MATERIALS AND METHODS
III. MATERIALS AND METHODS

GLASSWARE:

All the glassware made of Borosil and Corning were cleaned (using cleaning solution), sterilized and used.

Cleaning solution (Rangaswami, 1975)

Potassium dichromate 60 g
Conc. H₂SO₄ 60 ml
Dist. Water 1000 ml

Potassium dichromate was dissolved in warm water, cooled and sulphuric acid was added slowly. It was mixed thoroughly and used for cleaning glassware.

CHEMICALS

Analytical grade chemicals supplied by Loba, Hi-Media, S.D. Fine chemicals and BDH were used.

MEDIA

Potato Dextrose Agar (PDA) (Rangaswami, 1975)

Potato (200 g) extract 500 ml
Dextrose 20 g
Agar 20 g
Dist. water 500 ml
pH 5.6
SELECTIVE MEDIUM FOR *AZOSPIRILLUM* (SMA) (Okon et al., 1977)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malic acid</td>
<td>5.0 g</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>0.5 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>0.4 g</td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>0.2 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1 g</td>
</tr>
<tr>
<td>CaCl</td>
<td>0.02 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.05 g</td>
</tr>
<tr>
<td>Sodium molybdate</td>
<td>0.002 g</td>
</tr>
<tr>
<td>MnSO$_4$</td>
<td>0.001 g</td>
</tr>
<tr>
<td>Ca(NO$_3$)$_2$</td>
<td>0.0014 g</td>
</tr>
<tr>
<td>ZnSO$_4$</td>
<td>0.0021 g</td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>0.002 g</td>
</tr>
<tr>
<td>Bromothymol blue</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Agar</td>
<td>1.75 g</td>
</tr>
<tr>
<td>Dist. water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.0</td>
</tr>
</tbody>
</table>

BUFFER USED

<table>
<thead>
<tr>
<th>Buffer</th>
<th>pH</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate buffer</td>
<td>5.8</td>
<td>0.25 M</td>
</tr>
<tr>
<td>Borate buffer</td>
<td>6.4</td>
<td>0.10 M</td>
</tr>
<tr>
<td>Sodium phosphate buffer</td>
<td>6.0</td>
<td>0.10 M</td>
</tr>
<tr>
<td>Sodium acetate buffer</td>
<td>8.8</td>
<td>0.10 M</td>
</tr>
<tr>
<td>Tris-HCl buffer</td>
<td>9.7</td>
<td>0.25 M</td>
</tr>
</tbody>
</table>
FAA

Formaline 13 ml
Glacial acetic acid 5 ml
50% ethanol 200 ml

TRYPAN/COTTON BLUE IN LACTOPHENOL
(Phillips and Hayman, 1970)

Phenol 20 g
Glycerine 40 g
Lactic acid 20 g
Trypan/cotton blue 5 ml (Aqueous solution 1%)

STUDY SITE

The study was carried out in an experimental plot of Botany Department, Bharathiar University, Coimbatore, Tamil Nadu, India.

SOIL

The soil for the experiments was collected from the experimental fields of Botany Department, Bharathiar University, Coimbatore, after ploughing it to the depth of 30 cm. The sandy loam soil (pH 6.8, 9.48 mg of N kg\(^{-1}\), 0.95 mg of P kg\(^{-1}\) and 37.79 mg of K kg\(^{-1}\)) was air dried and passed through 2 mm sieve. It was then steam sterilized at 121°C for 1 h on three consecutive days and subsequently used to fill the polybags (30x15 cm) of 2.5 kg capacity after the addition of organic matter.
ORGANIC MATTER

Dried and powdered form of organic matter viz., sheep manure, was amended at the rate of 10 g kg\(^{-1}\) soil. The sheep manure contained 14 mg N g\(^{-1}\), 0.75 mg of P g\(^{-1}\) and 11.8 mg of K g\(^{-1}\). The organic matter was added to the soil and mixed thoroughly before (steam) sterilizing the soil.

HOST GENOTYPES AND SOURCES

Seeds of two cotton (Gossypium hirsutum L.) varieties, MCU-9 and MCU-5 were obtained from the Central Institute of Cotton Research, Regional Station, Coimbatore, Tamil Nadu, India.

MICROBIAL SOURCES

VAM FUNGI

The indigenous vesicular-arbuscular endomycorrhizal fungus, Glomus geosporum (Nicol. and Gerd.) Walker obtained from Department of Botany, Bharathiar University, Coimbatore, was maintained in pot cultures with maize.

Azospirillum

Azospirillum brasilense (a cotton specific strain) in peat carrier was obtained from the Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, India.
Verticillium

A highly virulent isolate of *Verticillium dahliae* Kleb. was obtained from the Central Institute of Cotton Research, Regional Station, Coimbatore, Tamil Nadu, India.

DEVELOPMENT OF COTTON SEEDLINGS

The experiments were laid out in a completely randomized block design consisting of eight treatments. The polybags arranged in 2x2m² sandpits were fumigated (with 0.4% formaldehyde applied at the rate of 21 ml/m² for 48 h), covered with polyethylene sheets and then exposed to air for 15 d. The cotton seeds were surface sterilized with 0.01 per cent mercuric chloride and washed in several changes of sterile water. Two seeds of each variety were sown in each polybag containing sterile soil and microbes according to the treatments. The microbes were inoculated alone and in combination. The sandy pits were drenched with water twice a day to maintain the soil temperature between 20 and 30°C. After germination, one seedling was removed from each polybag.

TREATMENTS

1. Con - Control
2. G - *Glomus geosporum*
3. A - *Azospirillum brasilense*
4. GA - *G. geosporum* + *A. brasilense*
5. GV - *G. geosporum* + *V. dahliae*
6. AV - *A. brasilense* + *V. dahliae*
7. GAV - *G. geosporum* + *A. brasilense* + *V. dahliae*
8. V - *V. dahliae*
INOCULATION PROCEDURE

VAM FUNGI

*Glomus geosporum* was inoculated by layering method (Hetrick *et al.*, 1984) using soil based inoculum containing infected maize root bits and chlamydospores (400 spores/100 g of soil).

**Azospirillum**

The cotton seeds of 2 varieties were first surface sterilized with 0.01% mercuric chloride solution followed by several washing in sterile water. The sterilized seeds were then treated with equal proportions of *Azospirillum brasilense* (Negi *et al.*, 1990) and sown in the polybags.

**Verticillium**

Five ml of conidial suspension of the pathogen containing $63 \times 10^3$ propagules ml$^{-1}$ prepared in sterile water was poured on the soil around the base of the seeds 3 days after sowing (Sivaprakasam, 1972; Schnathorst, 1981).

Each treatment including control was replicated 5 times.

**ASSESSMENT OF *GLOMUS GEOSPORUM* COLONIZATION**

The per cent colonization of *G. geosporum* was assessed following a modified method of Phillips and Hayman (1970). The roots were fixed in FAA with water, cut into 1 cm long bits. They were cleaned in 2.5% potassium hydroxide solution, acidified in 5N hydrochloric acid and were left in lactophenol trypan/cotton blue (0.05%) at room
temperature overnight. The per cent colonization was estimated according to the magnified intersection method of McGonigle et al. (1990).

**QUANTIFICATION OF *AZOSPIRILLUM BRASILENSE* POPULATION**

The bacterial population in the rhizosphere was estimated by following the most probable number (MPN) method of Alexander (1965). From each sample 10 g of soil was mixed in 100 ml of sterile dist. water. From this, 1 ml was transferred to another tube containing 10 ml dist. water to make dilution (10⁻¹). Serial dilutions were made up to 10⁻⁹ dilution factor. The dilution sample were transferred to sterile (100x15 mm) petri plates to which sterile selective medium for *Azospirillum* (SMA), (Okon et al., 1977) was poured and mixed well by rotating the petri plates. The plates were incubated at room temperature (24±1°C) for 15 d and examined for colonies and the results were expressed as number of cells/g of soil. For each dilution 5 petri plates were maintained.

**QUANTIFICATION OF *VERTICILLIUM DAHLIAE* POPULATION**

The fungal population in the soil samples was estimated by following the most probable number method of Alexander (1965) as described under quantification of *Azospirillum brasilense* population. The results were expressed as number of microsclerotia/g of dry soil.

**EVALUATION OF DISEASE INCIDENCE**

Disease incidence of cotton plants inoculated with *V. dahliae* was evaluated by grading plant and leaf symptoms according to Dimond et al. (1952).
Grade 0: no disease symptom
Grade 1: epinasty and/or slight yellowing of leaf
Grade 2: 20 to 50 per cent yellowing of leaf area or stunted growth with small leaves
Grade 3: complete yellowing and/or partial wilting
Grade 4: leaf fallen or completely non-functional

The average grade was computed for the plant as a whole and divided by 0.04 to give a maximum value of 100.

EVALUATION OF PLANT GROWTH

Plant samples were collected with their root system intact at 20, 40, 60, 80, 100 and 120 d after the emergence. The leaf area was measured by using T area meter (Delta-T devices). An average of 5 leaves for each treatment was accounted. Shoot and root length was measured after washing the roots free of soil and respective dry weights were recorded after drying in a hot-air oven at 80°C for 72 h.

PHYSIOLOGICAL STUDIES

MOISTURE CONTENT

Known quantity (5 g) of leaves or roots was taken in a preweighed crucible and dried at 80°C. Five replicates were maintained for each treatment. The crucible was weighed until a constant weight was obtained. The moisture content was calculated using the formula,

\[\text{Moisture content (mg/g)} = \frac{(W_2 - W_1) - (W_3 - W_1)}{W_2 - W_1}\]
where,

\[ W_1 : \text{Weight of the crucible} \]
\[ W_2 : \text{Weight of the crucible + fresh weight of sample} \]
\[ W_3 : \text{Weight of the crucible + dry weight of sample} \]

**ESTIMATION OF CHLOROPHYLL** (Yoshida *et al.*, 1971)

In a glass mortar and pestle, one g of leaf was minced and homogenised in 80% acetone. The homogenate was filtered through cheese cloth. The residue was reextracted with 80% acetone and filtered. The filtrates were pooled and centrifuged at 7000 g for 10 min. The clear supernatant was made up to 20 ml with 80% acetone and its optical density (OD) was measured at 645 and 663 nm. Total chlorophyll was calculated by using the following formula and the results were expressed in mg/g dry weight.

\[
\text{Total chlorophyll (mg/g)} = \frac{20.2 \, A_{645} + 8.02 \, A_{663}}{a \times 1000 \times W}
\]

\[
\text{Chlorophyll a (mg/g)} = \frac{12.7 \, A_{663} - 2.69 \, A_{645}}{a \times 1000 \times W}
\]

\[
\text{Chlorophyll b (mg/g)} = \frac{22.9 \, A_{645} - 4.68 \, A_{663}}{a \times 1000 \times W}
\]

where,

- \(a\) : Length of path light in the cell (1cm)
- \(V\) : Volume of the extract in ml
- \(W\) : Dry weight of the sample in g
MEASUREMENT OF PHOTOSYNTHESIS

Photosynthetic oxygen evolution by the leaves was measured at 25°C according to Allen and Holmes (1986) using a Clark type (YSI model 53). Oxygen electrode at a light intensity of 100 Em⁻² s⁻¹ and the illumination being provided by a 150 W incandescent bulb were utilised. Leaf disc with 5 mm dia was used to measure oxygen evolution. Before each series of measurement 5.0 ml of 0.1 M phosphate buffer (pH 7.0) was placed in the sample chamber, allowed to equilibrate and aerated for 5 min adjusting the amplifier to read 100%. Then the leaf disc was added and photosynthetic rate was calculated from percentage of saturation. Oxygen evolved was expressed as µ moles of O₂/ h⁻¹ mg chl. g⁻¹.

The table gives oxygen concentration (100% saturation) at various temperatures and at normal atmospheric pressure.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>O₂ concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.442</td>
</tr>
<tr>
<td>5</td>
<td>0.386</td>
</tr>
<tr>
<td>10</td>
<td>0.341</td>
</tr>
<tr>
<td>15</td>
<td>0.305</td>
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<tr>
<td>20</td>
<td>0.276</td>
</tr>
<tr>
<td>25</td>
<td>0.253</td>
</tr>
<tr>
<td>30</td>
<td>0.230</td>
</tr>
<tr>
<td>35</td>
<td>0.219</td>
</tr>
</tbody>
</table>

µ mole of O₂ evolved h⁻¹ mg chl.a⁻¹ was calculated using the following formula:
\[ \frac{C \times v \times 60 \times X}{\text{Chl.a} \times 5 \times 100} \]

where \( C = \text{O}_2 \) conc (mM) at air saturation at that temperature.

\( v = \) Volume of phosphate buffer (ml).

\( \text{chl.a} = \) Chlorophyll a present in the leaf disc (mg).

\( 60 = \) for expressing \( \text{O}_2 \)/hour.

\( 5 = \) Experimental time in 5 min.

\( 100 = \) Air saturation.

or a single conversion factor was used. The factor

\[ \frac{0.6 \times C \times v}{\text{chl.a}} \]

where \( C = \text{O}_2 \) conc (mM) at air saturation at that temperature.

\( v = \) Volume of phosphate buffer (ml).

\( \text{chl.a} = \) Chlorophyll a present in the leaf disc (mg).

From the result, \( \text{O}_2 \) evolved at a particular percentage of saturation was calculated.

**BIOCHEMICAL STUDIES**

For biochemical studies such as reducing sugar, total soluble sugar, free amino acids, o-dihydric phenol and phenols, the sample was extracted with alcohol and used.

**ALCOHOL EXTRACT** (Mahadevan and Sridhar, 1986)

Roots or leaves, 5 g were cut into small pieces (1-2 cm), plunged in boiling ethyl alcohol for 10 min. It was cooled and homogenized for 5-10 min in a glass mortar with
pestle. The homogenate was filtered through cheese cloth and the residue was reextracted with 2-3 ml of 80% alcohol. The filtrates were pooled and centrifuged at 10,000 g for 15 min. The clear supernatant was made up to 10 ml with 80% alcohol. The content of reducing sugars, total sugars, free amino acids, O-dihydric phenol and phenol was estimated from the alcohol extract.

**ESTIMATION OF REDUCING SUGARS (Nelson, 1944)**

The alcohol extract was evaporated to dryness and the residue was dissolved in dist water to a final volume of 1 ml/100 mg dry plant material.

**Reagents:**

a. Alkaline copper mixture:

**Copper reagent 'A'**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium carbonate</td>
<td>25 g</td>
</tr>
<tr>
<td>Sodium potassium tartrate</td>
<td>25 g</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>20 g</td>
</tr>
<tr>
<td>Sodium sulphate</td>
<td>200 g</td>
</tr>
</tbody>
</table>

The chemicals were dissolved in 800 ml of dist water and made up to 1 L.

**Copper reagent 'B'**

Copper sulphate, 15 g, was dissolved in dist water and made up to 100 ml. To this 1 or 2 drops of conc H₂SO₄ were added.

**Alkaline copper mixture**

This mixture was prepared by mixing copper reagent A and B in a ratio 25:1.

b. Arsenomolybdate colour reagent:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium molybdate</td>
<td>5 g</td>
</tr>
<tr>
<td>Dist water</td>
<td>95 ml</td>
</tr>
<tr>
<td>Conc H₂SO₄</td>
<td>4.2 ml</td>
</tr>
</tbody>
</table>
The ingredients were mixed thoroughly, to which 0.6 g Na$_2$HSO$_4$. 7 H$_2$O was added. The solution was incubated at 37° C for 24 h in a brown bottle.

**Method:**

To 1 ml of extract, 1 ml of alkaline copper mixture was added in a test tube. It was heated for 20 min on a boiling water bath and cooled under running tap water. One ml of arsenomolybdate reagent was added. After 15 min the mixture was diluted to 25 ml with dist water after 15 min and the colour intensity was measured at 500 nm. Blank was maintained without plant extract. The amount of reducing sugars was calculated by using a glucose standard graph and expressed as glucose equivalents in mg/g dry plant material.

**ESTIMATION OF TOTAL SOLUBLE SUGAR**
(Mahadevan and Sridhar, 1986)

Since alcohol interferes with colour development during anthrone-sugar reaction, the alcohol was evaporated by using hot water bath. The residue was dissolved in dist water and the final volume was adjusted to 1.0 ml for every 100 mg of plant material. The amount of total sugar present in the extract was estimated by anthrone method.

**Reagent:**

Anthrone reagent, 0.2% was prepared by dissolving 200 mg of anthrone in 100 ml conc H$_2$SO$_4$.

**Method:**

The extract, 0.2 ml was pipetted into a clean test tube, to which 4 ml of anthrone reagent was added by sliding down along the wall of the test tube. A glass marble was placed on the top of the tube to prevent loss of water by evaporation. The tube was heated in boiling water bath for 10 min and cooled to room temperature (28±1°C) in a water
bath. A blank was prepared without the plant extract. The intensity of the developed colour was measured at 625 nm in UV-VIS spectrophotometer (Beckman DU-40). The amount of soluble sugars present in the extract was calculated by using a standard glucose curve and expressed as glucose equivalents in mg/g dry plant material.

**ESTIMATION OF AMINO ACIDS** (Mahadevan and Sridhar, 1986)

**Reagent:**

Commercial grade ninhydrin reagent, 0.2 g was dissolved in 80% ethanol.

**Method:**

Alcohol extract 1 ml was pipetted into a test tube. Then 1 ml of ninhydrin reagent was added and mixed thoroughly. The tube was covered with a glass marble and heated for 20 min in a boiling water bath. The content was made up to 5 ml with dist water. Blank was maintained with 1 ml of dist water. The intensity of purple colour was read at 570 nm in spectrophotometer (Beckman DU-40). The amount of amino acid was calculated from a glycine standard curve.

**ESTIMATION OF O-DIHYDRIC PHENOL** (Bray and Thorpe, 1954)

**Reagents:**

a. Arnow's reagent

Sodium nitrate 10 g

Sodium molybdate 10 g

Dist water 100 ml

Mixed thoroughly and stored in a brown bottle.

b. HCl 0.5 N

c. NaOH 1.0
Method:

One ml of alcohol extract was taken in a test tube, to which 1 ml of 0.5 N HCl, 1 ml Arnow's reagent, 10 ml dist water and 2 ml 1 N NaOH were added. Pink colour developed immediately after the addition of NaOH. The colour intensity was read at 515 nm. Blank was prepared with all reagents and 80% alcohol. The amount of o-dihydric phenol was calculated by using catechol as standard.

ESTIMATION OF TOTAL PHENOL (Bray and Thorpe, 1954)

Reagents:

a. Folin-Ciocalteu reagent (commercial) - diluted with dist water in 1:1 ratio
b. Sodium carbonate, 20%

Method:

To 1 ml of the alcohol extract in a graduated test tube, 1 ml of Folin-Ciocalteu reagent was added followed by 2 ml of 20% sodium carbonate. The content was thoroughly mixed, heated in a boiling water bath for 1 min and cooled. The volume was made up to 25 ml with dist water and the colour intensity was read at 650 nm. Blank was prepared with reagents and alcohol. Phenol was estimated by using a standard graph employing catechol standards.

ESTIMATION OF TOTAL SOLUBLE PROTEIN (Lowry et al., 1951)

Leaf or root samples were randomly collected from each experimental polybag on 20, 40, 60, 80, 100 and 120 d after emergence of the plants.
Extraction buffer (pH 7.0):

- Tris HCl 50 mM
- Sucrose 250 mM
- EDTA 3 mM
- NaCl 50 mM
- Polyvinylpyrrolidone 1%

Plant materials were thoroughly washed in water and homogenized in ice cold extraction buffer (5 ml/g) in an ice bath. The homogenates were centrifuged at 40,000 g for 20 min at 4°C. The clear supernatant containing the soluble proteins were estimated. The protein content was estimated according to Lowry et al. (1951).

Reagents:

a. Alkaline sodium carbonate solution (2% Na$_2$CO$_3$ in 0.1N NaOH)
b. CuSO$_4$- Sodium potassium tartrate solution (0.5% CuSO$_4$.5H$_2$O in 1% Na K tartrate)
c. Alkaline copper reagent: reagents a and b were mixed freshly in the ratio of 50 : 1.
d. Folin phenol reagent: (commercial) the reagent was diluted with equal volume of dist water.

Method:

To 0.1 ml of protein extract, 5 ml of reagent c was added. After 10 min, 0.5 ml of reagent d was added, mixed well and incubated for 30 min at room temperature (28±1°C). The colour was read at 610 nm in VIS spectrophotometer (Beckman DU-40). Blank was maintained with dist water. The protein was calculated from a standard graph using bovine serum albumin (BSA).
EXTRACTION OF CYTOKININ (Sridhar and Ou, 1972)

Fresh leaves or roots, 5 g were homogenized in 5 ml of buffer. Thirty ml of Ethanol was added. After 6 to 8 h, the content was centrifuged at 2000 g for 20 min. The volume of the supernatant was reduced to 5 ml under reduced pressure in a rotary evaporator at 40° C. The pH was adjusted to 2.9 with 1 N HCl. The chlorophyll, auxins and gibberellins were removed by extracting thrice with ethyl ether. The pH of the aqueous solution was adjusted to 7.8 with 1N NaOH. Cytokinin was extracted from this by using n-butanol. The extraction was made for six times, 5 ml/time. The butanol layers were pooled and dried under reduced pressure at 40° C. The residue was dissolved in 2 ml of buffer and subjected to thin layer chromatography (TLC).

The cytokinin extract was streaked on TLC plate and the chromatogram was developed in the solvents containing n-butanol : 1 N NH₄OH : H₂O (7:1:2). The fluorescing spots were marked and the chromatogram was divided into 10 equal parts. The cytokinin from each part was eluted, using n-butanol and the solvent was evaporated. The residue was dissolved in a known amount of dist water and used for bioassays.

ESTIMATION OF CYTOKININ (Sridhar and Ou, 1972)

Cytokinin present in the extract was assayed by radish cotyledon test. Uniform sized cotyledons of 3 d old radish seedlings grown in dark were selected. The preweighed cotyledons were floated either in dist water (control) or test solutions in petri plates. The plates were incubated under fluorescent light for 3 d. After incubation, the leaves were taken out, plotted on filter paper to remove the moisture and weighed. Same experiment was done by using known concentrations of kinetin and a dosage response graph was
drawn. The amount of cytokinin presented in the extract was calculated from the standard curve.

**ESTIMATES OF NUTRIENTS**

Dried leaf and root samples were ground separately and 0.5 g ground material was digested in triple acid mixture (nitric acid, sulphuric acid and 60 per cent of perchloric acid). P concentration was determined by vanadomolybdate blue method (Jackson, 1958). Total N was determined by microKjeldahl method (Humphries, 1956) and K was determined by flame photometric method (Davis, 1962) and the values were expressed as μg/g dry material.

**ENZYME ASSAYS**

**Enzyme extraction by acetone** (Mahadevan and Sridhar, 1986)

Ten g of leaf or root material was ground in 50 ml of prechilled (-20°C) acetone. The resulting slurry was centrifuged at 2000 g for 10 min at 4°C. The pellet was washed with excess of chilled acetone till the residue was completely pigment free. The residue was dried in vacuo condition and stored at -15°C.

**Estimation of enzymes from acetone powder**

Fifty mg of acetone powder was kept overnight in 10 ml of 0.1 M Tris HCl buffer (pH 7.2) at 0-4°C. It was filtered through cheese cloth and the filtrate was centrifuged at 5,000 g at 4°C for 10 min. The supernatant was used as enzyme source. Protein content of the supernatant was estimated according to Lowry et al. (1951).
ESTIMATION OF PHENOL OXIDASE (Kar and Mishra, 1976)

Reagents:

Phosphate buffer 0.1 M pH 6.0
Catechol 0.01 M dissolved in 0.1 M phosphate buffer (pH 6.0).

Method:

Enzyme extract, 2 ml was mixed with 3 ml phosphate buffer, to which 1 ml catechol in phosphate buffer was added and mixed. Changes in the absorbance at every 30 sec up to 3 min were recorded in a UV scanning spectrophotometer (Philips PU 8740) at 495 nm. The changes in the absorbance in between 30-150 sec of incubation were plotted and the enzyme activity was calculated.

ESTIMATION OF PHENYLALANINE AMMONIA-LYASE (Neish, 1961)

Reagents:

Boric acid - borax buffer 0.1 M pH 8.8
L-Phenylalanine 0.1 M

Method:

Borate buffer, 2 ml was pipetted into a clean test tube, to which 1 ml of enzyme extract, 0.6 ml of phenylalanine and 2.4 ml of dist water were added. The mixture was incubated at 30°C in a water bath for 15 min to allow for an initial non-enzymatic
decrease in absorbance. The optical density of the reaction mixture was read at 290 nm at 20 min interval for an hour. Heat killed enzyme extract was used as control. Enzyme activity was expressed as μg cinnamic acid formed h⁻¹ mg⁻¹ protein.

STATISTICAL ANALYSIS

The data were subjected to analysis of variance and the means were separated according to Duncan's Multiple Range Test (DMRT).