PN-ABD-380
62910

USAID # 3.A49 : NARESA # RG/USAID/02 - FINAL REPORT

IMPROVING N FIXATION IN GRAIN LEGUMES BY SELECTED STRAINS OF RHIZOBIA

BY

PROF. J.M.R.S.BANDARA.

B.Sc.Agric.(Cey.); Ph.D.(London); D.I.C.(London).

Department of Agricultural Biology, Faculty of Agriculture
University of Peradeniya, Peradeniya, Sri Lanka.

Rec'd in SCI APR 28 1989

C O N T E N T S

1.0 Introduction

2.0 Methodology

2.1 collection of rhizobial strains 4

2.2 authentication of rhizobial isolates 5

2.3 purification of rhizobial isolates 6

2.4 characteristics 8

2.5 evaluation of isolates for N fixation 10

2.5.1 green house experiments 10

2.5.2 Leonard jar experiments 15

2.6 evaluation of rhizobial strains under field conditions 17

2.6.1 field layout 17

2.6.2 soil tests 18

2.6.3 field preparation 21

2.6.4 inoculation of seeds 22

2.6.5 observations 24

2.7 survival of rhizobia in soil 25

2.7.1 soil core tests 25

2.7.2 agglutination test 27

2.8 competitive ability of rhizobia for nodulation 30

3.0 Results and Discussion 32

3.1 collection of root nodules 32

3.2 isolation of rhizobia 37

3.3 authentication of rhizobial isolates 38

3.4 purity of isolates 38

3.4.1 gram stain 40

3.4.2 keto lactose test 40

3.4.3 growth on GPA 40

3.5 characteristics of rhizobia 41

3.5.1 growth 41

3.5.2 acid/alkaline production 43

3.5.3 antibiotic sensitivity 44

3.6 evaluation of rhizobia 44

3.7 evaluation of rhizobia under field conditions 59

3.7.1 population of rhizobia in field soil 59

3.7.2 population in inoculated seeds 59

3.7.3 effect of P on N fixation 62

a. response after 20 DAS at MI. 62

b. response after 40 DAS at MI. 84

c. response after 60 DAS at MI. 110

d. grain yield 120

3.8 competitive ability of rhizobia for nodulation 125

3.9 survival of rhizobia in the field 131

3.10 evaluation of rhizobia under wetzone field conditions 133

references 145

acknowledgement 148

SUMMARY

Nodule samples from mung bean and cowpea were collected from various locations in the main grain legume growing districts of Sri Lanka. Isolates of rhizobia obtained from these nodules were purified and authenticated using their ability to nodulate *Macroptilium atropurpureum.*

Eight of the isolates collected in this study, two other isolates previously collected from Girandurukotte in Badulla district *viz:* MC 6 and YC 6 were evaluated for N fixing ability on cowpea and mung bean. NifTAL strain TAL 209 was used as the standard check treatment.

Results of the initial evaluation using pot tests and Leonard jar experiments indicated that strains YC 6, MC6 and CP 30 were superior strains and hence used in field evaluation under both dry and wetzone conditions.

Population of rhizobia in soil at Maha Illuppallama was estimated as 230 cells/g with CL 6.6 - 80.3 x 100 at 95% level using most probable number method.

Effect of different levels of soil added P as 0,60 and 180 kg/ha and interaction with different strains on N fixation was also tested.

Isolate MC 6 showed high N fixation ability in mung bean under dry zone conditions at 0 and 60 kg of P /ha. However cowpea when inoculated with Sri Lankan strains did not performed better than TAL 209 inoculated plants under dryzone conditions.

When soil is not enriched with P strains CP 30 and MC 6 increased the yield of mungbean over TAL 209. The trend of higher grain yield in CP 30 and MC 6 over TAL 209 was seen at other levels of P but was significantly higher only in MC 6 at 180 kg/ha.

performance of YC 6 and CP 30 was inferior to TAL 209 at average P levels (60kg/ha) in cowpea. However strain YC 6 produced very high yield 2778 kg per hectare in comparison to 1773kg per ha by TAL 209 inoculated cowpea at 180 kg of P per ha.

Nodulating capability of all test stains were similar. Survival ability of introduced rhizobia over the cropless (inter-season period) period was highest in YC 6. Both MC 6 and CP 30 were poor survivors. The survival ability of TAL 209 was poor and its occupancy in nodules was reduced by 66.5% and 55.98% in mungbean and cowpea nodules respectively in the interim cropless period

between two seasons.

1. Introduction

The main objective was to study the effectiveness of selected indigenous strains of rhizobia on biological nitrogen fixation in two widely used grain legumes namely *Vigna unguiculata* (cowpea) and *Vigna radiata* (mung bean) in Sri Lanka.

Nitrogen fixing ability of the rhizobial strains is one of the main biological parameters that influence BNF. Therefore the main aim of the project was to select and evaluate highly effective strains to improve nitrogen fixation capacity in both cowpea and mung bean. It was also postulated that the effective strains may not be wide spread among the native population of rhizobia and as such survival ability of selected strains are of paramount importance in evaluating different strains.

It was postulated that the highly effective rhizobial strains alone will not fix nitrogen at its maximum capacity unless other factors that influence the crop growth are sufficiently fulfilled. Among these the nutrient status of the soil are of greater importance and it is well within the management of the farmers. Therefore the influence of one of the major limiting nutrient PHOSPHORUS was also studied.

2.METHODOLOGY

2.1 collection of rhizobial strains.

Samples of root nodules were collected from uninoculated field crops of mung bean (*Vigna radiata*) and cowpea (*Vigna unguiculata*) grown during the Yala season from various regions of Sri Lanka. (Table 3.1 and Table 3.2). Host plants were carefully removed from soil and the following observations were recorded at the time of collection of nodules.

i. Date of collection

ii. Location of field

iii. Species and variety of the host

iv. Growth stage of the host

v. Degree of nodulation

vi. Size and shape of nodules.

Samples collected were placed in (155 x 30)mm sample tubes containing moisten cotton wool. Tubes were loosely capped and placed in containers to avoid desiccation during transportation from distant field plots. samples collected were stored at 4C at Department of Agric. Biology laboratory until isolations were done.Cultures isolated from the nodules were maintained on Yeast Manitol Agar with regular subculturing until authentication of Rhizobial strains were completed.

2.2. authentication of rhizobial isolates.

Authentication of rhizobial strains were done by direct inoculation of Siratro (*Macroptilium atropurpureum*) grown on nitrogen free nutrient medium. In addition to the rhizobium isolates collected (Tables 3.1 and 3.2) two NifTAL strains viz. TAL 173 and TAL 209 of cowpea group rhizobia were used along with four rhizobial strains collected from Mahaweli c area Girandurukotte and tested at the Soybean research unit at Gannoruwa.

Healthy siratro seeds of uniform size were scarified by immersing in conc. sulphuric acid for 20 min., rinsed in eight changes of sterile distilled water before soaking in sterile distilled water for one hour. Seeds were then dried under aseptic conditions under a stream of sterile air in a laminar flow cabinet.

Germinated siratro seeds were then transferred to nutrient agar slants previously streaked with rhizobial strains under test. A single seed per test tube were placed at the upper edge of the slant. Each strain was replicated five times. Five slants were maintained as uninoculated control. Slants were maintained under 12h illumination with daylight bulbs and an incandescent bulb in a complete randomized pattern at room temperature. Plants were irrigated with sterile Brockwells nutrient medium (Gibson,1980).

The root system of siratro seedlings were examined under dissecting microscope for nodulation at seven day intervals. The absence or presence of nodules and the number of nodules when present were recorded (table 3.5).The experiment was terminated on the 42nd day after planting of seedlings.

The isolates which nodulate siratro seedlings were designated as authenticated Rhizobium species.
The authenticated isolates were considered different strains of rhizobia until specific identification tests were done. Rhizobia reisolated from siratro nodules were preserved at 4C.

2.3. purification of rhizobium isolates

Authenticated strains of rhizobia were purified to ensure that the isolates were free of contamination mainly of *Agrobacterium spp.* using the following test.

a. Gram stain to ensure that the cultures are gram negative.

b. Keto Lactose test (Bearnaerts and Delay, 1963). Agrobacterium contaminations were identified from Rhizobial cultures with response to keto lactose test as follows. Two day old Rhizobial cultures grown in sterile medium containing 1% lactose, 0.1% yeast extract and 2% agar were flooded with a shallow layer of Benedicts reagent was incubated for one hour at 25 C. The plates were then observed for a yellow ring around the cell mass that will be produced by *Agrobacterium spp.*

c. Response to culturing in Glucose peptone agar (Vincent,1970) was assessed in order to eliminate other bacterial contamination. Isolates were grown in a medium containing 0.5% glucose, 1.0% peptone,1.5 % agar supplemented with alcohol solution of Bromocresol purple. Plates were incubated at 30C and growth rate and change in pH were recorded.

2.4. characteristics of the rhizobial isolates.

a. Growth Characteristic

Growth characteristics of the isolates were studied on YMA medium containing congo red and rose bengal. Parameters recorded were:

Number of days from inoculation to appear visible colonies.

Colour, turbidity of the colonies

Transparency and gum production.

b. Acid/Alkaline production.

Yeast manitol agar medium containing bromothymol blue (5 ml of 0.5% alcoholic solution per litre) were inoculated with the test cultures. Colour change in the medium was recorded after one week of incubation at 26C.

c. Antibiotic Sensitivity

The sensitivity of eight authenticated isolates of rhizobia and the two NifTAL strains were tested with eight antibiotics at three different concentrations.

A multiple inoculator (Josey *et al.*,1979) was used to inoculate the plates containing different antibiotics as follows. The rhizobial strains used were TAL 173, TAL 209, YC6 R2-1, YC 1 R3-3, MC6 R2-2, CP8, CP 25, CP 30 GG6 and GG 17.The multiple inoculator was sterilized by autoclaving at 15 psi, 121 C for 30 min. prior to adding the YM broth containing rhizobial strains to the wells of the inoculator. The prongs of the inoculator was sterilized before every transfer by heating the prongs to red hot with a bunsen flame.

Inoculation was done by dipping the prongs to the matching wells and then touching the plates. the plates were were incubated at 26C for ten days and the growth of the isolates in different antibiotics were recorded as

0 no growth

1 weak growth

2 Medium growth

3 Good growth

2.5.evaluation of rhizobial isolates for nitrogen fixation

2.5.1. Green House Experiments

The effectiveness of the authenticated isolates of Rhizobia were tested by seed inoculation of cowpea (*Vigna unguiculata*) cv. MI 35 and mung bean (*Vigna radiata*) cv.MI 5.

a.PREPARATION OF THE PLANTING MEDIUM.

River sand sieved with a 2mm mesh was used as the substrate for plant growth.. The nutrients adhered to sand particles were removed by saturating the sand with 3% HCl solution by placing sand for seven days with regular stirring in plastic buckets. Acid washed sand was then leached with tap water until the leachate is free of acid and finally with demineralized water. The sand was then transferred to ploypropylene bags and autoclaved at 15 psi, 121 C for two hours before transferring to 7"x7" plastic pots previously surface sterilized with 95% ethanol.

b. PREGERMINATION OF SEEDS AND SOWING

cowpea and mung bean seeds of uniform size were selected for tests. Seeds were surface sterilized by immersing in 3% NaOCl solution for 10 minutes followed by rinsing in eight changes of sterile distilled water. Seeds were finally soaked i sterile distilled water for one hour and transferred aseptically to petriplates containing moistened sterile filter paper. Seeds were then incubated at 27± 1C. Germinated seeds were then placed in sterile sand medium in pots with the help of a sterile forceps about one cm below the surface and they were slightly covered with sand. Pots were then kept in the green house maintained at 27±2 C.

c. INOCULATION OF POTS.

The authenticated isolates of rhizobia were grown on YM broth in 250 ml conical flasks. The inoculated broth were incubated at 25C. The broth cultures were shaken for two hours at three day intervals. Ten days after inoculation of cultures the aliquots were pipetted uniformly onto the seedling roots, when the plants are three days old. Each pot received ten ml of the broth culture. each treatment was replicated ten times and placed in the green house in a completely randomized design.

Five days after sowing seedlings were thinned down so as to leave three plants per pot. pots were irrigated daily with sterilized solution of Bergersens nitrogen free nutrient medium (Gibson, 1980).

d. OBSERVATIONS

On the 42nd day plants were carefully dug up from the rooting medium, roots were washed and the following observations were recorded.

i. Number of nodules
ii. Dry weights of roots and shoot.
iii. Total nitrogen content.
iv. Nitrogenase activity of the nodules.

Dry Weight of the Plant Samples

Dry weight of the plant samples were measured after drying at 65 C for 24 hours.

Total Nitrogen Percentage of Plants

Kjeldahl method was used to determine the total nitrogen content. Two grams of oven dried, ground plant materials were digested by adding five ml of conc. sulphuric acid and a small amount of metallic selenium in digestion tubes (30x2.5 cm) for 45 min. at 320-330 C. After cooling each tube was added with 25 ml distilled water.

Recovery and estimation of Ammonia.

Kjeldtec Auto analyzer was used for the distillation and estimation of ammonia. The releasing ammonia was trapped in boric acid solution and titrated with 0.1N HCl.

Nitrogenase activity of Nodules

Acetylene reduction assay was used to estimate the nitrogenase activity of nodules. Root system with intact nodules of test plants were carefully washed with water to remove soil debris and placed in 100ml conical flask with gas tight caps.

A volume of air equals to 10% of each flask was removed with an injection syringe by piercing caps and equal volume of acetylene (1000 ppm) was injected in. Gas samples withdrawn from flasks after one and two hours of incubation were stored in 5ml vaccutainers for analysis. Ethylene produced by the activity of the nitrogenase enzyme in root nodules were estimated by gas chromatography using a "Varian 3300" gas chromatograph with a poropak N (1.5m x 1/8", 80/100 mesh) column and a flame ionization detector under the following conditions.

Column temperature	- 60C
Injector temperature	- 85C
Detector temperature	- 85C
Nitrogen pressure	- 5.5 bars
Hydrogen pressure	- 2.5 bars
Air pressure	- 4.0 bars
Instrument pressure under above conditions	- 125 - 130 kpa
Retention time	- 3.5 minutes

2.5.2. Leonard Jar Experiments

The effectiveness of the authenticated isolates along with the the 2 Nif TAL strains were also tested by Leonard Jar Assembly method. Both hosts namely cowpea and mungbean were employed in these tests.

Leonard jar assembly (Leonard,1944) were prepared using transparent clear glass coconut arrack bottles (750 ml) collected from market place. Empty tin cans (10 x 12 cm - Nestomalt) lined with polypropylene were used as the lower vessel of the assembly which carried the nutrient solution. Bottomless glass bottles were placed inverted on the lower vessel.Bottles were filled with acid washed river sand (sieved with 2mm mesh) through which a 30 cm absorbent wick was passed through to the bottom vessel containing nutrients to facilitate nutrient uptake by the substrate.. The whole assembly wad then autoclaved at 15 psi for 2 hours.

Three pregerminated seeds were placed in the rooting medium in Leonard jar assembly at a depth of one cm from the surface, with the help of a sterile forceps.Three day old seedlings were inoculated with ten old broth culture as 10 ml of bacterial suspension per each assembly.Leonard Jars were then kept in an illuminator rack fixed with day light bulbs and a incandescent bulb with a 12 h illumination cycle.

Dry weight,total nitrogen content and nitrogenase activity of plant samples inoculated with different strains of rhizobia were recorded as described above.

2.6 evaluation of rhizobial strains under field conditions

Different strains of rhizobia selected by pot experiments and by the Leonard Jar tests were subjected to field evaluation for their ability to fix nitrogen under field conditions.

Field evaluations were done at two sites namely

i. *Dry zone* location - at MahaIlluppallama in the Mahaweli H area in the district of Anuradhapura.

ii. *Wet zone* location - Peradeniya in the upcountry in Kandy district.

Field experiments at Maha Illuppallama was conducted during Oct- Dec (*Maha*), 1986 and at Meewatura, Peradeniya during *Yala* season in 1987.

2.6.1 Field layout

Field trial was conducted in a randomized complete block design (RCBD) as 3 x 3 factorial with 3 levels of super phosphate and three strains of rhizobia.

The three levels of super phosphate used were

P1 = 0 kg/h

P2 = 60 kg/h

P3 = 180 kg/h

The three strains of rhizobia used were with Cow pea:

S1 - TAL 209

S2 - CP 30

S3 - YC6-R21

with mung bean:

S1 - TAL 209

S2 - CP 30

S3 - MC 6 -R22

2.6.2 Soil test

i. Soil sampling

Five sub-samples of soil from each plot in a randomized manner from 8 - 10 cm depth using a heat sterilized soil auger. Samples were collected in polythene bags and stored at 4C until tests were done.

sub-samples were air dried and mixed thoroughly in order to obtain a composite sample.Resultant soil sample was sieved with 0.2mm mesh and tested for moisture content, pH an native rhizobial population.

ii. Sol moisture content

Moisture content was estimated by difference in weight of air dried samples after oven drying soil at 105C for 24 h.

iii. soil pH

The pH of soil suspension made by ten grams of soil suspended in 25 ml of 1N KCl for half an hour was estimated using a pH meter.

iv. Soil texture

Soil texture was determined by the hydrometer method using sodium hexameta phosphate as the dispersing agent.

v. Organic matter

Organic carbon content was estimated by Walkey and Black (1934) method. Total carbon values were multiplied by factor 1.732 in the computation of organic matter in samples.

vi. Cation exchange capacity

Soil samples were saturated with 1.0 M sodium acetate and adsorbed sodium ions were extracted by treating the soil samples with ammonium acetate of pH 7.0. Sodium concentration of the lechate was

measured using an atomic absorption spectrophotometer (Chapmann , 1965).

vii. Total nitrogen content

The method used for the determination of total nitrogen content was described in 2.5.1.d above.

viii. Native population of Rhizobia

Plant infection tests conducted in Leonard jars planted with either cowpea or mungbean were used to assessed the native population. Ten grams of sieved soil was suspended in sterile distilled water in a sterilized volumetric flask (250 ml). the suspension was then shaken at 200 - 300 rpm for 10 minutes on a mechanical shaker. This suspension was then used to prepare a series of dilutions varying from 1 in 100 to 1 in 10 million.

Tests were done in triplicate with six dilution levels of soil. Each jar was inoculated with one ml of soil dilution and kept under a 12 h illumination cycle as described above. Three weeks after planting the presence or absence of nodules were observed.

2.6.3 Field preparation

Total weedicide Gramoxone was applied at the rate of 450 l/ha, four days prior to ploughing the field. While preparing 6" raised beds *weedicide "round up"* was applied to control *Panicum repens* . Four weeks after spraying gramoxone plots were fertilized with urea (46%N) and Muriate of Potash (60% K2O). Nitrogen and Potassium rates added were 40 an 80 kg/ha respectively.

As no gradient existed for the available nutrients topography and shade were the main factors considered in the layout of the field plots.The experiment was laid in 3 x 3 factorial randomized block design with 4 replicates. Beds were separated with 800 cm wide drains and each block was consisted of nine plots. Blocks were separated from each other by two meter wide drains. Plot size used for cowpea was 3 x 1 m while that is used for mung bean was 3.4 x 1m. The inoculated seeds were planted at the rate of 3 seeds/hill at a spacing of 15 x 30 cm for cowpea and 10 x 30 for mung bean. Seedlings were thinned down to obtain 2 plants/hill. Plots were irrigated when needed.

2.6.4 Inoculation of seeds.

i Preparation of inoculant

Both cowpea and mung bean seeds were inoculated with 3 strains of rhizobia as single strain peat inoculant. Four isolates of rhizobia used to prepare the peat inoculant were TAL 209, CP 30, YC 6-r2-1 and MC6-r2-2. Individual strains were grown in 100 ml of yeast manitol broth in 250 ml conical flasks incubated at 26 C . Cultures were shaken at 100 - 150 rpm every three days for 2 hours.

Peat obtained from Hortonplains, Nuwaraeliya was sundried, ground and sieved with 0.05 mm sieve was mixed with filter mud (less than 0.2mm) at a ratio 3:1 (peat:filtermud). Mixture was then autoclaved at 121 C and 15 psi for 2h in polypropylene bags. Bags were sealed with adhesive tapes. Peat medium at room temperature was inoculated with rhizobial strain as 15 ml of rhizobium broth culture/20 g of peat medium using sterile syringes. Inoculated peat bags were then incubated at 26 C for ten days for the multiplication of rhizobia. Inoculant bags were then stored at 4C in the refrigerator until they are used for seed inoculation.

ii. Inoculation of seeds

Cowpea and mung bean seeds were surface sterilized prior to inoculation by dipping the seeds with 3% NaOCl for 10 min. and rinsing several times with sterile distilled water. Seeds were then inoculated with single strain inoculant as follows.

Gum arabic (40g) were dissolved gradually in 100ml of distilled water at near boiling point. To this suspension 25 g of calcium carbonate was added and stirred well. An aliquots (3.5 ml) of this solution was added to 100 g of seeds of cowpea and 30g of mungbean separately in polythene bags and mixed well until the seeds were completely coated with gum arabic. Peat inoculant was then added to seeds at coated with gum arabic.

iii. Population of rhizobia on inoculated seeds

The rhizobial population on inoculated seeds were estimated by plate enumeration method.

Ten seeds of each of cowpea and mungbean were taken randomly from the inoculated seeds and were added to 100 ml of sterile distilled water in 250 ml conical flasks. The flasks were shaken vigorously in order to disperse the inoculant attached to seeds into

water.While shaking 10ml of the suspension was pipetted into 90 ml of sterile distilled water and the resultant solution was further diluted to obtain a series of dilutions from 1 in hundred to of 1:10 million.

Aliquots (0.1 ml) of each dilution was pipetted on to solidified yeast manitol agar plates and evenly spreaded on the surface of agar using a sterile bent glass rod. Plates were then incubated at 26C for 6-7 days before counting the number of colonies.

2.6.5 Observations

Plant samples were taken at 20,40 and 60 days after planting by uprooting 4 plants randomly selected from each plot.These samples were used to assess the following

i. Number of nodules per plant
ii. Dry weight of roots and shoots per plant
iii. Total nitrogen percentage of the plant
iv. Nitrogenase activity of the root systems 40 days after planting.
v. Grain yield.

2.7 survival of rhizobia in soil

The ability of the inoculated strains of rhizobia to survive in soil in the absence of the host plant at the experimental site at Maha Illuppallama was tested using soil cores obtained from the field after harvest.

Soil cores collected in empty tin cans (7.5 x 11.0 cm) were used as the planting medium for test plants and rhizobial strains isolated from nodules were identified by serological methods.

2.7.1 Soil core tests

Soil cores were taken from experimental plots that were not treated with phosphorus fertilizer , after harvesting was done, in pre-sterilized tin cans.Plants in these plots were detopped at the color region leaving the root system with nodules in the field. Soil cores were taken again from these plots at the beginning of the following season. Soil cores were assembled as shown in fig. by fitting the core into a plastic bottle to which the drain water was collected.

a. SOWING OF SEEDS

The soil cores taken from plots with cowpea and mung bean were planted with the same cultivar as in the previous season. Surface sterilized pre-germinated seeds were planted at a rate of 2 seeds per core at a depth of 1cm and the seeds were slightly covered with soil. Plants were grown in the green house and irrigated with sterile distilled water when necessary.

b. PRESERVATION OF NODULES FOR AGGLUTINATION

Nodules were harvested from the plants grown in soil cores when the plants were 42 days old.Nodules were washed and wiped with blotting paper to remove any moisture adhered to nodules before packing in small polythene bags. Bags were sealed and immersed in gently boiling water to inactivate enzymes. After cooling the bags were stored at 4C.

2.7.2 Agglutination test

a. ANTIGEN

Four strains TAL 209,CP30, MC6 and YC6 were purified by plating on Congo Red Yeast Manitol Agar (CRYMA). Purified cultures were inoculated on 20 ml YMA flats in 125 ml culture bottles with screw caps and incubated at 26 C. Cultures were stored at 4C after checking the purity of these cultures on CRYMA for the use in preparation of antiserum.

b. PREPARATION OF ANTISERUM

Each culture bottle was added with 10 ml of sterile saline solution and 20 sterile glass beads. Bottles were then tilted back and forth to dislodge the cells of rhizobia into the saline solution. The suspension of rhizobia were transferred into sterile McCarteney bottles. The antigen concentration was adjusted to the equivalent of tube number 4 of Mc.Farlands barium sulphate standard (Lennette, Spaulding and Truant,1974) The antigens were prepared on the same day as when the injections were done.

Following injection schedule was used for the preparation of antiserum using six to 12 months old 1.5 - 2.0 kg body

weight rabbits.

Antigen injection schedule

day		procedure
1	injection	0.5ml intra muscular
2	injection	1.0 ml IM
3	injection	1.5 ml IM
12	injection	1.5 ml IM
13	injection	2.0 ml IM
14	injection	2.0 ml IM and 2ml SubCut.
21	Test bleeding and titre determination	
23	Ear bleeding and serum collection	
	booster injection	2.0 ml SC
30	Ear bleeding and serum collection	
	booster injection	2.0 ml Sc
37	Ear bleeding and serum collection	
	booster injection	2.0 ml SC
42	Coordinate bleeding and serum collection	

Sterile disposable syringes were used throughout the preparation of antiserum. When injecting rabbits were immobilized by rolling the animal tightly with a clean towel. Intra muscular injection were made to the left or right thigh muscle.

Test bleeding of rabbits were done as follows. Small area along the marginal ear vein was shaved and cleaned with a cotton swab dipped in 70% ethyl alcohol . About 1 - 2 ml of blood was collected from the marginal vein with the help of a pointed sterile blade. Bleeding was stopped

by applying light pressure with the thumb on to the wound made by the scalpel.

Blood sample was allowed to clot at room temperature for 2h and serum separated was used to test the agglutination titre.

During process of ear bleeding blood volume collected was around 10 - 15 ml. Blood clotting at the time of bleeding was prevented by the application of xylene on to the wound. At the end of bleeding the injury was covered with a drop of vaseline.Blood samples were collected in sterile vaccutainers were allowed to clot before centrifuging at 3000 - 5000 rpm for 15 minutes for the separation of serum from red blood cells.

Serum collected in sterile capped vials were stored under refrigeration.

c. AGGLUTINATION TEST

The agglutination tray method (Somasegaren and Hoben,1985) was used for the identification of rhizobial strains in the nodules. Polythene bags with frozen nodules were cut opened and each nodule was placed in a well on a polyvinyl chloride agglutination tray with 60 wells commencing at well A1 to A10 . To each well containing a nodule was

added with six drops (0.03 ml) of saline solution (0.8% NaCl) using sterilized pasture pipettes.

Nodules placed in " A" wells were crushed in saline solution by pressing with a sterile glass rod. Remaining nodule tissue was then pushed out against the wall of the wells. Three drops each of these antigen solutions were then pipetted into respective "B" wells to which 0.03 ml of antiserum of a test strain was added and mixed well before covering with cellophane tapes to prevent evaporation during incubation. Trays were incubated at 36C for 2 hours before observation for agglutination.

2.8 competitive ability of rhizobia for nodulation

a. SOIL CORE TEST

Soil cores collected prior to land preparation at maha Illuppallam site were used in this study. Soil cores were collected in pre-sterilized empty cans as described above.

b. TREATMENTS

Three sets of soil cores were heat treated to kill native rhizobia in order to estimate the nodulation ability of introduced rhizobia in the absence of native strains. Temperature at the centre of soil cores were maintained at 55 C for one hour by placing them in an oven after moistening the cores.

Strains of rhizobia used are the same as in the field experiments. Three soil cores were kept unheated and uninoculated as check treatments to determine the presence of rhizobia in the field prior to inoculation.

Following treatments were used in the study.

1. Natural soil core inoculated with S1- TAL 209
2. Heat treated Inoculated with S1- TAL 209
3. Natural soil Inoculated with S2- CP 30
4. Heat treated Inoculated with S2- CP 30
5. Natural soil Inoculated with S3-YC6 or MC6
6. Heat treated Inoculated with S3-YC6 or MC6
7. Natural soil, uninoculated

The soil cores were assembled as describe earlier. Seeds were sown three days after the heat treatment at the rate of two seeds per core. The cultivars used were the same as in the field tests. Seeds were pre-sterilized and pre-germinated as described in 3.5.1. The cores were then kept in the green house and irrigated with sterile distilled water when necessary.

Three days after planting soil cores were inoculated with rhizobial strains. Each strain was grown separately in YMA broth. the concentrations of broth cultures were adjusted to a similar value by diluting with uninoculated broth. Each core was inoculated with 10 ml of the broth culture.

3.0 RESULTS AND DISCUSSION

3.1 collection of root nodules

Nodule samples were collected from various locations in the main legume growing districts namely *Anuradhapura, Mahanuwara (Kandy)* and *Matale*. Sample sites are listed in table 3.1. and 3.2 Forty four cowpea and eighteen mung bean nodule samples were collected. Samples were collected from plants at or prior to flowering. It was observed that the nodules samples obtained at Kundesale did not bear functioning nodules. Nodule count per plant were not taken into account in the collection phase. However the nodule size, shape and their distribution on the root system were recorded from those plants where nodules were collected (Table 3.3 and 3.4)

Most nodules were found on lateral roots although few nodules borne on tap root were also observed in both crops.Nodule size varied from 4-7 mm (medium) to less than 4 mm (small) in diameter. Nodules in general were round in shape some were borne in clusters. Cowpea plants with larger irregular shaped nodules were also observed. These nodules were well attached to roots.

Table 3.1 Location of sampling sites of cowpea nodules.

Sample Number	Month-85	Place	District	Zone
CP1 CP2 CP3	Aug.	Dodangolla	Kandy	wet
CP4	Aug.	Kundasale	Kandy	dry
CP5 to CP13	Aug.	Kolabissa	Kandy	wet
CP14 CP15	Sep.	Thotagamuwa	Matale	wet
CP16 CP17	Sep.	Thanne	Matale	wet
CP18 CP19	Sep.	Kumbiyangoda	Matale	wet
CP20	Sep.	Galwehera	Matale	dry
CP21 CP22	Sep.	Nalanda	Matale	dry
CP23 - CP29	Sep.	Galawadukumbura	Matale	dry
CP30 - CP32	Sep.	Nalanda	Matale	dry
CP33	Sep.	Bulagala	Matale	dry
CP34 CP35	Sep.	Madatugama	A'pura	dry
CP36	Sep.	Gonapathirawa	A'pura	dry
CP37 - CP41	Sep.	M'Illuppallama	A'pura	dry
CP42 CP43	Sep.	Galmaduwa	A'pura	dry
CP44	Sep.	Gelioya	Kandy	wet

Table 3.2 Location of sampling sites of mungbean nodules

Sample Number	Month 85	Place	District	Zone
GG1	August	Kundasale	Kandy	dry
GG2 & GG3	August	Kolabissa	Kandy	wet
GG4 GG5 & GG6	Septem	Thotagamuwa	Matale	wet
GG7	Septem	Dambulla	Matale	dry
GG8 GG9 & GG10	Septem	Galawadukumbura	Matale	dry
GG11 & GG12	Septem	Nalanda	Matale	dry
GG13 & GG14	Septem	Eppawala	A'pura	dry
GG15	Septem	Ipalagama	A'pura	dry
GG16 & GG17	Septem	Gonapathirawa	A'pura	dry
GG18	Septem	Madatugama	A'pura	dry

Table 3.3 Nodules collected from cowpea plants.

Sample number	Cultivar	Stage of host	nodule distrbn.	nodule size	nodule shape
CP1	MI 35	PR	LR	M	R
CP2	Arlinton	PR	LR	S	R
CP3	MI 35	PR	M&LR	L	I
CP4	MI 35	PR	LR	S	R
CP5,6,10,11,14	Arlinton	PF	LR	M	R
CP16,29,33	Arlinton	PF	LR	M	R
CP17,22,30,32	Bushita	PF	LR	M	R
CP38,42,43	Bushita	PF	LR	M	R
CP18,39,44	Hawari me'	PF	LR	M	R
CP35,25	Polon me'	PF	LR	M	R
CP36,40	MI35	PF	LR	M	R
CP7,9,15,34	Arlinton	PF	LR	S	R
CP19	Hawari me'	PF	LR	S	R
CP28,37	Bushita	PF	LR	S	R
CP41	Eta	PF	LR	S	R
CP8	Arlinton	PF	LR	L	IA
CP12	Arlinton	SS	LR	S	R
CP13	Arlinton	PF	MR	S	R
CP20	Eta	PF	MR	L	IA
CP21	Bushita	PF	LR	S	RC
CP22	Bushita	PF	LR	M	RC
CP23,23	Polon me'	PF	LR	M	RC
CP26	Bushita	PF	LR	L	R
CP31	Hawari me'	PF	M&LR	M	R

Note:

SS: Seedling stage
PF: Pod Filling stage
PR:Pod Ripening Stage

MR: on main root
LR: on laterals
M&LR: on both

S: Smaller than 4mm dia
M: Medium 4-7mm diam.
L: Large more than 7mm

Table 3.4 Nodules collected from mung bean plants

Sample number	Cultivar	Stage of host	Nodule distrbn.	Nodule size	Nodule shape
GG1	MI 2	PR	LR	S	I
GG 2	T 51	PR	M & L	M	RS
GG3	MI 1	PF	MR	L	RC
GG4	MI 35	PR	LR	S	RS
GG5 and GG6	MI 1	PR	LR	M	RS
GG7	MI 2	PR	LR	M	IA
GG8 and GG9	MI 2	PR	LR	S	RS
GG10	MI 2	SS	M & L	M	RS
GG11,GG12,GG14	MI 2	PF	LR	M	RS
GG16,GG17,GG18	MI 2	PF	LR	M	RS
GG13	MI 2	PF	LR	S	RS
GG 15	MI 2	PF	LR	L	RS

Note:
SS: Seedling Stage
PF: Pod filling Stage
PR: Pod Ripening Stage

MR: on main root
LR: on laterals

S: Smaller than 4mm d
M: Medium 4-7 mm diam

RS: Round and Single
RC: Round & in clusters
I: Irregular
IA: Irregular and well attached to roots

3.2 isolation of rhizobia

It was observed in the preliminary studies that the surface sterilent recommended by most authors, 0.1 % mercuric chloride inhibited the growth of cowpea group rhizobia. In subsequent isolations 95% ethanol was used as a surface sterilizing agent.

The bacterial colonies isolated from nodules that failed to absorb congo red pigments when grown in CRYMA were identified as rhizobia. In order to further purify the isolates these were grown in CRYMA in four successive serial transfers while discarding associated contaminants that absorb congo red at various rates .

The nodule samples CP1, CP2,CP3,CP4,CP6,CP10, CP11, CP12, CP19, CP44, GG1, GG7, GG9,GG16 AND GG18 failed to produce colonies on YMA medium. This failure may be due to the absence of viable cells of rhizobia in these nodules as the nodules were collected in the late reproductive phase of the crops.

3.3 authentication of rhizobial isolates

Authentication of isolates collected from nodules were necessary to obtained pure single strain cultures.In the authentication tests conducted with the 52 isolates collected only TEN were able to nodulate *Macroptilium atropurpureum* (siratro) seedlings (Table 3.5). Five of these were the isolates collected from cowpea and mung bean plants while three cultures were soybean isolates and two were NifTAL strains. The isolates which nodulated siratro seedlings were authenticated a Rhizobial strains. These isolates were considered as different strains of Rhizobium until systematic strain identifications were done.

3.4 purity of isolates.

Vincent (1982) stated that *Agrobacterium spp* are the most likely to be confused with fast growing rhizobia. The positive identification of Rhizobia cannot be achieved by the ability of isolates to nodulate and reisolation of the strain from the formed nodule. Vincent *et al.* (1979) therefore recommended following tests that distinguish rhizobia from nonrhizobial cultures.

Table 3.5 Number of nodules per plant on siratro seedlngs inoculated with Rhizobia in authentication tests.

Treatments	Days after Inoculation					
TAL 173	1.2	2.4	4.4	5.8	6.4	7.2
TAL 209	0.8	0.8	1.8	2.8	4	4.4
YC 6	0.6	2.2	3.6	3.6	3.6	3.8
MC 6	0.2	0.6	1.8	2.4	2.8	3.4
YC 1	0	0	0.2	1	2.4	3.6
CP 8	0	1	1.4	2.4	3.4	3.6
CP 25	0	0	0.8	1.2	2.4	2.8
CP 30	0	0	0.4	0.8	1	1.6
GG 6	0	0.2	1.4	2.4	3	3.4
GG 17	0	0	0.2	0.4	0.8	1.6

3.4.1 Gram stain

All authenticated strains were stained pink ie. gram negative and the bacteria were found to be rod shaped under oil immersion (x 100).

3.4.2 Ketolactose test

Agrobacterium radiobacter and *Agrobacterium tumefaciens* metabolize lactose and sucrose with the production of 3-ketoglucoside substances which are readily detected using Benedicts reagent (Bernaerts and de lay,1963) and the reaction is not known to occur in other organisms. Among the ten isolates tested none were able to produce a yellow ring around the cell mass growing in medium containing Benedict's reagent

3.4.3 growth on glucose peptone agar

Agrobacterium spp. grows well in in GPA with a marked change in pH while *Rhizobium spp.* show a poor growth with no change in pH (vincent,1982). All the isolates of rhizobia tested showed a poor growth on GPA in comparison to YMA. They fail to change the color Bromocrysol purple indicating that the strains tested were not contaminated with *Agrobacterium* spp.

3.5 characteristics of rhizobia

Graham (1976) showed that several growth characters of rhizobial cultures are important in the strain identification.

3.5.1 growth characteristics

Vincent (1982) reported that fast growers of rhizobium produce medium or large colonies of 1-5 mm in diameter after 3-5 days when incubated at 25 - 28C while colonies of slow growers will be barely detectable at 3-5 days and will produce colonies not exceeding 1mm even after 10 days.

The growth characteristics of authenticated strains of rhizobia studied on YMA medium added with congo red and rosebengal are summarized in table 3.6. Two NifTAL strains were found to produce visible colonies after 7 days of incubation. The isolates CP8 , CP25, CP30, GG6, YC1, and MC6 grew faster producing visible colonies in 3-4 days after inoculation. Isolates GG 17 nad YC6 showed n intermediate growth rate taking 4-5 days to appear visible colonies.

Table 3.6 Growth characteristics of Rhizobial isolates

Isolate	Growth rate!!	Colony color	Gum prodn.	Turbidity
CP 8	3	yellowish	low	translucent
CP 25	3	yellowish	medium	
CP 30	3	whitish	medium	less trans.
GG 6	3	yellowish	high	less trans.
GG 17	3	whitish	medium	translucent
YC 1	3	whitish	medium	translucent
MC 6	4	whitish	medium	translucent
YC 6	4.5	whitish	medium	translucent
TAL 173	7	whitish	medium	translucent
TAL 209	7	whitish	high	less trans.

!! number of days to appear 1mm diam colonies

The isolates CP 8 and GG 6 were yellowish milky while all others were whitish milky in color. The gum production in TAL 209 and GG6 was higher where as in in CP 8 it is relatively low. All other isolates were medium gum producers.

The turbidity of the colonies are lower in CP 30 and TAL 209 compared to all others tested.

3.5.2 acid/alkaline production

Ability to change the pH of YMA medium under well aerated conditions such as on the surface of a poured plate is a useful basis of distinction between slow and fast growers. Under these conditions fast growers characteristically drop the pH sufficiently to change bromothymol blue to yellow where as slow growers fail to change the indicator or move it in the direction of alkalinity changing it color to blue (Vincent,1982).

The isolates CP8, CP25, CP30 , GG6, YC 1 and MC 6 grew faster on YMA were acid producing. The NifTAL strains were slow growers and found to be alkaline producers. GG7 and YC 6 which showed medium growth also produced acids in YMA.

3.5.3 antibiotic sensitivity

Out of the many isolates of rhizobia collected from various locations in Sri lanka some strains could be identical or related. In order to determine their relationship antibiotic sensitivity of the isolates were investigated.The isolates with similar pattern of sensitivity were considered to be identical.

The sensitivity of test strains to antibiotics are listed in table 3.7. The growth of the isolates on different antibiotics at very low concentrations were found to be different from each other indicating that test strains are unrelated.

3.6 evaluation of rhizobia

It is recommended by most researchers that Rhizobium for legume inoculants should be tested for their effectiveness in nitrogen fixation under controlled conditions and field conditions. It is also suggested that their ability to survive under field conditions, competitiveness, pH and pesticide tolerance should be evaluated before recommending for commercial use.

Table 3.7 Antibiotic sensitivity of rhizobial isolates tested

ANTIBIOTIC		GROWTH OF COLONIES									
compound	con.mg/l	GG6	GG17	CP8	CP25	CP30	T.173	T.209	MC6	YC6	YC1
nalidixic acid	2.5	3	2	1	3	3	2	2	1	0	3
nalidixic acid	10	3	2	0	3	3	2	2	2	0	3
nalidixic acid	15	3	1	0	2	3	1	1	1	0	3
erythromycin	2.5	3	3	0	3	3	2	0	1	0	3
erythromycin	10	3	3	0	3	3	1	0	1	0	3
erythromycin	15	3	3	0	3	3	1	0	0	0	3
refampicin	0.25	3	3	2	3	3	3	2	0	2	3
refampicin	0.5	3	3	1	3	3	3	2	0	0	3
refampicin	2.5	3	3	0	3	3	3	1	0	0	3
luramycin	2.5	3	3	0	1	0	2	0	0	0	0
luramycin	10	3	3	0	0	0	2	0	0	0	0
luramycin	20	3	2	0	0	0	1	0	0	0	0
streptomycin	0.025	3	3	0	0	3	2	0	0	0	0
streptomycin	0.1	3	3	0	0	3	2	0	0	0	0
streptomycin	0.2	3	2	0	0	3	2	0	0	0	0
tetracyclin	0.2	3	2	0	1	2	2	2	0	0	2
tetracyclin	0.5	3	2	0	0	1	1	1	0	0	1
tetracyclin	2.0	3	2	0	0	0	0	0	0	0	0
neomycin	1.25	3	3	1	3	3	2	1	0	1	3
neomycin	2.5	3	2	0	3	3	1	0	0	0	3
neomycin	10	3	2	0	3	3	1	0	0	0	3
chloremphenico	5.0	3	3	3	3	3	2	2	1	2	3
chloremphenico	10	3	3	3	3	3	2	2	0	1	3
chloremphenico	20	3	2	3	3	3	2	1	0	0	3
Control		3	3	3	3	3	3	3	3	3	3

0 = no growth 1= weak growth 2 = medium growth 3 = Good growth

The effectiveness is usually measured by either direct means using total nitrogen uptake, protein and non protein content in inoculated plants or by indirect means such as nodule count, plant dry weight gain and nitrogenase activity of inoculated plants.

The direct assessment of nitrogen estimate the quantities of nitrogen itself that is found in the system. The fixation of atmospheric nitrogen by living systems leads to demonstratable net increase of nitrogen content. Bergersen (1980) reported that this increase could be detected by direct analysis of total nitrogen.

The effect of inoculation of cowpea and mungbean with the selected strains on nodulation, shoot and root dry weight, total nitrogen percentage, nitrogen uptake and nitrogenase activity observed in pot experiments are summarized in table 3.8 and 3.9 . Data were collected at the early reproductive phase of the crops ie. six weeks after planting when the efficiency of the nitrogen fixation is considered to be at maximum.

Table 3.8 Response of cowpea (*Vigna unguiculata*) to inoculation with Rhizobia in pot experiments.

Rhizobial strains	Number of nodules per plant	Shoot dry weight g	Root dry weight g
control	0.2	0.167	0.085
TAL 173	11.6	0.261	0.091
TAL 209	20.2	0.278	0.112
YC 6	19.9	0.354	0.143
MC 6	11.0	0.187	0.106
YC 1	16.5	0.205	0.087
CP 8	12.4	0.187	0.101
CP 25	10.9	0.191	0.117
CP 30	26.1	0.371	0.164
GG 6	6.8	0.172	0.107
GG 17	12.2	0.228	0.107
L.S.D	6.806	0.0023	0.0374
CV%	50.9	32.3	37.9
S.D.	3.058	0.0341	0.0188

P level used in statistical analysis was 0.05
All alues are mean of ten replicates.

Table 3.9 Effect of inoculation of cowpea with Rhizobia on nitrogen fixation.

Rhizobial strains	Total Nitrogen percentage	Nitrogen uptake *mg*	Acetylene reduction *n mol/h/plt*
Control	3.341	8.382	70.31
TAL 173	3.369	12.423	203.19
TAL 209	3.495	13.822	532.58
YC 6	3.335	16.445	327.98
MC 6	3.515	10.431	211.21
YC 1	3.570	10.374	206.14
CP 8	3.460	9.432	177.81
CP 25	3.462	10.764	178.74
CP 30	3.403	18.21	646.24
GG 6	3.50	9.368	210.69
GG 17	3.451	11.259	198.49
L.S.D	ns	2.942	22.57
CV%	7.49	29.17	14.95
S.D.	0.1328	1.4785	32.905

P level used in statistical analysis was 0.05
Acetylene reduction assay as mean of three replicates.

In the pot experiment significantly higher (p=0.05) nodulation and top dry weight were observed in all inoculated plants compared to those that were not inoculated (Fig 3.1 & 3.2). The isolate CP 30 was the highest nodulator producing 26.1 nodules/plant followed by TAL 209 and YC6 even though the dry matter production of TAL 209 was lower than that of YC 6. In Leonard Jar experiments all inoculated plants had a significantly higher nodulation than that of uninoculated control (Fig. 3.1). Nodulation was highest in CP 30 inoculated plants whereas YC 6 & TAL 209 came second and third respectively. Many isolates significantly enhanced the shoot dry matter production over uninoculated controls. Even in Leonard experiments CP 30 was the best drymatter producer followed by YC 6 and TAL 209, similar in response to pot experiments.

In pot experiments highest shoot dry matter production was observed in plants inoculated with strain CP 30. Isolate CP 25 although was a very poor nodulator showed relatively high shoot dry matter production which was significantly higher than that of TAL 209.

Fig 3.1 Nodulation of inoculated cowpea

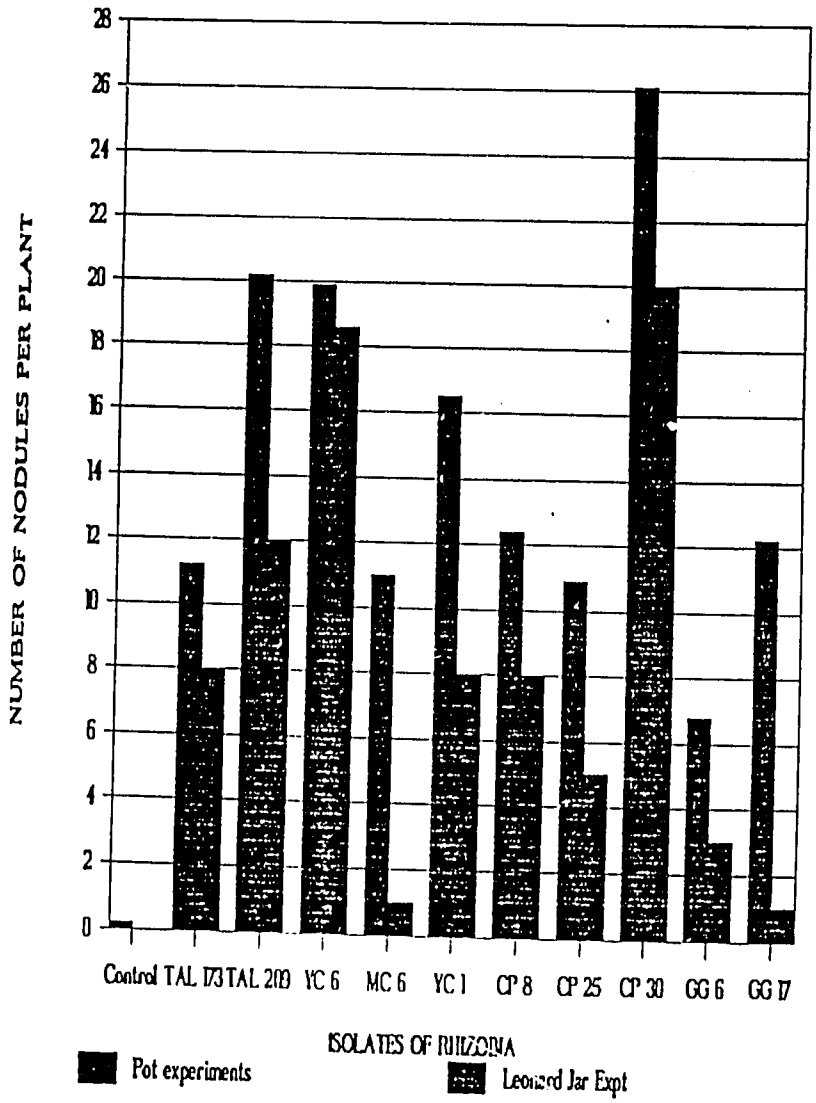
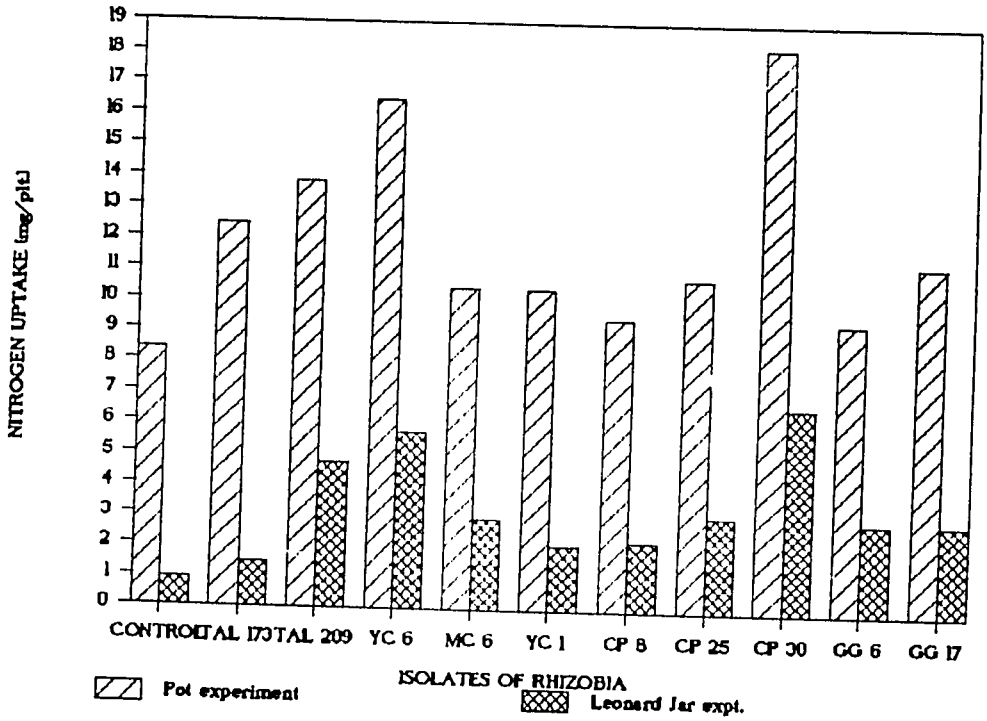


Fig 3.2 N fixation in inoculated cowpea



In both pot and Leonard jar experiments no significant difference were observed in the percentage of total nitrogen content in inoculated plants over that of uninoculated controls but the nitrogen uptake was significantly increased in inoculated plants. (Fig 3.2). In both experiments a good correlation wa observed between nitrogen uptake and shoot dry weight (table 3.9 and table 3.10). Among the strains CP 30 performed at highest efficiency in nitrogen uptake.

All the inoculated treatments showed significantly higher level of nitrogenase activity over the uninoculated controls. The highest acetylene reduction activity was observed in CP 30 inoculated plants in both Leonard jar and pot experiments (Fig.3.4).

Response of mungbean to inoculation by rhizobial strain are summarized in table 3.11 and 3.12. Significantly higher rate of nodulation was seen in plants inoculated with TAL 209 or CP 30 or MC 6. Although these differences varied with respect to other parameters in nitrogen uptake ad acetylene reduction assay strains CP30, TAL 209 and MC 6 were significantly superior in performance. Strain CP 30 emerged as the best strain for mungbean.

Results of the pot and Leonard jar experiments were essentially similar. Accordingly the isolates CP 30, YC 6

Table 3.10 Effect of inoculation of cowpea with Rhizobia in Leonard Jar experiments.

Strains	Nodule #/plt	Dry weight g Shoot	Dry weight g Root	N % Total	N mg Uptake	Acetylene reduction
Control	0.0	0.035	0.007	2.92	0.93	38.6
TAL 173	8.0	0.036	0.04	3.24	1.46	86.46
TAL 209	12.0	0.094	0.04	3.56	4.75	316.95
YC 6	18.6	0.132	0.031	3.53	5.74	363.24
MC 6	1.0	0.074	0.115	3.24	2.96	134.90
YC 1	8.0	0.047	0.067	3.32	2.10	117.97
CP 8	8.0	0.046	0.223	3.30	2.29	131.09
CP 25	5.0	0.074	0.021	3.26	3.09	205.16
CP 30	20.0	0.144	0.035	3.71	6.63	506.02
GG 6	3.0	0.071	0.02	3.21	2.94	172.89
GG 17	1.0	0.074	0.019	3.16	2.97	82.25
L.S.D	0.662	0.003	ns	ns	0.260	19.83
CV%	28.06	15.8	10.05	3.8	14.26	18.07
SD	1.763	0.009	0.031	0.218	0.379	28.92

P level used was 0.05

* Acetylene reduction assay recorded in n moles/h/plant as a mean of three replicates.

Fig 3.3 Growth of inoculated cowpea.

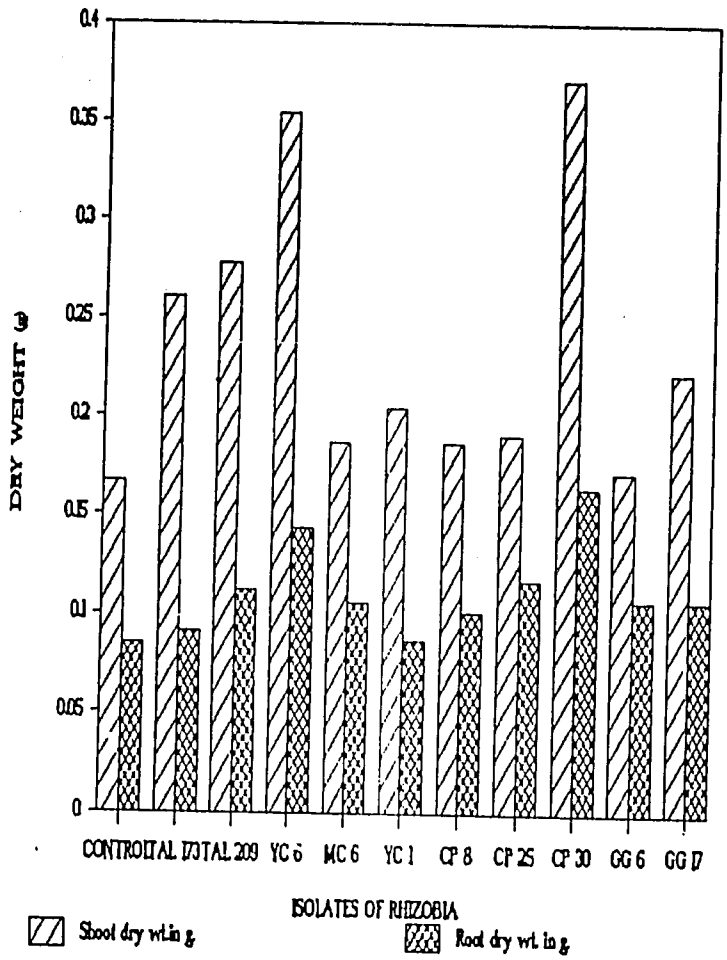


Fig 3.4 Nitrogenase activity in cowpea

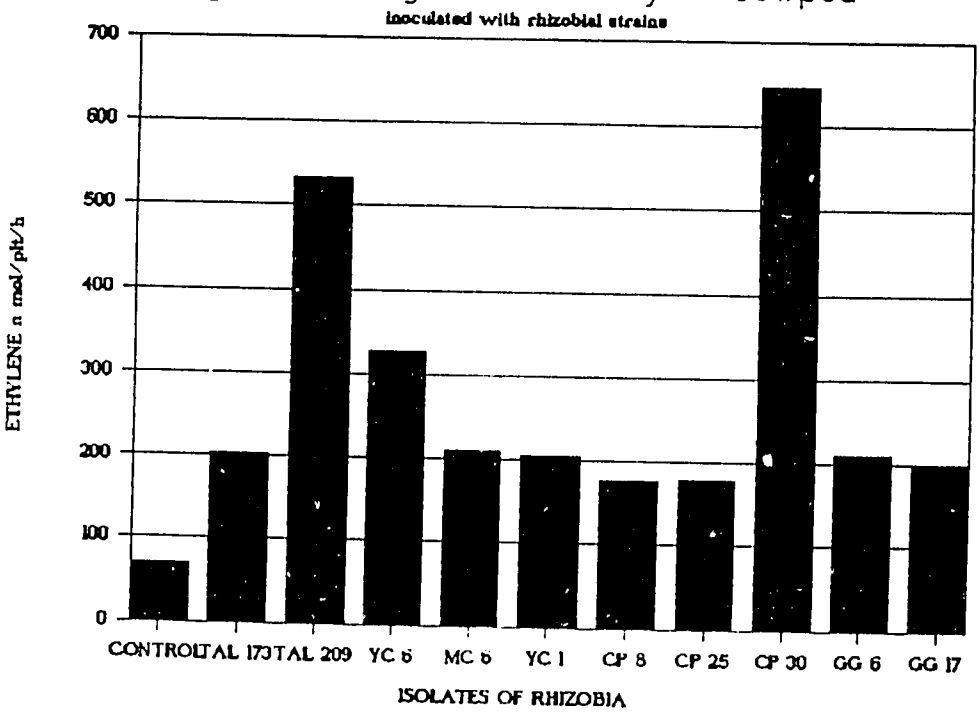


Table 3.11 Response of mung bean *(Vigna radiata)* to inoculation with Rhizobia in pot experiments.

Rhizobial Strains	Number of nodules per plant	Shoot dry weight g	Root dry weight g
Control	0.4	0.079	0.035
TAL 173	5.8	0.083	0.031
TAL 209	16.0	0.116	0.049
YC 6	4.2	0.079	0.033
MC 6	9.8	0.114	0.038
YC 1	5.6	0.104	0.037
CP 8	4.7	0.098	0.053
CP 25	4.4	0.089	0.029
CP 30	11.2	0.131	0.067
GG 6	4.2	0.081	0.031
GG 17	5.7	0.104	0.036
L.S.D	2.28	0.017	0.0135
CV%	39.18	19.9	38.1
SD	1.147	0.008	0.006

P level used 0.05
All values are mean of ten replicates.

Table 12 Effect of inoculation on the nitrogen fixation of mung bean.

Rhizobial Strains	Total Nitrogen %	Nitrogen uptake *mg*	Acetylene reduction *nmol/h/plt*
Control	3.27	3.74	23.55
TAL 173	3.39	3.85	44.25
TAL 209	3.44	5.65	79.61
YC 6	3.54	3.98	62.68
MC 6	3.27	4.93	149.27
YC 6	3.40	4.82	94.40
CP 8	3.48	4.90	89.44
CP 25	3.24	3.90	54.5
CP 30	3.38	6.69	206.94
GG 6	3.30	3.69	70.6
GG 17	3.29	4.55	100.14
LSD	ns	0.795	10.53
CV%	7.49	19.46	19.2
SD	0.113	0.399	15.36

Values are means of ten replicates except in acetylene reduction assay only three replicates were used.

and TAL 209 were selected as the superior and effective isolates in nitrogen fixation on cowpea and were used in the field experiment for further evaluation.

3.7 Evaluation of Rhizobia Under Field Conditions

Details of the soil properties at the two field sites are summarized in table 3.13.

3.7.1 population of rhizobia in field soil

In the ten fold soil dilution series for nodulation tests it was found that plants grown in dilutions 1:100 and 1:1000 were well nodulated. None of the other dilutions did not produce nodules. Therefore the most probable number of rhizobia in soils is 230 cells/g with a confidence limit of 6.6 - 80.3 x 100 at 95% level.

3.7.2 population of rhizobia in inoculated seeds.

The results of the plate enumeration tests are given in table 3.14 and 3.15. No rhizobial colonies were produced at 10-7 and 10-8 dilutions.

Table 3.13 Properties of soil at field sites

Parameter	Meewatura	Maha Illupplallama
1. Texture		
sand %	55	70
silt %	15	10.8
clay %	32	19.2
2. Texture class	sandy clay	sandy loam
3. pH		
H_2O	5.45	6.5
1N KCl	4.6	5.58
4. CEC (me/100g soil)	3.34	11.4
5. Base saturation %	65.8	94.0
6. Organic matter %	1.12	1.25
7. Total Nitrogen %	0.098	0.099
8. NH_4^+ -- N (ppm)	12.57	7.53
9. NO_3^- -- N (ppm)	11.78	62.91
10. Available		
P (ppm)	9.45	15.06
Na(mg/100 g soil)	10.62	4.832
Mg ,,	9.6	7.73
K ,,	1.6	26.4
11. W.H.C.	29.0	26.4

Table 3.14 Population of Rhizobia per seed based on plate enumeration test#

STRAIN	RHIZOBIA PER SEED	
	Mung bean	cowpea
TAL 209	36.3×10^4	59×10^4
CP 30	41×10^4	70×10^4
MC 6	43.4×10^4	-
YC 6	-	52.5×10^4

Results reported here are based on dilution 10^{-4} among a series of dilutions 10^{-2} to 10^{-6}.

3.7.3 effect of P on nodulation and N fixation in inoculated cowpea and mungbean.

a. RESPONSE AFTER 20 DAYS - MAHA ILLUPPALLAMA FIELD

i. nodulation

Effect of P on nodulation in mung bean plants are summarized in table 3.15. In the absence of P fertilizer strain MC6 showed significantly higher nodulation than the other two strains tested viz TAL 209 and CP 30. All strains responded positively for the addition of P fertilizer and the differences were significantly higher from 0 to 60 kg/h P and also from 60 to 180 kg/ha. Interaction between P level and strain for nodulation were found to be significant at P=0.05 (Fig 3.6) However the there was no significant difference in nodulation among strains at P level of 60 kg/ha. Strain MC 6 performed extremely well and produced very high nodulation of 15.56 nodules / plant compared to 8.43 nodules per plant in TAL 209 inoculated plots at 180 kg/ha P levels.

Table 3.15 Effect of P on mean number of of nodules per plant in mungbean at 20 DAS.

Rhizobial strains	P added as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	3.687	6.00	8.437
CP 30	5.062	6.812	8.562
MC 6	6.25	6.562	15.562

L.S.D. for each column = 1.38 at P = 0.05

Fig 3.5Nitrogenase activity in mungbean

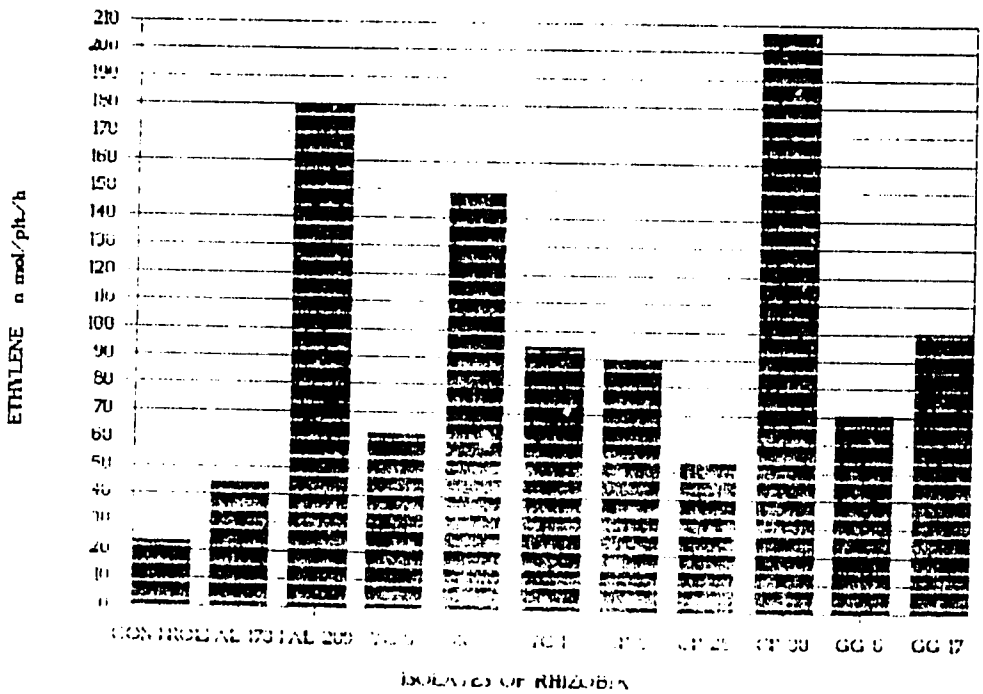
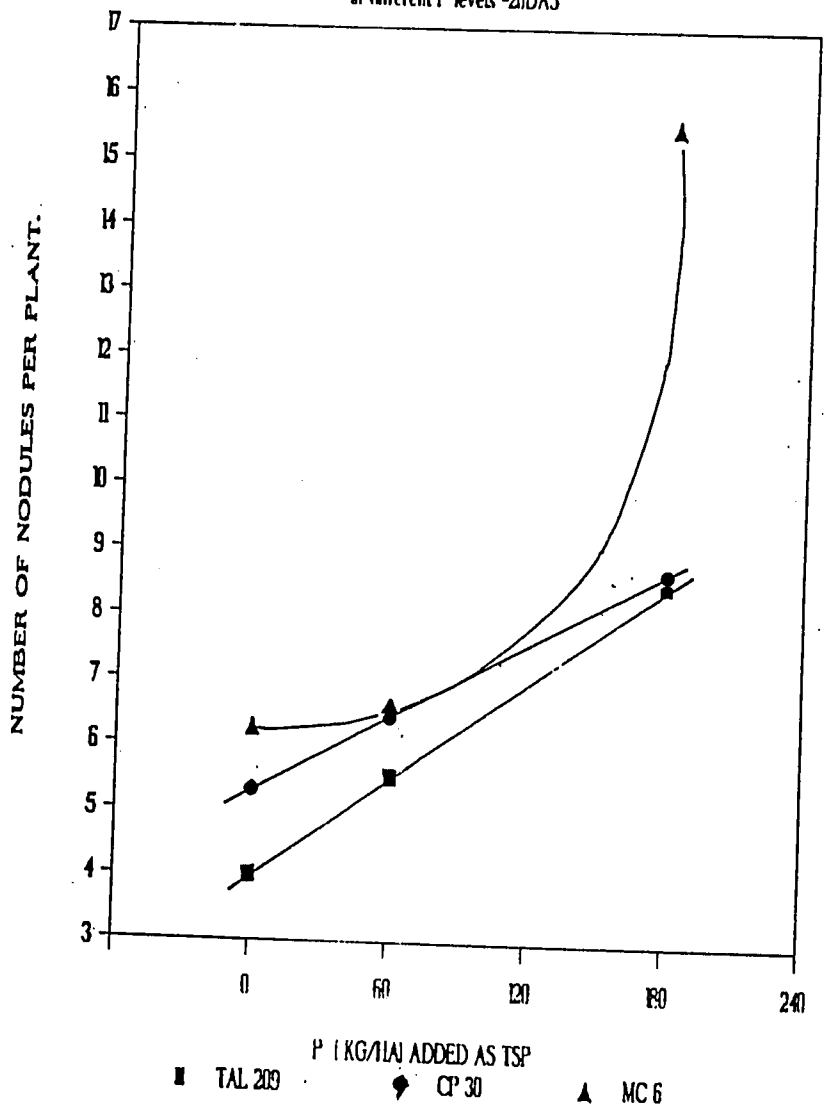


Fig 3.6 Nodulation in mung bean

at different P levels -20DAS



TAL 209 : $y = 4.0 + 2.2 \times 10^{-2} p$

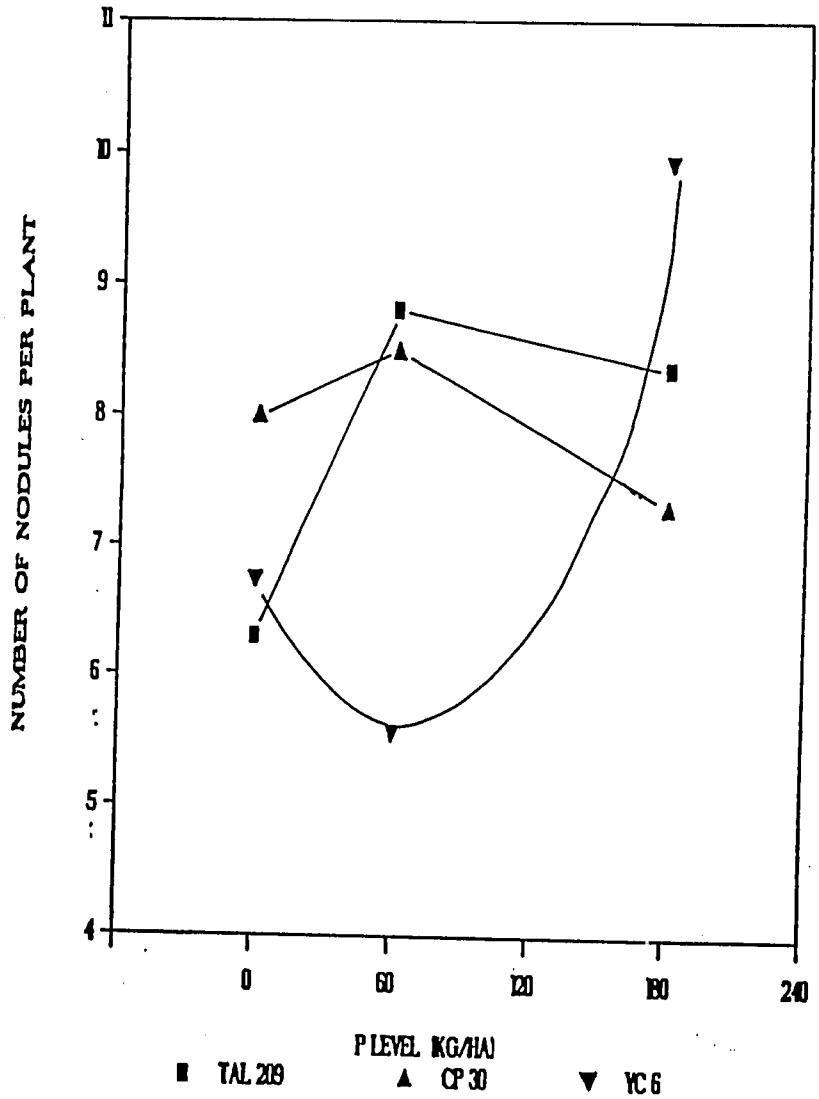
CP 30 : $y = 5.31 + 1.8 \times 10^{-2} p$

MC 6 : $y = 6.25 + 1.8 \times 10^{-2} p + 3.9 \times 10^{-4} p^2$

In cowpea strain CP 30 increased the nodulation significantly without addition of P fertilizer (0 P level) in comparison to standard NifTAL strain TAL 209. Surprisingly YC 6 showed a reduction in nodulation at 60kg/ha P level however nodulation increased to very high level of 9.93 per plant at 180 kg of P per ha. Regression analysis revealed that there is no significant statistical response in nodulation to inoculation with TAL 209 or CP 30 with increase in P level in fertilizer (Fig. 3. 7)

Shaw *et al.* (1966) reported that increased nodulation as number of nodules, volume and dry weight of nodules occurred in legumes with applied phosphorus fertilizer. This is attributed to as an indirect effect of P on nodulation, associated with growth response by the legume. A beneficial effect of P on nodule initiation on *Stylosanthes humilis* was reported by Gates in 1974. He observed early nodule initiation in higher P soil than those of low P soil. Increased nodule dry weight under high P fertilization was reported in *Glycine max* (Geopfort, 1971). Increase in nodulation in cowpea with higher P fertilizer (from 0 to 40 kg/h) was reported by Ayanabe & Nangja,1971 and Tewari, 1965.

Fig 3.7 Nodulation in cowpea – 20DAS



YC 6 : $y = 6.75 - 0.03\,p + 3.1 \times 10^{-4}\,p^2$

Table 3.16 Mean number of nodules in cowpea inoculated with different strains at 20 DAS

Rhizobial strains	P added as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	6.31	8.81	8.37
CP 30	8.00	8.50	7.31
YC 6	6.75	5.56	9.93

LSD = 0.8894 for each column at P 0.05
Each value is a mean of 4 replicates

ii.shoot dry weight

In mungbean , inoculation with strain MC 6 showed a significant increase in shoot dry weight, both at P level of0 and 60 kg/h (Table 3.17. At 180 kg of P /h no significant difference was seen among strains in dry matter production.. Even P x N (nodulation) was found to be non-significant. Regression analysis indicated that the response of TAL 209 was at the highest at 148.75 kg of P /ha with a shoot dry weight of 0.427 g and for CP 30 it was at the maximum at 125.45 kg of P/ha yielding 0.422 g of shoot (dry weight) . The strain MC6 showed a continuous increase in dry matter up to 180 kg of P/ha (Fig. 3.8).

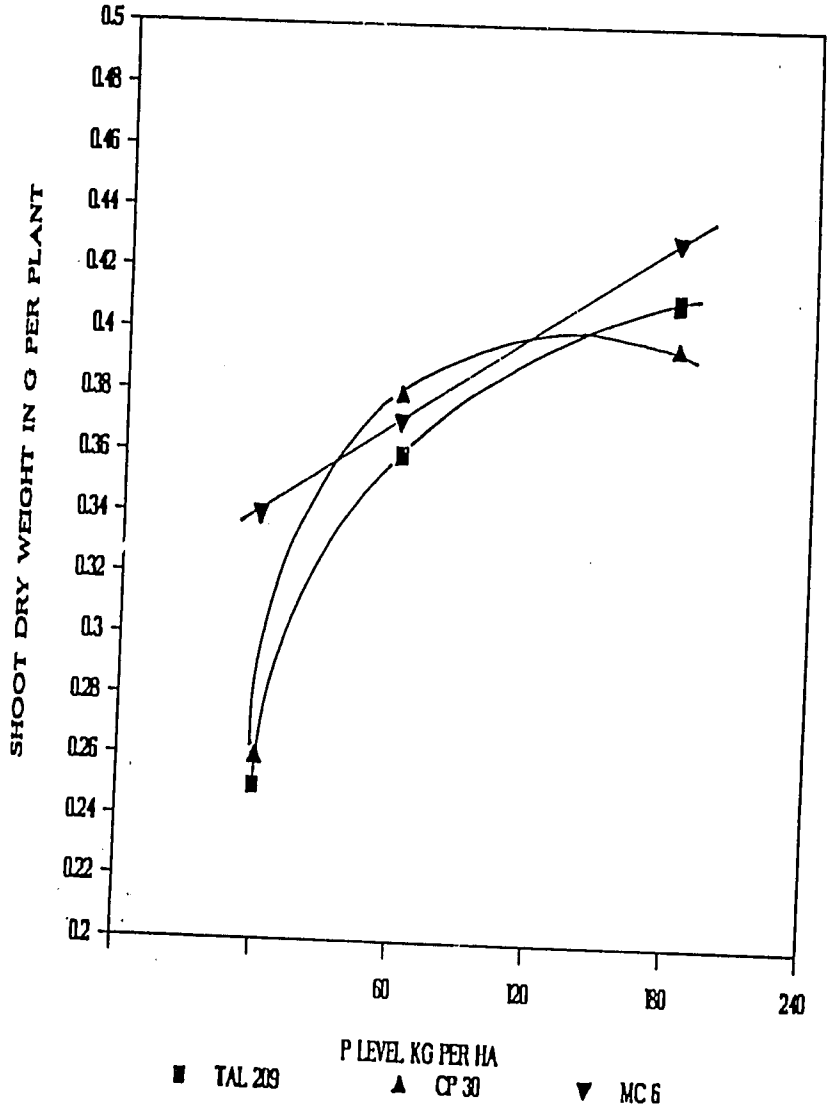
It is evident from the table 3.18 that the shoot dry weight of cowpea in soil without added P was significantly low when inoculated with CP 30, also the increase observed in YC 6 over TAL 209 was not significant. Strain TAL 209 performed better than both YC 6 and CP 30 with increase in P in TSP. Regression analysis reveals that TAL 209 and CP 30 reached maximum values at 115.0 and 100.83 kg of P /ha respectively. No significant response was observed in YC 6 (Fig. 3.9).

Table 3.17 Mean shoot dry weight of inoculated mungbean plants at 20 DAS at MI

Rhizobial strains	Quantity of P added as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
	(shoot dry wt. g/plt)		
TAL 209	0.2375	0.3625	0.4062
CP 30	0.2562	0.3812	0.4125
MC 6	0.3187	0.3939	0.4187

LSD = 0.0266 g for each column at P = 0.05

Fig 3.8 Shoot dry weight of mung bean



TAL 209: $y = 0.25 + 2.38 \times 10^{-3} p - 8.0 \times 10^{-6} p^2$

CP 30 : $y = 0.25 + 2.76 \times 10^{-3} p - 1.1 \times 10^{-5} p^2$

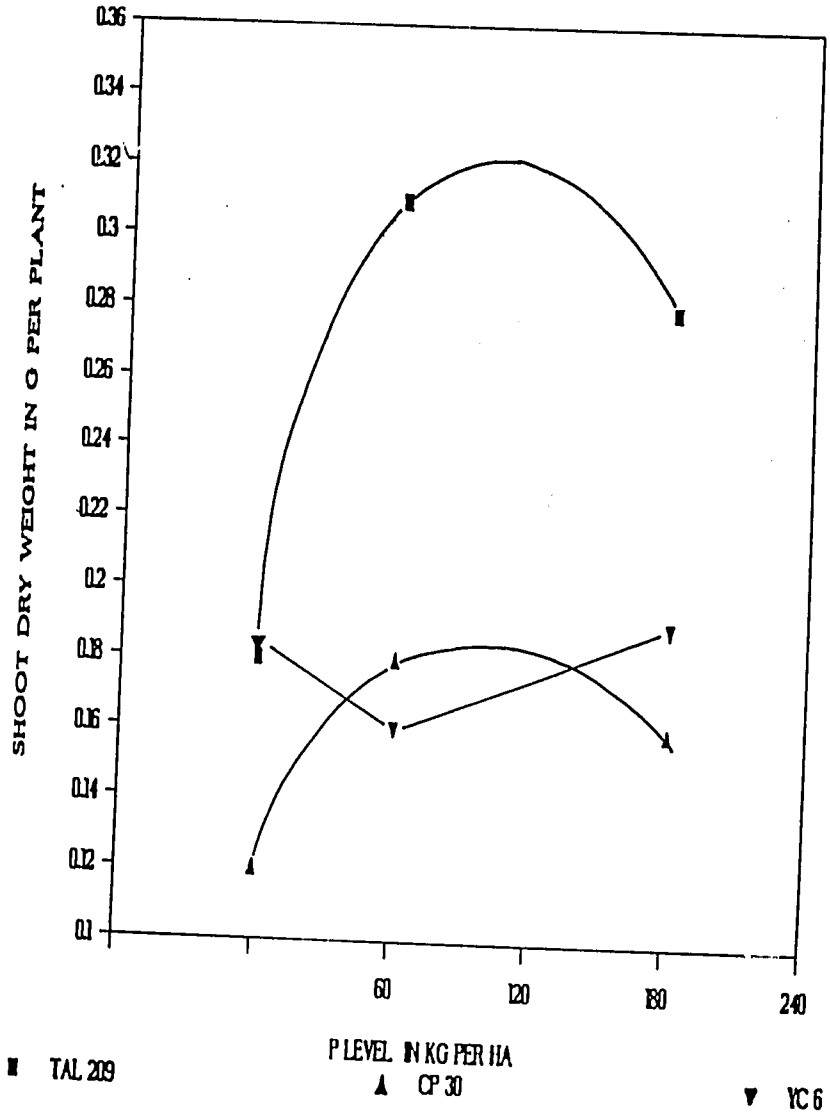
MC 6 : $y = 0.34 + 5.06 \times 10^{-4} p$

Table 3.18 Mean shoot dry weight in inoculated cowpea plants at 20 DAS at Maha Iluppallama

Rhizobial strains	Quantity of P added as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
	(shoot dry wt. g/plt)		
TAL 209	0.175	0.306	0.281
CP 30	0.125	0.177	0.157
YC 6	0.181	0.156	0.187

LSD = 0.00686 for each column at P =0.05

Fig 3.9 Shoot dry weight of cowpea 20 D



TAL 209: $y = 0.17 + 2.99 \times 10^{-3} p - 1.3 \times 10^{-5} p^2$

CP 30 : $y = 0.12 + 1.21 \times 10^{-3} p - 6.0 \times 10^{-6} p^2$

iii. root dry weight

At zero and 180 kg of P/ha both CP 30 and MC 6 has significantly increased the root dry weight of mungbean compared to the standard strain used TAL 209 (Table 3.19). Only strain MC 6 inoculated plants showed a significantly higher increase in root dry weight at 60 kg of P/ha. The P x Strain interaction was found to be non - significant.. Regression analysis of root dry weight on P level illustrated in fig. 3.10 indicate that maximum root dry weight of plants inoculated with TAL 209 & CP 30 are 115.08 and 123.78 g/plt respectively.

In *Vigna unguiculata* at zero P level CP 30 had significantly low root dry weight.Regression analysis indicated that TAL 209, CP 30 and YC6 produced maximum root dry weight at 86.75; 90.56 and 106.0 kg of P/ha respectively (Fig. 3.11). Tal 209 produced the highest root dry weight at both 60 and 180 kg of P/ha.

iv. total nitrogen content

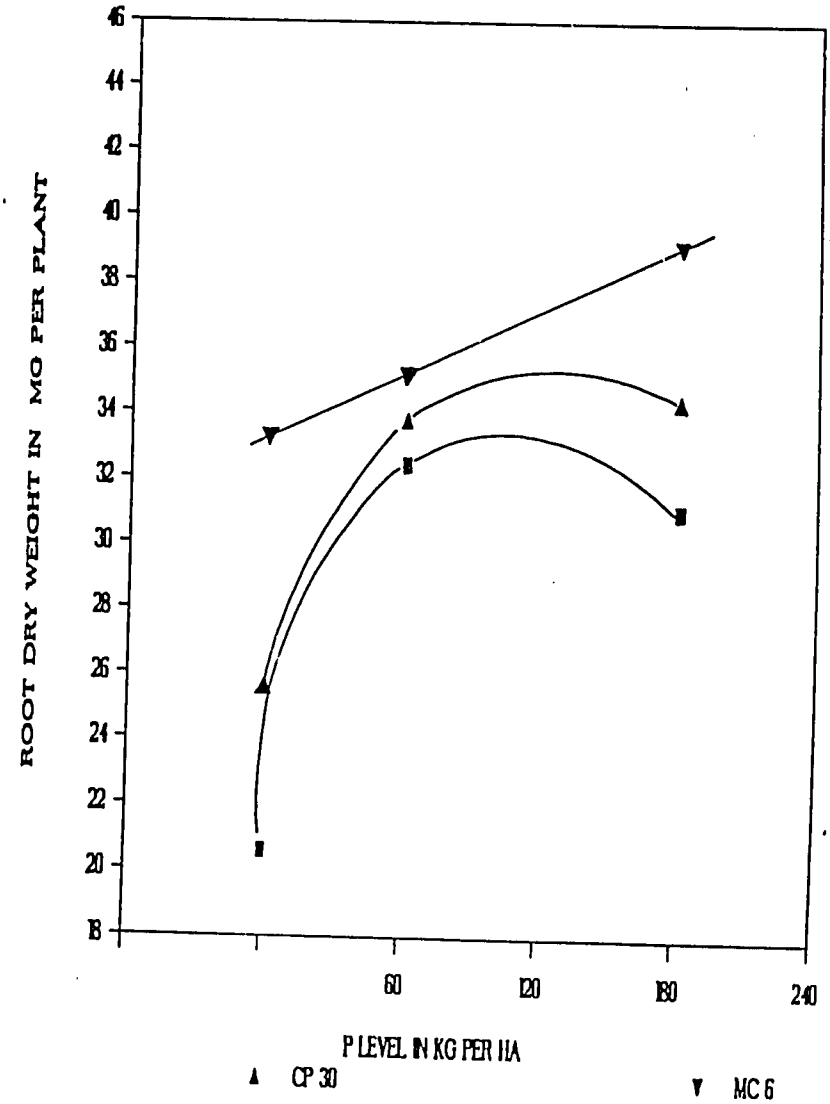
Neither interactions (P x S) nor main effects were significant in total nitrogen content of both crops. No significant response was observed in nitrogen content of inoculated treatments with increase in P level.

Table 3.19 Mean root dry weight (mg/plt.) of mung bean at 20 DAS, inoculated with rhizobia at MI.

Rhizobial strains	Quantity of P added as TSP		
	0 kg/ha	60 kg/ha (mg/plt)	180 kg/ha
TAL 209	20.62	32.5	31.25
CP 30	25.0	33.75	34.37
MC 6	32.5	36.25	38.75

LSD = 1.988 (among strains)

Fig 3.10 Root dry weight of mung bean

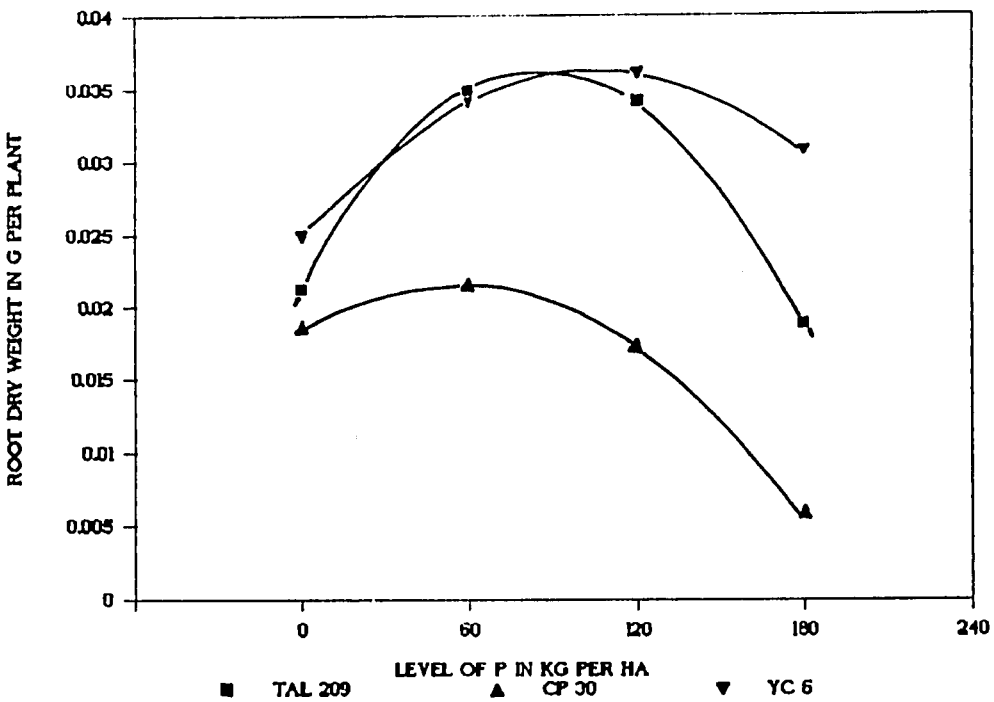


TAL 209 : $y = 20.6 + 0.27\,p - 1.1 \times 10^{-3}\,p^2$

CP 30 : $y = 25.6 + 0.17\,p - 7.2 \times 10^{-4}\,p^2$

MC 6 : $y = 33.2 + 0.03\,p$

Fig 3.11 Root dry weight of cowpea–20D



TAL 209 : $y = 0.0213 + 3.47 \times 10^{-4} p - 2.0 \times 10^{-6} p^2$

CP 30 : $y = 0.0187 + 1.09 \times 10^{-4} p - 1 \times 10^{-6} p^2$

MC 6 : $y = 0.025 + 2.12 \times 10^{-4} p - 1 \times 10^{-6} p^2$

Table 3.20 Mean root dry weight(g/plt.) in cowpea at different P levels at 20 DAS in MI experiment.

Rhizobial strains	P added as T.S.P.		
	0 kg/ha	60 kg/ha	180 kg/ha
	(root dry wt. g/plt)		
TAL 209	0.0212	0.3625	0.0312
CP 30	0.0187	0.0231	0.0187
YC 6	0.0251	0.3375	0.0275

LSD = 0.00016 g among strain

v. nitrogen uptake

Increases in nitrogen uptake was observed in both crops on inoculation with rhizobia. *V. radiata* inoculated with MC 6 showed a significantly higher N uptake over TAL 209 inoculated crops at zero and 60 kg of P/ha. (Table.3.21). P x S interaction was non significant.. No significant difference was observed between strain for N uptake at 180 kg of P/ha. The maximum N uptake of TAL 209 and CP 30 inoculated plots were at 115 and 130.22 kg of P/ha yielding 16.05 and 16.3 mg of nitrogen per plant respectively (Fig. 3.12).

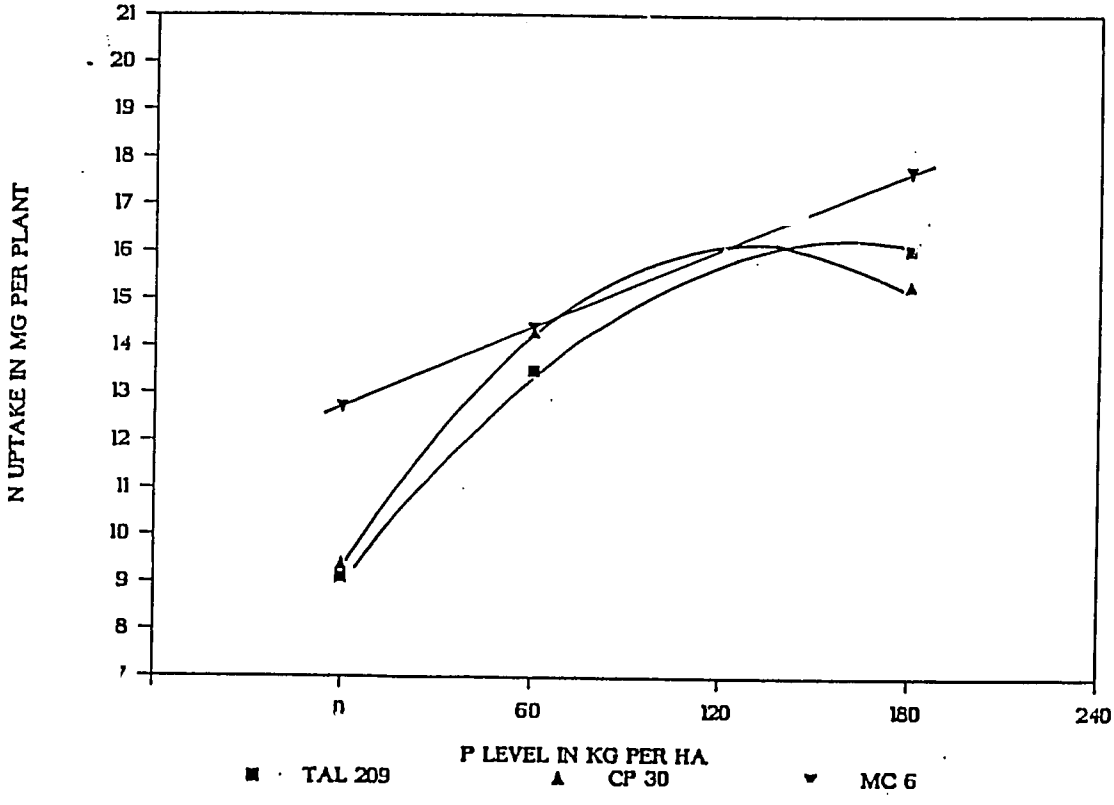
N uptake in cowpea inoculated with CP 30 and YC 6 were significantly lower than TAL 209 (Check treatment) at 60 and 180 kg of P/ha. N uptake in the check strain TAL 209 was best at all levels of P 0 - 180 kg/ha (Fig. 3.13). indicating the high BNF activity of TAL 209 at higher P level when cowpea plants are inoculated with. The high BNF activity of TAL 209 with improved agronomic practices are of lesser importance to Sri lankan agriculture where fertilizers are costly. The medium values of P levels could of immense value in the increase of BNF

Table 3.21 Mean N uptake in mung bean plants at 20 DAS at Maha Illuppallama.

Rhizobial strain	Amount of P added as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
	(mg/plt)		
TAL 209	9.123	13.482	16.051
CP 30	9.399	14.266	15.256
MC 6	11.989	15.479	17.382

LSD = 1.621mg among strains

Fig 3.12 N uptake in mungbean – 20DAS

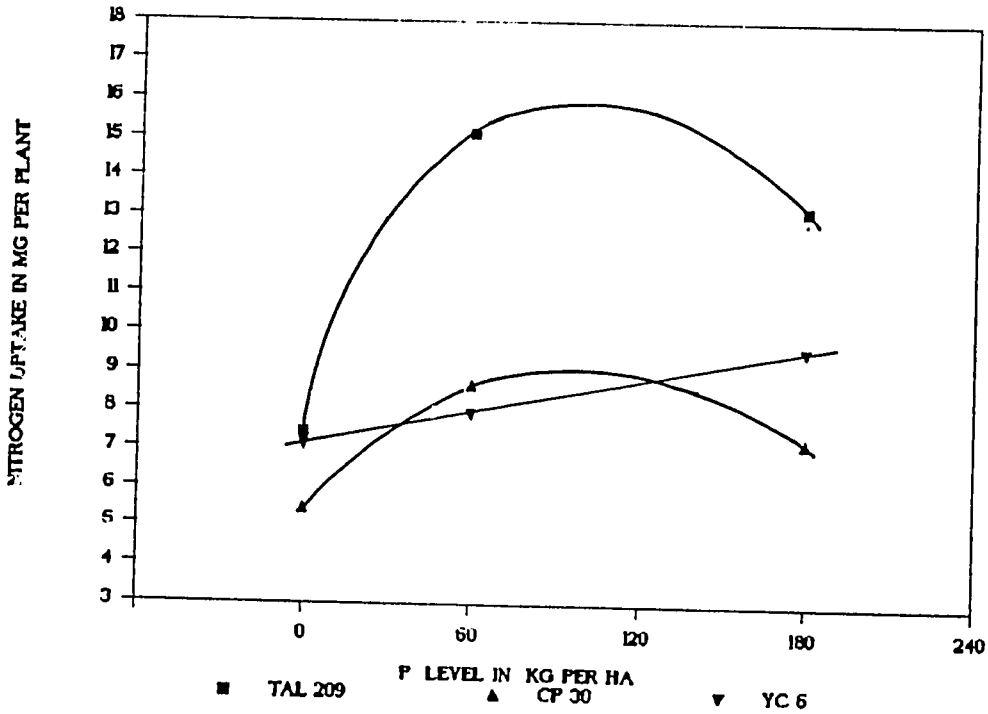


TAL 209: $y = 9.12 + 8.99 \times 10^{-2} p - 2.9 \times 10^{-4} p^2$

CP 30 : $y = 9.4 + 10.6 \times 10^{-2} p - 4.0 \times 10^{-4} p^2$

MC 6 : $y = 12.7 + 0.027 p$

Fig 3.13 N uptake in cowpea – 20DAS



TAL 209 : $y = 7.41 + 0.177\,p - 8.03 \times 10^{-4}\,p^2$

CP 30 : $y = 5.43 + 0.0759\,p - 3.67 \times 10^{-4}\,p^2$

YC 6 : $y = 7.1 + 0.0135\,p$

Table 3.22 Mean Nitrogen uptake in mg/plt in cowpea at 20 DAS.

Rhizobial strains	Amount of P added as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	7.411	15.117	13.179
CP 30	5.427	8.862	7.212
YC 6	7.457	7.373	9.707

LSD = 0.9472 mg among strains

activity in both cowpea and mung bean using local strains as inoculant. It is interesting to note that TAL 209 or CP30 inoculated cowpea plants had the maximum N-uptake at 110.21 or 103.45 kg of P/ha respectively.

Andrew and Robin (1969) reported that the N concentration in the tops of legumes deficient in P are low. They establish a good correlation between the Phosphorus and Nitrogen concentration in tropical legumes. Results from this study could attribute these effects to root development, nodulation and nodule efficiency.

Incorporation of P as T.S.P. resulted in higher Nitrogen fixation in *V.radiata* by MC 6 and *V. unguiculata* by TAL 209 at 20 days after sowing (DAS).

b. RESPONSE AFTER 40 DAYS - MAHA ILLUPPALLAMA FIELD.

i. nodulation

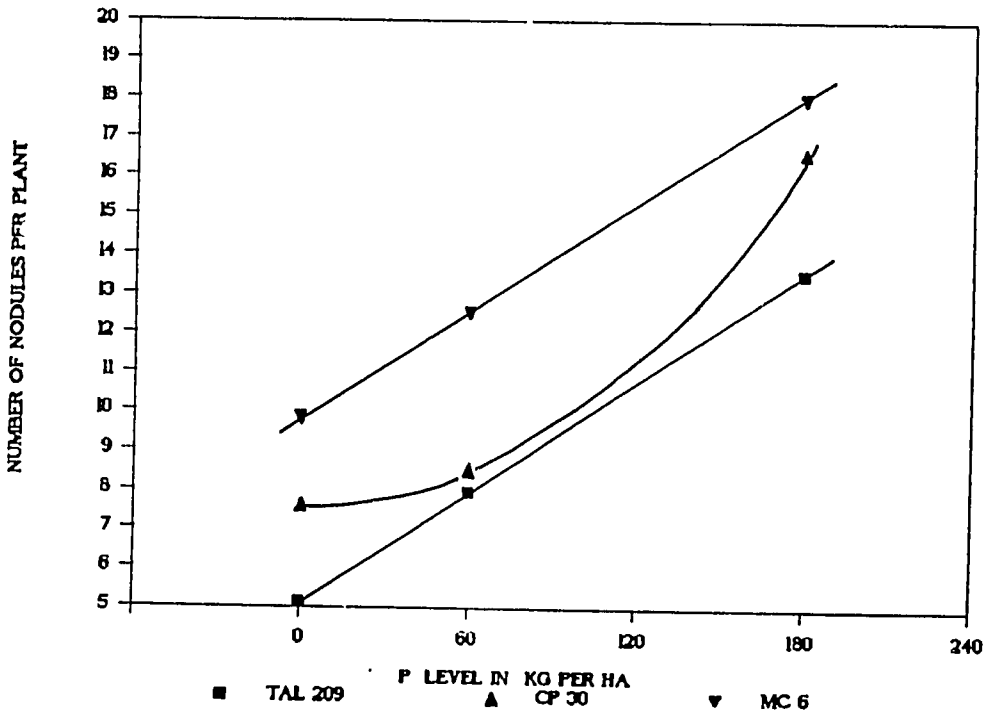
Incorporation of TSP has resulted in significant increase in nodulation *V. radiata* inoculated with MC 6 when compared with standard TAL 209 at all levels of P. (Table.3.23). In general CP 30 inoculated mung bean plants were superior in nodulation to TAL 209 both at zero and 180 kg of P/ha. The P x S interaction was not significant (Fig.4.14)

Table 3.23 Mean number of nodules/plant in mungbean plants at 40 DAS at Maha Illuppallama

Rhizobial strain	Quantity of P added as T.S.P.		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	4.062	9.312	13.01
CP 30	7.562	8.511	16.625
MC 6	8.687	14.31	17.437

L.S.D. = 1.582 among strains

Fig 3.14 Nodulation in mung bean:40 DAS



TAL 209 : $y = 5.14 + 0.0467\ p$

CP 30 : $y = 7.56 - 1.7 \times 10^{-3}\ p + 2.89 \times 10^{-4}\ p^2$

MC 6 : $y = 9.85 + 4.5 \times 10^{-2}\ p$

Effect of level P on nodulation of cowpea is summarized in table 3.24. Nodulation trend in CP 30 inoculated plants appears to be unique where maximum nodulation was seen at 60 kg of P/ha. In general CP 30 could be considered as the best performer in dry zone soil where maximum nodulation was seen at 110 kg of P/ha was added (Fig. 3.15)

ii. Shoot dry weight

Effect of P on shoot dry weight of mung bean is shown in table 3.25. No significant difference between strains were seen when no P were added or even at 60 kg of P per ha. However when P is added at 180 kg /ha MC 6 inoculated plants yielded the highest shoot dry weight/plant (5.25). P x s interaction was not significant. All three strains showed a positive linear relationship (Fig. 3.16).

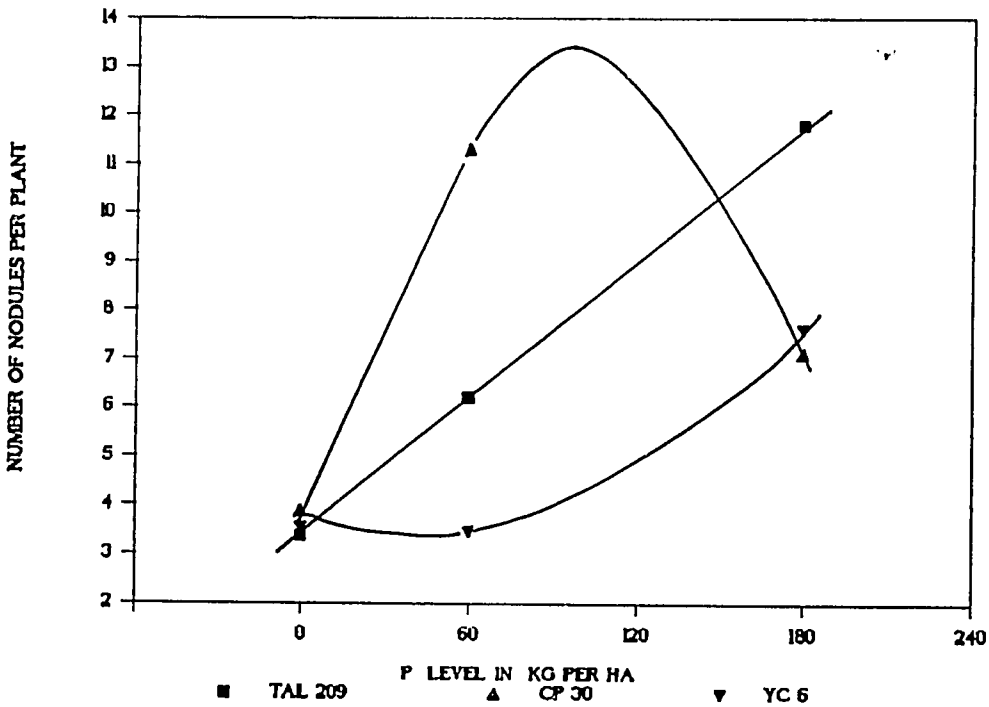
Although a higher nodule number was observed in CP 30 inoculated plants the shoot (dry weight) dry matter yielded by cp 30 at 60 kg of P/ha in cowpea was not significantly different to other strains.

Table 3.24 Mean number of nodules in inoculated cowpea at 40 DAS, grown under different P levels at MI.

Inoculant strain	Amount of P added to soil as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	2.751	7.187	11.501
CP 30	3.875	11.312	7.125
YC 6	3.562	3.437	7.625

LSD = 0.8159 among strains

Fig 3.15 Nodulation in Cowpea–40DAS



TAL 209 : $y = 3.4 + 0.0468\ p$

CP 30 : $y = 3.87 + 0.177\ p - 8.83 \times 10^{-4}\ p^2$

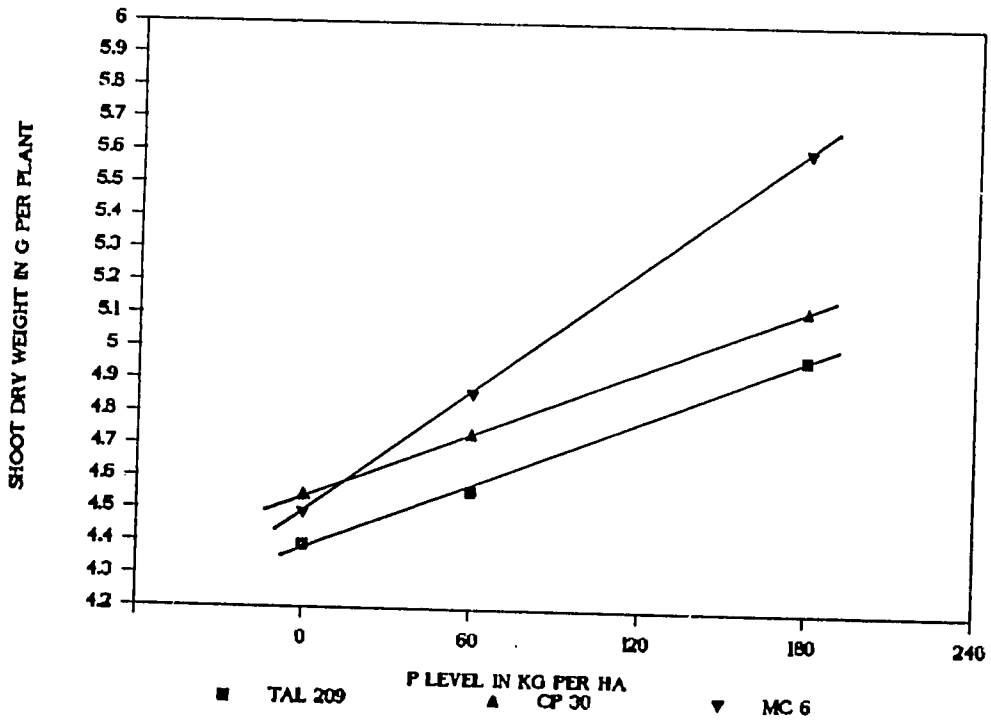
YC 6 : $y = 3.56 - 1.4 \times 10^{-2}\ p - 2.05 \times 10^{-4}\ p^2$

Table 3.25 Effect of inoculation of mungbean at differen P levels on the shoot dry weight in g/plt.

Inoculant strain	Quantity of P added as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	4.287	4.742	4.925
CP 30	4.541	4.757	5.121
MC 6	4.507	4.827	5.625

LSD = 0 .2379 g among strains

Fig 3.16 Shoot dry weight of mungbean



TAL 209 : $y = 4.39 + 0.00325\ p$

CP 30 : $y = 4.56 + 0.0319\ p$

MC 6 : $y = 4.49 + 0.00627\ p$

This may be due to low or non effective nodules produced by strain CP 30. The shoot dry matter production (3.48 g/plt) was observed in TAL 209 at 112.2 kg of P/ha while YC 6 needed 180 kg of P/ha to produce 3.25 g of dry matter /plant. Significant difference between standard and other strains were found only at 60 kg of P /ha.(table 3.26 and Fig.3.17)

iii. Root dry weight.

It is evident from regression analysis that there is no significant response in root dry weight of mungbean plants to increase in P level. On the analysis of pooled data no significant difference was detected in root weight yielded by the treatments at different P levels.

Effect of P level on root dry weight of cowpea is summarized in table 3.27. At zero P YC 6 has shown a a significant increase over TAL 209 .

iv total nitrogen

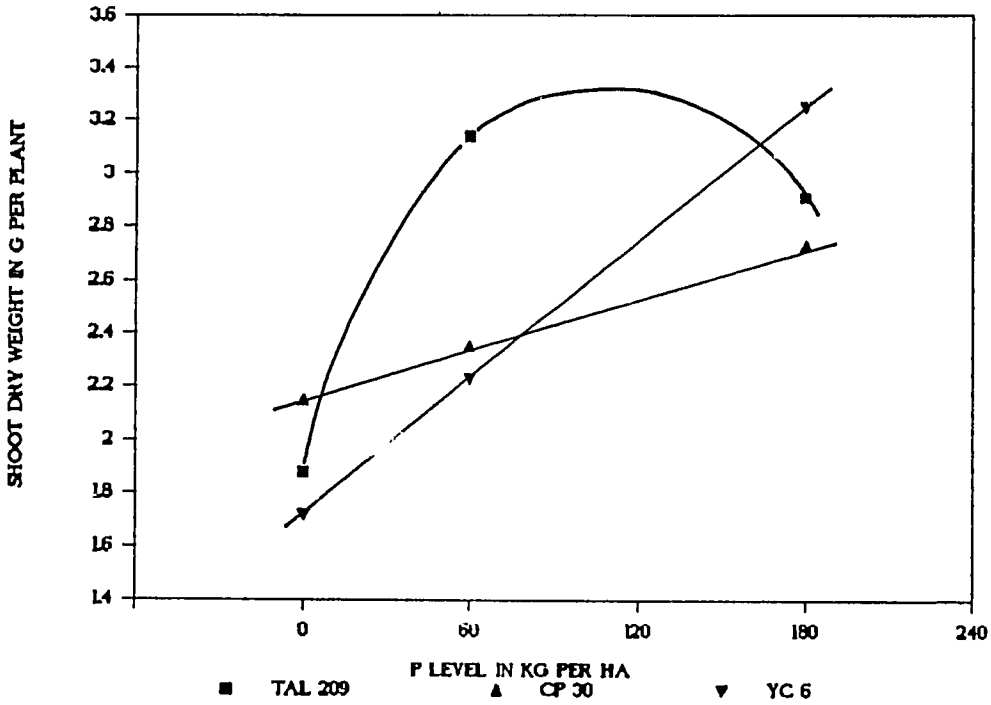
In mungbean the total nitrogen content was found to be non significant. It is seen by the regression analysis data that there is no significant response of total nitrogen to increase in P level. However the pooled data for all three strains showed a positive linear relationship between total nitrogen and P level used (Fig 3.18)

Table 3.26 Effect of inoculation of cowpea grown under different P levels on shoot dry weight(g/plt) at 40DAS.

Inoculant strains	Quantity of P added to soil as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	1.88	3.14	2.91
CP 30	2.16	2.96	2.73
YC 6	1.91	1.96	3.09

LSD = 0.3398 g among strains

Fig 3.17 Shoot dry weight of cowpea:40



TAL 209 : $y = 1.88 + 0.0285\ p - 1.27 \times 10^{-4}\ p^2$

CP 30 : $y = 2.15 + 0.0032\ p$

YC 6 : $y = 1.73 + 0.00849\ p$

Table 3.27 Mean root dry weight (g/plt) of cowpea plants cultivated at different P levels, at 40 DAS.

Inoculant strain	Amount of P added to soil as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	0.175	0.201	0.256
CP 30	0.163	0.231	0.225
YC 6	0.206	0.206	0.237

LSD = 0.02638 g among strains

Fig 3.18 Total N content of mungbean:40

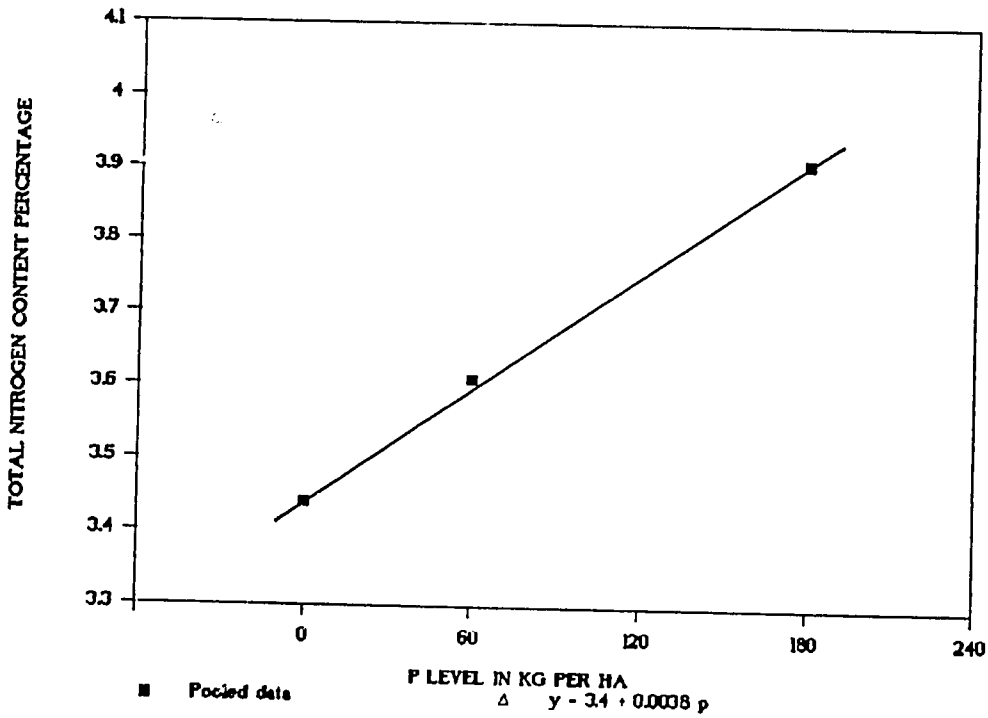


Table 3.28 and Fig 3.19 illustrate the effect of phosphorus on total N content of cowpea plants. Strain YC 6 showed a significant increase in N content over the standard TAL 209 in soils where no P was added. P x s interaction was not significant. The standard strain TAL 209 yielded maximum N content at 110.48 kg of P/ha.

v. N uptake

None of the strains showed significantly higher N uptake in mungbean compared to the standard strain TAL 209 when no P was added.At 180 kg/ha, MC 6 inoculated plants had a significantly higher N uptake. Linear relation was observed in regression analysis of N uptake/plant on P level (table 3.29 and Fig. 3.20)

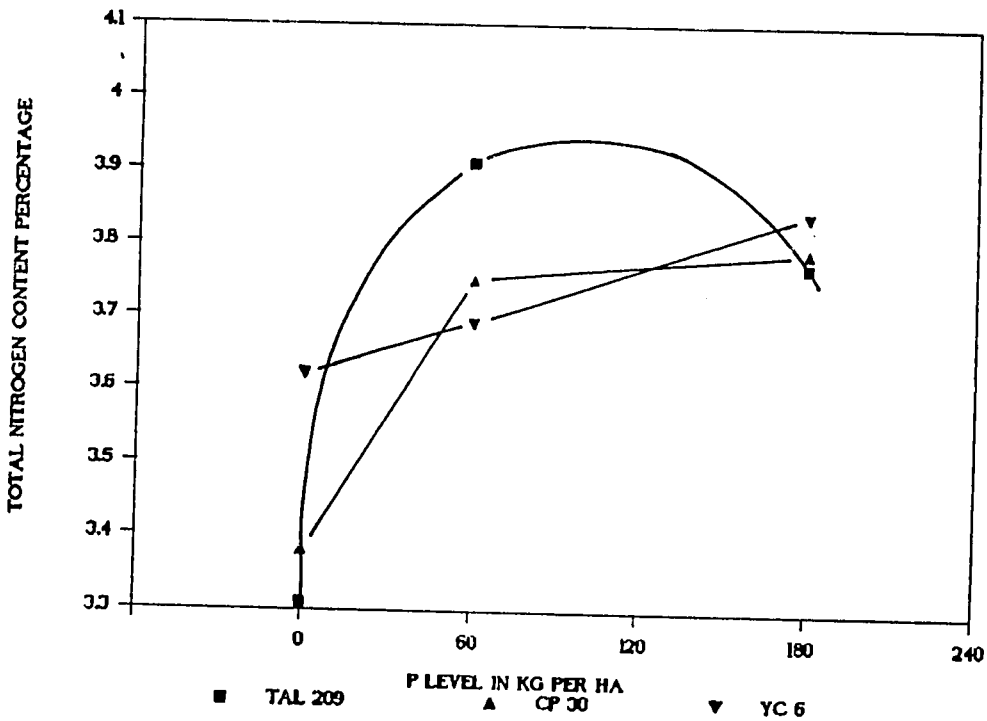
Table 3.30 summarizes the effect of P on the N uptake in cowpea. Standard strain TAL 209 reached the maximum value of N uptake at 111.63 kg of P/ha to decline 117 30 mg/plant at 180 kg of P/ha (Fig3.21).

Table 3.28 Mean total nitrogen content (%) of inoculated cowpea plants at different P level, at 40 DAS.

Inoculant strain	Amount of P added to soil as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	3.31	3.91	3.77
CP 30	3.39	3.74	3.79
YC 6	3.67	3.69	3.84

LSD = 0.221 among strains

Fig 3.19 Total N content of cowpea:40D



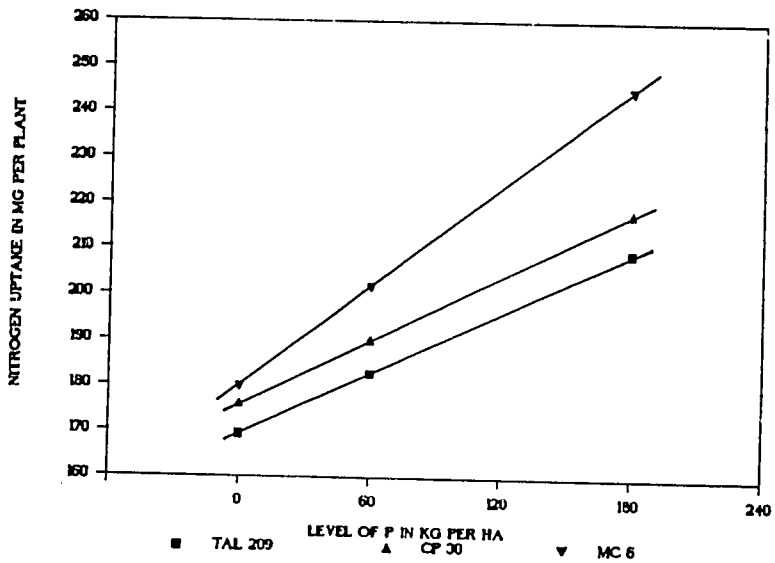
$$TAL\ 209 : y = 3.31 + 0.0137\ p - 6.2 \times 10^{-5}\ p^2$$

Table 3.29 Mean nitrogen uptake (mg/plt.) of inoculated mung bean plants grown at various P levels, at 40DAS.

Inoculant strain	Amount of P added to soil as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	166.251	187.362	207.646
CP 30	176.199	189.636	218.146
MC 6	176.987	205.431	243.552

LSD = 22.425 mg among strains

Fig 3.20 Nitrogen uptake in mungbean:40



TAL 209 : $y = 169 + 0.221\ p$

CP 30 : $y = 176 + 0.234\ p$

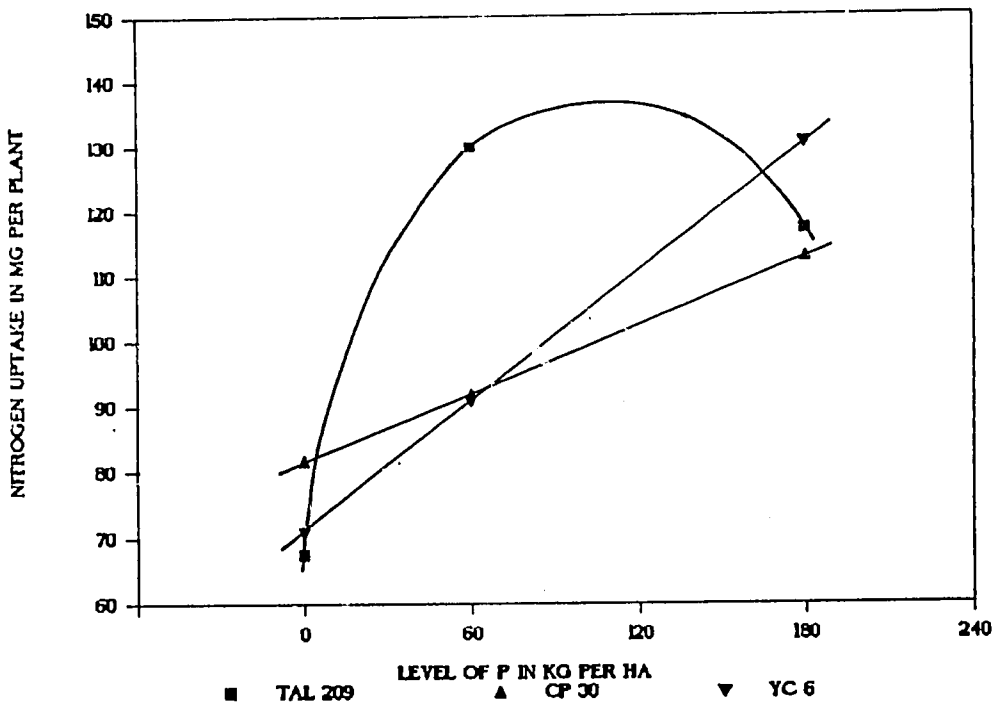
MC 6 : $y = 180 + 0.362\ p$

Table 3.30 Mean nitrogen uptake (mg/plt.) by inoculated cowpea plants at different P level at 40 DAS

Rhizobial strains	Amount of P added as TSP		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	67.514	129.951	117.306
CP 30	78.669	96.556	111.399
YC 6	77.964	79.915	134.07

LSD = 13.588 mg among strains.

Fig 3.21 Nitrogen uptake in cowpea:40



TAL 209 : $y = 67.5 + 1.42\ p - 6.36 \times 10^{-3}\ p^2$

CP 30 : $y = 81.7 + 0.174\ p$

YC 6 : $y = 70.8 + 0.332\ p$

vi <u>nitrogenase activity</u>

It is evident from table 3.31 that nitrogenase activity of the CP 30 and MC 6 in mungbean nodules were although higher than that of TAL 209 in soils where no P was added, values were not significantly different to TAL 209. However with added P both strains showed significantly higher acetylene reduction than TAL 209. Fig 3.22 illustrates the positive relationship with nitrogenase activity and P level in soil in mungbeans.

In cowpea although there is a gradual increase in nitrogenase activity with addition of P fertilizer the response by strain CP 30 is more or less static.Cp 30 was almost similar inactivity to TAL 209 when no P was added to soil. YC 6 showed a significant increase in activity at 180 kg of P /ha (Table 3.32 and Fig. 3.23). Tal 209 and CP 30 reached the maximum values in Nitrogenase activity at 112.79 and 155.28 kg of P/ha respectively.

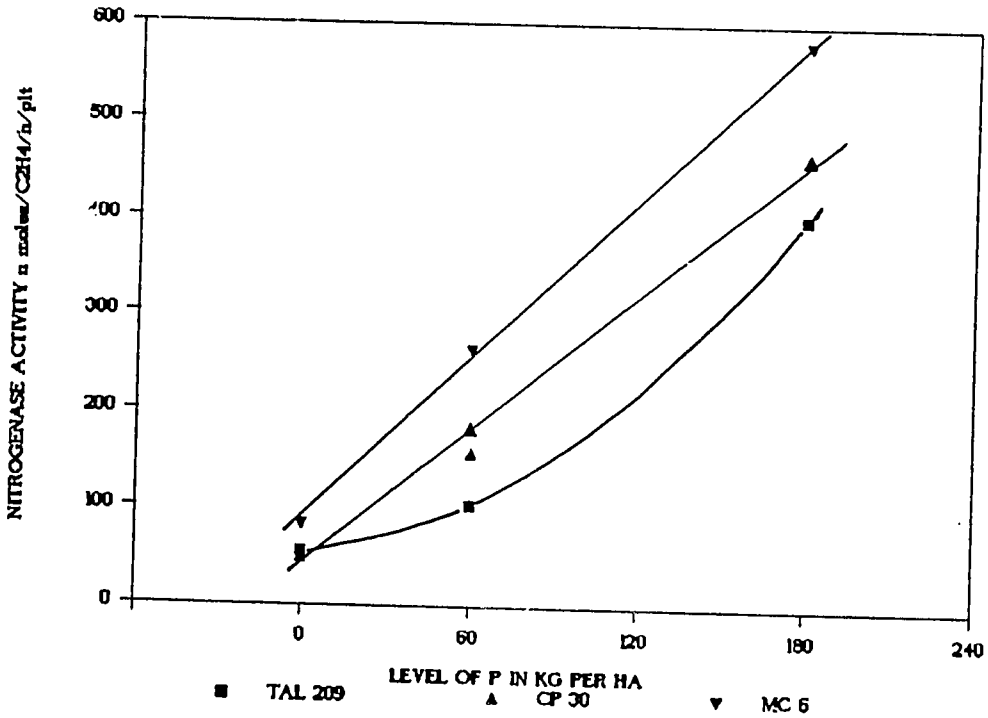
Increase nodulation and higher values for acetylene reduction in *Stylosanthes guianensis* and *Centrocema pubescens* with mycorrhizal infections which improves phosphorus supply to the plant were reported by Crush (1974) and Mosse (1977).

Table 3.31 Nodule Nitrogenase activity (mean n moles ethylene/h/plt.) in mungbean.

Inoculant Strain	Amount of P added to sol		
	0 kg/ha	60 kg/ha	180 kg/ha
TAL 209	54.571	103.535	401.731
CP 30	67.515	156.805	463.385
MC 6	74.027	262.561	599.908

LSD = 32.201 nmoles among strains

Fig3.22 Nitrogenase activity– mungbean



TAL 209 : $y = 54.8 + 0.259\ p + 9.27 \times 10^{-3}\ p^2$

CP 30 : $y = 49.2 + 2.25\ p$

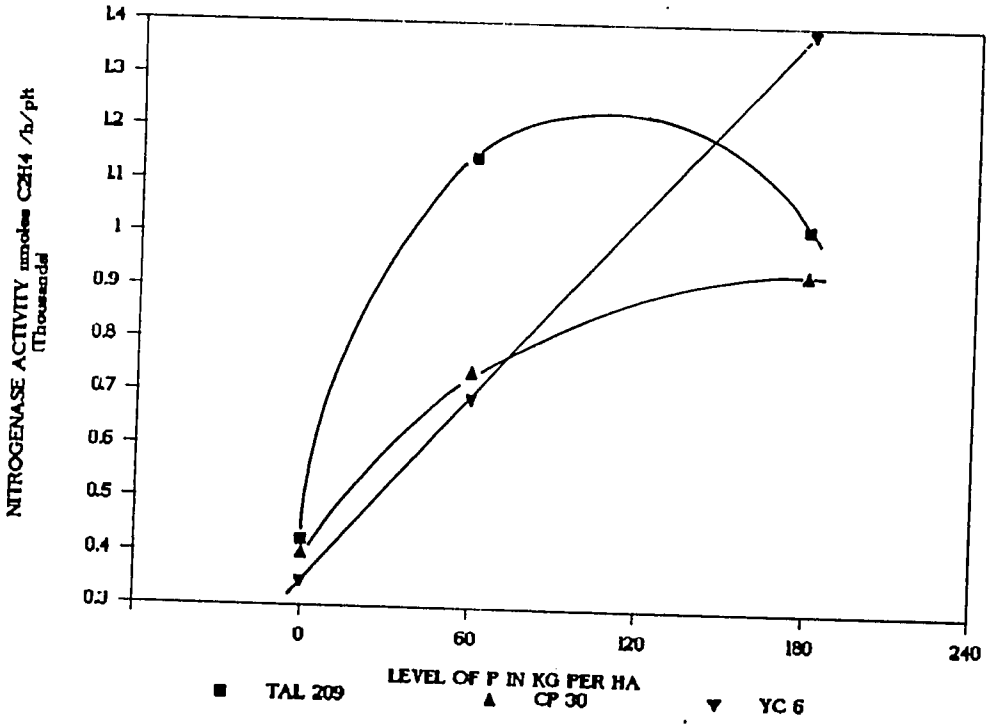
MC 6 : $y = 82.6 + 2.79\ p$

Table 3.32 Nitrogenase activity (nmoles of C_2H_4 /h/plt) in cowpea nodules under different P levels.

Quantity of P added (kg/ha)	RHIZOBIAL STRAINS		
	TAL 209	CP 30	YC 6
	(n moles of ethylene/h/plant)		
0	423.13	400.15	377.35
60	1144.91	741.08	645.95
180	1018.01	711.85	1401.15

LSD = 96.428 n moles among strains

Fig 3.23 Nitrogenase activity in cowpea



TAL 209 : $y = 423 + 16.4\ p - 7.27 \times 10^{-2}\ p^2$

CP 30 : $y = 400 + 7.05\ p - 2.27 \times 10^{-2}\ p^2$

YC 6 : $y = 346 + 5.78\ p$

The strain MC 6 emerges as the best N fixing strain by the results at 40 DAS in mungbean, for all levels of P (0 to 180 kg/ha) as reported above. TAL 209 was still the best nodulating strain for cowpea. none of the ot . strains could not beat the capability of TAL 209 at high level of P . However strain YC 6 was efficient at high P level 180 kg/ha.

c. RESPONSE AFTER 60 DAYS - MANA ILLUPPALLAMA FIELD

i. shoot dry weight

The shoot dry weight of mung bean plants after 60 days of cropping, inoculated with various strains at three different levels of P is summarized in table 3.33 Although the dry matter produced increased with increase in P level added as fertilizer no significant difference in the performance of strains were detected except at 60 kg of P /ha where both CP 30 and MC 6 inoculated plants yielded significantly higher dry mater than that of standard TAL 209. (Fig 3.24)

In cowpea however while CP 3 produced significantly higher shoot dry weight (table 3.34) in sol where no P was added , strain TAL 209 at 60 kg of P/ha and strain YC6 at 180 kg of P/ha produced the highest shoot dry weight. TAl 209 yielded the highest at 166.39 kg/ha as depicted in Fig 3.25.

ii. Root dry weight

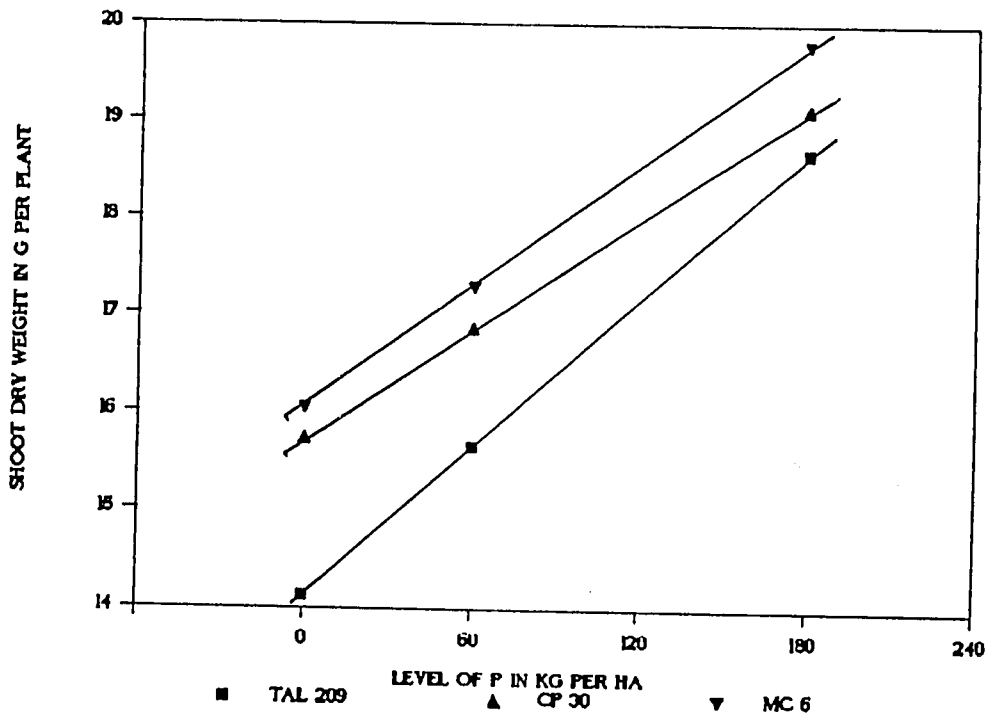
Root dry weight of mung bean plants are summarized in table 3.35. Effect of inoculation with MC 6 wa significantly different to standard strain or CP 30. Better root growth was seen with MC 6 at all levels of P (Fig.3.26)

Table 3.33 Shoot dry weight of 60 day old mungbean plant inoculated with different strains at various P levels.

Amount of P added (kg/ha)	STRAINS OF RHIZOBIA		
	TAL 209	CP 30 (g/plt.)	MC 6
0	14.081	15.231	15.112
60	15.710	17.618	18.680
180	18.662	18.870	19.318

LSD = 1.3229 g among strains

Fig3.24 Shoot dry weight of mungbean:60



TAL 209 : $y = 14.1 + 0.0253\ p$

CP 30 : $y = 15.7 + 0.0188\ p$

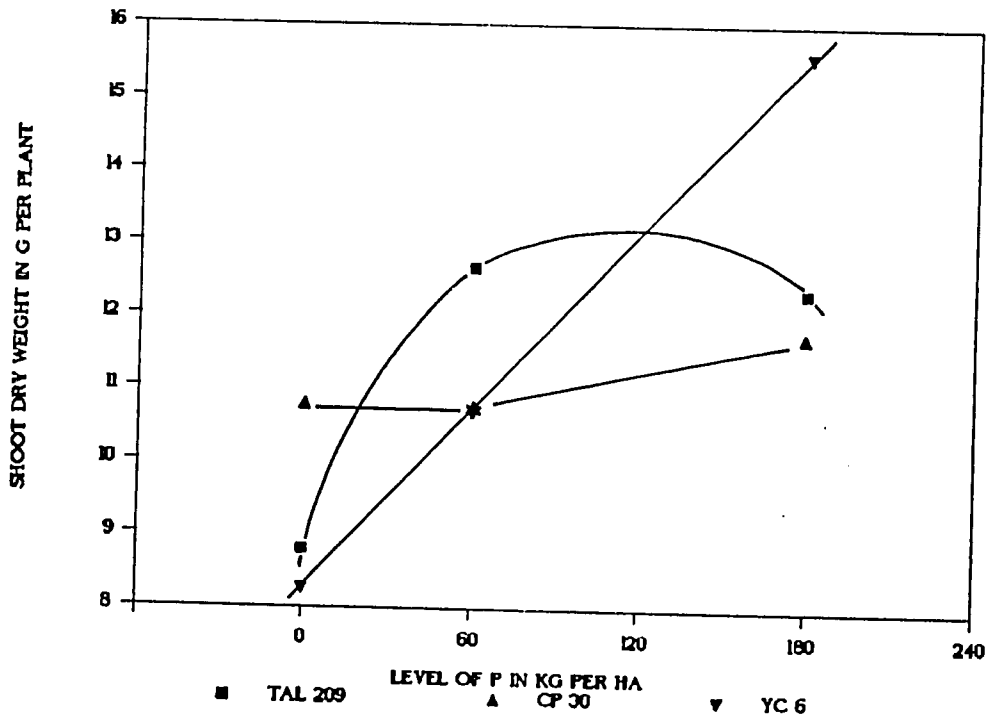
MC 6 : $y = 16.0 + 0.020\ p$

Table 3.34 Mean shoot dry weight of 60 day old cowpea inoculated with different strains at different P levels

Amount of P added (kg/ha)	INOCULANT STRAINS		
	TAL 209	CP 30 (g/plt)	YC 6
0	8.801	10.77	9.37
60	12.65	10.76	10.5
180	12.33	11.73	16.67

LSD = 21.094 g among strains.

Fig 3.25 Shoot dry weight of cowpea:60D



TAL 209 : $y = 8.8 + 0.0866\ p - 3.72 \times 10^{-4}$

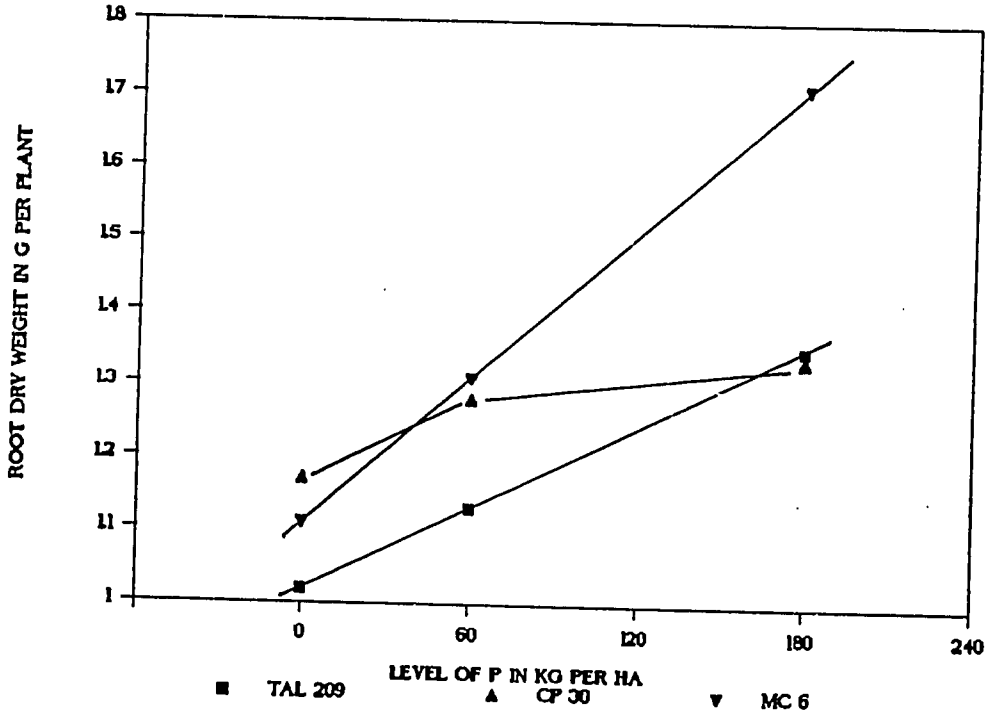
YC 6 : $y = 8.25 + 0.0406\ p$

Table 3.35 Mean root dry weight in g/plt in mungbean inoculated with different strains at three levels of P.

Rhizobial Strains	AMOUNT OF P ADDED KG/HA		
	0	60	180
	(Root D.wt. g/plt.)		
TAL 209	0.951	1.240	1.318
CP 30	1.173	1.278	1.335
MC 6	1.122	1.281	1.718

LSD = 0.114 g among strains

Fig 3.26 Root dry weight of mungbean:60



TAL 209 : $y = 1.02 + 0.0018\ p$

MC 6 : $y = 1.11 + 0.0034\ p$

In cowpea both interaction S x P and their main effects on the root growth were not significantly different over TAL 209. No significant difference were recorded even in pool data with increase in P level.

iii. <u>total N content</u>.

No significant difference was seen among strains in the total N content in plants after 60 days of cropping in mung bean at all levels of P except Mc 6 which differed significantly from others at 180 kg of P /ha with a mean total N content of 2.9878% compared to 2.72% in TAL 209.(table 3.36).Fig 3.27 shows the regression analysis of N content % on P level with different inoculant in mung bean.

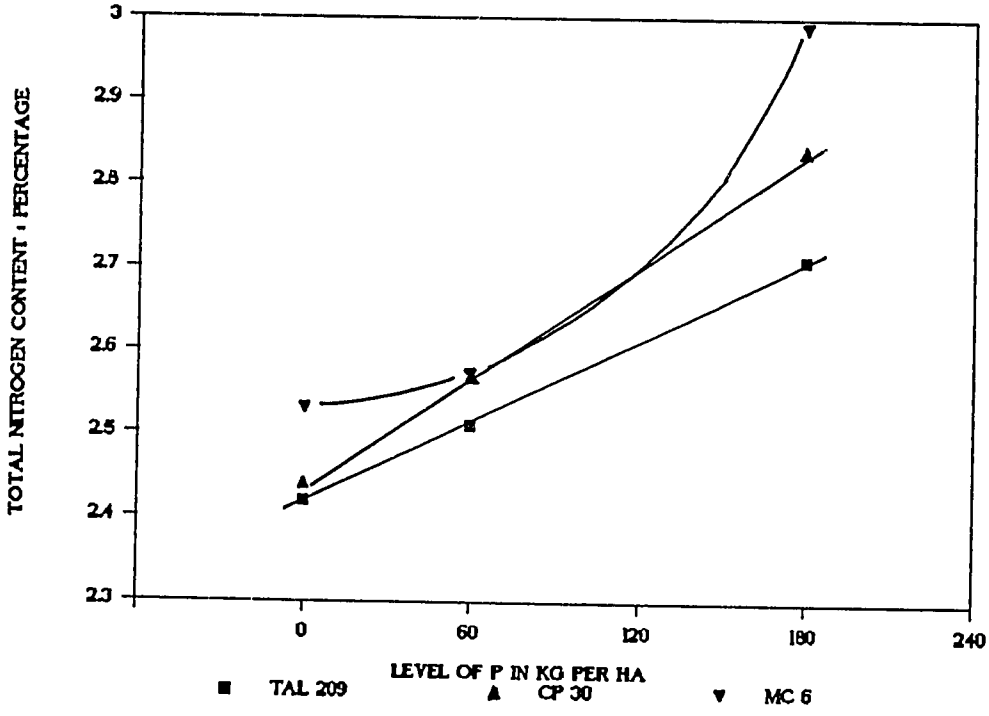
In cowpea however no significant difference among strains were obtained in N content at all levels of P.

Table 3.36 Mean N content (%) of mung bean at 60 DAS.

Rhizobial strains	Amount of P added		
	0 kg/ha	60 kg/ha (% N)	180 kg/ha
TAL 209	2.417	2.506	2.71
CP 30	2.441	2.569	2.843
MC 6	2.531	2.567	2.987

LSD = 0.2331 % among strains

Fig3.27 Nitrogen content of mungbean:60



MC 6 : $y = 2.53 + 3.25 \times 10^{-4}\, p + 1.59 \times 10^{-6}\, p^2$

iv. Nitrogen uptake.

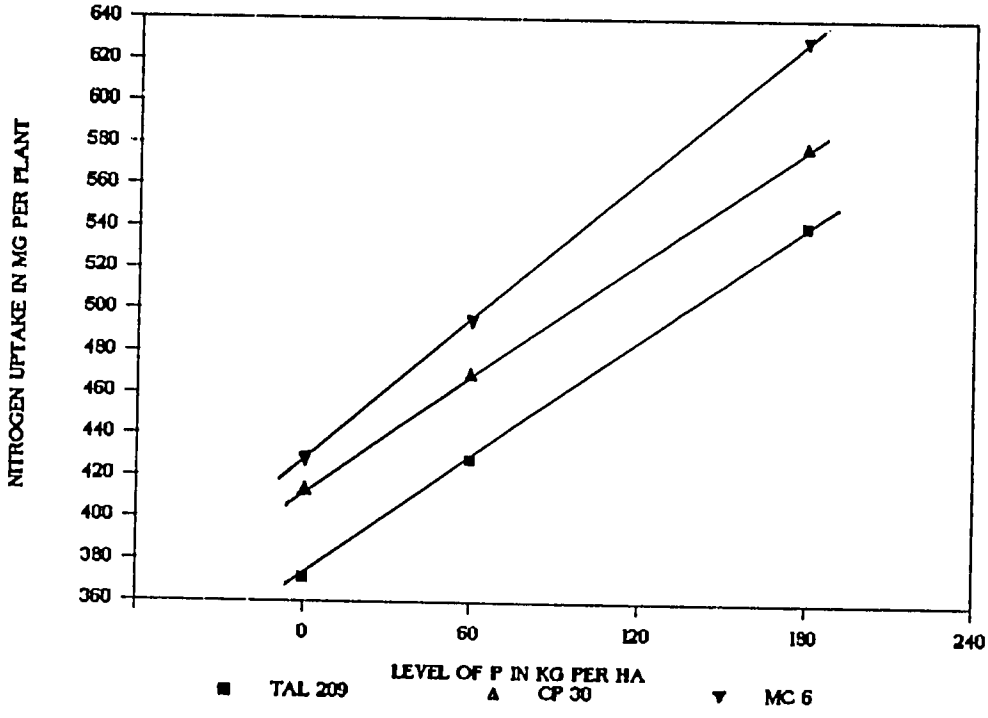
Positive linear relationships were observed for all levels of P for N uptake when mung beans were inoculated with test strains Fig 3.28). Both MC 6 and CP 30 had a significant influence in increasing N uptake in inoculated mungbean plants at 60 and 180 kg of P/ha. Performance of MC 6 was consistently better at all levels of P. (Fig 3.28 and table 3.37).

N uptake in cowpea is summarized in table 3.38. although CP 30 had a significant influence when no P was added YC 6 inoculated cowpea plants showed significantly higher N uptake at highest P level of 180kg /ha. Regression analysis revealed that TAL 209 reached maximum at 114.7 kg of P /ha (Fig. 3.29).

d. GRAIN YIELD

It is evident from table 3.39 that significantly higher mean grain yields were produced by CP 30 and MC 6 over standard strain TAL 209 when soil is not enriched with P fertilizers. The trend of higher grain yield in CP 30 and MC6 over TAL 209 was seen at other levels of P but it was significantly higher only in MC 6 at 180kg of P/ha. Higher rate of increase in yield was seen in MC6 inoculated plants (Fig 3.30) but the P x S interaction was non significant.

Fig 3.28 Nitrogen uptake in mungbean:60



TAL 209 : $y = 372 + 0.941\ p$

CP 30 : $y = 414 + 0.92\ p$

MC 6 : $y = 428 + 1.12\ p$

Table 3.37 Mean N uptake (mg/plt.) in mung bean at 60 DA

Rhizobial strains	Amount of P added in kg/ha		
	0	60	180
		(mg/plt)	
TAL 209	363.085	441.340	536.875
CP 30	403.167	485.217	574.083
MC 6	412.510	517.127	626.753

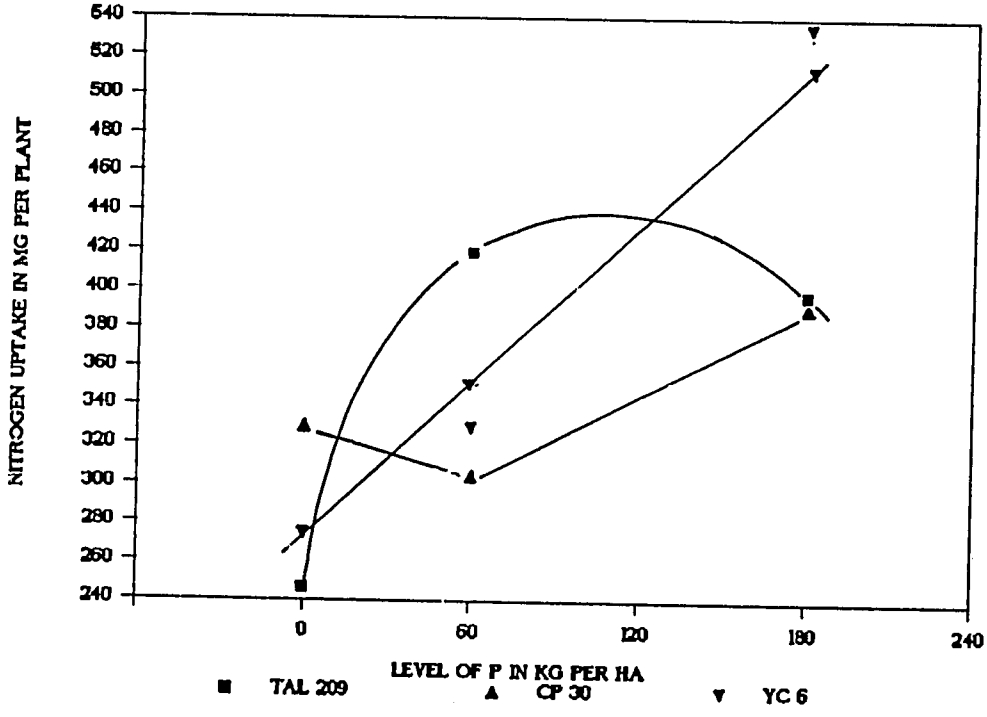
LSD = 53.819 mg among strains.

Table 3.38 Mean P uptake in cowpea (mg/plt.) at 60 DAS

Rhizobial Strains	AMOUNT OF P ADDED IN KG/HA		
	0	60	180
		(mg/plt.)	
TAL 209	246.822	419.569	397.818
CP 30	328.837	303.995	391.418
YC 6	275.103	328.751	535.028

LSD = 49.271 mg among strains

Fig 3.29 Nitrogen uptake in cowpea :60



$$TAL\ 209 : y = 247 + 3.9\ p - 0.017\ p^2$$

$$YC\ 6 : y = 261 + 1.48\ p$$

Table 3.39 shows the mean grain yield in cowpea inoculated with different strains at three P levels.As shown above the influence of strains CP 30 and YC 6 more prominent and significantly higher than standard TAL 209 when no P was added to soil. However their performance at average agronomic practices with 60 kg of P/ha was inferior and significantly lower than that of TAL 209. Yield increase by TAl 209 is significantly higher with addition of 60 kg of P/ha.(Table 3.39 and Fig. 3.31).

Strain YC 6 produced very high yield 2778.34 kg/ha in comparison to 1773.9 kg/ha by TAL 209 at 180 kg of P/ha.

3.8 Competitive ability of Rhizobia for nodulation

The results of the soil core tests conducted in order to evaluate the competitive ability of rhizobia for nodulation is summarized in table 3.40. No nodulation was seen in sterilized soils and as such only nodulation in other treatments are reported.

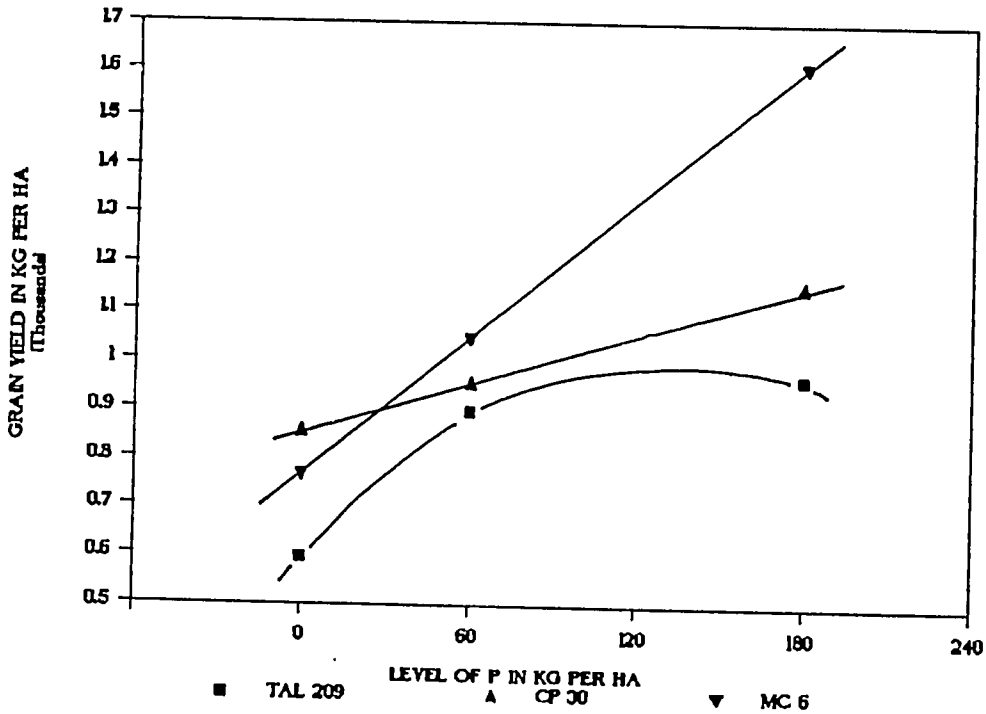
Introduced strains namely CP 30, TAL 209 and MC 6 or YC 6 were not detected in uninoculated plants. It is therefore evident from these results that strains used in inoculant were not found in native rhizobial population at test sites.

Table 3.39 Mean grain yield of mungbean and cowpea inoculated with different strains of rhizobia at various P levels at MI.

RHIZOBIAL STRAINS	AMOUNT OF P (KG/HA) ADDED TO SOIL AS TSP		
	0	60	180
		(yield kg/ha)	
MUNGBEAN			
TAL 209	595.94	896.396	965.606
CP 30	823.484	978.556	1141.231
MC 6	830.178	951.015	1669.859
COWPEA			
TAL 209	1311.515	2091.376	1773.913
CP 30	1475.256	1572.528	2045.423
YC 6	1497.871	1578.308	2778.343

LSD for mung bean = 176.411 kg among strains
cowpea = 158.347 kg among strains

Fig 3.30 Grain yield of mungbean

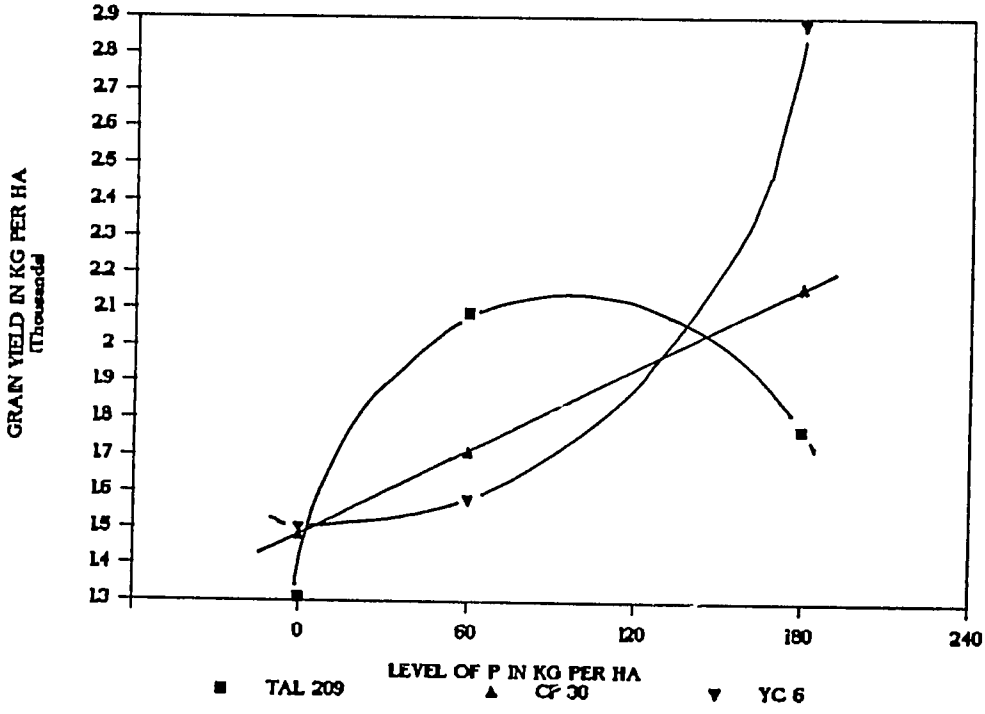


TAL 209 : $y = 596 + 6.52\ p - 0.225\ p^2$

CP 30 : $y = 854 + 1.68\ p$

MC 6 : $y = 766 + 4.68\ p$

Fig 3.31 Grain yield of cowpea



TAL 209 : $y = 1312 + 18.2\ p - 0.086\ p^2$

CP 30 : $y = 1484 + 3.78\ p$

MC 6 : $y = 1498 - 1.82\ p + 0.0527\ p^2$

Table 3.40 Competition of introduced strains of rhizobia for nodulation on mung bean.

Inoculant	Percentage occupancy of nodules		
	TAL 209	CP 30	MC 6
Uninoculated	0	0	0
TAL 209	64.9	0	0
CP 30	0	65.67	0
MC 6	0	0	68.1

LSD = 11.8 CV = 13.3% SD = 6.77

Table 4.41 Competition of introduced strains for nodulation on cowpea.

Inoculant	Percentage occupancy of nodules		
	TAL 209	CP 30	YC 6
Uninoculated	0	0	0
TAL 209	70.49	0	0
CP 30	0	72.37	0
YC 6	0	0	67.33

LSD = 20.75 CV = 21.96 SD = 11.74

Plants grown in all treatments were well nodulated. In mungbean 64.9%, 65.17% and 68% of nodules were found due to TAL 209,CP30 and MC6 respectively. In cowpea 70.49% was due to TAL 209, 72.36% was due to CP 30 and and 67.33 % due to YC 6.

3.9 Survival of Rhizobia in the field.

The results of the soil core tests conducted for the evaluation of survival ability of the rhizobia introduced at M.I.are summarized in table 3.42. The populations of the four strains tested were significantly reduced in number from one cropping season to other. The survival ability was highest in YC 6 a strain collected at Girandurukotte, Badulla district. Both MC 6 (collected from Girandurukotte) and CP 30 (Nalanda) were poor survivors. The survival ability of TAL 209 standard strain tested was poor and its occupancy in nodules was reduced by 66.54% and 55.98% in mung bean and cowpea nodules respectively in the interim cropless period between one season to the next.

Table 3.42 Survival of Introduced Rhizobia in MI soil during inter season period (end of cropping to begining of next season) !!

Rhizobial strain	Crop	% reduction of occupancy $
TAL 209	Mungbean	66.54
	Cowpea	55.98
CP 30	Mungbean	30.26
	Cowpea	31.29
MC 6	Mungbean	33.8
YC6	Cowpea	22.52

!! Survival is expressed as a percentage reduction in occupancy of nodules by introduced strain

$ Reduction in the percentage occupancy of nodules by test strain computed by difference in nodulation at the end of cropping season and at the begining of following season

3.10 Evaluation of rhizobia under wetzone field conditions

a. growth parameters.

Effect of inoculation of cowpea and mungbean with selected strains of rhizobia at various P levels on the growth parameters at Meewatura - Peradeniya field is summarized in table 3.43 and 3.44.

i. mungbean

Effect of inoculation with various strains of rhizobia on the increase in shoot dry weight was significantly different to each other at 20 DAS.

Strain CP 30 and MC 6 showed a significant influence in increase in shoot dry weight than the standard strain TAL 209. This was true for all P levels except at 60 P where CP 30 produced significantly low shoot dry weight.

Table 3.43 Root and shoot growth of mung bean inoculated with various rhizobial strains at different P levels at Meewatura field.

D A S	RHIZOBIUM	LEVEL OF P IN KG/HA					
		0		60		180	
		Shoot	Root	Shoot	Root	Shoot	Root
20							
	TAL209	0.275	0.025	0.407	0.375	0.48	0.05
	CP 30	0.338	0.029	0.33	0.035	0.525	0.065
	MC 6	0.305	0.03	0.41	0.365	0.605	0.065
	LSD	0.0651	0.0053				
40							
	TAL209	2.362	0.268	2.9	0.275	4.375	0.372
	CP 30	2.38	0.274	2.656	0.279	3.425	0.394
	MC 6	2.306	0.256	2.981	0.331	3.413	0.38
	LSD	0.6535	0.0651				
60	TAL209	4.855	0.375	5.387	0.443	6.281	0.856
	CP 30	4.825	0.425	5.868	0.462	8.931	0.85
	MC 6	5.097	0.394	5.531	0.43	6.637	0.706
	LSD	1.0687	0.0921				

LSD values are at p = 0.05 for main effects among strains.
Root and shoot dry weight is given as g/plt.

Table 3.44 Shoot and root growth of cowpea inoculated with various rhizobial strains at different P levels at Meewatura field

D A S	RHIZOBIUM	LEVEL OF P KG PER HA.					
		0		60		180	
		shoot	root	shoot	root	shoot	root
20							
	TAL209	0.294	0.04	0.387	0.059	0.668	0.080
	CP 30	0.35	0.03	0.7187	0.0438	0.919	0.088
	YC 6	0.381	0.047	0.406	0.038	0.456	0.063
	LSD	0.1063					
40							
	TAL209	2.944	0.281	3.263	0.388	3.513	0.488
	CP 30	1.919	0.3	3.025	0.419	3.819	0.394
	YC 6	2.819	0.294	2.238	0.381	3.363	0.481
	LSD	0.502	0.046				
60							
	TAL209	9.568	0.819	11.569	0.906	11.05	0.938
	CP 30	5.247	0.844	11.162	1.06	16.15	0.910
	YC 6	8.644	0.875	11.1	0.944	12.168	1.113
	LSD	1.333	ns				

LSD values at p = 0.05 among strains
Shoot and root dry weight are given as g/plant.

With maturity at 40 DAS however, TAL: 209 inoculated plants produced higher shoot dry weight 4.437 g/plant.

By and large the effect of CP 30 on the shoot growth was significantly higher at 60 DAS at 180 kg of P/ha. However no difference was detected at other Plevels. No significant difference was observed in P x Strain interaction either at 20,40 or 60 DAS. Similar trends were shown in root growth of mung beans.

ii. Cowpea

Increase in shoot growth was significantly higher in CP 30 inoculated plants at 20,40 and 60 DAS. These differences are prominent at higher P level (180) although at 0, P strain TAL 209 produced the highest shoot growth. In general root growth was not significantly different among different inoculant strains.

iii.biological nitrogen fixation.

Although the interaction at 60 DAS was not significant in cowpea higher N uptake was seen in both strains CP 30 and YC 6 inoculated plants. CP 30 performed equally well at 0,60 and 180 kg/ha. Both CP 30 and YC 6 were better adopted to wetzone conditions than TAL 209 (table 3.45 and 3.46).

iv. Grain yield

The grain yield (kg/ha) of inoculated mungbean and cowpea are shown in figures 3.32 and 3.33. Yield of cowpea crop inoculated with YC 6 was higher than TAL 209 and CP 30 at all P levels. Although no significant different in yield was seen between TAL 209 and CP 30 at 0,60 P levels the yield at 180 P approached that of YC 6 inoculated crops.

The interaction effects at 0.60 and 180 with all three strains were significantly different. Each strain performed better with increase in addition of P.

Table 3.45 Total nitrogen content and nitrogen uptake in mung bean plants inoculated with different strains of rhizobia at various P levels , at Meewatura.

D A S	RHIZOBIAL STRAIN	L E V E L O F P KG P E R HA					
		0		60		180	
		Total N %	Nitrogen uptake mg/plt	Total N %	Nitrogen uptake mg/plt	Total N %	Nitrogen uptake mg/plt
20							
	TAL209	2.58	7.949	2.79	12.47	2.81	15.24
	CP 30	1.95	7.101	2.21	7.893	3.31	19.44
	MC 6	2.10	7.100	2.73	12.24	4.01	26.92
	LSD	0.448	3.277				
40							
	TAL209	2.30	60.2	2.47	77.63	2.79	150.28
	CP 30	2.45	64.53	2.55	73.52	2.96	112.29
	MC 6	2.17	55.2	2.46	81.83	2.89	110.17
	LSD	0.285	21.08				
60							
	TAL209	1.37	68.97	1.43	84.61	1.48	107.88
	CP 30	1.38	72.09	1.52	96.25	1.99	196.01
	MC 6	1.34	73.98	1.48	88.06	1.59	112.05
	LSD	ns	22.97				

LSD values are at p= 0.05 among strains

Table 3.46 Nitrogen content (%) and N-uptake (mg/plt.) in cowpea plants inoculated with different rhizobial strains at various levels of P.

D A S	RHIZOBIAL STRAIN	L E V E L O F P KG P E R HA					
		0		60		180	
		Total N %	Nitrogen uptake mg/plt	Total N %	Nitrogen uptake mg/plt	Total N %	Nitrogen uptake mg/plt
20							
	TAL209	2.938	9.83	3.96	17.51	4.14	30.54
	CP 30	3.91	14.85	4.07	30.67	4.23	42.38
	YC 6	3.89	16.59	4.09	18.50	4.08	21.17
	LSD	0.378	4.573				
40							
	TAL209	2.14	69.44	2.67	98.66	2.32	92.4
	CP 30	2.28	49.29	2.29	78.82	2.64	11.34
	YC 6	2.28	71.37	2.72	69.94	2.82	109.76
	LSD	0.325	18.79				
60							
	TAL209	1.378	142.78	1.55	193.12	1.41	169.81
	CP 30	1.39	84.45	1.41	167.92	1.52	236.04
	YC 6	1.27	120.42	1.68	203.68	2.02	261.70
	LSD	0.312	43.97				

Fig. 3.32 Grain yield in cowpea -wz

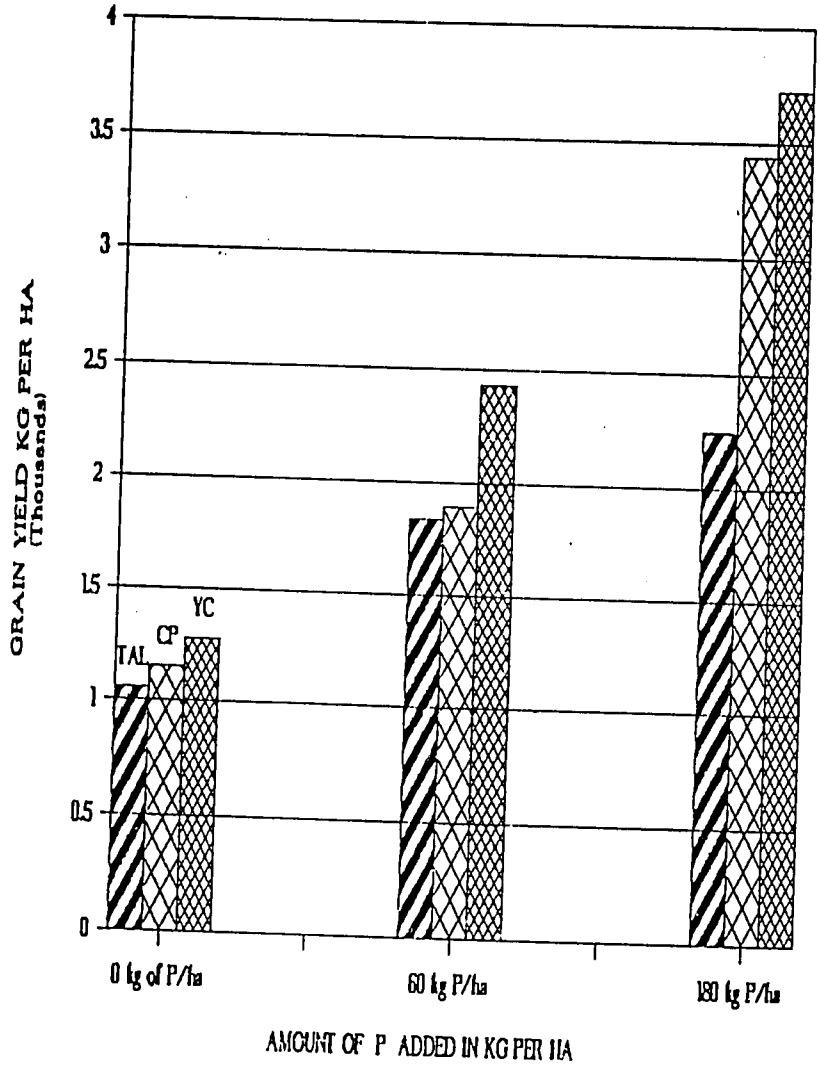


Fig 3.33 Grain yield of mung bean

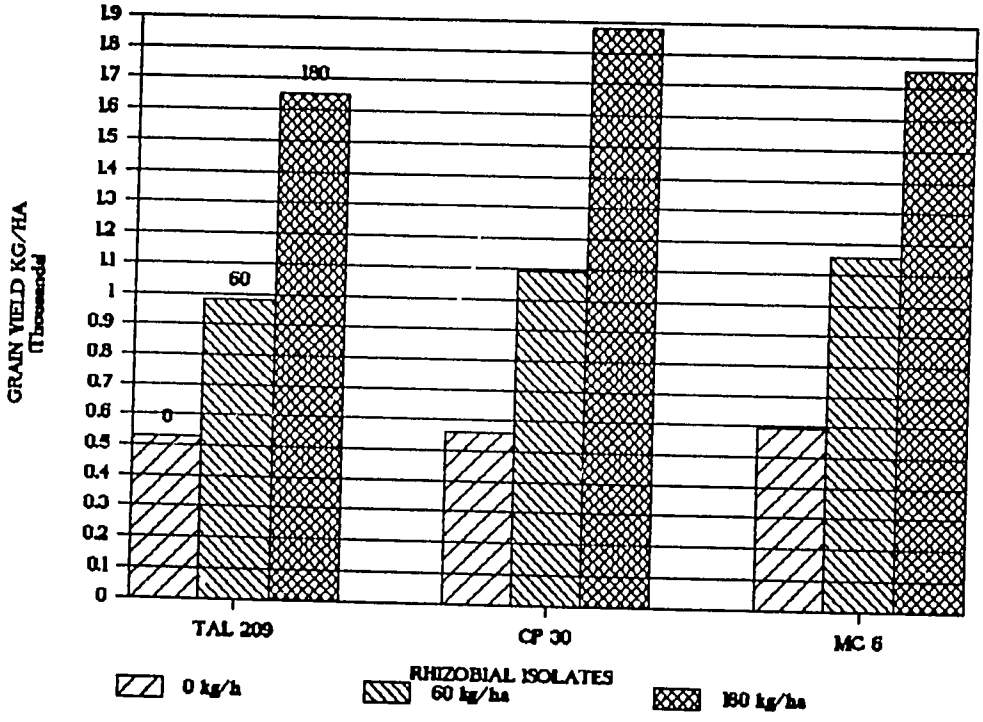


Table 3.47 Percentage of Phosphorus in cowpea plants inoculated with different strains at three P levels.

D A S	INOCULANT	LEVELS OF P KG PER HA		
		0	60	180
20				
	TAL 209	0.391	0.467	0.484
	CP 30	0.411	0.483	0.611
	YC 6	0.439	0.536	0.494
	LSD	ns		
40				
	TAL 209	0.382	0.418	0.406
	CP 30	0.385	0.386	0.422
	YC 6	0.313	0.472	0.459
	LSD	0.046		
60				
	TAL 209	0.183	0.288	0.229
	CP 30	0.178	0.204	0.231
	YC 6	0.178	0.234	0.261
	LSD	0.026		

LSD values are at p =0.05 among strains.

Table 3.48 Amount of P (%) in mung bean plants inoculated with different strains at three P levels.

D A S	INOCULANT	LEVEL OF P ADDED TO SOIL kg/ha 0	60	180
20				
	TAL 209	0.388	0.433	0.461
	CP 30	0.355	0.411	0.496
	MC 6	0.386	0.412	0.426
	LSD	0.065		
40				
	TAL 209	0.249	0.283	0.324
	CP 30	0.264	0.294	0.397
	MC 6	0.283	0.333	0.373
	LSD	ns		
60				
	TAL 209	0.203	0.304	0.328
	CP 30	0.281	0.302	0.372
	MC 6	0.281	0.297	0.355
	LSD	0.037		

All LSD values are at p = 0.05 among strains.

Percentage P in cowpea and mungbean plants are given in table 3.47 and 3.48 respectively. it is evident from these data that 180 kg of P /ha appears to in surplus of the requirements. Both Cp 30 inoculated mungbean and cowpea has utilized more P at all levels.

In mung bean however no difference in yield was seen among strains when no Phosphorus is added to soil. Both CP 30 and MC 6 inoculated crops produced a higher yield than TAL 209 inoculated plots at 60 kg of P per ha. With the increase in P level to 180 kg /ha differences were reduced to such an extent that only CP 30 was significantly different from TAL 209.

REFERENCES

Andrew, C.S. and Robins, M.F. 1969. *Australian Journal of Agric. Research.* 20, 665 - 674.

Bergersen F.J. 1980 . *Methods for evaluating Biological Nitrogen Fixation.* John Wiely Press, New York.

Bernaerts, M . J. and Deley, J. 1963. *Nature (London).* 197, 406 -407.

Chapmann, H.D. 1965. *Monograph: American Society of Agronomy.* 8,891

Gates,C.J. 1974. *Australian Journal of Botany* 22, 45 - 55

Geopfort,C.F. 1971. *Agron. Sulriogr* 7, 5-9.

Gibson,A.H. 1980. In Methods for Evaluating Biological Nitrogen Fixation ed. P.J.Bergerson, John Wiley, New York

Graham, P.H. 1976. In Symbiotic nitrogen fixation in plants ed. P.S.Nutman Cambridge Univ. Press. London

Josey, D.P., Beynon,J.L.,Jonston.A.W.B. and Beringer J.E. 1979. *Journal of Applied Bacteriology*.46,343 - 353.

Leonard, L.T. 1944 *USDA Circ*. No. 703. 8 pages.

Lennette, F.H.; Spaulding, E.H. and Truant, J.P. 1974 Manual for clinical microbiology. Am. Soc. for Micr. Washington.

Mosse, B. 1977 *New Phytol*. 78, 278 -279

Shaw N.H.; Gates, C.T. and Wilson J.R. 1966. *Australian Journal of Experimental Agriculture and Animal Husbandary* 6,149 -156.

Somasegaren, P. and Hoben, H.J. 1985.Methods in Legume Rhizobium Technology. University of Hawaii, NifTAL, 67-147

Tewari, J.P. 1965 *Experimental Agriculture* 1, 257 - 259

Vincent, J.M. 1970. IBP Hand Book No. 15. Blackwell Sci. Pubs. Oxford.

Vincent J.M. 1982. Nature and Basic Properties of Rhizobia in Nitrogen Fixation in Legumes. Academic Press, London

Vincent, J.M.,Whitney A.S. and Bose, J. (Eds) 1977. Exploiting the Legume Rhizobium symbiosis in Tropical Agriculture. Univ. of Hawaii Coll. Trop. Agric. *Misc. Publ.* 145. 469pp.

Walkey, A. and Black. I.A. 1934. *Soil Science* 37, 29

Acknowledgement

Helpful and kind assistance by NARESA officials especially Additional Director General Mr. L C de S Wijesinghe, Dr. Mahendra Watson and Ms. Swarna Prelis are gratefully acknowledged. The helpful attitude of the Accountant and the officers of the financial administration needs special mention.

I am grateful to Mr Oswin Silva of USAID Colombo and USAID office in Washington for their kind assistance and the financial help.