MATERIALS AND METHODS

DESCRIPTION OF EXPERIMENTAL SITE

In the present study, the spent wash (treated effluent) and the sludge directly collected from the Trichy Distilleries and Chemicals Private Limited, Senthaneerpuram, Tiruchirappalli, Tamilnadu were used in all the experiments.

The Trichy Distilleries and Chemicals Pvt. Ltd. produces a huge quantity of alcohol (17 million litres per year) and thereby generates a voluminous molasses spent wash (treated effluent/stillage) (2,00,000 lakh litres per day) as well as sludge in mammoth proportions. The treated effluent is let into the Uyyakondan canal, a tributary of the river Cauvery and the sludge gets piled up in heaps. The present study was conducted in the University Botanical Garden in open field conditions to precisely understand the effects of foliar application of the spent wash (treated effluent) and soil application of the sludge on growth and economic productivity of crop plants.
PLANT MATERIALS

Five commonly cultivated leguminous crops in Tamilnadu such as

\textit{Vigna radiata} L.var.CO-123

\textit{Vigna mungo} L.var.Vamban-1

\textit{Vigna unguiculata} (L) Wasp. var.CO-4

\textit{Cajanus cajan} (L.) Millsp.var.SA-1

\textit{Arachis hypogaeae} L.var.Vamban-1

and two monocots

\textit{Sorghum vulgare} Stauf.

\textit{Pennisetum typhoides} Stauf. and Hubb

were employed in the present study. The aforesaid crop plants were selected mainly because of their economic importance in the district of Tiruchirappalli, Tamilnadu, India. The leguminous seeds were procured from the National Pulses Research Centre,Vamban, Pudukkottai, Tamilnadu, India and the seeds of \textit{Sorghum} and \textit{Pennisetum} were supplied by the Tamilnadu Agricultural University, Coimbatore, Tamilnadu, India.
EXPERIMENTAL DESIGN

To unravel the ways and means of utilising the distillery wastes (spent wash and sludge), the following experiments were designed:

(i) Presowing soaking treatment:

To find out whether presowing soaking treatment of seeds with the spent wash could promote germination and growth of crops, healthy and uniform seeds of crop plants were selected and soaked in the spent wash at different time interval (3, 6, 12, and 24 h). The soaked seeds after thorough washing in tap water were raised in pots.

(ii) Foliar application of the spent wash:

To evaluate the effects of the spent wash as a foliar supply of nutrients, the 7-day old seedlings grown in the open field were sprayed uniformly with a hand sprayer until the spent wash ran down the leaf. The strength of the spent wash spray ranged from 0% to 100% (0, 30, 50, 80, and 100%). The spray was given on alternative days. The control plants were sprayed with tap water.
(iii) Soil application of sludge:

To understand whether the sludge could affect seed germination and seedling growth, the sludge was powdered and mixed with the garden soil thoroughly at increasing concentrations (0-100%) as specified below and the crop plants were raised in earthen pots. Plants raised in pots containing the garden soil served as the control. The pots were uniformly watered to avoid running down of water once in two days.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sludge (g)</th>
<th>Soil (g)</th>
<th>Sludge (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>25</td>
<td>250</td>
<td>750</td>
<td>25</td>
</tr>
<tr>
<td>50</td>
<td>500</td>
<td>500</td>
<td>50</td>
</tr>
<tr>
<td>75</td>
<td>750</td>
<td>250</td>
<td>75</td>
</tr>
<tr>
<td>100</td>
<td>1000</td>
<td>-</td>
<td>100</td>
</tr>
</tbody>
</table>

(iv) Composted manure:

To evaluate the use of distillery wastes as a source microbial inoculum to decompose farmyard manure, the spent wash, sludge and farmyard manure were mixed in combinations viz. spent wash and FYM (1:1), sludge and FYM (1:1) and spent wash, sludge and FYM (1:1:1) in earthen pots covered with a layer of sterilized
soil on the top and the pot was covered with guny bags. The pots were sprinkled with water daily and the mixtures were allowed to decompose for one month. The use of composted manure for growth of crop plants was also studied.

The following aspects were investigated in detail as influenced by the application of the sludge/effluent in all the crop plants.

1. Physico-chemical analyses of the garden soil and the distillery wastes (spent wash and sludge)

2. Elemental analyses of the distillery wastes and the garden soil.

3. Germination studies

4. Agrobotanical characters of the 14-day, 30-day, 50-day, 80-day and 120-day old crop plants

5. Economic yield

6. Photosynthetic studies

7. Quantification of biomolecules in leaves and seeds

8. Elemental analyses in leaves

9. Assay of enzymes

10. Root nodulation pattern

11. Screening of microflora in the distillery wastes.
STUDY AREA, GEOLOGY AND CLIMATE

The experimental site is situated in the botanical garden of the Bharathidasan University, Tiruchirappalli, Tamilnadu, India. The study site is between latitude 8.46' E and 10.5' N at an elevation of 65 meters above Mean Sea Level. 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 temperatures of 28 - 32° C and 22 - 25° C respectively. The average annual relative humidity is 71.75 % at 8.30 am with an average wind speed of 13.86 kmph. The soil used for potted experiment was red loamy sandy with pH of 8.0 ± 0.1 and EC of 0.09 ± 0.01 mS cm⁻¹, major elements such as N (10.36 mg g⁻¹), P (0.63 mg g⁻¹) and K (0.88 mg g⁻¹) and total organic matter of 3.12 % and total organic carbon of 1.99 %. The soil had a bulk density of 0.62 g cc⁻³ and water holding capacity of 21.87 % with a percentage pore space of 36.67.

ELEMENTAL ANALYSES OF THE DISTILLERY WASTES AND GARDEN SOIL

The sludge was 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 sludge, soil and 5 ml of spent
wash 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 concentrated hydrochloric acid) was added. The tubes were heated until complete cease-over 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 Whatman No.1 filter paper. The filtered samples were analysed for the following elements through Flame photometer Systronics, India) and Atomic Absorption spectrophotometer (GBC Pvt. Ltd., Australia).

Potassium (K)
Iron (Fe)
Calcium (Ca)
Manganese (Mn)
Sodium (Na)
Zinc (Zn)
Copper (Cu)
Cromium (Cr)
Nickel (Ni)
The ppm values thus obtained were converted to mg/g using the following formula

\[
\text{mg/g} = \frac{\text{ppm} \times \text{volume of the sample}}{\text{wt of the sample} \times 1000000} \times 10
\]

**PHYSICO-CHEMICAL ANALYSIS OF THE GARDEN SOIL AND THE DISTILLERY WASTES**

The following physico-chemical parameters of the garden soil and the distillery wastes were analysed:

1. pH
2. Electrical conductivity (EC)
3. Colour
4. Biological Oxygen Demand (BOD)
5. Chemical Oxygen Demand (COD)
6. Water Potential
7. Water holding capacity (WHC)
8. Porosity
9. Bulk density
10. Real specific gravity
11. Dissolved carbonate
12. Hydrogen carbonate
13. Total organic matter
14. Chlorides
15. Nitrates

DETERMINATION OF EC, pH, BOD, COD AND WATER POTENTIAL

The pH and electrical conductivity of the soil and the distillery wastes were measured by addition of enough distilled water to the samples to produce a sample water ratio of 1:2 (20 g soil in 40 ml \( H_2O \)). 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). BOD and COD of the treated effluent (spent wash) were determined using standard APHA methods (1980).

WATER HOLDING CAPACITY (WHC) (Piper, 1966)

The soil and sludge samples were powdered and dried in an oven at 105 °C for 24 h. The weight of the circular soil cylinder (internal diameter of 5.4 cm and a height of 12.7 cm respectively) was noted initially (W0) and a circular filter paper (Whatman No 1) was placed inside the perforated bottom of the cylinder, the cylinder was filled with the dried soil and again the second weight of the cylinder was noted (W1). The cylinder was placed in the petridish of 10 cm diameter containing water, for
about 12 h to enable the water that enters the cylinder and saturate the sample. The cylinder was taken out, wiped on the outer surface and the final weight (W2) was noted.

\[
\text{WHC(\%)} = \frac{(W2 - W0) - (W1 - W0)}{(W1 - W0)} \times 100
\]

Where,

\(W0\) - weight of the empty cylinder (g)

\(W1\) - weight of the cylinder with the sample (g)

\(W2\) - weight of the cylinder with the sample saturated with water (g)

**DETERMINATION OF BULK DENSITY, PARTICLE DENSITY AND PORE SPACE**

Twenty grams of the sludge sample were weighed accurately and transferred to a 100ml measuring cylinder by gently tapping it. The volume of the sample gently packed was noted. Fifty ml of water was added till the sample was completely soaked and there was still 5 ml of water above the sample surface. The cylinder was kept undisturbed for half an hour in order to fill the pore space with water. The volume of the sample was noted at the end of the experiment. The percentage pore space, apparent specific gravity and real specific gravity were calculated using the formulae
suggested by the Piper (1966).

Pore space volume \( t = (p + q) - r \) (ml)

\% Pore space = \( \frac{r}{p} \times 100 \)

Bulk density (apparent specific gravity) = \( \frac{w}{p} \)

Real specific gravity = \( \frac{w}{p - t} \)

where,

\( w \) = weight of the soil taken (g)

\( p \) = volume of soil (ml)

\( q \) = volume of water added (ml)

\( p + q \) = volume of soil + water (ml)

\( r \) = volume of soil+water at the end of the experiment (ml)

**DETERMINATION OF WATER SOLUBLE CARBONATE AND HYDROGEN CARBONATE** (Hesse, 1971)

Two grams of air dried distillery waste sample were taken in an Erlenmayer flask and 100 ml of distilled water was added. The samples were shaken well for 30 min and filtered until a clear extract was obtained. 10 ml of aliquot of the extract was taken in a white porcelain basin and added a few drops of phenolphthalein solution. The samples were titrated against concentrated sulphuric acid until the pink colour is appeared and the titre value \( (Y \text{ ml}) \) was noted. Three to four drops of methyl orange solution were added and continued the titration until the indicator turned red. The titre value \( (Z \text{ ml}) \) was noted and the titrated solution was saved for determination of...
chloride.

\[2(Z - 2Y) \times \text{Con. of acid (mol)}\]

\[\text{Bicarbonate (milliequivalents per litre)} = \frac{\text{Chlorides 0.02 M AgNO}_3 \text{ used (ml)}}{\text{aliquot (ml)}} \times 1000\]

\[4Y \times \text{Con. of acid (mol)}\]

\[\text{Carbonate (milliequivalents per litre)} = \frac{\text{Carbonate (milliequivalents per litre)}}{\text{aliquot (ml)}} \times 1000\]

**DETERMINATION OF WATER SOLUBLE CHLORIDES**

The aliquot of extract used for carbonate determination was used for chloride determination. The aliquot of extract was titrated with 0.02 M silver nitrate solution using potassium dichromate as an indicator. The end point was revealed by a permanent reddish brown precipitate.

\[\text{Chlorides 0.02 M AgNO}_3 \text{ used (ml)}\]

\[\text{(milliequivalents/litre)} = \frac{\text{Chlorides 0.02 M AgNO}_3 \text{ used (ml)}}{\text{aliquot (ml)}} \times 20\]

**DETERMINATION OF NITRATE**

The soil/distillery waste samples were dried in an air and 50g of the sample was taken in 500 ml Erlenmeyer flask. 250 ml of extraction reagent [a] 12.5 g CuSO\(_4\) dissolved in 100 ml of water and b) 0.6 g of AgSO\(_4\) dissolved in 100 ml of distilled
water. Twenty 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 15 min. 0.4 g of Ca (OH)$_2$ was added and again the mixture was shaken for 5 min prior to addition of 1.0 g of MgCO$_3$. The contents were filtered through the filter paper (Whatman No. 1) and the total volume of the filtrate was measured. Twenty five ml of the filtrate was evaporated to dryness over a water bath and 0.5 ml of phenol-disulphonic acid was added to the residue and dissolved the latter with the help of glass spatula. Five ml of distilled water and 1.5 ml of 12 N KOH solution were added and stirred for thorough mixing. The yellow colour supernatent was taken and the absorbance was read at 410 nm. Distilled water was used as blank. The nitrate content of the filtrate was determined from the standard curve using anhydrous KNO$_3$. The total nitrate content of the sample was determined by the following formula

$$\frac{F \times V}{1000 \times W}$$

where,

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

$V = \text{Total volume of the filtrate (ml)}$

$W = \text{Weight of the dried sample}$
Preparation of phenol-disulphonic acid reagent:

Twenty five g of white phenol was dissolved in 150 ml of concentrated sulphuric acid. Eighty five ml of concentrated H₂SO₄ was further added and heated for about 2 h on a water bath, cooled and stored in a dark bottle.

GERMINATION STUDIES

EFFECT OF ADDITION OF SLUDGE ON SEED GERMINATION

The sludge was thoroughly mixed with the garden soil at different proportions ranging from 0 -1000 g sludge Kg⁻¹ soil. Healthy and uniform seeds were surface sterilized in 0.1 % HgCl₂ for 3 min and then washed thoroughly in distilled water. After surface sterilization, the seeds were sown in earthen pots and watered daily for germination studies. The seedlings raised in the sludge free soil served as the control. Time taken for emergence radicle was noted. Percentage of germination was recorded using the following formula in all the treatments after 7 days of germination.

Radicle emergence was taken as an indication of germination.

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

% of germination =

64
MEASUREMENT OF ROOT AND SHOOT TOLERANCE INDICES

Root and shoot tolerance indices were calculated at different time intervals during the growth of the plant (7-day, 50-day and 80-day) using the following formulae of Taylor and Foy (1985). The plants were uprooted carefully, the roots washed with tap water to remove the soil particles, rinsed thrice with excess distilled water and blotted dry. Roots and shoots were separated individually and dried in a hot air oven at 60 ± 2 °C for 48 h and weighed.

\[
RTI = \frac{\text{Underground biomass of the treated plants}}{\text{Underground biomass of the control plants}}
\]

\[
STI = \frac{\text{Aerial biomass (shoot) of the treated plants}}{\text{Aerial biomass (shoot) of the control plants}}
\]

MEASUREMENT OF SEEDLING VIGOUR INDEX (SVI)

After 7 days of growth, the seedlings were uprooted carefully, the roots washed with tap water to remove soil particles, rinsed thrice with excess distilled water and blotted dry. The length of the embryonic axis was measured using a meter.
scale. The seedling vigour index was measured by the following formula suggested by Abdul-Baki and Anderson (1973).

\[ SVI = \text{Length of embryonic axis (cm)} \times \% \text{ of germination.} \]

**DETERMINATION OF DRY MATTER PRODUCTION (DP)**

After 14, 30, 50, 80 and 120 days of growth, the seedlings were uprooted carefully, the roots washed with tap water to remove soil particles, rinsed thrice with excess distilled water and blotted dry. Roots, shoots and leaves were separated individually and dried in an hot air oven at 60 ± 2 °C until a constant dry weight was obtained. Total dry matter production was inclusive of both above- and underground biomass.

**AGROBOTANICAL CHARACTERS OF 14-DAY, 30-DAY, 50-DAY, 80-DAY AND 120-DAY OLD CROPS**

The plants after 14, 30, 50, 80 and 120 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 agrobotanical characters like shoot length (cm), root length (cm), leaf area (cm²), number of root nodules, fresh and dry weight of the nodule, root, stem and leaf (g), root tolerance index, shoot tolerance index, total dry matter production, relative growth rate (mg day⁻¹), net
assimilation rate (mg m\(^{-2}\) day\(^{-1}\)), leaf area ratio, specific leaf weight (mg cm\(^{-2}\)) and harvest index (%) were analysed in both the control and treated plants.

**DETERMINATION OF LEAF AREA**

From the plants after 14, 30, 50, 80 and 120 days of growth, the leaves were collected and the leaf area per plant was analysed using leaf area meter (Systronics, India).

**DETERMINATION OF DRYMATTER PRODUCTION**

Different plant parts were separated, dried in an hot air oven at 60 ± 2 °C for 48 h and weighed to determine dry matter production.

**RELATIVE GROWTH RATE (RGR)**

RGR was determined following the formula suggested by Williams (1946) and expressed in mg per day.

\[
RGR = \frac{\log e W2 - \log e W1}{t2 - t1}
\]
where,

\( W_1 \) and \( W_2 \) are dry weight of whole plant at time \( t_1 \) and \( t_2 \) respectively

\( t_1 \) and \( t_2 \) are time in days

**NET ASSIMILATION RATE (NAR)**

The formula proposed by Williams (1946) was employed for calculating the NAR and the values are expressed in mg m\(^{-2}\) day\(^{-1}\)

\[
NAR = \frac{W_2 - W_1}{t_2 - t_1} \times \frac{\log_e L_2 - \log_e L_1}{L_2 - L_1}
\]

where,

\( W_1 \) and \( W_2 \) are dry weight of whole plant at time \( t_1 \) and \( t_2 \) respectively

\( L_1 \) and \( L_2 \) are leaf areas at times \( t_1 \) and \( t_2 \) respectively

**SPECIFIC LEAF WEIGHT (SLW)**

From the leaf area and the leaf dry weight, SLW was derived from the formula suggested by Pearce et al. (1968) and the results are expressed in mg cm\(^{-2}\).

\[
SLW = \frac{\text{Leaf dry weight}}{\text{Leaf area}}
\]
LEAF AREA INDEX (LAI) AND LEAF AREA RATIO (LAR)

After determining the leaf area of the plant, the LAI was arrived at by using the formula by Williams (1946).

\[
\text{LAI} = \frac{\text{Leaf area of the plant}}{\text{Ground area occupied}}
\]

and the leaf area ratio was arrived at by using the formula

\[
\text{LAR} = \frac{\text{Leaf area of the plant}}{\text{Leaf dry weight}}
\]

CROP GROWTH RATE (CGR)

Crop growth rate was determined following the formula

\[
\text{CGR} = \text{LAI} \times \text{NAR}
\]
PRODUCTIVITY STUDIES

To evaluate the effects of foliar application of the spent wash and sludge as a soil supplement on productivity, harvest index was worked out at maturity (120 days for all plants and 180 days for *C. cajan*) by using the following formula:

\[
\text{Harvest index (\%)} = \frac{\text{Total dry weight of the seeds per plant}}{\text{Total dry weight of the plant}} \times 100
\]

The other economic yield characters studied were:

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

SCREENING OF MICROFLORA IN THE DISTILLERY WASTES

The sludge and spent wash (treated effluent) as such was collected and dried at room temperature (28 ± 2 °C) with a relative humidity of 50-55 % for determination of population of soil *Rhizobia, Azospirillum, Azotobacter* and total fungi. The sludge
was collected from the 50-day old potted plants of *Vigna mungo*.

**DETERMINATION OF RHIZOBIAL POPULATION**

One g of sludge/spent wash was suspended in 100 ml of sterile distilled water. This suspension was serially diluted from $10^{-1}$ to $10^{-8}$. 0.5 ml of suspension from each dilution was pipetted into yeast extract mannitol agar (YEMA) medium incorporated with congo-red in sterile glass petridishes (9x9 cm).

**Composition of yeast extract mannitol agar medium** (Vincent, 1970)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>3.0 g</td>
</tr>
<tr>
<td>Calcium sulphate</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Dipotassium hydrogen phosphate</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Magnesium sulphate. $7\text{H}_2\text{O}$</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>1.5 g</td>
</tr>
<tr>
<td>Agar</td>
<td>15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>6.8 - 7.0</td>
</tr>
</tbody>
</table>
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 48 h (Gambaks BOD incubator, Madras) for development of colonies. The colonies were counted and total number of colonies g\(^{-1}\) soil was calculated by multiplying the total number of colonies with the dilution factor. Those single colonies characterized by watery and translucent of white opaque appearance without absorbing the congored, were picked up and transferred to yeast extract mannitol agar (YEMA) slants. After they attained a substantial growth, they were removed to a refrigerator and stored at 4 °C until further use.

**ISOLATION AND IDENTIFICATION OF AZOSPIRILLUM**

One g of sludge/spent wash was mixed with 10 ml of sterile distilled water. This was diluted from 10\(^{-1}\) to 10\(^{3}\). 0.5 ml suspension from each dilution was pipetted on the culture plates already containing nitrogen free malate medium (NFb) (Dobereiner, 1980).

**Composition of NFb medium:**

- Malic acid - 5.0 g
- K\(_2\)HPO\(_4\) - 0.5 g
- MgSO\(_4\) \(\cdot\) 7H\(_2\)O - 0.2 g
NaCl - 0.1 g  
CaCl$_2$ - 0.02 g  
Na$_2$MoO$_4$. 2H$_2$O - 0.002 g  
MnSO$_4$. 2H$_2$O - 0.01 g  
Fe EDTA (1.64 % w/v - 4.0 ml (aqueous))  
Bromothymol blue - 3.0 ml (0.5 % w/v in ethanol)  
KOH - 4.5 g  
Biotin - 0.1 mg  
Distilled water - 1000 ml

The pH was adjusted to 6.8 with NaOH and then agar was added. The medium was green at the initial pH. For semi-solid medium 1.75 g agar and for solid medium 15 g agar was added and 20 mg of yeast extract was also added. After incubation for 40-48 h thin initially veil like but later very dense, white undulated pellicles were formed. From the most active cultures, a portion of the pellicle was transferred to a new semisolid NFb medium containing 1 mg of yeast extract. Colonies of white small dry and often slightly merging with the agar were transferred to semi solid medium again (without yeast). After they attained a substantial growth, they were stored at 4 °C in a refrigerator until further use.
Preparation of Fe-EDTA

Fe-EDTA was prepared by boiling 0.69 g ferrous sulphate and 0.93 g disodium salt of EDTA in 80 ml of double distilled water. After cooling, the volume was made up to 100 ml with distilled water and the reagent was sterilized and stored in a refrigerator.

ISOLATION AND IDENTIFICATION OF AZOTOBACTER

One g of sludge/spent wash was mixed with 10 ml of sterile distilled water. This suspension was diluted from $10^{-1}$ to $10^{-3}$. 0.5 ml of suspension from each dilution of different soils was pipetted separately into modified Burk's medium (Rennie, 1981) in glass petridish and rotated to facilitate uniform spread of the inoculum over the medium. The plates were kept in an inverted position in an incubator (Gambaks BOD incubator, Madras) at 30 °C for 3 days.

Composition of modified Burk's medium

- Sucrose - 20 g
- $\text{K}_2\text{HPO}_4$ - 0.08 g
- $\text{KH}_2\text{PO}_4$ - 0.20 g
- $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ - 0.41 g
- $\text{CaCl}_2$ - 0.30 g
Na$_2$MoO$_4$. 7H$_2$O - 0.10 mg
FeSO$_4$. 7H$_2$O - 0.70 mg
Agar - 15 g
Distilled water - 1000 ml
pH - 7.3

After 25-48 h, larger very soft colonies appeared and they were picked out and transferred to slants containing modified Burk's medium. After they attained a substantial growth, they were stored at 4 °C in a refrigerator until further use.

GROWTH CURVE

Purified cultures of *Rhizobium* were inoculated into YEM, modified Burk's and NFb liquid media respectively for time-course study on growth of the microbes. Inoculations were made by adding 1 ml of a culture having an absorbance of 0.1 OD to 100 ml of the medium. At different time intervals ranging from 0-72 h after inoculation, growth was monitored by measuring turbidity at 520 nm.

ISOLATION AND QUANTIFICATION OF TOTAL FUNGI

One g of sludge/spent wash was mixed with 10 ml of sterile distilled water. This was serially diluted from $10^{-1}$ to $10^{-3}$. 0.5 ml suspension from each dilution was pipetted into culture plates already containing Czapek's agar medium.
Composition of the Czapek's agar medium: (Balasubramanian and Kumar, 1989)

- NaNO₃ - 3.0 g
- KH₂PO₄ - 1.0 g
- MgSO₄ 7H₂O - 0.5 g
- KCl - 0.5 g
- FeSO₄ 7H₂O - 0.01 g
- Sucrose - 30 g
- Agar - 15 g
- Distilled water - 1000 ml
- pH - 7.3

Culture plates were incubated in an incubator (Gambaks BOD incubator, Madras) for 24 h. After incubation, the colonies were counted and the total number of colonies per g soil was obtained by multiplying the number of colonies with the dilution factor.

IDENTIFICATION OF VESICULAR ARBUSCULAR MYCORRHIZAL (VAMF) FUNGUS IN ROOT SEGMENTS
(Phillips and Hayman, 1970)

Root segments were collected, washed thoroughly and fixed in Formalin Acetic and Alcohol (FAA) and transferred to distilled water. After thorough washing, root segments were transferred to 10 % KOH and heated at 90 °C in an hot air oven.
for 5 min in a steam pressure cooker. After cooling, the segments were washed in distilled water, bleached with 3 % alkaline H$_2$O$_2$ for 10 min and rinsed in distilled water. The root segments were then treated with 5 N HCl for 5 min and rinsed thoroughly in distilled water. The roots were then stained with 0.5 % tryphan blue in lactophenol and the excess stain was removed in clear lactophenol. The root segments were mounted on a slide in clear lactophenol solution with a gentle pressure on the coverslip to flatten the roots for microscopic observations. The percentage of root infection was calculated by slide technique using the following formula (Daft and Nicolson, 1966).

\[
\text{Number of cells infected} \\
\text{% of infection} = \frac{\text{Number of cells infected}}{\text{Total number of cells observed}} \times 100
\]

MICROPHOTOGRAPHY

Microphotographs of the endophytic VAMF infection thread and vesicles of 80-day old plant roots were taken using Nikon microscope (Nikon HFX, Japan).

ANALYSIS OF LEAF PHOTOSYNTHESIS

After 50 days of growth, the rate of leaf photosynthesis was measured using portable Infrared Gas Analyser (IRGA) (LICOR, Inc, USA).
DETERMINATION OF LEAF WASH pH AND EC

One g of fresh leaves of 50-day old control and treated plants were thoroughly washed in 10ml of double glass distilled water (pH and EC of 5.0 ± 0.1 and 0.009 ± 0.001 mS cm⁻¹ respectively). The pH and EC values were determined as mentioned earlier.

DETERMINATION OF LEAKAGE OF SOLUTES THROUGH MEMBRANES

One g of leaf discs of 50-day old control and treated plants were incubated in 20 ml of double glass distilled water (pH and EC of 5.0 ± 0.1 and 0.009 ± 0.001 mS cm⁻¹ respectively) at room temperature (28 ± 2 °C) for 24 h. After incubation the pH and EC values were determined using digital pH meter (Hanna Instruments, USA) and electronic digital conductivity meter (Global electronics, Hyderabad, India) and the absorbance of the solution was read at 254 nm using UV-visible spectrophotometer (Bausch and Lomb, USA).

QUANTIFICATION OF CHEMICAL CONSTITUENTS IN LEAVES

The leaves from the 8th node were harvested from 50-day old plants were shade-dried in laboratory temperature of 28 ± 2 °C and relative humidity of 50-55 %
for about a week. The samples were powdered and the following chemical substances were quantified and expressed as mg g\(^{-1}\) dw.

a) Total soluble proteins
b) Free amino acids
c) Total phenols
d) Total soluble sugars
e) Sucrose
f) Total soluble starch
g) Free proline
h) Total water soluble SH compounds
i) Total ureides
j) Total allantoin
k) Total allantoic acid
l) Total nitrate
m) Total nitrite
n) Total nitrogen

For determination of chloroplast pigments and total ascorbic acid fresh and healthy leaves from the 3rd node from the top were used.
DETERMINATION OF CHLOROPLAST PIGMENTS

One g of leaf tissue was washed in distilled water, blotted dry with filter paper and ground with 15 ml of 80 % (v/v) aqueous acetone using a pinch of MgCO₃ in a ceramic pestle and mortar. The homogenate was centrifuged at 5000 rpm for 10 min using refrigerated centrifuge (K - 24, West Germany) at 5 °C. The supernatent was saved and the pellet re-extracted repeatedly with the same solvent until the pellet retained no green colour. The supernatents were pooled and made upto a total volume of 25 ml with 80 % aqueous acetone. The absorbance was read in a spectrophotometer (Spectronic 2000, Baush and Lomb, USA) at 663 and 645 nm and the contents of chlorophylls a and b and total chlorophyll were calculated using the following formulae (Arnon, 1949).

\[
\text{Total Chlorophyll} = \frac{20.2 \times \text{OD} \, 645 + 8.02 \times \text{OD} \, 663}{(mg \, g^{-1} \, fw) \times a \times 1000 \times w}
\]

\[
\text{Chlorophyll a} = \frac{12.7 \times \text{OD} \, 663 - 2.69 \times \text{OD} \, 645}{(mg \, g^{-1} \, fw) \times a \times 1000 \times w}
\]

\[
\text{Chlorophyll b} = \frac{22.9 \times \text{OD} \, 645 - 4.68 \times \text{OD} \, 663}{(mg \, g^{-1} \, fw) \times a \times 1000 \times w}
\]
where,

\[ a = \text{length of light path in the cell (1 cm)} \]
\[ v = \text{volume of acetone extract in ml} \]
\[ w = \text{fresh weight of the sample in g} \]

**DETERMINATION OF TOTAL CAROTENOIDS**

The content of total carotenoids in the 80% aqueous acetone extract was measured at 473 nm using an extinction value of \( E_{1 \text{cm}} 2500 \) as an average value (Goodwin, 1954).

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

One g of leaf was ground with 10 ml of 80% methanol using pestle and mortar and the homogenate was centrifuged at 5000 rpm using Remi centrifuge (R8C, Bombay, India) for 10 min. The supernatant was saved and the pellet re-extracted twice with the same volume of the solvent. The supernatents were pooled and used for estimations of total soluble sugars, total free amino acids, total phenols and total sucrose. When the supernatent was rich in chloroplast pigments, to the 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 1.0 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. 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 up to 10 ml volume with 60 % (v/v) ethanol and the absorbance was read in a spectrophotometer (Spectronic 2000, Bausch and Lomb, USA) at 575 nm. The concentration of free amino acids was determined using L-Glycine as the standard (Troll and Canan, 1953).

ESTIMATION OF TOTAL SOLUBLE SUGARS

To one ml of the sample (80 % methanol extract), 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 (Spectronic 2000, Bausch and Lomb, USA) 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 1.0 ml of the sample (80 % methanol extract), 3.0 ml of 10 % (v/v) Folin phenol reagent and 1.0 ml of saturated NaHCO$_3$ were added. The reaction mixture
was kept at 50 °C for 15 min and the absorbance was measured in a spectrophotometer (Spectronic 2000, Bausch and Lomb, USA) at 660 nm using catechol as the standard (Swain and Hillis, 1959).

ESTIMATION OF SUCROSE

To one ml of the sample (80 % methanol extract), 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 (Spectronic 2000, Bausch and Lomb, USA) at 620 nm. Total content of sucrose was calculated using glucose as the standard (Van Handel, 1968).

ESTIMATION OF TOTAL SOLUBLE STARCH

To the resulting pellet after methanol extraction, 6.5 ml of 52 % (v/v) perchloric acid (PCA) and 5.0 ml of distilled water were added and the mixture was kept at 0 °C for 20 min and then centrifuged at 5000 rpm for 10 min using remi centrifuge (R8C, Bombay, India). The supernatent was saved and the residue re-extracted with the same volume of the solvent mixture and the supernatents were combined and used for starch estimation. To 0.5 ml of the sample, 4.5 ml of distilled
water and 10.0 ml of cold anthrone - sulphuric acid reagent (200 mg anthrone dissolved in 100 ml of cold 95 % H₂SO₄) were added and kept in a boiling water bath for 8 min. The reaction mixture was cooled and the absorbance read in a spectrophotometer (Spectronic 2000, Bausch and Lomb, USA) at 630 nm. Total soluble starch was calculated using glucose as the standard and multiplying the equivalent by 0.90 (McCready et al., 1950).

**ESTIMATION OF TOTAL SOLUBLE PROTEINS**

One g of plant material was ground with 10 ml of distilled water using pestle and mortar and the homogenate was centrifuged at 5000 rpm for 10 min using Remi centrifuge (R8C, Bombay, India). The supernatant was stored and the pellet re-extracted, and to the combined supernatant an equal volume of 10 % (w/v) trichloroacetic 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) CuSO₄, 2.5 ml of 12.5 % (w/v) Na₂CO₃ and 0.5 ml of 25 % (w/v) folin phenol were added and the final volume was adjusted to 5.0 ml with distilled water. The reaction mixture was kept in dark for 20 min and the colour development was read in a spectrophotometer (Spectronic 2000, Bausch and Lomb, USA) at 740 nm. Total soluble proteins were calculated using bovine serum albumin (BSA) as the standard (Lowry et al., 1951).
DETERMINATION OF WATER SOLUBLE SH COMPOUNDS

For determination of water soluble SH compounds, leaves were homogenised in 0.15 % sodium ascorbate solution (w/v; 5 ml for gdw) at 0°C. The homogenate was filtered through one layer of miracloth and the filtrate was centrifuged at 6000 rpm for 15 min at 0°C using refrigerated centrifuge (K-24, West Germany). To one ml of the clear supernatant, 1.2 ml of 0.2 M potassium phosphate buffer pH 8.0 and 0.2 ml of Sigma, 5' - Dithio - bis - (2-Nitrobenzoic acid) (DTNB) (10 mM in 0.02 M potassium phosphate buffer pH 7.0) were added. The developing yellow colour was determined at 412 nm. Absorbance was coloured of the supernatent and DTNB. Corrections were made for the absorbance of the deproteinized supernatent (DTNB replaced by water) and that of DTNB (deproteinized supernatent) replaced by 0.15 % sodium ascorbate (w/v). For determination duplicate aliquots from each extract were analysed and averaged. (De Kok et al., 1981)

DETERMINATION OF FREE PROLINE

500 mg of leaf was ground with 2 % (w/v) sulfosalicylic acid using pestle and mortar and the homogenate was centrifuged at 5000 rpm using remi centrifuge (R8C, Bombay, India) for 10 min at 5°C. The supernatent was saved and the pellet re-extracted twice with the same volume of the solvent. The supernatents were pooled.
and used for estimations of free proline. To 5 ml of the supernatent, 5 ml of glacial acetate acid and 5 ml of freshly prepared acid ninhydrin (prepared by dissolving 1.25 g ninhydrin in a mixture of 30 ml warm glacial acetic acid and 20 ml 6 M phosphoric acid with agitation) were added and kept in a boiling water bath for one hour. The samples were cooled to the room temperature and the mixture was extracted with 5.0 ml of toluene. The samples were allowed to stand for 20 min at room temperature and the absorbance of the toluene layer was recorded at 520 nm. The concentration of free proline was determined using L-proline as the standard (Bates et al., 1973).

DETERMINATION OF ASCORBIC ACID (Barakat et al., 1973)

One g of fresh leaf was ground with 5 ml of 0.4 % oxalic acid using pestle and mortar and the homogenate was centrifuged at 6000 rpm using Remi centrifuge (Remi R8C, Bombay, India) for 10 min. The supernatent was saved and the pellet re-extracted twice with the same volume of the solvent. The supernatents were pooled and used for estimation of total content of ascorbic acid. The supernatent was titrated with 0.025 % 2,6 Di-chlorophenol-indophenol (DCPIP). The faint pinkish colour developed during titration was taken as the end point. The titre value was noted and the concentration of total ascorbic acid was determined using ascorbic acid as the standard. The total ascorbic acid was calculated from the following formula

\[
Mg \text{ ascorbic acid/100 g tissue} = I \times S \times D/A \times 100 / W
\]
where,

\[ I = \text{ml of indophenol reagent used in the titration} \]
\[ S = \text{mg of ascorbic acid reacting with one ml of the indophenol reagent.} \]
\[ D = \text{volume of the extract in ml} \]
\[ A = \text{the aliquot titrated in ml} \]
\[ W = \text{weight of the sample in g} \]

**DETERMINATION OF NITRATE AND NITRITE**

One g of dried powder of leaves was weighed separately, boiled for 10 min in 5 ml of distilled water. Nitrate and nitrite contents were estimated as outlined by Wolley et al. (1960). One ml of the aqueous extract was added to 9 ml of 20 % (v/v) acetic acid solution containing 0.2 ppm of copper as copper sulphate. To each sample was added 1.0 g of the salt mixture described by Nelson et al. (1954). The mixture was made by mixing thoroughly the finely ground chemicals given below

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barium sulphate</td>
<td>100.0 g</td>
</tr>
<tr>
<td>Citric acid</td>
<td>75.0 g</td>
</tr>
<tr>
<td>MnSO₄·7H₂O</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Sulphanilic acid</td>
<td>4.0 g</td>
</tr>
<tr>
<td>Zinc powder</td>
<td>2.0 g</td>
</tr>
<tr>
<td>1-naphthyl amine</td>
<td>2.0 g</td>
</tr>
</tbody>
</table>
Blank (without extract) was also run simultaneously. Tubes containing the assay mixtures were shaken at least thrice at 3 min interval and centrifuged at 5000 rpm using remi centrifuge (Remi R8C, Bombay, India) for 10 min. The absorbance of the clear supernatant was read at 520 nm against a reagent blank.

The same procedure was followed omitting zinc, manganese sulphate and copper sulphate for determination of nitrite, present in the samples. By deducting the value of the first reading from that of the second, the quantity of nitrate present in the sample was obtained. Nitrate and nitrite amounts were calculated from standard graphs constructed using analar potassium nitrate and sodium nitrite respectively.

**DETERMINATION OF TOTAL UREIDES, TOTAL ALLANTOIN AND TOTAL ALLANTOIC ACID (Vogels and VanderDrift, 1970)**

One g of dried plant material was taken in 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 (Remi centrifuge, R8C, Bombay, India) and the supernatant used for analysis of total ureides, total allantoin and total 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 hydrolysed 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 formazen

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 - Ureidohydantoin).
**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.

**ESTIMATION OF TOTAL NITROGEN**

The dried plant material was ground in a porcelain mortar and pestle and the total nitrogen content was estimated following the modified microkjeldahl method (Umbreit et al., 1972). Ten mg of powdered plant material was taken in a microkjeldahl flask. A pinch (About 50 mg) of catalyst (Humphries, 1956) (CuSO₄, 5 H₂O - 1 g; K₂SO₄ - 8 g; SeO₂ - 1 g ground separately and mixed together) and 0.5 ml of concentrated sulphuric acid were introduced into the kjeldahl flask. The flask was gently heated on a digestion rack until the fumes of sulphuric acid 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 up to 20 ml with double glass distilled water. To one ml of the above diluted digest, 2 ml of distilled water, 2 ml of colour reagent and 3 ml
of 2 N NaOH were added in series. After 15 min, the absorbance of the solution was read at 490 nm against a reagent blank. Total nitrogen in the sample was determined with reference to a standard graph prepared using NH₄Cl.

**Preparation of colour reagent**

Four g of KI and 4 g of Hgl were dissolved in 25 ml distilled water. About 1.75 g of light coloured gum ghatti was powdered and dissolved in 750 ml of boiling distilled water. Then the solution of KI and Hgl was mixed with the gum ghatti solution. This solution was made upto 1000 ml with double distilled water and then filtered through Whatman No.1 filter paper.

**ASSAY OF ENZYMES**

Fresh leaves 50-day old leguminous crops were collected and assayed for the following enzymes

a) Nitrate reductase (NR)
b) Nitrite reductase (NIR)
c) Glutamine synthetase (GS)
d) Glutathione reductase (GR)
e) Catalase
f) Ascorbate peroxidase (ASCPO)
g) Superoxide dismutase (SOD)
h) Carbonic anhydrase

ISOLATION OF INTACT CHLOROPLASTS

The isolation and purification of intact chloroplasts was carried out following the method of Gnanam and Kulandaivelu, (1969). 100 mg of fresh leaves were ground in semi frozen grinding medium of 20 mM Tris HCl pH 7.5 containing 330 mM sorbitol, 5 mM MgCl₂, 10 mM NaCl, and 2 mM sodium isoascorbate. The homogenate was filtered through eight layers of muslin cloth and centrifuged by accelerating to approximately 6000 rpm and returned to rest in 90 sec, using refrigerated centrifuge (K-24, West Germany). After centrifugation the supernatant was discarded and the pellet surface washed with the grinding medium which was then decanted. Small quantities of resuspension medium (1 ml/tube) contained 330 mM sorbitol, 2 mM EDTA, 5 ml MgCl₂, 10 mM NaCl in 20 mM Tris HCl ph 7.5 were added and the chloroplasts were resuspended by mild shaking of the tubes. The chloroplast suspension was then stored on ice.
ASSAY OF IN VIVO NITRATE REDUCTASE ACTIVITY

In vivo NR activity was determined by the method of Hagemen and Hucklesby (1971). Freshly harvested leaves of 50-day old plants were washed in distilled water and cut into small leaf discs (1 cm). The leaf discs of leaves (100 mg) were vacuum infiltrated for 3 min in 5.0 ml of 0.1 M 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 (NEDH) to 1.0 ml of sample from the incubation medium. After 10 min, the optical density was measured at 550 nm in a spectrophotometer (Spectronic 2000, Bausch and Lomb, USA) with potassium nitrite as the standard. NR activity was expressed as μmole nitrite released mg⁻¹ protein hour⁻¹.

ASSAY OF NITRITE REDUCTASE

The activity of this enzyme was measured by measuring the disappearance of nitrite from the reaction mixture. Preparation of Intact chloroplasts the isolation and purification of intact chloroplasts was carried out by the following method of Gnanam and Kulandaivelu (1969) as described earlier. The assay mixture contained 0.3 ml of
intact chloroplast as a crude enzyme source and 1.5 ml of the reaction mixture. The reaction mixture contained 6.25 ml of 0.5 M 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 et al., 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 one ml of water to which 1 ml of 1 % (w/v) sulphanilamide in 3N HCl and 1.0 ml of 0.02 % (w/v) NEDH were added. The absorbance of the 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 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 (K - 24, West Germany). The reaction mixture in 0.75 ml of volume contained 15 μmol ATP, 20 μmol MgSO₄, 5 μmol hydroxylamine, 60 μmol L-glutamate and 37.75 μmol 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.

**Preparation of ferric chloride reagent** (Black and Wright, 1955).

10 % FeCl₃·6H₂O in 4 ml 24 % TCA - 1 ml
6N HCl - 1.5 ml Distilled water - 6.5 ml
Total Volume -13.0 ml

**ASSAY OF ASCORBATE PEROXIDASE**

Leaves were harvested from 50-day old plants and ground separately at 4 °C by mortar and pestle with polyvinyl-polypyrrolidone (25 % weight) and 4 volumes of buffer consisting of 50 mM KH₂PO₄/K₂HPO₄ 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 (K - 24, West Germany) for 20 min at 4 °C to remove cell
debris. The supernatent was used as a source of crude enzyme. Ascorbate peroxidase was measured by a modified spectrophotometric procedure based on the rate of decrease in absorbance at 265 nm using ascorbate peroxidation (Asada, 1984). The assay was performed in a 1.5 ml quartz cuvette containing 0.25 mM ascorbate, 50 mM KH$_2$PO$_4$/K$_2$HPO$_4$ at pH 7.0, 1.0 mM H$_2$O$_2$ and 37.5 µl of extract containing 50 µg of protein. Corrections were made for the low rates of ascorbate disappearance due to nonenzymatic and H$_2$O$_2$ independent oxidation. Rate of ascorbate disappearance was determined during the linear phase of the reaction (20-60 S). Enzyme activity was expressed in terms of n mole of ascorbate utilised mg$^{-1}$ protein min$^{-1}$.

ASSAY OF CATALASE

Leaves were harvested from 50 days old plants and ground separately at 4 °C by using mortar and pestle with polyvinyl-polypyrrolidone (25 % weight) and four volumes of buffer consisting of 50 mM KH$_2$PO$_4$/K$_2$HPO$_4$ 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 (K -24, West Germany) for 20 min at 4 °C to remove the cell debris. The supernatent was used as a source of crude enzyme. Catalase was measured by a modified spectrophotometric procedure based on the rate of decompositon of H$_2$O$_2$ by following the decline in absorbance at 240 nm (Clairborne, 1985). The assay was performed in a 3 ml quartz cuvette containing 200 µl enzyme
extract, 50 mM potassium phosphate buffer pH 7.0, and 37.5 mM H₂O₂. Rate of H₂O₂ decomposition was determined during the linear phase of the reaction (20 - 60 s). Enzyme activity was expressed as units mg⁻¹ protein min⁻¹ (Beers and Sizer, 1952). One unit of activity of catalase was equal to one μmole of hydrogen peroxide decomposed per minute at 25 °C.

Absorbance / min x 1000

Units mg⁻¹ protein = \frac{\text{Absorbance / min X 1000}}{43.6 x \text{mg protein / ml reaction mixture}}

*43.6 = Molar absorbancy index for hydrogen peroxide at 240 nm in a 1cm cuvette.

ASSAY OF GLUTATHIONE REDUCTASE

The same crude enzyme extract prepared for assay of ascorbate peroxidase was used for 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 K₂HPO₄ buffer pH 8.0, 0.13 mM Na₂ 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 oxidised. Rate of NADPH oxidation was determined during the linear phase of the reaction (20 - 60 s). Enzyme activity was expressed in terms of OD units mg⁻¹ protein min⁻¹.
ASSAY OF SUPEROXIDE DISMUTASE (Beauchamp and Fridovich, 1971)

Intact chloroplasts were used as a crude enzyme source, as was prepared for nitrite reductase activity. The reaction mixture in a final volume of 3 ml contained 2.5 ml of 50 mM phosphate buffer pH 7.8, 0.1 ml of 30 mM methionine, 0.1 ml of 2.25 mM nitroblue tetrazolium (NTB), 0.1 ml of 3 mM EDTA, 100 μl enzyme extract and 0.1 ml 90 μM riboflavin. Riboflavin was added at last and the tubes were shaken and placed 30 cm below a light bank consisting of (300 μE m⁻² S⁻¹, PAR) four 15 W fluorescent lamps. The reaction was started by switching on the light and the reaction was allowed to run for one h. The reaction was linear during this period. The reaction was stopped by switching off the light and the tubes were covered with black cloth. The absorbance of the reaction mixture was read at 560 nm. A non-irradiated reaction mixture which did not develop blue colour served as the control. The volume of the enzyme extract corresponding to 50% inhibition of the reaction was considered as one enzyme unit. Maximum colour was observed in the reaction mixture lacking enzyme. The activity of enzyme was expressed as number of units mg⁻¹ protein min⁻¹.
ASSAY OF CARBONIC ANHYDRASE

Carbonic anhydrase activity was measured on the basis of the time taken for pH change from 8.3 to 7.3 after the addition of 2 ml of CO₂ saturated water to 12 mM veronal -H₂SO₄ buffer pH 8.3 containing the intact chloroplast in a total volume of 5 ml (Dionisio et al., 1989). The intact chloroplast suspension served as a crude enzyme source. The temperature was maintained at 4 °C during the reaction. Enzyme activity in unit was calculated as follows. One unit = To/T-1 where T and To represent the time (sec) needed for the pH change with and without the samples. The activity was expressed as number of units mg⁻¹ protein min⁻¹.

STUDY ON NODULATION POTENTIAL OF SLUDGE-RHIZOBIA

Rhizobia isolated from garden soil, root nodules of *V. unguiculata* and distillery sludge were cultured in YEM medium independently. The respective Rhizobia were independently added to the 7-day old seedlings of *V. unguiculata* raised in pots filled with garden soil, sterilized soil, sludge and sterilized sludge. When the plants attained 30 days of growth, nodulation pattern was studied.
STATISTICAL ANALYSIS

The data recorded in most of the experiments were the mean values ± standard error. The % control values are given in parenthesis in some experiments. Certain experiments were tested for significance using Students 't'test and the agrobotanical characters were subjected to two-way Analysis of Variance (Zar, 1974).

SOURCE OF CHEMICALS AND GLASSWARES

Glasswares from Borosil and Analar Grade Chemicals by BDH and Merck (India) were used in all experiments. Fine chemicals obtained from Sigma (USA) were used in enzyme studies.