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COMPETITION BETWEEN STRAINS OF RHIZOBIA FOR NODULATION OF LEUCAENA LEUCOCEPHALA

The University of Arizona M.S. 1985

COMPETITION BETWEEN STRAINS OF RHIZOBIA FOR NODULATION OF LEUCAENA LEUCOCEPHALA

by

Lucinda Faith Salo

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5/7/85

Date

This thesis is dedicated to the girl I left behind

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ABSRACT

Two strains of rhizobia effective on Leucaena leucocephala (Lam.) de Wit. were examined in soil and sterile sand to determine their abilities to compete for nodulation sites. Strains TAL1145 from NifTAL, and 5B2, collected in Veracruz, Mexico, were fast-growing strains and produced acid in laboratory media.

Treatments applied to plants in sand included each strain singly, plus mixtures of the two in various proportions. Plants in soil from Jalapa (pH 5.0), and La Balsa, Veracruz (pH 7.1), were inoculated with each strain singly.

Neither strain was able to consistently predominate in sand, where no significant differences in plant growth were found between treatments. Strain 5B2 was able to survive better than TAL1145 in the La Balsa soil, while TAL1145 was more successful in the Jalapa soil.

INTRODUCTION

As the world's population increases, both food and energy are becoming increasingly difficult to obtain. The extensive cutting of wood in the less developed countries, where it is the major source of fuel for 90% of the people (Eckholm, 1975), has lead to widespread deforestation. This in turn leads to serious erosion problems which reduce the agricultural productivity of the land. The use of animal manure for fuel, as firewood becomes harder to find, further reduces the soil fertility as badly needed nutrients and organic matter are not returned to the soil (Eckholm, 1975).

As the cost of industrially fixed nitrogen fertilizer increases, biological systems capable of fixing nitrogen are becoming more important as low cost alternatives requiring only small enery inputs. Leguminous plants, able to use atmospheric nitrogen because of their symbiotic associations with rhizobia bacteria, provide high protein plant products useful as human food, feed for animals, and fertilizer for other crops.

Leucaena leucocephala (Lam.) de Wit., a fast-growing woody legume, has great potential as a renewable

source of fuel, wood products, and high protein foliage, useful for forage and as fertilizer. It is also an excellent plant for use in reforestation and erosion control, and for windbreaks, firebreaks, and as a shade tree for other crops.

Inoculating legume seeds with selected strains of rhizobia should result in increased nitrogen fixation, and therefore increased productivity of the plants. An ideal inoculant would provide the plant with rhizobia able to survive and grow well over a wide range of biotic and abiotic conditions in the soil, compete successfully with the indigenous strains of rhizobia for nodulation sites on the roots, and, once inside the roots, fix large amounts of nitrogen.

Although much progress has been made in developing inoculants for more traditional crops, little work has been done in selecting strains of rhizobia to increase biological nitrogen fixation by Leucaena leucocephala. The purpose of this study is to evaluate strains of rhizobia for suitability as competitive inoculants for this plant. The specific objectives are to:

1. Screen strains collected in Veracruz, Mexico for effectiveness of nitrogen fixation, and compare them to other effective strains.

2. Investigate the growth rate and pH change in media

of rhizobia effective on Leucaena leucocephala.

3. Determine the relative abilities of two highly effective strains to compete for nodulation sites under sterile conditions.

4. Evaluate the success of these strains as inoculants when competing with indigenous strains in soil.

LITERATURE REVIEW

Leucaena leucocephala

From its center of diversity in southern Mexico, Leucaena leucocephala had spread throughout Mexico and Central America by the time that the Spanish arrived in 1517. The galleon trade then moved the plant from ports on the west coast of Mexico to islands in the Pacific, and from there it spread to southeast Asia, India, Australia and Africa (Dijkman, 1950; National Academy of Sciences, 1977).

Now world-wide in distribution, leucaena is most common at elevations below 750m (Whyte, Nilsson-Leissner and Trumble, 1953; Brewbaker, 1980), as its growth is limited by the cool nights of higher regions. However, it is planted with success up to 1500m, and has long been used as a shade tree for crops in the tropical highlands of the Pacific regions (Oakes, 1968; Hill, 1971). Leucaena is able to grow on coral formations down to sea level, and is thought to be highly salt tolerant and to

have potential as a crop for saline soils (Brewbaker and Hutton, 1979; Felker et al., 1981).

Leucaena flourishes where rainfalls range from 500 to 5000mm per year (Oakes and Skov, 1967; Bogdan, 1977), although it is also the dominant vegetation on Diamond Head, Hawaii which receives only 250mm per year (National Academy of Sciences, 1977). Growth is thought to be limited in regions of higher precipitation because of the more acid soils in those regions (Brewbaker, 1982). The plants cannot tolerate waterlogging, and the faster runoff on steep, rocky slopes is felt to be one reason why leucaena grows well in such terrain (Takahashi and Ripperton, 1949).

Although native to the tropics, leucaena has become naturalized in many areas of the semitropics. It is found as far north as Texas and Florida (Dijkman, 1950), and can survive winters in Tucson, Arizona (Pepper, personal com-munication, 1984). The trees will drop their leaves when subjected to frosts, and, in extremely cold weather, may die back to the ground, but will survive better than other tropical forage legumes (Jones, Jones and Cooksley, 1982).

Leucaena is able to grow in a wide variety of soils of quite low fertility, and, unlike many tropical legumes, it is adapted to clay soils (Oakes, 1968; Jones

et al., 1982). It is especially noted for its ability to grow in shallow, rocky soils, similar to those of the Yucatan Penninsula to which it is native (Oakes, 1968; Hill, 1971). This area was formed by the uplift of a large limestone shelf, and is covered with shallow, calcareous soils of high pH. Leucaena prefers neutral to alkaline soils, altough some workers have had promising results on acid soils, especially when fertilizers are applied and the seeds are pelleted with lime (Esquivel Salazar, 1965; Morales, Graham and Cavallo, 1973; Halliday, 1981). Brewbaker (1980) stated that the optimum pH for growth is 5.5 to 8.5, and liming of acid soils often gives dramatic responses (Munns and Fox, 1977; Hutton and Andrew, 1978). Vesicular-arbuscular mycorrhiza associatd with the roots allow leucaena to grow in soils of quite low phosphorus status (Possingham, Groot Obbink and Jones, 1971).

Intraspecific Variation

Hutton and Gray (1959) described three types of Leucaena leucocephala on the basis of growth habit, maturity and yield: Hawaiian, Salvador, and Peru. Although known as "Hawaiian", the shortest and seediest type is actually native to southeastern Mexico, and was introduced to Hawaii where its early maturity and profuse seeding have made it a weed. This from rarely reaches

more than 5m in height, branches strongly from the base to form rounded shrubs, and yields relatively low amounts of wood and foliage.

The Salvador type, native to southern Mexico and Central America, is tall and slender, reaches heights of 20m with only sparse branching, and produces some of the highest annual wood yields ever recorded (National Academy of Sciences, 1977). It is faster growing than the Hawaiian type, but, as it reaches maturity later and produces fewer seeds, it does not have the same reputation for weediness. This form is a larger version of the Hawaiian type in all respects, having larger stems, leaves, pods, and seeds, and producing higher yields of foliage and wood (Oakes and Skov, 1967; Brewbaker, 1975).

The Peru type reaches 15m in height, branches from the base, and is late flowering. It is thought to be the result of hybridization between the first two types, having the height and vegetative vigor of the Salvador type, plus the strong branching of the Hawaiian type (Brewbaker and Hutton, 1979). The Peru form is capable of producing the highest forage yields of any type of leucaena (Hutton and Bonner, 1960; Hill, 1970).

Wood Production

Leucaena produces excellent charcoal and firewood which have long been used in the Philippines and Indonesia (Whyte et al., 1953; Dijkman, 1950). The wood is very dense and has an exceptionally high calorific value for a fast-growing tree. This ranges from 4640 to 4673cal/kg for the Hawaiian type, and from 4167 to 4445cal/kg for the Salvador type (Bawagan and Semana, 1978). When the Hawaiian wood is made into charcoal the calorific value is increased to 7250cal/kg, which is 70% that of fuel oil (Dijkman, 1950). Due to its greater density, the Hawaiian type produces more heat when burned, but the Salvador type is capable of far greater yields.

Pure stands can produce from 24 to over $100m^3$ wood/ha/yr, with the average thought to be about $40m^3$ wood/ha/yr (National Academy of Sciences, 1977; Brewbaker, Van Den Beld and Macdicken, 1982). However, researchers in the Philippines claim that annual growth increments of $312m^3$ wood/ha have been reached (Bawagan and Semana, 1978). A study of the feasability of leucaena tree farms as an energy source in Hawaii estimated that it is possible to produce the equivalent of 30bbl oil/ha/yr with this crop (Brewbaker, 1980). Due to its ability to coppice readily from harvested stumps,

leucaena provides a renewable energy source without the need for frequent replanting.

Foliage Production

The development of leucaena as a forage has been hampered by the fact that it is toxic to monogastrics, causing hair loss by interfering with growth in the follicle (Jones et al., 1982). The toxic substance has been identified as mimosine, an uncommon amino acid (Yoshida, 1945), which is present in the foliage at 2 to 5% of the dry matter, ranging from 10% in the youngest tips to 1% in the oldest leaves (Jones et al., 1982). In ruminants it is metabolized to 3,4-dihydroxypyridine (DHP), which affects the thyroid gland, causing goiter and supressing production of thyroxine, which leads to low feed intake and low weight gain (Jones, 1978). However, this is not a serious problem when introdued into the diet gradually and used as less than 30% of the ration (Jones, et al., 1982). Wide variations in mimosine contents have been found in segregating generations of interspecific crosses of Leucaena sp., offering promise for the breeding of low-mimosine lines to eliminate these problems (Brewbaker and Hylin, 1965; Gonzalez, Brewbaker and Hamill, 1967).

Leucaena has great potential for supplying badly needed high protein forage to supplement grasses in the

seasonally dry tropics, as its strongly developed taproot allows it to reach water unavailable to more shallowly-rooted crops, and to remain green into the dry season. The use of this high quality forage has resulted in some of the highest weight gains measured with forage fed cattle (National Academy of Sciences, 1977). The foliage is equal or superior to alfalfa in total digestile nutrients, carotene, and crude protein, which ranges from 15 to 30% of the dry matter (Oakes, 1968; Hutton and Bonner, 1960; Oakes and Skov, 1967).

Yields of forage have ranged from 22 to 29 tons dry matter/ha/yr (Kinch and Ripperton, 1952; Brewbaker, Plucknett and Gonzalez, 1972), and yields of protein have ranged from 3600 to 4850kg/ha (Hutton and Bonner, 1960; Brewbaker et al., 1972). However, the potential yield of forage in Hawaii has been estimated at 41 tons dry matter/ha/yr, containing more than 6700kg of protein (Brewbaker et al., 1972). When compared with four other tropical forage legumes in the U.S. Virgin Islands, leucaena was found to be the most suitable based on yield, protein content, palatability, ensiling quialities, persistence, and ease of handling (Oakes and Skov, 1962).

Hawaiian studies have shown leucaena foliage to contain 44kg P/ha and 187kg K/ha, plus calcium and

micronutrients. When this foliage was applied to corn, the yield increases approached those seen with commercial fertilizers (National Academy of Sciences, 1977). Due to its deep taproot, leucaena is able to reach nutrients not available to other crops, and, through leaf drop, these are added to the soil along with considerable amounts of organic matter. Because of its rooting style, leucaena is an excellent shade or nurse tree, as competition with other crops is minimal.

Nitrogen Fixation

The amounts of nitrogen fixed by pure stands of Leucaena leucocephala are among the highest recorded. Quantities range from 74 to 584kg N/ha/yr, with an average figure considered to be 277kg N/ha/yr (Nutman, 1976). A four-year-old stand growing in alluvial sand in Tanzania reduced 35umol C_2H_4/g nodule dry weight/hr. These trees were estimated by auger sampling to support a nodule biomass of 51±16kg/ha on a dry weight basis, and to fix 110±30kg N/ha/yr (Hogberg and Kvarnstrom, 1982). This rate is higher than that for other tropical woody legumes. Inga jinicuil in Mexico was estimated to support a nodule biomass of 71±14kg dry nodule weight/ha, but extrapolation from samples containing three nodules determined nitrogen fixation to be only 35kg/ha/yr (Roskoski, 1981), and Acacia pellita in Australia was

able to fix only 12±4kg N/ha/wet season (Lamgkamp, Swiden and Dalling, 1979).

Rhizobial Symbiont

Rhizobia have traditionally been divided into species on the basis of host infectivity. The original classification system was based on the study of only a small number of agriculturally important species of legumes, and now appears to be in need of revision. Fred, Baldwin, and McCoy (1932) divided rhizobia into *R. meliloti*, *R. trifolii*, *R. leguminosarum*, *R. phaseoli*, *R. japonicum*, and *R. lupini*, with the cowpea cross-inoculation group not given species status. The first four species are fast-growing types with generation times of 2 to 4 hours that produce acid in laboratory media, and the last three groups are slow-growing types with generation times of more than six hours that produce alkali in laboratory media (Fred et al., 1932; Vincent, 1974).

There are many differences between these two types of rhizobia in addition to growth rates and reaction in media. Slow growers generally have a single subpolar flagella (Leifson and Erdman, 1958), and are more exacting in their carbohydrate requirements (Graham, 1964a; Vincent, 1974) than the peritrichously-flagellated fast growers that are able to use a wider variety of

carbohydrates, as they have more metabolic pathways at their disposal.

Slow-growing rhizobia have higher levels of intrinsic antibiotic resistence (Graham, 1963a), will remain effective after mutating to resistance to some antibiotics that will cause fast growers to lose effectiveness (Pankhurst, 1977), and are better able to survive high temperatures and dessication (Marshall, 1964; Bushby and Marshall, 1977; Mahler and Wollum, 1981). Fast-growing types produce a wider variety of enzymes than slow growers, including dehydrogenases (Martinez-De Drets and Arias, 1972), and use glutamic acid as a tryptophan deaminating substrate in the production of indole acetic acid in preference to the 2-ketoglutaric acid prefered by the slow growers (Garcia-Rodriguez et al.; Werner and Berghauser, 1976).

The classification of rhizobia on the basis of host specificty is becoming less satisfactory as more is learned about these bacteria. Fast-growing strains have been isolated from soybeans (Keyser et al., 1982) and lupins (Miller, personal communication, 1982), both previously thought to be nodulaed only by slow-growing types.

Serological studies have shown very little relationship between the fast- and slow-growing rhizobia,

but a close relationship between some fast growers and the agrobacteria. Based on anitgenic similarities, it is felt that R. trifolii, R. leguminosarum, and R. phaseoli are closely related but different from: R. meliloti, rhizobia from Leucaena spp. which do not fit into any of the accepted species, fast-growing strains from Lotus spp., Agrobacterium tumifaciens, and A. radiobacter. These are in turn different from the slow-growing types including R. japonicum, slow growing strains of R. lupini, and Rhizobium sp. (Graham, 1963b; Vincent and Humphrey, 1970). Graham (1963b, 1964b) concluded that the fast and slow growers are the result of evolution from completely different types of soil bacteria, and that they should be divided at the generic level, with Agrobacterium tumifaciens and A. Radiobacter included with the fast-growing rhizobia.

These ideas are now reflected in the taxonomy given in Bergey's Manual of Systematic Bacteriology (Jordan, 1984). The genus Rhizobium now contains only the fast-growing types, and includes three species: R. leguminosarum which consists of three biovars, trifolii, phaseoli and viceae, R. melitoti which forms nodules on sweet clover and alfalfa, and R. loti which is effective on some types of lotus plus other plants. The new genus Bradyrhizobium contains the slow-growing types, and

includes one species, B. japonicum. The bacteria forming nodules on cowpeas, siratro and certain species of lotus are also included in this genus, but are not yet classified as a species or biovar.

Graham's conclusions about the separate evolution of slow- and fast-growing types of root-nodule bacteria are in disagreement with Norris (1965a, 1965b), who felt that the fast-growers evolved from the slow growers. Norris hypothesized that the slow-growing, alkali-producing bradyrhizobia originated in the acid soils of the humid tropics concurrently with the legumes. The production of acid would have been selected against in these soils, but as the legumes advanced and moved out of the tropics into the more alkaline soils of the temperate regions, this restriction would have been lifted. The bacteria would then have been able to exploit more efficient metabolic pathways that produce acid and allow faster growth rates. As the legume hosts moved into less acid soils and formed symbiotic relationships with fast-growing rhizobia, Norris felt that they also became more specialized in their rhizobial requirements, and lost the ability to cross-inoculate with a variety of strains of root-nodule bacteria.

Norris (1965a) found that the strongest acid producers were generally associated with the most

advanced and strain-specific hosts growing in the highest pH soils. Leucaena leucocephala was the only member of the relatively primitive subfamiy Mimosaceae with associated acid-producing rhizobia similar to the fast-growing strains associated with temperate legumes. Norris (1967) pointed out that leucaena prefers alkaline soils and responds to liming, and hypothesized that, although native to the tropics, it has become adapted to non-acid soils and developed specific rhizboial requirements, much as the clovers and medics.

Cross-inoculation Grouping

Although leucaena has repeatedly been shown to require specific rhizobia with limited host ranges (Galli, 1958; Trinick, 1968; Staphort and Strijdom, 1970), nodulation by native strains has been found in India (Basak and Goyal, 1980; Basavaraju and Hegde, 1983), Senegal (Dreyfus and Dommergus, 1981a), and Hawaii (Halliday, 1981). Leucaena was found to be effectively nodulated by fast-growing rhizobia isolated from Mimosa invisa Marth., which formed effective nodules when inoculated with leucaena rhizobia (Ishizawa, 1955).

Trinick (1968) tested 99 strains of rhizobia representing all seven accepted cross-inoculation groups and found that only strains from Leucaena leucocephala, Mimosa invisa, M. pudica L., and Acacia farnesiana (L.)

Pers. were able to nodulate leucaena effectively, while strains from Sesbania grandiflora (L.) Poir. were able to produce ineffective nodules. The only rhizobia infective on leucaena were fast-growing strains from tropical woody legumes, and Trinick concluded that these formed a cross-inoculation group. These bacteria resembled strains from temperate legumes such as Medicago sativa L., also strongly adapted to alkaline, calcareous soils, which had previously been shown to form ineffective nodules with leucaena rhizobia (Trinick, 1965). Neither fast-growing strains from temperate legumes, or the slow-growing cowpea types typically associated with tropical legumes were able to nodulate leucaena (Trinick, 1968).

Leucaena rhizobia effectively nodulated Mimosa invisa and Acacia farnesiana, plus Vigna sinensis (L.) Savi ex Hassk. spp. sesquipedalis (L.) van Eseltine, usually nod- ulated by slow-growing strains (Trinick, 1968). In addition, these strains were able to form ineffective nodules on Sesbania grandiflora, Medicago sativa, and many tropical legumes typically nodulated by slow growers (Trinick, 1968). A later study (Trinick and Galbraith, 1980) showed that leucaena rhizobia were able to nodulate Parasponia andersonii Planch., a member of the Ulmaceae family that is effectively nodulated by slow-growing rhizobia.

Further study with strains from leucaena, Mimosa invisa, M. pudica, Acacia farnesiana, and Sesbania grandiflora (Trinick, 1980) helped confirm the existence of a cross-inoculation group. These strains were similar in colony characteristics, precipitation in calcium glycerophosphate medium, and utilization of carbon sources to fast-growing types such as R. meliloti. Serological studies showed close antigenic relationships between strains from the five woody legumes.

Investigations of rhizobia from 13 species of acacia showed that the host species could be divided into three groups based on patterns of effective nodulation with strains of root-nodule bacteria (Dreyfus and Dommergus, 1981a, 1981b). The first group was nodulated only by slow-growing strains, the second by fast-growing strains, and the third by both fast- and slow-growing strains. Leucaena rhizobia were found to be similar to strains associated with the second group which included A. farnesiana and A. senegal (L.) Willd. A high degree of cross infectivity was found between leucaena and acacia of this group.

Jarvis (1981) pointed out that the cross-inoculation group concept is based on only a small part of the rhizobial genetic material that may be carried on plasmids. Determination of the DNA homology of 25

strains of rhizobia capable of nodulating leucaena showed that they represented five distinct homology groups, and that even strains originally isolated from leucaena were from three different groups. Jarvis concluded that host infectivity is not a suitable basis for the classification of rhizobia.

Morphological and Metabolic Properties

Although they are fast growers and produce acid in laboratory media, leucaena are anomolous in many respects, and seem to be intermediate between the fast- and slow-growing rhizobia. Tan and Broughton (1981, 1982) found that although the strain investigated had a generation time of 3.0 hours, reduced the pH of labotatory media after an initial increase, and used sugars in preference to nitrogenous compounds as do other fast growers, it had a single, subpolar flagella and used relatively large amounts of glutamate like the slow growers. A study in Thailand showed that the survival of leucaena rhizobia in flooded paddy soil was significantly higher than that of slow-growing R. japonicum (Rerkasem and Tongkumdee, 1981)

Interstrain Competition for Nodulation

Strains of rhizobia that are noninfective (will not form nodules), or ineffective (will not fix nitrogen)

with a particular host are easily eliminated during greenhouse screening. The amount of nitrogen that a strain is capable of fixing can be evaluated by measuring the nitrogen content of the plant or the H_2 evolved, or through the use of isotopically labeled N_2 or the acetylene reduction assay. Once an infective, effective strain capable of fixing large amounts of nitrogen is seclected, its ability to compete for nodulation of the host must be evaluated.

To form a large percentage of the nodules on a plant, a strain must first be able to survive and grow in the soil. This involves the ability to survive antagonism, predation, competition for substrates, and the absence or inactivity of the host, and to grow over a range of pH conditions, water and oxygen contents, and temperature. The ability to establish numbers in the rhizosphere adequate for nodulation in spite of competition and adverse conditions was termed "incursive ability" by Harris (1954), who pointed out that a poorly incursive strain, although it may be able to form a large number of nodules, would appear to be poorly competitive.

Competitive ability and the capacity to fix nitrogen have long been thought to be independent traits in rhizobia (Nicol and Thornton, 1941; Vincent and Waters, 1953). Mytton and de Felice (1977) confirmed

this using competitive diallel analysis, but stated that concurrent selection for these two characteristics could produce strains that are both effective and competitive.

Multiple Infection

Early reports (Dunham and Baldwin, 1931; Hughes and Vincent, 1942) suggested that nodules can only contain one strain of rhizobia, although multiple strains in different nodules on plants exposed to mixtures of strains were common. Vincent (1954) found nodules containing two strains on subterranean clover growing in agar tubes, although it was much more common to find only one. He pointed out that this did not necessarily prove double infection of one root hair, as two separate infections could have coalesced to form one nodule.

Since Vincent's findings, double infections have been reported on a variety of legumes inoculated with mixtures of strains including soybeans (Skrdleta and Karimova, 1969) and clover (Labandera and Vincent, 1975). The percentage of double infections has ranged from as low as 0.7% on plants growing in sand (Means, Johnson, and Erdman, 1961), to 25% in agar tubes (Marques Pinto, Yao, and Vincent, 1974), and 36% in vermiculite (May and Bohlool, 1983). Marques Pinto et al. (1974) hypothesized that the large numbers of bacteria surrounding the roots in a continuous film in tube

culture allowed a high percentage of double infections, although they did not rule out the possibility of the coalesence of two infection threads.

The number of double infections in plants growing in the field has generally been found to be low, ranging from less than 1.0 to 3.5% (Brockwell, Schwinghamer, and Gault, 1976; Bromfield and Jones, 1980). However, May and Bohlool (1983) found double infections to be as frequent as 38% in field-grown lentils, depending on the strain mixture and soil type. These workers also found a close relationship between competitiveness and the presence of a strain in double infections.

Using genetically marked strains, Johnson and Beringer (1975) found that the strain forming the majority of nodules on peas was also in the majority in mixed nodules. This raised the possibility of inaccuracies in earlier reports due to the inability to detect low numbers of bacteroids using standard serological techniques.

Basis of Competitive Ability

Chen (1941) found that a given strain of rhizobia had the ability to form a set number of nodules per gram of root, and termed this the "relative infectivity" of the strain. Nicole and Thornton (1942) added that this ability was also reflected in the speed of nodulation,

with more infective strains producing nodules more quickly. Although these workers felt that the relative infectivity of a strain was important, they found that it could be overcome by competition outside the root. Growth rate in the rhizosphere was seen as the major factor in this competition, with a very fast-growing strain being able to check the growth of slower strains to the extent that almost all of the nodules would be formed by the fast-growing strain.

After studying the R. trifolii-red clover symbiosis, Nutman (1946) concluded that the number of nodules and speed of nodulation were determined by the interaction of genetically controlled factors of both the bacteria and the host. Inbreeding of the host plant increased nodule number, and greatly delayed nodulation. Vincent and Waters (1953, 1954) could not explain the relative success in nodulation of clovers in terms of the number of nodules produced, the speed of nodulation, or the growth rate in the rhizoshere. Rather, they hypothesized that the host plant determined the relative success of strains by selecting among the strains in mixed inocula.

Differences in Competitive Ability

Differences in competitive ability between strains are common, and there are many reports of strains

able to form a large proportion of the nodules even when present as only a small percentage of the inoculum. With soybeans (Means, Johnson, and Erdman, 1961), the chlorosis-inducing strain 76 was responsible for 85% of nodules when accounting for only 1.1% of the inoculum. One strain of R. japonicum formed 80 and 100% of nodules when used in ratios of 1:60 and 3:5, respectively (Skrdleta and Karimova, 1969), and strain TA1 has produced up to eleven times as many nodules on clover as competing strains (Dudman and Brockwell, 1968). Strain Viking I formed 100% of nodules on Phaseolus vulgaris when competing with indigenous strains of R. paseoli in the field (Robert and Schmidt, 1983). Aside from competitive interactions, mixtures of effective strains have been found to be superior to single strains of rhizobia, although this effect has not been found to be statistically significant (Hofer, 1945; Burton and Allen, 1950; Russell and Jones, 1975b).

The relative competitive ability of strains can be modified by growing conditions, host species and cultivar. After studying the competitivenss of strains of R. japonicum in the greenhouse, Johnson, Means, and Weber (1965) found that, although strain 110 was outstanding in both the greenhouse and the field, the second ranked strain in the greehouse screening was

unsuccessful in the field. Early work by Read (1953) indicated that competition between inoculated strains of R. trifolii and native strains as reflected in the establishment of the inoculated types was influenced by soil conditions at 13 sites in England, and by variations in weather between years. More recent studies by Roughley, Blowes and Herridge (1976) found that interstrain competition for nodulation of subterranean clover was modified by location at five sites in Australia.

Differences in host selectivity at the generic level are reflected in the cross-inoculation group concept (Fred et al., 1932). Labandera and Vincent (1975) illustrated differences in the selection of strains by hosts at the species level with three kinds of clover, and variations have been found between cultivars of subterranean clover (Roughley et al., 1976), soybeans (Caldwell and Vest, 1968), and lentils (May and Bohlool, 1983). Studies with red and white clover, both cross-pollinated species, showed that there was a considerable variation within and between cultivars in the selelction of strains (Russell and Jones, 1975a; Jones and Hardarson, 1979). This variation was thought to be the result of the genetic heterogeneity of these species.

Competition Between Strains of Different Effectivity

When mixtures of effective and ineffective strains were used to inoculate subterraneun clover, Robinson (1969a, 1969b) found that the effective strains formed a higher percentage of nodules than would be expected from the relative numbers of each strain present in both agar tubes and field studies. A ratio of 10,000:1 in favor of the ineffective strain was necessary before it could overcome this selective effect to form a substantial number of nodules (Robinson, 1969a). The ineffective strain was sometimes found to be slightly later in producing nodules than the effective strain, supporting earlier ideas about the basis of competitive ability. Robinson hypothesized that the host was able to distinguish between the effective and ineffective strains, and that the compatability of the nitrogen-fixing symbiosis somehow determined the compatability of nodule formation.

There are other instances of effective strains having an advantage in nodulation, although researchers have not agreed on what the basis of that advantage is. Studies of Medicago and Trifolium spp. inoculated with various proportions of paired strains also showed that the less effective strains were poorly competitive, although no correlation was found between competitive

ability and speed of nodulation (Marques Pinto et al., 1974). The effective strain TA1 was found to be a stronger competitor than ineffective strains of R. trifolii, although competing strains were better able to colonize plant roots (Labandera and Vincent, 1975). The competitive ability of TA1 was not related to the number of nodules formed when used singly, but it consistently produced nodules slightly earlier than other strains.

Evidence for the selection of effective strains over ineffective ones has not been conclusive, as there are many findings to the contrary. Data from Vincent and Waters (1953) and Nicol and Thornton (1941) showed that less effective strains could form a larger proportion of nodules than more effective strains. Although white clover plants generally selected the more effective strains from a 50:50 mixture, there was great variation between individual plants, and some actually preferred the much less effective strain (Jones and Russell, 1972). This departure from Robinson's hypothesis was thought to be at least in part due to the genetic heterogeneity of white clover, in contrast to the homogeneity of the subterranean clover used in the earlier work.

In studies with two tropical legumes, Franco and Vincent (1976) could not relate success in nodulation to any one factor. Although a slow-growing, effective

strain was far more competitve than a fast-growing, ineffective strain on siratro, an ineffectice strain was superior on stylo. The reason for the greater competitive ability of the ineffective strain in the latter case was considered to be either its significantly earlier nodulation or a greater compatability with the host.

The selection by host legumes for effective or ineffective strains is subject to modification by external conditons. The proportion of nodules formed by an effective strain of R. trifolii was higher than would be expected under acid conditions, but the opposite was true under neutral and alkaline conditions (Russell and Jones, 1975b).

No preference was shown by Medicago sativa when inoculated with effective and ineffective strains of the same origin (Arerger, 1981), although the plants did select strains from mixtures of equally effective and ineffective strains. Amerger hypothesized that work showing a preference for effective strains resulted from the use of wild-type strains that had, through natural selection, become both effective and competitive.

Interstrain Competition for Nodulation of Leucaena leucocephala

The development of inocula for leucaena has been

hindered by its adaptation to alkaline soils and perennial habit. If this plant's potential as a source of firewood, green manure, and forage for the tropics is to be realized, inocula must be developed that will supply strains able to survive in acid soils and to reinfect the trees in subsequent years.

Although strains isolated from leucaena are typically fast-growing, acid-producing types, at least one strain has been found that grows more slowly and is a mild alkali-producer. Norris (1973) compared these two types of strains on a loam soil of pH 5.0. The alkali-producing strain CB81 was able to form nodules whether pelleted with lime or unpelleted, and produced plants that significantly outyielded those inoculated with the acid-producing strains NGR8, which was only able to form nodules when pelleted with lime. However, Norris cautioned that it was highly unlikely that the nodules produced around the crowns of plants by seed pelleting would be able to ensure success for perennials growing in acid soils. When Bushby (1982) reexamined Norris' plots three years later, NGR8 was not found, while CB81 was recovered from 33% of tested nodules. After three years, there were no differences between pelleted and unpelleted treatments.

In further studies with these two strains, Bushby (1982) found that both survived better in a sandy spodosol of pH 5.2 than in a mollisol of pH 6.3. CB81 was again superior, being able to survive longer, multiply more readily, and form more nodules on plants in both soils. Tests in two Philippine soils showed CB81 to be superior to strain L when both strains were used singly (Paterno and Tilo, 1980). When the two were used together, the indigenous strains were able to form a larger proportion of nodules, indicating that the two strains were incompatible.

The fast-growing strain TAL582 was quite unsuccessful when used to inoculate seeds in dibble tubes for transplanting and for direct seeding into a Hawaiian soil of pH 6.0 (South, 1982). The percentage of nodules containing TAL582 when harvested after ten weeks in the soil ranged from 0 to 4%.

TAL1145 was found to be the strongest competitor among six strains investigated at two sites in Hawaii (Moawad and Bohlool, 1984). The inocula studied included the NifTAL-recommended inoculum for leucaena, containing strains TAL82, TAL582, and TAL1145, this mixture plus each of three strains isolated from the field sites, and a mixture of the three native strains.

TAL1145 was the best competitor in both an oxisol and a mollisol, and showed the most consistent competitive performance, forming 64% of the nodules when the NifTAL mixture was used, and from 28 to 66% when the native strains were also used (Moawad and Bohlool, 1984). Soil type was found to affect competitive ability, with strains performing best at the site from which they were isolated.

MATERIALS AND METHODS

This project was a joint venture by the University of Arizona and the Instituto Nacional de Investigaciones sobre Recursos Bioticos in Jalapa, Veracruz, Mexico. Nodules were collected in Mexico, and the initial isolations of rhizobia were done there, as were later field and pot studies. Greenhouse screening for effectiveness of biological nitrogen fixation, and the remainder of the competition, laboratory, and antigenic studies were done at the University of Arizona in Tucson.

Strains Used

Mexican isolates of rhizobia were obtained from Dr. Joann Roskoski. Nodules were collected from Leucaena leucocephala at three sites in southern Veracruz state (Appendix A), and rhizobia were isolated using Vincent's method (Vincent, 1970). After rinsing with 95% ethanol, sterilizing in 0.1% acidified mercuric chloride, and rinsing with sterile tap water, nodules were crushed and streaked on yeast extract mannitol plates (Appendix B). Single colonies provided seven isolates from each site.

Other strains effective on leucaena were obtained from the NifTAL Project, Paia, Hawaii. Seeds were collected from Hawaiian-type leucaena growing near Jalapa, Veracruz.

The rhizobia from Mexico were tested using the method of Bernaerts and de Ley (1963) to eliminate Agrobacterium spp. Clumps of cells were placed on lactose plates (Appendix B), and after two days a small amount of Benedict reagent (Appendix B) was poured over the surface of the agar. The plates were examined after one half hour for the presence of a yellow ring of cuprous oxide that would indicate the production of the reducing sugar 3-ketolactose by a strain of agrobacteria. Two of the isolates from Carrizal gave a positive reaction to this test, and were not considered further.

Greenhouse Screening

Two isolates from each Mexican site and four strains from NifTAL were chosen for greenhouse screening of effectiveness of bioloigcal nitrogen fixation as evaluated by biomass production and nitrogenase activity. Rhizobia were grown in yeast extract mannitol broth (Appendix B) on a rotary shaker at room temperature for 3 to 5 days. Seeds were inoculated with 1ml of broth, and the exact numbers of cells were calculated from plate counts of dilution series done at planting (Table 1).

Table 1. Origin and inoculation rates for greenhouse screening

Strain	Origin	Number cells seed^{-1}
5B2	Tlacotalpan, Ver.	1.02×10^{7}
5ZC	Tlacotalpan, Ver.	2.45×10^{7}
10A2	Veracruz, Ver.	2.80×10^{9}
10ZE	Veracruz, Ver.	3.14×10^{8}
13ZB	Carrizal, Ver.	8.75×10^{8}
13ZD	Carrizal, Ver.	1.94×10^{9}
TAL82	NifTAL	4.53×10^{7}
TAL582	NifTAL	3.17×10^{8}
TAL1145	NifTAL	1.46×10^{9}
RL52	NifTAL	5.50×10^{8}

Seeds were rinsed with 95% ethanol, then surface sterilized with 0.2% acidified mercuric chloride (Vincent, 1970), and scarified by rinsing in 90°C autoclaved tap water. Glass petri dishes with damp filter paper in the bottoms were autoclaved and used for germinating the sterilized seeds.

Layers of pea gravel 2cm deep were put into the bottoms of 15cm plastic pots, which were then filled with

20 grit silica sand, and autoclaved for 14 hours. Three pregerminated seeds were planted in each pot, inoculated with 1ml of rhizobia culture each, and later thinnned to one per pot. To reduce airborne contamination, the surface of the sand was covered with a 1.5cm layer of pea gravel that had been autoclaved for 14 hours. Modified Jensen's nutrient solution (Vincent, 1970; Appendix B) was added as needed through elbow straws to plastic trays snapped to the bottoms of the pots. To further reduce cross contamination, the pots and trays were set into a plastic bag, and a rubber band was added around the top of the pot to secure the bag and straw.

Plants were grown in the greenhouse from November 12, 1981 to May 20, 1982. During the coolest part of the winter, greenhouse temperatures averaged 15 and 30^{o}C in the early morning and late afternoon, repectively. A randomized complete block design with five blocks was used. At harvest two missing plants in the 5ZC treatment, and one missing control plant resulted in three and four replications for these treatments, respectively.

After 14, 17, 22, and 28 weeks, visual ratings were assigned to each treatment by 3 to 4 people, and plant heights were measured. The plants were harvested after 28 weeks by cutting at the level of the gravel.

Plant tops and roots were oven dried at 60°C for three days, and the dry weights of tops, roots, and nodules were determined. Each root was separated from the sand and put into a 0.95 liter (1 quart) canning jar to assay for nitrogenase activity (Hardy et al., 1968).

A septum from a blood collection tube (Vacutainer 10 ml, 100x16mm) installed in the canning jar lid allowed gas sampling. Fifty ml of air was removed from the jar with a syringe, and 50ml of acetylene was added. Gas samples were taken after one hour with a 22 gauge double-ended needle and blood collection tubes (Vacutainer 10ml). One ml samples were taken from the tubes with a gas-tight syringe (Precision Sampling Corp. Series A-2 "Pressure-Lok") and analyzed with a gas chromatograph (Varian 3700) equipped with a flame ionization detector. The umoles of acetylene reduced/plant/hr were then calculated (Appendix C).

TAL1145 and 5B2 from Tlacotalpan were chosen for further study. TAL1145 had the highest biomass production and visual rating scores, and was able to reduce large amounts of acetylene. Strain 5B2 was selected as representative of rhizobia associated with leucaena in the areas of neutral to alkaline soils to which the tree is native after some of the other Mexican strains lost effectiveness.

Production of Fluorescent Antibodies

Cultures were grown as for greenhouse screening. The cells were washed by centrifuging, then resuspending them three times in filter sterilized 0.85% NaCl containing 0.01% merthiolate, then heated in a boiling water bath for one hour to inactivate flagellar antigens. The concentration of cells was adjusted to about $1x10^9$/ml by adding filtered saline until an absorbance of 0.60 at 640nm was reached (Bausch and Lomb Spectronic 100). The cell suspensions were stored in blood collection tubes at 4^oC until needed.

Cells were injected into the marginal ear vein and the loose skin at the back of the neck of virgin female New Zealand White rabbits with 25 guage 16mm (5/8 in) needles according to the schedule in Table 2. Up to 50ml of blood was collected in a syringe from the main ear artery every two weeks with a 23 guage 19mm (3/4 in) infusion set.

The blood was transferred to a jar, allowed to clot at room temperature for 1 to 2 hours, and the serum was poured off. The clot was stored in the refrigerator overnight, and the additional serum extruded was collected. The serum was then kept in the refrigerator for one day to allow blood cells to settle out, then pipetted off and stored at -20^oC. The titer of the

Table 2. Injection schedule for rabbits

Day	Amount
1	0.5ml IV, 2.0mlSC
2	1.0ml IV
3	1.5ml IV
4-6	Rest
7	1.0ml SC
8	1.5ml SC
9	2.0ml SC
10	2.0ml SC
11	2.0ml SC
12	2.0ml SC
Then 1.5ml SC every 14 days.	

antiserum was checked against the homologous antigen at dilutions of 1/400, 1/800, 1/1600, 1/3200, and 1/6400 in 0.85% NaCl. After reacting overnight at 35^{o}C, the highest dilution that agglutinated the antigen and left a clear supernatant gave the titer of the antiserum.

Antiserum with a titer of 1/1600 or greater was used to prepare fluorescent antibodies using a modification of the procedures of Hill and Gray (1967),

Schmidt, Bankole, and Bohlool (1968), and Trinick (1969). The antiserum was fractionated in an ice bath with saturated ammonium sulfate (706g/liter) and the gamma globulins were collected by centrifugation. This was repeated two to three times until a clean white precipitate was obtained.

The protein fraction was then suspended in deionized water and dialyzed against 0.85% NaCl (VWR dialysis tubing 6.35mm (1/4 in) diameter) for 14 hours at 4°C to remove ammonium sulfate. The protein content was determined (Bio-Rad Protein Assay Kit I) and adjusted to 1% with deionized water before the addition of phosphate buffers and 50ug fluorescein isothiocyanate (FITC)/mg protein.

After stirring in the dark overnight at 4°C to conjugate the serum and FITC, the fluorescent antiserum was dialyzed against pH 7.0 phosphate buffer (Appendix B) in the dark for three days to remove excess FITC. To prevent bacterial contamination, merthiolate was added to 0.01%, and the conjugate was centrifuged and filtered (Millipore Corp. .45um) to remove particulate matter. The antiserum was stored at -20°C until needed.

The fluorescent antisera were each tested against both strains of rhizobia to assure that the reactions were specific and without cross-reaction.

Covering the bacterial smear with gelatin-rhodamine isothiocyanate (RhITC) containing 8mg RhITC/g of gelatin (Bohlool and Schmidt, 1968, Appendix D) reduced background fluorescence when the slides were examined under a microscope (Zeiss) equipped with an epifluorescent ultraviolet lamp. The two strains, TAL1145 and 5B2 were found to be serologically distinct.

Metabolic Properties

To learn more about the rhizobia effective on leucaena and their relationships to other types of rhizobia, the growth rate and pH change in media of the two strains used in these studies were examined. Growth rates were studied in yeast extract mannitol broth (Appendix B), and pH change was studied on yeast extract mannitol plates with added bromocresol blue (Appendix B).

For growth rate studies strains were first grown as before to log phase, then 0.5ml of the cultures were used to inoculate 250ml of broth in 500ml erlenmeyer flasks with two replications. The cultures were grown at 25^{o}C in a water bath shaker (American Optical Scientific Instrument Division model #406015) at 80 strokes per minute. Each flask was sampled every six hours, and the generation times were calculated (Appendices E and F; Figure 1). Spectrophotometric anaylsis (Bausch and Lomb

Spectronic 100) at 640nm allowed calibration with cell concentrations found using serial dilution plates.

Dilution series of log phase cultures of the strains were plated out on media with and without bromocresol blue. Color change of bromocresol blue from blue to green was accepted as an indication of acid production.

Competition Study in Sand

To determine their relative abilities to compete for nodulation sites, the selected strains were used to inoculate seeds singly, and together in cell ratios of 50:50 and 1:100 in favor of each. The ends of dibble tubes (Ray Leach Cone-Tainer Nursery, Canby, Oregon) were wrapped with a strip of tape until large enough to stand upright in 16 dram vials with only the bottom portion of the tube inside the vial. The cone-tainers and vials were soaked in 20% bleach, rinsed with sterile tap water, then assembled with two elbow straws tucked together end-to-end running through the center of the tube into the vial. A small amount of pea gravel that had been autoclaved for 2.5 hours was put in the bottom of the cone-tainer, and the tube was filled with autoclaved 20 grit silica sand.

Seeds were surface sterilized and germinated as for the greenhouse study. One seed was planted per tube, and sterile deionized water was added to keep the sand

moist until the seedlings were 3cm tall. During this time, the cone-tainers were stored inside a large translucent plastic garbage bag to reduce cross contamination. Bacterial cultures were grown as for the greenhouse study. As the growth rates of the two strains were very similar, the cultures were mixed by volume when used, and the exact numbers of cells applied were calculated from plate counts of dilutions of the inocula (Table 3).

One ml of inocula was added to each 3cm-tall seedling, and the sand was covered with autoclaved pea gravel to reduce airborne contamination. The vial at the bottom of the tube was filled with sterile nutrient solution modified from Norris and Date (1976, Appendix B) then taped to the cone-tainer to reduce cross contamination. Nutrient solution was added through the straw as needed to keep the vial full.

The plants were grown for eleven weeks in a growth chamber (Percival walk-in growth chamber model #PGW-9675) with day and night temperatures of 27 and 16^{o}C, respectively, with 12 hours of light each day. Each treatment was replicated four times, and six uninoculated controls were used. Two of the controls remained nodule-free throughout the experiment, and only data from these two were used.

Table 3. Inoculation rates for competition study in sand

Treatment	Number cells seed^{-1}		Ratio of cells
	5B2	TAL1145	5B2:TAL1145
5B2	$6.9x10^8$	0	-
TAL1145	0	$5.2x10^8$	-
50:50	$3.4x10^8$	$2.6x10^8$	1.3:1
5B2 100:1	$6.8x10^8$	$5.2x10^6$	130.8:1
TAL1145 100:1	$6.9x10^6$	$5.1x10^8$	0.013:1

This study was harvested in the same manner as the greenhouse screening, with acetylene reduction data, and top, root, and nodule dry weights collected. The tops plus roots were analyzed for total nitrogen using micro-Kjeldahl techniques (Bremner, 1965).

One hundred mg samples were digested for three hours after clearing in 3ml sulfuric acid with 2g catalyst mixture containing 100g K_2SO_4, 6.39g $CuSO_4$, and 10g Se. Ten ml of 50% NaOH were added, the samples were distilled, and 35 ml of distillate were collected in 6ml of 2% boric acid indicator solution containing 1% methyl red-methylene blue (2:1) indicator (Appendix B).

Back-titration with 0.02N and 0.005N potassium diiodate (Appendix G) gave the percentage of nitrogen in the samples.

The nodule contents were identified using fluorescent antibodies to determine the percentage formed by each strain. The dried nodules were soaked in a small amount of deionized water in individual wells of spot plates, and crushed with a clean glass rod. Smears were heat fixed on glass slides, then covered with RhITC which was dried to a film at 60°C. Staining was done in moist chambers for 30 minutes using fluorescent antisera diluted to half-strength with pH 9.0 phosphate buffer (Appendix B). The stained slides were rinsed in 0.85% NaCl, then deionized water, and air-dried. All nodules were checked with antisera against both strains to identify double infections or contamination with other than inoculated strains.

Pot Competition Study

Strains were grown as before, and 20ml of each culture were mixed into 40g of autoclave-sterilized peat with 2g of calcium carbonate in a whirl-pak bag (NASCO 18oz [532 ml]). The peat inocula were sent to Veracruz where they were used to inoculate surface-sterilized seeds using 20% gum arabic containing 2% calcium carbonate as a sticker. Seeds were planted in dibble

tubes filled with a mixture of equal parts by volume of vermiculite, sand, and gravel, that had been autoclaved for four hours. The plants were grown outside in Jalapa, Veracruz, with modified Jensen's nutrient solution (Appendix B) added as needed.

Seedlings were planted in 57 liter plastic pots in December, 1982, after 22 weeks growth in cone-tainers. A factorial design replicated four times was used which included both strains of rhizobia and soil from both field sites: Jalapa (elevation 1225m, soil pH 5.0) and La Balsa (elevation 450m, soil pH 7.1). The pots were located outdoors in Jalapa, and watered as necessary. At harvest there were 14 surviving plants, with one missing from the TAL1145 in Jalapa soil and 5B2 in LaBalsa soil treatments.

This study was harvested in July, 1983, and acetylene reduction assays (Appendix C) were carried out as before (Carle model 9500 gas chromatograph). Leaf, stem, root, and nodule dry weights were found, and the total nitrogen contents of leaves, stems, and roots were determined using Kjeldahl techniques. A random subsample of nodules from each plant was taken to Tucson, and the contents identified as in the sand competition study. When positive reactions were found to the antisera of the non-inoculated strains, a study was done to determine

the presence or absence of indigenous strains of rhizobia reacting with the antisera used. Fresh soil samples were collected from each of the field sites, and care was taken that they were from areas away from the inoculated plots, and outside traffic patterns.

Each soil was added to four Leonard jars, with aluminum foil covering the necks of the jars and secured with rubber bands. Two small holes in the foil allowed drainage, and the addition of bleach to the reservoir jars helped to reduce cross-contamination. Several (four to six) pregerminated seeds were sown in each jar, and tap water was added as needed. The plants were grown for five weeks in the growth chamber under the same condition as the competition study in sand. Several nodules were found on each plant, and the contents were examined as before using both antisera. None of the nodules were found to contain bacteroids reacting with antisera to either inoculated strain.

RESULTS AND DISCUSSION

Greenhouse Screening

Trees inoculated with the investigated strains were evaluated during growth in the greenhouse by periodic visual ratings and height measurements. At harvest, the acetylene reduction assay was used to evaluate the efficiency of biological nitrogen fixation, and the top, root, nodule and total dry weights were used as a measure of biomass production. Much variation in biomass production and efficiency of biological nitrogen fixation was found among strains collected in Mexico (Tables 4, 5 and 6). Some compared favorably with the proven strains, while one, strain 10ZE, produced plants smaller than the uninoculated controls.

TAL1145 had consistently high visual ratings and height measurements. It also produced the largest top, root, and total biomass of any strain, had the highest rate of acetylene reduction on a per mg nodule dry weight basis, and the highest of any Hawaiian strain on a per plant basis. This strain, already known to be a superior strain, was judged to be the best overall strain in the study, and was selected as a comparision for the Mexican strain chosen.

Table 4. Visual ratings of trees during greenhouse screening

	Visual rating[1]			
Strain	13 wks	16 wks	21 wks	27 wks
5B2	3.30	3.25	3.00	3.25
5ZC	2.67	2.88	2.75	3.13
10A2	3.83	3.88	3.50	3.13
10ZE	1.00	1.12	1.25	2.06
13ZB	4.50	4.62	3.75	3.50
13ZD	3.67	3.62	4.00	4.25
TAL82	3.83	4.50	2.75	4.13
TAL582	4.00	3.75	4.00	4.38
TAL1145	5.00	4.62	5.00	4.88
RL52	4.30	4.38	4.25	3.88
Control	2.00	1.75	2.00	2.75

[1] 1 to 5, 5 high

Table 5. Heights of trees during greenhouse screening

	Height (cm)[1]			
Strain	13 wks	16 wks	21 wks	27 wks
5B2	13.12	18.68	28.70	43.60
5ZC	12.17	17.90	27.43	43.80
10A2	13.74	22.45	32.40	48.94
10ZE	9.46	12.80	16.92	26.80
13ZB	15.50	19.64	35.34	43.20
13ZD	13.18	17.08	28.96	53.84
TAL82	14.98	21.28	27.83	57.30
TAL582	14.04	19.36	38.66	63.66
TAL1145	14.88	23.56	37.94	60.60
RL52	14.40	20.14	36.66	60.36
Control	9.64	15.48	29.00	39.72

Table 6. Biomass and acetylene reduction from greenhouse screening

	biomass (dry weight)				nmol acetylene reduced hr^{-1}	
Strain	tops(g)	roots(g)	nodules(mg)	total(g)	$plant^{-1}$	mg $nodule^{-1}$
5B2	6.23	6.59	317	13.1	5600	18
5ZC	5.52	6.29	330	12.2	6000	18
10A2	5.75	7.80	363	13.9	8500	23
10ZE	2.21	2.83	142	5.2	3500	25
13ZB	5.34	5.35	208	10.9	4500	22
13ZD	8.11	7.39	347	15.9	10000	29
TAL82	7.65	7.34	434	15.6	6800	15
TAL582	8.66	7.46	370	16.5	8600	23
TAL1145	8.89	9.62	312	18.6	9300	30
RL52	7.68	8.78	339	16.8	7900	23
Control	4.70	4.47	263	9.6	3500	13

Strain 5B2, from southern Veracruz state, was quite high in visual ratings, height measurements, biomass production and rates of acetylene reduction. This strain seemed to be typical of rhizobia effective on leucaena in the areas where the plant is native, and was selected for further study after some of the Mexican strains lost effectiveness.

Metabolic Propeties

Generation times in yeast extact mannitol broth were 3.3 hours for 5B2, and 2.9 hours for TAL1145 (Figure 1, Appendices E and F). These rates were similar to the 3.0 hours found by Tan and Broughton (1981, 1982), and indicated that both strains were fast growing strains, as they had generation times between 2 and 4 hours (Fred et al., 1932; Vincent, 1974).

TAL1145 and 5B2 both produced a yellow color on yeast extract mannitol plates with added bromocresol blue, indicating the production of acid in laboratory media. This is in keeping with other findings for fast-growing strains (Fred et al., 1932; Vincent, 1974). These strains are of the genus Rhizobium described by Jordan (1984), and are similar to root-nodule bacteria previously found associated with leucaena, being fast growers and producing acid.

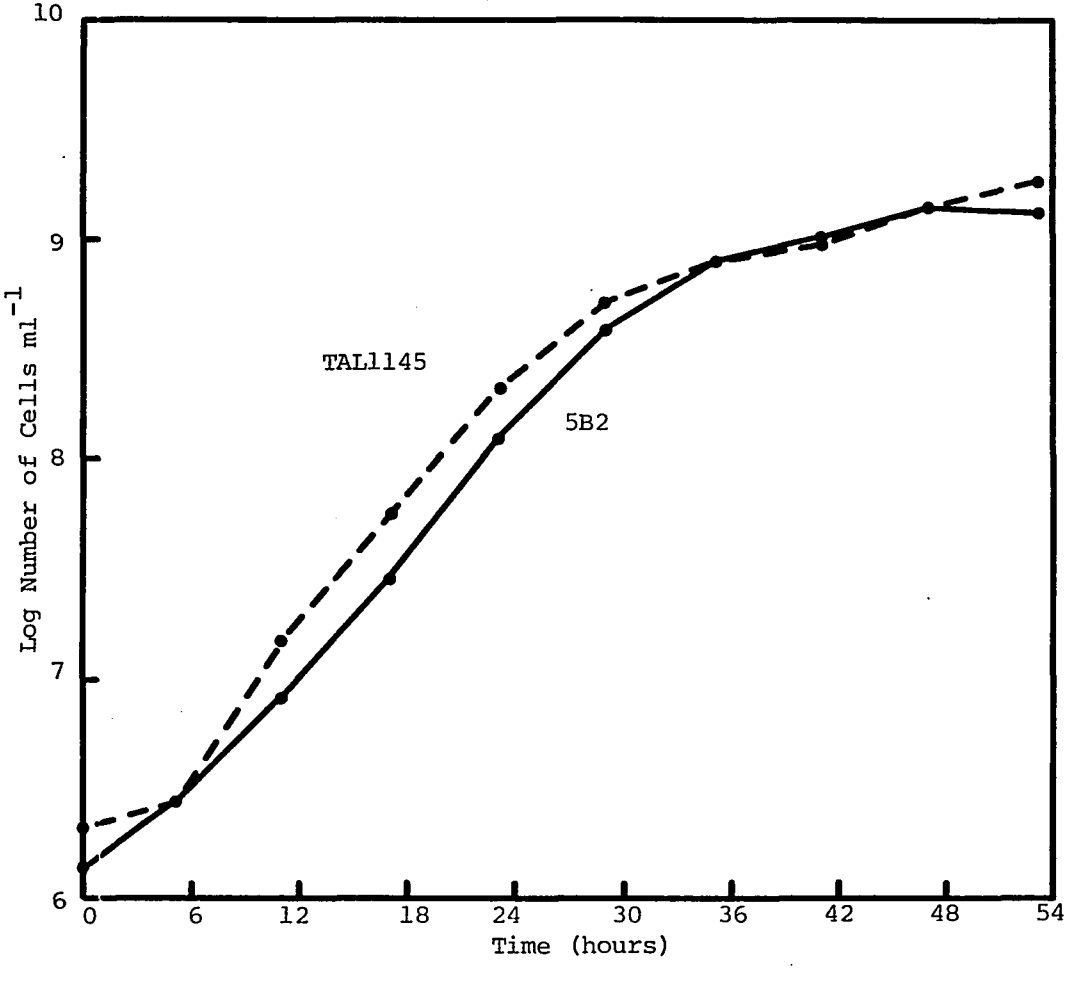

Figure 1. Growth of Rhizobia in Yeast Extract Mannitol Broth

Competition Study in Sand

At harvest, the rates of acetylene reduction on a per plant and per mg nodule dry weight basis were determined for this study. In addition, the top, root, nodule and total dry weights, and the nitrogen content of the tops plus roots on a percentage and a total weight basis were determined. Analysis of the nitrogen content of the nodules was not possible as they were used in determining the representation of the strains on the plants.

The effects of the ratios of the numbers of cells of the two strains used to inoculate the seeds were analyzed using the MSUSTAT statistical package (Lund, 1983). The AV1W program for a one-way analysis of variance was used to determine differences between treatments in the representation of the strains in the nodules after log transformations of the data, and in the plant growth variables examined (Appendices I and J, respectively). Significance was determined at the 5% confidence levels, and mean separations were determined using the Least Significant Difference Test in the AV1W program of MSUSTAT (Lund, 1984).

Table 7 and Appendix H show the nodule contents from this study. The reason for the positive reaction with TAL1145 serum of some of the nodules on the 100% 5B2

Table 7. Identification of nodule contents from competition study in sand,

Ratio cells seed^{-1} 5B2:TAL1145	Nodule Number	% Nodules Reacting With 5B2	TAL1145	Both	Unknown
100% 5B2	18	85.0c	15.0a	0a	0a
100% TAL1145	19	0a	100b	0a	0a
1.3:1	20	10.8b	71.4b	2.8a	15.0bc
130.8:1	10	51.5c	41.8ab	2.0a	4.7ab
0.013:1	19	43.2c	23.6ab	8.1a	25.1c

Columns of values followed by the same letter are not significantly different at the 5% confidence level.

treatment is not known. It is unlikely that TAL1145 actually formed nodules on this plant, as all of the other 100% treatments were completely free from the uninoculated strain, and the controls were largely nodule-free. The nodules that gave the "wrong" response were all in one batch of nodules processed together on one spot plate, all of the nodules on that plate gave the same response, and none of the other nodules from that plant gave a positive response with TAL1145 antiserum. It appears that this problem was due to operator error, although attempts to locate the source of the problem were unsuccessful.

TAL1145 formed an increasing percentage of nodules as it increased in the inoculum from a ratio of 1:131 to 1:1.3 to 100%. It formed 42% of the nodules when present as 1% of the inoculum, 71% when present as 50%, and 100% when forming all of the inoculum. This trend was seen in all of the treatments except where TAL1145 formed a majority of the inoculum by a ratio of 1:0.013, where if formed only 24% of the nodules.

However, 5B2 formed more nodules than TAL1145 when it was present as both 99% and 1% of the inoculum, forming 52 and 43% of the nodules, respectively. TAL1145 was able to form more nodules than 5B2 only in the 50:50 mixture where it was present as 43.0% of the incoulum. Here it formed a large majority, (71%) of the nodules.

Double infections ranged from 2.0% in the 100:1 treatment in favor of 5B2, to 8.1% in the 100:1 treatment in favor of TAL1145.

Tables 8 and 9 show the results of the determination of the plant growth variables examined. The uninoculated controls produced significantly less biomass of all kinds, contained less nitrogen, and had lower rates of acetylene reduction than any of the inoculated treatments.

Although a 50:50 mixture of the two strains gave the highest top, root, nodule and total plant weights, the highest rate of acetylene reduction on a per plant basis, and the largest nitrogen content on a weight basis, this treatment was not significantly different from any of the others in any of the variables. Hofer (1945), Burton and Allen (1950), Russell and Jones (1975b) and Josephson and Pepper (1984) found similar trends although these differences were also not found to be statistically significant.

The amounts of acetylene reduced ranged from 23 to 43nmoles/mg dry nodule/hr. This was slightly higher than the 15 to 30nmoles/mg dry nodule/hr for the older plants in the greenhouse screening, and compares favorably with the 35nmoles/mg dry nodule/hr obtained by Hogberg and Kvarnstrom (1982) for a four-year-old stand in Tanzania. Although the rates of

Table 8. Biomass from competition study in sand

Ratio cells seed^{-1}	Biomass (mg)			
5B2:TAL1145	tops	roots	nodules	total
Control	78a	212a	0a	291a
100% 5B2	528b	461ab	40b	1029b
100% TAL1145	576b	539b	42b	1157b
1.3:1	631b	625b	51b	1307b
130.8:1	548b	545b	33b	1126b
0.013:1	442b	410ab	36b	888b

Columns of values followed by the same letter are not significantly different at the 5% confidence level.

Table 9. Nitrogen content and acetylene reduction from competition study in sand

Ratio cells seed^{-1}	N content		nmol C_2H_2 reduced hr^{-1}	
5B2:TAL1145	%N	mgN	plant^{-1}	g dry nod^{-1}
Control	0.83a	2.4a	0a	0a
100% 5B2	2.19b	21.2b	1748c	43.095c
100% TAL1145	2.07b	23.3b	1022b	23.435b
1.3:1	2.08b	25.5b	1816c	36.495c
130.8:1	2.16b	23.7b	1182bc	35.472bc
0.013:1	2.22b	18.7b	1378bc	39.557c

Columns of values followed by the same letter are not significantly different at the 5% confidence level.

acetylene reduction on a per mg nodule dry weight basis were on the same order as those in this study, the rates per plant were higher with the older trees in the greenhouse screening, where they ranged from 3500 to 10,000nmoles/plant/hr, compared with 1000 to 1800 nmoles/plant/hr for the competition study in sand.

The root biomass of the 100% 5B2 treatment was the lowest found, although it was not significantly different from either the controls or the rest of the inoculated treatment. No significant differences were found between treatments for nitrogen content, and the only significant difference found in the rates of acetylene reduction was that of a significantly lower rates of acetylene reduction, both on a per plant and per mg nodule dry weight basis for the 100% TAL1145 treatment.

It is difficult to draw any conclusions about either strain's competitive ability as there are no clear-cut relationships apparent between the ratios of numbers of cells used in the inocula and relative numbers of nodules formed. As TAL1145 formed a larger proportion of the inoculum it was able to form an increasing percentage of nodules in several treatments, although it was unable to form a significant percentage of nodules when at an advantage of 1:0.013.

Although the highest values for biomass production, nitrogen content on a total weight basis, and rate of

acetylene reduction of a per plant basis were found in the 50:50 treatment which contained a high percentage of nodules formed by TAL1145, there does not appear to be a direct relationship between the representation of TAL1145 in the nodules and plant performance. Although the 100% TAL1145 treatment, made up of nodules containing only TAL1145, showed higher levels of all types of biomass production and a higher total weight of nitrogen than the 100% 5B2 treatment, the percent nitrogen content and the rates of acetylene reduction on both a per plant and per mg nodule dry weight basis were higher for the plants with only 5B2 nodules. In fact, the rate of acetylene reduction on a per mg nodule dry weight basis for the 100% TAL1145 treatment was found to be significantly lower than that for any of the other treatments.

Pot Competition Study

The variables analyzed in this study included the leaf, stem, root, nodule and total dry weights, the rate of acetylene reduction on a per plant and per g nodule dry weight basis, and the nitrogen contents of the leaves, stems and roots on a percentage basis, and the total nitrogen content on a percentage and a total weight basis. Again, the nodules were used in determing the representation of the strains on the plants, so were not available for nitrogen analysis.

The effects of the strain, the soil, and interactions between the two were analyzed using the AVMF program from MSUSTAT for a 2 X 2 factorial design (Lund, 1983). Analysis of variance tables were prepared with values for significance at the 5% confidence levels for the representation of strains in the nodules after log transformations of the data, and the plant growth variables (Appendices L and M, respectively). Means were separated using the Least Significant Difference Test in the AVMF program of MSUSTAT (Lund, 1983).

Identification of nodule contents (see Table 10 and Appendix K) showed that only 5B2 was recovered in significant numbers from the La Balsa soil, and only TAL1145 in significant numbers from the Jalapa soil, regardless of which strain had been used to inoculate the seeds. If this had been the result of cross-reacting strains in the two soils, it would have been highly unlikely that a nonrandom pattern such as this would have been seen. As an additional study failed to show the presence of bacteria in either soil reacting with the antisera used, it was concluded that this was the result of cross-contamination while the seedlings were growing in the cone-tainers prior to being planted in the large pots of soil. The would have been very possible, as the trees were growing out-of-doors and standing very close to each other where cross-contamination due to wind, or

Table 10. Identification of nodule contents from pot competition study, treatment means

Soil and Strain	Nodule Number	% Nodules Reacting With 5B2	TAL1145	Both	Unknown
Jalapa Soil					
5B2	49	1.0a	37.6a	1.0	60.3
TAL1145	50	2.0a	32.1a	5.6	60.4
La Balsa Soil					
5B2	29.7	27.8b	0a	0	72.2
TAL1145	39.0	9.5b	2.0a	0.5	88.0

Columns of values followed by the same letter are not significantly different at the 5% confidence level. No significant differences found at the 5% level for columns with no letters.

during rains or the addition of nutrient solution would have been very easy.

The different numbers of nodules formed by each strain in the two soils must then be a reflection of the strain's differing abilities to survive and compete against the indigenous strains present in the two soils. Read (1953), and Roughley, et al. (1976) found that soil and environmental conditions could influence a strain's competitive ability at sites in England and Australia. It must be remembered that treatments in soil from both sites were grown at Jalapa, where the weather is typically cool and wet, rather than under the climatic conditions associated with each field site.

TAL1145 was either more successful at competing with the native strains than 5B2, or the indigenous strains in the Jalapa soil were more competitive than those in the La Balsa soil. This is shown by the higher percentages of nodules containing TAL1145 in the Jalapa soil, than containing 5B2 in the La Balsa soil. This resulted in there being more nodules formed by unknown strains in the La Balsa soil: 72 and 88% when seeds were inoculated with 5B2 and TAL1145, repectively, versus 60% for both treatments in the Jalapa soil.

Moawad and Bohlool (1984) found TAL1145 to more successful when competing against the indigenous strains at two sites in Hawaii. When applied in mixtures with

two to three other strains it was able to form between 28 and 66% of the nodules.

TAL1145 was better able to survive and compete for nodulation sites in the Jalapa soil, and 5B2, isolated from a site in the same area of Veracruz state as the La Balsa site, was more successful in the La Balsa soil. TAL1145 was more successful in the acid (pH 5.0), and was isolated from a low pH soil in Hawaii. This is similar to the results Moawad and Bohlool (1984) obtained with leucaena at two sites in Hawaii. When native strains were used as inoculants, the strains performed best when they were used at the sites from which they had been isolated.

Double infections were quite low, ranging from 0.5 to 5.6% of the nodules. This is slighlty higher than other findings of <1.0 to 3.5% for field-grown plants (Brockwell et al. 1977; Bromfield and Jones, 1980), but lower than that for the sand competition study, where double infections ranged from 2.0 to 8.1% of the nodules.

Tables 11 and 12 show the results of this study. It is not surprising that effects of the strain were uncommon. However, significant effects of the soil were found for leaf dry weight and stem nitrogen content, with the plants growing in Jalapa soil having higher levels of

Table 11. Biomass and acetylene reduction from pot competition study

Soil and strain	Biomass (grams)					nmol C_2H_2 reduced hr^{-1}	
	leaves	stems	roots	nodules	total	$plant^{-1}$	g dry nod^{-1}
Jalapa soil							
5B2	7.28a	5.18	8.09	0.5842	21.13	1.66b	4.39
TAL1145	7.76a	6.73	9.45	0.4656	24.41	1.82b	4.08
LaBalsa soil							
5B2	13.53b	9.80	9.16	0.2274	32.72	0.500a	2.10
TAL1145	12.25b	9.35	10.58	0.5404	32.72	1.55a	3.28

Columns of values followed by the same letter are not significantly different at the 5% confidence level. No significant differences found at the 5% level for columns with no letters.

Table 12. Nitrogen content from pot competition study

Soil and strain	leaves(mg)	stems(mg)	roots(mg)	total(mg)	total(%)
Jalapa soil					
5B2	331.8	80.03a	117.6	529.3	2.430
TAL1145	354.5	97.23a	118.1	569.8	2.433
LaBalsa soil					
5B2	573.0	186.7b	121.0	880.7	2.746
TAL1145	485.2	135.3b	134.7	755.2	2.350

Columns of values followed by the same letter are not significantly different at the 5% confidence level. No significant differences found at the 5% level for columns no letters.

both. In addition, the rate of acetylene reduction/ plant/hr was significantly higher in the La Balsa soil.

This shows that, under Jalapa's climatic conditions, trees growing in the La Balsa soil with nodules containing primarily the 5B2 strain had higher rates of acetylene reduction. These trees also produced larger leaves, and contained larger amounts of stem nitrogen than those growing in the Jalapa soil with nodules containing primarily TAL1145.

The rates of acetylene reduction on both a per plant and per mg nodule dry weight basis were lower in this study than in either the greenhouse or growth chamber competition study. This is problably due to the presence of the nodules formed by the less-efficient indigenous strains. Rates per plant/hr ranged from 3500 to 10,000nmoles/plant/hr in the greenhouse screening, and from 1000 to 1800nmoles/plant/hr in the competition study in sand, while amounts reduced per plant/hr in the pot competition study were between 0.50 and 1.82nmoles/plant/hr. Rates per mg nodule dry weight were also much lower for the pot competition study, ranging from 2.10 to 4.39nmoles/hr, compared with from 15 to 43nmoles/hr for the greenhouse screening and competition study in sand.

CONCLUSIONS

More similarities than differences were found in these studies. Strains 5B2 and TAL1145 were both found to be fast-growers, with generation times of 3.3 and 2.9 hours, respectively, and both produced acid in laboratory media.

When the two strains were used singly and in varying proportions to inoculate seeds in sterile sand culture, TAL1145 was able to form an increasing percentage of nodules as it increased in the inoculum from a ratio of 1:131 to 1:1.3 to 100%. However, it was unable to form a significant portion of the nodules when it formed a majority of the inoculum by a ratio of 1:0.013. Neither strain was found to be consistently superior in any of the plant growth variables examined.

In the competition study in soil, striking differences were seen in the strains' abilities to survive in the two soils used. Strain 5B2 was better able to survive, and therefore compete for nodulation sites in the relatively high pH La Balsa soil, and TAL1145 was better suited to the more acid Jalapa soil.

APPENDIX A

ADDITIONAL INFORMATION ON RHIZOBIA COLLECTION SITES

Table A. Analysis of soils at rhizobia collection sites in Mexico

	pH[1]	N (%)[2]	Available P (ppm)	Exchangeable Cations (meq/100g)			
				Na[5]	K[5]	Ca[6]	Mg[6]
Tlacotalpan	7.0	0.254	12.6[3]	0.154	0.238	15.5	1.69
Veracruz	6.3	0.354	6.14[4]	0.224	0.391	21.8	1.75
Carrizal	7.1	0.460	3.65[3]	0.424	1.41	31.5	2.55

[1]In water, 1:1

[2]Kjeldahl nitrogen

[3]Olsen method

[4]Bray I method

[5]Flame emission photometry

[6]Atomic absorption spectrophotometry

APPENDIX B

MEDIA, BENEDICT REAGENT, NUTRIENT SOLUTIONS, BUFFERS AND INDICATOR

Yeast Extract Mannitol Medium
(modified from Vincent, [1970] p.3)

K_2HPO_4	0.5g
$MgSO_4.7H_2O$	0.2g
NaCl	0.1g
Mannitol	10.0g
Yeast extract	.4g
Deionized water	1 liter

For solid yeast extract mannitol medium add 15g agar/liter.

Autoclave 15 minutes at 120°C.

Lactose Agar Medium
(Bernaerts and DeLey, 1963)

Lactose	10g
Yeast extract	1g
Agar	15g
Deionized water	to 1 liter

Autoclave 15 minutes at 120°C.

Benedict Reagent
(Orum, personal communication, 1981)

I.	$CuSO_4.5H_2O$	17.3g
	Deionized water	100ml
II.	Na citrate	173g
	$NaHCO_3$	100g
	Deionized water	500ml

Dissolve indgredients in II. with heat. Filter, dilute to 700ml with deionized water. Add I. with constant stirring. Dilute to 1000ml.

Jensen's Nutrient Solution
(Vincent, [1970] p. 75)

$CaHPO_4$	1.0g
K_2HPO_4	0.2g
$MgSO_4.7H_2O$	0.2g
NaCl	0.2g
$FeCl_3$[1]	0.1g
Micronutrient solution[2]	1.0ml
Deionized water	to 1 liter

[1]Replaced with 0.5ml iron chelate (Hamp-Iron 845 5% Iron Chelate Solution, Organic Chemical Division, W. R. Grace & Co.

[2]See below

Micronutrient Solution
(from Vincent, [1970] p. 75)

H_3BO_3	0.2864g	(.05%)
$MnCl_2.4H_2O$	0.1802g	(.05%)
$ZnSO_4.7H_2O$	0.0220g	(.005%)
MoO_3	0.0075g	(.005%)
$CuSO_4$	0.0050g	(.002%)
Deionized water	to 100ml	

Add ingredients to deionized water one at a time, stirring to dissolve between additions.

Norris and Date's Nutrient Solution
(modified from Norris and Date, [1976] p. 163)

$CaSO_4.2H_2O$	0.34g
K_2HPO_4	0.17g
$MgSO_4.7H_2O$	0.25g
KCl	0.074g
Iron chelate[1]	0.5ml
Micronutrient solution[2]	1.0ml
Deionized water	to 1 liter

[1]Hamp-Iron 845 5% Iron Chelate Solution, Organic Chemical Division, W. R. Grace & Co.

[2]See above

Phosphate Buffers

Stock solutions

Solution A

$NaH_2PO_4.H_2O$	27.8g
Deionized water	to 1 liter

Solution B

$Na_2PO_4.7H_2O$	53.6g
Deionized water	to 1 liter

For pH 7.0 buffer

Solution A	19.5ml
Solution B	30.5ml
Deionized water	to 1 liter

pH 9.0 Phosphate Buffer

Na_2HPO_4	14.196g
Deionized water	to 1 liter

Boric Acid
Methyl Red - Methylene Blue
Indicator Solution

Dissolve 200mg methyl red Na salt in deionized water and dilute to 100ml. Dissolve 100mg methylene blue in deionized water and dilute to 50ml. Mix these two solutions together and store in the refrigeratore until needed.

To prepare the indicator, dissolve 10g boric acid in deionized water, add 5ml of the mixed indicator solution and dilute to 500ml. Prepare fresh boric acid indicator solution each day.

APPENDIX C

CALCULATIONS FOR ACETYLENE REDUCTION ASSAY

CALCULATIONS FOR ACETYLENE REDUCTION ASSAY

Method used for greenhouse screening and competition study in sand:

peak height x attenuation = sample units

ethylene in acetylene used = 7.14 units/ml

ethylene standard = 6.99×10^{-6} umoles/unit

jar size = 950ml

(sample units - 7.14 units/ml)(6.99×10^{-6} umoles/unit)(950ml) = umoles/plant/hr

Method used for pot competition study:

log mole C_2H_4 = .9921(log peak height) - 12.209

APPENDIX D

PREPARATION OF RHODAMINE ISOTHIOCYANATE

One gram of gelatin was mixed into 50ml of deionized water with gentle heating. The suspension was brought to 25°C, and the pH was adjusted to 11.0 with 1N NaOH. After autoclaving for 10 minutes at 120°C the pH was readjusted to 11.0.

Ten mg of rhodamine isothiocyanate were added to 2ml of acetone and as much as possible was dissolved. This was then added to the gelatin mixture, and stirred overnight at room temperature to allow conjugation to take place. The conjugated rhodamine isothiocyanate was then dialyzed with continuous stirring to remove excess dye. This was done against a solution of 200ml pH 7.0 phosphate buffer, 1800ml deionized water and 15g NaCl which was changed daily until no more pink color appeared.

The dye was then filtered through a .45um filter and thimersol was added to a final concentration of 1:10,000. Storage was at -10°C.

APPENDIX E

CALCULATIONS FOR

DETERMINATION OF GENERATION TIME

$$u = \frac{\log_{10}a - \log_{10}b \ (2.303)}{n}$$

u = specific growth rate

a = concentration of cells in #/ml at time 2

b = concentration of cells in #/ml at time 1

n = number of hours between time 1 and time 2

$$g = \frac{.693}{u}$$

g = generation time in hours

u = specific growth rate

APPENDIX F

GROWTH OF RHIZOBIA

IN YEAST EXTRACT MANNITOL BROTH

Table F. Growth of rhizobia in yeast extract mannitol broth

Time	5B2		TAL1145	
(hrs)	cells ml^{-1}	log cells ml^{-1}	cells ml^{-1}	log cells/ml^{-1}
0	$1.39x10^{6}$	6.14	$2.06x10^{6}$	6.31
5	$2.76x10^{6}$	6.44	$2.86x10^{6}$	6.46
11	$6.92x10^{6}$	6.84	$1.11x10^{7}$	7.04
17	$3.24x10^{7}$	7.51	$5.30x10^{7}$	7.72
23	$1.20x10^{8}$	8.08	$2.14x10^{8}$	8.33
29	$3.10x10^{8}$	8.49	$4.00x10^{8}$	8.60
35	$7.96x10^{8}$	8.90	$7.70x10^{8}$	8.89
41	$8.50x10^{8}$	8.93	$8.70x10^{8}$	8.94
47	$1.42x10^{9}$	9.15	$1.35x10^{9}$	9.13
53	$1.30x10^{9}$	9.11	$1.85x10^{9}$	9.27

APPENDIX G

CALCULATIONS FOR DETERMINATION OF NITROGEN

$$(\text{ml } .02\underline{N}_{\text{sample}} \times .02\text{meq/ml}) - (\text{ml } .02\underline{N}_{\text{blank}} \times .02\text{meq/ml}) + (\text{ml } .005\underline{N}_{\text{sample}} \times .005\text{meq/ml}) - (\text{ml } .005\underline{N}_{\text{blank}} \times .005\text{meq/ml}) = \text{meq of titrant used}$$

$$\frac{\text{meq titrant} \times \text{equivalent weight of nitrogen}}{\text{sample size}} \times 100 = \text{mg N}$$

$$\frac{\text{meq titrant} \times 14\ \text{meq/mg}}{100\text{mg}} \times 100 = \%\ \text{N}$$

APPENDIX H

IDENTIFICATION OF NODULE CONTENTS FROM COMPETITION STUDY IN SAND

Table H. Identification of nodule contents from competition study in sand

Ratio cells seed^{-1} 5B2:TAL1145	Rep	Nodule Number	% Nodules Reacting With 5B2	TAL1145	Both	Unknown
100% 5B2	1	24	100	0	0	0
	2	14	100	0	0	0
	3	20	40	60	0	0
	4	12	100	0	0	0
	mean	17.5	85.0	15.0	0	0
100% TAL1145	1	21	0	100	0	0
	2	21	0	100	0	0
	3	27	0	100	0	0
	4	8	0	100	0	0
	mean	19.2	0	100	0	0
1.3:1	1	27	3.7	40.7	11.1	44.4
	2	16	25.0	68.7	0	6.2
	3	21	14.3	76.2	0	9.5
	4	15	0	100	0	0
	mean	19.8	10.8	71.4	2.8	15.0
130.8:1	1	4	50.0	50.0	0	0
	2	12	91.7	0	8.3	0
	3	16	50.0	31.2	0	18.8
	4	7	14.3	85.7	0	0
	mean	9.8	51.5	41.8	2.0	4.7
0.013:1	1	14	42.9	21.4	0	35.7
	2	44	22.7	38.6	18.2	20.4
	3	10	50.0	20.0	0	30.0
	4	7	57.2	14.3	14.3	14.3
	mean	18.8	43.2	23.6	8.1	25.1

APPENDIX I

ANALYSIS OF VARIANCE TABLES FOR NODULE CONTENTS FROM COMPETITION STUDY IN SAND

Table I. Analysis of variance for nodule contents from competition study in sand. Log transformations.

Source	Degrees of Freedom	Sum of Squares	Mean Square	Obs. F	Req. F 5%
Reacting with 5B2 antiserum (%)					
Total	19	11.57			
Treat'ts	4	9.835	2.459	21.26	3.06
Error	15	1.735	0.1156		
Reacting with TAL1145 antiserum (%)					
Total	19	8.658			
Treat'ts	4	3.865	0.9661	3.023	3.06
Error	15	4.793	0.3195		
Reacting with both antisera (%)					
Total	19	3.901			
Treat'ts	4	0.9838	0.2459	1.265	3.06
Error	15	2.917	0.1945		

Table I. continued

Unknown (%)					
Total	19	8.376			
Treat'ts	4	5.686	1.421	7.926	3.06
Error	15	2.690	0.1793		

APPENDIX J

ANALYSIS OF VARIANCE TABLES FOR COMPETITION STUDY IN SAND

Table J. Analysis of variance tables from competition study in sand

Source	Degrees of Freedom	Sum of Squares	Mean Square	Obs. F	Req. F 5%
Top Biomass (mg)					
Total	21	945.6			
Treat'ts	5	474.0	94.8	3.216	3.26
Error	16	471.6	29.5		
Root Biomass (mg)					
Total	21	625.7			
Treat'ts	5	279.0	55.8	2.576	3.26
Error	16	346.6	21.7		
Nodule Biomass (mg)					
Total	21	6.267			
Treat'ts	5	3.675	0.7350	4.536	3.26
Error	16	2.592	0.1620		
Total Dry Matter (mg)					
Total	21	3271			
Treat'ts	5	1582	316.5	2.998	3.26
Error	16	1689	105.6		

Table J. Continued

Source	Degrees of Freedom	Sum of Squares	Mean Square	Obs. F	Req. F 5%
Nitrogen Content (%N)					
Total	21	4.082			
Treat'ts	5	0.8760	0.6412	11.71	3.26
Error	16	3.206	0.0548		
Nitrogen Content (mgN)					
Total	21	1409			
Treat'ts	5	839.6	167.9	4.718	3.26
Error	16	569.5	35.59		
Acetylene Reduction (nmol plant^{-1} hr^{-1})					
Total	21	9032			
Treat'ts	5	5636	1127	5.219	3.26
Error	16	3446	216.0		
Acetylene Reduction (nmol g dry nod^{-1} hr^{-1})					
Total	21	4268			
Treat'ts	5	3188	637.7	9.448	3.26
Error	16	1085	67.80		

APPENDIX K

IDENTIFICATION OF NODULE CONTENTS FROM POT COMPETITION STUDY

Table K. Identification of nodule contents from pot comptetition study

Soil	Strain and rep	Nodule Number	% Nodules Reacting With Antisera to 5B2	TAL1145	Both	Unknown
Jalapa						
	5B2 1	55	0	29.1	0	70.9
	2	41	0	36.6	0	63.4
	3	48	2.1	41.7	4.2	52.0
	4	51	2.0	43.1	0	54.9
	mean	49	1.0	37.6	1.0	60.3
	TAL1145 1	48	0	33.3	0	66.7
	2	49	4.1	20.4	0	75.5
	3	53	1.8	42.6	16.7	38.9
	mean	50	2.0	32.1	5.6	60.4
La Balsa						
	5B2 1	42	9.5	0	0	90.5
	2	9	55.6	0	0	44.4
	3	38	18.4	0	0	81.6
	mean	30	27.8	0	0	72.2
	TAL1145 1	31	6.4	3.2	0	90.4
	2	51	15.7	2.0	2.0	80.3
	3	36	2.7	2.7	0	94.6
	4	38	13.2	13.2	0	86.8
	mean	39	9.5	2.0	0.5	88.0

APPENDIX L

ANALYSIS OF VARIANCE TABLES FOR NODULE CONTENTS FROM POT COMPETITION STUDY

Table L. Analysis of variance for nodule contents from pot competition study. Log transformations.

Source	Degrees of Freedom	Sum of Squares	Mean Square	Obs. F	Req. F 5%
Reacting with 5B2 antiserum (%)					
Total	13	1.2099			
Soil	1	0.8263	0.8263	30.68	4.96
Strain	1	0.0164	0.0164	0.6073	4.96
Strain x soil	1	0.0978	0.0978	3.632	4.96
Error	10	0.2694	0.0269		
Reacting with TAL1145 antiserum (%)					
Total	13	1.8559			
Soil	1	1.542	1.542	110.3	4.96
Strain	1	0.0796	0.0796	5.696	4.96
Strain x soil	1	0.0945	0.0945	6.759	4.96
Error	10	0.1398	0.0140		

Table L. Continued

Reacting with both antisera (%)					
Total	13	0.4780			
Soil	1	0.0480	0.0480	1.136	4.96
Strain	1	0.0075	0.0075	0.1775	4.96
Strain x soil	1	0.0001	0.0001	0.0030	4.96
Error	10	0.4224	0.0422		
Unknown (%)					
Total	13	0.0534			
Soil	1	0.0138	0.0138	4.107	4.96
Strain	1	0.0014	0.0014	0.4027	4.96
Strain x soil	1	0.0047	0.0047	1.395	4.96
Error	10	0.0336	0.0034		

APPENDIX M

ANALYSIS OF VARIANCE TABLES FROM POT COMPETITION STUDY

Table M. Analysis of variance tables from pot competition study

Source	Degrees of Freedom	Sum of Squares	Mean Square	Obs. F	Req. F 5%
Leaf Biomass (g)					
Total	13	70.71			
Soil	1	28.85	28.85	7.050	4.96
Strain	1	0.1567	0.1567	0.038	4.96
Strain x soil	1	0.7877	0.7877	0.192	4.96
Error	10	40.92	4.092		
Stem Biomass (g)					
Total	13	41.82			
Soil	1	13.11	13.11	4.785	4.96
Strain	1	0.3071	0.3071	0.112	4.96
Strain x soil	1	1.008	1.008	0.368	4.96
Error	10	27.40	2.740		
Root Biomass (g)					
Total	13	27.98			
Soil	1	1.204	1.204	0.485	4.96
Strain	1	1.925	1.925	0.775	4.96
Strain x soil	1	0.001	0.001	0.0004	4.96
Error	10	24.85	2.485		

Table M. Continued

Source	Degrees of Freedom	Sum of Squares	Mean Square	Obs. F	Req. F 5%
Nodule Biomass (mg)					
Total	13	328.10			
Soil	1	19.89	19.89	0.789	4.96
Strain	1	9.44	9.44	0.374	4.96
Strain x soil	1	46.57	46.57	1.847	4.96
Error	10	252.2	25.22		
Total Biomass (g)					
Total	13	365.17			
Soil	1	98.97	98.97	3.794	4.96
Strain	1	2.699	2.699	0.104	4.96
Strain x soil	1	2.698	2.698	0.103	4.96
Error	10	260.8	26.08		
Acetylene reduced (nmol $plant^{-1}$ hr^{-1})					
Total	13	2.0225			
Soil	1	0.5066	0.5066	5.325	4.96
Strain	1	0.3642	0.3642	3.828	4.96
Strain x soil	1	0.2003	0.2003	2.105	4.96
Error	10	0.9514	0.0951		

Table M. Continued

Source	Degrees of Freedom	Sum of Squares	Mean Square	Obs. F	Req. F 5%
Acetylene Reduced (nmol g dry nodule^{-1} hr^{-1})					
Total	13	16.37			
Soil	1	2.384	2.384	1.801	4.96
Strain	1	0.1884	0.1884	0.142	4.96
Strain x soil	1	0.5556	0.5556	0.420	4.96
Error	10	13.24	1.324		
Leaf Nitrogen Content (mg N)					
Total	13	111,638			
Soil	1	34,590	34,590	4.742	4.96
Strain	1	1,057	1,057	0.145	4.96
Strain x Soil	1	3,051	3,051	0.418	4.96
Error	10	72,940	7,294		
Stem Nitrogen Content (mg N)					
Total	13	13,658.5			
Soil	1	5235	5235	7.526	4.96
Strain	1	292.5	292.5	0.420	4.96
Strain x Soil	1	1176	1176	1.691	4.96
Error	10	6955	695.5		

Table M. Continued

Source	Degrees of Freedom	Sum of Squares	Mean Square	Obs. F	Req. F 5%
Root Nitrogen Content (mg N)					
Total	13	5361.5			
Soil	1	100.7	100.7	0.195	4.96
Strain	1	50.87	50.87	0.098	4.96
Strain x Soil	1	42.96	42.96	0.083	4.96
Error	10	5167	516.7		
Total Nitrogen Content (mg N)					
Total	13	250,312			
Soil	1	72,020	72,020	4.245	4.96
Strain	1	1,806	1,806	0.106	4.96
Strain x soil	1	6,886	6,886	0.406	4.96
Error	10	169,600	16,960		
Total Nitrogen Content (%N)					
Total	13	0.3218			
Soil	1	0.0136	0.0136	0.590	4.96
Strain	1	0.0385	0.0385	1.676	4.96
Strain x soil	1	0.0400	0.0400	1.742	4.96
Error	10	0.2297	0.0230		

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