Materials & Methods
MATERIALS AND METHODS

COLLECTION OF SOIL SAMPLES FROM DIFFERENT ENVIRONMENTS

In the present study soil samples were collected from different environments (locations) such as, botanical garden (Bharathidasan University), polluted land (Tannery effluent), virgin land (cultivated) and coastal saline land.

The garden soil was collected at the botanical garden, Bharathidasan University, Suriyur campus, Tiruchirappalli - 620 024, Tamil Nadu. The soil was red loamy sand with a pH value of 7.9 and an EC value of 0.27 mS/cm. Polluted soil was collected in Sembattu village which is on the Trichy-Pudukkottai highway. The tanneries release a huge volume of waste water (effluent) containing organic and inorganic impurities. The wastes released from these industries are let out into barren lands and canals and sometimes infiltrate into neighbouring rice fields as well. The soil was clay in nature with a pH of 8.7 and EC of 4.4 mS/cm. The virgin soil sample with no history of cultivation before was collected in Kottappattu village near Airport, Tiruchirappalli. The soil was red loam with pH 7.6 and EC 1.20 mS/cm. The coastal saline soil was collected from Sembodai village of Vedaranyam, a small town in Nagapattinam District of Tamil Nadu at a distance of about 140 km from Tiruchirappalli. Sembodai (north) was comparatively more saline than other neighbouring areas with a pH of 8.2 and an EC of 2.09 mS/cm.
PLANT MATERIALS

Three commonly cultivated leguminous crops in Tamil Nadu such as

\textit{Vigna mungo} L.
\textit{Vigna radiata} L.
\textit{Vigna unguiculata} L.

belonging to the family Fabaceae (Leguminosae) were employed as experimental materials in the present study. The aforesaid pulses were selected mainly because of their economic importance and popular cultivation in the District of Tiruchirappalli, Tamil Nadu, India.

The leguminous seeds were procured from the National Pulses Research Centre, Vamban, Pudukkottai, Tamil Nadu, India. The Bharathidasan University, Botanical Garden was used for the field study.

EXPERIMENTAL DESIGN

a. Isolation of rhizobial strains from different soil samples as well as from root nodules.
c. Preparation of Bio-inoculants
The following aspects were investigated in detail:

1. Chemical analysis of the collected soil samples
2. Elemental analysis of soil samples
3. a) Determination of soil rhizobial population
   b) Determination of root nodule rhizobial population
4. Germination studies
5. Agrobotanical characters of 50 days old (Blackgram, green gram and cowpea).
6. Quantification of chemical constituents in leaves and nodules.
7. Assay of enzymes related to nitrogen metabolism in leaves and nodules
8. Economic yield and productivity.

PREPARATION OF THE EXPERIMENTAL PLOT

The experimental Plot was given a deep ploughing twice to a depth of 40 - 45 cm in order to loosen the soil. Nursery beds were prepared and watered regularly for one week before sowing the seeds. Bio-inoculated seeds (Inoculated with respective rhizobial strains) of *Vigna mungo*, *V. radiata* and *V. unguiculata* were sown in rows in nursery beds. The spacing between each row was 2'. The experimental plot has an area of 15' x 10' containing 5 rows. Systematic irrigation was given until harvest.
STUDY AREA, GEOLOGY AND CLIMATE

The field experiments were carried out during 1995 - 98 in the Botanical garden of Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. The study site is between latitude 8.64' E and 10.5' N at an elevation of 65 meters above MSL. Air, temperature, rainfall and percentage of relative humidity were obtained from the meteorological department, Government of India, Airport Weather Report Station, Meenambakkam, Madras, India. The climate is dry with an average annual rainfall of 2.27 mm. The area has a mean annual maximum and minimum temperatures of 34 and 24 ± 1°C respectively. The experimental site has a natural photoperiod of 26 ± 1 watts m⁻² S⁻¹ with day and night temperature of 28 ± 32°C and 22 ± 25°C respectively. The average annual relative humidity was 71.25% at 8.30 am.

ELEMENTAL ANALYSIS OF THE COLLECTED SOIL SAMPLES

Soil samples, collected from different locations were dried in an hot air oven at 100 ± 2°C for 48 h and then sieved (0.2 mm) to produce a homogeneous sample. 500 mg of each sample was taken separately in a boiling test tube and 15 ml of triple acid mixture (10 ml of concentrated nitric acid, 4 ml of perchloric acid and 1 ml of con. hydrochloric acid was added). The tubes were heated until complete ceaseover of the brown fumes. The samples were cooled to room temperature and diluted to 100 ml using
double glass distilled water. The samples were filtered through Whatmann No.1 filter paper (Singh, 1988). The filtered samples were analysed for the following elements through atomic absorption spectrophotometer (GBC pty Ltd., Australia).

Nitrogen (N)  
Phosphorous (P)  
Potassium (K)  
Calcium (Ca)  
Magnesium (Mg)  
Sodium (Na)  
Zinc (Zn)  
Copper (Cu)  
Manganese (Mn)  
Sulphur (S)  
Iron (Fe)  
Lead (Pb)

The PPM values thus obtained were converted to mg/g using the following formula:

\[
\text{mg/g} = \text{Percentage} \times 10
\]

\[
\% = \frac{\text{PPM} \times \text{Volume of the sample} \times 100}{\text{Weight of the sample} \times 100000}
\]

The following chemical analysis were performed in the collected soil samples.

pH  
Electrical Conductivity
Oxidisable Organic Carbon
Total organic matter
Nitrates
Nitrites

DETERMINATION OF ELECTRICAL CONDUCTIVITY (EC) AND pH OF THE SOIL

The pH and electrical conductivity of the soil samples were measured by addition of enough distilled water to soil samples to produce a soil : water ratio of 1 : 2 (20 g soil in 40 ml dist. H₂O). For measurement of pH, a digital pH meter (Hanna Instruments, USA) was used and EC was measured using an electronic digital conductivity meter (Global Electronics, Hyderabad, India).

DETERMINATION OF ORGANIC CARBON [Rapid titration method, Walkey and Black (1934)]

Five grams of soil sample were ground using pestle and mortar and the homogenate was passed through a 0.5 mm filter. 1 g of accurately weighed soil was transferred to 500 ml Erlenmayer flask. 10 ml of 1 N Potassium dichromate was added from a burette with an automatic zero and the sulphuric acid from an automatic pipette. The flask was shook for one minute and left for 30 min. Then 20 ml of water, 10 ml of phosphoric acid and 1 ml of diphenylamine indicator (0.5 g of diphenylamine dissolved in
mixture of 100 ml concentrated sulphuric acid and 20 ml water) solution, were added and titrated with ferrous sulphate from an automatic burette until the solution became purple. The titration was continued slowly until the colour flashed to green to which 0.5 ml of 1 N potassium dichromate was added and completion of titration was achieved by adding ferrous sulphate drop by drop until the last trace of blue colour disappeared. The amount of carbon oxidised was expressed as a percentage of the soil using the formula.

\[
\frac{V_1 - V_2}{W} \times 0.03 \times 100
\]

Where

\[V_1\] - Volume of 1 N potassium dichromate solution (ml)
\[V_2\] - Volume of 1N ferrous sulphate (ml)
\[W\] - Weight of the soil taken (g)

Total organic carbon = oxidisable organic carbon x 1.33
Percent organic matter = oxidisable organic carbon x 2

**DETERMINATION OF NITRATE**

The soil samples were dried in an hot air oven at 50 g of soil sample was taken in 500 ml Erlenmayer Flask. 250 ml of extraction reagent [(a)
12.5 CuSO₄ dissolved in 100 ml of water and (b) 0.6 g of AgSO₄ dissolved in 100 ml distilled water [20 ml of (a) and 100 ml of (b) were mixed and diluted with distilled water to make one litre of extraction reagent) was added and shaken for 5 min prior to addition of 1.0 g of Mg, CO₃. The contents were filtered through the filter paper (Whatman No.1) and the total volume of the filtrate was measured. 25 ml of the filtrate was evaporated to dryness over a water bath and 0.5 ml of phenol disulphuric acid was added to the residue and dissolved the later with the help of glass spatula. 5 ml of distilled water and 1.5 ml of 12 N KOH solution were added and stirred for thorough mixing, the yellow colour supernatant was taken and the absorbance read at 410 nm. Distilled water was used as blank. The nitrate conent of the filtrate was determined from the standard curve using anhydrous KNO₃. The total nitrate content of the soil was determined by the following formula.

\[
\text{NO}_3^- - N \text{ (mg g}^{-1}) = \frac{F \times V}{1000 \times W}
\]

Where,

\[
F = \text{NO}_3^- - N \text{ determined in the filtrate (mg L}^{-1})
\]

\[
V = \text{Total Volume of the filtrate (ml)}
\]

\[
W = \text{Weight of the dried soil}
\]
PREPARATION OF PHENOL - DISULPHURIC ACID REAGENT

20 g of white phenol was dissolved in 150 ml of conc. sulphuric acid. 85 ml of conc. H$_2$SO$_4$ was further added and heated for about 2 h over a waterbath, cooled to room temperature and stored in a dark bottle.

DETERMINATION OF SOIL RHIZOBIAL POPULATION

Soil samples collected from different environments was used to determine the rhizobial population. 1 g of respective soil was suspended in 10 ml sterile distilled water (1 : 10). This suspension was serially diluted from $10^{-1}$ to $10^{-8}$. 0.5 ml of suspension from $10^{-6}$ dilution was pipetted into yeast extract mannitol agar (YEMA) medium incorporated with congored in sterile glass petridishes.

COMPOSITION OF YEAST EXTRACT MANNITOL AGAR MEDIUM
(VINCENT, 1970)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>g/100ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>10.0</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>3.0</td>
</tr>
<tr>
<td>Calcium sulphate</td>
<td>0.1</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>0.5</td>
</tr>
<tr>
<td>Magnesium sulphate</td>
<td>0.2</td>
</tr>
</tbody>
</table>
Sodium chloride - 0.1 g
Yeast extract - 1.5 g
Agar - 15 g
Distilled water - 1000 ml
pH - 6.8 - 7.0

Sterilized congored solution (2.5 ml of 1% solution) was added aseptically to each litre of the melted medium, just prior to use. The plates were incubated at 26°C for 72 h for development of colonies.

The single colonies characterised by water and translucent of white opaque appearance without absorbing congored, were counted and total number of colonies g⁻¹ soil was calculated by multiplying the total number of colonies with the dilution factor.

ISOLATION OF RHIZOBIAL STRAINS FROM SOIL SAMPLES THROUGH PLANT ROOT NODULES

The collected soil samples approximately 1 kg were filled in polythene bags (30 cm x 20 cm). Uniform sized black gram, green gram and cowpea seeds were surface sterilized and sown in polythene bags. After 4 weeks of growth the plants were uprooted carefully and the roots washed in tap water to remove soil particles. A well formed healthy pinkish nodule on the tap root was carefully cut out with a portion of the root attached to the nodule.
They were exposed momentarily (5 - 10 sec) to 95% ethanol and then immersed in 0.1% mercuric chloride. The nodules were then transferred to sterile glass tubes containing 1 - 2 ml of sterilized distilled water. Using one end of the presterilized glass rod, the nodules were crushed and the milky juice, extract was allowed to mix up with the sterile distilled water. From this serial dilutions were made and 0.5 ml from eight fold diluted series was pipetted into yeast extract mannitol agar (YEMA) medium incorporated with congo red as described earlier on sterile petriplates. The plates were incubated at 26°C for 48 to 72 h (Gambaks BOD incubator, Madras) for the development of colonies. Those single colonies characterised by watery translucent white opaque appearance without absorbing the congo red were picked out and streaked on other petriplates thrice for purity and then transferred to YEMA slants.

**PURITY CHECK ON RHIZOBIUM**

The following tests were carried out to check the purity of the cultures.

1. *Growth in Hofer's Alkaline Broth* (Hofer, 1935)

A loopful of the isolate was inoculated into 25 ml of Hofer's alkaline broth (yeast extract mannitol broth adjusted to pH 11.0) and the growth was observed. The positive reaction for Rhizobium was indicated by the absence of growth in the medium.
Hofer's alkaline broth was prepared by adjusting the pH of YEM broth to 11.0 by adding 28 ml of 1 N NaOH and 1 ml of 1.6% Bromothymolblue (alcoholic solution).

2. *Production of Acid or Alkali*

Bromothymolblue was incorporated in 5 ml of a 0.5% alcoholic solution per litre of YEM medium giving a final concentration of 25 ppm and green colour (pH 6.8). A loopful of the isolate was streaked into sterile petriplates containing the above medium and incubated at 26°C for 4 - 6 days. Change of colour of the medium to deep blue as the colonies developed indicated production of alkali by slow growing rhizobia whereas the development of yellow colouration indicated acid production by the fast growers.

3. *Growth in Glucose Peptone Broth*

A loopful of isolate was transferred to 25 ml of glucose - peptone broth of the following composition.

*Composition of Glucose peptone Broth:*

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Bromocresol Purple</td>
<td>10.0 ml</td>
</tr>
<tr>
<td>(1% alcoholic solution)</td>
<td></td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

37
Rhizobium grows poorly in this medium causing little change in pH, poor growth indicates the presence of Rhizobium.

**GROWTH IN NUTRIENT AGAR MEDIUM**

Purity of the *Rhizobium* culture was checked by plating on nutrient agar medium. Colonies did not grow on nutrient agar indicating purity of rhizobia.

*Composition of nutrient agar medium*:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef extract</td>
<td>1.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Sodium Chloride</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

The pH of the medium was adjusted to 6.0.

**IDENTIFICATION OF RHIZOBIAL STRAINS**

Purified cultures of root nodule rhizobia of greengram, blackgram and cowpea, isolated from different soils were identified at the Centre for Advanced Studies (CAS) in Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India.
GROWTH CURVE

Purified cultures of rhizobia were inoculated in YEM media for time-course study on growth of the microbes. Incubations were made by adding 1 ml of the inoculum having an absorbance of 0.1 O.D to 100 ml of the medium. At different time intervals ranging from 8 - 96 h after inoculation, growth was monitored by measuring turbidity at 520 nm.

Rhizobial cells and root nodules were observed and photomicrographed by using Nikon SMZ 2T stereo microscope and Nikon Eclipse E 400 with Nikon FD X 35 H III photographic unit.

NODULATION TEST BY LEONARD'S JAR METHOD

The modified Leonard Jar assembly consisted of 700 ml capacity beer bottle with lower portion cut off. This was inverted into a glass bottle of half litre capacity. The beer bottle was filled with 900 grams of sterilised sand, after plugging the neck with cotton wool. This was the growth medium. The growth medium was irrigated by a centrally positioned cotton wick which ran through the length of the bottle and extended out of the mouth of the bottle into the reservoir containing the nutrient solution. The complete assemble was sterilized.
Preparation of Jensen's nutrient solution

- \( \text{CaHPO}_4 \) - 1.0 g
- \( \text{K}_2\text{HPO}_4 \) - 0.2 g
- \( \text{MgSO}_4 \) - 0.2 g
- \( \text{NaCl} \) - 0.2 g
- \( \text{FeCl}_3 \) - 0.1 g
- Dist Water - 1000 ml
- pH - 6.8

STERILIZATION OF SEEDS

Undamaged clean greengram, blackgram and cowpea seeds were selected to a reasonably uniform size and rinsed with 95% ethanol and immersed for 3 minutes into 0.1% HgCl solution. The seeds were then washed thoroughly with three changes of sterile water and planted directly into the Leonard jar, bottle. A petridish was placed on the top of the inverted bottle to protect against contamination and maintain the surface in a moist condition. A black paper was wrapped around the bottles to protect the root from the light and algal growth. The units were placed in a favourable place for germination and growth.

As soon as the seedlings were established, the petridish was removed from the top and 10 ml of one week old rhizobial inoculum was introduced.
around the seedlings and the units were left open. Uninoculated and nitrate controls were provided in the usual way. The diluted nutrient solution in the reservoir was maintained until the completion of the experiment.

The plants were uprooted from the units carefully after five weeks of growth, infection and nodulation were analysed.

PREPARATION OF BIO-INOCULANTS

The inoculation of legume seeds prior to sowing involves spreading a sufficient number of specifically invasive and effective rhizobia on the seed surface so as to increase the chances of the young plant being nodulated by selected bacteria. The aim is to establish in the rhizosphere of the seedling a vigorous population of the applied strain in order to achieve early and effective nodulation.

Respective strains of rhizobia were grown on YEM medium independently in large flasks on a shaker for five days. The farm yard manure (FYM) was powdered, sieved (through 100 mesh sieve) and autoclaved at 15 lbs pressure for 4 hours. After cooling, a high count rhizobial broth was mixed so as to attain a 40% moisture holding capacity of the carrier. The carrier and the broth were mixed manually and cured in trays for 2 days and packed in polythene bags.
METHODS OF INOCULATION

Carrier based, respective cultures (inoculum) were independently mixed with minimum amount of water to form a slurry (gum arabic) and seeds were added to the slurry so as to uniformly coat the seeds with the inoculant.

PREPARATION OF ADHESIVE

100 g gum arabic was dissolved in 250 ml distilled water and boiled for half an hour. The boiled solution was vigorously stirred for 30 min and cooled before adding the inoculum. This solution served as a good adhesive for rhizobial cell on the seeds.

PREPARATION OF PELLETED SEED

Finely ground calcium carbonate sieved through 300 mesh was added to freshly inoculated wet seeds in a container and mixed rapidly for 2 min until seeds were evenly coated. The lime coated seeds appeared as white tablets and they were allowed to harden for an hour by spreading on a clean surface.

SEED GERMINATION

Healthy and uniform-sized pelleted seeds (Bio-inoculated with respective rhizobial strains) were sown in the field and watered daily for
germination studies. The percentage of germination was recorded using the following formula in all treatments after 7 days of germination.

\[
\% \text{ of germination} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds sown}} \times 100
\]

AGROBOTANICAL CHARACTERS OF 50 DAYS OLD LEGUME CROPS

The plants after 50 days of growth were uprooted carefully the roots washed with tap water to remove soil particles rinsed thrice with excess distilled water and blotted dry. The following agrobacterial characters like shoot length (cm) root length (cm) leaf area (cm\(^2\)) number of root nodules, and total dry matter production were analysed in both the control and bio-inoculant-treated plants.

DETERMINATION OF LEAF AREA

After 50 days of growth, the leaves were collected and the leaf area was analysed using leaf area meter (Systronics).

DETERMINATION OF DRY MATTER PRODUCTION

Plant parts were separated individually and dried in an oven at 60.2°C for 48 h and weighed to determine dry matter production.
PRODUCTIVITY STUDIES

To evaluate the effects of inoculation, productivity was worked out at maturity (120 days old blackgram, green gram and cowpea plants).

The Economic yield characters studied were:

- Total number of pods plants$^{-1}$
- Total dry weight of the pods (g plant$^{-1}$)
- Total dry weight of seeds (g plant$^{-1}$)
- Total dry weight of the pod wall (g plant$^{-1}$)
- Dry weight of 100 seeds.

INDUCED SALT-STRESS EXPERIMENT (POT CULTURE EXPERIMENT)

To find out optimal level of tolerance of rhizobia to salinity, pot culture experiment was undertaken. Earthen pots were lined with polythene bags and each pot was filled with garden soil. The seeds of the two legumes, greengram and cowpea were inoculated with different soil rhizobial strains isolated through root nodules (garden, virgin, polluted and saline soil) and sown at the rate of 10 seeds per pot. Thinning was done after the establishment of seedlings so as to have only four plants of uniform size in each pot. The pots were arranged in rows and each pot was treated with saline solutions of various concentrations i.e. 2, 4, 6, 8 and 10 mS/cm
respectively after one week of establishment of seedlings. Different levels of salinity were prepared by adding NaCl, CaCl$_2$ and Na$_2$SO$_4$ in appropriate quantity (Na$^+$, Ca$^{2+}$ and Cl$^-$ : SO$_4^{2-}$ ratio as 4 : 1) using digital conductivity meter (Global electronics, Hyderabad, India). The solutions were added in excess of the volumes required for saturation of soil in each pot. Irrigation with artificial saline water at different strengths was done once in 3 days. The control set of pots were irrigated with tapwater only.

On the 50th day the following parameters were studied:

a. Number of Nodules  
b. Leghaemoglobin content of nodules  
c. Nodular nitrogen content

**QUANTIFICATION OF CHEMICAL CONSTITUENTS IN NODULES AND LEAVES**

The leaves and nodules were harvested from 50 days old blackgram, greengram and cowpea plants and shade-dried for about a week in laboratory temperature of 28 ± 2°C and relative humidity of 50 - 55%. The samples were powdered and the following chemical substances were quantified and expressed as mg g$^{-1}$. 
For determination of chloroplast pigments, fresh and healthy leaves from the 5th node from top were used.

The content of leghaemoglobin was determined in fresh and healthy nodules.

DETERMINATION OF CHLOROPLAST PIGMENTS

100 mg of leaf tissue was washed in distilled water blotted dry with filter paper and ground with 5 ml of 80% (v/v) aqueous acetone using mortar and pestle. The homogenate was centrifuged at 5000 rpm for 10 min at 5°C using a refrigerated centrifuge (plastocrafts). The supernatant was saved and the pellet reextracted repeatedly with the same solvent until the pellet
retained no green colour. The supernatants were pooled and made upto a total volume of 25 ml with 80% aqueous acetone. The absorbance was read in a spectrophotometer at 663 and 645 nm and the contents of chlorophyll, 'a' and 'b' and total chlorophyll were calculated using the following formulae (Arnon, 1949).

\[
\text{Total chlorophyll (mg/g fw)} = \frac{20.2 \times \text{OD} 645 + 8.02 \times \text{OD} 663}{a \times 1000 \times w} x V
\]

\[
\text{Chlorophyll 'a' (mg/g fw)} = \frac{12.7 \times \text{OD} 663 - 2.69 \times \text{OD} 645}{a \times 1000 \times w} x V
\]

\[
\text{Chlorophyll 'b' (mg/g fw)} = \frac{22.9 \times \text{OD} 645 - 4.68 \times \text{OD} 663}{a \times 1000 \times w} x V
\]

Where,

- \( a \) = length of light path in the cell (1 cm)
- \( V \) = volume of acetone extract in ml
- \( w \) = fresh weight of the sample in g.
DETERMINATION OF LEGHAEMOGLOBIN (LHb)

Fresh and healthy root nodules were collected. About 500 mg of nodules was extracted three times, each time with 3 ml of Drabkin's solution prepared by adding 52 mg of potassium cyanide, 198 mg of potassium ferri cyanide and 1 g of sodium bicarbonate to 1000 ml of distilled water (Wilson and Reisenauer, 1963). The nodule samples were centrifuged at 5000 rpm using refrigerated centrifuge at 5°C for 15 min to remove the nodule debris. The resulting supernatant was again centrifuged at 10,000 rpm for 20 min at 5°C and the resulting clear supernatant was used for LHb determination.

Leghaemoglobin was quantified colorimetrically by reading the absorbance at 540 nm of the nodule soluble fraction in a spectronic 2000 (Baush and Lomb, USA). Two corrections were made for the contribution of turbidity and biliverdin like pigments to absorption at 540 nm namely filtration through a millipore filter (0.7 μM pore diameter) and measurement of A540-½ (A540 + A560). The result was expressed as O.D. Units per gram fresh weight nodules.

EXTRACTION OF TOTAL SOLUBLE SUGARS, FREE AMINO ACIDS AND TOTAL PHENOLS

500 mg nodule / leaf was ground with 10 ml of 80% methanol using pestle and mortar and the homogenate was centrifuged at 5000 rpm using Remicentrifuge (RXC, Bombay) for 10 min. The supernatant was saved
and the pellet reextracted twice with the same volume of the solvent. The supernatants were pooled and used for estimates of total soluble sugars, total free amino acids, total phenols and sucrose. When the supernatent was rich in chloroplast pigments, methanol fraction was added half the volume of petroleum ether and through a separating funnel the petroleum ether layer containing chloroplast pigments was decanted and discarded.

ESTIMATION OF FREE AMINO ACIDS

To 0.5 ml of the above sample (80% methanol extract) was added 0.1 ml of 80% (v/v) phenol and kept in a boiling water bath for 10 min and to the mixture ninhydrin, 0.2 ml of 0.5% (w/v) was added and again kept in a boiling water bath for 10 min. The mixture was then cooled and made upto 10 ml volume with 60% (v/v) ethanol and the absorbance was read in a spectrophotometer at 575 nm. The concentration of free amino acids was determined using L - Glycine as the standard (Troll and Canon, 1953).

ESTIMATION OF TOTAL SOLUBLE SUGARS

To 0.5 ml of the sample, 1.0 ml of 5% (v/v) phenol and 5.0 ml of Conc. H$_2$SO$_4$ were added and the final volume was adjusted to 15 ml with double distilled water and the absorbance was read in a spectrophotometer at 490 nm. The concentration of total soluble sugars was calculated using glucose as the standard (Dubois et al., 1956).
ESTIMATION OF TOTAL PHENOLS

To 0.5 ml of the sample 3.0 ml of 10% (v/v) Folin-phenol reagent and 1.0 ml of saturated NaCO₃ were added. The reaction mixture was kept at 50°C for 15 min and the absorbance was measured in Spectrophotometer at 660 nm using catechol as the standard (Swain and Hillies, 1959).

ESTIMATION OF SUCROSE

To 0.5 ml of the sample 0.1 ml of 30% (w/v) aqueous KOH was added and kept in a boiling water bath for 10 min. The samples were cooled and 3.0 ml of anthrone reagent (200 mg anthrone dissolved in 100 ml of cold 95% H₂SO₄) was added and kept at 40°C for 10 min. The absorbance was read in a spectrophotometer at 620 nm. Total content of sucrose was calculated using glucose as the standard (Van Handel, 1968).

ESTIMATION OF TOTAL SOLUBLE PROTEINS

500 mg of plant material was ground with 10 ml of distilled water using pestle and mortar and the homogenate was centrifuged at 5000 rpm in a refrigerated centrifuge (Plastocrafts) for 10 min. The supernatant was stored and the pellet reextracted and to the combined supernatant, an equal volume of 10% (w/v) trichloro acetic acid (TCA) was added, centrifuged at 5000 rpm and the supernatant discarded. The precipitate was dissolved in 2 ml of 0.1 N NaOH and used for protein estimation. To 0.5 ml of the sample 0.5 ml
of 0.1% (w/v) CuSO$_4$, 2.5 ml of 12.5% (w/v) Na$_2$CO$_3$ and 0.5 ml of 25% (v/v) folin-phenol were added and the final volume was adjusted to 5.0 ml with distilled water. The reaction mixture was read in a spectrophotometer at 740 nm. Total soluble protein were calculated using Bovine serum albumin (BSA) as the standard (Lowry et al., 1951).

ESTIMATION OF TOTAL NITROGEN (Umbriet et al., 1972).

The dried plant material was ground in a porceline mortar and pestle and the total nitrogen content was estimated following the modified microkjeldahl method.

10 mg of powdered plant material was taken in a microkjeldahl flask. A pinch (about 50 mg) of catalyst (Humphries, 1956) (CuSO$_4$, 5H$_2$O - 1g, K$_2$SO$_4$ - 8 g; SeO$_2$ - 1g ground separately and mixed together) and 0.5 ml of Con. H$_2$SO$_4$ were introduced into the kjeldahl's flask. The flask was gently heated on digestion rack until the fumes of the H$_2$SO$_4$ were evolved. It was then heated strongly until the digest in the flask turned to an apple green colour. After cooling the digest was made upto 20 ml with double distilled water.

To 1 ml of the above diluted digest, 2 ml of water, 2 ml of colour (Nessler's) reagent and 3 ml of 2 N NaOH were added in series. After 5 min, the absorbance of the solution was read at 490 nm against a reagent blank. Quantities of nitrogen in the samples were determined with reference to a standard prepared using NH$_4$Cl.
EXTRACTION AND ESTIMATION OF NITRATE AND NITRITE (Wooley et al., 1960).

Dried powder (100 mg) of leaves were boiled for 10 min in 5 ml of distilled water. The extract was filtered and the filtrate was used for nitrate and nitrite estimation.

1 ml of the aqueous extract was added to 9 ml of 20% (v/v) glacial acetic acid, containing 0.2 ppm of copper as CuSO₄. To each sample was added 1.0 g of salt mixture (Nelson et al., 1954). The salt mixture was prepared by mixing thoroughly the finely ground chemicals as given below.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Amount (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barium sulphate</td>
<td>100</td>
</tr>
<tr>
<td>Citric acid</td>
<td>75</td>
</tr>
<tr>
<td>Sulphanitic acid</td>
<td>4</td>
</tr>
<tr>
<td>MnSO₄·7H₂O</td>
<td>10</td>
</tr>
<tr>
<td>Zinc powder</td>
<td>2</td>
</tr>
<tr>
<td>1-Naphthylamine</td>
<td>2</td>
</tr>
</tbody>
</table>

Blank (without the extract) was also run simultaneously. Tubes containing the assay mixtures were shaken at least thrice at every 3 min interval using shaker and finally centrifuged at 3000 rpm for 10 min. The absorbance of the clear, supernatant was read at 520 nm against a reagent blank. The same procedure was repeated for the same sample omitting zinc, manganese sulphate, copper sulphate and this second run gave the
quantity of nitrite alone present in the leaf tissue. The first reading (optical density) minus the second reading (optical density) gave the quantity of nitrate present in the sample. Nitrate and nitrite amounts were calculated from the standard graphs constructed using analar potassium nitrate and sodium nitrite respectively.


1 g of dried plant material was taken in a large test tube and 10 ml of extraction solution containing equal volumes of ethanol and 0.1 M potassium phosphate buffer pH 7.0 was added. The test tubes were kept over a boiling water bath for 5 - 10 min and then cooled to room temperature. The sample was centrifuged at 1000 rpm and the supernatant used for analysis of total ureides, allantoin and allantoic acid.

1. CONVERSION OF ALLANTOIN TO ALLANTOATE

To 5.0 ml of the above sample, 1.0 ml of 0.5 N NaOH was added, mixed and heated at 100°C for 5 min in a boiling water bath.

2. CONVERSION OF ALLANTOATE TO GLYOXYLIC ACID

To the sample from step 1, 1.0 ml of 0.65 N HCl was added, mixed and heated at 100°C for 10 min.
3. **NEUTRALISATION OF HYDROLISED SAMPLE**

To the sample from step 2, 1.0 ml of 0.4 M potassium phosphate buffer pH 7.0 was added.

4. **CONVERSION OF GLYOXYLATE TO GLYOXYL PHENYLHYDRAZONE**

To the neutralised sample 1.0 ml of phenyl hydrazine solution (0.1 g of phenylhydrazine HCl in 30 ml of water) was added and allowed to stand at room temperature for 5 min.

5. **CONVERSION OF GLYOXYL PHENYLHYDRAZONE TO DIBENZYL FORMATION**

The sample from step 4, were cooled in an ice bath and 5 ml of pre-cooled concentrated HCl was added and stirred for a while and then added 1.0 ml of freshly prepared ferric cyanide solution (0.5 g of potassium ferric cyanide dissolved in 30 ml of water) and stirring was continued. The pink colour developed was measured at 545 nm. The concentration of total ureides was determined from the standard using allantoin (sigma - pfs) 5 - ureidohydration).
ALLANTOIC ACID DETERMINATION

For allantoic acid determination step 1 was deleted and 1.0 ml of 0.5 N NaOH was immediately added prior to step 3.

ALLANTOIN DETERMINATION

Allantoin determination was made from the difference between the total ureide and allantoic acid contents.

ANALYSIS OF LEAF PHOTOSYNTHESIS

After 50 days of growth, the rate of leaf photosynthesis was measured on fully expanded leaves using a closed gas exchange portable Infrared Gas Analyser (IRGA) (Li-Cor 6252, Inc, USA). The chamber position after inserting the leaf was identical to the natural position of the leaf. Care was taken to avoid any moisture on the leaf surface. The leaves were exposed between 11.00 and 13.00 hours under direct natural sunlight. The CO₂ uptake was expressed as μ moles m² Sec⁻¹.

ASSAY OF ENZYMES

Fresh leaves and nodules from 50 days old (Blackgram, greengram and cowpea) leguminous crops were collected and assayed for the following enzymes.
a. Nitrogenase (N\textsubscript{2} - ase)
b. Nitrate reductase (NR)
c. Nitrite reductase (NIR)
d. Glutamine synthetase (GS)
e. Glutathione reductase (GR)

ASSAY OF NITROGENASE IN ROOT NODULES

Nitrogenase activity was determined by the acetylene reduction assay (Hardy et al., 1968). 1 g of healthy and fresh nodules were taken in experimental vials of 18 ml capacity and sealed with serum caps. The gas phase was replaced by acetylene using a clean syringe and the nodules were incubated for 1 h at 30°C. Gas samples (1 ml) were analysed in a gas chromatograph (Hewlett Packard 5890 USA) fitted with stainless steel column packed with Poropak N and flame ionization detector and with dry nitrogen as a carrier gas. The peak height of ethylene formed was measured and the results were expressed as n moles of ethylene produced g\textsuperscript{-1} f w h\textsuperscript{-1}.

ASSAY OF IN VIVO NITRATE REDUCTASE ACTIVITY

In vivo NR activity was determined by the method of Hageman and Hucklesby (1971). Freshly harvested leaves/nodules of 50-day plants were washed in distilled water and cut out into small leaf discs (1 Cm) or thin sections of nodules. The leaf discs or slices of leaves/nodules (100 mg)
were vacuum- infiltrated for 3 min in 5.0 ml of 0.1 N phosphate buffer (pH 7.5) containing 0.1 M KNO₃ and 5% n-propanol. After infiltration the vials were incubated for 30 min in dark at 30°C.

For blank, 1.0 ml of sample was taken from the 0.1 M phosphate buffer after vacuum infiltration ('0' time). The concentration of nitrite released to the incubation medium was determined by adding 1.0 ml of 1% (w/v) sulphanilamide in 3 N HCl and 1.0 ml of 0.02% (w/v) N- (1-Napthyl) ethylenediamine dihydrochloride to 1.0 ml of sample from the incubation medium. After 10 min the optical density was measured at 550 nm in a spectrophotometer with potassium nitrite as the standard. NR Activity was expressed as μ moles nitrite released mg⁻¹ protein h⁻¹.

**ASSAY OF NITRITE REDUCTASE**

The reaction mixture contained 6.25 ml of 0.5 N Tris HCl pH 7.5, 4.32 mg of NaNO₂, 6.01 mg of methyl viologen and 25 ml of distilled water. The blank was without the enzyme extract. The reaction was initiated by the addition of 0.2 ml of a freshly prepared sodium dithionite solution containing 25 mg of Na₂S₂O₄ in 1.0 ml of 25 mg/ml NaHCO₃ solution. The reaction mixture turned blue as methyl viologen got reduced by sodium dithionite. The reduced methyl viologen acts as the donor for the reduction of NO₂ to NH₄ catalysed by nitrite reductase (Finka and Warner, 1977). The blue coloured reaction mixture was incubated for 10 min and at the end of the incubation period, the reaction was stopped by vigorously shaking
the reaction mixture in a vortex until the blue colour disappeared. From this an aliquot of 20 μl was taken and added to 1 ml of water to which 1 ml of 1.1 (w/v) sulphanilamide in 3 N HCl and 1.0 ml of 0.02% (w/v) N - (1-Napthyl) ethylenediamide dihydrochloride were added. The absorbance of pink colour developed was read at 540 nm and the amount of nitrite disappeared was calculated from the standard graph. The enzyme activity was expressed as μ moles nitrite disappeared mg⁻¹ protein min⁻¹.

ASSAY OF GLUTAMINE SYNTHETASE (Robinson et al., 1991).

Cell free extracts were prepared from leaves and nodules by grinding them separately in chilled pestle and mortar using 0.01 M potassium phosphate - KOH buffer pH 7.5 containing 5 mM cysteine with 1 : 4 (w/v) ratio of the tissue to the extraction medium. The samples were filtered through eight layers of muslin cloth and centrifuged at 6000 rpm for 15 min using refrigerated centrifuge (Plasto crafts). The reaction mixture in 0.75 ml of volume contained 15 μ mol ATP, 20 μ MgSO₄, 5 μ Mol hydroxylamine, 60 μ mol L - glutamate and 37.75 μ mol Tris HCl buffer pH 7.6. The reaction was initiated by the addition of 0.25 ml enzyme extract and terminated after 30 min by the addition of 0.75 ml of ferric chloride reagent. After centrifugation the absorbance was determined at 500 nm. The enzyme activity was expressed as OD Units mg⁻¹ protein min⁻¹.
ASSAY OF GLUTATHIONE REDUCTASE

500 mg of nodules and leaves were harvested in 50 days old (Blackgram, Green gram and cowpea) plants ground separately at 4°C by mortar and pestle with polyvinyl - polypyrrolidone (25% weight) and 4 volumes of buffer consisting of 50 mM KH$_2$PO$_4$ and 0.1 mM EDTA at pH 7.0. The macerate was filtered through muslin cloth and then centrifuged at 8000 rpm using refrigerated centrifuge for 20 min at 4°C to remove cell debris and bacteroids. The supernatent was used as a source of crude enzyme and glutathione reductase activity expressed as the difference in the rate of NADPH oxidation with and without GSSG (Jablonski and Anderson, 1978).

The assay was performed in a 3 ml quartz cuvette containing 0.13 M K$_2$HPO$_4$ buffer pH 8.0, 0.13 mM Na$_2$ EDTA, 0.33 mM GSSG (oxidised) and 200 µl of the enzyme extract. Reaction was initiated by the addition of 33 µm NADPH and the reaction followed by monitoring the decline in absorbance at 340 nm in view of NADPH being oxidized. Rate of NADPH oxidation was determined during the linear phase of the reaction (20 - 60s). Enzyme activity was expressed in terms of OD units mg$^{-1}$ protein min$^{-1}$. 