Materials and methods
MATERIALS AND METHODS

The seeds of *Amaranthus lividus* L., obtained from Sutton Seed Co. Calcutta, West Bengal, have been used as experimental material to study the effects of different abiological stress during early imbibitional stage.

**Methods of heat shock study:**
To study the effect of heat shock, the seeds of *Amaranthus lividus* were washed with sterile distilled water and treated with 0.1% HgCl2 for 5 minutes twice and finally washed twice with sterile distilled water for 15 mins. Surface sterilised *Amaranthus* seeds were then allowed to imbibe water for six hours and thereafter were sown on moist filter paper in petriplates.

Petriplates with *Amaranthus* seeds on moist filter papers were divided into different batches (30 seeds per plate) and were kept at 45°C for 4, 8 and 12 hrs in darkness. The end of heat shock (different duration) was considered zero hr of germination. The seeds were allowed to grow at 25±2°C with 12 hr photoperiod (270 Em-2s-1) and 78±2% RH. Germination behaviour was calculated after 24, 48 and 72 hr respectively. For all biochemical estimations, qualitative protein profile, membrane deterioration studies of 72 hr old germinating seedlings were used.

**Methods of cold shock study:**
To study the effect of chilling, the seeds of *Amaranthus lividus* were washed with sterile distilled water and treated with 0.1% HgCl2 for 5 minutes twice and finally washed twice with sterile distilled water for 15 mins. Surface sterilised *Amaranthus* seeds were then allowed to imbibe water for six hours and thereafter were sown on moist filter paper in petriplates.

Petriplates with *Amaranthus* seeds on moist filter papers were divided into different batches (30 seeds per plate) and were kept at 45°C for 4, 8 and 12 hrs in darkness. The end of chilling shock (different duration) was considered zero hr of germination. The seeds were allowed to grow at 25±2°C with 12 hr photoperiod (270 Em-2s-1) and 78±2% RH. Germination behaviour was observed after 24, 48 and 72 hr respectively. For all biochemical estimations, qualitative protein profile, membrane deterioration studies of 72 hr old germinating seedlings were used.

**Methods of salinity stress study:**
Surface sterilised seeds of *Amaranthus lividus* (surface sterilization done using the same procedure as described earlier) were imbibed in sterile distilled water for 6 hours and allowed to germinate in petriplates on filter paper soaked with different conc. of NaCl (50, 75, 100, 125 and 150 μM, pH 6.8). The Electrical conductivity (EC) was recorded as 0.69, 0.97, 1.25, 1.56 and 1.80 S respectively. The control experiment was with distilled water. The seeds were allowed to grow at 25±2°C with 12 hr photoperiod (270 Em-2 S-1) and 78±2°C RH. Germination behaviour was observed after 24, 72 and 120 hours, respectively. For all biochemical estimations and membrane deterioration studies 120 hr old seedlings were used. For qualitative protein profile 120 hr old seedlings were used.
Methods of heavy metal stress study:
Surface sterilised and water imbibed (process described earlier) seeds of *Amaranthus lividus* were germinated in petriplates (30 seeds per plate) on filter paper soaked with different concentrations of PbCl₂ and CdCl₂ (10⁻⁶, 10⁻⁵, 10⁻⁴, and 10⁻³ M, pH 6.8). For control set glass distilled water was used. The seeds were allowed to grow at 25±2°C with 12 hr. photoperiod (270 E m⁻² s⁻¹) and 78±2% RH. Germination behaviour was observed after 24, 72, 120 and 168 hours, respectively. For all biochemical estimations and membrane deterioration studies 168 hr old seedlings were used. For qualitative protein profile 168 hr old seedlings were used.

GERMINATION BEHAVIOUR STUDIES

(i) Percent germination of seeds:
To calculate the % of germination of seeds of *Amaranthus* under imbibitional heat shock, chilling shock, salinity stress and heavy metal stress the number of seeds germinated at different interval of times (as specified in the methods of study) were studied. For heat shock and chilling shock studies the moisture content in the petriplates were checked time to time, so as to keep the uniform moisture level in each petriplates.

For salinity and heavy metal stress studies the different solutions for treatment were added time to time (or changed) in petriplates to maintain the uniform and desired concentrations of treatment and the pH was maintained at 6.8.

(ii) Root shoot length:
Root and shoot lengths of germinating seedlings (stressed and control) were measured after desired time in terms of mm. For each treatment, 5 replicates were studied. They are expressed in terms of Root/ Shoot length.

(iii) Biomass:
**Fresh weight**: To estimate fresh weight, ten healthy seedlings were taken. They were blotted properly and weighed by a micropan balance (Adhir Dutt and Co.). For each treatment five replicates were taken into account.

**Dry weight**: To estimate dry wt., ten healthy seedlings were packed inside a paper and kept inside the hot air oven maintained at 70°C for 48 hrs. and then the respective weights of dried seedlings were noted. For each treatment five replicates were designed.

(iv) Relative growth index (RGI):
Relative growth index was calculated using the following formula (Paliouris & Hutchinson, 1991).

\[
RGI = \frac{\text{average dry matter of a seedling in treatment}}{\text{average dry matter of a seedling in control}} \times 100
\]

For each treatment, 5 replicates were considered.

MEMBRANE DETERIORATION STUDIES

Cell viability assay:
The cell viability assay was performed following the method of Chen et al (1982). Root and shoot segments (5 mm length) were transferred to test tubes containing 3 ml of 0.8% (w/v) triphenyl tetrazolium chloride in 50 mM sodium phosphate buffer, pH 7.4. Samples were incubated for 18-20 hr in dark. The leaf segments were removed after incubation
and rinsed with distilled water and placed in test tubes containing 3 ml of 95% ethanol. Samples were boiled to dryness and resuspended in 3 ml of 95% ethanol. Absorbance was recorded at 486 nm. Cell viability was determined in terms of OD.

**A₂₈₀ values, relative leakage ratio and UV absorbance spectra**

For the estimation of A₂₈₀ values of tissue leachate the method of Redmanney et al. (1986) was followed with some necessary modifications. 200 mg of tissues (shoot/root) from each type of treatment and control were placed in 15 ml sterile double distilled water in 50 ml Erlenmeyer's flasks. Flasks were placed on a rotary shaker for 24 hr in order to rinse exhaustively. At the end, 1.5 ml of aliquot of bathing solutions from each flask was taken out and absorbance at 280 nm was recorded using Beckman DU64 UV-Vis spectrophotometer.

For examining the absorbance spectra of UV-absorbing tissue leachate, the same aliquotes were scanned using scan speed of 750 cm sec⁻¹ in the wavelength range between 300-250 nm in a Beckman DU64 UV-VIS spectrophotometer. The aliquots were again placed back to the mother flask containing the bathing solutions and concerned tissue (seedling/leaf/root) and were autoclaved at 15 lb/cm² pressure for 15 mins to destroy the membrane integrity. The second aliquot of 1.5 ml (after autoclaving) from each flask was taken and absorbance was measured at 280 nm (A'₂₈₀). The relative leakage ratio (RLR) of UV-absorbing substances were measured as -

\[
\text{RLR} = \frac{A_280 \text{ (before autoclaving)}}{A'_280 \text{ (after autoclaving)}}
\]

**Electrical conductivity of tissue leachate**

For the measurement of leakage of electrolytes, 200 mg of concerned tissues (seedling/leaf/root) was taken and washed thoroughly in 25 ml of deionised water for 6 hr. The electrolytes in the tissue leachate were determined by measuring the electrical conductivity (EC) in a direct reading Systronics conductivity meter and finally, EC of tissue leachate was expressed in terms of mS cm⁻¹ g⁻¹ d.m.

To estimate membrane injury index, 200 mg of concerned tissue from each treatment was placed in glass vials containing 15 ml deionised water and incubated at 25°C for 24 hr. The EC of the bathing medium was measured at 25°C in direct reading Systronics conductivity meter. The tissue with leachate was then autoclaved (at 15 lb/cm² for 15 mins) and at the end, it was brought to 25°C and EC was measured again. The injury index was calculated using the formula of Sullivan (1972) as

\[
\text{Percent injury} = \frac{1 - \frac{C_1}{C_2}}{1 - \frac{T_1}{T_2}} \times 100
\]

where C₁/C₂ is the EC of control sample before and after autoclaving and T₁/T₂ is the EC of stressed sample before and after autoclaving.

**Superoxide determination**

For the determination of O₂⁻, the process of Chaitanya & Naithani (1994) was followed with some modifications. Weighed amount of tissue (root & shoot) was homogenized in cold (0°C) with 0.2 M sodium phosphate buffer, pH 7.2 with the addition of diethyldithiocarbamate (10⁻³ M) to inhibit SOD activity. The homogenate was immediately centrifuged for 1 min at 3,000 rpm at 40°C. In the
supernatant, $O_2^-$ anion was measured by its capacity to reduce nitroblue tetrazolium (2.5 x $10^{-4}$ M). The absorbance of the end product was measured at 540 nm. Formation of $O_2^-$ was expressed as AOD ($A_{540}$) min$^{-1}$ g$^{-1}$ d.m. of the sample.

**Estimation of mean tolerance index:**

The mean tolerance indices (MTI) of different stressed samples were estimated following the procedure of Paliouris and Hutchinson (1991). Root and shoot length of different stressed seedlings were recorded against their control values. Mean tolerance index was calculated using the following formula:

$$MTI = \frac{\text{mean root/shoot length of an individual in a treatment}}{\text{mean root/shoot length of an individual in control soln.}} \times 100$$

**Estimation of $\alpha$-NH$_2$ and soluble carbohydrate content in tissue leachate:**

For the measurement of $\alpha$-NH$_2$ ($\alpha$-amino nitrogen) and soluble carbohydrate in tissue leachate, 200 mg of concerned tissue (shoot/root) was taken and washed thoroughly in 25 ml of deionised water for six hours. The $\alpha$-NH$_2$ present in the tissue leachate was estimated by Ninhydrin (Moore and Stein, 1948). For this estimation 2 ml of leachate was added with 2 ml of 1% Ninhydrin solution, and boiled it for 20 mins. Finally, developed colour was measured at 590 nm.

The soluble carbohydrate present in tissue leachate was estimated by anthrone reagent (McCreaday et al., 1950). For this estimation, 1 ml of tissue leachate was added to 3 ml of anthrone reagent in cold condition and kept in the hot water bath for 5 mins and again cooled down to room temperature and final colour was measured at 610 nm. To prepare anthrone reagent, 100 mg of anthrone was dissolved in 1.5 ml ethanol and finally the volume was made 50 ml with conc. H$_2$SO$_4$. $\alpha$-NH$_2$ and soluble carbohydrate content in the tissue leachate were expressed in terms of mg g$^{-1}$ dry mass.

**Estimation of membrane lipid peroxidation (in terms of malondialdehyde, MDA, accumulation):**

The membrane lipid peroxidation of stressed tissues was estimated in terms of malondialdehyde accumulation. To estimate MDA content, the TBA (thiobarbituric acid) test was performed using the procedure of Heath and Packer (1968).

Sample (200 mg) was homogenised in 5 ml of 0.1% TCA, then centrifuged at 10,000 rpm for 5 min and finally supernatant was taken. To 1 ml of supernatant, 3 ml of 5% TCA (trichloroacetic acid) containing 1% TBA was added and mixture was heated in a hot water bath for 30 min and cooled quickly in the cold bath and finally centrifuged at 10000 rpm for 10 min. Thereafter, absorbance of supernatant was measured at 530 nm. The non-specific turbidity (if any) was corrected by subtracting $A_{600}$ from $A_{530}$ value. The conc. of MDA was calculated from its extinction coefficient of 155 $\mu$M cm$^{-1}$. The formula employed as:

$$\text{Concentration of unknown} = \frac{\text{absorbance of unknown at 530 nm}}{\text{diameter of cuvette} \times 155} \times \text{moles L}^{-1}$$

The MDA content is finally expressed in nmole g$^{-1}$ dry mass of tissue.
Extraction and Estimation of lipoxygenase (LOX) activity:

For the extraction and estimation of LOX, the process of Peterman and Siedow (1985) was followed. Tissue (200 mg) was homogenised with 5 ml of 50 mM sodium phosphate buffer (pH 6.5). Homogenate was centrifuged at 5,000 rpm for 5 min. Supernatant was taken and re-centrifuged at 1,7000 rpm for 10 min in cold and assayed for LOX. Assay mixture consisted of 1 ml enzyme extract, 1 ml of 1.65 mM sodium phosphate buffer (pH 6.5) and 1 ml of 1.3 mM linoleic acid (Sigma). Incubated at 25°C for one hour. Finally, absorbance was measured at 234 nm.

Estimation of ethylene (C2H4) evolution:

For the measurement of quantitative ethylene evolution the method of Bhattacharjee & Mukherjee (1995) was followed. One gram of seedlings was transferred to a 8.8 cm3 glass vial (lined inside with moist cotton) and made air tight by cap sealing and was kept in darkness at 25±2°C for 24 hr. Finally, 1 ml of head space was withdrawn from vial using gas tight Hamilton syringe and amount of C2H4 was determined by GLC (Nucon) using Porapack-T column and flame ionising detector (FID).

Extraction and Estimation of Hydrogen peroxide:

The extraction and estimation of hydrogen peroxide was done following the process of Mac Nevin & Arnon (1953) using Titanic sulphate. For this, one gram of tissue was extracted with 5 ml of cold acetone. Then, filtered through Whatman No. 1 filter paper and volume made up to 10 ml with distilled water. To this, 1 ml of 5% Titanic sulphate (in 20% H2SO4) was added. After that 2 ml of conc. NH4OH was added, and finally centrifuged at 6,000 rpm for 10 min. Pellet obtained was washed with 5 ml of acetone (thrice) and then centrifuged at 5000 rpm for 10 min. Pellet was then dissolved in 3 ml of 2(N) H2SO4 and read at 420 nm against a blank.

Extraction and Estimation of Catalase (CAT):

For the extraction and estimation of CAT, the process of Snell & Snell (1971) was used with some necessary modifications. For this, 100 mg of tissue was homogenised with 0.1 M sodium phosphate buffer (pH 7) containing 1% polyvinylpolpyrrolidone (PVPP). Homogenate was centrifuged at 5,000 rpm for 10 min at 40°C. For the enzyme assay, 1 ml of enzyme extract was added to 1 ml of 0.5 mM H2O2 and kept it as such for 15 min at 37°C. The reaction was stopped by 2 ml of 1% TiSO4 (in 25% H2SO4). The assay mixture was further centrifuged at 5,000 rpm. Supernatant was taken and read at 420 nm. The enzyme activity was expressed according to Fick and Qualset (1975) which has been described later.

Extraction and estimation of Peroxidase (POD):

For the extraction and estimation of peroxidase the method of Kar and Mishra (1976) was followed with some necessary modifications. Tissue (100 mg) was homogenised with 5 ml of pre-chilled Na-phosphate buffer (.05 M, pH 6.5) at 0°C and centrifuged at 40°C & 1,000 rpm for 10 min. Supernatant was used as enzyme source. The assay mixture consisted of 2 ml of 125 mM sodium phosphate buffer (pH 6.8), 1 ml pyrogallol (94.5 mg pyrogallol in 50 ml H2O), 1 ml of H2O2 (0.56 ml 30% H2O2 in 100 ml distilled water) and 1 ml of enzyme source. The
assay mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 0.5 ml of 5% H₂SO₄. Finally, the colour was read at 420 nm. The enzyme activity was expressed according to Fick and Qualset (1975).

**Extraction and estimation of Superoxide dismutase (SOD):**

For the determination of SOD activity the enzyme was extracted by homogenizing 500 mg of tissue in prechilled 10 ml of 100 mM Na-phosphate buffer (pH 6.8) containing 1% w/v PVPP and centrifuged at 6000 rpm at 4°C for min. Supernatant was used as enzyme source. The enzyme activity was determined by measuring the photochemical reduction ability of nitroblue toluene (NBT) according to Giannopolitis & Ries (1977) with some modifications (Bhattacharjee & Mukherjee, 1996). Reaction mixture (3 ml) contained 0.05 M Na₂CO₃, 0.1 mM EDTA, 63 mM NBT and 13 mM riboflavin. Riboflavin was added at last. Test tubes were placed under two 40 w fluorescent lamp at a distance of 30 cm at 25°C. After 30 min, light was switched off and absorbance at 560 nm was recorded. The non-irradiant sample served as control. The enzyme activity was expressed according to Fick & Qualset (1975), described later.

**Determination of enzyme activity (POD, CAT, SOD) using the method of Fick and Qualset (1975):**

In all cases, the enzyme activity was determined as

\[ \frac{\Delta A \times T_v}{t \times v} \]

where

\[ \Delta A = \text{absorbance of the sample after incubation minus absorbance at zero time (control)} \]
\[ T_v = \text{total volume of filtrate} \]
\[ t = \text{incubation time in minutes} \]
\[ v = \text{volume of filtrate taken for incubation} \]

And the activities in all the cases were expressed as enzyme unit min⁻¹ g⁻¹ dry mass of tissue.

**Extraction and estimation of membrane protein thiol (MPT):**

For the determination of MPT, the membrane preparation was performed according to Singh (1997) with some necessary modifications. The plant tissue was homogenized in ice cold buffer (0.05 M Tris-HCl, pH 7.0). The homogenate was centrifuged at 10,000 x g for 30 minutes at 4°C and the pellet was discarded. The membranes were sedimented by centrifugation at 100,000 x g for 3 hr at 4°C and the pellet containing membranes were resuspended in ice cold buffer (0.05 M Tris-HCl, pH 7.0). The membrane fractions were stored under ice cold condition and used for analysis of MPT. Membrane associated protein-bound thiol groups were assayed after precipitation by TCA and quantifying by DTNB following the procedure of Ellman (1959). The precipitated membrane protein was mixed with 2 ml of 0.5 M sodium phosphate buffer, pH 8.25 and 0.1 ml of 10 mM DTNB in 25 mM sodium phosphate, pH 7.0 and allowed to stand at room temperature for 2 minutes. The total MPT compound was determined at 415 nm according to Ellman (1959) and Dekok & Kuiper (1986).
BIOCHEMICAL ESTIMATION

Photosynthetic pigments:
Photosynthetic pigments (chlorophyll a & b, carotenoids) were extracted and estimated following the process of Lichenthaler & Welburn (1983). For the extraction of photosynthetic pigments, 50 mg of leaf tissues were homogenised with 5 ml of 96% ethanol and then centrifuged at 5000 rpm for 10 min. Then the absorbance was measured at 665, 649 and 470 nm by Beckman DU64 UV-Vis spectrophotometer. Finally, different pigment contents were calculated using following formulas:

\[
\text{Chla} = (13.95 \times A_{665} - 6.88 \times A_{649}) \mu g/ml
\]

\[
\text{chl b} = (24.96 \times A_{649} - 7.32 \times A_{665}) \mu g/ml
\]

\[
\text{carotenoids} = (1000 \times A_{470} - 2.05 \times \text{chl a} - 11.48 \times \text{chl b}) / 245 \mu g/ml
\]

Chl a, Chl b and Carotenoid pigments were then expressed in terms of mg/g dry mass of tissue.

Extraction and estimation of reducing sugar:
For the extraction and estimation of reducing sugar, Nelson-Somoyagi method modified by Sadasivam & Manikam (1996) was followed. Plant material (500 mg) was extracted with 80% ethanol (10 ml x 2). The supernatant was collected and evaporated on a water bath at 80°C followed by the addition of 45 ml of water. Taking 0.2 ml of the above, the working solution was prepared and 1 ml of copper tartarate was added to it and kept in boiling water bath for 10 minutes. Now, to the cold solution, 1 ml of arsenomolybolic acid reagent was added (a) 2.5 g of ammonium molybdate dissolved in 45 ml of water followed by the addition of concentrated sulphuric acid and mixed well. (b) 0.3 g of sodium hydrogen arsenate dissolved in 25 ml of water was added. Reagent (a) and (b) were mixed well and kept at 37°C for 24 to 48 hours to obtain the actual reagent. The volume was made up to 10 ml with distilled water. The absorbance of the colored solution was read at 610 nm.
absorbance was read at 620 nm after 10 minutes. For standard curve, glucose was used. Finally, the content was expressed in terms of mg g\(^{-1}\) dry mass.

**Relative water content (RWC):**
For the determination of RWC, 10 primary leaves of equal diameter (4 mm) were weighed and floated on distilled water for 4 hr at 25\(^{\circ}\)C. Then, the leaves were blotted dry and weighed prior to oven drying at 80\(^{\circ}\)C for 24 hr and RWC was calculated as

\[
\frac{\text{Fresh weight} - \text{Dry weight}}{\text{Turgid weight} - \text{Dry weight}} \times 100
\]

**Extraction and estimation of proline:**
Proline was extracted and estimated following the procedure of Bates et al. (1973). Tissue (200 mg) was homogenised with 10 ml of 3% sulfosalicylic acid, centrifuged at 4,000 rpm for 10 min. Then, 2 ml of supernatant, 2 ml of glacial acetic acid and 2 ml of ninhydrin reagent [To prepare ninhydrin reagent, 315 mg of ninhydrin was dissolved in 7.5 ml acetic acid glacial and 5 ml of dil. orthophosphoric acid (7 ml stock orthophosphoric acid, AR + 5 ml of dist \(H_2O\))] were mixed and kept in hot water bath at 100\(^{\circ}\)C for an hour. After, the test tubes were kept inside the chiller of refrigerator for 10 min. Again, kept at room temperature for 2 min. Finally, the chromophore was extracted with 4 ml of toluene and absorbance was measured at 520 nm. Proline content was finally expressed in mg g\(^{-1}\) dry mass.

**Root metabolic activity (RMA):**
RMA was determined following the procedure of Bhattacharjee & Mukherjee (1995). Root tips (5 mm) were floated in 0.1\% (w/v) TTC solution and kept in incubator for 3 hr at 35\(^{\circ}\)C. After incubation, the root tips were weighed and crushed with 4 ml of cold ethyl acetate buffer and centrifuged at 4,000 rpm for 70 min at 4\(^{\circ}\)C. Intensity of red colour was measured at 486 nm using UV-VIS spectrophotometer. For blank, 4 ml of ethyl acetate was used. Activity was measured in terms of OD.

**Extraction and estimation of Amino acids:**
The amino acids were extracted and estimated following ninhydrin method (Moore & Stein, 1948). 100 mg of tissue was homogenised with 5 ml of 80% boiled ethanol. Homogenate was centrifuged at 5,000 rpm for 10 mins. Then sup. was taken as such and pellet was washed twice with boiled 80% ethanol (2 ml). The total sup. was taken and volume made upto 10 ml. To 1 ml of sup, 3 ml of 0.5% Ninhydrin (in ethanol) was added and boiled for 30 mins. Finally, the absorbance was measured at 590 nm.

**Estimation and extraction of Ca, Na and K:**
For the extraction of Ca, Na & K, 25 mg of dried tissue was homogenised in 5 ml of 1(N) HCl. Homogenate was filtered through Whatman No.1 filter paper. Taking the filtrate, readings for different metals (Ca, Na and K) were taken in the flame photometer (Systronics) using respective filters Prat & Fathi-Ettai, 1990). Finally, the content was expressed in terms of mg g\(^{-1}\) dry mass of tissue.

**Extraction and estimation of soluble protein:**
Protein was extracted following the method of Kee and Nobel (1986) with some necessary modifications. Tissue (2 g) was homogenised in a mortar containing neutral sand, 25 ml of 50 mM Tris-HCl
(pH 7.0), 2% SDS, 0.05% (w/v) β-mercaptoethanol, 2.5 mM p-hydromercuribenzoate, 1 mM phenyl methyl sulfonyl fluoride (PMSF). Coarse debris was removed by filtration followed by centrifugation at 3,000 x g for 15 min. Five volumes of cold acetone (at -20°C) were added to the decanted supernatant and mixture was stored overnight at -20°C. The resulting precipitate was collected by centrifugation (12,000 x g for 15 min), the pellet was washed once with cold acetone. The final pellet was freeze-dried. Protein content of the powder was determined by Bradford's dye binding method (Bradford 1976). Defatted BSA was used as protein standard. To determine protein, 0.1 ml of 0.15 M NaCl was added to 5 ml of Bradford reagent (100 mg of Coomasie Brilliant Blue G-250 dissolved in 50 ml of 95% ethanol, 100 ml of 85% H₃PO₄ was added and finally diluted to 1000 ml with glass distilled H₂O). Protein soln. with Bradford reagent was mixed well and read just after 2 min at 595 nm. Finally the content was expressed in terms of mg g⁻¹ dry mass.

**Extraction and estimation of protease:**

For the extraction and estimation of protease, the process of Snell and Snell (1971) was followed with some modifications (Bhattacharjee & Mukherjee, 1995). Tissue (200 mg) was homogenised with 5 ml of chilled 0.1 M phosphate buffer (pH 6.5) at 0°C and centrifuged at 10,000 rpm for 10 min under cold condition. Supernatant was used as crude enzyme source. The assay mixture consisted of 1 ml enzyme extract, 0.1 ml MgSO₄, 7H₂O (0.1 M) and 1 ml of BSA (0.5 mg/ml). The assay mixture was kept as such at 37°C for one hour. The reaction was stopped by adding 1 ml of 50% TCA and the residual protein was pelleted by centrifugation and measured by Bradford's method. After centrifugation at 5,000 rpm for 10 min, the pellet obtained was dissolved in 0.1 ml of 0.15 M NaCl and to this, 5 ml of Bradford reagent was added (as described before). The colour developed was measured at 595 nm. The enzyme activity was expressed as enzyme unit min⁻¹ g⁻¹ d.m. according to the formula of Fick & Qualset (1975) as described earlier.

**Extraction and estimation of α-amylase:**

The enzyme α-amylase was extracted and estimated following the combined methods of Khan & Faust (1967) with some necessary modifications. 300 mg of tissue was homogenised with 10 ml of pre-chilled Na-phosphate buffer (0.1 M, pH 6.5). Homogenate was centrifuged at 5000 rpm for 10 min and supernatant was used as crude enzyme source. The assay mixture consisted of 0.5 ml of enzyme source and 0.1% starch solution. Then, incubated at 37°C for 10 min. Reaction was stopped by 3 ml of I₂-HCl solution (60 mg of KI and 16 mg of I₂ was dissolved in 100 ml of 0.5 N HCl). The intensity of color developed was measured at 620 nm. The enzyme activity was expressed as enzyme unit min⁻¹ g⁻¹ d.m. according to the process of Fick and Qualset (1975), described earlier.

**Extraction and estimation of nucleic acids (DNA and RNA):**

For the extraction and estimation of DNA and RNA the process of Jayaraman (1985) was followed with some necessary modifications. Tissue (200 mg) was homogenised with 10 ml of 10% ice cold trichloroacetic acid (TCA). Homogenate was centrifuged at 3,000 rpm for 15 min. Supernatant was discarded. Pellet was suspended in 5 ml of ethanol-ether soln. (1:1). Again centrifuged at 3,000 rpm for
Pellet was mixed with 5 ml of 0.5(N) NaOH and was mixed well and incubated at 37°C for 18 hr. After, the mixture was again centrifuged at 3,000 rpm for 15 min. Supernatant obtained, contained RNA in hydrolysed form as well as protein. To the supernatant, equal volume of 10% TCA was added and centrifuged at 3,000 rpm for 15 min. Supernatant contained RNA only and was estimated by orcinol reagent. The pellet obtained from the centrifugation of NaOH treated material contained DNA and most proteins. The pellet was hydrolysed with 1 ml of HClO₄ in a boiling H₂O bath for an hour and cooled down and volume made upto 10 ml. Finally, that was centrifuged at 3,000 rpm and the supernatant obtained contained DNA, which was estimated by diphenylamine reagent.

Preparation of orcinol reagent: Orcinol (250 mg) was dissolved in 1.5 ml of ethanol and then added to 25 ml of conc. HCl containing 0.1% FeCl₃ (w/v). This reagent was always made fresh.

Preparation of Diphenylamine reagent: Diphenyl amine (1 g) was dissolved in 100 ml of glacial acetic acid. To this, 2.75 ml of conc. H₂SO₄ was added. This reagent was always made fresh.

Estimation of RNA:
To 3 ml of sample, 3 ml of freshly prepared orcinol reagent was added and heated in a boiling water bath for 20-30 min, cooled to room temperature and absorbance was measured at 700 nm. For making standard curve, authentic yeast RNA (Sigma) sample was used.

Estimation of DNA:
To 3 ml of sample, 5 ml of diphenylamine reagent was added, heated in a water bath for 20 min and finally absorbance was measured at 610 nm. For making standard curve authentic yeast DNA (Sigma) sample was used. Standard curves for DNA was made from authentic Herring sperm DNA sample following the same procedure.

Extraction and estimation of ALAD (5-aminolevulinic acid dehydratase):
ALAD was extracted and the activity was estimated according to Schneider (1970) with some necessary modifications. Primary leaf (50 mg) segments were homogenized in 10 ml of cold 0.05 mM Tris-HCl buffer, pH 8.2 containing 10 mM DTT. The homogenate was centrifuged at 15,000 x g at 4°C and the supernatant was assayed for enzyme activity. Assay mixture contained 1 ml enzyme source, 0.27 ml 1 mg ml⁻¹ ALA, 1.35 ml 0.05 M Tris-HCl buffer containing 0.02 M MgCl₂ and was subsequently incubated for 1 hr at 37°C. After incubation, the reaction was stopped by adding 0.3 ml of 3 M TCA and the contents were centrifuged. Porphobilinogen content of the supernatant was estimated by using Ehrlich’s reagent (Mauzerall & Granick, 1956) and absorbance was measured at 553 nm. Finally, ALAD activity was expressed in terms of n mole PBG min⁻¹ g⁻¹ d.m.

Extraction and estimation of chlorophyllase:
For the extraction and estimation of chlorophyllase the modified process of Nag et al. (1981) was used. For the substrate, the primary leaves were extracted with 80% alkaline acetone (4 ml g⁻¹ leaf material) and centrifuged in cold condition twice at 4,000 rpm. The combined supernatant was stored at 0°C. Then, 100 mg of experimental leaf material was extracted with acetone (50% w/v) and centrifuged at 15,000 x g at 4°C.
The supernatant, used as enzyme source, was incubated at 37°C with 5 ml of substrate and 4 ml of sodium citrate solution (0.04 M) and pH was maintained at about 8 and kept for 2 hr. After incubation, mixture was centrifuged and residue was washed with 80% acetone. To the supernatant 5 ml of 2% NaCl was added. Volume was made upto 24 ml with 80% acetone. Half of the solution was taken for the determination of total chlorophyll and other half was shaken twice in a separating funnel with 5 ml of petroleum ether. Acetone layer containing protochlorophyllides was again made upto 12 ml with 80% acetone and the absorbance was recorded at 607 nm. The enzyme activity was expressed as % chlorophyll degraded g \(^{-1}\) d.m. min\(^{-1}\).

**Protein extraction for electrophoresis:**
Germinating seedlings (2 g) were homogenised in a mortar containing washed neutral sand (a pinch) with 25 ml of extraction buffer [200 mM Tris-HCl, pH 7.5, 2% (w/v) SDS, 0.05% (w/v) β-mercaptoethanol, 2.5 mM p-hydromercuribenzoate, 1 mM PMSF (PMSF was added just before homogenisation from a 10 mM solution in DMSO) and one of antifoam C emulsion (Sigma)]. Coarse debris was removed through centrifugation at 3,000 x g for 15 min. Five volumes of cold acetone (-20°C) was added to the decanted sup. and the mixture was stored overnight at -20°C. Resulting precipitate was collected by centrifugation (12,000 x g for 10 min), the pellet was washed twice with cold acetone and the final pellet was freeze dried (Virtis). Protein content was determined by Bradford’s dye binding method (Bradford, 1976) as described earlier.

**Gel electrophoresis:**
Proteins were analysed using Laemmli's (1970) one-dimensional gradient (8 to 15%) SDS-PAGE. The protein-aqueous powder was dissolved in 6 mM Tris-HCl (pH 6.8), 2% w/v SDS, 10% (w/v) glycerol, 5% β-mercaptoethanol, 0.001% bromophenol blue. After heating to 100°C for 2 min and spinning at 1,000 x g for 5 min, acetone precipitated protein (30 µg) was loaded on each well. Electrophoresis was performed in a slab gel apparatus (Biotech) with a linear 8 to 15% (w/v) polyacrylamide separating gel and a 5% (w/v) stacking gel. Electrophoresis was at 50v. Gradient separating gel was made using a Biorad gradient maker. After electrophoresis, the proteins were made visible by silver staining. For mol. wt. determinations, the marker (Myosin heavy chain, 200 kDa; CaATPase, 100 kDa; Myofibril, 68 kDa; Actin, 42 kDa; Myosin, 25 kDa; Cytochrome C, 13.3 kDa) proteins were run in the same condition.

**Silver staining:**
Silver staining of protein bands was done following the process of Oakley et al. (1980). The gel was first fixed in 50% methanol and 10% acetic acid for 30 min. Then washed in 5% methanol and 7% acetic acid solution overnight. After, the gel was soaked in 10% glutaraldehyde solution for half an hour, rinsed in distilled water (twice) and kept in deionised water overnight. Finally the water was decanted and the gel was placed in ammoniacal AgNO\(_3\) solution (To make 100 ml of ammoniacal AgNO\(_3\) solution, 1.4 ml of fresh ammonia solution was added with 21 ml of 0.36% NaOH with vigorous agitation and 4 ml of 19.4% AgNO\(_3\) was added slowly). The gel was photographed through Agfa Copex panchromatic 35 mm film and dried in a Biorad gel dryer.

**Ameliorating effect of Triadimefon (50**
mg L\(^{-1}\)) on imbibitional heat shock and chilling stress induced injury in *Amaranthus lividus*

**Method of pretreatment:**

*A. lividus* seeds, obtained from sutton seed Co., were washed with sterile distilled water and treated with 0.1% HgCl\(_2\) for 5 mins (twice) for surface sterilization. And finally washed twice with sterile distilled water for 15 mins. Triadimefon [1(4-chlorophenoxy)-3, 3 diethyl-1-(1,2,4-trizol-1-yl)-2 butanone] pretreatment was performed by imbibing the surface sterilized seeds in 50 mg L\(^{-1}\) solution for 18 hour. Seeds were then air-dried at room temperature and finally shown in petriplates on moist filter papers (30 seeds per plate) and kept at 25°C in darkness for 24 hour. After that germinatig seeds were subjected seperately to heat shock at 45°C for 8 hours and chilling stress at 4°C for 8 hours. Finally they were allowed to grow at 25°C with 12 hour photoperiod (270 E m\(^{-2}\) s\(^{-1}\)) and 78±2% RH. Germination behavior, biochemical estimations and membrane deterioration studies, 5 day old seedlings were used. For qualitative protein profile also 5 day old seedlings were used.

**Ameliorating effect of Triadimefon (50 mg L\(^{-1}\)) on NaCl-salinity stress induced injury during early germinations**

In order to study the ameliorating effects of Triadimefon on NaCl-salinity stress induced injury, surface sterilized seeds (Surface sterilization done using the same procedure as described earlier) were imbibed in sterile distilled water for 6 hours and allowed to germinate seperately in petriplates on filter paper soaked with the following solutions -

(i) Triadimefon (50 mg L\(^{-1}\))
(ii) 100 mM NaCl (EC 1.26)
(iii) 100 mM NaCl + Triadimefon (37.5 mg L\(^{-1}\))
(iv) 100 mM NaCl + Triadimefon (50 mg L\(^{-1}\))
(v) Distilled water (Control).

The seeds were allowed to grow at 25°C±2°C with 12 hour photoperiod (270 E m\(^{-2}\) s\(^{-1}\)) and 78±2% RH. For germination behavior, biochemical estimations and membrane deterioration studies, 5 day old seedlings were used. For qualitative protein profile also 5 day old seedlings were used.

**Effect of Ca\(^{2+}\) on membrane stabilization under imbibitional chilling and heat shock. Methods of pretreatment:**

In order to study the involvement of divalent Calcium (Ca\(^{2+}\)) on free radical induced membrane damage in imbibitionally heat and chilling stressed *Amaranthus lividus*, surface sterilized seeds were inbibed in the following solution sperately, for 16 hours:

- i) 20 mM CaCl\(_2\)
- ii) 2 mM EGTA
- iii) 1 mM LaCl\(_3\)
- iv) glass distilled water

Seeds were then air dried in room temperature and finally sown in petriplates on moist filter papers (30 seeds per plate) and kept at 25°C in darkness for 24 hours. Subsequently, the germinating seeds were subjected seperately to chilling at 4°C and
heat shock at 45°C for 8 hours. Finally, they were allowed to grow at 25°C. For assessment of qualitative protein profile and free radical induced membrane deterioration, 2 days old seedlings (after stress imposition) were used.