CHAPTER III

MATERIALS AND METHODS

Plant Material

Seeds of sunflower (*Helianthus annuus* L.) var CO-2 and cotton (*Gossypium hirsutum* L.) var MCU-9, employed in the present study, were obtained from the Tamil Nadu Agricultural University, Coimbatore, India. The sunflower variety, CO-2 developed recently and released for wider cultivation in Tamil Nadu, possesses higher yield potential and oil content and better tolerance to rust disease. Its duration is 87 days. It is a herbaceous annual growing to a height of about 134 cm. The broadly ovate to elliptic leaves are generally opposite below but alternate above. The inflorescence is a solitary terminal capitulum of about 15 cm in diameter. The disc florets are perfect with a tubular 5-limbed corolla. The ray florets are neutral with yellow corolla. The capitulum yields about 806 seeds (Mohamed Sheriff et al., 1987).

*Gossypium hirsutum* L. is commonly known as upland cotton. It is an annual shrub with a few spreading and ascending branches; stem usually upright; stem tip and leaves glabrous. Leaves are cordate and half or less cut into 3–5 divergent lobes; lobes broadly triangular with acuminate tips. Flowers are pale yellow and turn pink on
the second day of blooming. The bolls are large and rounded, green and smooth with oil glands. The seeds are covered all over with white fuzzy coating (Kochhar, 1981).

**Pre-sowing soaking treatment**

Seeds free from visible defects were selected. They were washed in tap water and surface sterilized with 0.1% mercuric chloride for one minute. Then washed thoroughly with distilled water several times to remove all traces of mercuric chloride. The surface sterilized seeds of sunflower and cotton were separately soaked either in distilled water or aqueous solutions of NaCl and KCl of the following strength:

<table>
<thead>
<tr>
<th>NaCl (%)</th>
<th>EC (mmhos/cm)</th>
<th>KCl (%)</th>
<th>EC (mmhos/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.02\times10^{-1}</td>
<td>0.1</td>
<td>0.02\times10^{-1}</td>
</tr>
<tr>
<td>0.5</td>
<td>0.09\times10^{-1}</td>
<td>0.5</td>
<td>0.08\times10^{-1}</td>
</tr>
<tr>
<td>1.0</td>
<td>0.17\times10^{-1}</td>
<td>1.0</td>
<td>0.16\times10^{-1}</td>
</tr>
<tr>
<td>1.5</td>
<td>0.25\times10^{-1}</td>
<td>1.5</td>
<td>0.25\times10^{-1}</td>
</tr>
<tr>
<td>2.0</td>
<td>0.33\times10^{-1}</td>
<td>2.0</td>
<td>0.32\times10^{-1}</td>
</tr>
<tr>
<td>2.5</td>
<td>0.40\times10^{-1}</td>
<td>2.5</td>
<td>0.39\times10^{-1}</td>
</tr>
<tr>
<td>3.0</td>
<td>0.55\times10^{-1}</td>
<td>3.0</td>
<td>0.45\times10^{-1}</td>
</tr>
</tbody>
</table>
In 100 ml of aqueous solutions of different strength of NaCl and KCl, 75 seeds of sunflower and Cotton were soaked independently. The duration of soaking was 10h.

**Preliminary screening experiments**

After treatment, the seeds were thoroughly washed in tap water and sown in earthen pots in the open field. Pots of uniform size (height - 30 cm; diameter - 38 cm) were taken and filled with an equal volume of vermiculite. The pre-treated seeds were sown in pots (20 per pot) and allowed to germinate. The pots were irrigated on alternate days with equal volume of water. Each treatment was replicated thrice. Percentage of germination was calculated on the 7th day. After 15 days of germination, length of shoot and root, vigour index, shoot and root tolerance indices, fresh and dry weight of the seedlings were calculated.

**Percentage of germination**

Percentage of germination was calculated on the 7th day using the following formula.

\[
\text{Percentage of germination} = \frac{\text{No of seeds germinated}}{\text{Total number of seeds sown}} \times 100
\]

**Length of shoot and root**

The seedlings were carefully removed from each pot and washed gently in tap water. The shoot length was measured from the ground level to the tip. Root length was also
measured from the ground level to the tip. Mean value ±SD was calculated and the length of shoot/root was expressed as cm per seedling.

**Vigour Index**

Seedling vigour index was calculated by the method suggested by Abdul-Baki and Anderson (1973).

\[
\text{Vigour Index} = \text{Length of embryonic axis (cm)} \times \text{percentage of germination.}
\]

**Shoot and root tolerance indices**

Shoot and root tolerance indices were calculated by the method adopted by Taylor and Foy (1985).

\[
\text{Shoot/Root Tolerance} = \frac{\text{Length of shoot/root of treated plants}}{\text{Length of shoot/root of control plants}}
\]

**Fresh and dry weight of seedlings**

Shoot and root samples were separated from a set of 10 seedlings collected from each pot. Fresh weight was taken. The samples were then kept in an hot air oven at 80°C for 48 h to a constant weight and the dry weight was taken using electrical balance (Sartorius BA210S, West Germany). Mean weight ±SD was calculated and recorded as mg per plant.

On the basis of the results obtained in the preliminary screening experiments with reference to percentage of
germination, shoot height, root length, vigour index, shoot and root tolerance indices, fresh and dry weight of the seedlings, lower, optimal and higher concentrations of each salt were worked out to narrow down the strength of the solution for further experiments. The concentrations thus determined for sunflower and cotton were as follows:

<table>
<thead>
<tr>
<th>Plant Name</th>
<th>Salts</th>
<th>Low</th>
<th>Optimum</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sunflower</td>
<td>NaCl</td>
<td>0.1</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>0.1</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Cotton</td>
<td>NaCl</td>
<td>0.1</td>
<td>0.5</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>KCl</td>
<td>0.1</td>
<td>1.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Mobilization of seed reserves under pre-sowing salt treatment

The experiments for mobilization studies were carried out under laboratory conditions (30±1°C; RH-76%). Surface-sterilized sunflower and cotton seeds were pre-treated with lower, optimal and higher concentrations of NaCl and KCl solutions for 10h. The soaked seeds were washed thoroughly and then allowed to germinate in sterilized petridishes (13 cm diameter). About 20 seeds were placed in each petridish lined with Whatman No. 1 filter paper and moistened with
distilled water. Seeds soaked in distilled water served as the control. Each treatment was replicated three times.

During the course of germination, the germinating seeds were collected and assorted into cotyledons and embryonic axis at four different periods following soaking viz., 0, 24, 48 and 72 h.

<table>
<thead>
<tr>
<th>Hours after soaking</th>
<th>Seedling Organs</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Cotyledons and Embryonic axis (not differentiated)</td>
</tr>
<tr>
<td>24</td>
<td>Cotyledons and Embryonic axis (not differentiated)</td>
</tr>
<tr>
<td>48</td>
<td>Cotyledons and Embryonic axis (differentiated into hypocotyl and radicle)</td>
</tr>
<tr>
<td>72</td>
<td>Cotyledons and Embryonic axis (differentiated into hypocotyl and radicle)</td>
</tr>
</tbody>
</table>

Samples were collected at different time intervals after soaking and homogenised either in glass distilled water or 80% methanol or appropriate buffers depending on different kinds of analyses.
Effects of pre-sowing soaking treatment on function of leafy cotyledons

For the study on function of leafy cotyledons, pot culture experiments were carried out under natural conditions. Pots of uniform size (height – 30 cm; diameter-38 cm) were taken and filled with garden soil. The pH of the soil was 8.3 and the EC Value was 0.8 mmhos/cm. The seeds soaked in lower, optimal and higher concentrations of each salt were sown about 20 per pot and allowed to germinate. Seeds soaked in distilled water served as control. The pots were irrigated carefully to avoid running down of water. Each treatment was replicated three times.

Following emergence from the soil, the cotyledons expanded between 5 and 7 days after sowing. Plant samples were periodically collected during the persistent period of the cotyledonary leaves at an interval of 5 days upto 25 days. In sunflower after 25 days of growth, the cotyledonary leaves shrivelled and got abscised. Chemical estimations of chloroplast pigments, total soluble starch, total soluble sugars and total water soluble proteins at indicated time intervals were carried out in the cotyledonary and primary leaves. Coupled to this investigation, growth parameters viz., shoot height from the ground level, root length, fresh and dry weight of the seedlings were also determined.
Plot design

40' x 10' x 2'

N
Field study

The experimental plots for the field study were designed and prepared in the college botanical garden. The soil was red sandy loam with the following chemical characteristics. The pH of the soil was 8.3 and the EC value was 0.8 mmhos/cm. The available soil nutrients were nitrogen (10.36 mg/g), phosphorus (0.63 mg/g) and potassium (2.44 mg/g). The important micronutrients of the soil were iron (2.44 mg/g), zinc (0.06 mg/g), manganese (0.28 mg/g) and copper (0.06 mg/g). The ridges and furrows of the plot were formed at an interval of 2 feet. Each plot consisted of five rows and each row consisting of 50 plants approximately with a row to row spacing of 2 feet and plant to plant spacing of 9 inches (Plot design).

Sunflower and cotton seeds were pre-treated with lower, optimal and higher concentrations of each salt. The treated seeds were washed thoroughly with tap water and sown on both the sides of the ridges. The first irrigation was done on the following day of sowing and subsequent irrigations were done periodically once in two days approximately to the field capacity. The other necessary plant protection measures such as weeding, manuring and application of insecticides were carried out as required. For each treatment, three replicates of 50 seeds were used.

Agrobotanical characters
For analyses of agrobotanical characters, 20 plants were randomly collected from each row for each treatment. The following growth parameters at two stages of growth (30 days after sowing and at the time of harvest, i.e., 90 days after sowing) were analysed in the control and treated plants.

1. Height of the plant (cm)
2. Length of the root (cm)
3. Total leaf area (cm²)
4. Fresh weight of the plant (g)
5. Dry Weight of the plant (g)
6. Shoot root ratio
7. Shoot and root tolerance indices.

Relative growth rate (RGR)

RGR is the increase in dry weight per unit of original weight over a time interval 't'. RGR was calculated using the formula of Williams (1946).

\[ RGR = \frac{W_2 - W_1}{t_2 - t_1} \times \frac{1}{W_1} \]

\( W_1 \) = Dry weight of the plant on previous Sampling

\( W_2 \) = Dry weight of the plant on succeeding sampling

\( t_1 \) = Time of previous sampling

\( t_2 \) = Time of succeeding sampling.
For RGR calculation, 10 plants were collected randomly from each row for each treatment and control at six different stages of growth viz., 5, 15, 25, 35, 45 and 55 days after sowing. Total dry weight was measured. Average was calculated and dry weight per plant was determined. The dry weight of plant samples on two successive stages was used for calculation. The results are expressed as mg day\(^{-1}\).

**Analyses of yield components**

For each treatment as well as for the control, 20 plants (90-day old) were randomly collected and analysed for the following yield components.

**Sunflower**

1. Number of seeds per capitulum
2. Fresh weight of 100 seeds (g)
3. Dry weight of 100 seeds (g)
4. Total seed dry weight per plant (g)
5. Harvest index (%)

**Cotton**

1. Number of bolls per plant
2. Total number of seeds per plant
3. Number of seeds per boll
4. Dry weight of 100 seeds (g)
5. Total seed dry weight per plant (g)
6. Harvest index (%)

**Harvest Index**

Harvest index was calculated using the following formula

\[
\text{Harvest index} (%) = \frac{\text{Total seed dry weight/plant}}{\text{Total dry weight/plant}} \times 100
\]
Elemental analyses (Jackson, 1967)

Elements such as Na and K were determined in germinating seedlings using Flame photometer (Systronics, India)

The samples were dried in an hot air oven at 100±2°C for 48 h and powdered with pestle and mortar. 500 mg of desired sample was taken separately in a boiling test tube, to which 15.0 ml of triple acid mixture (10.0 ml of conc. HNO₃, 4.0 ml of HClO₄ and 1.0 ml of conc. HCl) was added. The tubes were heated until complete ceaseover of the brown fumes. The samples were cooled to room temperature and diluted to 100 ml using double distilled water. The samples were filtered through Whatman No.1 filter paper. The filtered samples were used for elemental analyses through Flame photometer (Na and K) (Systronics, India) using respective standard and characteristic filter for each element.

The ppm values thus obtained were converted to mg g⁻¹ dw using the following formula.

\[
\text{Percentage} = \frac{\text{PPm \times Volume of the sample \times 100}}{\text{Weight of the sample \times 100 000}}
\]

mg g⁻¹ dw = Percentage value \times 10
Chemical estimations

For all estimations of chemical constituents, the primary leaves of the first node were collected from the bottom of 30-day old plants. The leaf samples were dried in shade and used for the following estimations.

1. Chloroplast pigments (Chlorophylls and Carotenoids)
2. Total lipids
3. Total water soluble proteins
4. Total soluble sugars
5. Free amino acids
6. Proline

Similarly for estimations of chemical constituents in germinating seedlings, the assorted seedling organs namely colyledons and embryonic axes were dried in shade and used for the following analyses.

1. Total lipids
2. Oil
3. Total water soluble proteins
4. Free amino acids
5. Proline
6. Total soluble sugars
7. Free fatty acids

Estimation of chlorophyll pigments

Chlorophyll pigments were estimated according to the procedure of Arnon (1949)

Hundred mg dry leaf tissue (Colyledonary/ primary) was homogenised with 10.0 ml of ice cold aqueous 80% acetone. The extract was centrifuged for 5 min at 5000 rpm using Remi centrifuge (Remi - R8C, India) The supernatant was decanted
and stored. The pellet was again washed with 5.0 ml of 80% acetone, centrifuged and the procedure was repeated until the pellet became colourless. The supernatants were pooled and the total volume was measured. The absorbance was measured using UV-VIS Spectrophotometer (Systronics-118, India) at 663 and 645 nm. Quantification of chlorophyll a, chlorophyll b and total chlorophylls was done using the following formulas.

\[
\text{Chlorophyll a} = \frac{(12.7 \times \text{OD 663}) - (2.69 \times \text{OD 645})}{1000 \times W \times a} \\
\text{Chlorophyll b} = \frac{(22.9 \times \text{OD 645}) - (4.68 \times \text{OD 663})}{1000 \times W \times a} \\
\text{Total Chlorophyll} = \frac{(20.2 \times \text{OD 645}) + (8.02 \times \text{OD 663})}{1000 \times W \times a}
\]

\text{OD} = \text{Absorbance at respective wavelength}

\text{V} = \text{Volume of the extract (ml)}

\text{W} = \text{Dry weight of the sample (g)}

\text{a} = \text{Length of the light path (1.0 cm)}

Estimation of carotenoids

The optical density of the same 80% acetone extract was read at 473 nm and for quantitative determination of carotenoids, an extinction of \(E_{1\%}^{1cm} 2500\) at 473 was used as an average value (Goodwin, 1954).
Estimation of total water soluble proteins

Total water soluble proteins were estimated following the procedure of Lowry et al. (1951).

Five hundred mg dried sample (Colyledons/embryonic axis/leaf) was ground with 5.0 ml of distilled water and centrifuged at 5000 rpm using Remi centrifuge (Remi -R8C, India) for 10 min. The pellet was re-extracted with the same volume of the solvent. The supernatants were saved and the total soluble proteins were precipitated with an equal volume of 10% trichloroacetic acid (TCA) and centrifuged at 3000 rpm for 10 min using Remi centrifuge (Remi-R8C, India). The pellet was dissolved in 5.0 ml of 0.1 N NaOH and used as sample for protein estimation.

To 0.5 ml of sample, 0.5 ml of 0.1% CuSO₄ (W/V) and 2.5 ml of 12.5% Na₂CO₃ and 0.5 ml of 25% folin reagent (V/V) were added. 1.0 ml of distilled water was added to make up the final volume of 5.0 ml. The reaction mixture without the sample served as the blank. The reaction mixture was kept for 20 min in dark and the intensity of the colour developed was read in UV-VIS Spectrophotometer (Systronics-118, India) at 740nm. Total soluble proteins were calculated using bovine serum albumin (BSA) as the standard.
Estimation of free amino acids

The estimation of free amino acids was carried out by the method of Troll and Canan (1953).

Hundred mg dry tissue (cotyledon/embryonic axis/leaf) was ground with 5.0 ml of 80% methanol at 0°C, filtered and centrifuged at 5000 rpm using Refrigerated centrifuge (Susperspin-R., Plastocraft, India) at 5°C for 15 min. The supernatant was stored and the pellet was re-extracted twice with the same solvent. The supernatants were pooled and to the pooled supernatant an equal volume of petroleum ether was added to remove the chloroplast pigments. Lower methanol phase was used for amino acid estimation.

To 1.0 ml sample, 1.0 ml of 80% phenol (80 ml of phenol dissolved in 20 ml absolute ethanol) was added and kept in a boiling water bath for 10 min. Then 0.2 ml of 5% ninhydrin (W/V) (ninhydrin dissolved in methanol) was added and kept in a boiling water bath for 10 min for colour development. The sample was made up to 10.0 ml with 60% ethanol. Absorbance was measured at 575 nm in UV-VIS Spectrophotometer (Systronics-118, India). Leucine was used as the standard.

Estimation of free proline

Free proline from the tissues was selectively extracted in aqueous sulfosalicylic acid and its concentration measured using ninhydrin method (Bates et al., 1973)
Two hundred mg dry tissue (Colyledon/embryonic axis/leaf) was homogenised in a mortar and pestle with 10.0ml of 3.0% sulfosalicylic acid. The homogenate was filtered through Whatman No-2 filter paper. The extraction was repeated twice and the filtrates were pooled and made upto a known volume.

To 2.0 ml of the filtrate, 2.0 ml of acid ninhydrin and 2.0 ml of glacial acetic acid were added and incubated for 1 h at 100°C in an hot waterbath. The tubes were then kept in an ice bath to terminate the reaction. Four ml of toluene was added and mixed vigorously using a vortex mixer for about 20 seconds. The toluene containing chromophore was aspirated from the aqueous phase and the absorbance was read at 520 nm in UV-VIS Spectrophotometer (Systronics -118, India) using toluene as blank. The proline concentration was calculated from a standard curve prepared with authentic proline.

Acid ninhydrin: 1.25 g ninhydrin was dissolved in a mixture of glacial acetic acid (30 ml) and 6M phosphoric acid (20 ml) with agitation. The reagent was stable for 24h when stored at 4°C.

Estimation of total soluble starch

Total soluble starch was estimated using the procedure outlined by McCready et al. (1950).
One gram dry leaf tissue was homogenised with 10.0 ml of 80% methanol. The homogenate was centrifuged at 5000 rpm in Remi Centrifuge (Remi-R8C, India) for 5 min. The pellet was retained and washed repeatedly with 80% methanol to remove all traces of soluble sugars. 50.0 ml of water and 6.5 ml of 52% perchloric acid (PCA) were added to the pellet and mixed thoroughly. The extract was centrifuged at 4000 rpm for 10 min and the supernatant was saved. The pellet was again treated with PCA and centrifuged. The supernatant was collected and the above step was repeated. All the supernatants were pooled and made up to a known volume.

For estimation of starch, 0.5 ml of sample was taken in a test tube. To this, 4.5 ml of distilled water and 10.0 ml of cold anthrone-sulphuric acid (200 mg anthrone dissolved in 100 ml of cold H$_2$SO$_4$) reagent were added, mixed thoroughly and kept in a boiling water bath for colour development. The intensity of colour was read in UV-VIS Spectrophotometer (Systronics -118, India) at 630 nm. Glucose was used as the standard. The total value was multiplied by a factor of 0.9.

*Estimation of total soluble sugars*

Total soluble sugars were estimated following the method of Dubois et al. (1956).
One gram of dry sample (Cotyledon/embryonic axis/leaf) was ground in a mortar and pestle with 10.0 ml of 80% ice-cold methanol. It was then centrifuged at 5000 rpm for 10 min using Remi Centrifuge (Remi- R8C, India) for 5 min. The supernatant was saved. Another 10.0 ml of 80% methanol was added to the pellet-and re-extracted and the combined supernatants were pooled and stored in an ice-bucket.

In a test tube 0.4 ml of the methanolic extract was taken. One ml of 5% phenol was added. It was followed by the addition of 5.0 ml of conc. H₂SO₄. The volume was then made upto 15.0 ml with distilled water. The mixture was kept at room temperature for 15 min. The colour developed was measured at 490 nm in UV-VIS Spectrophotometer (Systronics-118, India). Glucose was used as the standard.

Quantification of lipoid compounds

Quantification of certain lipoid compounds such as total lipids, oil, glycerol and free fatty acids was carried out in germinating seedlings as well as in mature, healthy and dry seeds of the control and treated plants.

Total lipids

Total lipids were determined gravimetrically following the method outlined by Folich et al. (1957).
Hundred mg dry tissue (cotyledon/embryonic axis/leaf/mature seed) was homogenised with 10.0ml of chloroform: methanol (2:1) solvent mixture. The extract was taken in a separating funnel. Using 0.72% NaCl solution, the extract was washed to remove the water soluble impurities. The lower layer of chloroform-methanol containing the total lipids was separated and taken in a crucible of known weight ($W_1$) and it was allowed to evaporate to dryness in an hot air oven at 80°C. The weight of total lipids was determined by reweighing the crucible ($W_2$).

$$\text{Total lipids} = W_2 - W_1$$

Oil estimation

The method adopted for extraction and determination of oil content was the one described by Meara (1955).

Extraction of one g dry tissue (Cotyledon/embryonic axis/mature seed) with petroleum ether in a soxhlet apparatus was continued for 18h. The extract was then quantitatively transferred to a pre-weighed flask and the solvent evaporated. The last traces of the solvent and moisture were removed by heating at 100°C under reduced pressure. The flask was then allowed to cool in a dessicator and reweighed to obtain the weight of the oil in the sample.
Glycerol estimation

Glycerol was estimated by the method of Mansour (1972). This method is based on the oxidation of glycerol in the presence of sulphuric acid and an oxidising agent namely potassium dichromate.

Extraction of glycerol was carried out by boiling one g dry tissue (seed) in 10.0ml of water. After boiling, the tissue was homogenised in a mortar and pestle. The homogenate was centrifuged in a Remi centrifuge (Remi-R8C, India) and the supernatant was taken and made upto a known volume.

To 10.0 ml of the extract in a boiling tube, 5.0 ml of 7.5% K$_2$CrO$_7$ followed by 30.0 ml of H$_2$SO$_4$ (50% V/V) were added. The contents were mixed and boiled for 15min and then cooled. The mixture was made upto 50 ml. The intensity of the colour developed was measured at 600 nm in UV-VIS Spectrophotometer (Systronics - 118, India). The results were expressed as OD units g$^{-1}$ dw.

Estimation of free fatty acids

Total free fatty acids were estimated following Anderson and McCarty (1972).

Five hundred mg dry tissue (Cotyledon/embryonic axis/mature seed) was homogenised with 10.0 ml of a solvent mixture composed of chloroform, petroleum ether and ethyl
ether (1:1:1). The homogenate was centrifuged at 4000 rpm using Remi Centrifuge (Remi-R8C, India) for 15 min. The supernatant was taken in a separating funnel, to which equal volume of chloroform was added, mixed thoroughly and allowed to separate. The lower chloroform layer was taken for estimation. To 3.0 ml of chloroform sample, 0.5 ml of 0.1% Rhodamine 6 G solution (100 mg Rhodamine dissolved in 100 ml benzene) was added. After 15 min the absorbance was read at 515 nm using UV-VIS Spectrophotometer (Systronics-118, India). Stearic acid was used as standard.

Assay of enzymes

A few enzymes like total lipases, nitrate reductase, protease and catalase were assayed in the germinating seedlings as well as in the leaf samples of 30-day old plants.

Assay of total lipases (Colowick and Kaplan, 1955)

One g fresh tissue (Seedling organs/leaf) was homogenised in a pre-chilled mortar and pestle with 10.0 ml of M/15 phosphate buffer (pH 7). The homogenate was centrifuged at 10000 rpm at 4°C in a Refrigerated Centrifuge (Superspin - R., Plastocraft, India). The crude supernatant was taken as the enzyme source.
To one ml of the enzyme in a test tube, 2.0 ml of M/15 phosphate buffer (pH 7) and 5.0 ml of distilled water were added. After one min 2.0ml of p-nitrophenol acetate (PNPA) (63.0 mg p-nitrophenol acetate in 10.0 ml methanol) was added. Zero time OD was measured at 410nm in a UV-VIS Spectrophotometer (Systronics - 118, India). The blank consisted of reagents without PNPA. The tubes were then incubated at 25°C for 20 min. OD was again measured at 410 nm in UV-VIS spectrophotometer. The difference between the final and initial OD values was calculated. Using the extinction coefficient of p.nitrophenol $E_{410}^M = 1.62 \times 10^4$, the amount of p-nitrophenol hydrolysed from PNPA was calculated.

The results are expressed as m moles of p-nitrophenol released mg$^{-1}$ protein h$^{-1}$.

Assay of in vivo nitrate reductase activity (NRA)

NRA was determined by the in vivo assay method of Hageman and Hucklesby (1971).

Five hundred mg fresh leaves/seedling organs were washed and cut into 5.0 mm discs/segments. The discs/segments were vacuum infiltrated for 2 min in 5.0 ml of 0.1 M phosphate buffer (pH 7.5) containing 100mM KNO$_3$ and 5.0% n-propanol. After infiltration, vials were incubated for 30 min in dark. The control contained the same
constituents omitting plant tissue. Nitrite formed was determined by adding 1.0 ml of 1.0% sulphanilamide in 3.0 N HCl and 1.0 ml of 0.02% N-Ethylene diamine dihydrochloride (NEDH) to 1.0ml of the sample from the incubation medium. After 10 min, the OD was measured at 540 nm in UV-VIS Spectrophotometer (Systronics-118, India) KNO₂ was used as the standard.

Nitrate reductase activity is expressed as μ moles of nitrite produced mg⁻¹ protein h⁻¹.

Assay of protease

Protease was assayed according to the procedure of Penner and Ashton (1967) in combination with the method of Mahadevan and Sridhar (1982).

One g of fresh tissue (Seedling organs/leaves) was ground in a pre-chilled mortar and pestle and extracted with 6 volumes of 0.1 M phosphate buffer (pH 6.5). The homogenate was strained through four layers of muslin cloth and centrifuged at 12000 rpm for 20 min at 4°C using Refrigerated centrifuge (Superspin -R., Plastocraft, India). The supernatant was used as the crude enzyme source.

The assay mixture was prepared by taking 2.0 ml of the crude enzyme, 1.0 ml of 0.1 M phosphate buffer (pH 6.5) and 1.0ml of 1.0% casein. The reaction mixture was incubated at
37°C for 2 h and then the reaction terminated by addition of 3.0 ml of 5.0% TCA. The reaction mixture was centrifuged at 4000 rpm for 10 min using Remi Centrifuge (Remi - R8C, India). The supernatant was used for determination of free aminoacids by ninhydrin method.

The reaction mixture in a final volume of 2.0 ml containing 0.1 ml of the supernatant, 1.4 ml of 0.5 M citrate buffer (pH 5.0) and 0.5 ml of 1.0% ninhydrin was heated in a waterbath for 15 min, cooled and diluted to 10.0 ml with 50% n-propanol and the colour intensity was measured in UV-VIS Spectrophotometer (Systronics -118, India) at 570 nm.

The activity is expressed as ug aminoacids (Leucine equivalent) released mg⁻¹ protein h⁻¹

Assay of Catalase (Clairborne, 1985)

One g fresh tissue (Seedling organs/ leaves) was ground at 4°C using mortar and pestle with polyvinylpoly- pyrrolidone (25% by weight) and 4 volumes of buffer consisting of 50 mM KH₂PO₄/K₂H PO₄ and 0.1 mM EDTA at pH 7.0. The homogenate was filtered and centrifuged at 8000 rpm for 20 min at 5°C using Refrigerated centrifuge (Superspin -R., Plastocraft, India). Crude supernatant was used as the enzyme source.
Catalase was assayed in a total volume of 3.0 ml reaction mixture containing 200 μl crude enzyme, 50 mM KH$_2$PO$_4$ buffer (pH 7.0) and 37.5 mM H$_2$O$_2$. The decomposition of H$_2$O$_2$ was followed by the decline in absorbance at 240 nm using UV-VIS Spectrophotometer (Systronic-118, India). The activity was calculated using the following formula.

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\text{Units mg}^{-1}\text{ protein min}^{-1} = \frac{\text{Absorbance/min} \times 1000}{43.6 \times \text{mg protein/ml reaction mixture}}
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*43.6 = Molar absorbancy index for hydrogen peroxide at 240 nm in a 1 cm cuvette.

The activity is expressed in units mg$^{-1}$ protein h$^{-1}$. 