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CHAPTER 3

MATERIAL AND METHODS

This chapter deals with the description of material used for the study and the methods adopted for the experimentation and determination of various traits during the course of the investigation.

3.1 Plant Material

The experimental material selected for the study was mustard (*Brassica juncea* L. Czern & Coss.). Mustard cultivars, ‘Type 59’, ‘Rayada’, ‘Chutki’, ‘Trishul’ and ‘Anmol 555’ were obtained from the local market, Aligarh, India.

3.1.1 Mustard

3.1.1.1 Nomenclature

Mustard is a common name for several herbaceous plants in the genera *Brassica*. The three main plants associated with mustard are *Sinapis hirta*, *Brassica juncea* and *Brassica nigra*.

*Brassica nigra* is known as black mustard; *Brassica juncea* as brown or Indian (Oriental) mustard; *Sinapis hirta* (or *Sinapis alba*) as white or yellow mustard. Seeds of *Brassica nigra* and *Brassica juncea* are considered to have the same pungency, seeds of white mustard are considered to be different in pungency. Indian Oleiferous Brassica is divided into four groups:

2. Sarson
   (i) Yellow sarson- *Brassica campestris* L.
   (ii) Brown Sarson- *Brassica campestris* L.
3. Toria (Lahi or maghi lahi)- *Brassica campestris* L.
4. Taramir or tara- *Eruca sativa* Mill.
In addition, two more species namely, *Brassica juncea* var. Rugosa (Pahadi rai) and *Brassica nigra* Koch (Banarasi rai) do not fall under any of the four groups.

*Brassica juncea* is the dominant species grown in India (Prakash 1980; Yadava and Singh 1999). It is an amphidiploid with a chromosome number of 18 and is believed to have derived from natural interspecific hybridization between *Brassica nigra* (n=8) and *Brassica rapa* (n=10). *Brassica juncea* is likely to have originated where distributions of *Brassica nigra* and *Brassica rapa* overlap, such as in the Middle East and neighbouring regions (Prakash 1980).

### 3.1.1.2 Botanical Description

*Brassica juncea* is an annual glabrous herb with broadly ovate, coarsely dentate basal leaves. The upper leaves are linear and entire. Roots, in general, are long and tapering. Toria is more or less surface feeder, but brown mustard has more or less long roots with limited spreads. Yellow sarson has both extensive and lateral spreads. The height of stem varies from 45 cm (in some varieties of Toria) to 190 cm (in Yellow sarson). In Toria and Brown sarson, the branches arise at an angle of 30º to 40º. In Yellow sarson, the branches arise laterally at an angle of about 10º to 20º and give the plant a narrow and pyramidal shape. The flowers are small and yellow coloured in corymbose recemes with cruciform petals. In yellow sarson the four petals are spread apart, whereas in brown sarson and toria, the petals overlap or may be placed apart, depending upon the cultivar. Fruit is a siliqua, breaking away from below upwards. The siliqua are two-valved, three-valved or four-valved, depending upon the number of carpels in the ovary. Seeds are exalbuminous. The flower begins to open from 8:00 h and continue up to 12:00 noon.

### 3.2 Experimentation

The experiments were conducted in pots filled with reconstituted soil (peat and compost, 4:1 (v/v); mixed with sand, 3:1 (v/v)) during the winter season of 2010-11, 2011-12 and 2012-13 on mustard (*Brassica juncea* L. Czern & Coss,) in the greenhouse of the Department of Botany, Aligarh Muslim University, Aligarh, India (27º53'N latitude, 78º40'E longitude and 187.45 m altitude above sea level) under natural day/night conditions. The experimental period extended from early October to late November.
3.2.1 Soil Physio-chemical Properties

To ensure maximum soil-aeration, thoroughly ploughed soil was collected from University Agriculture Farm, Aligarh Muslim University, Aligarh for the filling of earthen pots. Soil samples were collected randomly from different areas of plots and mixed thoroughly for filling the pots. Before pots filling the soil was analyzed for selected characteristics which had pH 7.6; available N at 100 mg kg\(^{-1}\) soil and sufficient P and K.

3.2.2 Treatments

Before the start of each experiment, earthen pots of 23-cm diameter were filled with mixture of soil. An approach of utilizing chemicals to modify ethylene synthesis and action was considered to assess the sensitivity of plants to ethylene. Ethephon and NBD were selected as the tool chemicals to modify ethylene synthesis and ethylene action, respectively. Mustard plants were treated with 200 µL L\(^{-1}\) ethephon and 100 µM NBD to modulate plant metabolism. Each of the treatment was given in 50 mL together with 0.5% surfactant teepol. The control group of plants was sprayed with an equal amount of deionized water plus 0.5% teepol. A high soil phosphorus (P) status was maintained by adding 30 mg P kg\(^{-1}\) soil as single superphosphate so that this nutrient may not influence the ethephon effects as ethephon on hydrolysis releases ethylene and P. Further, Ni and Zn were applied as nickel sulfate (NiSO\(_4\)) and zinc sulfate (ZnSO\(_4\)) at the time of sowing. Appropriate amount i.e., 0 or 126.41 or 252.83 or 505.67 mg NiSO\(_4\) or 126.15 or 252.30 or 504.60 ZnSO\(_4\) was mixed thoroughly with soil to achieve 0 or 50 or 100 or 200 mg Ni/Zn kg\(^{-1}\) soil, respectively. The specific treatments are elaborated separately under experiment headings. Experiment treatments were as follows:
Table 9: Explanation of treatments in Experiments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>200 µL L⁻¹ Etephon</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>50 mg Ni kg⁻¹ soil</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>100 mg Ni kg⁻¹ soil</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>200 mg Ni kg⁻¹ soil</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>50 mg Zn kg⁻¹ soil</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>100 mg Zn kg⁻¹ soil</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>200 mg Zn kg⁻¹ soil</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>100 µM NBD</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Cultivars</td>
<td>Type 59; Rayada; Chutki; Trishul; Anmol 555</td>
<td>Type 59 (Ethylene-sensitive)</td>
<td>Type 59 (Ethylene-sensitive)</td>
</tr>
<tr>
<td>Replicates</td>
<td>Five</td>
<td>Five</td>
<td>Five</td>
</tr>
<tr>
<td>Plant sampling</td>
<td>30 DAS</td>
<td>30 DAS</td>
<td>30 DAS</td>
</tr>
</tbody>
</table>

3.2.3 Sowing of Seeds

Healthy seeds of mustard cultivars were surface sterilized with 0.01 g L⁻¹ HgCl₂ solution followed by repeated washings with distilled water. Before sowing light application of deionized water was given in each pot to provide necessary moisture for germination of the seeds. Ten seeds per pot were sown to avoid germination failure and after the establishment of seedlings, thinning was done to retain only three healthy plants of nearly equal size in each pot.

3.3 Experimental Set-up

The experimental details are provided separately for each experiment and for clear and easy understanding they are presented (Scheme of treatments 1-3).
3.3.1 Experiment 1

Experiment 1 was conducted in the winter season of 2010-11. Surface sterilized seeds of mustard cultivars, ‘Type 59’, ‘Rayada’ ‘Chutki’ ‘Trishul’ and ‘Anmol 555’ were sown on October 15, 2010. The aim of the experiment was to select ethylene-sensitive and ethylene-insensitive mustard cultivars. To assess the sensitivity of plants to ethylene an approach of utilizing chemicals to modify ethylene biosynthesis and action was considered. Ethephon (ethylene source) and NBD (ethylene action inhibitor) were selected as tool chemicals to modify ethylene biosynthesis. Ethephon releases ethylene inside the cell on hydrolysis, while NBD inhibits ethylene action. Mustard cultivars were treated with 0, 200 µL L\(^{-1}\) ethephon, 100 µM NBD at 20 DAS. Each of the treatment was given in 50 mL together with 0.5% surfactant teepol. The control group of plants was sprayed with an equal amount of deionized water plus 0.5% teepol. Ethephon on hydrolysis results in ethylene and phosphoric acid. Therefore, to account for the effects of ethylene alone, sufficient amount of phosphate was applied with the other ethephon concentrations and control so that equivalent amount of phosphate is present in all the treatments. The treatments were arranged in a factorial randomized design and the number of replicates for each treatment was five. At 10 days after treatment i.e. 30 DAS, plants sampling was done to record various parameters as given below and on these basis ethylene-sensitive and ethylene-insensitive cultivars were selected. There were total 75 pots used in the experiment (3 treatments x 5 cultivars x 5 replicates x 1 sampling). For sampling, one plant from each pot was selected for ethylene biosynthesis and photosynthetic characteristics, while the other plant was used for determination of growth. Following parameters were studied at the sampling time.

A. Ethylene Biosynthesis
   (i) ACS Activity
   (ii) Ethylene

B. Photosynthetic Characteristics
   (i) Chlorophyll Content
   (ii) Rubisco Activity
   (iii) Net Photosynthesis
(iv) Stomatal Conductance
(v) Intercellular CO₂ concentration

C. Growth characteristics
   (i) Leaf Area
   (ii) Plant Dry Mass

3.3.2 Experiment 2

Experiment 2 was conducted in the winter season of 2011-12. This experiment was conducted using the ethylene-sensitive cultivar ‘Type 59’, based on the findings of Experiment 1. Seeds of ‘Type 59’ cultivar were sown on October 16, 2011. The aim of the experiment was to study the influence of 0, 50, 100 and 200 mg kg⁻¹ soil of Ni and Zn on the metabolism of mustard plant and concurrent changes in photosynthetic and growth characteristics. All studied parameters were sampled at 30 DAS. There were total 40 pots used in the experiment (8 treatments x 1 cultivar x 5 replicates x 1 sampling). Treatments were arranged in a complete randomized block design. The number of replicates for each treatment was five. At 30 DAS, plants were selected for the determination of S-assimilation, N-assimilation, proline metabolism, ethylene biosynthesis, photosynthetic characteristics and growth. At 30 DAS, one plant from each pot was selected for the determination of ethylene biosynthesis, S-assimilation, N-assimilation and photosynthetic characteristics. Second plant was used to determine antioxidant metabolism, proline metabolism and oxidative stress measurements. Another plant was uprooted and leaf area was determined. Subsequently, leaves and the rest of the plant were dried separately. Leaf dry mass was recorded and plant dry mass was recorded. The dried leaf powder was used for leaf S and N determination. The following parameters were recorded at 30 DAS in this experiment.

A. Oxidative Stress
   (i) H₂O₂ Content
   (ii) TBARS Content

B. Antioxidant Metabolism
   (i) Superoxide Dismutase Activity
**SCHEME 1**: Diagrammatic representation of the arrangement of pots for Experiment 1. Scheme shows the arrangement of pots for five cultivars sampled at 30 DAS. Each row represents the arrangement of pots for one cultivar. Treatments were applied on leaf foliage of plants at 20 DAS. At 30 DAS, two plants from each pot in a cultivar and replicate was used for determination of various parameters studied.
Material and Methods

(ii) Ascorbate Peroxidase Activity

(iii) Glutathione Reductase Activity

(iv) Reduced Glutathione Content

C. Sulfur Assimilation

(i) ATP-sulfurylase Activity

(ii) Sulfur Content

(iii) Serine Acetyltransferase Activity

(iv) Cysteine Content

(v) Methionine Content

D. Nitrogen Metabolism

(i) Nitrate Reductase Activity

(ii) Nitrogen Content

E. Proline Metabolism

(i) Proline Content

(ii) Glutamyl Kinase Activity

(iii) Proline Oxidase Activity

F. Photosynthetic Characteristics

(i) Rubisco Activity

(ii) Chlorophyll Content

(iii) Net Photosynthesis

(iv) Stomatal Conductance
Material and Methods

(v) Intercellular CO$_2$ Concentration

(vi) Water Use Efficiency

(vii) Actual PS II Activity

(viii) Maximum PS II Activity

(ix) Intrinsic PS II Activity

(x) Photochemical Quenching

(xi) Non-photochemical Quenching

(xii) Electron Transport Rate

G. Photosynthetic Nutrient Use Efficiency

(i) Photosynthetic-Nitrogen use Efficiency

(ii) Photosynthetic-Sulfur use Efficiency

H. Ethylene Biosynthesis

(i) ACS Activity

(ii) Ethylene

I. Water Relations

(i) Leaf Water Potential

(ii) Leaf Osmotic Potential

J. Growth Characteristics

(i) Leaf Area

(ii) Relative Growth Rate

(iii) Plant Dry Mass
SCHEME 2: Diagrammatic representation of the arrangement of pots for Experiment 2. Scheme shows the arrangement of pots at different levels of heavy metals concentrations. Heavy metals were mixed in the soil. At 30 DAS, three plants from each treatment was used for determination of various studied parameters.
3.3.3 Experiment 3

Experiment 3 was conducted in the winter season of 2012-13 with the purpose to study the role of ethylene in mediating Ni and Zn-induced metabolism of ethylene-sensitive mustard cv. ‘Type 59’. To study the significance of ethylene in Ni stress and Zn stress amelioration, the selected, Type 59 ethylene-sensitive cultivar was subjected to 0 (control), 200 µL L⁻¹ ethephon, 200 mg Ni/Zn kg⁻¹ soil, or 200 µL L⁻¹ ethephon + 200 mg Ni/Zn kg⁻¹ soil grown for 30 DAS under the same plant growth conditions described for earlier experiment. Furthermore, to confirm the ethylene response of plants for metals tolerance, we used ethylene action inhibitor 100 µM NBD in the presence of Ni or Zn. Seeds were sown on October 18, 2012. Treatments were arranged in a factorial randomized design. The number of replicates for each treatment was five. Number of the pots used for Experiment 3 was 40 (8 treatments x 1 cultivar x 5 replicates x 1 sampling). The selection of plants for determination of various characteristics at 30 DAS were same as described for Experiment 2. At 30 DAS, one plant from each pot was selected for the determination of ethylene biosynthesis, S-assimilation, N-assimilation and photosynthetic characteristics. Second plant was used to determine antioxidant metabolism, proline metabolism and oxidative stress measurements. Another plant was uprooted and leaf area was determined. Subsequently, leaves and the rest of the plant were dried separately. Leaf dry mass was recorded and plant dry mass was recorded. The dried leaf powder was used for leaf S and N determination. The parameters were same as described for earlier experiment (Experiment 2).

3.4 Chemicals

Chemicals used in the study were obtained from Sigma-Aldrich. Other major and minor salts and buffer components were procured from MERCK, HIMEDIA and/or SRL.
3.5 Parameters Studied

Protein was estimated according to Bradford (1976) using BSA as standard.

3.5.1 Sulfur Assimilation

- ATP-sulfurylase Activity
- Sulfur Content
- Serine Acetyltransferase Activity
- Cysteine Content
- Methionine Content
- Photosynthetic-Sulfur Use Efficiency

3.5.1.1 Assay of ATP-sulfurylase Activity

The method of Lappartient and Touraine (1996) was followed for the assay of ATP-sulfurylase activity (ATP-S; EC, 2.7.7.4). The details of the procedure are given below.

3.5.1.1.1 Enzyme Assay

One gram fresh leaf tissue was ground at 4°C in a buffer consisting of 10 mM Na₂EDTA, 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol (DTT), and 0.01 g mL⁻¹ insoluble PVP, using a 1:4 (w/v) tissue to buffer ratio. The homogenate was centrifuged at 20,000 × g for 10 min at 4°C. The supernatant (crude extract) was used for the in vitro ATP-sulfurylase assay. The enzyme activity was measured using molybdate-dependent formation of pyrophosphate. The reaction was initiated by adding 0.1 mL of crude extract to 0.5 mL of the reaction mixture, which contained 7 mM MgCl₂, 5 mM Na₂MoO₄, 2 mM Na₂ATP, and 0.032 units mL⁻¹ of sulfate-free inorganic pyrophosphate in 80 mM Tris-HCl buffer (pH 8.0). Another aliquot from the same extract was added to the same reaction mixture except that Na₂MoO₄ was absent. Incubations were carried out side by side at 37°C for 15 min, after which phosphate was determined on a spectrophotometer (UV 2080 plus, Analytical Technologies, India).
**SCHEME 3:** Diagrammatic representation of the arrangement of pots for Experiment 3. Scheme shows plants were grown with/without Ni and Zn stress and treated with 200 µL L⁻¹ ethephon or 100 µM norbornadiene at 20 DAS. At 30 DAS, three plants from each treatment was used for determination of various studied parameters.
3.5.1.2 Sulfur Content

3.5.1.2.1 Digestion of Leaf Powder

Oven-dried leaf powder (100 mg) was taken in digestion tube of 75 mL capacity. In digestion tube, 4.0 mL acid mixture (consisted of concentrated nitric acid and perchloric acid in the ratio of 1:1) and 7.5 mg of selenium dioxide as catalyst was added. The digestion was carried out till the digested solution became colourless. Following digestion, the volume was made up to 75 mL with de-ionized water. The interference of silica was checked by filtering the contents of the tube.

3.5.1.2.2 Estimation of Sulfur

Total sulfur in plant samples was estimated according to the turbidimetric method of Chesnin and Yien (1950). A 5 mL aliquot was pipette out from the digested solution for turbidity development in 25 mL volumetric flask. Turbidity was developed by adding 2.5 mL gum acacia (0.25%) solution, 1.0 g BaCl$_2$ sieved through 40-60 mm mesh and the volume was made up to the mark with de-ionized water. The contents of 25 mL volumetric flask were thoroughly shaken till BaCl$_2$ completely dissolved. Turbidity was allowed to develop for 2 min. The values were recorded at 415 nm within 10 min after the turbidity development. A blank was also run simultaneously after each set of determination.

The amount of sulfate was calculated with the help of a calibration curve drawn afresh using a series of K$_2$SO$_4$ solutions.

3.5.1.3. Determination of Serine Acetyltransferase Activity

Serine acetyltransferase activity (SAT; EC, 2.3.1.30) in the crude leaf extract was determined by the method of Kredich and Tomkins (1966). Fresh leaf tissues (0.5 g) were ground with chilled mortar and pestle in 2 mL of ice cold extraction buffer Tris-HCl 100 mM (pH 8.0), 100 mM KCl, 20 mM MgCl$_2$, 1% Tween 80 and 10 mM DTT. The samples were transferred to microcentrifuge tubes and spun at 11, 600 × g for 10 min at 4° C. The supernatant obtained was used for SAT assay. The enzyme reaction mixture (1 mL) contained 0.1 mM acetyl CoA, 50 mM Tris–HCl (pH 7.6), 1 mM DTNB, 1 mM EDTA and 1 mM L-serine. Subsequent to reaction initiation by
addition of enzyme at 25°C, the initial rate was estimated by monitoring the increase in absorbance at 412 nm and the rates were calculated using an extinction coefficient of 13,600 for thionitrobenzoic acid. A blank containing all materials except L-serine was run simultaneously and subtracted from the reaction rate obtained with L-serine.

### 3.5.1.4. Determination of Cysteine Content

The content of cysteine in leaves was determined spectrophotometrically adopting the method of Giatonde (1967).

#### 3.5.1.4.1. Procedure

Fresh leaf (0.5 g) was homogenized in 5% (w/v) ice-cold perchloric acid. The final volume of 4 mL g⁻¹ of plant tissue was used. The suspension was centrifuged at 2,800 g for 1 h at 5°C and supernatant was filtered through Whatman No. 30 paper. One mL of filtrate was treated with acid ninhydrin reagent. The extinction was read at 580 nm and the amount of cysteine was calculated with reference to a calibration curve obtained under similar conditions for the amount of cysteine.

### 3.5.1.5. Determination of Methionine Content

Methionine content was determined following the method of Horn et al. (1946). The details of the procedure are given below.

#### 3.5.1.5.1. Procedure

Fresh leaf tissues (0.5 g) was weighed and transferred to a flask. To this sample 20 mL of 6.0 N HCl was added. The material was refluxed for 20 to 24 h then transferred into china disk. It was evaporated on water bath with the addition of 1 g activated charcoal. Evaporation was continued until the content of china disk became viscous. Warm de-ionized water was added and filtered through Whatman filter paper No. 1. The filtrate was collected into 25 mL volumetric flask and made up to 25 mL. The china disk was washed with little amount of hot water for about 5-6 times. Filtrate was collected in a flask and this hydrolyzate was transferred to a 10 mL beaker and 4 mL de-ionized water and 2 mL of 5 N-NaOH were added. Further, 0.1 mL sodium nitropruside and 2.0 mL glycine solution (3%) were also added. Finally, 4 mL
metaphosphoric was added to develop colour. The intensity of colour was read at 450 nm on spectrophotometer with simultaneous blank readings.

3.5.1.6 Photosynthetic-Sulfur Use Efficiency

Photosynthetic sulfur use-efficiency was calculated by the ratio of photosynthesis rate to N content per unit leaf area.

3.5.2. Nitrogen Assimilation

- Nitrate Reductase Activity
- Nitrogen Content
- Photosynthetic-Nitrogen Use Efficiency

3.5.2.1. Nitrate Reductase Activity

The activity of nitrate reductase (EC 1.7.99.4) in leaves was measured by preparing an enzyme extract using the method of Kuo et al. (1982). Leaf tissue (1.0 g) was frozen in liquid N₂, ground to a powder with a chilled mortar and pestle, and then stored at -80°C. The powder was thawed for 10 min at 4°C and was homogenized in a blender in 250 mM Tris-HCl buffer (pH 8.5), containing 10 mM cysteine, 1 mM EDTA, 20 µM FAD, 1 mM DTT and 10% (v/v) glycerol. The homogenate was centrifuged at 10,000 × g for 30 min at 4°C. The activity of NR was assayed as the rate of nitrite production at 28°C adopting the procedure of Nakagawa et al. (1984). The assay mixture contained 10 mM KNO₃, 0.065 M HEPES (pH 7.0), 0.5 mM NADH in 0.04 mM phosphate buffer (pH 7.2) and enzyme in a final volume of 1.5 mL. The reaction was initiated by adding NADH. After 15 min the reaction was terminated by adding 1 mL of 1 N HCl solution containing 1% sulfanilamide followed by the addition of 1 mL of 0.02% aqueous N-1-napthylethylenediamine dihydrochloride (NED). The absorbance was read at 540 nm using a spectrophotometer after 10 min.

3.5.2.2. Nitrogen Content

Leaf nitrogen content was estimated by the Kjeldahl digestion method as described by Lindner (1944).
A 10 mL aliquot of the digested material was taken in a 50 mL volumetric flask. To this, 2 mL of 2.5 N sodium hydroxide and 1 mL of 10% sodium silicate solutions were added to neutralize the excess of acid and to prevent turbidity, respectively. The volume was made up to the mark with de-ionized water. In a 10 mL graduated test tube, 5 mL aliquot of this solution was taken and 0.5 mL Nessler’s reagent was added. The final volume was maintained with de-ionized water. The contents of the tubes were allowed to stand for 5 min for maximum color development. The optical density of the solution was read on a spectrophotometer at 525 nm.

3.5.2.2 Photosynthetic-Nitrogen Use Efficiency

Photosynthetic nitrogen use-efficiency was calculated by the ratio of photosynthesis rate to N content per unit leaf area.

3.5.3. Enzymatic and Non-enzymatic Antioxidants

- Superoxide Dismutase
- Ascorbate Peroxidase
- Glutathione Reductase
- Reduced Glutathione and Redox State

3.5.3.1 Activity of Antioxidant Enzymes

3.5.3.1.1 Enzyme Extraction

Fresh leaf tissue (200 mg) was homogenized with an extraction buffer containing 0.05% (v/v) Triton X-100 and 1% (w/v) polyvinylpyrrolidone (PVP) in potassium-phosphate buffer (100 mM, pH 7.0) using chilled mortar and pestle. The homogenate was centrifuged at 15,000 × g for 20 min at 4°C. The supernatant obtained after centrifugation was used for the assay of superoxide dismutase (SOD; EC 1.15.1.1) and glutathione reductase (GR; EC 1.6.4.2) enzymes. For the assay of ascorbate peroxidase (APX; EC 1.11.1.11), extraction buffer was supplemented with 2 mM ascorbate.
3.5.3.1.1 Superoxide Dismutase

Activity of SOD was determined by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT), according to the methods of Beyer and Fridovich (1987) and Giannopolitis and Ries (1977).

3.5.3.1.1.1 Enzyme Assay

A 5 mL reaction mixture containing 5 mM HEPES (pH 7.6), 0.1 mM EDTA, 50 mM Na₂CO₃ (pH 10.0), 13 mM methionine, 0.025% (v/v) Triton X-100, 63 µmol NBT, 1.3 µmol riboflavin, and the enzyme extract was illuminated for 15 min and a control set was not illuminated to correct for background absorbance. A unit of SOD was defined as the amount of enzyme required to cause 50% inhibition of the reaction of NBT at 560 nm.

3.5.3.1.1.2 Ascorbate Peroxidase

Ascorbate peroxidase activity was determined by the method of Nakano and Asada (1981).

3.5.3.1.1.2.1 Enzyme Assay

Ascorbate peroxidase activity was determined by the decrease in the absorbance of ascorbate at 290 nm due to its enzymatic breakdown. The volume of 1 mL of 50 mM K-phosphate buffer (pH 7.2) contained 0.5 mM ascorbate, 0.1 mM H₂O₂, 0.1 mM EDTA and 0.1 mL enzyme extract. The reaction was allowed to run for 5 min at 25° C. Ascorbate peroxidase activity was calculated by using the extinction coefficient 2.8 mM⁻¹ cm⁻¹. One Unit of enzyme activity is defined as the amount necessary to decompose 1 µmol of substrate consumed per min at 25° C.

3.5.3.1.1.3 Glutathione Reductase

Glutathione reductase activity was determined by the method of Foyer and Halliwell (1976) and modified by Rao (1992).
3.5.3.1.3.1 Enzyme Assay

Glutathione reductase activity was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm. The assay mixture (3 mL) contained phosphate buffer (25 mM, pH 7.8), 0.5 mM oxidized GSH, 0.2 mM NADPH and the enzyme extract. The activity of GR was calculated by using extinction coefficient at 6.2 mM$^{-1}$ cm$^{-1}$. One unit of enzyme was the amount necessary to decompose 1 µmol of NADPH min$^{-1}$ at 25° C.

3.5.3.1.2 Reduced and Oxidized Glutathione

Glutathione was assayed by an enzymic recycling procedure (Griffith 1980) in which it was sequentially oxidized by 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) and reduced by NADPH in the presence of GR.

3.5.3.1.2.1 Procedure

For specific assay of GSSG, the GSH was masked by derivatization with 2-vinylpyridine. Fresh leaf tissue (0.5 g) was ground in liquid N$_2$ using a mortar pestle. The ground tissue was suspended in 0.5 mL 5% sulphosalicyclic acid and centrifuged at 12000 × g for 10 min. A 300 µL aliquot of supernatant was removed and neutralized by addition of 18 µL 7.5 M triethanolamine. A 150 µL sample was then used to determine concentrations of GSH plus GSSG. Another sample was pretreated with 3 µL 2-vinylpyridine for 60 min at 20° C to mask the GSH by derivatization to allow the subsequent determination of GSSG alone. In each case 50 µL aliquots of the two samples were mixed with 700 µL 0.3 mM NADPH, 100 µL DTNB and 150 µL buffer containing 125 mM sodium phosphate, 6.3 mM EDTA (pH 6.5). A 10 µL aliquot of GR (5 U mL$^{-1}$) was then added and the change in absorbance at 412 nm was monitored at 30° C. Standard curve for calculations was prepared from GSH covering a range of 10-100 nmol. Redox state was presented as the ratio of GSH to GSSG.

3.5.4. Proline Metabolism

- Proline Content
- Glutamyl Kinase Activity
- Proline Oxidase Activity
3.5.4.1 Determination of Proline Content

Proline content was determined spectrophotometrically by adopting the ninhydrin method of Bates et al. (1973). Fresh leaf samples (300 mg) were homogenized in 3 mL of 3% sulphosalicylic acid. The homogenate filtrate was reacted with 1 mL each of acid ninhydrin and glacial acetic acid for 1h in a test tube placed in a water bath at 100° C. The mixture was extracted with toluene and the absorbance was measured on a spectrophotometer at 520 nm using L-proline as a standard.

3.5.4.2 Assay of Proline Metabolizing Enzymes

To determine the activity of GK (EC 2.7.2.11) and PROX (EC 1.5.99.8), enzyme extract was prepared by homogenizing 500 mg leaf sample in 0.1 M Tris-HCl buffer (pH 7.5) at 4° C. The homogenate was centrifuged at 30,000 × g for 30 min and pellet was collected and used as extract for assay of GK and PROX. For GK enzyme activity extract was stored at -20° C.

3.5.4.2.1 Determination of Glutamyl Kinase and Proline Oxidase Activity

Activity of GK was assayed by the method of Hayzer and Leisinger (1980) with slight modification. The frozen sample was suspended in 10 mL of 0.1 M Tris–HCl buffer containing 1 mM 1, 4 DTT to rupture the cell and centrifuged at 30,000 × g for 30 min. The assay mixture contained 50 mM L-glutamate, 10 mM ATP, 20 mM MgCl₂, 100 mM hydroxylamine HCl and 50 mM Tris–HCl (pH 7.0) with 200 µL of desalted extract in a final volume of 500 µL. The reaction was started by the addition of enzyme extract. After 30 min of incubation at 37° C, the reaction was stopped by the addition of 1.0 mL FeCl₃·3H₂O (2.5% w/v) and trichloroacetic acid (TCA) (6%, w/v) in 2.5 M HCl. Protein was precipitated and removed by centrifugation at 12,000 × g (4° C) and absorbance was recorded at 540 nm. Activity of glutamyl kinase was expressed in U mg⁻¹ protein. One Unit of the enzyme activity is defined as µg of glutamyl hydroxamate min⁻¹ mg⁻¹ protein. Glutamyl hydroxamate was used as standard.

Proline oxidase activity was determined adopting the method of Huang and Cavalieri (1979) with slight modification. The pellet was mixed with 1 mL Tricine, KOH buffer (pH 7.5) containing 6 M sucrose. This extract was used for the enzyme
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assay. The assay mixture contained 1.2 mL of 50 mM Tris–HCl buffer (pH 8.5), 1.2 mL of 5 mM MgCl₂, 0.1 mL of 0.5 mM NADP, 0.1 mL of 1 mM KCN, 0.1 mL of 1 mM phenazine methosulfate (PSM), 0.1 mL of 0.06 mM 2, 6 dichlorophenol indophenols (DCPIP) and 0.1 mL of 0.1 M proline in a final volume of 3 mL. The increase in absorbance was recorded at 600 nm at 25°C using proline to initiate the reaction. Proline oxidase activity was expressed in U mg⁻¹ protein. One Unit of the enzyme activity is defined as mM DCPIP reduced min⁻¹ mg⁻¹ protein.

3.5.5. Oxidative Stress

- H₂O₂ Content

- TBARS Content

3.5.5.1 Determination of H₂O₂ Content

The content of H₂O₂ was determined following the method of Okuda et al. (1991). Fresh leaf tissues (200 mg) were ground in ice-cold 200 mM perchloric acid. After centrifugation at 1, 200 × g for 10 min, perchloric acid of the supernatant was neutralized with 4 M KOH. The insoluble potassium perchlorate was eliminated by centrifugation at 500 × g for 3 min. In a final volume (1.5 mL), 1 mL of the eluate, 400 µL of 12.5 mM 3-(dimethylamino) benzoic acid in 0.375 M phosphate buffer (pH 6.5), 80 µL of 3-methyl-2-benzothiazoline hydrazone, and 20 µL of peroxidase (0.25 Unit) were added. The reaction was started by the addition of peroxidase at 25°C, and the increase in absorbance was recorded at 590 nm.

3.5.5.2. Determination of TBARS Content

The level of lipid peroxidation products in the leaf sample was determined by estimating thiobarbituric acid reactive substances (TBARS) as described by Dhindsa et al. (1981). Fresh leaf tissue (200 mg) was ground in 0.25% 2-thiobarbituric acid (TBA) in 10% TCA using mortar and pestle. After heating at 95°C for 30 min, the mixture was quickly cooled on ice bath and centrifuged at 10,000 × g for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for non-specific turbidity by subtracting the absorbance of the same at 600 nm. The blank was also run. The TBARS content was calculated using the extinction coefficient (155 mM⁻¹ cm⁻¹).
3.5.6. Ethylene Biosynthesis

- ACS activity
- Ethylene

The activity of ACS (EC 4.4.1.14) was measured by adopting the methods of Avni et al. (1994) and Woeste et al. (1999). Leaf tissue (5.0 g) was ground in 100 mM HEPES buffer (pH 8.0) containing 4 mM DTT, 2.5 mM pyridoxal phosphate and 25% PVP. The homogenized preparation was centrifuged at 12,000 × g for 15 min. One mL of the supernatant was placed in a 30 mL tube and 0.1 mL of 5 mM AdoMet was added and incubated for 2 h at 22° C. The ACC formed was determined by its conversion to ethylene by the addition of 0.1 mL of 20 mM HgCl₂ followed by the addition of 0.1 mL of a 1:1 mixture of saturated NaOH/NaCl and placed on ice for 10 min. for control set, AdoMet was not added.

Ethylene evolution was measured by cutting 0.5 g leaf material into small pieces that were placed in 30 mL tubes containing moist paper to minimize evaporation from the tissue and were stoppered with secure rubber caps and placed in light for 2 h under the same condition used for plant growth. Earlier experiment showed that 2 h incubation time was adequate for ethylene detection without the interference of wound-induced ethylene, which began after 2 h of leaf incubation. A 1 mL gas sample from the tubes was withdrawn with a hypodermic syringe and assayed on a gas chromatograph (Nucon 5700, New Delhi, India) equipped with a 1.8 m Porapack N (80-100 mesh) column, a flame ionization detector and data station. Nitrogen was used as the carrier gas. The flow rates of nitrogen, hydrogen and oxygen were 30, 30 and 300 mL min⁻¹, respectively. The detector was at 150° C. Ethylene identification was based on the retention time and quantified by comparison with the peaks from standard ethylene concentration.

3.5.7. Water Relations

Leaf water potential was measured on second leaf from top (fully expanded youngest leaf) of the plant using water potential system (Psypro, WESCOR, USA). The leaf used for water potential measurement was frozen in liquid nitrogen in sealed polythene bags which was thawed and cell sap was extracted with the help of a
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disposable syringe. The extracted sap was used for the determination of osmotic potential using a vapour pressure osmometer (5520, WESCOR, USA).

3.5.8. Photosynthetic Characteristics

Following photosynthesis-related parameters were studied

- Net Photosynthetic Rate
- Stomatal Conductance
- Intercellular CO₂ Concentration
- Chlorophyll Content
- Rubisco Activity
- Actual PS II Activity
- Maximum PS II Activity
- Intrinsic PS II Activity
- Photochemical Quenching
- Non-photochemical Quenching
- Electron Transport Rate
- Water Use Efficiency

Net photosynthetic rate, stomatal conductance and intercellular CO₂ concentration were measured in fully expanded uppermost intact leaves of plants in each treatment using infrared gas analyzer (CID-340, Photosynthesis System, BioScience, USA). The atmospheric conditions during measurement were photosynthetically active radiation (PAR), 850 ± 22 µmol m⁻² s⁻¹, relative humidity 62 ± 3%, atmospheric temperature, 22 ± 1°C and atmospheric CO₂, 360 µmol mol⁻¹.

Chlorophyll was measured in intact leaves with the help of SPAD chlorophyll meter (502 DL PLUS, Spectrum Technologies, USA).

Rubisco activity (EC 4.1.1.39) was determined spectrophotometrically by monitoring NADH oxidation at 30°C at 340 nm (Usuda 1985). Leaf tissue (1.0 g) was homogenized using a chilled mortar and pestle with ice-cold extraction buffer
containing 0.25 M Tris-HCl (pH 7.8), 0.05 M MgCl₂, 0.0025 M EDTA and 37.5 mg DTT. The homogenate was centrifuged at 4° C at 10,000 × g for 10 min. The resulting supernatant was used to assay the enzyme. The reaction mixture contained 100 mM Tris-HCl (pH 8.0), 40 mM NaHCO₃, 10 mM MgCl₂, 0.2 mM NADH, 4 mM ATP, 0.2 mM EDTA, 5 mM DTT, 1 U of 3-phosphoglycerate kinase. The activity was estimated after the addition of enzyme extract and 0.2 mM RuBP.

For chlorophyll fluorescence measurements fully expanded leaves were allowed to adapt under dark condition for 30 min and then measurements were made using Junior-PAM chlorophyll fluorometer (Heinz Walz, Germany). Minimal fluorescence (F₀) and maximum fluorescence (Fₘ) were measured in dark-adapted leaves with a low measuring beam at a light intensity of 125 µmol m⁻² s⁻¹, whereas under light-adapted condition, minimal fluorescence (F₀’) and maximum fluorescence (Fₘ’) were measured in the same leaves with a saturating light intensity (625 µmol m⁻² s⁻¹) together with steady-state fluorescence (Fₛ). The variable fluorescence (Fᵥ and Fᵥ’) was calculated as Fₘ–F₀ and Fₘ’–F₀’, and Φ PS II was determined as Fₘ’–Fᵥ/Fₘ’, maximal efficiency of PS II by using Fᵥ/Fₘ and intrinsic efficiency of PS II was by using Fᵥ/Fₘ’. Using fluorescence parameters determined in both light- and dark-adapted states, the photochemical quenching (qP) and non-photochemical quenching (NPQ) were calculated. Photochemical quenching was calculated as (Fₘ’–F₀)/Fᵥ and NPQ as (Fₘ–Fₘ’)/Fₘ’ (Maxwell and Johnson 2000). Electron transport rate (ETR) was calculated by following formula: Φ PS II × photosynthetic photon flux density × 0.5 × 0.84 as suggested by Krall and Edwards (1992).

Water use efficiency (WUE) was calculated as the ratio of net photosynthetic rate to stomatal conductance to avoid effects of small differences in vapour pressure between measurements (Von Cammerer and Farquhar 1981).

3.5.9. Growth Characteristics

Following growth-related parameters were studied

- Leaf Area
- Relative Growth Rate
- Plant Dry Mass
Plants were uprooted carefully from the pots, washed to remove dust. Leaf area was measured with a leaf area meter (LA 211, Systronics, New Delhi, India).

Relative growth rate (RGR) was calculated using the following formula given by Radford (1967).

\[
RGR = \frac{\ln(W2) - \ln(W1)}{(t2 - t1)} \text{ Where, } W1 \text{ and } W2 \text{ are plant dry mass at times } t1 \text{ and } t2.
\]

Dry mass of plants was measured after they were dried in a hot air oven at 80ºC till constant weight and weight of the samples was recorded.

3.6. Statistical Analysis

Data were analyzed statistically and standard errors were calculated. Analysis of variance was performed on the data using SPSS (ver. 17.0 Inc., USA) to determine the significance at \( P < 0.05 \). Least significant difference (LSD) was calculated for the significant data to identify difference in the mean of the treatment. Data are presented as mean ± SE (n=5).
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Instruments used in the experimentation (A) Chlorophyll fluorescence meter (B) Chlorophyll fluorescence meter (C) SPAD chlorophyll meter and (D) IRGA
Instruments used in the experimentation (A) Gas chromatograph (B) Osmometer (C) Ultracentrifuge and (D) Water potential system
Instruments used in the experimentation (A) Leaf area meter (B) Micropipettes (C) Spectrophotometer and (D) Deep freezer
Working in Lab