ESTABLISHMENT AND NODULATION IN *Leucaena glauca*

by

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to my mother

to the memory of my father

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BIOGRAPHY

The author was born at Uralla, New South Wales, Australia, on January 13, 1936. He attended Rocky River Primary School, Armidale High School and Barker College, Hornsby, terminating secondary studies in 1952. In 1955 he entered St. Andrew's College within the University of Sydney, and the Faculty of Agriculture, leaving in 1958, and graduating in May 1959 as Bachelor of Science in Agriculture.

From March, 1959 he has held a position of Agronomist with the Department of Agriculture, Stock and Fisheries of the Territory of Papua and New Guinea, working initially on cocoa and annual crops, but more recently concentrating on pasture agronomy.

In October, 1962 he entered the Inter-American Institute of Agricultural Sciences, for post-graduate studies on tropical pastures in the Department of Animal Industry, having been granted one year of overseas study-leave by his Department in T.P. & N.G. He returned to Australia in October, 1963.

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INTRODUCTION

The establishment of legume/grass pasture mixtures for grazing animals has become a standard practice in many temperate regions of the world. The same cannot yet be said of most tropical regions, although the advantages of such mixtures are no less important in the tropics.

Investigations on the establishment, nodulation and nitrogen fixation of pasture legumes have similarly been confined largely to temperate species, until comparatively recently. Results obtained with these species, although offering a guide, cannot be applied directly to their tropical counterparts. Similar basic studies are required on promising tropical legumes.

Because of the rapid growth rate and tall habit of many "improved" tropical grasses, the development and maintenance of a satisfactory grass/legume mixture is prejudiced unless optimum conditions are provided, as far as possible, for the germination, establishment and nodulation of the legume component.

In this study, the following factors were investigated, which could be expected to affect variously the germination, nodulation and early growth and nitrogen fixation of the plant.

1. Seed scarification with hot water or concentrated sulfuric acid.
2. Strain of Rhizobium for inoculation of the seed.
3. Effect of the seed coat on the viability of Rhizobium.
4. The nature of the suspending medium used for the application of the rhizobial inoculum.

5. Small amounts of nitrogen added to the soil about the time of first nodule development.

6. The sulphate level in the soil.

Leucaena glauca (L.) Benth. (= L. leucocephala (Lam.) de Wit) was used for this study. It has considerable potential as a pasture and fodder legume. While there is some published information on its germination, and Rhizobium strain requirements, it was considered that further studies were warranted.

LITERATURE REVIEW

I. LEUCAENA GLAUCA

1. Scientific name, origin and description

The name Leucaena glauca is very well established for this species, and frequently used as the common name, so that it has been retained in the title of this study. However, de Wit (73) after a thorough examination of type material, found that the species should be named Leucaena leucocephala. The new complete name is therefore Leucaena leucocephala (Lam.) de Wit. For want of a more universal common name, "leucaena" will be used throughout this study when referring to L. leucocephala.

Leucaena originates in tropical America, but has become pan-tropical, especially in moist regions at low latitudes (69). It has become naturalized in many Pacific Islands, West and East Indies, Philippines, Indo-China, Ceylon, Queensland and parts of Africa.

Leucaena is a shrub or small tree, two to ten metres high. The compound leaves are 15 - 25 cm. long, with numerous paired leaflets that are narrow and 7 - 12 mm. long. The white flowers occur in dense rounded heads 2-5 cm. in diameter. Seed pods are thin, flat, 12-18 cm. long, 1.4-2.0 cm. wide and contain 15-25 elliptical, shiny, brown seeds. (from Farinas (10)..

2. Nutritional value, yield and toxic properties

The value of leucaena as a browse plant for cattle has been recognized in many countries (5, 6, 11, 16, 36, 37, 50, 67), while in Hawaii the plant is grown extensively as a forage and pasture crop for both beef and dairy cattle (29, 30, 31, 32, 41, 60).

That leucaena is a plant worthy of more attention can be seen from comparisons of chemical analyses of leucaena and alfalfa, which have been made in the Philippines (16), Ceylon (39) and, together with other grasses and legumes, in Hawaii (60). The data in Table 1 are derived from these sources. For ease of comparison, all results are converted to dry matter basis.

TABLE No. 1 Comparison of the nutritive value of leucaena with alfalfa, elephant grass and para grass.

Forage	Yield per acre per year lb.	Crude protein %	Crude fibre %	Total digestible nutrients %
Leucaena [a]	-	27.98	12.43	71*
Leucaena [b]	-	25.56	11.35	71*
Leucaena [c]	15,184	22.67	-	57
Alfalfa [a]	-	23.44	27.38	60*
Alfalfa [b]	-	19.29	32.14	56*
Alfalfa [c]	14,925	20.40	-	59
Elephant grass [c] (Napier)	29,920	4.88	-	59
Para grass [c]	29,792	6.36	-	58

a - data from Farinas (10)
b - data from Joachim (39)
c - data from Takahashi and Ripperton (60)
* - estimated values, by method of Glover, Duthrie and Dougall (24)

The value of total digestible nutrients for leucaena will vary according to the amount of stem included, but appears to be generally superior to alfalfa. Yields of dry matter and T.D.N. per acre of leucaena were about half that of elephant grass, under Hawaiian conditions.

The Hawaiian variety appears to be a relatively low yielding type. In Queensland (35) under 40 inches of rainfall, a Peruvian variety produced 11,236 lb. dry matter per acre, while the Hawaiian variety produced only 1,335 lb. per acre. Strains from El Salvador and Guatemala were intermediate. Protein percentages were about equal in all strains.

Part of the high nitrogen content of leucaena is in the form of mimosine, an unbound alpha amino acid, which is toxic to horses and pigs, but not to cattle or sheep under normal conditions (8, 9, 41, 60). Mimosine is partly destroyed by the action of microbes in the rumen. Sheep transferred from ordinary pasture to a sole diet of leucaena shed the fleece completely in about a week, but when fed gradually increasing amounts of leucaena they become completely resistant to the toxic substance (9). Hutton and Gray (36) reported apparent differences in mimosine content among leucaena varieties grown in Queensland, and were hopeful of breeding low-mimosine strains. However, more precise analytical techniques have shown a lack of variation in mimosine content in the species, and the breeding program for low mimosine has been discontinued (8).

3. Leucaena in pasture mixtures

There are few published results of establishment of leucaena in mixtures with high producing pasture grasses. Takahashi and Ripperton (60) report that leucaena is used quite extensively for pasture purposes in Hawaii, with several thousand acres containing appreciable amounts. Most of this area is the result of natural seeding or of extensive broadcasting of seed without any land preparation. For prepared fields and planted pastures, guinea (Panicum maximum), bermuda (Cynodon dactylon)

and dallis (Paspalum dilatatum) grasses were recommended, sown between double rows of leucaena, i.e. two rows of leucaena three feet apart, followed by a six foot strip of grass. Farinas (16) gives similar recommendations. In Rhodesia, leucaena drilled in double rows two feet apart with 10 feet strips of buffel grass (Cenchrus ciliaris) between, gave a satisfactory stand in the first season, and was very productive in the second (68). A later report notes that leucaena/grass mixtures were difficult to graze in a mixture because of the high palatability of the leucaena, which resulted in animals over-grazing it (54).

Similar problems are recorded by Nogales (48) in investigations on mixtures of several grasses and legumes in Venezuela. Animals selectively grazed the leucaena in preference to pangola or para grass. With guinea and elephant grasses, the leucaena was choked out during its early growth. The grasses and legumes were sown in alternate single rows 1 metre apart which would place leucaena at a disadvantage when combined with tall-growing grasses such as these. The leucaena was also attacked by insects and a fungus. Nogales implied that economic use of leucaena would be confined to dry regions, but this is contradicted by the fact that leucaena thrives as a shade tree for cacao and as a roadside weed in regions with 180 inches annual rainfall in New Guinea.

4. Seed treatment and inoculation

Seed of leucaena shows poor germination unless the very impervious seed coat is treated in some way to render the testa permeable to water. Methods most commonly used are (a) mechanical scarifications, (b) treatment with hot water, and (c) treatment with concentrated sulphuric acid. Akamine (1) found that mechanical scarification or treatment

with 52 per cent sulphuric acid for 1 hour produced a germination of more than 80 per cent. Hot water treatment (75-80°C initial temperature; seed and water allowed to cool to 38°C) produced about 70 per cent germination. Untreated seed showed 10-15 per cent germination. Venkataratnam (65) recommended hot water treatment at 70-80°C for 5 minutes, which gave 74 per cent germination. Temperatures below 10°C or above 80°C damaged the seeds.

Rios et al (55) studied the effect of various concentrations and times of immersion in sulphuric acid on the germination of four legumes, including leucaena. Immersion for 20 minutes in 100 per cent sulphuric acid resulted in 92.6 per cent germination of leucaena seeds. At this concentration, the duration of treatment (10, 20 or 30 minutes) hardly affected germination percentage, but lower concentrations of acid (75, 50, 35, 20 per cent) resulted in progressively lower germination percentages. More recently, Gray (25) studied the effects of hot water treatment (80°C and 100°C) for various periods on two samples of leucaena. Best results (94-100 per cent germination) were obtained with treatment for two to five minutes at 80°C. The same seed treated with commercial sulphuric acid for 20 minutes gave 32 and 70 per cent germination in the two samples. When the hot water-treated seeds were dried rapidly after treatment, germination was 98 per cent at the end of 15 months' storage in air-tight containers. This method is thus superior to that recommended by Takahashi and Ripperton (60), in which the seed is soaked for several hours after placing in hot water. Seed treated in this way deteriorated rapidly in storage. Gray noted that the effect of the hot water was to produce a network of tiny cracks in the testa, which apparently allowed entry of water into the seed, but did not noticeably affect its smooth glossy surface.

The rather specific rhizobial strain requirements of leucaena are apparently not widely known. Norris (49) mentions that Leucaena glauca will nodulate with very few strains of rhizobia. Similarly Galli (19), in a study of legume "cross-inoculation groups", noted that Leucaena glauca belonged to a group to which no other species studied belonged. Knowledge of this fact is important when introducing leucaena to a new region.

II. SOME FACTORS AFFECTING VIABILITY OF RHIZOBIA AND NODULATION

1. The seed coat

Haas and Fred (26) in 1919 studied the effect of germinating seed of soybean on its nodule forming bacteria, and found that the seeds excreted no substance toxic to the growth of the nodule bacteria, but favour this growth. They noted that, when mercuric chloride is used to sterilize soybean seed, sufficient is retained by the seed to retard development of its nodule bacteria on agar plates in the vicinity of the seed. This could explain some of the earlier reports on toxicity of legume seed coats to nodule bacteria.

More recently, however, Thompson (61) conclusively demonstrated that the seed of Trifolium subterraneum L. var. Tallarook contain a thermostable, water soluble antibiotic which was active against a strain of nodule bacteria normally incorporated in commercially prepared inocula. In a series of field experiments Thompson (62) confirmed that physical separation of the rhizobial inoculum and the seed coat of subterranean clover resulted in improved nodulation, when sown in a soil of pH 6 or higher.

In more acid soils the nature of the chemical constituents of the seed pelleting material was more important than the actual separation, suggesting that soils differ considerably in their ability to inactivate the antibiotic.

Bowen (12) obtained similar results with seed coat diffusates of subterranean clover, and also of Centrosema pubescens, while lucerne (Medicago sativa) seed diffusates showed little activity. The toxicity of C. pubescens was due almost entirely to the seed coat, but the effect was not evident until the seeds began to germinate. Seed sterilization was effected by applying equal parts of ethyl alcohol and 20 volume per cent hydrogen peroxide, followed by three washings with sterile distilled water. Tests showed no residual toxicity from the sterilizing agents.

Masterton (45) noted inhibition of strains of Rhizobium on agar plates by seeds or discs of filter paper soaked with seed extracts of Trifolium pratense, T. hybridum, T. repens, Lotus corniculatus, L. uliginosus, Medicago sativa, Pisum sativum, Vicia faba, Phaseolus vulgaris and P. multiflorus, but not by Lupinus luteus. The degree of inhibition varied, and strains of Rhizobium differed in resistence to inhibition, but none was completely resistent.

2. The inoculum suspension

Vincent (66) noted that the death rate of clover Rhizobium deposited on clover seeds or glass beads from liquid yeast mannitol suspension containing 10 per cent sucrose was of the same magnitude as on peat-inoculated clover seeds, and was superior (i.e., lower)

Sankaram (57) determined the viability of rhizobia on subterranean clover seed stored over a period of 12 days in cloth bags at room temperature. The peat inoculum was applied to the seed by two methods, (a) by the addition of water and (b) by addition of a dilute sucrose solution (concentration not stated). The viable count (rhizobia per g. of seed, x 10^3) declined from 70 to 20 after 12 days, using water. Using the sugar solution, the count increased from 70 to 130 at 6 days, dropping to 100 at 12 days.

Loneragan et al (42) included 10 per cent of sucrose or maltose in solutions when applying rhizobia to seeds by vacuum impregnation, and noted that the form of sugar did not affect the degree of nodulation.

3. Combined nitrogen

Numerous studies have been made on the effects of combined nitrogen, particularly nitrate, on the nodulation of legumes by rhizobia, and on subsequent nitrogen fixation. These have been reviewed by Fred, Baldwin and McCoy (17), van Schreven (58), and more recently, by Raggio and Raggio (52).

It is generally accepted that both nodulation and nitrogen fixation are inhibited by high available nitrogen levels. The site of inhibition of nodule formation is still not known with certainty. Some studies suggest that the effect is local in character. Using a divided-root-system technique, Wilson (70) showed that nodules failed to develop on roots growing in solutions containing ammonium nitrate at concentrations greater than 1/300 (266 parts per million).

Other roots of the same plant growing in nitrogen-free solution nodulated freely. From this observation it was concluded that the effect of nitrates in depressing nodule formation is only local in character.

Other experiments have led to the conclusion that combined nitrogen exerts an effect internally in the plant tissues. Allison and Ludwig (2) report data of Stroud, which suggests that nodule numbers can be related to the supply of carbohydrates in the roots. They present additional data to support the theory that decreased nodulation in the presence of abundant combined nitrogen is due to inadequate carbohydrate supply in the roots; where nitrogen is abundant the carbohydrates synthesized are used for top growth, and little is available for the growth of roots and nodules, as demonstrated by top root ratios. Later investigations indicated that the depressing effect of combined nitrogen could be offset by addition of mannitol (33), or sucrose (43) to the substrate, which lends support to the 'available carbohydrate' or 'C:N' hypothesis. Allison and Ludwig (3) reviewed the subject briefly, stressing the importance of carbohydrate supply, rather than the relation of carbohydrate to nitrogen in the plant.

Raggio *et al* (53) studied nodulation in excised bean roots. They concluded that NO_3-nitrogen inhibits nodulation if it is supplied in the medium common to root and bacteria, but not if supplied through the base of the root. This would support Wilson's hypothesis (70) of the localized effect of nitrate. However, an examination of the data of Raggio et al (p. 330), comparing "nitrate-free" and

"nitrate supplied via the base", shows that there was a decline in nodule numbers from 22 to 14 respectively, while number of roots nodulated declined from 71 to 58 per cent. Both treatments received 10 per cent sucrose via the base of the root. Also the inhibitory effect of nitrate when supplied in the medium was reduced considerably when sucrose supplied via the base was increased from 2 to 10 per cent. Nodule numbers increased from 1 to 10, and number of roots nodulating rose from 8 to 31 per cent, respectively. The data can therefore equally support the 'carbohydrate supply' theory.

Although high levels of combined nitrogen have a depressing effect, there is considerable evidence that low levels of added combined nitrogen are beneficial for nodulation and nitrogen fixation. Göbel (23) obtained increases in fresh weight of nodules on soybeans in sand culture when sodium nitrate was applied at rates up tc 200 pounds per acre. Higher levels depressed nodulation. Nodule numbers generally decreased with increasing nitrate, but the numbers were very erratic. Ammonium nitrate and ammonium sulphate both depressed nodulation at the rates used, especially ammonium sulphate. This may have been a pH effect, as no mention was made of controlling reaction. Ludwig and Allison (43), found one to five milligrams of potassium nitrate beneficial to nodule numbers on soybeans grown in pots containing six pounds of sand.

In Ulex europaeus, a legume, nodule dry weight was increased by the inclusion of 10 mg. per litre of ammonium-nitrogen, but decreased by 50 mg. per litre, in water culture where there was provision for

the control of the level of combined nitrogen and pH. In the same experiment Alnus glutinosa and Myrica gale (non-legumes) produced greater dry weights of nodules per plant in the presence of up to 50 mg. of ammonium-nitrogen per litre than plants without added nitrogen. However, relative to the enhanced growth of the plants as a whole, nodule development was continuously depressed as the level of combined nitrogen was increased (44).

Gibson and Nutman (22) studied the effect of combined nitrogen in several forms on time of initial nodulation, nodule number and nitrogen uptake of white clover grown on nutrient agar slopes. Nitrogen was supplied at the rate of 20 parts per million. Potassium nitrate, sodium nitrate, ammonium nitrate and sodium nitrite significantly delayed the time of initial nodulation, but asparagine, urea and ammonium sulphate had no effect. However, nodule number (at 54 days) was increased by all the nitrogenous compounds at the concentration used. Although nitrate at low concentration delayed nodule initiation, it increased the rate at which nodules were formed, so that final nodule numbers were increased.

Pate and Dart (51) studied the effects of ammonium nitrate on the nitrogen fixation of four legumes grown in sand culture. In purple vetch (Vicia atropurpurea) the strain of Rhizobium used proved to be important. Two point five to 10 mg. of combined nitrogen applied at sowing stimulated nitrogen fixation in one host-strain combination, while in others, all levels of combined-N depressed fixation. Time of application was also found to be important. In

poona cowpea (Vigna sinensis), greatest fixation efficiency (mg. N fixed per gram dry weight of nodules) occurred when small quantities of combined-N were added about 10 days after sowing, which was when the first nodules were appearing on the roots. About this time, the seedling often goes through a period of so-called "nitrogen hunger", before the nodules can begin to supply the increasing nitrogen requirements of the growing plant.

4. Sulphur and nodulation

There is evidence that the main role of sulphur in nodulation is indirect, being required for protein formation from absorbed combined nitrogen.

However, Wilson (70) obtained results indicating that many sulphates actually reduced nodule numbers, although manganese sulphate and several metal-complex sulphates increased nodule numbers. Nodule weights were not recorded. Gaw and Soong (20) found that calcium sulphate and ferrous sulphate increased nodule numbers and plant dry weight in peas, while sulphates of potassium, chromium, copper, barium and magnesium depressed nodule formation and reduced plant dry weight. Ammonium sulphate reduced nodule numbers but increased plant dry weight.

Anderson and Spencer (4) compared the role of sulphur in the nitrogen metabolism of legumes and non-legumes. Sulphur was needed by the non-legumes for protein formation from the absorbed combined nitrogen. In legumes, nodule numbers and symbiotic nitrogen fixation was greatly reduced by sulphur deficiency, but this was a

reflection of the reduced nitrogen demand of the S-deficient plants. The restricted growth of sulphur-deficient clover was not due to poor nitrogen fixation consequent upon defective nodulation, but to a deficiency of sulphur in the host legume. Ashford and Bolton (7) confirmed that sulphur deficiency restricts growth through its effects on the nutrition of the host legume, rather than through stimulation of nodule bacteria. The depressing effect of ammonium nitrate on nodulation was markedly offset by the application of sulphur (as potassium sulphate), but this was a reflection of the greater nitrogen demands of plants supplied with adequate sulphur.

MATERIALS AND METHODS

I. LOCATION

The studies were conducted in the Department of Animal Industry of the Inter-American Institute of Agricultural Sciences, Turrialba, Costa Rica. Seed germination tests and studies of the growth of Rhizobium cultures were conducted in the laboratory. Pot experiments were conducted in the glasshouse of the Department's climatic chamber. Day temperatures can be kept from exceeding 35°C, except for occasional short periods, by forced ventilation of outside air. The field trial was commenced in the experiment farm of the Department.

II. SEED AND RHIZOBIA

The seed of Leucaena glauca used in all trials was obtained from El Salvador by Mr. Arthur Semple. In addition a sample of a variety of L. glauca from Perú (CPI 18614), supplied by the Plant Introduction Section of the Commonwealth Scientific and Industrial Research Organization, Australia, was used in Experiment 2. The seed of the Centrosema species used in the same experiment was obtained by Mr. Semple from a grass plot at La Hulera Experiment Station of the Institute.

Strains of Rhizobium nodule bacteria were obtained from the following sources:

Leucaena strains NGR 8, NGR 31 and Centrosema strain NGR 26 was supplied by the Department of Agriculture, Stock and Fisheries

of the Territory of Papua and New Guinea. The third Leucaena strain was supplied by the Agricultural Research Service of the United States Department of Agriculture, and is believed to be the same Australian strain, CB 81, as used by Esquivel (15) in his studies on L. glauca.

III. SOILS

The sand used in Experiments 1 and 4 was part of a load obtained from the Turrialba river in November, 1962, of the kind used regularly in the Institute for concrete work.

The soils used in Experiments 5 and 6 were as follows:

Soil "A" - Virgin forest soil (0-6 ins.) from within the Gamma Field of the Institute. This soil is a senile latosol of the Colorado series (27), remote from the source of radiation.

Soil "B" - Of the same origin as soil A, but taken from a paddock near the entrance to the Gamma Field, which has been under Guinea grass for a number of years. The sampled depth of this soil was also 0-6 ins.

The sand and soils were air dried on the glasshouse floor, then sifted through a 3 mm. square-mesh wire sieve, and thoroughly mixed before rebagging.

The soil granules of soils A and B which would not pass through the 3 mm. sieve were sifted again through a 4 mm. mesh sieve. The granules thus obtained was used to provide drainage in the base of each pot in the experiments in which these soils were used.

Approximate field capacity was determined for the sand and the two soils.

The field trial (Experiment 7) was located on Institute Claystony phase (27).

IV. THE EXPERIMENTS

Experiment 1. The time of first nodule formation

Seeds of Leucaena glauca were grown in sand culture to determine the time or stage at which nodules first form on the roots.

The two treatments were:

1. Control (seed uninoculated)
2. Inoculated (Rhizobium strain NGR 8)

The experiment was commenced on November 10, 1962. Six mechanically scarified seeds of L. glauca were sown into sand contained in undrained waxed-cardboard milk cartons, of approximately one litre capacity. For treatment 1, sixteen pots were sown with uninoculated seed, while for treatment 2, 16 pots were sown with seed inoculated with a water suspension of Rhizobium. Fifty ml. of a nutrient solution, containing per litre, 0.5 g. K_2HPO_4, 0.4 g. $MgSO_4$, 4.0 g. $Ca_3(PO_4)_2$ and 1.0 g. fritted trace element mixture, was applied to all pots on November 23.

To observe the stage at which the first nodules formed, two pots from each treatment were selected at random and harvested on the days November 21, 23, 26, 29, December 2 and December 6. The occurrence of those nodules which could be seen by the naked eye, the length of time for nodulation to occur, and the stage of development of the plants was noted.

Experiment 2. The growth of Rhizobium in the presence of legume seeds

Part (a): Four 4-inch Petri plates of sterile yeast mannitol agar were inoculated with cultures of Rhizobium, two each with Leucaena strains NGR 8 and NGR 31. Immediately after inoculation, five surface-sterilized seeds of L. glauca were distributed evenly over each agar plate. On one plate of each strain of Rhizobium were seeds of L. glauca var. Perú, while on the remaining plates were seeds of L. glauca from El Salvador.

Part (b): Eight yeast mannitol agar plates were inoculated with rhizobia, four with Leucaena strain NGR 8 and four with Centrosema strain NGR 26. Five surface-sterilized seeds of L. glauca (El Salvador) or of Centrosema sp. were distributed over each plate, so that two plates of each strain received L. glauca, and two Centrosema sp. Fifty per cent of the seed of each species had been lightly scarified with sand paper to ensure that some seeds on each plate would germinate.

Surface sterilization of all seed was accomplished by the method described by Bowen (12), in which seeds are shaken for 10-15 minutes in a solution of equal parts of ethyl alcohol and 20 volume per cent hydrogen peroxide, followed by three washings with sterile distilled water over a further 10 minutes.

The Petri dishes were incubated at 28°C and periodic examinations made on the growth of the rhizobia and germination of the seeds.

Part (a) was conducted from December 5-15, 1962 and part (b) from August 30 to September 9, 1963.

Experiment 3. Hot water and sulphuric acid treatment of seed L. glauca.

Six identical batches of L. glauca seed were treated as follows, and the per cent germination subsequently determined from duplicate samples of 50 seeds from each treatment.

Treatment 1. Control - untreated

Treatment 2. Hot water (80°C) for two minutes

Treatment 3. Hot water (80°C) for four minutes

Treatment 4. Concentrated sulphuric acid for 12 minutes, with only sufficient acid to moisten the seed.

Treatment 5. Concentrated sulphuric acid for 10 minutes, with enougn acid to completely immerse the seed

Treatment 6. As for treatment 5, but for 20 minutes.

Seeds were germinated in Petri dishes on a moist filter paper overlying several layers of soft paper towelling. The dishes were left in the laboratory where the temperature range was approximately 21-27°C. Counts of the number of seeds germinated was made after 14 days. The tests were conducted during the period March 29 - April 25, 1963.

Experiment 4. Nodulation and growth of leucaena in response to the factors nitrogen, sulphate and inoculum suspension

This pot trial consisted of a 2 x 2 x 2 factorial with six replications, the 48 pots being arranged in a randomized block design. The three factors, which were studied singly and in all combinations were:

Factor I - A solution of 10 per cent sucrose (versus water) as the suspending medium for the Rhizobium inoculum applied to the seed.

Factor S - Sulphate (versus no sulphate) included in the nutrient solution applied to each pot.

Factor N - The addition of four mg. of nitrogen per pot, as urea, at the time of first nodule formation (versus no nitrogen application).

The resulting eight treatment were:

(1) - control. Inoculum in water, no sulphate, no urea

i - inoculum in 10 per cent sucrose solution, no sulphate, no urea

s - inoculum in water, plus sulphate, no urea

n - inoculum in water, no sulphate, plus urea

i s - inoculum in 10 per cent sucrose, plus sulphate, no urea

i n - inoculum in 10 per cent sucrose, no sulphate, plus urea

s n - inoculum in water, plus sulphate, plus urea

i s n - inoculum in 10 per cent sucrose, plus sulphate, plus urea.

Six mechanically scarified seeds of L. glauca, inoculated according to the respective treatment of the trial, were sown in each pot in 1550 g. of sand. Leucaena strain NGR 8 of Rhizobium was used throughout.

A complete nutrient solution, minus nitrogen and sulphur, was applied to all pots in the trial. The composition of the solution was based on that used by Dart and Pate (14), with the modification that two solutions were required, one with sulphates and one without sulphates.

Nutrient Solution No. 1 - with sulphate

Calcium sulphate, $CaSO_4 \cdot 2H_2O$	1.00 g.
Di-potassium phosphate, K_2HPO_4	0.40 g.
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$	0.20 g.
Versenol (HEEDTA)	0.25 g.
Micro-nutrient stock solution	1.00 ml.
Iron chelate stock solution	10.00 ml.

made up to one litre with distilled water.

Nutrient Solution Nº 2 - without sulphate

Calcium chloride, $CaCl_2$	0.65 g.
Di-potassium phosphate, K_2HPO_4	0.40 g.
Magnesium chloride, $MgCl_2$	0.165 g.
Versenol (HEEDTA)	0.25 g.
Micro-nutrient stock solution	1.00 ml.
Iron chelate solution	10.00 ml.

made up to one litre with distilled water.

Micro-nutrient Stock Solution

Boric acid, H_3BO_3	2.86 g.
Manganese chloride, $MnCl_2 \cdot 4H_2O$	1.81 g.
Zinc chloride, $ZnCl_2$	0.11 g.
Copper chloride $CuCl_2 \cdot 2H_2O$	0.05 g.
Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	0.025 g.

made up to one litre with distilled water.

Iron Chelate Stock Solution

Ferric chloride, $FeCl_3 \cdot 6H_2O$	2.42 g.
Versenol (HEEDTA)	2.69 g.

made up to one litre with distilled water.

Versenol was added to nutrient solution No. 2 to reduce the precipitation of calcium and magnesium phosphates, and also to solution No. 1, so that both solutions contained equivalent quantities.

Solution No. 1 or No. 2 according to the experimental treatment involved, was applied at the rate of 75 ml. per pot.

Urea was also applied in solution. Twenty millilitres of a solution containing 0.4287 g. of urea per litre (200 mg. nitrogen per litre) were applied to the pots included in the treatments requiring nitrogen.

Pots were brought to near field capacity by adding distilled water at the beginning of the experiment, and daily, to make up for losses by evapotranspiration.

At the end of seven and a half weeks, all plants were harvested. Plant tops and roots were harvested separately, and the number of plants in each pot noted. Nodules were removed from the roots, and the number of nodules per pot recorded. Plant tops, roots and nodules were dried in a vacuum oven at 70°C for 3 hours, and the respective dry weights per pot recorded after the plant material had come to a constant weight in the dry-room atmosphere of 50 per cent relative humidity.

Determinations of nitrogen content were made on ground material of the tops and roots of each pot. To obtain sufficient material to analise for nitrogen content of the nodules, replications were combined thus: - I + II + III, and IV + V + VI, to give two combined replications of each treatment for analysis.

Experiment 5. Time and rate of application of nitrogen and its effect on nodulation and yield of leucaena

In this pot experiment two levels of nitrogen (as urea) were applied at sowing or at the time of first nodule formation, to L. glauca grown in the two senile latosols described previously. The five treatments were:

A. Control, no nitrogen

B. 2 mg. nitrogen per pot, applied at sowing

C. 4 mg. nitrogen per pot, applied at sowing

D. 2 mg. nitrogen per pot, applied at first nodule formation

E. 4 mg. nitrogen per pot, applied at first nodule formation

Five replications on each soil type were arranged in two 5 x 5 latin square designs.

The polyethylene-lined pots were filled with 1400 g. of soil, after 100 g. of granules (pseudo gravel) of the respective soil had been placed in the bottom of the pot for drainage.

A complete nutrient application, minus nitrogen, was made to all pots at the following rates per pot, based on calculations from Mitscherlich standards (28):

Triple superphosphate	0.81 g.
Potassium chloride, KCl	0.685 g.
Magnesium sulphate, $MgSO_4 \cdot 7H_2O$	0.286 g.
Calcium sulphate, $CaSO_4 \cdot 2H_2O$	0.57 g.
Manganese chloride, $MnCl_2 \cdot 4H_2O$	35.5 mg.
Zinc sulphate, $ZnSO_4$	25.7 mg.
Copper sulphate, $CuSO_4 \cdot 5H_2O$	20.0 mg.
Borax, $Na_2B_4O_7$	31.4 mg.
Sodium molybdate, $Na_2MoO_4 \cdot 2H_2O$	11.4 mg.
Iron, as chelate	10.0 mg.

The triple superphosphate was granulated, the granules being in the range 2.0 - 1.0 mm. diameter. This and the calcium sulphate were applied dry to the soil and thoroughly mixed before being added to the pot. A stock solution was made up of potassium chloride plus magnesium sulphate. The salts of manganese, zinc, copper, boron and molybdenum were also made up in solution and stabilized with 10.0 g. of Versenol per litre. The iron chelate was from the same stock

solution as that used in Experiment 4. The required quantities of the stock solutions were applied to each pot in 300 ml. of distilled water.

Twelve scarified seeds, inoculated with a 10 per cent sucrose suspension of Rhizobium strain NGR 8, were sown in each pot on July 9, 1963 for soil "A" and July 12, 1963 for soil "B". Five extra pots of soil "A" were treated in the same way as those in the experiment proper. One of these was harvested on the days July 21, 24, 26 and 28, and examined for the formation of nodules.

The nitrogen treatments were applied in 100 ml. of distilled water at the time of sowing for treatments B and C; and for the treatments D and E, on August 1, 1963 for soil "A" and August 10, 1963 for soil "B".

When all seedlings had emerged, and the first leaf was developing, the number of seedlings in each pot was reduced to eight. An application of 0.1 g. of Arasan 75 (T.M.T.D.) in 70 per cent alcohol was made to all pots of soil "B" when a number of plants was seen to be affected by a damping-off disease.

On September 20, 1963, harvesting of plants from both soil types was commenced, and completed on September 26, 1963. The harvest of any replication was always completed on the same day. Plant tops, roots and nodules were harvested separately, dried at 85°C for 24 hours, and weighed after coming to equilibrium with the dry-room atmosphere of 50 per cent relative humidity.

Experiment 6. The relative effectivenes of three strains of rhizobia in the nodulation of leucaena

In this experiment five treatment were replicated twice on each of soils "A" and "B", and the pots disposed in a randomized block design. The treatments were:

1. Control, no treatment
2. Inoculation with Leucaena strain NGR 8
3. Inoculation with Leucaena strain NGR 31
4. Inoculation with Leucaena strain CB 81
5. Application of urea, equivalent to 120 kg. of nitrogen per hectare.

Twelve scarified seeds of L. glauca, dusted with Arasan 75 (T.M.T.D.), were sown into the pots of soil on August 13, 1963. The pots had been prepared in an identical manner to that described for Experiment 5, with the exception that only 1300 g. of soil "A" (Plus 100 g. of pseudo gravel) were used for each pot, to leave more space at the top of the pot for adding water.

The inocula in treatments 2, 3 and 4 were applied to the soil as heavy suspensions in 100 ml. of distilled water, on August 18, 1963. At the same time urea was applied to the pots of treatment 5, at the rate of 117.4 mg. per pot, equivalent to 60 kg. of nitrogen per hectare. This application of urea was repeated on September 4, 1963.

Plants in soil "B" were harvested on September 11, 1963, and those in soil "A" on September 27, 1963. Plant material from each

pot was separated into tops, roots and nodules, dried at 85°C for 24 hours, and weighed after coming to constant weight in the dry-room atmosphere of 50 per cent relative humidity.

Experiment 7. A field trial to compare the production of a leucaena/pangola grass mixture with that of Pangola grass alone

This trial was commenced, but had to be abandoned before completion, for reasons which will be discussed later in this study.

The experiment was laid down in a randomized block design, with three treatments and six replications. Each plot measured 7.3 x 30.5 metres (24 feet x 100 feet). A preliminary sample cut was taken of the Pangola grass in each plot before any treatments were applied. This consisted of a two-metre strip down the centre of each plot, cut by a tractor-mounted mower. Three random samples, each from an area two metres x five metres were collected from each plot, and weighed. A composit sub-sample was collected from the three within-plot samples for dry matter determination. The remaining part of the plots was then mowed.

Treatment 1. Untreated pangola grass

Treatments 2 and 3. Plots in which six longitudinal strips were cultivated with an 18 inch rotary hoe. In treatment 3, seeds of L. glauca, previously acid treated, and inoculated with Leucaena Rhizobium strain NGR 8, were sown by hand into the cultivated strips on April 26, 1963.

An overall dressing of a nitrogen-free, but otherwise complete fertilizer mixture of triple superphosphate, potassium chloride, magnesium sulphate, calcium carbonate and fritted trace elements, was envisaged. On June 7, 1963 triple superphosphate at 448 kg/ha, and fritted trace elements at 28 kg/ha. were applied to all plots. The remaining fertilizers were not applied before the experiment was abandoned. Hand weeding of the rows containing leucaena was carried out periodically.

V. ANALYSIS OF SOILS AND PLANT MATERIAL

1. Soils. pH and soil nitrogen were determined for the sand used in Experiment 4, and for soils "A" and "B". Total sulphur was determined only on the sand.

(a) pH. Normal pH was measured potentiometrically on a 2:5 soil: water suspension. Exchange pH was also determined, by Schofield and Taylor's method, as described by Sáiz del Río and Bornemisza (56), using 0.01 M calcium chloride solution, and a soil:solution ratio of 1:2.

(b) Soil nitrogen. Determination of soil nitrogen were carried out on a macro-Kjeldahl apparatus, using the method described by Jackson (38). Nitrates were not included in the determination.

(c) Total sulphur. The turtidimetric method of Bardsley and Lancaster (10) was used.

2. Plant material. Nitrogen determinations were made by the micro-Kjeldahl method described by Müller (47).

VI. THE USE OF THE GLASSHOUSE

The glasshouse used in this study has a forced-air ventilation system. During all the pot trials the ventilating system was switched on during the day only while the sun was shining, to keep the temperature from exceeding 38°C. Outside air was blown in along the sides and across the ends of the glasshouse, and escaped through the open windows at the peak of the roof and also along the sides. Strips of white paint applied to the underside of the roof also reduced some of the insolation.

In all trials the replications, or blocks, were orientated along the north-south axis of the glasshouse, so that differences in ventilation and changes in light intensity would largely be accounted for in the between-blocks variance during the statistical analysis of the results.

A thermograph, in which the bimetallic strip was shaded from direct insolation, recorded the temperature inside the glasshouse continuously.

VII. METHOD OF EXAMINATION OF THE ROOTS

To harvest the roots and nodules so that a minimun of plant material was lost, plant tops were first cut off at the soil level, then the pot was completely immersed in a bucket of water and gently shaken. With the plants grown in sand, very few roots or nodules were broken off by this method, and the plants were separated easily.

The two soils were also fairly effectively treated by the same method. In Experiments 5 and 6, the outer waxed cardboard was carefully torn off, and the polyethylene tube, containing the soil still in a single mass, was immersed in the water and the soil slipped gently out of the tube. Some roots and nodules were broken off with the agitation required to loosen the soil, but these were recovered by passing a piece of fine nylon gauze through the water several times, and then straining the water out of the bucket. The mass of roots was transferred to a bucket of clean water for examination and separation of the nodules.

VIII. STATISTICAL ANALYSIS

In Experiment 4 the factor effects were calculated by the method described by Yates (74). Where interactions occurred the simple effects of each factor involved were examined by the method described by Steel and Torrie (59).

In Experiment 5, missing values in the data for soil "B" were estimated by the use of Yates' formula, and Duncan's multiple range test was used to detect significant differences between rows (59).

RESULTS

I. SOIL ANALYSIS

Chemical analyses

The results of analyses of the sand and two soils used in this study are shown in Table 2.

TABLE No. 2 pH, nitrogen and sulphur content of sand and two soils

	Reaction		Nitrogen	Total Sulphur
	Normal pH	Exchange pH	%	p.p.m.
Sand	6.6	5.8	0.01	50
Soil "A"	4.8	4.3	0.53	-
Soil "B"	5.8	5.2	0.39	-

Additional pH determinations were made on the sand in Experiment 4, after addition of the nutrient solutions:

	pH
Sand with Nutrient Solution No. 1 (plus sulphate)	7.5
Sand with Nutrient Solution No. 2 (minus sulphate)	7.4

A complete chemical analysis of a sample of soil taken from the same site as that of soil "A" was provided by the Soils section of the Department of Plant Industry and Soils of the I.A.I.A.S. This is shown in Table 3.

Field capacity and apparent specific gravity

The field capacity and the apparent specific gravity of the sand and two soils are shown in Table 4. The apparent specific gravity refers to the weight of soil per volume in the pot.

TABLE No. 3 Analysis of soil "A" (0 - 6 ins). Gamma Field (Colorado Series). Senile latosol.
(Soils Section, I.A.I.A.S.)

Reaction		O.M.	Total N	C/N	P_2O_5 (Truog)	Exchangeable Cations				Degree of Satn.	Nutrient Ratios		
Normal pH	Exchange pH				p.p.m.	Sat. Cap.	Ca	Mg	K		Ca/Mg	Mg/K	Ca+Mg / K
						m.e./100 g.							
4.1	3.8	9.6	0.43	12.7	9	41	Tr	0.25	0.28	1.3	low	0.9	0.9
Standards for comparison													
High	7.5	6.5	5.2	0.26	11.5	90	-	24	6	0.55	-		
Med.	6.5	5.5	2.6	0.15	10.0	37	-	12	3	0.35	-	4.0	8.0	4.0
Low	5.0	4.0	1.3	0.04	8.5	15	-	4	1	0.20	-			

TABLE No. 4 Field capacity and apparent specific gravity

	Field Capacity % by volume	Apparent Specific Gravity
Sand	44.4	1.55
Soil "A"	70.0	0.91
Soil "B"	70.8	0.89

II. GLASSHOUSE TEMPERATURE

Temperature records for the periods of the various glasshouse esperiments are recorded in Table 5.

TABLE No. 5 Glasshouse temperature records, in degrees Centigrade

Temperature recorded	Experiment			
	Nº 1	Nº 4	Nº 5	Nº 6
Average daily maximum	32.9	32.6	35.1	35.6
Average daily minimum	18.1	16.1	17.9	17.9
Average mean day (6 am - 5 pm)	25.7	26.1	27.3	27.7
Average mean night (6 pm - 5 am)	19.6	19.3	20.1	20.0
Highest temperature recorded	40.0	36.1	38.9	38.9
Lowest temperature recorded	15.6	13.3	14.4	14.4

III. RESULTS OF EXPERIMENTS

Experiment No. 1. The time of first nodule formation

(a) Uninoculated. No nodules were found on any of the plants examined which had not been inoculated with Rhizobium.

(b) Inoculated. One nodule was found on one plant 19 days after sowing. After 22 days, one nodule was found on each of three plants. At this stage the second leaf, or first bi-pinnately compound leaf, was developing. After 26 days, nodules were found on four plants, the seedlings having their second leaf fully expanded.

Experiment No. 2. The growth of rhizobia in the presence of legume seeds

In both part (a) and part (b) of this experiment, no inhibition of growth occurred of the Rhizobium strains NGR 8 and NGR 26 in the presence of seeds of Leucaena glauca or Centrosema sp. Figure No. 1 shows the growth of cultures of two rhizobia strains on agar plates in the presence of both germinating and dormant seeds of L. glauca and Centrosema sp.

Experiment No. 3. Hot water and sulphuric acid treatment of Leucaena seeds

Treatment with concentrated sulphuric acid for 20 minutes resulted in a germination after 14 days of 99 per cent, compared with four per cent for the untreated seed. Acid treatment was more effective if seeds were completely immersed, than if only sufficient acid was used to wet the seed coats. With the variety of leucaena used in this experiment, acid treatment was much more effective than treatment with hot water at 80°C. The results of the different treatments are set out in Table No. 6.

TABLE No. 6. Per cent germination, after 14 days, of seeds of Leucaena glauca treated in various ways. (Duplicate samples of 50 seeds).

Untreated	Hot water, 80°C		Concentrated sulphuric acid		
	2 minutes	4 minutes	12 munites+	10 munites*	20 minutes*
6	26	16	22	46	100
2	32	20	20	40	98
Mean 4	29	18	21	43	99

\+ Seed moistened only

* Seed completely immersed

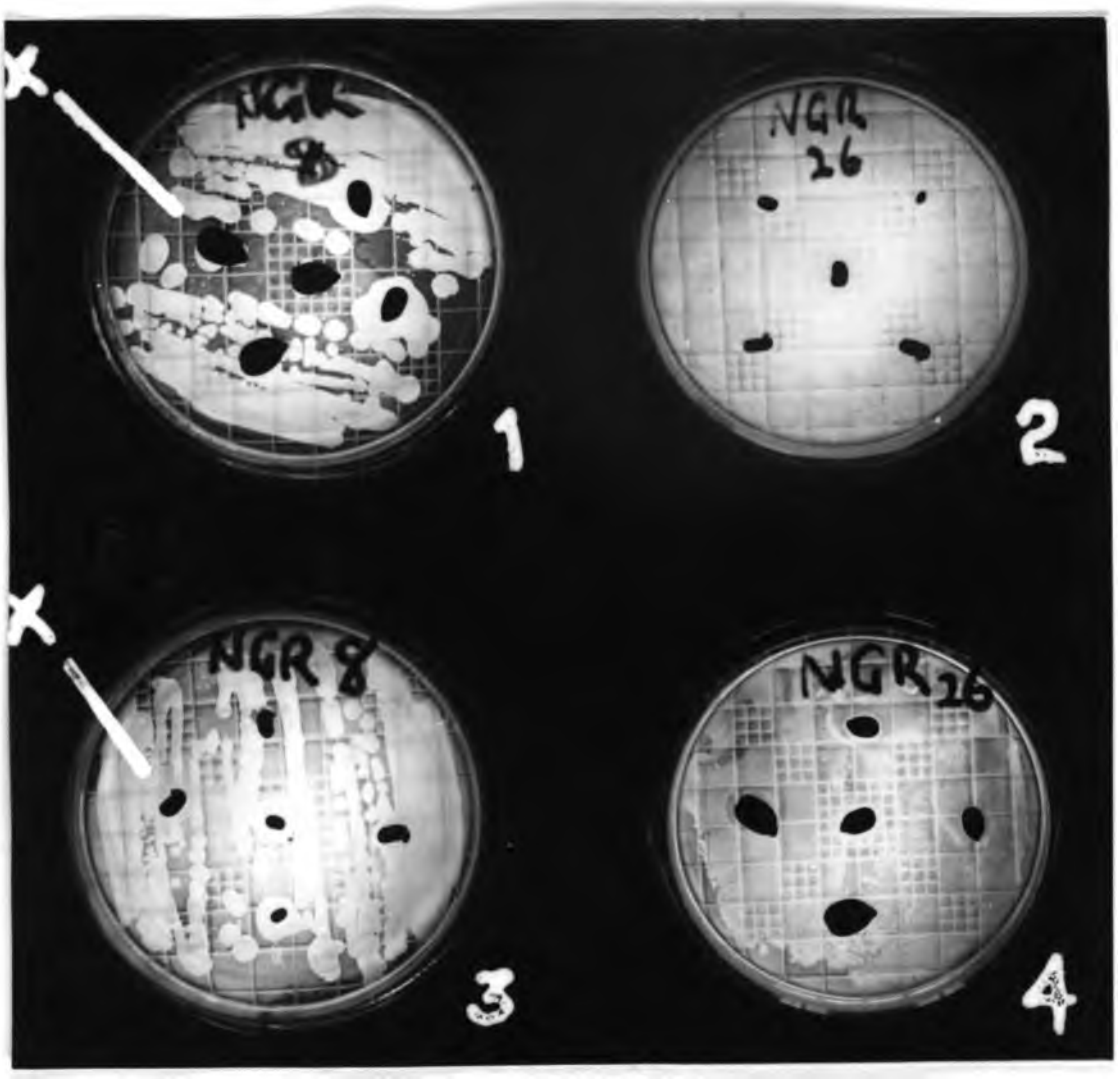

FIGURE 1 The growth of Rhizobium in the presence of legume seeds.

1. Strain NGR 8 and seeds of Leucaena glauca
2. Strain NGR 26 and seeds of Centrosema sp.
3. Strain NGR 8 and seeds of Centrosema sp
4. Strain NGR 26 and seeds of Leucaena glauca

X indicates germinating seeds.

Experiment Nº 4. Nodulation and growth of leucaena in response to the factors nitrogen, sulphur and inoculum suspension

The growth of the plants in this trial was not particularly vigorous, but no nutrient deficiencies of any kind were noted.

The yield of plant tops was affected when the pots were accidentally allowed to dry out excessively on one occasion. This caused considerable leaf fall in all pots, but some were more affected than others. This would add to the error variance and reduce the chance of detecting significant differences due to the presence ~~of~~ or absence of a factor. However, it is considered that the yield results, especially of roots and nodules, are not invalidated.

Since the experiment was designed as a factorial, the results are presented in terms of the factor effects, rather than on the basis of differences between treatment means per se. The factors are considered in terms of their main effects, i.e., the average effect of the factor in both the presence and absence of the other factors. Where there is a significant interaction between two factors, the simple effects are considered, i.e. the separate effects of one factor at each level of the interacting factor. Some significant interactions also occurred even when neither of the factors involved showed a significant main effect, and these are considered separately.

The yields of dry matter and nitrogen in the plant tops, roots and nodules, analysis of variance of the data, and the calculations of the factorial effect means are presented in the Appendix.

Factor I. Ten per cent sucrose solution as the suspending medium for the application of *Rhizobium* inoculum to the seed of *L. glauca* significantly increased the yield of nitrogen in the nodules ($P < 0.01$). This factor was not independent in its action, however, since the I x N interaction was significant ($P < 0.05$). Examination of the simple effects of I in the presence and absence of N reveals that the beneficial effect of the sucrose based inoculum was significant only when combined nitrogen was also added.

Factor S. Considered in terms of its main effects, the inclusion of sulphate in the nutrient solution applied to the sand culture did not significantly affect the dry matter or nitrogen yield in plant tops, roots or nodules, or nodule numbers of *L. glauca*.

Factor N. The application of four mg. of nitrogen (as urea) per pot significantly increased the dry matter yields of the roots ($P < 0.01$), but depressed the number of nodules per plant ($P < 0.01$). With respect to nodule dry matter yield, the main effect of N was to depress the yield significantly ($P < 0.05$), but the I x N interaction was also significant ($P < 0.05$). An examination of the simple effects of N reveal that its depressing effect was only significant in the absence of sucrose in the inoculum suspension.

Interaction I x S. Factors I and S in combination interacted to produce significant increases in the dry matter yield of roots and nodules, and in nodule numbers ($P < 0.05$). An examination of the simple effects of the two factors shows the following significant ($P < 0.05$) effects: Factor S depressed nodule numbers and dry matter

yield if I was absent, but not if present. Factor S increased root dry matter yield if I was present.

I increased nodule numbers if S was present, but depressed nodule dry matter yield if S was absent.

Interaction I x N. The combination of factors I and N resulted in increased dry matter and nitrogen yields of the plant tops and nodules. An examination of the simple effects of the two factors reveals the following significant ($P < 0.05$) effects:

Factor I depressed yields of dry matter and nitrogen in the plant tops in the absence of factor N. In the nodules, factor I depressed dry matter yield in the absence of factor N, but increased the nitrogen yield when factor N was also present ($P < 0.01$).

Factor N depressed nitrogen yield in the plant tops unless factor I was present. In the nodules, both dry matter and nitrogen yields were depressed unless factor I was present.

Interactions S x N and I x S x N. Factors S and N were probably independent in their effects, since the S x N interaction failed to reach the 5% level of significance for any of the data considered. The third order interaction I x S x N was also non-significant statistically.

The factor effects and interactions are presented in Table 7, and in Figures 2 and 3. The effect of a factor on yield, nodule number, etc. is shown as per cent increase or decrease from the general mean of all pots.

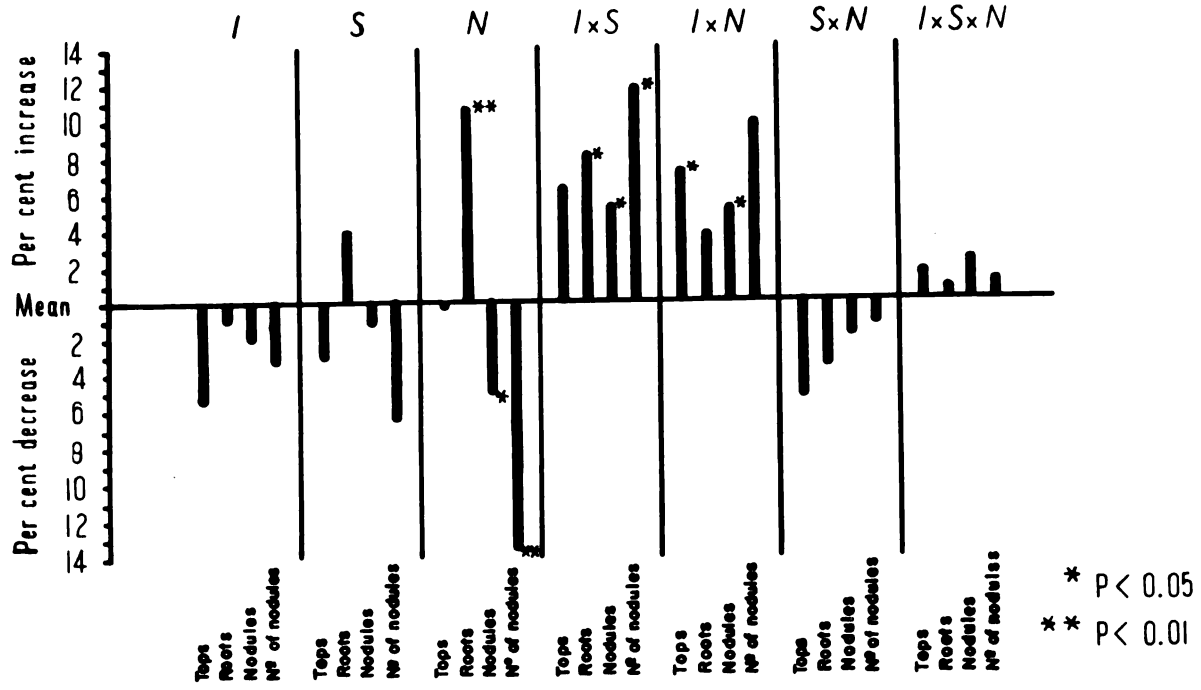

FIGURE 2 Factor and interaction effects on dry matter yield and nodule number
Per cent variation from the general mean

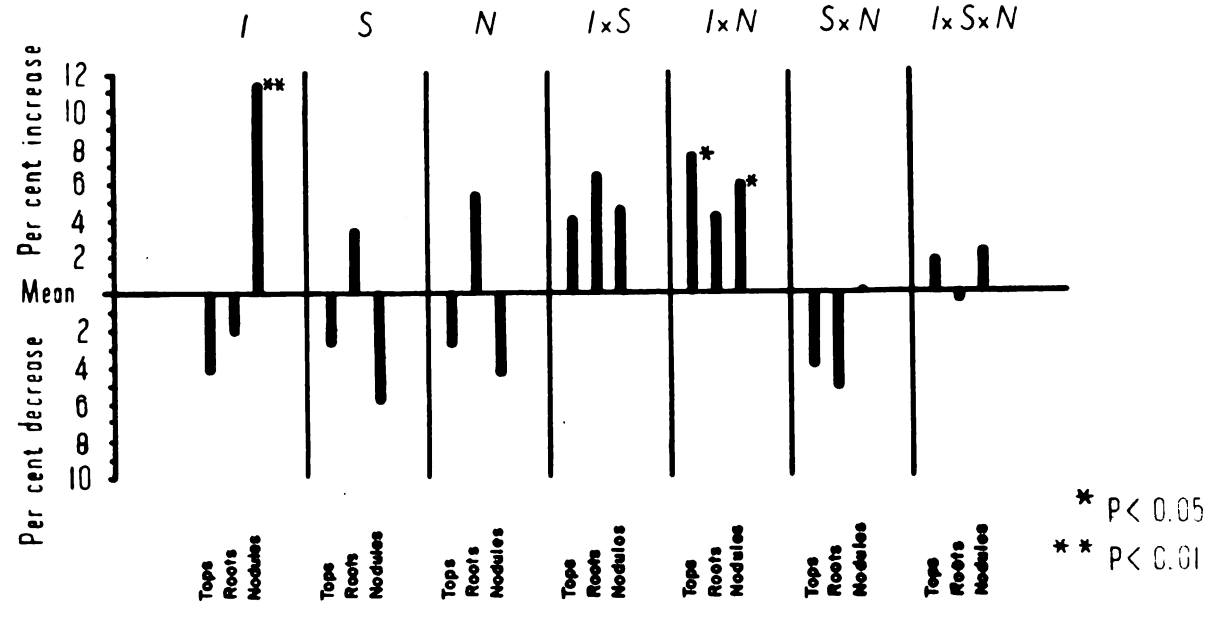

FIGURE 3. Factor and interaction effects on yield of nitrogen (mg. N per plant)
Per cent variation from the general mean

TABLE No. 7 Factor and interaction effects on dry matter yield per pot, nitrogen yield per plant, and nodule number per plant. Per cent variation from the general mean

Factor	I	S	N	IxS	IxN	SxN	IxSxN
Dry matter/pot							
Tops	-5.3	-3.0	-0.3	+6.1	+7.2*	-5.3	+1.5
Roots	-1.1	+3.9	+10.5**	+8.0*	+3.7	-3.6	+0.7
Nodules	-1.9	-1.3	-5.1*	+5.2*	+5.1*	-1.9	+2.3
Nitrogen/plant							
Tops	-4.3	-2.8	-2.8	+4.0	+7.5*	-4.1	+1.8
Roots	-2.2	+3.5	+5.4	+6.5	+4.2	-5.2	-0.5
Nodules	+11.4**	-5.9	-4.4	+4.6	+6.0*	+0.2	+2.3
No. nodules	+3.3	-6.5	-13.7**	+11.8*	+9.8	-1.3	+1.1

* = $P < 0.05$ ** = $P < 0.01$

Experiment No. 5. Time and rate of application of nitrogen, and its effect on nodulation and growth of leucaena

Soil "A". On July 28, 1963 a few nodules were found on the roots of plants harvested from one of the five extra pots planted for the purpose of determining the time of first nodule formation. These were very small and not easily visible to the naked eye. The plants had developed to the stage of expanding the second leaf.

Growth of the aerial parts of the plant was more vigorous in this soil than in the sand culture in Experiment 4. There were no noticeable differences between treatments, and all plants were uniformly dark green.

The plant tops were harvested from September 20 to 26, 1963. When the soil was washed from around the roots it was discovered that the development of nodules had been almost completely inhibited in all treatments. Only two very small nodules were observed from among all 25 pots examined. The effect of the treatment on nodulation could not therefore be determined. As might be expected under these circumstances, there were no significant differences between treatments in dry matter yield of plant tops or roots. There were, however, significant differences ($P < 0.01$) in dry matter yield between the rows, which were disposed along the north-south axis of the glasshouse. Pots in the row nearest the open windows and the ventilating duct (Row V) showed significantly higher yields than the inner-most row (Row I):

Plant tops

Row No.	I	II	IV	III	V
Mean yield per pot, g.	4.47	4.84	4.97	5.08	5.51

Roots

Row No.	I	IV	II	III	V
Mean yield per pot, g.	3.52	3.63	3.85	3.93	4.49

Means bracketed are not significantly different; means not bracketed are significantly different at the 1% level.

Soil "B". Plants grown in this soil were harvested during the same period as those in soil "A". The plants had been reduced in

numbers by an attack of a damping-off disease, and the T.M.T.D. treatment against this disease scorched some of the leaves. In spite of this the growth and yield of both tops and roots was consistently higher, on a per-pot basis, than that of soil "A". Most plants in this soil were quite well nodulated, but the yield of nodules was very variable. No significant differences were detected between treatments with regard to dry matter yield of plant tops, roots or nodules.

Significant differences ($P < 0.05$) in the yield of roots and nodules were detected between rows. As with the trial on soil "A", these rows were orientated along the north-south axis of the glasshouse, but on the opposite side. It was found that the row nearest the open windows and ventilating duct again showed the highest yield of roots and the innermost row showed the lowest. Yield of nodules was also lowest in pots of the innermost row, but the centre row showed the highest nodule yields (see Appendix).

Figures 4 and 5 demonstrate the differences in growth of roots and plant tops, respectively, in the two soils "A" and "B". As shown in Figure 4, tap root development in soil "A" was generally restricted, whereas in soil "B" the tap-root extended the full depth of the pot in most cases.

The roots grown in soil "A" also had an unusually crimped appearance, compared with the smooth texture of the surface of the roots from soil "B". The tops of plants growing in soil "A" were shorter, yielded less, but were darker green in colour than those of soil "B".

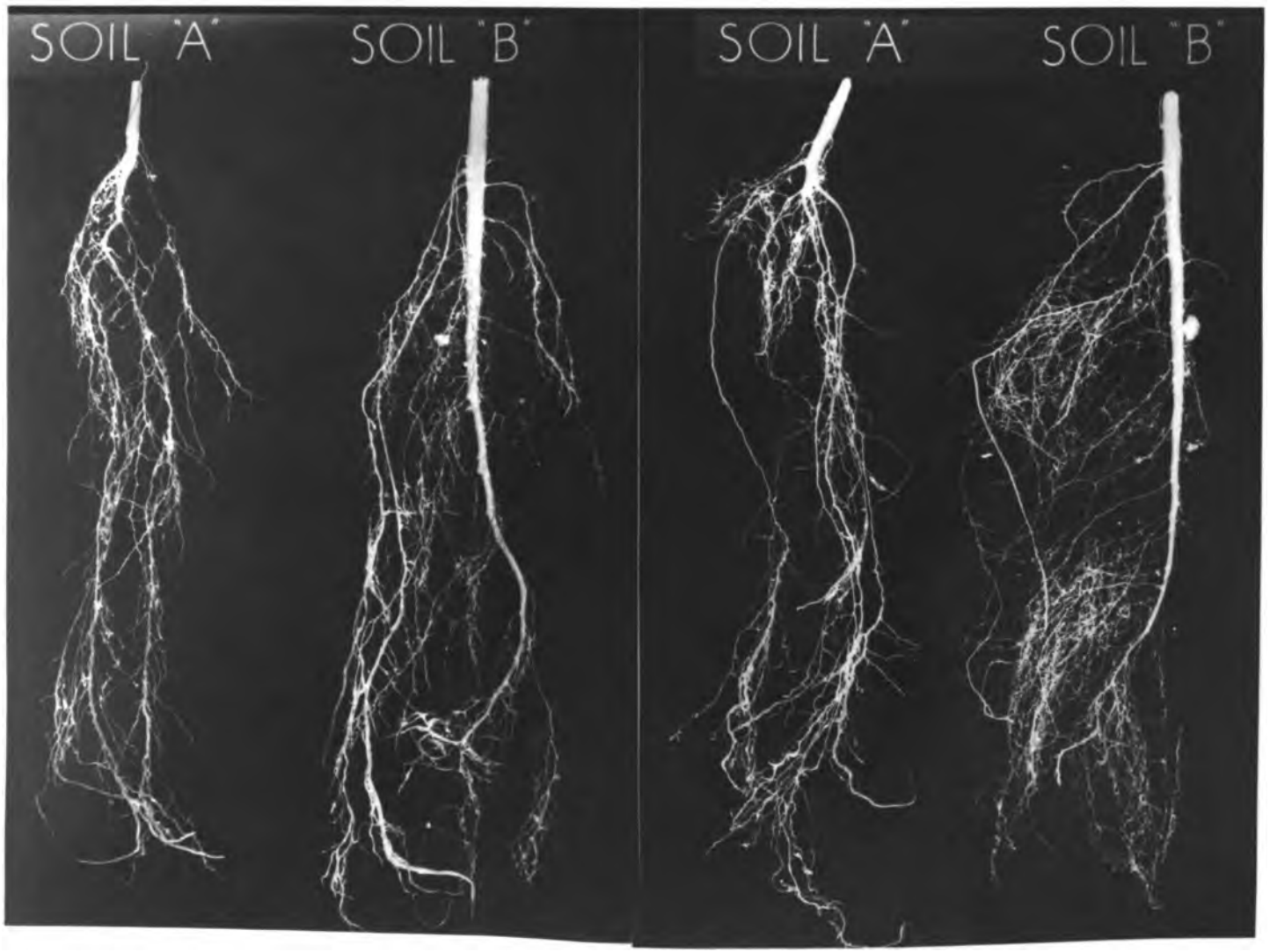

Figure 4 Root systems of typical plants grown in soils A and B
Left hand pair - Treatment B. Right hand pair - Treatment C.

FIGURE 5 Top growth of Leucaena glauca grown in soils "A" and "B" (Treatment A- control)

Experiment No. 6. The relative effectiveness of three strains of rhizobia in the nodulation of leucaena

There were no visible differences between treatments in the outward appearance of the plants in either soil "A" or soil "B".

Plants growing in soil "B" were harvested four weeks after sowing, on September 11, 1963. This was earlier than intended originally, but there was some evidence of an inpeding attack by the same damping-off type of disease which affected plants in this soil in Experiment 5.

Plants in soil "A" were harvested six weeks after sowing, on September 29, 1963. As occurred in Experiment 5, nodulation was comply inhibited in this soil, so that the effect of the treatments on nodulation could not be determined.

Plant grown in soil "B" were nodulated in the inoculated Treatments 2, 3 and 4, but no nodules were detected in any plants in the control Treatment 1, or in Treatment 5. The yield of nodules, however, was very small. Analysis of variance of the dry matter yield of the nodules show a significance at the 1% level according to the standard F test. This is because Treatments 1 and 5 had zero yield of nodules. Application of Duncan's multiple range test to the treatment means revealed that the yield of nodules from the three strains of rhizobia did not differ significantly at the 1% level. At the 5% level, strains NGR 8 and NGR 31 did not differ significantly from one another, but both were superior to the strain CB 81, while the yield of nodules from the CB 81 treatment was not significantly different from the control (zero):

Treatment	Control	Urea	CB 81	NGR 31	NGR 8
Mean yield of nodules, mg. per pot	0	0	1.45	3.65	4.15
P < 0.01	⌊	———	⌋		⌊ ——— ⌋
P < 0.05	⌊	———	——— ⌋	⌊	——— ⌋

Means bracketed do not differ significantly

Since only the plants in soil "B" were nodulated, and these had to be harvested after only one month, the amount of nodular tissue was too small to produce any measurable effect on the dry matter yield of the plants.

Experiment No. 7. A field trial to compare the production of a leucaena/pangola grass mixture with that of pangola grass alone

Germination of the acid-treated inoculated seed was excellent, and a number of plants which were dug up from time to time were effectively nodulated. However, the leucaena received a number of setbacks which resulted in the experiment finally having to be abandoned. Firstly, attacks by leaf-harvesting ants decimated the initially promising stand of seedlings. Secondly, the experiment was laid down during a reasonably dry period, but soon after the seedlings were established it was evident that during the wetter periods of the year the soil on which this experiment was sited has a water table only 12 to 18 inches below the soil surface. This would

effectively prevent the normally deep-penetrating roots of leucaena from reaching below this level. Competition from the roots of the vigorously growing pangola grass was therefore much greater than expected.

DISCUSSION

1. The use of milk cartons as pots

The waxed cardboard milk cartons used in the pot trials in this study are satisfactory for such experiments, but only with certain modifications. The carton, if unprotected from the soil, begins to decompose after about four weeks. This can be easily overcome by providing a drain hole in the base, and lining the carton with a tube of polyethylene film which is slightly larger in diameter than the top of the carton. This permits the plastic liner to be folded over the top edge of the pot, thus preventing any water from penetrating between the carton and the plastic. This was done in Experiments 5 and 6 with very satisfactory results. It was found also that the carton allows enough light to pass through for a skin of algae to grow on the outside of the soil against the plastic liner. This could easily be overcome by using a liner of black plastic instead of the clearer plastic which was used in these experiments.

The tall narrow shape of the fully opened milk carton appeared to provide sufficient root room for six to eight leucaena plants for up to 12 weeks.

Because of its low field capacity and high porosity, the sand used in Experiment 4 was a difficult medium to maintain in a satisfactory state of moisture. In the undrained milk cartons, blackening of the roots at the base of the carton indicated that anaerobic conditions prevailed, while the top of the pot dried out very rapidly, requiring application of water more than once a day.

2. The glasshouse

Temperature conditions were generally satisfactory in the glasshouse used in this study. The temperature rarely exceeded 38°C, and then only for a short period. In the study of Bowen and Kennedy (13) 81 per cent of the strains of rhizobia from tropical legumes examined had a maximum temperature for growth in the range 32.6 - 38.6°C. Control of abnormally high temperatures must be taken into account in glasshouse experiments, especially with legumes.

3. Seed scarification

While treatment with concentrated sulphuric acid was found to be by far the most effective treatment, its application to large quantities of seed is more difficult and involves greater hazards than hot water treatment or mechanical scarification.

A reasonably simple and safe method of using concentrated acid would be to place the acid in a strong flexible plastic container. A plastic-coated wire basket in which the seeds were completely enclosed could then be immersed in the acid and agitated to ensure that all the seed were covered by the acid. After the specified period of treatment the acid could be quickly washed off in running water with a minimum wastage of acid.

4. The seed coat and survival of rhizobia

Although the seed coats of Centrosema pubescens have been shown to contain an antibiotic (12), the seed of the Centrosema species used in this trial (unidentified, but not C. pubescens) as

well as that of L. glauca, do not have this attribute. A study of a wider range of tropical legumes is needed to determine whether the characteristic is common in tropical legumes, or largely confined to the temperate legumes, which are generally accepted as being more recent or advanced in the evolutionary scale. Masterton (45) included a wide range of temperate legume species in his study of this factor and found inhibition of growth of Rhizobium caused to some extent by all but one of the species studied.

Possibly the possession of such an antibiotic is an advantage to the species under natural conditions. The problem of seed coat toxicity to Rhizobium only arises when the plant is provided with an artificial supply of the nodule-forming bacteria by means of inoculation of the seed.

5. Comparison of strains of rhizobia in the nodulation of leucaena

Strain testing of rhizobia is usually carried out under sterile conditions, either in a medium of agar or in sand culture. Under field conditions the environment in which Rhizobium must invade the legume roots would be very different from those obtained in sterile agar or sand culture.

It was known with reasonable certainty that neither of the soils used in Experiment 6 would contain any native Leucaena Rhizobium strains, since leucaena is not native to the region, and its rhizobial requirements are quite specific (19, 49). To keep the environment of the rhizosphere in this experiment somewhat near those prevailing in the field, the soil was not sterilized or specially treattreated in any way.

Any gross contamination of one treatment by strains of Rhizobium from another was avoided, but apart from this the heavy suspension of the respective strain of Rhizobium was considered sufficient to ensure that nodulation by extraneous rhizobia would be most unlikely to occur. This hypothesis was vindicated by the complete absence of nodules in the control treatment of soil "B" after one month. If it had been possible to continue the treatment longer, some nodulation might have occurred in the control treatment, however.

Since there were only two replications available for statistical analysis, and the plants had to be harvested earlier than was desirable, the apparent superiority ($P < 0.05$) of strains NGR 8 and NGR 31 over strain CB 81 requires more definite confirmation.

6. Nodule initiation on leucaena

In both the sand and soil "A", nodule initiation in L. glauca occurred during the expansion of the second leaf. Nodules were easily visible when the second leaf was fully expanded, at least in the sand culture. Nodule initiation can be affected by many factors, however, and under other conditions the stage of growth of the plant when nodules can first be observed may de different. Gibson and Nutman (22) showed that nitrate delayed nodule initiation; soils with differing nitrification capacities may therefore be expected to affect nodule initiation differently.

7. The effect of sucrose in the Rhizobium inoculum applied to seeds of leucaena

On the basis of the results of Sankaram (57) and those quoted by Vincent (66), the inclusion of sucrose solution in the inoculum applied to legume seeds reduces the death rate of the nodule-forming bacteria, and may even cause an increase in the number of viable cells of Rhizobium during storage over several days. The subsequent effect of the sucrose when the seed is sown in the soil can only be surmised, but one might expect an increase in nodule numbers per plant, or an increase in the yield of nodules, as a result of greater numbers of bacteria available for nodule formation.

The results of Experiment 4 suggest, however, that there may be more involved than mere survival or multiplication of the bacteria. The only significant main effect of Factor I alone was to substantially increase the nitrogen yield in the nodules. This suggests that the sucrose may have acted as a source of energy for the invading rhizobia. However, it is estimated that only about 3.3 mg. of sucrose would have been included on each seed due to the inoculum applied, and this would be localized to a small area around the seed when it was sown. The significant I x S interactions are also difficult to explain.

The I x N interaction might be interpreted on the basis of the relation between supply of carbohydrate and nitrogen. Whereas N alone significantly depressed nodule numbers and dry nodule matter yields, the presence of sucrose in the inoculum prevented, and even

reversed the depressing effect of N. These results agree with Wilson's theory (72) on the carbohydrate:nitrogen balance in the legume-*Rhizobium* symbiosis, and are supported by the results of Hopkins and Fred (33), and of Ludwig and Allison (43).

8. The effect of small amounts of combined nitrogen added about the time of first nodule formation

The beneficial effects of small quantities of added combined nitrogen obtained by Pate and Dart (51) were not substantiated completely in Experiments 4 or 5 of this study. In sand culture four milligrams of nitrogen (as urea) added per pot depressed nodulation unless the rhizobia were applied to the seed in 10 per cent sucrose, in which case nodulation was improved by the added nitrogen.

The soils used in Experiment 5 (nitrogen rate and time of application) proved to be both very high in total nitrogen, and no treatment effects could be detected.

9. The role of sulphur in nodulation

Added sulphur in the sand culture of Experiment 4 did not result in any significant beneficial effects on nodulation. Although the value of 50 parts per million of total sulphur (Table 2) is quite a low value, there was apparently sufficient to provide most or all of the needs of the plants grown in this sand. Ashford and Bolton (7), in their study of *Melilotus alba* grown in vermiculite, suggested that the plant was able to utilise very small amounts of sulphur to fix appreciable amounts of nitrogen. The interaction of factors I and S in this experiment remain unexplained.

10. Number of nodules as a measure of nodulation and nitrogen fixation

In the analysis of data from Experiment 4 it was found that nodule numbers showed the highest coefficient of variation of all the measurements taken. Considering the difficulty and the time involved in counting nodules, especially from plants grown in soil or sand culture, the usefulness of this measurement is doubtful. This conclusion finds support in the work of Hopkins, Wilson and Peterson (34), who also encountered high variability in nodule numbers per plant. Gibson (21) also noted that the important criterion in assessing nodule function was not the number of nodules, but the volume and longevity of the central tissue containing the bacteroids.

The coefficients of variation for the different measurements used in experiment 4 are shown in Table 8.

TABLE No. 8 Means, standard errors and coefficients of variation of the data obtained in Experiment No. 4

	General mean $\bar{x}$	Standard error s^2	Coefficient of variation, % $s^2/\bar{x} \cdot 100$
Dry matter per pot			
Tops, g.	0.738	0.083	11.2
Roots, g.	0.562	0.068	12.1
Nodules, mg.	77.9	6.048	7.8
Nitrogen per plant, mg.			
Tops	2.764	0.297	10.7
Roots	1.646	0.207	12.6
Nodules	0.563	0.029	5.1
Number of nodules	29.34	4.981	17.0

11. Nodulation on the soils "A" and "B"

Both of these soils were selected partly because of their physical nature, which allowed for easy separation of the roots from the soil and examination for nodulation. Soil "A" was known to be high in total nitrogen, but no analysis for soil "B" was available. Because Guinea grass had shown considerable responses to applications of nitrogen fertilizer on this soil it was presumed to have a much lower nitrogen content than soil "A". Subsequent analysis, as shown in Table 2, revealed that although lower in nitrogen content than soil "A", soil "B" was still very high in total nitrogen, when compared with the standards for soil nitrogen given in Table 3.

A question then arises: why was nodulation quite satisfactory in soil "B", but completely inhitited in soil "A"?

A nutrient deficiency might be involved, although a complete fertilizer mixture was applied to both soils. Phosphorus is known to be strongly fixed in these soils, but triple superphosphate was added in granular form specifically to avoid complete fixation. Plants grown in soil "A" were generally smaller and a darker green than those in soil "B" however (Figure 5).

A possible explanation for the remarkable difference between the two soils may be in the relative nitrification rate. That the availability of the soil nitrogen in soil "B" may be quite low is supported by the previous observation that guinea grass responded to applications of nitrogenous fertilizer. If the nitrification rate in soil "A" was very high, sufficient nitrate may have been released to completely inhibit nodulation of leucaena. This possibility should be examined further.

The reaction of Soil "A" was also a full pH unit lower than that of soil "B" (Table 2). While any nutrient deficiencies induced by the pH of 4.8 in soil "A" should have been overcome by the complete nutrient mixture added to the soil, the possibility of toxic concentrations of aluminium, manganese or hydrogen ions cannot be overlooked (58). Against this possibility is that the growth of the plants themselves was quite healthy, with no symptoms of deficiency or toxicity that could be associated with the low pH.

CONCLUSIONS

1. Under conditions similar to those of this study, the formation of the first nodules on Leucaena glauca can be expected to occur during the development of the second leaf, i.e. the first bipinnately compound leaf.

2. The seed coats of L. glauca and the Centrosema species examined in this study did not contain any antibiotic which may adversely affect the viability of rhizobia applied to the seed.

3. Scarification of L. glauca seed to improve germination is most effectively accomplished by treatment with concentrated sulphuric acid for 20 minutes. Treatment of large quantities of seed would require plastic coated containers and equipment.

4. Ten per cent sucrose solution as the suspending mediun for the application of Rhizobium inoculum to the seed of L. glauca may have assisted the survival of the bacteria, and in the presence of sufficient nitrogen and sulphur, was beneficial to nodulation and nitrogen yield of the plants.

5. The beneficial effects of small quantities of nitrogen on nodulation was dependent in this study on the presence of sucrose in the inoculum applied to the seed.

6. The establishment of leucaena in grass pastures is dependent on the ability of the roots of the leucaena to penetrate below the main mass of grass roots as quickly as possible after establishment, thus avoiding severe competition from the grass.

Where leaf-harvesting ants are known to occur, some measure of insecticidal protection may be required before leucaena could be established.

7. Suggestions for further research arising out of this study are:

(a) to determine whether the inhibition of nodulation of L. glauca in one of the soils used in this study was due to low pH, high available nitrogen, or some other factor.

(b) that studies be made of the nitrification rate of the soils used in this study together with other soils in the vicinity of the Institute. Any information gained about the availability of the soil nitrogen may have important practical significance, since considerable amounts of nitrogenous fertilizers are applied by Departments within the Institute;

(c) that another attempt be made to establish L. glauca on a more suitable site, and to study its potential as a forage or pasture legume, either in a pure stand or mixed with a grass.

SUMMARY

Some factors involved in the establishment and nodulation of the forage legume Leucaena glauca were studied in laboratory and glasshouse experiments. For the latter, plants were grown in sand culture and in two senile latosol soils. Except for particular nutrients under study, a complete nutrient solution was applied to all pots.

The germination of seeds of L. glauca is improved by hot water (80°C) and sulphuric acid treatment. Treatment of seed with concentrated sulphuric acid for 20 minutes resulted in a germination of 99 per cent. Untreated seeds showed a germination of four per cent.

The seeds of L. glauca, and a species of Centrosema also studied, did not have any adverse effect on the growth of strains of Rhizobium on agar plates, whether the seeds were dormant or germinating.

The application of Rhizobium inoculum to L. glauca seeds by means of a 10 per cent solution of sucrose was beneficial to nodulation and nitrogen yield of plants in sand culture, provided combined nitrogen and sulphate were added at the same time.

The beneficial effect of small amounts of combined nitrogen on the nodulation of L. glauca in sand culture was dependent on the presence of sucrose in the inoculum applied to the seed.

In one of the soils used in this study nodule development was almost completely inhibited by an undetermined factor. High soil nitrogen content or low pH may equally well be involved.

A field trial to study the production of a leucaena/pangola grass mixture had to be abandoned because of the strong competition from the roots of the grass, aggravated by a high water table in the soil. Leaf-harvesting ants also decimated the seedlings at an early age.

RESUMEN

Algunos factores relacionados con el establecimiento y la nodulación de la leguminosa forrajera, Leucaena glauca fueron estudiados en experimentos del laboratorio e invernadero. Para las pruebas en macetas, las plantas se cultivaron en arena y en dos suelos del tipo latosol viejo. Excepto para los nutrientes individuales bajo estudio, se añadió una solución nutritiva completa a cada maceta.

La germinación de las semillas de L. glauca fue aumentada ~~por~~ por tratamientos con agua caliente (80°C) y ácido sulfúrico. El tratamiento con ácido sulfúrico concentrado durante 20 minutos resultó con una germinación de 99 por ciento. El testigo mostró una germinación de cuatro por ciento.

Las semillas de L. glauca y una especie de Centrosema, no tuvieron ningún efecto adverso sobre el crecimiento de cultivos de Rhizobium en agar, ya sea que las semillas estuvieron germinadas o inactivas.

La aplicación del inóculo de Rhizobium por medio de una solución de sacarosa al 10 por ciento, fue ventajosa a la nodulación y el rendimiento de nitrógeno en plantas desarrolladas en arena, cuando se agregó un compuesto de nitrógeno y sulfato al mismo tiempo.

El efecto beneficioso de cantidades pequeñas de un compuesto de nitrógeno sobre la nodulación en L. glauca fue condicional a la presencia de sacarosa en el inóculo aplicado a las semillas.

En uno de los suelos que fue usado en este estudio, el desarrollo de los nódulos fue prácticamente inhibido por un factor no determinado. Cantidades altas de nitrógeno en el suelo, o bajo pH pueden ser implicados igualmente.

Un experimento de campo para estudiar la producción de una mezcla de leucaena con zacate pangola fue abandonado, debido a la competencia de las raíces del zacate con las de leucaena. Esta competencia fue agravada por un alto nivel de la capa freática en el suelo. Además, hormigas atacaron a las hojas y disminuyeron en gran cantidad el número de plántulas.

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APPENDIX

Experiment 4. Statistical analyses

1. Plant tops - dry matter - grams per pot

Calculation of factor effects

Treatment	Total	(1)	(2)	(3)	Factor effect	Effect mean	Per cent
(1)	4.76	8.76	17.73	35.42	TOTAL	0.738	
i	4.00	8.97	17.69	-0.94	I	-0.039	-5.3
s	4.66	9.21	-1.11	-0.52	S	-0.022	-3.0
is	4.31	8.48	0.17	+1.08	IxS	+0.045	+6.1
n	4.73	-0.76	0.21	-0.04	N	-0.002	-0.3
in	4.48	-0.35	-0.73	+1.28	IxN	+0.053	+7.2*
sn	4.03	-0.25	+0.41	-0.94	SxN	-0.039	-5.3
isn	4.45	+0.42	+0.67	+0.26	IxSxN	+0.011	+1.5

Variation due to	Sum of squares	D.F.	Mean square	F
Total	0.40280	47		
Blocks	0.05905	5	0.01181	
Treatments	0.10283	7	0.01467	2.13
I	0.01840	1		
S	0.00563	1		
N	0.00003	1		
IxS	0.02430	1		
IxN	0.03413	1		4.95*
SxN	0.01840	1		
IxSxN	0.00140	1		
Error	0.24142	35	0.00689	

Examination of IxN interaction

Inoculum	Nitrogen		
	n_o	n_1	$n_1 - n_o$
i_o	9.42	8.76	-0.66
i_1	8.31	8.93	+0.62
$i_1 - i_o$	-1.11	+0.17	

Effect of N, within $i_o = \frac{-0.66^2}{24}$

$= 0.01815$

Effect of N, within $i_1 = \frac{+0.62^2}{24}$

$= 0.01601$

Effect of I, within $n_o = \frac{-1.11^2}{24}$

$= 0.05113^{**}$

Effect of I, within $n_1 = \frac{0.17^2}{24}$

$= 0.00120$

2. Roots - dry matter - grams per pot

Calculation of factor effects

Treatment	Total	(1)	(2)	(3)	Factor effect	Effect mean	Per cent
(1)	3.26	6.13	12.77	26.99	TOTAL	0.563	
i	2.87	6.64	14.22	-0.15	I	-0.006	-1.1
s	3.29	7.10	-0.33	+0.53	S	+0.022	+3.9
is	3.35	7.12	0.18	+1.03	IxS	+0.045	+8.0*
n	3.65	-0.39	0.51	+1.45	N	+0.059	+10.5**
in	3.45	0.06	0.02	+0.51	IxN	+0.021	+3.7
sn	3.37	-0.20	0.45	-0.49	SxN	-0.020	-3.6
isn	3.75	0.38	0.58	+0.13	IxSxN	+0.005	+0.9

* = $P < 0.05$, ** = $P < 0.01$

Variation due to	Sum of squares	D.F.	Mean square	F
Total	0.29085	47		
Blocks	0.00676	5	0.00135	
Treatments	0.08300	7	0.01185	2.58*
I	0.00046	1		
S	0.00585	1		
N	0.04380	1		9.52**
IxS	0.02210	1		4.80*
IxN	0.00541	1		
SxN	0.00500	1		
IxSxN	0.00035	1		
Error	0.16109	35	0.00460	

Examination of IxS interaction

Inoculum	Sulphur		
	s_o	s_1	$s_1 - s_o$
i_o	6.91	6.66	-0.25
i_1	6.32	7.10	+0.78
$i_1 - i_o$	-0.59	+0.44	

Effect of S, within i_o = $\frac{-0.25^2}{24}$ = 0.002

Effect of S, within i_1 = $\frac{0.78^2}{24}$ = 0.025*

Effect of I, within s_o = $\frac{0.59^2}{24}$ = 0.015

Effect of I, within s_1 = $\frac{0.44^2}{24}$ = 0.008

* = P < 0.05, ** = P < 0.0

3. Nodules - dry matter - milligrams per pot

Calculation of factor effects

Treatment	Total	(1)	(2)	(3)	Factor effect	Effect mean	Per cent
(1)	501	956	1917	3739	TOTAL	77.89	
i	455	961	1822	-35	I	-1.46	-1.9
s	490	926	-65	-25	S	-1.04	-1.3
is	471	896	30	+97	IxS	+4.04	+5.2*
n	473	-46	5	-95	N	-3.96	-5.1*
in	453	-19	-30	+95	IxN	+3.98	+5.1*
sn	423	-20	27	-35	SxN	-1.46	-1.9
isn	473	50	70	+43	IxSxN	+1.80	+2.3

Variation due to	Sum of squares	D.F.	Mean square	F
Total	2,422.48	47		
Blocks	467.86	5	93.57	2.56*
Treatments	674.25	7	96.32	2.63*
I	25.52	1		
S	13.02	1		
N	188.02	1		5.14*
IxS	196.02	1		5.36*
IxN	188.02	1		5.14*
SxN	25.52	1		
IxSxN	38.52	1		
Error	1,280.37	35	36.58	

* = $P < 0.05$

Examination of IxS interaction

Inoculum	Sulphur		
	s_o	s_1	$s_1 - s_o$
i_0	974	913	-61
i_1	908	944	+36
$i_1 - i_o$	-66	+31	

Effect of S, within i_o $= \frac{-61^2}{24}$

$= 155.04^*$

Effect of S, within i_1 $= \frac{36^2}{24}$

$= 54.0$

Effect of I, within s_o $= \frac{-66^2}{24}$

$= 181.5^*$

Effect of I, within s_1 $= \frac{31^2}{24}$

$= 40.0$

Examination of IxN interaction

Inoculum	Nitrogen		
	n_o	n_1	$n_1 - n_o$
i_o	991	896	-95
i_1	926	926	0
$i_1 - i_o$	-65	+30	

Effect of N, within i_o $= \frac{-95^2}{24}$

$= 376.04^{**}$

Effect of N, within i_1 $= 0$

Effect of I, within n_o $= \frac{-65^2}{24}$

$= 176.04^*$

Effect of I, within n_1 $= \frac{30^2}{24}$

$= 37.5$

$^* = P < 0.05$, $^{**} = P < 0.01$

4. Nodule numbers - mean number per plant

Calculation of factor effects

Treatment	Total	(1)	(2)	(3)	Factor effect	Effect mean	Per cent
(1)	207.9	385.5	752.4	1308.4	TOTAL	29.34	
i	177.6	366.9	656.0	+23.0	I	+0.96	+ 3.3
s	179.8	341.7	-23.0	-46.0	S	-1.92	- 6.5
is	187.1	314.3	46.0	+83.0	IxS	+3.46	+11.8*
n	170.7	-30.3	-18.6	-96.4	N	-4.02	-13.7**
in	171.0	7.3	-27.4	+69.0	IxN	+2.88	+ 9.8
sn	134.3	0.3	37.6	-8.8	SxN	-0.37	- 1.3
isn	180.0	45.7	45.4	+7.8	IxSxN	+0.33	+ 1.1

Variation due to	Sum of squares	D.F.	Mean square	F
Total	1564.317	47		
Blocks	201.837	5	40.37	
Treatments	494.297	7	70.61	2.85*
I	11.020	1		
S	44.083	1		
N	193.603	1		7.80**
IxS	143.521	1		5.78*
IxN	99.188	1		4.00°
SxN	1.163	1		
IxSxN	1.268	1		
Error	868.183	35	24.81	

° = P < 0.10, * = P < 0.05, ** = P < 0.01

Examination of IxS interaction

Inoculum	Sulphur		
	s_o	s_1	$s_1 - s_o$
i_o	378.6	314.1	-64.5
i_1	348.6	367.1	+18.5
$i_1 - i_o$	-30.0	+53.0	

Effect of S, within $i_o = \frac{-64.5^2}{24}$ Effect of I, within $s_o = \frac{-30.0^2}{24}$

$= 173.34^*$ $= 37.5$

Effect of S, within $i_1 = \frac{18.5^2}{24}$ Effect of I, within $s_1 = \frac{53.0^2}{24}$

$= 14.26$ $= 117.04^*$

Examination of IxN interaction

Inoculum	Nitrogen		
	n_o	n_1	$n_1 - n_o$
i_o	387.7	305.0	-82.7
i_1	364.7	351.0	-13.7
$i_1 - i_o$	-23.0	+46.0	

Effect of N, within $i_o = \frac{-82.7^2}{24}$ Effect of I, within $n_o = \frac{-23^2}{24}$

$=284.97^{**}$ $= 22.04$

Effect of N, within $i_1 = \frac{-13.7^2}{24}$ Effect of I, within $n_1 = \frac{46^2}{24}$

$= 7.82$ $= 88.17$

$^* = P < 0.05$, $^{**} = P < 0.01$

5. Plant tops - yield of nitrogen - milligrams per plant

Calculation of factor effects

Treatment	Total	(1)	(2)	(3)	Factor effect	Effect mean	Per cent
(1)	17.87	33.43	67.28	132.68	TOTAL	2.764	
i	15.56	33.85	65.40	-2.84	I	-0.118	-4.3
s	17.72	33.83	-3.90	-1.84	S	-0.077	-2.8
is	16.13	31.57	1.06	+2.64	IxS	+0.110	+4.0
n	17.13	-2.31	0.42	-1.88	N	-0.078	-2.8
in	16.70	-1.59	-2.26	+4.96	IxN	+0.207	+7.5*
sn	15.04	-0.43	0.72	-2.68	SxN	-0.112	-4.1
isn	16.53	1.49	1.92	+1.20	IxSxN	+0.050	+1.8

Variation due to	Sum of squares	D.F.	Mean square	F
Total	5.32897	47		
Blocks	1.08427	5	0.21685	2.49
Treatments	1.14957	7	0.16422	1.86
I	0.16803	1		
S	0.07053	1		
N	0.07363	1		
IxS	0.14520	1		
IxN	0.51253	1		5.79*
SxN	0.14963	1		
IxSxN	0.03000	1		
Error	3.09513	35	0.08843	

* = $P < 0.05$

Examination of IxN interaction

Inoculum	Nitrogen		
	n_o	n_1	$n_1 - n_o$
i_o	35.59	32.17	-3.42
i_1	31.69	33.23	+1.54
$i_1 - i_o$	-3.90	+1.06	

Effect of N, within i_0 = $\frac{-3.42^2}{24}$ = 0.48735*

Effect of I, within n_o = $\frac{-3.90^2}{24}$ = 0.63375*

Effect of N, within i_1 = $\frac{1.54^2}{24}$ = 0.09881

Effect of I, within n_1 = $\frac{1.06^2}{24}$ = 0.0468

6. Roots - yield of nitrogen - milligrams per plant

Calculation of factor effects

Treatment	Total	(1)	(2)	(3)	Factor effect	Effect mean	Per cent
(1)	9.84	18.36	38.44	79.02	TOTAL	1.646	
i	8.52	20.08	40.58	-0.88	I	-0.037	-2.2
s	10.01	20.45	-1.26	+1.40	S	+0.058	+3.5
is	10.07	20.13	0.38	+2.58	IxS	+0.108	+6.5
n	10.43	-1.32	1.72	+2.14	N	+0.089	+5.4
in	10.02	0.06	-0.32	+1.64	IxN	+0.068	+4.2
sn	9.67	-0.41	1.38	-2.04	SxN	-0.085	-5.2
isn	10.46	0.79	1.20	-0.18	IxSxN	-0.008	-0.5

* = $P < 0.05$

Variation due to	Sum of squares	D.F.	Mean square	F
Total	2.10073	47		
Blocks	0.16345	5	0.03269	
Treatments	0.43446	7	0.06206	1.26
I	0.01613	1		
S	0.04083	1		
N	0.09540	1		
IxS	0.13867	1		3.23
IxN	0.05603	1		
SxN	0.08670	1		
IxSxN	0.00067	1		
Error	1.50282	35	0.04293	

7. Nodules - yield of nitrogen - milligrams per plant

Calculation of factor effects

Treatment	Total	(1)	(2)	(3)	Factor effect	Effect mean	Per cent
(1)	1.169	2.372	4.609	9.015	TOTAL	0.563	
i	1.203	2.237	4.406	+0.515	I	+0.064	+11.4**
s	1.075	2.267	0.021	-0.263	S	-0.033	- 5.9
is	1.162	2.139	0.394	+0.209	IxS	+0.026	+ 4.6
n	1.074	0.034	-0.135	-0.023	N	-0.025	- 4.4
in	1.193	0.087	-0.128	+0.273	IxN	+0.034	+ 6.0*
sn	0.932	0.119	0.053	+0.007	SxN	+0.001	+ 0.2
isn	1.207	0.275	0.156	+0.103	IxSxN	+0.013	+ 2.3

* = $P < 0.05$, ** = $P < 0.01$

Variation due to	Sum of squares	D.F.	Mean square	F
Total	0.03579	15		
Blocks	0.00024	1	0.00024	
Treatments	0.03153	7	0.00502	6.0*
I	0.01657	1		20.0**
S	0.00432	1		
N	0.00257	1		
IxS	0.00279	1		
IxN	0.00465	1		5.6*
SxN	0.00001	1		
IxSxN	0.00066	1		
Error	0.00582	7	0.00083	

Examination of IxN interaction

Inoculum	Nitrogen		
	n_o	n_1	$n_1 - n_o$
i_o	2.244	2.006	-0.238
i_1	2.365	2.400	+0.035
$i_1 - i_o$	0.121	0.394	

Effect of N, within i_o $= \frac{-0.238^2}{24}$

$= 0.00708$*

Effect of N, within i_1 $= \frac{0.035^2}{24}$

$= 0.00015$

Effect of I, within n_o $= \frac{0.121^2}{24}$

$= 0.00244$

Effect of I, within n_1 $= \frac{0.394^2}{24}$

$= 0.01940$**

* = $P < 0.05$, ** = $P < 0.01$

Experiment 5. Statistical analyses

1. Soil "A", plant tops - dry matter - grams per pot

	Treatment					
	A	B	C	D	E	Mean
Totals	25.94	24.16	25.47	23.67	25.18	
Means	5.19	4.83	5.09	4.73	5.04	4.98

Analysis of variance

Variation due to	Sum of squares	D.F.	Mean square	F
Total	5.42235	24		
Columns	0.52039	4	0.13009	
Rows	2.85591	4	0.71397	6.4**
Treatments	0.70883	4	0.17720	
Error	1.33722	12	0.11143	

S.E. = 0.334, C. of V. = $\frac{0.334}{4.98} \cdot 100 = 6.7\%$

$S.E._{\bar{x}} = 0.149$

** = $P < 0.01$

2. Soil "A", roots - dry matter - grams per pot

	Treatment					
	A	B	C	D	E	MEAN
Totals	20.38	18.32	20.31	18.64	19.49	
Means	4.08	3.66	4.06	3.73	3.90	3.89

Analysis of variance

Variation due to	Sum of squares	D.F.	Mean square	F
Total	5.34122	24		
Columns	0.20910	4	0.05227	
Rows	2.84690	4	0.71172	5.41**
Treatments	0.70734	4	0.17683	
Error	1.57788	12	0.13149	

S.E. = 0.363, C. of V. = $\frac{0.363}{3.89} \cdot 100 = 9.3\%$

$S.E._{\bar{x}} = 0.162$

** = $P < 0.01$

3. Soil "B", plant tops - dry matter - grams per pot

	Treatment A	B	C	D.	E	MEAN
Totals	33.15	31.93	34.04	35.14	36.44	
Means	6.63	6.39	6.81	7.03	7.29	6.83

	Row I	II	III	IV	V
Totals	30.06	34.30	35.69	33.45	37.20

Analysis of variance

Variation due to	Sum of squares	D.F.	Mean square	F
Total	15.34000	22#		
Columns	2.90988	4	0.72747	
Rows	5.78284	4	1.44571	3.43°
Treatments	2.43284	4	0.60821	
Error	4.21444	10#	0.42144	

S.E. = 0.649, C. of V. = $\frac{0.649}{6.83} \cdot 100 = 9.5\%$

two degrees of freedom subtracted for 2 estimated values

° = $P < 0.10$

4. Soil "B", roots - dry matter - grams per pot

	Treatment					
	A	B	C	D	E	MEAN
Totals	24.18	21.38	23.67	26.33	29.17	
Means	4.84	4.28	4.73	5.27	5.83	4.99

	Row				
	I	II	III	IV	V
Totals	18.85	26.34	26.05	23.44	30.05

Analysis of variance

Variation due to	Sum of squares	F.D.	Mean square	F
Total	35.23890	22		
Columns	6.63307	4	1.65826	
Rows	13.72843	4	3.43210	4.32*
Treatments	6.93799	4	1.73444	
Error	7.93890	10	0.79389	

S.E. = 0.891, C. of V. = $\frac{0.891}{4.99} \cdot 100 = 17.9\%$

* = $P < 0.05$

5. Soil "B", nodules - dry matter - milligrams per pot

	Treatment					
	A	B	C	D	E	MEAN
Totals	805.2	524.4	1020.9	1271.6	1354.4	
Means	161.0	104.9	204.2	254.3	270.9	199.1

	Row				
	I	II	III	IV	V
Totals	326.9	1127.7	1392.5	840.8	1378.6

Analysis of variance

Variation due to	Sum of squares	D.F.	Mean square	F
Total	415,032.040	22		
Columns	32,409.096	4	8,102.27	
Rows	184,251.460	4	46,062.87	4.36*
Treatments	92,766.936	4	23,191.73	
Error	105,604.548	10	10,560.45	

S.E. = 102.76, C. of V. = $\frac{102.76}{199.1} \cdot 100 = 51.6\%$

* = $P < 0.05$

Experiment 6. Statistical analysis

Soil "B", nodules - dry matter - milligrams per pot

Block	Treatment 1	2	3	4	5	Total
I	0	4.8	4.6	1.4	0	10.8
II	0	3.5	2.7	1.5	0	7.7
Total	0	8.3	7.3	2.9	0	18.5

Analysis of variance

Variation due to	Sum of squares	D.F.	Mean square	F
Total	33.725	9		
Blocks	0.961	1	0.9610	2.27
Treatments	31.070	4	7.7675	18.34**
Error	1.694	4	0.4235	

S.E. = 0.651, C. of V. = $\frac{0.651}{1.85} \cdot 100 = 35\%$

** = P < 0.01