MATERIALS AND METHODS
3.1 **Source**— Seeds of *Vigna mungo* (L.) Hepper cv. T-9 (Black Gram) was obtained from National Seed Corporation Unit, Indian Agricultural Research Institute, PUSA, New Delhi.

3.2 **Growth Conditions**— Healthy seeds of uniform size were sorted and sterilized with 0.1% HgCl₂ solution for 5 min. and washed with distilled water. Then, seeds were germinated in petriplates containing Whatman filter paper no. -1, moistened with Arnon and Hoagland media (Control). Copper metal was added to the nutrient solution at concentration 0.05, 0.1 and 0.2 mM CuSO₄·5H₂O. Zinc metal was added as 0.25, 0.50, 1.00 and 1.50 mM of ZnSO₄·7H₂O. And, a mixture of both salts was added to study the interaction of both metals at the concentrations mentioned above. Various treatment conditions are mentioned below—

(1) Control  (2) 0.05 mM CuSO₄  (3) 0.1 mM CuSO₄  (4) 0.2 mM CuSO₄  
(5) 0.25 mM ZnSO₄  (6) 0.50 mM ZnSO₄  (7) 1.00 mM ZnSO₄  (8) 1.50 mM ZnSO₄  
(9) 0.05 mM CuSO₄ + 0.25 mM ZnSO₄  (10) 0.05 mM CuSO₄ + 0.50 mM ZnSO₄  
(11) 0.05 mM CuSO₄ + 1.00 mM ZnSO₄  (12) 0.05 mM CuSO₄ + 1.50 mM ZnSO₄  
(13) 0.1 mM CuSO₄ + 0.25 mM ZnSO₄  (14) 0.1 mM CuSO₄ + 0.50 mM ZnSO₄  
(15) 0.1 mM CuSO₄ + 1.00 mM ZnSO₄  (16) 0.1 mM CuSO₄ + 1.50 mM ZnSO₄  
(17) 0.2 mM CuSO₄ + 0.25 mM ZnSO₄  (18) 0.2 mM CuSO₄ + 0.50 mM ZnSO₄  
(19) 0.2 mM CuSO₄ + 1.00 mM ZnSO₄  (20) 0.2 mM CuSO₄ + 1.50 mM ZnSO₄  

Sterile conditions were maintained by adding 20µg/ml of streptomycin sulphate in the medium to suppress microbial growth. All experiments were carried out for seven days at 28±2°C in dark. At regular interval of time required number of seeds were withdrawn and used for analysis of various growth indices such as seed germination, seedling growth and enzymatic activities.
3.3 **Heavy metal assessment** - Heavy metals were determined by the method (EPA method 3050) as outlined by Gupta (2000). 10ml of 1:1 HNO₃ was added to 2g of dry cotyledons / embryonic axis in a 150ml beaker. It was placed on a hot plate, covered with a watch glass and heated at 95°C for 15 minutes. Cooled the digest and added 5.0ml of conc. HNO₃. It was refluxed for an additional 30 minutes at 95°C. Repeated the last step and reduced the solution to about 5.0ml without boiling. Cooled the sample again and added 2.0ml of deionized water and 3.0ml of 30% H₂O₂. With the beaker covered, heated the sample gently to start reaction. Continued to add 30% H₂O₂ in 1.0ml increments, followed by gentle heating until the effervescence subsided. Added 5.0ml of conc. HCl and 10ml of deionized water and refluxed the sample for an additional 15 minutes without boiling. Cooled and filtered the sample through Whatman No. 42 filter paper. Diluted to 100ml with deionized water and analyzed for copper and zinc by atomic absorption spectrophotometer (Z-6100, Hitachi).

3.4 **Seed germination and seedling growth** – At regular intervals required number of seeds were withdrawn and used for the analysis of various growth indices. The number of seeds germinated in a fixed time is expressed as % germination. The counting of the germinated seed was done after 24, 48 and 72 hrs. as there was no further germination after 72 hrs. Emergence of both radicle and plumule was taken as the initiation of germination. The seedling growth was measured in terms of length, fresh weight and dry weight of embryonic axis on 4th, 6th and 8th day after sowing. Seedling length was measured in mm and fresh and dry weights were measured in mg.

3.5 **Quantitative estimation of nucleic acids**- Nucleic acids were extracted by the method given by Schneider (1957). DNA and RNA were estimated according to Burton (1968) and Schneider (1957) respectively.

**Preparation of reagents** - 1) Diphenylamine Reagent- It was prepared by mixing 0.5 ml of 1.6% acetylaldehyde and 100 ml of diphenylamine solution. Diphenylamine solution was prepared by dissolving 1.5 g of diphenylamine in 100 ml of glacial acetic acid and 1.5 ml of concentrated H₂SO₄.
2) Orcinol Reagent- It was prepared by dissolving 100 mg of ferric chloride (FeCl₃·6H₂O) in 100 ml of concentrated HCl and then 3.5 ml of 6% solution of orcinol prepared in alcohol was added.

**Pre-processing** – The plant tissue (cotyledons and embryonic axis) were homogenized in 10% TCA (Trichloro acetic acid) in ratio 1:4 (w/v) and the contents were centrifuged at 3000 rpm for 10 min. Supernatant was discarded. The process was repeated thrice to remove acid soluble substances. The residue was re-suspended in 5.0 ml of 2:1 ethanol: ether mixture and contents were centrifuged at 3000 rpm for 10 min. for the elimination of lipoidal matter. The supernatant was discarded and pellet was suspended in 5 ml of 0.5 N NaOH. All the contents were mixed well and kept at 37°C for 18 hrs. The alkali treated preparation was centrifuged at 3000 rpm for 10 min. Then, supernatant containing RNA in hydrolysed form as well as proteins was collected in another test tube. To the precipitate which contained DNA and some proteins, 1.0 ml of 5% HClO₄ was added and contents were shaked properly. Then, samples were heated in boiling water bath for 1 hr. and cooled. It was followed by the centrifugation at 3000 rpm for 10 min. and supernatant was collected which contained deoxyribonucleotides released from the hydrolysis of DNA and made up to a known volume with 5% HClO₄. To the supernatant containing hydrolyzed RNA equal volume of 10 % TCA was added and centrifuged at 3000 rpm for 10 min. Supernatant was collected and made up to a known volume with 5% HClO₄.

**3.5.1 DNA content**- To 2.0 ml of the sample solutions in which DNA had to be estimated 4.0 ml of diphenylamine reagent was added. Contents were mixed properly and kept at room temperature in dark for 16-18 hrs. or overnight. The blue colour developed in the sample was read at 600 nm. Standard calibration curve was plotted by using herring sperm DNA. Results were expressed as mg of DNA/g.f.wt. of tissue.

**3.5.2 RNA content** – RNA content was estimated by orcinol reaction. 2.0 ml of sample solutions were taken in test tubes and 2.0 ml of 5% HClO₄ was used as blank. Then, 3.0 ml of orcinol reagent was added to all test tubes and mixed properly. The mixture was heated in a boiling water bath for 20 min. and cooled to
room temperature. Intensity of green colour developed was noted at absorbance 665 nm. RNA concentration (mg/g.f.wt.) of sample was calculated with the help of standard curve prepared by using yeast RNA.

3.6 Estimation of carbohydrate fractions

3.6.1 Total soluble sugars – Sugars were measured by anthrone reagent method of Chopra and Kanwar (1991).

**Preparation of Reagents** - 1) Anthrone reagent- Anthrone reagent was prepared by dissolving 200mg of anthrone in 100ml of 70 % chilled H$_2$SO$_4$.

**Procedure** - Plant material (cotyledon and embryonic axis) 4% (w/v) was extracted with 80% ethanol and centrifuged to collect supernatant. The extraction was repeated again. The supernatant was collected and the total volume was made upto 4.0 ml with distilled water. For estimating the amount of sugar, 0.1 ml of the supernatant and 0.1 ml of 30% KOH were added in the test tubes and covered with aluminium foil. The contents were heated at 100$^\circ$C for 10 min. Samples were cooled to room temperature. 3.0 ml of anthrone reagent was added and kept at 40$^\circ$C for 10-15 min. Took O.D. at 620 nm against blank and the sugar content (mg/g.f.wt.) was analysed by comparing with the standard curve prepared by using analytical sucrose. From the amount of sucrose, the glucose can be calculated according to the formula:

$$\text{Amount of glucose} = \frac{\text{Amount of sucrose}}{0.95}$$

3.6.2 Estimation of starch – Starch from the sugar free pellet was estimated by the method of Clegg (1956).

**Preparation of Reagents** - 1) Anthrone reagent- Anthrone reagent was prepared by dissolving 200mg of anthrone in 100ml of 70 % chilled H$_2$SO$_4$.

**Extraction** - Extraction of starch was carried out at room temperature. 5ml of water was added to the sugar free pellet and while stirring, 6.5 ml of 52% perchloric acid was also added. The contents were stirred continuously for 15 min. and centrifuged at 5000 r.p.m. for 15 min. The residual pellet was extracted with perchloric acid again and final volume of the pooled supernatant of each sample was raised to 100 ml.

**Procedure** - Took 1ml extract in test tube. Blank containing distilled water was also run simultaneously. To all the test tubes added 10 ml of anthrone reagent and
mixed thoroughly. After heating in water bath for 12 min., the test tubes were cooled to room temperature and absorbance was recorded at 620 nm with reference to blank. Amount of glucose was then calculated by comparison with the standard curve prepared using glucose (20µg / ml) as standard. Starch (mg/g.f.wt.) was calculated according to the formula given below-

\[
\text{Amount of Starch} = 0.9 \times \text{amount of glucose} \quad \text{(Because 0.9 g of starch produces about 1 g of glucose on hydrolysis.)}
\]

**3.7 Estimation of protein** – Protein was estimated by Bradford method (1976).

**Preparation of Reagent-** 1) Bradford reagent- It was prepared by dissolving 100 mg of Coomassie Brilliant Blue G 250 in 50 ml of ethanol, then 100 ml of 85% phosphoric acid was added and volume was raised up to 1L with distilled water.

Weighed amount of cotyledons and embryonic axis was macerated in phosphate buffer (1:4 w/v), (0.01 M, pH- 7.6) and transferred to centrifuge tubes. The homogenate was centrifuged at 8000 rpm for 20 min. Supernatant was collected and extraction was repeated 4-5 times. All supernatants were combined and volume was raised up to 50 ml with phosphate buffer. To 0.1 ml of the above extract 0.1 M phosphate buffer was added to make the volume 1 ml. Then, 5 ml of Bradford reagent was added to all the test tubes and mixed thoroughly. After addition of Bradford reagent, blue colour developed in test tubes and absorbance was taken at 595 nm against the reagent blank. Amount of protein per ml of the sample preparation was calculated from standard curve plotted by using bovine serum albumin.

**3.8 Estimation of Free amino acid** – Free amino acids were estimated by the method given by Moore and Stein (1963).

**Preparation of Reagent-** 1) Ninhydrin reagent was prepared by dissolving 2 g of ninhydrin in 25 ml of methyl cellosolve and then 25 ml of 0.2 M acetate buffer (pH- 5.5).

Free amino acids were extracted by grinding plant tissue (Cotyledons + Embryonic axis) in 70 % ethanol (1:4 w/v) in a pestle and mortar. Homogenate was centrifuged at 5000 rpm for 10 min. Decant the supernatant. Extraction was repeated 4-5 times and then, supernatants were combined and final volume was raised up to
50 ml with 70% (w/v) ethanol. 5 ml of this ethanolic extract was taken and evaporated to dryness in a boiling water bath and residue was dissolved in 5 ml of 0.2 M citrate buffer (pH-5.0). 2 ml of the above sample was taken in a test tube. For blank, 2 ml of 0.2 M citrate buffer (pH-5.0) was taken in place of the sample preparation. 1 ml of ninhydrin reagent was added to each tube and mixed thoroughly. All test tubes were kept in boiling water bath for 20 min., after cooling under running tap water and final volume was made up to 10 ml with distilled water. Purple colour was developed and absorbance was noted at 570 nm. Amount of amino acid was calculated by using standard graph plotted by using glycine.

3.9 Estimation of Inorganic phosphates- Fiske and Subbarow method (1925) was followed for the estimation of inorganic phosphates with slight modifications.

**Preparation of Reagents**

- **Solution-a)** 10 g of sodium bisulphite (NaHSO₃) in was dissolved in 200 ml of distilled water and mixed until a clear solution is obtained.
- **Solution-b)** 20 g of sodium sulphite (Na₂SO₃) was dissolved in 100 ml of distilled water and then, filtered to obtain clear solution.
- **Aminonaphthosulfonic acid** Took 500 mg of ANSA powder and 195 ml of sodium bisulfite solution was added and mixed. To this solution 5 ml of sodium sulphite was added and mixed thoroughly to get a clear solution. (If the resultant solution is not clear 0.5 ml of solution-b is added followed by thorough mixing. This can be repeated thrice beyond this it is not advisable). This reagent was stored in brown bottle in cold.

**Procedure** 4% of w/v homogenate of cotyledons and embryonic axis tissue were prepared in distilled water at room temperature. To 10 ml of 10% trichloroacetic acid in small flask, 2.5 ml of the homogenate was added slowly. After shaking well, the mixture was filtered through filter paper to remove protein precipitated. To 5.0 ml of protein free filtrate, 1.0 ml of acidic ammonium molybdate solution and 0.4 ml of aminonaphthosulfonic acid reagent were added. After shaking well, the mixture was diluted to 10 ml with distilled water and allowed to stand for 5 min. The coloured solution was transferred to a cuvette and absorbance was read at 660 nm. To set the instrument to zero, a blank was prepared with 5.0 ml of 10% TCA, 1 ml of molybdate solution and 0.4 ml of
aminonaphthosulfonic acid reagent and diluted with distilled water to 10 ml. A calibration curve was plotted with standard phosphate solutions.

3.10 Amylase activity – Amylases were extracted from germinating seeds by the method of Swain and Dekker (1966).

Preparation of Reagents- 1) 1% w/v 3,5-dinitro salicylic acid- This was prepared by dissolving 1g DNSA at room temperature in 20 ml of 2N NaOH and approximately 50 ml of water. After adding 30 g of sodium potassium tartarate to it the final volume was made to 100ml with distilled water.

2) 1% (w/v) starch solution- It was prepared by dissolving soluble starch in 0.1 M acetate buffer (pH-5.5), boiled for 1 min. and then centrifuged. Supernatant obtained was used as starch solution.

Extraction- The cell free extract was prepared in 25 mM Tris-HCl buffer (pH-7.4) containing 1mM CaCl₂ and 5 mM β-mercaptoethanol. The enzyme preparation was dialysed for 4 hrs. against the extraction buffer to remove endogenous sugars from the extract.

Activities of α-amylase and β-amylase were then selectively determined. For α-amylase (EC-3.2.1.1) extract was preincubated with 7.5 mM p-hydroxymercuric benzoate for 20 min. at 20°C to inactivate β-amylase. Activity of β-amylase (EC-3.2.1.2) was determined after regrading α-amylase inactive by complete removal of free Ca²⁺ by preincubating the dialysed extract with 10 mM EDTA at 20°C for 20 min. Both the enzymes were assayed colorimetrically by measuring the quantity of maltose liberated from starch according to the procedure of Sumner and Howell (1935).

Assay Procedure – The reaction mixture was containing 1.0 ml of 1% starch solution, 0.5 ml of water and 0.5 ml of an appropriately treated tissue extract. After 30 min. of incubation at 25°C, 2.0 ml of DNSA (dinitrosalicylic acid) reagent was added to stop the reaction. The tubes to which DNSA was added prior to the enzyme preparation served as blank. The tubes were then kept for 10 min. in a boiling water bath and cooling under running tap water. After adding 20 ml of water, the optical density of the solution was recorded at 540 nm. A calibration curve was prepared using maltose (0.2 to 2mg).
3.11 Protease activity – The enzyme was extracted and assayed according to the procedure of Beever (1968).

Preparation of Reagent- 1) Ninhydrin reagent- It was prepared by dissolving 2 g of ninhydrin in 25 ml of methyl cellosolve and then 25 ml of 0.2 M acetate buffer (pH- 5.5).

Procedure- Cotyledon and embryonic axis from each treatment was macerated in a chilled pestle and mortar in presence of 0.05 M Tris-HCl (pH-7.5), (1:4 w/v) containing 5 mM cysteine. The reaction mixture in a final volume of 2 ml contained: 1ml of 1% (w/v) casein dissolved in 0.2 M sodium citrate buffer (pH-5.5), 0.4 ml of enzyme extract and 0.6 ml of distilled water. After incubating at 40⁰C for 30 min., the reaction was stopped with 1 ml of 20% (w/v) TCA. The contents were thoroughly mixed and centrifuged at 5000 rpm for 15 min. Tubes to which TCA was added prior to enzyme preparation served as control. To 0.5 ml aliquot of the supernatant, 1.0 ml citrate buffer and 1.0 ml ninhydrin reagent were added. The mixture was kept in boiling water bath for 20 min., cooled to room temperature and its volume made to 5 ml with distilled water. The absorbance was read at 570 nm against reagent blank. The amount of free amino acids (as equivalent of leucine) 0-10 µmoles were determined from a standard graph of Leucine.

3.12 Acid phosphatase activity (E.C.-3.1.3.2)

The embryonic axis and endosperm extract was prepared separately in 0.5 M sodium acetate buffer (pH-5.5) (1:4 w/v) and assayed according to the method of Johnson et al. (1973). Reaction mixture in a final volume of 1.5 ml contained – 1.4 ml of 0.3 M sodium citrate buffer (pH-5.5) containing 0.5 mg of p-nitro phenol phosphate (PNP) and 0.1 ml of enzyme preparation. After incubation at 37⁰C for 20 min., the reaction was terminated with 2 ml of 10% Na₂CO₃. Absorbance due to formation of p-nitrophenol was recorded at 410 nm. A standard curve was prepared over a range of 10-100 µg p-nitrophenol.

3.13 Nitrate reductase activity – To study the variation in nitrate reductase activity seeds were grown in alternate light and dark periods of 8 and 16 hrs. respectively under the light intensity of 120µmolm⁻²s⁻¹. The enzyme activity in embryonic axis was assayed by in vivo method (Srivastava, 1975) with slight modifications. Freshly
harvested plant tissue was taken in black vials of 20 ml capacity containing 0.1 M sodium phosphate buffer (pH-7.4) in ratio 1:4 (w/v), 1.0 ml of 0.2 M KNO₃ and 1.0 ml of 25% n-propanol. These vials were sealed and incubated in dark for 30 min. at 30°C. Nitrite released in the incubation mixture due to enzyme enzyme activity was measured by colour development. For this 2.0 ml of aliquot from the incubation mixture (drawn immediately after the termination of assay), 2.0 ml of 1.0% sulphanilamide in 1 N HCl (w/v) and 2 ml of 0.02% N (1-Naphthyl) ethylene diamine-dihydrochloride (NED) (w/v) were added. After 20 min. absorbance was read by VIS-Spectrophotometer at 540 nm. A pink colour was developed due to the formation of di-azo compound with sulphanilamide and nitrite which is coupled with NED. The amount of nitrite was calculated as µmNO₂⁻ / hr/g. f. wt. with the help of standard curve prepared from sodium nitrite.

3.14 Extraction of Antioxidant enzymes (Catalase and Peroxidase)

Weighed amount of embryonic axis tissue was homogenized in sodium phosphate buffer (0.1 M, pH-7) 1:4 (w/v), containing 1% polyvinylpyrrolidone (PVPP) in a cold pestle and mortar. The homogenate was centrifuged at 10,000 X g for 20 min. at 4°C. The supernatant obtained was used for the estimation of catalase and peroxidase.

3.14.1 Catalase Activity – For its estimation, the decomposition of H₂O₂ was measured by recording the decline in absorbance at 240 nm for 3 min. following the method of Abei (1984). The reaction mixture contained 50 mM sodium phosphate buffer (pH-7.0), 50 mM H₂O₂ and 50 µl of enzyme extract in a 3ml volume. A mixture without H₂O₂ served as blank. Catalase activity was calculated by using mM extinction coefficient for H₂O₂ (39.4 mM⁻¹ cm⁻¹) and expressed as amount of H₂O₂ decomposed / min./g.fr.wt.

\[
\text{Catalase activity} = \frac{(\text{Initial} - \text{Final}) \times \text{Extinction coefficient} \times \text{Dilution factor}}{\text{Time}}
\]

3.14.2 Peroxidase Activity – This was assayed as described by Pundir et al. (1999). For the assay, mixture of 1.8 ml sodium phosphate buffer (0.05 M, pH- 7.0), 0.1 ml phenol (1mg/ml), 0.1 ml 4-aminophenazone (0.5 mg/ml) and 0.1 ml extract was
preincubated at 4\(^{0}\)C for 5 min. Then, 1.0 ml of 10 mM H\(_2\)O\(_2\) (30% w/v) was added followed by incubation at 4\(^{0}\)C for 10 min. and thereafter absorbance was read immediately at 520 nm. The amount of H\(_2\)O\(_2\) utilized was extrapolated from the standard curve between A\(_{520}\) and H\(_2\)O\(_2\) concentration. One unit of enzyme activity was defined as the amount of H\(_2\)O\(_2\) decomposed/min./g.fr.wt.

3.15 Polyacrylamide Gel Electrophoresis

The SDS-PAGE was performed on vertical slab (10 cm X 10 cm X 1mm) gel electrophoresis chamber by the method given by Laemmli (1970).

Materials and reagents-

1. Acrylamide- Bisacrylamide stock solution
   Acrylamide- 30g
   Bisacrylamide- 0.8 g
   Dissolved in Distilled water and made final volume to 100 ml. Filtered through Whatman filter paper No. 1 and stored in brown bottle at 0 – 4\(^{0}\)C.

2. Stacking gel buffer stock – (0.5 M Tris-HCl, pH-6.8)
   Tris – 6 g
   1M HCl – 48.0 ml
   pH was adjusted 6.8 and final volume was raised up to 100 ml with distilled water. Filtered it through Whatman filter paper No. 1 and stored at 0- 4\(^{0}\)C.

3. Resolving gel buffer stock – (1.5 M Tris- HCl, pH-8.8)
   Tris- 36.3g
   1M HCl- 48.0 ml
   pH was adjusted 8.8 and final volume was raised up to 100 ml with distilled water. Filtered it through Whatman filter paper No. 1 and stored at 0- 4\(^{0}\)C.

4. 1.5% (w/v) Ammonium Persulphate (APS) in Distilled water
   0.15 g of APS was dissolved in 10 ml water. This reagent was prepared just before use.

5. TEMED (N,N,N,N- Tetramethyl ethylene diamine)
It was used as supplied by the manufacturer.

6. **Reservoir buffer**- (Tris-glycine, pH-8.3) for 1000 ml

   Tris- 3g  
   Glycine-14.4  
   SDS- 1.0 g  

pH was adjusted 8.8 and final volume was raised up to 1000 ml with distilled water.

7. **Staining Solution**

   Coomassie Brilliant Blue R-250- 1.25 g  
   Methanol- 200 ml  
   Glacial acetic acid – 35 ml  

Final volume was made up to 500 ml with distilled water, filtered and stored at room temperature.

8. **Destaining Solution**

   Glacial acetic acid- 75 ml  
   Methanol- 50 ml  

Mixed and distilled water was added to make final volume 1 L.

9. **SDS (10% w/v)**

1 g of Sodium Do-decyl sulphate was dissolved in 10 ml of distilled water.

10. **Sample buffer**-

    1M Tris- HCl, pH-6.8 – 12.5 ml  
    SDS – 4.0 g  
    B-mercaptoethanol- 10.0 ml  
    Glycerol – 20.0 ml  
    1% Bromophenol Blue – 4.0 ml  

Then, distilled water was added to make final volume 100 ml.

11. **Standard Molecular Weight Marker Proteins**-

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<tr>
<th>Protein</th>
<th>Molecular Wt. (K.D)</th>
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Rabbit Phosphorylase 97.4
Bovine serum albumin 66.2
Ovalbumin 42.7
Carbonic anhydrase 31.0
Lysozyme 14.4

12. **Preparation of Stacking gel (2.5%)**

Acrylamide solution – 2.5 ml
Stacking gel buffer (Tris-HCl, pH-6.8) – 5.0 ml
10% SDS – 0.20 ml
1.5% APS – 1.00 ml
D. Water- 11.30 ml
TEMED – 0.015 ml

13. **Preparation of Resolving gel (12.5%)**

Acrylamide solution – 12.5 ml
Resolving gel buffer (Tris-HCl, pH-8.8) – 3.75 ml
10% SDS – 0.30 ml
1.5% APS – 1.50 ml
D. Water- 11.95 ml
TEMED – 0.015 ml

**Procedure**- Plant tissue (embryonic axis) was macerated with 0.1 M phosphate buffer (pH- 7.6) in ratio 1:4 (w/v) and centrifuged at 8000 rpm for 20 min. Supernatant was collected and used as sample. Protein sample was mixed with equal volume of sample buffer. Mixture was boiled in boiling water bath for 3 min. and cool to room temperature. Glass plates, comb and spacers were cleaned properly and dried. Casting plates were fixed and separating gel was poured into casting plates. Some water was added with pipette over the separating gel to prevent the oxidation of acrylamide. It was left undisturbed for 10 min. and water was removed with the help of tissue paper. Then, stacking gel was poured and comb was inserted. It was left for 5 min. Comb was removed and wells were washed with water. Casting plates were transferred from sandwitch to buffer tank containing running buffer Tris-
glycine. Wells were dipped in buffer; samples and Marker (10μl) were loaded in the wells. Current supply was switched on at 32 milliampere until dye crossed the stacking gel then 50 milliampere until the dye reached the bottom of the separating gel. After that gel was removed from casting plates and it was placed in staining solution overnight. Then, gel was transferred to destaining solution and bands were visualized.

3.16 Statistical Analysis- Statistical analysis was done by using Microsoft excel and Graphpad prism 5.0 software. All the experiments were conducted in triplicates. The obtained data were statistically analysed for the mean ± S.D. and differences in values for control and treated plants were analyzed by means of Dunnett’s multiple comparison test taking p≤ 0.05 as significant level. Further, data was subjected to two way ANOVA to determine the effect of treatment conditions, time period and their interaction on various parameters.