MATERIALS AND METHODS

Plants

Seeds of green gram (*Vigna radiata* (L.) Wilczek cv. ADT-1, CO-5 and Vamban) and black gram (*Vigna mungo* (L.) Hepper cv. CO-5 and T-9) were chosen for the study. They were procured from Pondicherry Agro Service Industries Corporation (PASIC) and Krishi Vigyan Kendra, Pondicherry. Viable seeds of uniform colour, size and weight were selected for the experiments.

Preparation of acidulated water

At present, simulated acidic rainfalls (SAR) are most frequently characterised by acidity or pH. In most experiments, *H₂SO₄* and *HNO₃* are used to provide the majority of the acid rain component in simulated rainfalls (*Evans, 1984a*), in the ratio of sulphate to nitrate, between 3:1 and 1:3 on an equivalent basis. Since Pondicherry is a coastal town where the air would be normally laden with chloride ions also, *HCl* was added to the other two acids normally used, following *Kohno and Kobayashi (1989a)*.

<table>
<thead>
<tr>
<th>pH</th>
<th>H⁺ (µeq l⁻¹)</th>
<th>EC µS</th>
<th>Concentration (mg l⁻¹)</th>
<th>Sulphate</th>
<th>Nitrate</th>
<th>Chloride</th>
<th>Sodium</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>0.10</td>
<td>29</td>
<td>BDL*</td>
<td>0.013</td>
<td>5.20</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>5.5</td>
<td>3.16</td>
<td>32</td>
<td>2.94</td>
<td>0.107</td>
<td>6.29</td>
<td>BDL</td>
<td></td>
</tr>
<tr>
<td>4.0</td>
<td>100.00</td>
<td>40</td>
<td>7.53</td>
<td>0.169</td>
<td>7.48</td>
<td>BDL</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>3162.20</td>
<td>950</td>
<td>139.40</td>
<td>0.798</td>
<td>9.08</td>
<td>BDL</td>
<td></td>
</tr>
</tbody>
</table>

*BDL = Below detectable limit

All simulated rain solutions contained the following inorganic ions:
- Calcium - 0.77,
- Magnesium - 0.013,
- Potassium - 0.30,
- Ammonium - 3.49 mg l⁻¹.

(Courtesy: Department of Science, Technology and Environment, Pondicherry.)
Double distilled water was adjusted to different pH levels viz. 5.5, 4.0 and 2.5 using a diluted mixture of $\text{H}_2\text{SO}_4$, $\text{HNO}_3$, and $\text{HCl}$ (4: 2: 1, v/v) in the molar ratios of 6: 3: 1. The glass distilled water had a pH of 6.5 - 6.8 and was adjusted to pH 7.0 by adding one or two drops of 0.1N NaOH and served as control. The other characteristics of simulated rain are presented in Table 3.

**Rain generating system**

The acidic rain was applied through a rain-generating unit modelled after Kohno and Kobayashi (1989a). It consisted of a reservoir, a compressor and a set of nozzle sprayers (pore size 0.35 mm). The acidulated water was served from a glass container and showered on the foliage from one metre above the ground level. The whole system was operated electrically. The potted plants were arranged on a platform and rotated on a turn table basis to ensure uniform leaf wetting. SAR was applied at the prefixed timing around 9.30 A.M. to avoid excess heat and irradiance. Each rain event lasted for 6 min at an effective flow rate of 7.8 mm h$^{-1}$ as measured by a rain-gauge. The rain drop size ranged from 0.35 to 1.35 mm. Depending upon the design of the experiments, the total number of showers varied from 10 to 20. About 200 ml of acidulated water was used in each shower. Showers were applied to the top of the plant and no efforts were made to prevent the 'run off' to soil (Kohno and Kobayashi, 1989a).

**Pot Culture**

The experiments were performed under conditions which closely approximate field environment under rain-fed conditions. Plants were grown in earthenware pots (25 x 25 cm) filled with a mixture of sand, red soil and farmyard manure (2: 1: 1 v/v). Ten healthy seeds
were sown at equidistant places at a depth of 2 cm in each pot. The pots were arranged randomly and treatments were replicated sufficiently to avoid possible experimental errors. Plants grown in earthen pots (3 to 4 per pot) were maintained under natural green house conditions (day-temperature, maximum 38° C; minimum night-temperature 18° C; relative humidity 60 ± 5 %; maximum irradiance (PAR) 1,400 μmol m⁻² s⁻¹; photo period 12 - 14 h). The sides were open allowing free air circulation but the covered roof excluded natural precipitations. As the SAR-showers contributed only a portion of normal rainfall, plants were irrigated with tap water to maintain soil moisture levels adequately. To check seasonal influences, the experiments were repeated in July-September and December-March rain-fed seasons.

For studies on nitrogen metabolism, ten-day-old seedlings in each pot were inoculated with 200 mg of the commercial preparation of Rhizobium inoculum (Department of Agriculture, Government of Pondicherry) suspended in 10 ml of water poured on the surface of the soil as suggested by Shriner and Johnston (1981).

**SAR applications**

Two types of short-term experiments were planned. In the first set of short-term experiments, all the five cultivars of two grain legumes were screened for their relative sensitivities in terms of growth and cell metabolite pools. Ten-day-old seedlings with the primary leaves (PL) fully expanded were exposed to SAR of 6 min duration each for 10 days. Biochemical analyses were restricted to the primary leaves.

In the second series of short-term experiments, studies on photosynthesis, nitrogen metabolism and surface perturbations were carried out and restricted to two green gram cultivars, ADT-1 and CO-5 only. Fifteen-day-old plants with PL and the emerging first trifoliate leaf
(TF1) were exposed to SAR of 6 min duration each for 10 days. The rain events were extended to another 10 days to monitor the progression of injury and sensitivities of leaves developing subsequently.

For monitoring the long-term effects of acidic precipitation on yield characteristics, the same two green gram cultivars ADT-1 and CO-5 were used. To study the effect of consecutive and episodic nature of rain events and to assess the sensitivity of the developmental stage to SAR, the consecutive showers were applied during early vegetative growth phase and flowering stages, respectively, as detailed below.

**Expt.I.** Plants (2-leaf stage) were exposed to SAR showers daily for 15 consecutive days - each of 6 min duration starting from 15 DAS.

**Expt.II.** Plants received showers of same intensity and duration (6 min) but only twice a week (Tuesday / Friday) for seven weeks starting from 15 DAS. This treatment commenced on young plants (two-leaf stage) and continued through the flowering stage.

**Expt.III.** Plants (5 leaf-stage) were exposed to SAR (6 min each) starting from the first sign of flowering, 35 DAS for cultivar CO-5 and 30 DAS for ADT-1 and continued for 15 days consecutively through the flowering stage.

The three treatment groups coincided with seedling, life time and flowering periods of the plant respectively. Though the timings of SAR differed, all the plants received the same amount of rainfall.
Assessment of growth

Ten plants per treatment were uprooted at the specified stage and the growth parameters viz. root and shoot length and the plant height were measured. The plant parts were weighed in Anamed Digital balance for fresh weight. They were then dried in an oven at 80°C for 48 h and weighed again for dry mass measurements.

Flowering index

The plants were tagged, numbered serially and checked periodically for flower buds. Beginning from the date on which the first plant was in flowers, flowering was assessed on every 5th day by recording the number of plants per treatment bearing flowers as well as the number of flowers / fruits in each plant. From the data obtained, the date on which 50% of the plants had flowers was extrapolated from them. Alongside the flowering vigour indices were also calculated using the following formula.

Flowering vigour index = \( \frac{\text{No of flowers in one stage} - \text{No of flowers in previous stage}}{\text{No. of flowers in previous stage}} \times 100 \).

Fruit harvest

Mature fruits were harvested periodically from each plant and the harvests were terminated by 75 DAS for green gram cultivar ADT-1 and 90 DAS for the other cultivar CO-5. Length, and fresh weight of the fruits, and number of seeds per pod were noted immediately, and the dry weight of seeds measured after 48 h at 80°C.

Harvest Index (H.I)

Harvest index was calculated using the following formula as suggested by Mohan et al. (1992).

\[
\text{Harvest index} = \frac{\text{Yield (mg)}}{\text{ Biomass (mg)}} \times 100.
\]
Biochemical analysis

Biochemical changes were observed in the primary or first trifoliate leaves.

Estimation of photosynthetic pigments

Chlorophyll (Chl)

Chlorophyll pigments were estimated following the non-destructive method of Shoaf and Liim (1976). This method was followed as it gave accurate results and can be done with ease than the conventional Arnon's method.

Fresh leaf discs (25 mg) were cut and placed in a tube containing 10 ml of Dimethyl sulfoxide (DMSO) and the pigments were extracted into the fluid without grinding by incubating in dark at 65°C for 1 hr as standardised previously. The complete extraction of chlorophyll in DMSO was visualised when the leaf tissues were free from the green pigments.

The absorbance was measured at 645 and 663 nm in a spectrophotometer with DMSO as blank. The pigments were quantified using the following formula:

\[
\text{chlorophyll a (mg g}^{-1}\text{ fw)} = \frac{(12.7 \times A_{663}) - (2.69 \times A_{645})}{1000 \times w \times 1} \times V \times df
\]

\[
\text{chlorophyll b (mg g}^{-1}\text{ fw)} = \frac{(22.9 \times A_{663}) - (4.68 \times A_{645})}{1000 \times w \times 1} \times V \times df
\]

where \( A = \) absorbance, \( w = \) fresh weight of the sample in mg, \( V = \) volume of the sample, \( df = \) dilution factor (if any).

The amount of total chlorophyll was calculated by adding the contents of Chl a and b.
**Carotenoids (Ikan, 1969)**

Absorbance values of pigments at 480, 645, and 663 were used to find the corrected optical density (OD) for carotenoids which was used for quantification.

\[
\text{Corrected OD} = A_{480} + [(0.114 \times A_{645}) - (0.638 \times A_{663})] \\
\text{Carotenoid (mg g\(^{-1}\))} = \text{corrected OD} \times \frac{1}{100} \times \frac{V}{W}
\]

where \(A\) = Absorbance, \(V\) = Volume of DMSO taken for extraction (ml), \(W\) = Fresh weight of the sample in mg.

**Estimation of soluble proteins (Lowry et al., 1951)**

**Extraction**

Fresh leaf material (300 mg) was macerated in a pre-chilled mortar and pestle with 10 ml of 20% TCA. The homogenate was centrifuged for 15 min at 600 rpm. The supernatant was discarded. To the pellet was added 5 ml of 0.1N NaOH, stirred well and centrifuged again for 15 min. The supernatant was saved (protein fraction) and used for the estimation of total proteins.

**Estimation**

Five ml of reagent `c` was added to 0.5 ml of protein extract and was allowed to stand for 10 min in dark. Then, 0.5 ml of Folin-Ciocalteau reagent was added to this solution and again kept in dark for 30 min. The absorbance was read at 660 nm in a spectrophotometer.

**Preparation of reagents**

1. Alkaline Sodium carbonate (Reagent A): Two grams of sodium carbonate were dissolved in 0.1N NaOH and made up to 100 ml.
2. Copper sulphate and potassium tartrate (Reagent B): Copper sulphate (1%) was mixed with equal volume of 2% sodium potassium tartarate solution freshly prepared.

3. Reagent C: 50 ml of reagent A was mixed with 1 ml of reagent B.

4. Folin-Ciocalteau Reagent: Commercial Folin-Ciocalteau was diluted with glass distilled water (1 : 2, v/v).

The protein content was calculated with reference to the standard curve for Bovine Serum Albumin.

**Preparation of Alcohol Extract for biochemical estimations**

Oven-dried leaves were powdered in a mortar and pestle. Fifty mg of the dried powder was boiled in a water bath with 10 ml of 80% ethyl alcohol. After cooling, the homogenate was centrifuged at 600 rpm for 15 min. The supernatant was saved and made up to 20 ml with 80% ethyl alcohol. This extract was used for quantitative estimation of reducing sugar, total sugar, ortho-dihydric (O.D) phenol and total phenol. The residue was saved for starch estimation.

**Estimation of reducing sugars**

The reducing sugars were estimated by the Nelson's modification of Somogyi's method (Nelson, 1944). To 1 ml of ethanolic extract, 1 ml of fresh copper reagent prepared by mixing copper tartarate and copper sulphate solution (25 : 1 v/v) was added. The mixture was heated in a boiling water-bath for 20 min and cooled. One ml of arsenomolybdate reagent was added and the contents incubated for 15 min. The solution was then diluted to 25 ml with distilled
water and the colour intensity was read at 500 nm in a spectrophotometer. The content of the reducing sugar was calculated using the standard graph for glucose.

**Preparation of reagents**

1. **Copper tartarate solution (A):** Twenty five g of anhydrous sodium carbonate, 25 g of sodium potassium tartarate, 20 g of sodium bicarbonate, 200 g of anhydrous sodium sulphate were dissolved in 800 ml of distilled water, diluted to 1 L, filtered and stored in a brown bottle.

2. **Copper sulphate solution (B):** Fifteen g of copper sulphate was added to 100 ml of distilled water. One or two drops of conc. H₂SO₄ was added to this reagent B.

3. **Copper reagent:** Twenty five ml of reagent A and 1 ml of reagent B were mixed.

4. **Arsenomolybdate reagent:** In 450 ml of distilled water 25 g of ammonium molybdate was dissolved. To this 21 ml of conc. H₂SO₄ was also added. Three grams of sodium arsenate dissolved in 25 ml of distilled water was also added to the above mixture. This was incubated at 37°C for 48 h. The reagent was stored in a glass stoppered brown bottle.

**Estimation of total sugar (Dubois et al., 1956)**

To 1 ml of ethanolic extract, 4 ml of cold anthrone reagent was added. This was shaken vigorously and boiled for 10 min in a boiling water bath. After cooling in running tap water, the absorbance was read at 620 nm in a spectrophotometer. A standard curve was prepared with known amounts of glucose to estimate the contents.
Anthrone reagent

To 40 ml of distilled water 100 ml of concentrated sulphuric acid was added. To 100 ml of the above mixture 200 mg of anthrone was added and mixed thoroughly until a golden yellow colour appeared.

Estimation of non-reducing sugars

The amount of non-reducing sugars was determined by the following formula suggested by Loomis and Shull (1937).

Non reducing sugars = Total sugars - Free reducing sugars x 0.95.

Estimation of starch (Mc Cready et al., 1950)

The residue left behind after alcoholic extraction of the leaf materials was dissolved in 5 ml of 52% perchloric acid for one h. The mixture was filtered through Whatman's filter paper (No. 42) and the filtrate was made up to 100 ml with distilled water. To 1 ml of the above extract, 4 ml of distilled water and 10 ml of freshly prepared cold anthrone reagent were added carefully along the sides of the tube. The contents of the tubes were shaken vigorously and heated in a boiling water-bath for 7.5 min. The tubes were then cooled immediately in running tap water and shaken well before reading the colour intensity at 630 nm in a spectrophotometer. The starch content was calculated with reference to glucose standard and multiplied by 0.9.

Preparation of reagents

1. Anthrone reagent: Anthrone (200 mg) was dissolved in 100 ml of cold 95% \( \text{H}_2\text{SO}_4 \).

2. Perchloric acid: To 18 ml of distilled water, 52 ml of commercial perchloric acid (70%) was added to get 52% perchloric acid.
Estimation of O.D. Phenols (Johnson and Shoal, 1952)

To 1 ml of alcoholic extract, 1 ml of 0.5N HCl and 1 ml of Arnow's reagent were added. To this, 2 ml of NaOH and 10 ml of distilled water were added. A pink colour appeared immediately on adding NaOH. The colour intensity was reduced by diluting it to 25 ml with distilled water and the absorbance read at 515 nm. The O.D. phenols were calculated using a standard curve with catechol.

Preparation of reagent

Arnow's reagent: Ten g of sodium nitrite and 10 g of sodium molybdate were mixed in 100 ml of distilled water. The reagent was stored in a brown bottle.

Estimation of total phenols (Bray and Thorpe, 1954)

To 1 ml of alcoholic extract, 1 ml of Folin-Ciocalteau reagent and 2 ml of 20% sodium carbonate were added and shaken well. The mixture was heated in a boiling water bath for 1 min and cooled under running tap water. The blue solution was diluted to 25 ml with distilled water and read at 650 nm in a spectrophotometer. Phenols were quantified using catechol as standard.

Preparation of reagents

1. 20% sodium carbonate: Twenty g of sodium carbonate was mixed with 100 ml of distilled water.

2. Folin-Ciocalteau reagent: Commercial Folin-Ciocalteau was diluted with distilled water in 1:2 ratio.
**Measurement of gas exchange in photosynthesis**

Photosynthetic CO$_2$ uptake and stomatal resistance were monitored using a LI 6200 portable infra-red gas analyser (Li-Cor Inc., USA) with a 1 litre leaf chamber. Ten readings were taken for each sample at 5 s intervals.

**Measurement of chlorophyll fluorescence**

*In vivo* Chl a fluorescence induction was followed in intact leaves after excitation with the broad-band blue radiation (100 W m$^{-2}$, 400-460 nm, Corning 5113) as described by Kulandaivelu and Daniell (1980). The photon flux density was 700μE. m$^{-2}$ s$^{-1}$. Leaves were dark incubated at 28° C for 10 min before fluorescence measurements. The photomultiplier (Hamamatsu R 375) placed at 90° to the excitation beam was protected by an interference filter (λ max 690 nm, half band width 12 nm, Schott, Germany). A leaf bit was placed in the black plexiglass frame and placed diagonally in a standard 4 ml glass cuvette. Photophore slide projector was used to produce the excitation light. An electromagnetic shutter with short opening time (ms) was used to control the excitation beam. The signal from the photomultiplier was directly displayed on a Hitachi recorder (Model 056) or stored in a digital oscilloscope (Iwatsu model SS-5802).

**Measurement of Electron transport activities.**

**Whole chain electron transport** (H$_2$O→MV) (Armond et al., 1978).

The rate of whole chain electron transport (H$_2$O→MV) in the isolated chloroplasts (mesophyll cells) was measured as O$_2$ uptake at 25° C using a Hansatech oxygen electrode (Hansatech, U.K). White actinic light (900 μE, m$^{-2}$ s$^{-1}$) from a slide projector was passed through a 10 cm water bath before illuminating the sample. The reaction mixture in a total
volume of 1.0 ml contained 20 mM phosphate (pH 7.5), 5 mM MgCl₂, 10 mM NaCl, 100 mM sucrose, 0.1 mM NaN₃, 5 mM NH₄Cl and mesophyll cells equivalent to 20 µg chlorophyll.

**PSII and PSI Electron Transport (Noorudeen and Kulandaivelu, 1982).**

**PSII electron transport (H₂O → BQ)**

PSII mediated O₂ evolution was continuously monitored at 25°C in the presence of BQ using the same electrode set up described above. The reaction mixture in a total volume of 1.0 ml contained 20 mM phosphate (pH 7.5), 5 mM MgCl₂, 0.5 mM BQ, 100 mM sucrose and chloroplasts (mesophyll cells) equivalent to 20 µg chlorophyll.

**PSI electron transport (DCIPH₂ → MV)**

PSI mediated electron transport from the artificial electron donors (DCPIP-ascorbate) was assayed polarographically as O₂ uptake. The reaction mixture in a final volume of 1.0 ml contained 20 mM phosphate (pH 7.5), 2 mM sodium ascorbate, 100 mM DCPIP, 5 µM DCMU, 5 mM NH₄Cl, 0.1 mM NaN₃, 1 mM MV and chloroplasts (mesophyll cells) equivalent to 10 µg chlorophyll. The other conditions were similar to whole chain electron transport experiment.

**Measurement of absorption spectra.**

Room temperature absorption spectra were recorded directly using a double beam spectrophotometer (Hitachi, Model 557). Chloroplasts were suspended in a medium containing 20 mM Tris-HCl (pH 7.5), 100 mM sucrose, 10 mM NaCl, 5 mM MgCl₂ and glycerol to a final concentration of 60%, v/v. Both the reference and sample cuvettes were placed in the
cuvette-holder with their ground surface facing the light beams, so as to scatter beams equally. The spectra were normalised at 540 nm.

**SDS-PAGE analysis of chloroplast polypeptides**

SDS-PAGE was performed as described by Laemmli (1970) using a polyacrylamide gradient of 7.5-15% gel.

Chloroplast proteins, both stromal and thylakoid were isolated after lysing the chloroplast using 10 mM Tris-HCl buffer (pH 7.8) containing 4 mM MgCl₂. The lysate was spun at 10,000 g for 10 min and thylakoids in this pellet are resuspended in the lysis buffer and again pelleted by centrifugation. The supernatants representing soluble and membrane fractions were added with 80% acetone and left at 20°C for 30 min. The proteins were separated by centrifugation and the pigments were washed off with repeated acetone wash. The pellet was treated with 10% TCA and the precipitated protein was separated by centrifugation. It was again washed with 80% acetone and ether respectively by centrifugation and the dry powder was finally suspended in a small volume of 5% SDS. After determining the protein content by the method of Lowry et al. (1951), aliquots of each sample were mixed with an equal volume of sample buffer and heated in boiling water bath for 2 min. It was then plunged into ice water for cooling immediately and spun in a microfuge for 5 min at 15,000 g before loading in the gel.

**Preparation of SDS-PAGE**

A 7.5-15% acrylamide linear gradient gel was prepared from solutions A and B using a gradient marker. Before casting, the glass plates with spacers (1 mm) were clipped and the sides and bottom were sealed with a 3% agar solution. The bottom of the gel was plugged
with a solution containing 2 ml of solution B, 20 µl of APS and 3 µl of TEMED. The mixture was slowly let into the chamber between the glass plates. A layer of isopropanol was laid over the gel solution slowly and the gels were allowed to polymerize for 30 min. The isopropanol was washed off with water. The gel comb was then inserted leaving a gap of 1 cm above the gel and then the stacking gel solution poured. After complete polymerisation in about 2 to 3 h the bottom spacers and the comb were removed gently. The wells were washed with water and the gels placed in a glass tank with running buffer avoiding air bubbles.

The protein concentration of all samples were equalised and 300 µg of sample was loaded in each slot. The run was started at 60 mV and increased to 125 mV subsequently and was continued until the marker dye reached the bottom, and the glass plates along with the gel were removed, the gels were slowly slid into a glass tray, washed once with water and then placed in the coomassie brilliant blue staining solution for 3-4 h. The gels were then destained. Marker proteins of known molecular weight were also run concurrently and the molecular weights of the polypeptide bands were then calculated from their Rf values.

**Preparation of reagents**

<table>
<thead>
<tr>
<th></th>
<th>Solution A (7.5% acrylamide)</th>
<th>Solution B (15% acrylamide)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% acrylamide solution</td>
<td>5.7 ml</td>
<td>11.4 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl pH 8.8</td>
<td>7.5 ml</td>
<td>7.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.6 ml</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>H₂O</td>
<td>14.0 ml</td>
<td>6.3 ml</td>
</tr>
<tr>
<td>10% APS</td>
<td>0.2 ml</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>10.0 ml</td>
<td>10.0 ml</td>
</tr>
<tr>
<td></td>
<td>38.0 ml</td>
<td>36.0 ml</td>
</tr>
</tbody>
</table>
APS was prepared freshly. The last two reagents were added just before casting the gel.

Sample buffer contained 125 mM Tris-HCl, pH 6.8; 25% glycerol (v/v); 25% β-mercaptoethanol and 0.1% bromphenol blue.

Stacking gel solution

- 0.5 M Tris-HCl, pH 6.8: 3.8 ml
- 40% acrylamide: 1.8 ml
- 10% SDS: 0.3 ml
- 10% APS: 0.25 ml
- TEMED: 15 μl
- Water: 8.85 ml

Running buffer

Prepared by mixing 6.0 g of Tris, 28.8 g glycine and 1.0 g SDS in 1 litre of water.

Staining solution

Prepared by mixing 200 mg Coomassie brilliant blue, 50 ml ethanol, 7 ml acetic acid and 43 ml water.

Destainer: Medium containing 20 ml ethanol, 7 ml acetic acid and 73 ml water.

Nodulation

Seeds of Vigna radiata cultivars ADT-1 and CO-5 were grown in earthenware pots as described earlier. Ten plants from each treatment and control meant for studying nitrogen metabolism were carefully uprooted from the soil at 30, 40 and 50 DAS as nodulation is known to be at peak by 5-7 weeks. The nodules were washed thoroughly with distilled water and the number recorded.
Estimation of leghaemoglobin (Bergerson, 1961)

Extraction

Fresh nodules (200 mg) were macerated in a pre-chilled mortar and pestle with 3 ml of phosphate buffer (pH 7.4) and filtered through two layers of cheese cloth. Nodule debris was discarded. The homogenate was centrifuged at 10,000 g for 30 min. The supernatant was diluted to 3 ml with phosphate buffer and saved. The extract was used for the quantitative estimation of the leghaemoglobin.

Estimation

To a suitable volume of the extract (3 ml) an equal volume of alkaline pyridine reagent was added. The solution became greenish yellow due to the formation of ferric haemochrome. The blank without extract was also run simultaneously. The greenish yellow solution was divided equally between two tubes. To one portion, a few crystals of sodium dithionite was added to reduce the haemochrome. Without aeration the solution was slowly stirred with the help of a rod. The absorbance was read at 556 nm after 5 min against a reagent blank. To the other portion was added a few crystals of potassium hexacyanoferrate to oxidise haemochrome and read at 539 nm.

Reagents

1. Diluent buffer: Sodium/potassium phosphate buffer of 0.1M (pH 7.4).

2. Alkaline pyridine reagent: Dissolve 0.8 g NaOH in 50 ml glass distilled water and cool. Add 33.8 ml of pyridine (33.2 g). Dissolve and dilute to 100 ml with water. This produces 4.2 M pyridine in 0.2 NaOH.
3. Sodium dithionite: Grind finely and store in small stoppered tubes in a
dessicator.

4. Potassium Hexacyanoferrate.

Calculation

The leghaemoglobin concentration was calculated using the following formula and ex-
pressed in percentage

\[ \text{LHb concentration (mM)} = \frac{A_{420} - A_{234} \times 2D}{23.4} \]

where D is the initial dilution.

Estimation of nitrogenase activity

Nodular nitrogenase activity was determined by the acetylene reduction technique
(Stewart et al., 1967)

Enzyme assays were carried out in 7 ml capacity bottles fitted with rubber serum stop-
pers. 500 mg sample was placed in each bottle and stoppered tightly and the assay was initi-
ated by injecting 0.6 ml (10%) acetylene gas using a disposable hypodermic syringe. The
bottles were incubated in light for 30 min and the reaction was then terminated by injecting 0.2
ml of 10% TCA. One ml of the gas phase was withdrawn and the ethylene formed was meas-
ured in a gas chromatograph (Aimil Nucon 5700 with FID using poropak T column model).
Nitrogen was used as a carrier gas and the flow rate was adjusted to 40 ml / min. Ethylene
standard was used as reference.

Nitrogenase activity was calculated using the following formula

\[ \text{Nitrogenase activity} = \frac{n \text{ moles of ethylene} \times 60 \times \text{volume of gas phase}}{\text{fresh weight} \times 30 \times \text{injection volume}} \]

where 30 = incubation time in min,

60 = for expressing the enzyme activity for one hour.
'n' moles of ethylene = \( \frac{\text{ethylene peak height} \times \text{attenuation}}{\text{sensitivity}} \) x 2.59

where attenuation = 32 and sensitivity = 1000

Nitrogenase activity was expressed as \( \mu \) moles of acetylene reduced h\(^{-1}\) \( \mu \)g fw\(^{-1}\).

**Estimation of nitrate reductase activity (NRA) - in vivo.**

Nitrate reductase activity was assayed by the method of Jaworski (1971) with suitable modifications (Muthucbdun et al., 1992). Freshly harvested leaves were washed and cut into 5 mm disks. Leaf bits / nodules corresponding to 100 mg fresh weight were incubated in vials containing 5 ml of incubation medium prepared by mixing 0.1N KNO\(_3\) (1 ml), 0.1M phosphate buffer of pH 7.5 (3.75 ml), 0.01 ml of Triton x-100 (0.1%) and 1% propanol (0.25 ml).

Incubation was carried out in dark for one hour at room temperature (28° C ± 2° C) giving occasional shakings. Aliquots of 0.2 ml from the incubation mixture were analysed for nitrite after 60 min. To 0.2 ml of incubation medium, 1.8 ml of distilled water, 1 ml of 3% sulphanilamide in 3N HCl and 1 ml of 0.02% N-(1-naphthyl) ethylene-diamine dihydrochloride were added in quick succession. This was incubated for 15 min in darkness for colour development and absorbance was read at 540 nm with suitable blank in a Schimatzu spectrophotometer. The amount of nitrite formed was expressed as 'n' moles of nitrite produced per min mg\(^{-1}\) fw using a sodium nitrite standard curve.

**Estimation of nitrate and nitrite (Woolley et al., 1960)**

About 50 mg of shade-dried powdered material was boiled for 10 min in 5 ml of distilled water. One ml aqueous extract was added to 9 ml of 20% (v/v) acetic acid solution containing 0.2 ppm of CuSO\(_4\). One gram of salt mixture as described Nelson et al (1954) was added to each sample. The salt mixture was made by mixing thoroughly the finely ground
chemicals viz. Barium sulphate 100 g, Citric acid 75 g, Manganous sulphate 10 g, Sulphanilic acid 4 g, Zinc powder 2 g and 1-Napthyl amine 2 g.

Blank without extract was also run simultaneously. Tubes containing the assay mixture were shaken at least thrice at 3 min intervals and finally centrifuged at 3000 rpm for 10 min. Absorbance of the clear supernatant was read at 520 nm against a reagent blank.

The procedure was repeated for another batch of samples omitting Zn, MnSO₄ and CuSO₄. The second run gave the quantity of nitrite alone present in the sample. The first value minus the second gave the quantity of nitrate present in the samples.

The amounts of nitrate and nitrite were calculated from standard graphs for potassium nitrate and sodium nitrite respectively.

**Estimation of nitrogen (Jackson, 1958)**

Dried leaf sample (100 mg) was placed in a 100 ml Kjeldahl flask and about 200 mg of the catalyst mixture (potassium sulphate, cupric sulphate and selenium dioxide mixed in the ratio of 50 : 10 : 1 by weight) and 3 ml of concentrated H₂SO₄ were added. The sample was digested for 2 h. When the solution became colourless or pale yellow, 10 ml of distilled water was added to the digest, cooled and decanted into the micro-Kjeldahl distillation flask. To that, 10 ml of 40% NaOH was added and distilled. The ammoniacal distillate was collected in a 25 ml conical flask containing 10 ml of 4% boric acid and 3 drops of mixed indicator (0.3 g bromocresol green and 0.2 g methyl red in 400 ml of 90% ethanol). This solution was titrated against 0.05N HCl. Nitrogen content was estimated using the following formula:

\[
\text{Nitrogen (\%)} = \frac{(\text{sample titre} - \text{blank titre}) \times \text{HCl normality} \times 14 \times 100}{\text{sample weight (g)} \times 1000}
\]
Soil pH and EC

Soil samples from the culture pots were collected at the end of the experiment and dried in an oven at 60°C. Twenty grams of dry soil was added to 50 ml of double-distilled water, stirred and allowed to stand for 30 min. The clear supernatant was decanted and used for pH and EC measurements in digital pH meter (Elico, India) and digital conductivity bridge (Elico, India), respectively.

Anatomical characteristics

Photography

Leaf surfaces were viewed through Nikon Labophot stereo microscope under incident and translucent light and photographed. Leaf bits of 0.5 x 1 cm were cut from middle interveinal region of the experimental samples. Thin free-hand sections were prepared using a sharp blade for observation under the microscope. Sectional views of developing lesions were photographed in various stages under a Nikon Labophot microscope (Type IV). Whole plants were photographed in daylight using a Pentax camera.

Epidermal cells

The size and number of epidermal cells were counted using a calibrated microscope. Calibration was done using a stage and ocular micrometer.

Stomatal frequency / index

Stomatal frequency was determined by examining the leaf impressions on polystyrene plastic film. The plastic medium (1 g of polystyrene in 100 ml of xylol) was applied on the leaf uniformly as a thin layer. After drying, the material was carefully removed and observed under magnification. Stomatal counts were made randomly on five selected regions on the adaxial surface. The same leaf impressions were also used for SEM analysis. Because stomatal
frequencies vary according to cell size, Salisbury (1928) introduced the term 'stomatal index' which relates the number of stomata per unit leaf area to the number of epidermal cells in the same area. Thus Stomatal index (S.I) = \( \frac{S}{S+E} \times 100 \) where \( S = \) number of stomata per unit leaf area, \( E = \) number of epidermal cells per unit leaf area.

**Mesophyll volume**

Mesophyll thickness in mm was multiplied by 100 to calculate the mesophyll volume in cm³ per dm² of leaf area (Patterson et al., 1978).

**Scanning electron microscopy (SEM studies)**

For observing the injuries and wax patterns, the leaf bits were first dehydrated in a graded series of ethanol. These were then coated with gold, using a Hitachi HUS-5GB vacuum coater and viewed in a Hitachi S-450 scanning electron microscope operated at 10 KV.

**Light microscopic studies**

**Determination of percent leaf area injured**

All plants under SAR-treatment were regularly examined for acid-rain injury. If visible injury was present in any of the test plants, control plants were checked for the same characteristics. When only the SAR-exposed plants showed a particular type of injury, it was attributed to acid rain rather than to other causes.

Lesions were scored for percent leaf area injured. The assessments were made using a dissecting microscope. Lesions were categorised into four groups depending upon the diameter of the injured area following Evans et al. (1977a).

- Type A injury - < 0.25 mm
- Type B injury - 0.25 to 1 mm
- Type C injury - 1 to 2 mm
- Type D injury - > 2 mm
The number of lesions per group per field was tabulated and related to the whole area to give the percentage of area injured.

**Determination of leaf contact angles and water-holding capacity**

**Leaf contact angle**

Contact angles were measured on adaxial surfaces of detached leaves of two cultivars (*Vigna radiata* cv. ADT-1 and CO-5). Since water of different pH levels did not influence the contact angle (Haines et al., 1985), only distilled water was used for the determinations. The leaves were sprayed with distilled water for 20 s from a distance of 1 m with a sprayer used for other experiments. Drop outlines were projected onto a wall using a 35-mm slide projector and traced on paper. Contact angle was measured as the angle between the surface of the leaf and the tangent plane of a water droplet at the circle of contact between air, liquid and leaf surface.

**Water-holding capacity**

As described for the leaf contact angle, the leaves were sprayed with distilled water and the droplet size and frequency were determined by micrometric method. The droplets were in the range between 0.34 and 1.40 mm in diameter. Water which remained on the adaxial surface after spraying was absorbed into a tissue paper and weighed. Leaf areas were determined for each leaf by outlining the leaves on the graph paper. Water-holding capacity was expressed as mg H₂O cm⁻².

**Statistical analysis**

At least five replicates were maintained for all treatments and control. The experiments were repeated to confirm the trends.
Application of a one-way ANOVA among the experimental groups, F-tests or two-tailed ANOVA is the standard practice and is less conservative: t-tests compared only pre-selected pairs i.e. treatment vs control rather than treatment vs treatment (Lee et al., 1981). The depth of significance between the treatments could be brought out clearly by a multiple range testing programme.

Hence Tukey's multiple range test (TMRT) was applied for the experimental data (at 5% level of significance). Wherever necessary, the data were evaluated by Pearson's product-moment correlation analyses (Zar, 1984).