Chapter – 3

MATERIALS AND METHODS
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MATERIALS AND METHODS

3.1 Proposed study

To achieve the objectives framed in chapter one, the following six experiments were conducted to elucidate the effect of proline and salicylic acid on cadmium-induced changes in chickpea (Cicer arietinum L.) cv. Avarodhi during 2007-2009.

3.2 Seeds

Certified seeds of Cicer arietinum L. cv. Avarodhi were obtained from National Seed Corporation Ltd., Indian Agricultural Research Institute, Pusa, New Delhi, India. Before the start of each experiment, healthy seeds of uniform size were tested for viability. Healthy seeds were surface sterilized with 0.01% mercuric chloride solution followed by repeated washings with double distilled water (DDW) to remove traces of adhered mercuric chloride.

3.2 Preparation of pots

Each earthen pot (25× 25 cm) was filled with an equal quantity of sandy loam soil mixed with farmyard manure, in a ratio of 6:1. A uniform basal starter dose of inorganic fertilizers (urea, single superphosphate and muriate of potash) were added at the rate of 40 mg, 138 mg and 26 mg respectively, per kg of the soil to each pot to maintain the fertility of soil. The pots were arranged in a randomized block design technique, in the net house of Department of Botany, Aligarh Muslim University, Aligarh.

3.3 Preparation of hormone and proline

Salicylic acid (SA) and proline were purchased from BDH Chemicals Ltd., Germany. A stock solution (10⁻³ M) of SA was prepared by dissolving its required quantity in 5 ml of ethanol, in a 100 ml volumetric flask and making to volume using DDW. The required concentrations of SA (10⁻⁴, 10⁻⁵ and 10⁻⁶ M) were prepared by
dilution of the stock solution. Surfactant “Tween-20” (0.5 ml) was added to each flask prior to application and final volume was maintained to 100 ml by using DDW.

Stock solution (0.1M) of proline was prepared by dissolving the required quantity of proline in DDW in a 100 ml volumetric flask and making to volume using DDW. Concentrations of 10, 20, 30, 40 and 50 mM were prepared by diluting the stock solution. Surfactant “Tween-20” (0.5 ml) was added to each flask prior to spraying and final volume was made to 100 ml using DDW.

3.4 Experiment 1

This experiment was prepared with 40 pots such that each treatment had 10 pots (replicates) and within each pot four plants were maintained, according to randomized block design technique, during the winter season (October, 2007 to March, 2008) to study the effect of varying doses of cadmium (Cd) on chickpea. At the start of the experiment, each set of pots was supplemented with different doses of Cd in the form of CdCl₂ (0, 25, 50 or 100 mg per kg of soil).

The surface sterilized seeds were inoculated with a uniform layer of *Rhizobium ciceri* and were sown in the pots at the rate of 8 seeds per pot. Thinning was done 10 days after sowing (DAS). Irrigation was done with tap water as and when required. At each stage of sampling (60 and 90 DAS) 10 plants were uprooted to assess the following characteristics:

1. Length of root and shoot per plant
2. Fresh and dry mass per plant
3. Number of nodules per plant
4. Nodule fresh and dry mass per plant
5. Nodule leghemoglobin content
6. Nodule carbohydrate content
7. Nodule nitrogenase activity
8. Glutamine synthetase (GS) activity in nodules
9. Glutamate synthase (GOGAT) activity in nodules
10. Glutamate dehydrogenase (GDH) activity in nodules
11. Nitrate content in roots
12. Nitrogen content in leaves
13. Nitrate reductase (NR) activity in leaves
14. Carbonic anhydrase (CA) activity in leaves
15. Stomatal conductance
16. Internal CO₂ concentration (Ci)
17. Water use efficiency (WUE)
18. Transpiration rate (E)
19. Photosynthetic rate (Pₙ)
20. Catalase (CAT) activity in leaves
21. Peroxidase (POX) activity in leaves
22. Superoxide dismutase (SOD) activity in leaves
23. Proline content in leaves
24. Leaf water potential

3.6 Experiment 2

This experiment was prepared with 60 pots such that each treatment had 10 pots (replicates) under a randomized block design technique, during the winter season (October, 2007 to March, 2008) to study the effect of different concentrations of proline on chickpea. All agronomic and cultural practices were the same as described in Experiment 1. At the stage of 30 DAS, the resulting plants were treated as:
(A) Plants were sprayed with DDW (control)
(B) Plants were sprayed with an aqueous solution of 10 mM of proline
(C) Plants were sprayed with an aqueous solution of 20 mM of proline
(D) Plants were sprayed with an aqueous solution of 30 mM of proline
(E) Plants were sprayed with an aqueous solution of 40 mM of proline
(F) Plants were sprayed with an aqueous solution of 50 mM of proline

Each plant was sprayed thrice. The nozzle of the sprayer was adjusted such that it pumped out 1 ml of the solution in one sprinkle. At each sampling stage (60 and 90 DAS) ten plants were uprooted to assess the same parameters as in Experiment 1.

3.7 Experiment 3

This experiment was prepared with 60 pots such that each treatment had 10 pots (replicates) under a randomized block design technique during the winter season (October, 2007 to March, 2008) to study the effect of different concentrations of salicylic acid (SA) on chickpea. All agronomic and cultural practices were the same as described in Experiment 1. At the stage of 30 DAS, the plants were treated as:
(A) Plants were sprayed with DDW (control)
(B) Plants were sprayed with 5% solution of ethanol
(C) Plants were sprayed with 0.5% solution of Tween-20
(D) Plants were sprayed with $10^{-4}$ M of salicylic acid
(E) Plants were sprayed with $10^{-5}$ M of salicylic acid
(F) Plants were sprayed with $10^{-6}$ M of salicylic acid
Each plant was sprayed thrice. The nozzle of the sprayer was adjusted such that it pumped out 1 ml of the solution in one spray. At each stage of sampling (60 and 90 DAS) ten plants were uprooted to assess the same parameters as in Experiment 1.

3.8 Experiment 4

This experiment was prepared with 50 pots such that each treatment had 10 pots (replicates), arranged under a randomized block design technique, during the winter season (October, 2008 to March, 2009) to study the interactive effect of Cd and proline. The concentration of proline was selected on the basis of Experiment 2. The scheme of the treatments is presented in table 1.

Table 1. Scheme of treatment for experiment 4:

<table>
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<th>Treatments</th>
<th>Cd doses (mg/kg soil)</th>
<th>Solution sprayed at 30 DAS</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>DDW</td>
</tr>
<tr>
<td>A</td>
<td>-</td>
<td>Proline (20mM)</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>Proline (20mM)</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>Proline (20mM)</td>
</tr>
<tr>
<td>D</td>
<td>100</td>
<td>Proline (20mM)</td>
</tr>
</tbody>
</table>

The rest of the agronomic and cultural practices remained the same as in experiment 1. Ten plants from each treatment were uprooted at 60 and 90 DAS to assess the same as in Experiment 1. The remaining plants were allowed to grow to maturity and were harvested after 150 DAS to study the following yield characteristics:

1. Number of pods per plant
2. Number of seeds per pod
3. Seed yield per plant
4. 100 seed mass
5. Seed protein content
3.9 Experiment 5

This experiment was prepared with 50 pots such that each treatment had 10 pots (replicates), arranged under a randomized block design technique, during the winter season (October, 2008 to March, 2009), to study the interactive effect of Cd and SA. The concentration of SA was selected on the basis of the data from Experiment 3. The scheme of the treatments is presented in table 2.

Table 2. Scheme of treatments for experiment 5.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cd doses (mg/kg soil)</th>
<th>Solution sprayed at 30DAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>DDW</td>
</tr>
<tr>
<td>A</td>
<td>-</td>
<td>$10^{-5}$ M of SA</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>$10^{-5}$ M of SA</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>$10^{-5}$ M of SA</td>
</tr>
<tr>
<td>D</td>
<td>100</td>
<td>$10^{-5}$ M of SA</td>
</tr>
</tbody>
</table>

The rest of the agronomic and cultural practices remained the same as in Experiment 1. Ten plants from each treatment were uprooted at 60 and 90 DAS to assess various parameters. The parameters studied were the same as in experiment 1. The remaining plants were allowed to grow to maturity and were harvested after 150 DAS to study the following yield characteristics:

1. Number of pods per plant
2. Number of seeds per pod
3. Seed yield per plant
4. 100 seed mass
5. Seed protein content
3.10 Experiment 6

This experiment was prepared with 50 pots such that each treatment had 10 pots (replicates), according to randomized block design technique, during the winter season (October, 2008 to March, 2009). At the start of the experiment, each set of pots was supplemented with different doses of cadmium as depicted in table 3. The rest of the agronomic and cultural practices remained the same as described in Experiment 1.

At the stage of 29 and 30 DAS each plant was sprayed with 20mM proline and \(10^{-5}\) M of SA, respectively, to elucidate the interactive effect of proline and SA on the cadmium-induced changes in chickpea.

Table 3. Scheme of treatment for experiment 6.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Cd doses (mg/kg soil)</th>
<th>Spray at 29 DAS</th>
<th>Spray at 30 DAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>DDW</td>
<td>DDW</td>
</tr>
<tr>
<td>A</td>
<td>-</td>
<td>20mM proline</td>
<td>(10^{-5}) M of SA</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>20mM proline</td>
<td>(10^{-5}) M of SA</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>20mM proline</td>
<td>(10^{-5}) M of SA</td>
</tr>
<tr>
<td>D</td>
<td>100</td>
<td>20mM proline</td>
<td>(10^{-5}) M of SA</td>
</tr>
</tbody>
</table>

Ten plants from each treatment were uprooted at 60 and 90 DAS, to assess various parameters i.e. the same as in Experiment 1. The remaining plants were allowed to grow to maturity and were harvested after 150 DAS to study the yield characteristics mentioned in Experiment 4.

The methods adopted to assess each parameter are described below in the following pages.
3.11 Growth parameters

3.11.1 Length of root and shoot

The whole mass of soil from each pot was removed and dipped in a bucket filled with tap water to uproot the plants with intact roots. The plants were gently moved to remove soil particles. This was followed by washing under running tap water. The length of roots and shoots was measured by using a meter scale.

3.11.2 Fresh and dry mass of plant

The plants were uprooted and washed gently with tap water. These plants were blotted in blotting sheets to remove the adhering water and weighed on an electronic balance to record their fresh mass. These plants were kept in an oven at 80°C for 72 hours and then weighed to obtain their dry mass.

3.12 Nodulation

3.12.1 Nodule number and their fresh and dry mass

The whole mass of soil was removed from the pot and placed in a bucket filled with tap water. The plants were moved gently to collect the intact root system with no damage to the nodules. The roots were then washed under running tap water and the number of nodules per plant was counted. The nodules were picked and weighed to record their fresh mass. Subsequently they were transferred to Petri-plates for overnight drying in an oven at 80°C. This dried material was weighed to obtain the dry mass of nodules per plant.

3.13 Biochemical analysis

3.13.1 Nodule Leghemoglobin content

The leghemoglobin content in fresh nodules was estimated following the method described by Sadasivam and Mannickam (1992).
Fresh nodules (200 mg) were mixed with 3 ml of 0.1M phosphate buffer (Appendix 1.1) and macerated in a mortar and pestle followed by filtration through two layers of cheese cloth. The nodule debris was discarded. The turbid reddish brown filtrate was centrifuged at 10,000 g for 10-30 minutes.

Three ml of alkaline pyridine reagent (Appendix 1.2) was added to 3 ml of nodule extract and mixed properly. The solution becomes greenish yellow due to formation of hemochrome. The hemochrome was divided equally into two test tubes. To one test tube, a few crystals of potassium hexacyanoferrate were added to oxidize the hemochrome and read at 539 nm on spectrophotometer (spectronic 20D, Milton Roy, USA). To the other test tube a few crystals of sodium dithionite were added to reduce the hemochrome. This mixture was read at 556 nm after an interval of 2-5 minutes, against a reagent blank. The leghemoglobin content (mM) was calculated using the formula:

\[ \text{Lb concentration (mM)} = \frac{A_{556} - A_{539}}{23.4} \times 2D \]

Where: D is initial dilution

\( A_{556} \) and \( A_{539} \) are absorbance at 556 and 539 nm, respectively.

3.13.2 Nodule carbohydrate content

Carbohydrates were extracted from the samples following the method of Yih and Clark (1965) and estimated by adopting the procedure of Dubois et al. (1956).

Dried nodule powder (50 mg) was transferred to a glass centrifuge tube containing 5 ml of 1.5 N H₂SO₄ (Appendix 2.1). The sample was centrifuged at 4,000 rpm for 10 minutes. The supernatant was decanted into 25 ml volumetric flask with two washings of the residue with DDW. The volume was made upto the mark by using DDW. Out of this extract, 1 ml was placed in a test tube to which 1 ml of 5%
distilled phenol (Appendix 2.2) was added. The test tube was placed in chilled water and 5 ml of H$_2$SO$_4$ (AR grade) was added. The absorbance was read at 490 nm on a spectrophotometer. A blank was run simultaneously with each set of samples. Standard curve was plotted by using known graded dilutions of glucose solution. The absorbance of each sample was compared with the calibration curve and per cent carbohydrate content was calculated on a dry mass basis.

3.13.3 Nitrogenase activity in nodules

Nitrogenase activity was assayed adopting the procedure of Hardy et al. (1968). Assays were carried out immediately after harvesting the plants. Nodulated roots were cut from the base and were shaken slowly to remove attached soil particles. Samples were assayed in 30 ml glass tubes sealed with a subseal to allow it to be pierced by a hypodermic needle bearing a syringe. Five ml (v/v) of air was withdrawn from the sample container and replaced by an equal volume of acetylene gas. After an incubation of 1h at room temperature, 0.5 ml of gas was injected into a gas chromatograph (Nucon Series 5500) equipped with a flame ionization detector to detect the ethylene gas. The results were expressed in terms of nano moles of ethylene formed/g nodule fresh mass/hour.

3.13.4 Glutamine synthetase (GS) activity in nodules

Glutamine synthetase (GS) enzyme was extracted and assayed as described by Thimmaiah (1999).

3.13.4.1 Enzyme extraction

Nodules (0.5 g) were macerated in a chilled mortar and pestle in 5 ml of grinding medium consisting of 0.1M potassium phosphate buffer (Appendix 3.1.1) pH 7.8, containing 0.4M sucrose (Appendix 3.1.2), 10 mM DTT (Appendix 3.1.3), 10 mM KCl (Appendix 3.1.4), 1 mM MgCl$_2$ (Appendix 3.1.5) and 10 mM EDTA.
(Appendix 3.1.6) followed by filtration through four layers of cheese cloth. The debris was discarded and the filtrate was centrifuged at 10,000 g for 20 minutes and the supernatant was used for the assay of glutamine synthetase (GS) activity.

3.13.4.2 Enzyme assay

A reaction mixture of 0.75 ml was prepared containing 50 mM Tris-maleate buffer pH 7.5 (Appendix 3.2.1), 67 mM hydroxylamine (Appendix 3.2.2), 80 mM L-glutamine (Appendix 3.2.3), 8 mM ATP (Appendix 3.2.4), 4 mM EDTA (Appendix 3.2.5), 50 ml crude enzyme and 33 mM Mg\(^{2+}\) as MgCl\(_2\) (Appendix 3.2.6). A blank was run with each set of samples containing buffer instead of L-Glutamine. The test tubes were incubated for 10 minutes at 25°C. The reaction was stopped by adding 0.2 ml of FeCl\(_3\) mixture (Appendix 3.3). This mixture was centrifuged and absorbance of brown colour developed was read at 540 nm.

A standard curve was plotted by using known graded dilutions of \(\gamma\)-glutamylhydroximate whose colour was developed by adding FeCl\(_3\) mixture. The absorbance of each sample was compared with the calibration curve and GS activity was measured.

3.13.5 Glutamate synthase (GOGAT) activity in nodules

Glutamate synthase (GOGAT) activity was assayed following the method described by Thimmaiah (1999).

3.13.5.1 Enzyme extraction

Nodules (0.5 g) were macerated in 3 cm\(^3\) of extraction medium consisting of 0.05M Tris-HCl buffer (pH 7.5) (Appendix 4.1.1) containing 0.4 M sucrose (Appendix 4.1.2) and 0.01 M \(\beta\)-mercaptoethanol (Appendix 4.1.3) in a mortar and pestle followed by filtration through four layers of cheese cloth. The debris was discarded the filtrate was centrifuged at 20,000 g for 30 minutes and the supernatant was used for the assay of glutamate synthase (GOGAT) activity.
3.13.5.2 Enzyme assay

A reaction mixture of 3 ml was prepared containing 0.7 ml of 0.1 M Tris-HCl buffer (Appendix 4.2.1), 1 ml of 0.3 M L-glutamine (Appendix 4.2.2), 0.1 ml of 0.33 M 2-oxoglutarate (Appendix 4.2.3), 0.2 ml of \(10^{-3}\) M NADH (Appendix 4.2.4) and 1 ml of crude enzyme. A blank was run simultaneously with each set of samples containing 0.1 ml of DDW instead of 2-oxoglutarate. The test tubes were incubated at 37°C for 15-30 minutes and absorbance was read at 340 nm. The GOGAT activity was measured as \(\mu\) moles NADH oxidized/h/g fresh weight of sample using formula:

\[
\frac{A_{340} \times \text{volume of assay solution} \times 1000}{6.22 \times \text{incubation time (minutes)} \times \text{mg protein in enzyme extract used}}
\]

Where: \(A_{340}\) is the absorbance at 340 nm.

3.13.6 Glutamate dehydrogenase (GDH) activity in nodules

Glutamate dehydrogenase (GDH) activity was assayed as described by Thimmaiah (1999).

3.13.6.1 Enzyme extraction

Nodules (0.5 g) were macerated in 3 ml of extraction medium consisting of 0.05 M Tris-HCl buffer of pH 7.5 (Appendix 4.1.1) containing 0.4 M sucrose (Appendix 4.1.2) and 0.01 M β-mercaptoethanol (Appendix 4.1.3) in a mortar and pestle followed by filtration through four layers of cheese cloth. The debris was discarded and the filtrate was centrifuged at 20,000 g for 30 minutes and the supernatant was used for the assay of glutamate dehydrogenase (GDH) activity.

3.13.6.2 Enzyme assay

Three ml of reaction mixture was prepared containing 1.6 ml of 0.1M Tris-HCl buffer of pH 7.5 (Appendix 4.2.1), 0.1 ml of 0.33 M 2-oxoglutarate of pH 6.0 (Appendix 4.2.3), 0.2 ml of \(10^{-3}\) M NADH (Appendix 4.2.4), 0.1 ml of 3M NH₄Cl
(Appendix 4.3), and 1 ml of enzyme extract. A blank was run simultaneously with each set of samples containing 0.1 ml of DDW instead of 2-oxoglutarate. The test tubes were incubated at 37°C for 15-30 minutes and absorbance was read at 340 nm. The GDH activity was measured as nano moles NADH oxidized h⁻¹ g⁻¹ fresh weight of the samples using formula:

\[
\frac{A_{340} \times \text{volume of assay solution} \times 1000}{6.22 \times \text{incubation time (minutes)} \times \text{mg protein in enzyme extract used}}
\]

Where: \(A_{340}\) is the absorbance at 340 nm.

### 3.13.7 Nitrate content in roots

The nitrate content in roots was estimated following the method of Singh (1988).

Dried root powder (200 mg) was macerated with 16 ml of 2% acetic acid (Appendix 5.1) in a mortar with pestle. Of this mixture, 0.5 ml was placed in a test tube to which 9.5 ml of DDW was added. To this mixture 0.5 g of powder mixture (Appendix 5.2) was added followed by immediate shaking. The solution turned pink which was filtered and absorbance was read at 540 nm. A blank was run simultaneously with each set of samples consisting of all the above components except the nodule material.

A standard curve was plotted by using known, graded dilutions of potassium nitrate solution. The absorbance of each sample was compared with that of the calibration curve and nitrate content was computed on dry mass basis.

### 3.13.8 Nitrogen content in leaves

The leaf nitrogen content was estimated by employing the method of Lindner (1944).
3.13.8.1 Digestion of powder

Oven dried leaf powder (50 mg) was transferred to a digestion tube to which 2 ml sulphuric acid (AR grade) was added. The digestion tube was heated on a temperature controlled digestion assembly for 2 h to allow the complete reduction of nitrogen present in the material. After cooling the digestion tube for about 15 minute 0.5 ml of 30% H₂O₂ was added drop wise and the solution was heated again until the colour turned from black to light yellow. After cooling for 30 minutes an additional 3-4 drops of 30% H₂O₂ were added followed by heating for about 15 minutes. The process was repeated until the contents of the digestion tube turned colourless. This digested material was transferred to a 50 ml volumetric flask after 2-3 washings. The final volume was made up to the mark by using DDW.

3.13.8.2 Estimation of nitrogen

Ten ml of the digested material was taken in a 50 ml volumetric flask and neutralized by adding 2 ml of 2.5N NaOH (Appendix 6.1) and 1 ml of 10% sodium silicate (Appendix 6.2). Volume was made up by using DDW. Out of this sample, 5 ml was pipetted into a graduated test tube to which 0.5 ml Nessler’s reagent was added drop wise, with repeated shakings. The final volume was made upto 10 ml with DDW. After waiting 5 minutes, to obtain optimum colour development, the absorbance of the solution was read at 525 nm on a spectrophotometer (Spectronic 20D, Milton Roy, USA).

A blank consisting of Nessler’s reagent and DDW was run simultaneously with each set of samples. A standard curve was plotted by using known, graded dilutions of ammonium sulphate solution. The absorbance of each sample was compared with that of the calibration curve and percent nitrogen, in each sample, was computed on a dry mass basis.
3.13.9 Nitrate reductase (NR) activity

The activity of nitrate reductase was measured following the method by Jaworski (1971), in fresh leaf samples.

Leaves were cut into small pieces (1 cm²) and 200 mg were weighed and transferred to plastic vials. To each vial 2.5 ml of phosphate buffer pH 7.5 (Appendix 7.1) and 0.5 ml of potassium nitrate solution (Appendix 7.2) was added followed by addition of 2.5 ml of 5% isopropanol (Appendix 7.3). The vials were incubated in BOD incubator for 2h at 30± 2°C in dark. 0.4 ml of incubated mixture was placed in a test tube to which 0.3 ml each of sulphanilamide solution (Appendix 7.4) and NED-HCl (Appendix 7.5) were added. The test tube was left for 20 minutes, for maximum colour development. The mixture was diluted to 5 ml with DDW. The absorbance was read at 540 nm on spectrophotometer. A blank was run simultaneously with each sample. A standard curve was plotted by using known graded concentrations of sodium nitrite solution. The absorbance of each sample was compared with that of a calibration curve and nitrate reductase activity (n mol NO₂ g⁻¹ h⁻¹) was noted on fresh mass basis.

3.13.10 Carbonic anhydrase (CA) activity

The carbonic anhydrase activity in the leaves was measured by following the method described by Dwivedi and Randhawa (1974).

Fresh leaf samples were cut into small pieces at a temperature of 25°C. These pieces were weighed (200 mg) and transferred to petriplates. The leaf pieces were cut further into smaller pieces in 10 ml of 0.2M cystein hydrochloride (Appendix 8.1) and left at 4°C for 20 minutes. The leaf pieces were blotted and transferred to a test tube containing 4 ml of phosphate buffer of pH 6.8 (Appendix
8.2). To this test tube 4 ml of 0.2M sodium bicarbonate (Appendix 8.3) solution and 0.2 ml of 0.002% bromothymol blue (Appendix 8.4) were added. The test tube was shaken gently and left at 4°C for 20 minutes. CO₂ liberated by the catalytic action of CA on NaHCO₃ was estimated by titrating the reaction mixture against 0.5N HCl (Appendix 8.5) using methyl red (Appendix 8.6) as an indicator. In each sample the quantity of HCl used to neutralize the reaction mixture was noted and difference was calculated. A blank consisting of all the above components of reaction mixture, except the leaf sample, was run simultaneously with each set of samples. The activity of the enzyme was calculated using the formula.

\[ \frac{V \times 22 \times N}{W} \]  

\[ \text{[Mol (CO₂) kg}^{-1} (\text{leaf.F.M.) S}^{-1}] \]

Where,  

- \( V \) = difference in volume (cm³ of HCl used in control and test sample titration)  
- 22 = equivalent weight of CO₂  
- \( N \) = Normality of HCl  
- \( W \) = Fresh mass of tissue used

3.13.11 Photosynthetic parameters

The photosynthetic parameters such as stomatal conductance, internal CO₂, water use efficiency, transpiration rate and net photosynthetic rate were measured by LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA), in clear sun during 11:00-13:00 hours.

3.13.12 Leaf peroxidase, catalase and superoxide dismutase activity

500 mg of leaf tissue was homogenized in 5 ml of 50 mM phosphate buffer (pH 7.0) containing 1% polyvinyl pyrrolidone. The homogenate was centrifuged at 15,000
rpm for 10 minutes at 5°C and the supernatant obtained was used as an extract for peroxidase, catalase and superoxide dismutase.

3.13.12.1 Leaf peroxidase activity

The activity of peroxidase was measured following the method of Chance and Maehly (1956) in fresh leaf samples.

To the 3 ml solution of pyrogallol phosphate buffer (Appendix 9.1), 0.1 ml of enzyme extract and 0.5 ml of 1% H₂O₂ were mixed in a cuvette and a change in absorbance, at 20 second intervals for a period of 3 minutes was read at 420 nm on a spectrophotometer. The control set was prepared by boiling the enzyme extract.

3.13.12.2 Leaf catalase activity

The activity of catalase was measured following the method by Chance and Maehly (1956).

The estimation of catalase was carried out by the permanganate titration method. 3 ml of phosphate buffer (pH 6.8) (Appendix 10.1), 1 ml of 0.1M H₂O₂ (Appendix 10.2) and 1 ml of enzyme extract were mixed and this mixture was incubated at 25°C for 1 minute. Then 10 ml of 2% H₂SO₄ (Appendix 10.3) was added. The mixture was titrated against 0.1N potassium permanganate (Appendix 10.4) to find the residual H₂O₂ until a purple colour persists for at least 15 sec. Similarly, a control set was maintained in which the enzyme activity was stopped by the addition of H₂SO₄, prior to the addition of enzyme extract.

3.13.12.3 Superoxide dismutase activity

The activity of superoxide dismutase was measured by the method of Beauchamp and Fridovich (1971) in the fresh leaf sample.
A 3 ml of reaction mixture containing 1 ml of 50 mM phosphate buffer (pH 7.8) (Appendix 11.1), 0.5 ml of 13 mM methionine (Appendix 11.2), 0.5 ml of 75 mM NBT (Appendix 11.3), 0.5 ml of 2 mM riboflavin (Appendix 11.4), 0.5 ml of 0.1 mM EDTA (Appendix 11.5) and 0.1 ml of enzyme extract was prepared. Riboflavin was added last. The absorbance of reaction mixture was read at 560 nm on a spectrophotometer.

3.13.13 Proline content in leaves

The proline content in the fresh leaf sample was measured following the method described by Bates et al. (1973).

Fresh leaf sample (0.5 g) was homogenized in mortar with 5 ml of 3% sulphosalicylic acid (Appendix 12.1). The homogenate was filtered through Whatman No. 2 filter paper and collected in a test tube with two washing each with 5 ml of sulphosalicylic acid. 2 ml each of glacial acetic acid and acid ninhydrin (Appendix 12.2) was added to 2 ml of the above extract. This mixture was heated in a boiling water bath for 1 h. The reaction was terminated by transferring the test tube to ice-bath. Four ml of toluene was added to the reaction mixture with vigorous shaking for 20-30 seconds. The chromophore (toluene) layer was aspirated and warmed to room temperature. The absorbance of red colour was read at 520 nm against a reagent blank. The amount of proline in the sample was calculated by using a standard curve prepared from pure proline (range 0.1 – 36 μ mol) and expressed on a fresh mass basis of the sample.

\[
\text{μ moles of proline g}^{-1} \text{ tissues} = \frac{\text{μ g proline cm}^{-3} \times \text{cm}^{-3} \text{ toluene}}{115.5} \times \frac{5}{\text{g (sample)}}
\]

where 115.5 is the molecular mass of proline.
3.13.14 Leaf water potential

Leaf water potential, at each selected stage, was measured in fresh, detached leaves of the sample plants by using Psypro water potential system (Wescor Inc. USA).

3.14 Yield parameters

3.14.1 Number of pods per plant

At harvest, 160 days after sowing, 9 plants (3 from each replicates) from each treatment were randomly sampled and counted for the number of pods plant$^{-1}$.

3.14.2 Number of seeds per pod

25 pods from each treatment were randomly selected for computing the number of seeds per pod.

3.14.3 Seed yield per plant and 100 seed mass

The pods from four plants, representing each treatment, were crushed and cleaned to assess the seed weight per plant. Hundred seeds were subsequently randomly picked and weighed to record 100 seed mass.

3.15 Seed protein content

The total protein content in the dry seeds, at harvest, was estimated by adopting the method of Lowry et al. (1951).

The oven dried seed powder (50 mg) was transferred to a mortar. The sample was ground with the addition of 1 ml of 5% trichloroacetic acid (Appendix 13.1). The pulp was transferred to a glass centrifuge tube with repeated washings and the final volume was made upto 5 ml. The mixture was centrifuged at 4,000 rpm for 15 minutes and the supernatant was discarded. Five ml of 1N NaOH (Appendix 13.2) was added to the residue. The tube was left in a water bath at 60$^\circ$C for 30 minutes.
After cooling for 15 minutes, the mixture was centrifuged at 4,000 rpm for 15 minutes. The supernatant was collected in 25 ml volumetric flask with repeated washings. Volume was made up to the mark using 1N NaOH that was used to estimate the total protein content.

1 ml of the above extract was transferred to a test tube and 5 ml of Reagent C (Appendix 13.3) was added to it. The solution was shaken well and allowed to stand at room temperature for 15 minutes. 0.5 ml of Folin's phenol reagent (Appendix 13.4) was added rapidly with immediate mixing. The blue colour developed whose intensity was read at 660 nm using spectrophotometer. A blank, consisting of all reagents of reaction mixture, except protein extract, was run simultaneously with each set of samples. The total protein content was calculated by comparing the absorbance of the samples with those of a calibration curve, plotted by using known graded concentrations of bovine albumin.

3.16 Statistical analysis

The experiment was conducted according to randomized block design technique. Each treatment was represented by ten pots where each pot was considered as a replicate. Three observations (from three different pots) were recorded per treatment. The treatment means were compared by analysis of variance using SPSS software version 10 (SPSS, Chicago, IL, USA). Least significant difference (LSD) was calculated at 5% level of probability.
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