia g^{-1} soil supports the finding in Chapter 3 that *A. mearnsii* is promiscuous for nodulation but specific for effectiveness within the cowpea cross-inoculation group. The lower MPN estimates of *A. mangium* relative to *A. auriculiformis*, *A. mearnsii*, and *M. atropurpureum* in two soils support the finding in Chapter 3 that *A. mangium* is specific for infectiveness relative to these species.

The fact that *A. mangium* in Waiakoa soil accumulated substantially less N in uninoculated and

inoculated pots relative to uninoculated *S. grandiflora* plants suggests that something other than rhizobia may have been limiting growth. The response of *A. mangium* to mineral N in this soil suggests that starter N may have been required.

Based on the tendency of MPN estimates of rhizobia in pure culture to be less than plate counts of the same cultures, a conservative recommendation would be to inoculate trees where MPN populations are less than 1000 rhizobia g^{-1} soil. If this recommendation is compared with actual results, inoculation would have been recommended for 14 of 24 tree-soil combinations, 10 of which responded to inoculation, but incorrectly recommending four species-soil combinations remain uninoculated where significant inoculation responses in fact occurred: *R. pseudoacacia* in Pane, Makawao, and Waiakoa soils, and *A. mearnsii* in Pane soil. When effectiveness groups are taken into consideration, *R. pseudoacacia* can be expected to respond to inoculation in all soils because no legumes within its effectiveness group (in this case only *R. pseudoacacia* and perhaps other species in the genus *Robinia*) are present at any of the sites. In the same way *A. mearnsii* can be expected to respond to inoculation in Pane soil. With Makawao soil, although no *A. mearnsii*

trees were present at the collection site, they do grow naturally in the general vicinity, which may explain the lack of response to inoculation in soil from this site.

In conclusion, leguminous trees respond to inoculation where rhizobial densities are low. As with grain and forage legumes, the magnitude of response to inoculation in tree legumes is inversely related to the density of rhizobia in the soil, with the greatest responses where rhizobial densities are less than 50 rhizobia g^{-1} soil. These experiments reveal that some tree legumes respond to inoculation despite the presence of high population densities of infective rhizobia in the soil, notably *R. pseudoacacia* and *A. mearnsii*. The reason appears to be that these species are specific for
effectiveness but promiscuous for nodulation relative to indigenous rhizobia.

Table 4.1

Soils used in inoculation experiments and MPN assays.

Soil Series[a]	Soil Classification[b]	Maui Net Site	Mean Annual Rainfall[c] mm year⁻¹	Mineralized N g pot⁻¹[d]	%NDF[e]	pH[f]	Legumes Present at Site
Pane	Andisol	Pasture Kekoa	1060	5.35	79.7	6.2	*Desmodium* sp., *Medicago* sp., *Trifolium repens, Vicia sativa*
Makawao	Humoxic Tropohumult	Haleakala Station	1800	2.32	75.7	5.4	*Desmodium* sp., *Trifolium repens, Leucaena leucocephala*
Keahua	Aridic Haplustoll	Pasture Waiakoa	380	2.21	86.8	6.2	*Crotalaria* spp., *Prosopis* sp.
Waiakoa	Aridic Haplustoll	Hashimoto farm	322	0.94	92.2	6.3	*L. leucocephala, Crotalaria* spp., *Indigofera* sp.

[a] from Soil Conservation Service, 1972.
[b] from Dr. H. Ikawa, personal communication.
[c] from Department of Land and Natural Resources, 1982.
[d] calculated as g N/g of soil/week * 9 weeks * g soil/pot.
[e] % Nitrogen derived from fixation for *Sesbaina grandiflora*.
[f] 1:1 with water.

Table 4.2

Strains used in inoculation experiments and MPN assays.

Species	TAL No.	Original Host	Other Names
Acacia auriculiformis	* 569	*Desmodium uncinatum*	MAR 472
	1388	*Acacia mearnsii*	-
	*1446	*Acacia auriculiformis*	-
Acacia mangium	569	*Desmodium uncinatum*	MAR 472
	1388	*Acacia mearnsii*	-
	*1867	*Acacia mangium*	LB 5
Acacia mearnsii	132	*Acacia mearnsii*	-
	940	*Acacia mearnsii*	NUM 777
	* 941	*Acacia mearnsii*	NUM 778
Leucaena diversifolia	583	*Leucaena leucocephala*	NGR 8
	*1145	*Leucaena leucocephala*	CIAT 1967
	1887	*Leucaena leucocephala*	MS 111
Robinia pseudoacacia	183	*Robinia pseudoacacia*	NIT 137A4
	*1889	*Robinia pseudoacacia*	USDA 3436
	1907	*Robinia pseudoacacia*	USDA 3112
Sesbania grandiflora	1113	*Sesbania sp.*	IC 70
	1114	*Sesbania sp.*	IC 71
	*1119	*Sesbania sp.*	IC91
Macroptilium atropurpureum	169	*Vigna unguiculata*	NIT 176A22
	* 209	*Vigna radiata*	-
	1000	*Arachis hypogaea*	-

* = strain used for comparison of pure culture MPN estimates with plate counts.

Table 4.3. Rhizobial densities of six tree legumes and *Macroptilium atropurpureum* in four tropical soils and associated comparisons of pure culture plate counts with pure culture MPN estimates.

Species	Growth System	Soil				Pure Rhizobial Culture	
		Pane	Makawao	Keahua	Waiakoa	Pane & Makawao soils	Keahua & Waiakoa soils
		-rhizobia g^{-1} soil-				-Plate Count:MPN Ratio-	
Acacia auriculiformis	Tube	> 2510	> 26300	8.6	366	5.0	5.2
Acacia mangium	Tube	21.9	> 35600	41.3	6.8	17.9	5.0
Acacia mearnsii	Tube	49400	>227000	135	320	0.6	3.0
Leucaena diversifolia	Tube	< 0.2	>338000	6.1	9670	1.0	3.0
Robinia pseudoacacia	Pouch	21600	> 21600	39.5	6480	2.0	15.4
Sesbania grandiflora	Pouch	< 0.2	< 0.2	1.0	< 1.1	6.5	i.f.
Macroptilium atropurpureum	Pouch	18400	10500	6.5	27.2	i.f.[c]	14.9

[a] i.f. = inoculant failure: colonies formed on plates but plants did not nodulate.

Table 4.4

Shoot dry weight response to mineral N of six tree legumes.

Species	Soil	+N	Inoculated	Uninoculated
			--g pot^{-1}--	
Acacia mearnsii	Pane	24.5 a	9.1 b	6.5 b
Sesbania grandiflora	Pane	23.5 a	17.5 b	6.7 c
Leucaena diversifolia	Makawao	25.2 a	12.1 b	11.0 b
Acacia auriculiformis	Keahua	14.3 a	5.4 b	5.7 b
Robinia pseudoacacia	Keahua	20.4 a	8.8 b	2.4 c
Acacia mangium	Waiakoa	14.5 a	1.0 b	1.2 b
Sesbania grandiflora	Waiakoa	40.2 a	21.9 b	3.6 c

For each species-soil combination, means followed by the same letter are not significantly different by Tukey's HSD.

Table 4.5

Models of shoot N response to inoculation using measures of rhizobial density, mineralized N, and BNF potential.

Model	Coefficients			Residual Mean Square
	a	b	c	
Models with rhizobial density alone				
$a + bx$	191.0	-0.001		77550
ax^{-b}	550.3	20.84		54137
$a + b(\log x) + c(\log x)^2$	525.8	-129.7	7.368	41861
a/x	596.3			38467
Models with rhizobial density and mineralized N pot^{-1}				
$(a + bm)/x$	1105	-149.1		24883
$(ae^{-bm})/x$	1461	0.322		22184
$(am^{-b})/x$	1106	0.755		19630
$(a + b/m)/x$	155.4	947.9		19360
Models with rhizobial density and %NDF				
$(a + b/n)/x$	5521	-408196		18339
$(a + bn)/x$	-4370	59.63		17248
$(ae^{bn})/x$	0.143	0.098		15455

$x = 1 + MPN$ estimate; $y =$ shoot nitrogen response to inoculation expressed as a % of the uninoculated treatment; $a =$ y-intercept; $m = N$ mineralized pot^{-1}, $n = $ %NDF of *S. grandiflora*.

Table 4.6. Effectiveness of indigenous vs. inoculant rhizobial strains on *Robinia pseudoacacia* in growth pouches.

Treatment	Shoot dry weight g pouch-1
Pane soil	
inoculant strains	0.356 a*
indigenous strains	0.071 b
uninoculated control	0.063 b
Makawao soil	
inoculant strains	0.353 a
indigenous strains	0.275 a
uninoculated control	0.064 b

For each soil, means followed by the same letter are not significantly different ($P < 0.05$) by Tukey's HSD

Figure 4.1. Shoot N of six tree legumes and *Macroptilium atropurpureum* grown in four soils with and without inoculation.
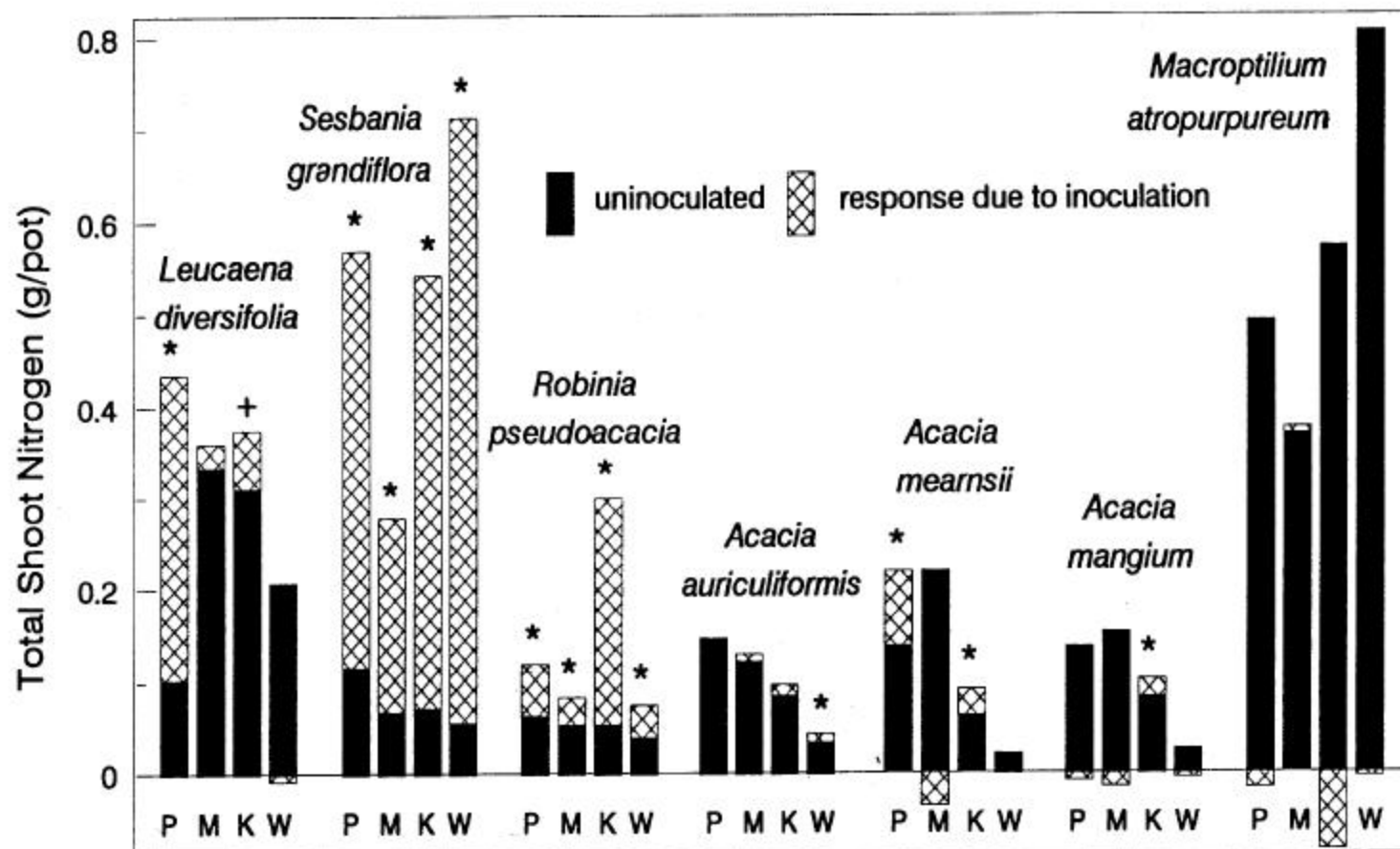
*, +: significant increase due to inoculation (P < 0.05 and P < 0.10 respectively)
soils: P = Pane, M = Makawao, K = Keahua, W = Waiakoa
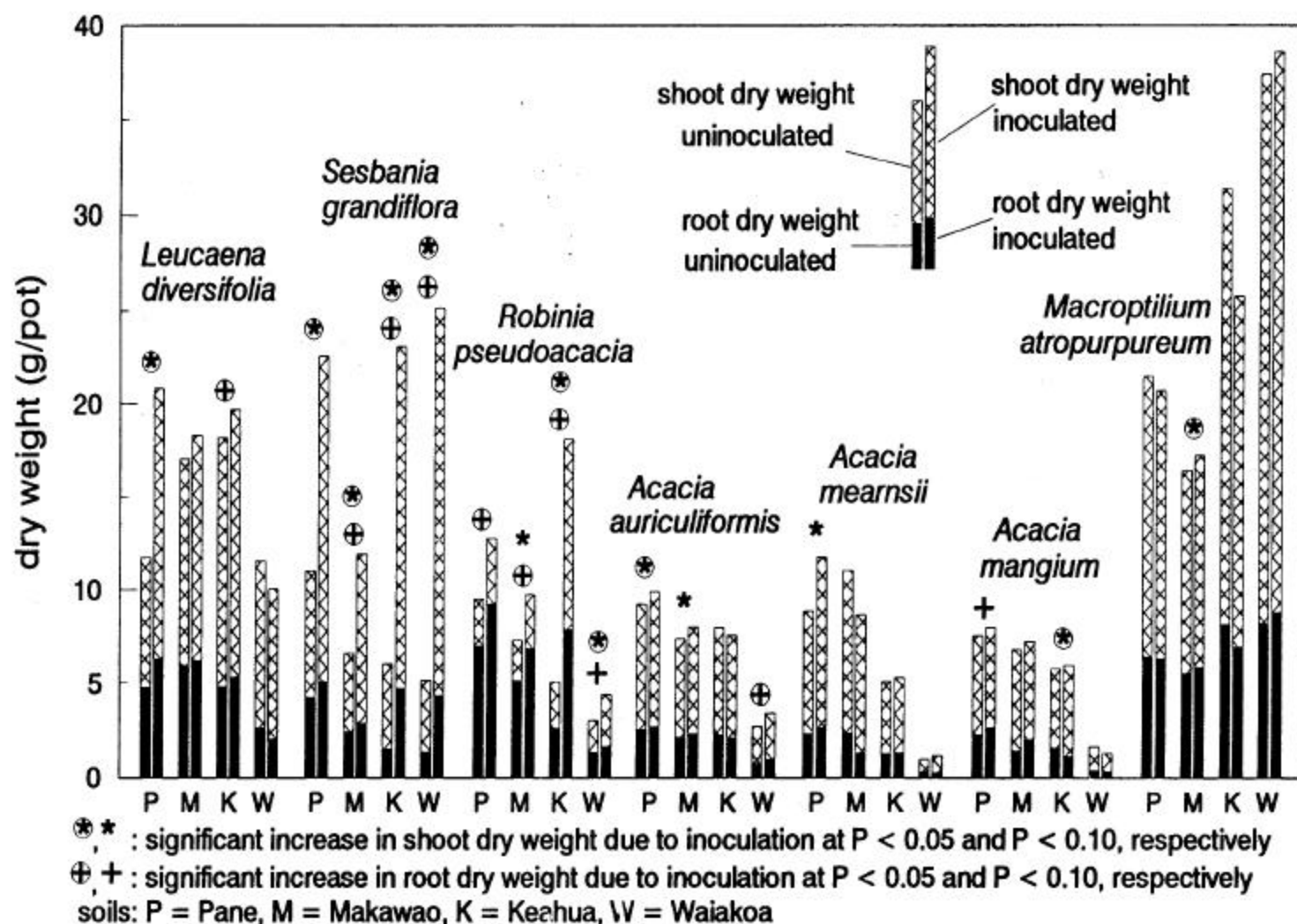
Figure 4.2. Shoot and root dry weight of six tree legumes and *Macroptilium atropurpureum* grown in four soils with and without inoculation.
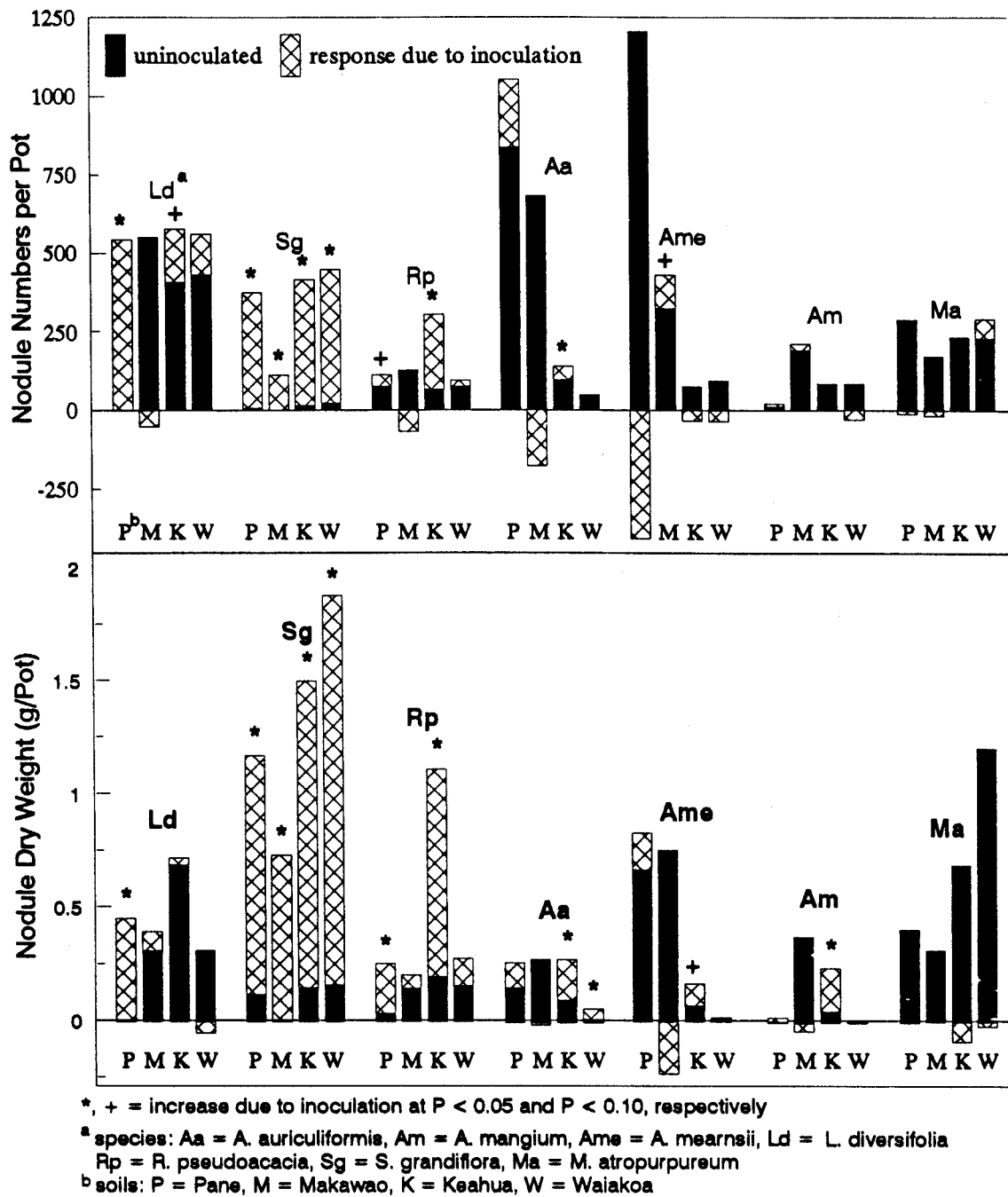
Figure 4.3. Nodule weight and numbers of six tree legumes and *Macroptilium atropurpureum* grown in four soils with and without inoculation.

*, + = increase due to inoculation at P < 0.05 and P < 0.10, respectively

[a] species: Aa = A. auriculiformis, Am = A. mangium, Ame = A. mearnsii, Ld = L. diversifolia
Rp = R. pseudoacacia, Sg = S. grandiflora, Ma = M. atropurpureum
[b] soils: P = Pane, M = Makawao, K = Keahua, W = Waiakoa

Chapter 5

Thesis Conclusion

Despite the passage of over a century since BNF in trees was first studied (Allen and Allen, 1981), knowledge of rhizobial relationships among tree species remains far behind that of grain and forage legumes. This thesis has added to the body of knowledge by taking techniques and approaches developed with grain and forage legumes and applying them to trees. Specifically, rhizobial effectiveness groups were determined for

a group of important tree legumes and this knowledge used in conjunction with measures of the density of indigenous rhizobial populations to determine the relationship between rhizobial density and response to inoculation.

Some of the contributions from this thesis are demonstration that reasonably accurate MPN estimates can be obtained with tree legumes, extension of the effectiveness group of *Leucaena* to include *Calliandra calothyrsus*, discovery that *Robinia pseudoacacia* and *Acacia mearnsii* are promiscuous for nodulation but specific for effectiveness, and confirmation that *Acacia mangium* is specific for both nodulation and effectiveness. Of practical importance, the data

indicate that the approach developed for predicting yield response with grain and forage legumes can also be used with trees.

As a general conclusion, trees appear fundamentally similar to grain and forage legumes with respect to their rhizobial relations. As with grain and forage legumes, one rhizobial cell appears sufficient to cause nodule formation, a basic assumption of the MPN assay. Like other legumes, trees that nodulate effectively with *Rhizobium* fall into rather distinct effectiveness groups and species that nodulate effectively with *Bradyrhizobium* exhibit a range of specificity for both nodulation and effectiveness, without sharply delineated effectiveness groups. The minimal rhizobial density required for maximal BNF appears to be the same for trees and other legumes, in the order of 50 to 100 rhizobia g^{-1} soil as determined by the MPN assay. As with other legumes, mineral N attenuates the response to inoculation as indicated by the improved fit of models that included an index of available mineral N.

Several aspects of tree-rhizobia relationships touched on in this thesis warrant further investigation. In Chapter 2, although fairly accurate MPN estimates were obtained with 11 of 14 species,

other growth systems need to be evaluated for the remaining species. Open tubes and Leonard jars might be worth consideration.

There has been very little published information pertaining to strain selection for tree legumes. Evidence of rhizobial specificity in tree legumes (Chapter 3), especially for homologous strains, emphasizes the need to evaluate strains likely to have evolved in symbiosis with the species in question.

The response to inoculation of *R. pseudoacacia* and *A. mearnsii* in soils where rhizobial density was high emphasizes the need to develop tests for effectiveness to identify situations where species are likely to respond to inoculation despite high rhizobial densities. In this regard, an effectiveness test was conducted in association with the experiments presented in Chapter 4, but for several reasons it failed to identify tree-soil combinations where responses to inoculation occurred despite high rhizobial densities (Appendix C).

Uninoculated and inoculated treatments of *A. mangium* grew very poorly in Waiakoa soil despite a dramatic response to mineral N (Table 4.4). The fact that Waiakoa soil had the lowest available soil N as measured by the N mineralization test, and that

inoculation increased yield of other tree species suggest that *A. mangium* has a higher requirement for starter N than species such as *S. grandiflora*. This and evidence that fast-growing species differ in their ability to fix N_2 in the presence of different levels of mineral N (George and Singleton, 1989) suggests that further work on relationships between N_2 fixation and mineral N might be fruitful, especially for species grown in association with food crops.

Appendix A

Generic determination of root nodule bacteria used in this thesis[*].

Strain		Genus[*]	BTB reaction[*]	XGal Reaction[*]	Growth[*] on:	
Tal No.	Original host				Sucrose	Lactose
1457	Acacia albida	Bradyrhizobium	alkaline	-		
1446	Acacia auriculiformis	Bradyrhizobium	alkaline	-		
1449	Acacia auriculiformis	Bradyrhizobium	alkaline	-		
1450	Acacia auriculiformis	Bradyrhizobium	alkaline	-		
1521	Acacia auriculiformis	Bradyrhizobium	alkaline	-		
1522	Acacia auriculiformis	Bradyrhizobium	alkaline	-		
881	Acacia koa	Bradyrhizobium	alkaline	-		
1867	Acacia mangium	Bradyrhizobium	alkaline	-		
1869	Acacia mangium	Rhizobium	acid	+		
63	Acacia mearnsii	Bradyrhizobium	alkaline	-		
111	Acacia mearnsii	Bradyrhizobium	alkaline	-		
112	Acacia mearnsii	Bradyrhizobium	alkaline	-		
126	Acacia mearnsii	Bradyrhizobium	alkaline	-		
132	Acacia mearnsii	Bradyrhizobium	alkaline	-		
133	Acacia mearnsii	Bradyrhizobium	alkaline	-		
134	Acacia mearnsii	Bradyrhizobium	alkaline	-		
179	Acacia mearnsii	Bradyrhizobium	alkaline	-		
180	Acacia mearnsii	Bradyrhizobium	alkaline	-		
940	Acacia mearnsii	Bradyrhizobium	alkaline	-		
941	Acacia mearnsii	Bradyrhizobium	alkaline	-		
942	Acacia mearnsii	Bradyrhizobium	alkaline	-		
1384	Acacia mearnsii	Bradyrhizobium	neutral	-		
1385	Acacia mearnsii	Bradyrhizobium	alkaline	-		
1386	Acacia mearnsii	Bradyrhizobium	alkaline	-		
1387	Acacia mearnsii	Bradyrhizobium	alkaline	-		
1388	Acacia mearnsii	Bradyrhizobium	alkaline	-		
1389	Acacia mearnsii	Bradyrhizobium	alkaline	-		
1852	Albizia caribaea	Bradyrhizobium	alkaline	-		
363	Albizia lebbeck	Bradyrhizobium		-	-	-
1536	Albizia lebbeck	Bradyrhizobium	alkaline	-		
1597	Albizia lebbeck	Bradyrhizobium	alkaline	-	-	-
1122	Albizia stipulata	Bradyrhizobium	alkaline	-		
1000	Arachis hypogaea	Bradyrhizobium	alkaline	-		
1371	Arachis hypogaea	Bradyrhizobium	alkaline	-		
33	Calliandra calothyrsus	Rhizobium		+		
1801	Calliandra calothyrsus	Rhizobium	acid	+		
1455	Calliandra surinamensis	Rhizobium	acid	+	+	+
651	Calopogonium mucunoides	Bradyrhizobium	alkaline	-		
201	Canavalia ensiformis	Bradyrhizobium	alkaline	-		
1380	Crotalaria paulina	Bradyrhizobium	alkaline	-		
850	Crotalaria sp.	Bradyrhizobium	alkaline	-		
569	Desmodium uncinatum	Bradyrhizobium	alkaline	-		
47	Enterolobium cyclocarpum	Bradyrhizobium	alkaline	-		
60	Enterolobium cyclocarpum	Bradyrhizobium	alkaline	-		
1530	Enterolobium cyclocarpum	Bradyrhizobium	alkaline	-		
69	Erythrina indica	Bradyrhizobium	alkaline	-		
749	Erythrina indica	Bradyrhizobium	alkaline	-		
1883	Flemingia macrophylla	Bradyrhizobium	alkaline	-		
1788	Gliricidia maculata	Rhizobium		+		
7	Gliricidia sepium	Rhizobium	acid	+		
1770	Gliricidia sepium	Rhizobium	acid	+		
1806	Gliricidia sepium	Rhizobium	acid	+	+	+
102	Glycine max	Bradyrhizobium	alkaline	-		
1908	Glycine max	Bradyrhizobium	alkaline	-		
82	Leucaena leucocephala	Rhizobium	acid		+	+
582	Leucaena leucocephala	Rhizobium		+	+	+
583	Leucaena leucocephala	Rhizobium	acid	+		
1145	Leucaena leucocephala	Rhizobium	acid	+	+	+
1887	Leucaena leucocephala	Rhizobium	acid	-	+	+

Generic determination of root nodule bacteria used in this thesis

Strain		Genus[a]	BTB[b] reaction	X-Gal[c] Reaction	Growth[e] on:	
Tal No.	Original host				Sucrose	Lactose
309	*Macrotyloma africanum*	*Bradyrhizobium*	alkaline	-		
45	*Paraserianthes falcataria*	*Bradyrhizobium*	alkaline	-		
644	*Phaseolus acutifolius*	*Bradyrhizobium*	alkaline	-		
22	*Phaseolus lunatus*	*Bradyrhizobium*	alkaline	-		
183	*Robinia pseudoacacia*	*Rhizobium*	neutral	-	+	+
1889	*Robinia pseudoacacia*	*Rhizobium*	acid	-	+	-
1907	*Robinia pseudoacacia*	*Rhizobium*	acid	+		
833	*Samanea saman*	*Bradyrhizobium*	alkaline	-		
1280	*Samanea saman*	*Bradyrhizobium*	alkaline	-		
1779	*Sesbania grandiflora*	*Rhizobium*	acid	-	+	+
1042	*Sesbania longifolia*	*Rhizobium*	acid	+		
1886	*Sesbania longifolia*	*Rhizobium*	acid	+		
674	*Sesbania rostrata*	*Rhizobium*	acid	+		
1829	*Sesbania rostrata*	*Azorhizobium[f]*	alkaline	-	-	-
1113	*Sesbania sp.*	*Rhizobium*	acid	+		
1114	*Sesbania sp.*	*Rhizobium*	acid	+		
1119	*Sesbania sp.*	*Rhizobium*	acid	+		
1137	*Sesbania sp.*	*Rhizobium*	acid	+		
658	*Stylosanthes sp.*	*Bradyrhizobium*		-	-	-
795	*Tephrosia glauca*	*Bradyrhizobium*	alkaline	-		
209	*Vigna radiata*	*Bradyrhizobium*	alkaline	-		
169	*Vigna unguiculata*	*Bradyrhizobium*	alkaline	-		
173	*Vigna unguiculata*	*Bradyrhizobium*	alkaline	-	-	-

[a] = tests conducted by H.H. Keyser.

[b] = Rhizobium if XGal reaction positive or strain grows on either sucrose or lactose; = Bradyrhizobium if BTB reaction is alkaline and XGal test is negative.

[c] = reaction on bromothymol blue agar determined between 3 and 7 days after plating.

[d] = IPTG -XGal test for detection of ß-galactosidase activity α-complementation (Sambrook et al., 1989). Determination made 10 days after plating.

[e] = growth on dissacharides determined after 7 days.

[f] = a characterized strain of *Azorhizobium*.

Data for individual MPN assays.

Species	Strain TAL #	Growth System	Dilution Factor	MPN Code	MPN Estimate	Factor for 95% C.I. of MPN	Plate Count	95% Confidence Interval for Plate Count	PC within C.I. of MPN	PC:MPN Ratio	Nodulated Uninoculated Controls	# of Weeks to Scoring
Acacia auriculiformis	569	pouch	3.9	43100000	4.22E+06	2.64	3.15E+09	2.510E+09 - 3.79E+09	N	746.4	0/8	7
	1446	pouch	3.9	43111000	2.61E+06	2.64	5.84E+09	5.100E+09 - 6.58E+09	N	2237.5	0/8	7
	1446	pouch	10.0	2100000	9.25E+04	3.80	1.96E+09	1.600E+09 - 2.32E+09	N	21189.2	1/4	5
	651	pouch	4.3	00000000	<1.73E+06	2.74	7.91E+08	6.90E+08 - 8.92E+08	N	>457.2*	0/8	5
	1446	tube	3.9	4434430	1.16E+09	2.64	5.84E+09	5.10E+09 - 6.58E+09	N	5.0	0/8	7
	569	tube	3.9	443412000	6.10E+08	2.64	3.15E+09	2.51E+09 - 3.79E+09	N	5.2	0/8	7
	651	tube	4.3	40000000	1.18E+07	2.74	7.91E+08	6.90E+08 - 8.92E+08	N	67.0	0/8	5
Acacia mangium	1867	pouch	3.9	44321100	1.27E+07	2.64	6.57E+09	5.92E+09 - 7.22E+09	N	517.3	0/8	7
	1867	pouch	4.0	441000	7.76E+05	2.68	5.90E+08	4.87E+08 - 6.93E+08	N	760.3	0/8	4
	1867	pouch	3.9	200000000	4.20E+05	2.64	2.45E+09	2.03E+09 - 2.87E+09	N	5833.3	0/8	7
	1867	pouch	4.1	000000	<1.30E+06	2.68	5.90E+08	4.87E+08 - 6.93E+08	N	>453.8*	0/8	5
	1867	tube	3.9	44433100	4.88E+08	2.64	2.45E+09	2.03E+09 - 2.87E+09	N	5.0	0/8	7
	1867	tube	3.9	4444332211	3.68E+08	2.64	6.57E+09	5.92E+09 - 7.22E+09	N	17.9	0/8	7
	1867	tube	4.1	433000	5.20E+07	2.68	1.04E+09	8.91E+08 - 1.19E+09	N	20.0	0/8	5
	1867	tube	4.1	441000	4.96E+07	2.68	1.04E+09	8.91E+08 - 1.19E+09	N	21.0	0/8	5
Acacia mearnsii	941	pouch	3.9	44444410	6.05E+08	2.64	3.34E+09	2.98E+09 - 3.70E+09	N	5.5	0/8	7
	941	pouch	3.9	43110000	2.03E+07	2.64	8.11E+08	6.64E+08 - 9.58E+08	N	40.0	0/8	7
	1388	pouch	4.0	430100	4.75E+05	2.68	3.13E+08	2.54E+08 - 3.72E+08	N	658.9	0/4	4
	940	pouch	4.1	0000000	<1.30E+06	2.68	1.32E+08	1.17E+08 - 1.47E+08	N	>101.5*	0/8	5
	941	tube	3.9	4444434210	5.84E+09	2.64	3.34E+09	2.98E+09 - 3.70E+09	Y	0.6	0/8	7
	941	tube	3.9	34400000	2.70E+08	2.64	8.11E+08	6.64E+08 - 9.58E+08	Y	3.0	0/8	7
	940	tube	4.1	43220000	6.07E+07	2.68	1.32E+08	1.17E+08 - 1.47E+08	Y	2.2	0/8	5
	940	tube	4.1	4321100	6.01E+07	2.68	1.32E+08	1.17E+08 - 1.47E+08	Y	2.2	0/8	5
Albizia lebbeck	1536	pouch	10.0	3412000	7.97E+06	3.80	9.47E+08	7.78E+08 - 1.12E+09	N	118.8	0/7	5
Albizia saman	833	pouch	10.0	1000000	2.55E+04	3.80	3.27E+08	2.51E+08 - 4.03E+08	N	12823.5	0/5	5
Calliandra calothyrsus	1455	pouch	10.0	4444100	3.59E+08	3.80	7.60E+08	6.57E+08 - 8.63E+08	Y	2.1	0/2	5
Flemingia macrophylla	1883	pouch	10.0	42110000	1.26E+06	3.80	1.09E+09	8.10E+08 - 1.37E+09	N	865.1	0/4	6
Gliricidia sepium	1145	pouch	10.0	44400000	2.31E+07	3.80	3.00E+08	1.65E+08 - 4.35E+08	N	13.0	0/5	6
	1806	pouch	10.0	4444300	1.12E+09	3.80	1.25E+09	1.06E+09 - 1.44E+09	Y	1.1	1/6	5

Data for individual MPN assays.

Species	Strain TAL #	Growth System	Dilution Factor	MPN Code	MPN Estimate	Factor for 95% C.I. of MPN	Plate Count	95% Confidence Interval for Plate Count		PC within C.I. of MPN	PC:MPN Ratio	Nodulated Uninoculated Controls	# of Weeks to Scoring
Leucaena diversifolia	1145	pouch	3.9	4444400000	4.01E+09	2.64	3.82E+09	3.26E+09	- 4.38E+09	Y	1.0	0/8	7
	1145	pouch	4.2	44311000	1.97E+08	2.72	3.36E+08	2.96E+08	- 3.76E+08	Y	1.7	0/8	5
	1145	tube	3.9	4444443200	7.56E+09	2.64	7.55E+09	5.98E+09	- 9.12E+09	Y	1.0	0/8	7
	1145	tube	4.2	444441000	1.06E+09	2.72	3.36E+08	2.96E+08	- 3.76E+08	N	0.3	0/8	5
	1145	tube	3.9	444301000	9.06E+08	2.64	3.82E+09	3.26E+09	- 4.38E+09	N	4.2	0/8	7
	1145	tube	4.2	444430000	4.96E+08	2.72	3.36E+08	2.96E+08	- 3.76E+08	Y	0.7	0/8	5
Leucaena leucocephala	1145	pouch	10.0	43320000	4.09E+06	3.8	2.71E+08	2.15E+08	- 3.27E+08	N	66.3	0/7	5
	1145	pouch	10.0	4433100	3.40E+07	3.8	1.22E+09	1.15E+09	- 1.29E+09	N	35.9	1/7	5
	1145	pouch	4.0	4444410000	5.01E+07	2.68	3.67E+07	3.07E+07	- 4.27E+07	Y	0.7	0/4	6
Paraserianthes falcataria	1536	pouch	4.2	11000000	7.35E+05	2.72	6.50E+08	5.04E+08	- 7.96E+08	N	884.4	0/8	5
	45	pouch	4.0	4422000000	1.75E+06	2.68	3.73E+08	2.26E+08	- 5.20E+08	N	213.1	0/4	6
	1536	tube	4.2	44420000	3.49E+08	2.72	6.50E+08	5.04E+08	- 7.96E+08	Y	1.9	0/8	5
	1536	tube	4.2	44431000	6.54E+08	2.72	6.50E+08	5.04E+08	- 7.96E+08	Y	1.0	0/8	5
Robinia pseudoacacia	1889	pouch	3.9	44442200	1.35E+09	2.64	2.72E+09	2.46E+09	- 2.98E+09	Y	2.0	0/8	7
	1889	pouch	4.0	44442000	6.82E+08	2.68	9.33E+07	7.40E+07	- 1.13E+08	N	0.1	0/8	5
	1889	pouch	3.9	444000100	2.70E+08	2.64	4.17E+09	2.85E+09	- 5.49E+09	N	15.4	0/8	7
	1889	tube	3.9	4444443300	2.52E+09	2.64	2.72E+09	2.46E+09	- 2.98E+09	Y	1.1	0/8	7
	1889	tube	4.0	44440100	4.73E+08	2.68	9.33E+07	7.40E+07	- 1.13E+08	N	0.2	0/7	5
	1889	tube	3.9	44100000	9.73E+07	2.64	4.17E+09	2.85E+09	- 5.49E+09	N	42.9	0/8	7
Sesbania grandiflora	1114	pouch	4.0	4322201	5.21E+07	2.68	4.58E+08	4.10E+08	- 5.06E+08	N	8.8	1/6	6
	1119	pouch	3.9	4444000	4.49E+08	2.64	2.93E+09	2.53E+09	- 3.33E+09	N	6.5	0/8	7
	1114	pouch	4.2	4443000	4.96E+08	2.72	9.60E+08	7.74E+08	- 1.15E+09	Y	1.9	0/8	5
	1114	tube	4.0	11100000	2.60E+06	2.68	4.58E+08	4.10E+08	- 5.06E+08	N	176.2	0/8	6
	1114	tube	4.2	00010000	<3.58E+05	2.72	9.60E+08	7.74E+08	- 1.15E+09	N	>2681.6	0/8	5
Sesbania sesban	1042	pouch	4.0	444444201	3.49E+08	2.68	6.13E+08	5.71E+08	- 6.55E+08	Y	1.8	0/3	7
	674	pouch	10.0	4444200	6.14E+08	3.8	6.73E+08	5.67E+08	- 7.79E+08	Y	1.1	0/7	5
	1042	pouch	10.0	44443012	1.20E+09	3.8	1.03E+09	8.46E+08	- 1.21E+09	Y	0.9	1/6	4

† = not used for means in Table 2.4.

Appendix C

Results of effectiveness tests in pouches[a].

Species	Soil	No. reps	Plants/ pouch	Steamed Soil (A)	Soil (B)	Soil + Rhizobia (C)	B vs. C	A vs. B
				Shoot dry weight (mg/plant)				
A. auriculiformis	Pane	8	1	66	90	112	0.087	0.041
A. mangium	Pane	8	1	16	12	24	0.000	1.000
A. mearnsii	Pane	7	2	72	41	83	0.000	1.000
L. diversifolia	Pane	7	2	74	46	170	0.000	1.000
R. pseudoacacia	Pane	6	2	121	73	300	0.0025	1.000
S. grandiflora	Pane	7	1	117	59	485	0.001	1.000
A. auriculiformis	Keahua	6	2	104	97	160	0.007	1.000
A. mangium	Keahua	6	2	30	20	48	0.002	1.000
A. mearnsii	Keahua	6	2	43	28	95	0.0025	1.000
L. diversifolia	Keahua	6	2	45	62	147	0.001	0.170
R. pseudoacacia	Keahua	6	2	107	79	342	0.0055	1.000
S. grandiflora	Keahua	4	2	111	121	566	0.0025	0.343
A. auriculiformis	Waiakoa	6	1	44	48	67	0.0015	0.241
A. mangium	Waiakoa	6	1	10	12	24	0.0125	0.253
A. mearnsii	Waiakoa	6	2	29	37	113	0.000	0.064
L. diversifolia	Waiakoa	6	1	18	97	61	1.000	0.001
R. pseudoacacia	Waiakoa	6	1	41	42	72	0.0395	0.418
S. grandiflora	Waiakoa	6	1	43	48	384	0.001	0.291

[a] Pouches were prepared as for MPN assays (Chapter 2), each pouch received an inoculum consisting of 10 g (dry weight equivalent) soil from an uninoculated pot in the inoculation experiment (Chapter 4) of one of three treatments: soil steamed for 45 min. at 110°C, unsteamed soil, or unsteamed soil to which rhizobia were added (approximately 10^6 rhizobia pouch[-1] of the same three-strain mixture used for inoculation in of the inoculation experiments). Plants were harvested after 6 weeks.

[b] = by one-tailed t test.

Literature Cited

Abdel Magid, H.M., P.W. Singleton, and J.W. Tavares. 1988. *Sesbania-Rhizobium* specificity and nitrogen fixation. Desert Plants 9(2):45-47.

Akkasaeng, R., P.C. Whiteman, and R.A. Date. 1986. *Rhizobium* requirements for two accessions of *Gliricidia maculata.* Tropical Grasslands 20(1):26-29.

Allen, O.N., and E.K. Allen. 1939. Root nodule bacteria of some tropical leguminous plants: II. Cross-inoculation tests within the cowpea group. Soil Sci. 47(1):63-76.

Allen, O.N. and E.K. Allen. 1981. The Leguminosae: a source book of characteristics, uses, and nodulation. The University of Wisconsin Press, Madison.

Barnet, Y.M., P.C. Catt, and D.H. Hearne. 1985. Biological nitrogen fixation and root-nodule bacteria *(Rhizobium sp.* and *Bradyrhizobium sp.)* in two rehabilitating sand dune areas planted with *Acacia spp.* Aust. J. Bot. 33:595-610.

Boonkerd, N. and R.W. Weaver. 1982. Cowpea rhizobia: comparison of plant infection and plate counts. Soil Biol. Biochem. 14:305-307.

Brewbaker, J.L., R. Van Den Beldt, and K. MacDicken. 1982. Nitrogen-fixing tree resources: potentials and limitations, p. 413-425. *In* P.H. Graham and S.C. Harris (ed.), Biological nitrogen fixation technology for tropical agriculture. CIAT, Cali, Colombia.

Brockweli, J. 1963. Accuracy of a plant-infection technique for counting populations of *Rhizobium trifolii.* Appl. Microbiol. 2:377-383.

Brockwell, J. 1980. Experiments with crop and pasture legumes-principles and practice, p. 417-488. *In* F.J. Bergersen (ed.) Methods for evaluating biological nitrogen fixation. John Wiley & Sons Ltd., Chichester.

Brockwell, J. 1982. Plant-infection counts of rhizobia in soil, p. 41-58. *In* J.M. Vincent (ed.) Nitrogen fixation in legumes. Academic Press, Sydney.

Brockwell, J., A. Diatloff, A. Grassia, and A. C. Robinson. 1975. Use of wild soybean *(Glycine ussuriensis* Regal and Maack) 3s a test plant in dilution-nodulation frequency tests for counting *Rhizobium japonicum. Soil* Biol. Biochem. 7:305-311.

Brockwell, J., R.A. Holliday, and A. Pilka. 1988. Evaluation of the symbiotic nitrogen-fixing potental of soils by direct microbiological means. Plant and Soil 108:163-170.

Burton, J.C. 1952. Host specificities among certain plants in the cowpea cross-inoculation group. Soil Sci. Soc. Am. Proc. 16:197-161.

Burton,   J.C. 1977. Associations of some uncommon leguminous plants with various rhizobia, p. 381-384. *In* J.M. Vincent, A.S. Whitney, and J. Bose (ed.) Exploiting
the legume-Rhizobium symbiosis in tropical agriculture. College of tropical agriculture miscellaneous publication
145, Department of Agronomy and Soil Science, University
of Hawaii.

Burton,   J.C. 1979. *Rhizobium species,* p. 29-58. *In* Microbial technology, 2nd ed., Vol. I, Academic Press.

Cochran, W.G. 1950. Estimation of bacterial densities by means of the "most probable number". Biometrics 6:105-116.

Crow, V.L., Jarvis, B.D.W., and R.M. Greenwood. 1981. Deoxyribonucleic acid homologies among acid-producing strains of *Rhizobium.* Int. J. Syst. Bacteriology 31:152172.

Date, R.A. 1977. Inoculation of tropical pasture legumes, p. 293-311. *In* J.M. Vincent, A.S. Whitney, and J.Bose (ed.) Exploiting the legume-Rhizobium symbiosis in tropical agriculture. College of Tropical Agriculture Miscellaneous Publication 145 Dept. of Agronomy and Soil Science, University of Hawaii.

Date, R.A. 1982. Assessment of the rhizobial status of the soil, p. 85-94. *In* J.M. Vincent (ed.), Nitrogen fixation in legumes. Academic Press, Sydney, Australia.

Date, R. A. 1991. Nitrogen fixation in *Desmanthus: strain* specificity of *Rhizobium* and responses to inoculation in acidic and alkaline soil. Tropical Grasslands 2547-2555.

Date, R.A. and J. Halliday. 1980. Relationships between *Rhizobium and* tropical forage legumes, p. 597-601. In R.J. Summerfield and A.H. Bunting (ed.) Advances in legume science, Royal Botanic Gardens, Kew.

Date, R.A., and D.O. Norris. 1979. *Rhizobium* screening of Stylosanthes species for effecitiveness in nitrogen fixation. Aust. J. Agric. Res. 30:85-104.

Davis, P.E. 1982. *Desmanthus* virgatus as a test plant for dilution-frequency infection tests using Leucaena leucocephala rhizobia. Soil Biol. Biochem. 14:313-314.

Department of Land and Natural Resources. 1982. Median rainfall. Circular CPS, Department of Natural Resources, Division of Water and Land Development, Honolulu, Hawaii.

Dommergues, Y.R. 1987. The role of biological nitrogen fixation in agroforestry, p. 245-271. In H.A. Steppler and P.K.R. Nair (ed.), Agroforestry, a decade of development. ICRAF, Nairobi, Kenya.

Dreyfus, B.L., H.G. Diem, J. Freire, S.O. Keya, and Y.R. Dommergues. 1987. *Nitrogen fixation* in tropical agriculture and forestry, p. 9-50. In E.J. DaSilva, Y.R. Dommergues, E.J. Nyns, and C. Ratledge (ed.) Microbial technology in the developing world. Oxford University Press, Oxford.

Dreyfus, E.L., and Y.R. Dommergues. 1981. Nodulation of Acacia species by fast- and slow-growing tropical strains of *Rhizobium.* Applied and Env. Microbiol. 41(1):97-99.

Duhoux, E., and Y. Dommergues. 1985. The use of nitrogen fixing trees in forest and soil restoration in the tropics, p. 384-400. In H. Ssali and S.O. Keya (ed.), Biological *nitrogen fixation* in Africa: proceedings of the first conference of the African association for biological nitrogen fixation. Matianum Press Consultants, Nairobi.

Galiana, A., J. Chaumont, H.G. Diem, and Y.R. Dommergues. 1990. Nitrogen-fixing potential of Acacia mangium and Acacia auriculiformis seedlings inoculated with *Bradyrhizobium and Rhizobium spp.* Biol. Fertil. Soils. 9:261-267.

George, T., and P.W. Singleton. 1989. Nodulation, N2 fixation and N assimilation during early growth by four tree legumes att different levels of mineral N supply. 12th North American Symbiotic Nitrogen Fixation Conference Proceedings. Ames Iowa. p. 120.

Gibson, A.H. 1980. Methods for legumes in glasshouses and controlled environment cabinets, p. 139-184. *In* F.J. Bergersen (ed.) Methods for evaluating biological nitrogen fixation. John Wiley & Sons Ltd., Chichester.

Gibson, A.H., B.L. Dreyfus, and Y.R. Dommergues. 1982. Nitrogen fixation by legumes in the tropics, p. 37-73. *In* Y.R. Dommergues and H.G. Diem (ed.) Microbiology of tropical soils and plant productivity. Martinus Nijhoff/Dr. W. Junk Publishers, The Hague.

Graham, P.H. and D.H. Hubbell. 1975. Legume *Rhizobium* relationships in tropical agriculture, p. 9-21. *In* E.C. Doll and G.O. Mott (ed.), Tropical forages in livestock production systems. ASA Special Publication No. 24.

Halliday, J., and P. Somasegaran. 1983. Nodulation, nitrogen fixation, and *Rhizobium* strain affinities in the genus *Leucaena, p.* 27-32. *In* Leucaena Research in the Asian Pacific Region, Proceedings of a workshop held in Singapore, 23-26 November, 1982, IDRC, Ottawa.

Jenkins, M.B., R.A. Virginia, and W.M. Jarrell. 1987. Rhizobial ecology of the woody legume mesquite *(Prosopis glandulosa)* in the sonoran desert. Appl. Environ. Microbiol. 53(1):36-40.

Johnson, M.D., and O.N. Allen. 1952. Nodulation studies with special reference to strains isolated from *Sesbania* species. Antonie van Leeuwenhjoek J. Microbiol. Serol. 18(1):1-12.

Kang, B.T., G.F. Wilson, and T.L. Lawson. 1984. Alley cropping, a stable alternative to shifting cultivation. International Institute of Tropical Agriculture, Ibadan, Nigeria.

Keeney, D.R. 1982. Nitrogen-availability indices. Agronomy 9:711-733.

Keyser, H.H., and P.B. Cregan. 1987. Nodulation and competition for modulation of selected soybean genotypes among *Bradyrhizobium japonicum* serogroup 123 isolates. Appl. Environ. Microbiol. 53:2631-2635.

Ndoye, I.; K.Tomekpe, B. Dreyfus, and Y.R. Dommergues. *1990. Sesbania* and *Rhizobium* symbiosis: nodulation and nitrogen fixation. in Macklin, B., and D.O. Evans (eds.) Perennial Sesbania species in agroforestry systems. NFTA, Waimanalo, Hawaii.

NifTAL and FAO. *1984.* Legume inoculants and their use. Rome.

Peoples, M.B., A.W. Faizah, B. Rerkasem, and D.F. Herridge. *1989.* Methods for evaluating nitrogen fixation by nodulated legumes in the field. ACIAR, Canberra.

Sadowsky, M.J., R.E Tullv, P.B. Cregan, and H.H. Keyser. *1987.* Genetic diversity in *Bradyrhizobium japonicum* serogroup *123* and its relation to genotype-specific nodulation of soybean. Appl. and Environ. Microbiol. *53(11):2624-2630.*

Sambrook, J., E.F. Fritsch, and T. Maniatis. *1989.* Molecular cloning: a laboratory manual. Second Edition, Vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Sanginga, N., K. Mulongoy, and A. Ayanaba. *1987.* Evaluation of indigenous strains of Rhizobium for *Leucaena leucocephala* (Lam.) De Wit in Nigeria conditions, p. *416436. In* Les arbres fixateurs d'Azote: l'amelioration biologique de la fertilite des sols. Ed. ORSTOM. ORSTOM, Paris.

Sanginga, N., K. Mulongoy, A. Ayanaba. *1985.* Effect of inoculation and mineral nutrients on nodulation and growth of *Leucaena leucocephala, p. 419-427. In* H. Ssali and S.O. Keya (ed.) Biological nitrogen fixation in Africa. Proceedings of the African Association for Biological Nitrogen Fixation, Matianum press consultants, Nairobi.

SAS Institute. *1986.* SAS users' guide: Statistics. SAS Institute Inc., Cary, N.C.

Scott, J.M. and F.E. Porter. *1986.* An analysis of the accuracy of a plant infection technique for counting rhizobia. Soil Biol. Biochem. *18:355-362.*

Singer, M.J., and D.N. Munns. *1987. Soils:* an introduction. Macmillan Publishing Company, N.Y.

Singleton, P.W. and J.W. Tavares. 1986. Inoculation response of legumes in relation to the number and effectiveness of indigenous rhizobium populations. Appl. and Env. Microbiol. 51(5):1013-1018.

Singleton, P.W., B.B. Bohlool, and P.L. Nakao. 1991a. Legume response to rhizobial inoculation in the tropics: myths and realities. In R.Lal and P. Sanchez (ed.), Myths and science of soils in the tropics. ASA special publication, in press. ASA-SSSA-CSSA, Madison, Wis.

Singleton, P.W., P.L. bloomer, J.E. Thies, P.L. Nakao, and B.B. Bohlool. 1991b. Protocols for field and greenhouse experimentation in legume BNF: Standardized network trials to develop models describing the ecology and performance of the rhizobium-legume symbiosis. University of Hawaii NifTAL Project, Paia.

Somasegaran, P., and H.J. Hoben. 1985. Methods in legume-Rhizobium technology. University of Hawaii NiFTAL Project, Paia.

Somasegaran, P., H.H. Keyser, and J.P. Roskoski. 1989. Rhizobial requirement and inoculation response of Gliricidia sepium (Jacq.) Walp. 12th North American Symbiotic Nitrogen Fixation Conference Proceedings. Ames Iowa. p. 77.

Thies, J.E., P.W. Singleton, and B.B. Bohlool. 1991a. Influence of the size of indigenous rhizobial populations on establishment and symbiotic performance of introduced rhizobia on field-grown legumes. Appl. and Env. Microbiol. 57(1):19-28.

Thies, J.E., P.W. Singleton, and B.B. Bohlool. 1991b. Modeling symbiotic performance of introduced rhizobia in the field by use of indices of indigenous population size and nitrogen status of the soil. Appl. and Env. Microbiol. 57(1):29-37.

Thies, J.E., B.B. Bohlool, and P.W. Singleton. 1991c. Subgroups of the cowpea miscellany: symbiotic specificity within *Bradyrhizobium* spp. for Vigna unguiculata, Phaseolus lunatus, Arachis hypogaea, and Macroptilium atropurpureum. Appl. Environ. Microbiol. 57(5):1540-1545.

Thompson, J.A., and J.M. Vincent. 1967. Methods of detection and estimation of rhizobia in soil. Plant and Soil 26(1):72-84.

Toomsan, B., O.P. Rupela, S. Mittal, P.J. Dart, and K.W. Clark. 1984. Counting *cicer-Rhizobium using* a plant infection technique. Soil Biol. Biochem. 16(5):503-507.

Trinick, 1982. *Host-Rhizobium Associations,* p. 111-122. In J.M. Vincent (ed.) Nitrogen fixation in legumes. Academic Press, Sydney.

Tuzimura, K., and I. Watanabe. 1961. Estimation of number of root-nodule bacteria by a nodulation-dilution frequency method. Soil Science and Plant Nut. 7(2):61-65.

Vincent, J.M. 1970. A manual for the practical study of, root-nodule bacteria. IBP Handbook No. 15. Burgess and s^n (Abingdon) LTD, Berkshire.

Virginia, R.A., M.B. Jenkins, and W.M. Jarrell. 1986. Depth of root symbiont occurrence in soil. Biol. Fertility Soils 2:127-130.

Weaver, R.W., and L.R. Frederick. 1972. A new technique for most-probable-number counts of Rhizobia. Plant and Soil 36:219-222.

Wilkinson, L. 1990. SYSTAT: The system for statistics. Evanston, IL, SYSTAT, Inc.

Wilson, J.K. 1944. Over five hundred reasons for abandoning the cross-inoculation groups of the legumes. w Soil Sci. 58:61-69.

Woomer, P., P.W. Singleton, and B.B. Bohlool. 1988a. Ecological indicators of native rhizobia in tropical soils. Applied and Env. Microbiol. 54(5):1112-1116.

Woomer, P., P.W. Singleton, and B.B. Bohlool. 1988b. Reliability of the most-probable-number technique for enumerating rhizobia in tropical soils. Applied and Env. Microbiol. 54(6):1494-1497.

Woomer, P., J. Bennett, and R. Yost. 1990. Overcoming the inflexibility of most-probable-number procedures. Agron. J. 82:349-353.