MATERIAL AND METHODS

3.1 Plant material

3.1.1. Collection

The seeds of different genotypes of *Cicer arietinum* L. (Plate II) required for the experimental purpose were obtained from the seed germplasm bank of International Crop Research Institute for Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, India. Seedlings of different genotypes were then raised from this seed stock in the experimental garden, Department of Botany, University of North Bengal.

3.1.2. Propagation

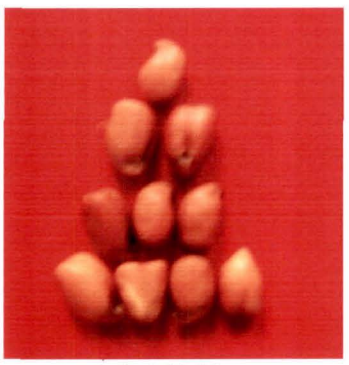
The propagation of the crop was done from the fresh seeds obtained from ICRISAT initially and then from the fresh seeds harvested (after 120-150 days of sowing) from the plants grown in the experimental garden. The seeds harvested were labeled and preserved carefully in air tight containers or sealed paper packets for future use.

3.1.3. Plantation

Chickpea seeds were sown in sandy-loam soil during middle of October to first week of November. Seeds were sown in lines at a distance of 25-30 cm between the rows and at a depth of 7-10 cm for better grain yield in the field. For pot culture, the seedlings were grown in 10 inch size clay pots containing steam sterilized soils. The soil used was thoroughly powdered and mixed with sand and farmyard manure in the proportion of 2:2:1 by volume (Plate III).

3.1.4. Maintenance

Because of slow initial growth the crop is often badly infested by weeds which suppress the crop growth and results into poor yield, therefore, one or two weedings or spraying of a herbicide was practiced for increasing the yield. Further Endosulfan (0.7% solution) was used once/twice during fruiting stage to prevent the crop from the attack of pod borer at pod filling stage. Plants were regularly watered



ICC 14340

ICC 10035

ICC 5003

ICC 4918

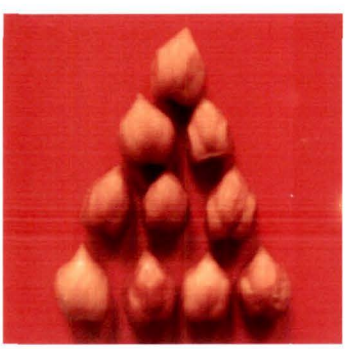
ICC 4969

ICC 16359

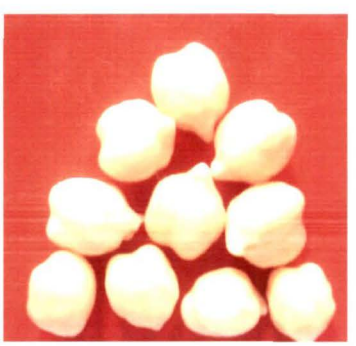
ICC 5319

ICCV 2

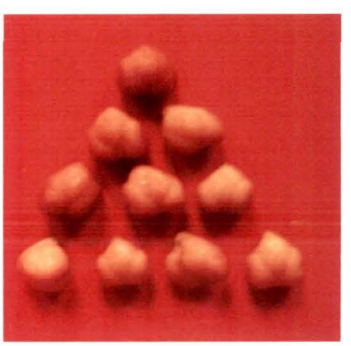
ICCC 37 (Kranthi)



ICCV 10 (Bharathi)



ICC 7344



ICC 1852

Plate II: Seeds of different genotypes of *Cicer arietinum* L.

Plate III (A-D): Growth of chickpea seedlings of different genotypes in pots
A: ICC V10 **B**: ICC C37 **C**: Different genotypes and **D**: ICC 5003

and maintained at normal temperatures of 25 ± 2°C. Plants were also fed regularly with Hoagland solution at 15days interval.

3.1.5. Selection of genotypes

On the basis of germination percentage and field performance the following 15 genotypes were selected for the purpose of study from the 37 genotypes obtained from the seed germplasm bank of ICRISAT, Patancheru, Andhra Pradesh, India. Selected genotypes included the common desi types, kabuli, green gram and a pod mutant genotype (Plate IV).

ICC 1852, ICC 2042, ICC 4918, ICC 4969, ICC 5003, ICC 5319, ICC 6119, ICC 7344, ICC 10035, ICC 14340, ICC 16359, ICCV 10 (Bharathi), ICCV 1, ICC C 37 (Kranthi) and ICCV 2 (Swetha).

3.2. Seed treatment

3.2.1. Chemical treatment of seeds

Selected seeds of various genotypes were imbibed in 100 µM salicylic acid (SA), 50 µM abscisic acid (ABA) and 10mM solution of $CaCl_2$ overnight. The seeds imbibed in different solutions were blotted dry on filter paper and were allowed to germinate at room temperature. The effect of various chemical treatments on seed germination was analyzed by recording the percentage of germination of treated seeds with respect to untreated control seeds imbibed in distilled water.

3.2.2. Seed bacterization with *Bacillus megaterium*

Chickpea seeds were washed gently with distilled water and surface sterilized before bacterization. Seeds of different genotypes were separately inoculated with PGPR strain *Bacillus megaterium* having a concentration of $2x10^6$cfu/ml suspended in 50 ml of 0.2% carboxy methyl cellulose (CMC) solution. The seeds soaked in sterile distilled water served as control. The seeds were imbibed for 24 hrs in bacterial suspension on a shaker to ensure uniform coating of bacterial cells on seed surface. After 24 hrs the seeds were blotted dry on filter paper (Whatman no.1), and incubated for 10 days for growth. The effect of seed bacterization on germination

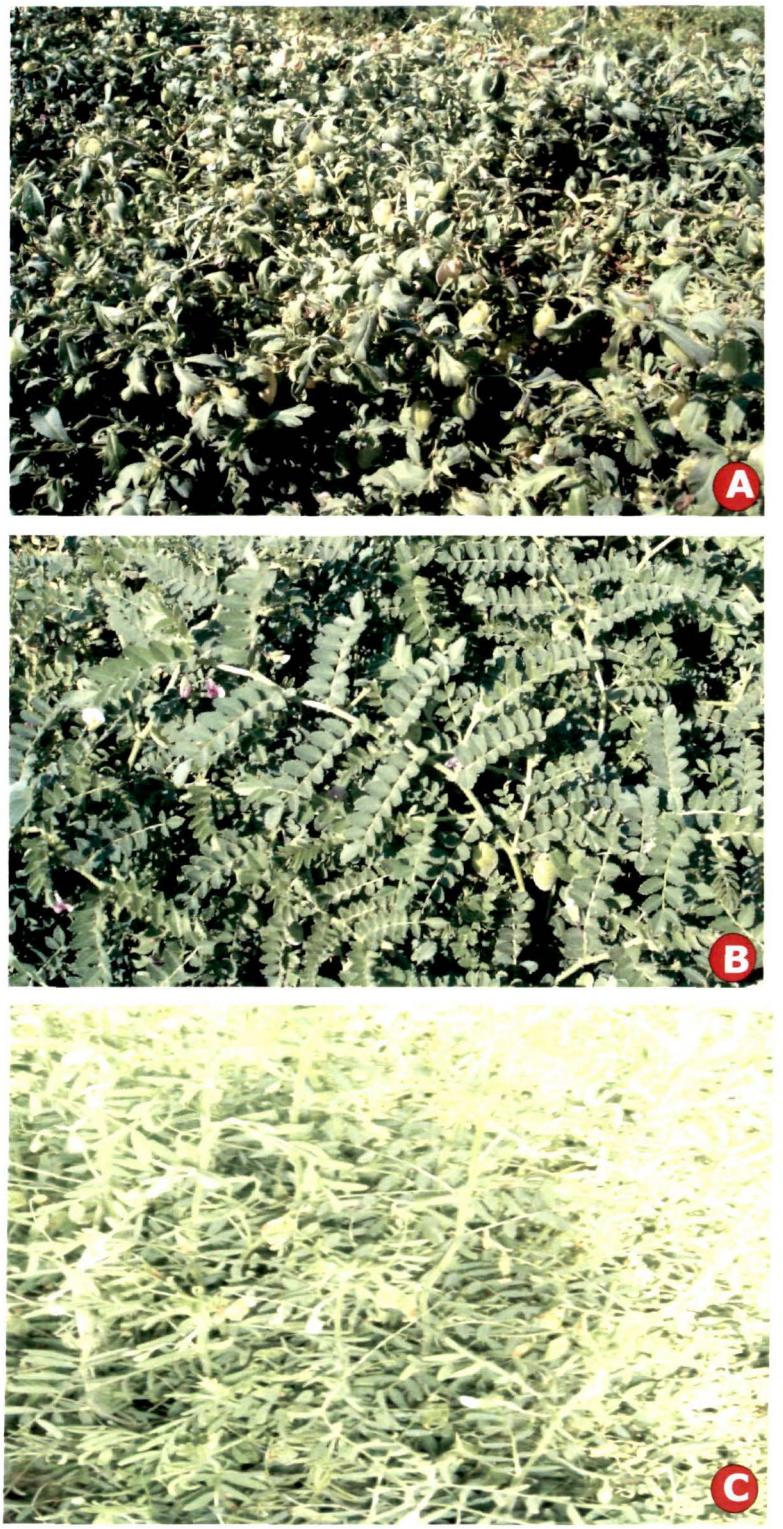


Plate IV (A-C): Chickpea plants bearing flowers and fruits **A**: ICC 7344 (Kabuli);**B:** ICC 1852 (Desi black gram) and **C:** ICC 14340 (Pod mutant)

was tested by recording the percentage of germination of bacterized seeds with respect to untreated control seeds after 24, 48 and 72 hrs.

3.3. Temperature treatment

3.3.1. Seeds: Chickpea seeds of different genotypes soaked overnight in sterile distilled water after surface sterilization with 0.1% $HgCl_2$ were treated at elevated temperatures of 30, 35, 40, 45, 50 and 55°C for 2 hrs duration in moist petri plates following which they were allowed to germinate at room temperature.

3.3.2. Seedlings: 20 days old seedlings were exposed to elevated temperatures of 30, 35, 40, 42, 44 and 46°C for 2 hrs duration which was followed by a recovery time of 96 hrs. The seedlings exposed to elevated temperatures were allowed to recover at room temperature of 25°C ± 2°C and relative humidity (RH) of 60-70% under 11-12 hrs of photoperiod.

3.4. Heat acclimation treatment

For heat–acclimation (HA) treatments of seedlings, the seedlings were pre-exposed to elevated but sub-lethal temperatures of 35, 40, 42 and 44°C for 2 hrs duration prior to lethal temperature treatment. Best heat acclimation was achieved with the exposure of seedlings to 42°C for 2 hrs. Hence this treatment of 42°C for 2 hrs was considered as heat acclimation treatment in all experiments.

3.5. Foliar application of chemicals

3.5.1. Salicylic acid (SA)

Seedlings were sprayed twice a day with 100 µM solution of salicylic acid (SA). The spray treatment was carried out for five consecutive days and finally just prior to exposure to heat stress. The pre-treated seedlings and distilled water treated (control) seedlings dipped in respective solutions in conical flasks were then subjected to heat stress (46°C for 2hrs) and sampled for experimental purposes.

3.5.2. Abscisic acid (ABA)

Abscisic acid (ABA) solution of strength 50 µM was used as a foliar spray on seedlings. The same volume (50 mL.) of distilled water was sprayed on control seedlings. The spraying treatment was carried out exactly in the same manner as mentioned in case of SA.

3.5.3. Calcium chloride ($CaCl_2$)

Calcium chloride solution of 10mM strength was sprayed on test seedlings in the form of foliar spray. Controls consisted of plants on which the same volume of distilled water was sprayed and treated in the same manner as test plants. The seedlings were immersed in respective solutions in conical flasks for heat treatments. After heat treatments, plants were returned to the room conditions and allowed to recover for up to 96 hrs.

3.6. Determination of tolerance index (TI) of seedlings

Variation in heat tolerance of the seedlings of various genotypes was calculated as the tolerance index (TI) which gives the percentage of shoot and/or root fresh biomass (g per plant) of treated (FW_t) over untreated control (FW_c) plants according to the following equation as suggested by Metwally *et al*. (2005):

$$TI\ (\%) = (FW_t / FW_c \times 100) - 100$$

3.7. Determination of plant growth promoting activity of *Bacillus megaterium*

The experiment was conducted under greenhouse conditions (in potted plants) and in the open field to assess the efficacy of *Bacillus megaterium* on plant growth promotion. The growth promotion was assessed in seedlings and field grown plants on the basis of vigour index (VI) by comparing the increase in root and shoot length of the treated plants with the untreated control plants under the same environmental conditions (temperature of 25± 2°C, 60-70% RH and 10-11hrs photoperiod (Baki and

Anderson, 1973). The experiment consisted of ten replicates with 10 seeds in each treatment in completely randomized design. The vigour index of seedlings and mature plants was calculated by applying the following formula :

Vigour index = (mean shoot length + root length) × % of germination

3.8. Protein analysis

3.8.1. Extraction of soluble proteins

Soluble proteins were extracted from seeds, leaves, stem and roots of different genotypes of *Cicer arietinum* L. following the method of Chakraborty *et al.* (1995) with modifications. Plant tissues were frozen in liquid nitrogen and ground in 0.05 M sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$ and 2 mM PMSF added at the time of crushing and centrifuged at 4°C for 20 min at 10000 g. The supernatant was used as crude protein extract.

3.8.2. Estimation

Soluble proteins were estimated following the method as described by Lowry *et al.* (1951). To 1 ml of protein sample 5 ml of alkaline reagent (1 ml of 1% $CuSO_4$ and 1 ml of 2% sodium potassium tartarate, dissolved in 100 ml of 2% Na_2CO_3 in 0.1 N NaOH) was added. This was incubated for 15 min at room temperature and then 0.5 ml of 1 N Folin Ciocalteau reagent was added and again incubated for further 15-20 min following which absorbance was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.8.3. SDS-PAGE analysis

Total soluble proteins extracted in 0.05 M sodium phosphate buffer were used as crude protein extract for analysis of protein pattern. Analysis was carried out on 10% SDS- PAGE gels following the method of Sambrook *et al.* (1989). Protein samples were loaded on the gel and separated for 3 hours at 200 V and 15-20 mA

current. Following electrophoresis the gel was fixed, stained in Coomassie Brilliant Blue (R-250) solution and finally destained in a solution of methanol, glacial acetic acid and water (4.5: 4.5: 1).

3.8.3.1. Preparation of stock solution

For the preparation of gel the following stock solutions were prepared:

(A) Acrylamide and N' N'- methylene bis acrylamide

A stock solution containing 29% acrylamide and 1% bisacrylamide was prepared in water. As both of these amides are slowly deaminated to acrylic and bis acrylic acid by alkali and light the pH of the solution was kept below 7.0. The stock solution was then filtered through Whatman No. 1 filter paper, kept in brown bottle and stored at 4°C and used within one month.

(B) Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

(C) Tris Buffer

i) 1.5 M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4°C for use.

ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4°C for use.

(D) Ammonium Persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

(E) Tris –Glycine electrophoresis buffer

Tris running buffer consists of 25 mM Tris base, 250 mM Glycine (pH 8.3) and 0.1% SDS. A 1X solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10 % SDS in 1 L of distilled water.

(F) SDS gel loading buffer

This buffer contains 50 mM Tris-HCl (pH 6.8), 10 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1X solution was prepared by dissolving 0.5 ml of 1M

Tris buffer (pH 6.8), 0.5 ml of 14.4 M β-mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue,1 ml glycerol in 6.8 ml of distilled water.

3.8.3.2. Preparation of gel

Mini slab gel (plate size 8 cm x10 cm) was prepared for the analysis of protein patterns by SDS-PAGE. For gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed with high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by Pasture pipette leaving sufficient space for comb in the stacking gel (comb + 1cm).

Composition of solutions

10% resolving gel

Name of the compound	Amount (ml)
Distilled water	2.85
30 % acrylamide	2.55
1.5M Tris (pH 8.8)	1.95
10 % SDS	0.075
10 % APS	0.075
TEMED	0.003

5 % stacking gel

Name of the compound	Amount (ml)
Distilled water	2.10
30 % acrylamide	0.50
1.5M Tris (pH 6.8)	0.38
10 % SDS	0.030
10 % APS	0.030
TEMED	0.003

After pouring the resolving gel solution, it was immediately overlayered with isobutanol and kept for polymerization for 2 hrs. After polymerization of the

resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and overlayered with water. Finally the gel was kept for polymerization for 30-45 min. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris- Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

3.8.3.3. Sample Preparation

Sample (50 µl) was prepared by mixing the sample protein (35 µl) with 1X SDS gel loading buffer (15 µl) in cyclomixer. All the samples were floated in boiling water bath for 3 min to denature the protein sample. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a microliter syringe. Along with the samples, protein markers consisting of a mixture of eleven proteins ranging from high to low molecular masses (Phosphorylase b-97; Fructose-6- phosphate kinase- 84; Bovine Serum Albumin-66; Glutamic dehydrogenase-55; Ovalbumin-45; Glyceraldehyde-3-phosphate dehydrogenase-35; Carbonic anhydrase- 29; Trypsinogen-24; Trypsin inhibitor-20; α Lactalbumin- 14.2 and Aprotin 8.5 kDa) was treated as the other sample and loaded in a separate well.

3.8.3.4. Electrophoresis

Electrophoresis was performed at a constant 15 mA current for a period of three hours until the dye front reached the bottom of the gel.

3.8.3.5. Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: water (10:20:70) for overnight. The gel was removed from the fixer and stained in Coomassie blue stain for 4 hrs at 37°C with constant shaking at low speed. The staining solution was prepared by dissolving 250 mg of Coomassie

brilliant blue (Sigma R 250) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No. 1 filter paper.

After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5: 4.5: 1) at 40°C with constant shaking until the background became clear.

3.9. Extraction of enzymes from seedlings

3.9.1. Peroxidase (POX; EC.1.11.1.7)

For the extraction of POX the plant tissues were ground to powder in liquid nitrogen and extracted in 0.1 M Sodium borate buffer (pH 8.8) containing 2 mM β mercaptoethanol under ice cold conditions. The homogenate was centrifuged immediately at 15000 rpm for 20 min at 4°C. After centrifugation the supernatant was collected and after recording its volume was used immediately for assay or stored at -20°C (Chakraborty *et al.*, 1993).

3.9.2. Ascorbate peroxidase (APOX; EC. 1.11.1.11)

APOX enzyme extract was prepared by powdering the tissues in liquid nitrogen and extracting in 0.05 M Na_2HPO_4/NaH_2PO_4 buffer (pH 6.9) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1mM phenylmethanesulphonylfluoride (PMSF) and 2mM polyvinylpyrrolidone (PVP) following the method of Asada (1984). The homogenate was centrifuged at 4°C for 20 min at 15000 g. The supernatant obtained was used for enzyme assay and estimation of total soluble protein content.

3.9.3. Catalase (CAT; EC 1.11.1.6)

The green tissues were ground to powder in liquid nitrogen and extracted with 2 ml of 0.05 M sodium phosphate buffer (pH 6.8) following the method of Chance and Machly (1955). The homogenate was centrifuged at 4°C for 20 min at 15000 g. The supernatant was used for the assay of CAT and total soluble proteins.

3.9.4. Superoxide dismutase (SOD; EC 1.15.1.1)

The SOD enzyme extract was prepared by grinding the plant tissue in liquid nitrogen to powder form and extracting in 0.1 M Potassium phosphate buffer (pH 7.8) containing 1% (W/V) insoluble Polyvinylpyrrolidone (PVPP), 1mM EDTA and 1mM PMSF following the method of Dhindsa *et al.* (1981). Insoluble material was removed by centrifugation at 12,000 g for 15 min at 4°C.

3.9.5. Glutathione reductase (GR; EC 1.6.4.2)

The enzyme extract for GR was prepared by crushing the plant tissue in liquid nitrogen and extracting with 0.1 M Sodium Phosphate buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid (EDTA),1mM phenylmethanesulphonylfluoride (PMSF) and 20 mM polyvinylpyrrolidone (PVP) and activity was determined following the method of Lee and Lee (2000).

3.10. Assay of enzyme activities

3.10.1. Peroxidase

For determination of peroxidase activity, 100 µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1ml of 0.2 M Sodium phosphate buffer (pH 5.4), 100 µl of 4mM H_2O_2 ,100 µl of O-dianisidine (5 mg/ml methanol) and 1.7 ml of distilled water. Peroxidase activity was assayed spectrophotometrically in UV VIS spectrophotometer (DIGISPEC-200GL) at 460 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakraborty *et al.*,1993). Specific activity was expressed as the increase in absorbance at 460 nm/g tissue/min.

3.10.2. Ascorbate peroxidase

Ascorbate peroxidase activity was assayed as decrease in absorbance by monitoring the oxidation of ascorbate at 290 nm according to the method of Asada *et al.* (1984) with some modification. The reaction mixture consisted of 0.1 ml of

enzyme extract, 0.1 ml of 0.5 mM ascorbic acid, 0.1 ml of H_2O_2 and 2.7 ml of sodium phosphate buffer (pH 7.2). Enzyme activity was finally expressed as change (decrease) in absorbance (ΔA_{290}) mg protein $^{-1}$ min $^{-1}$.

3.10.3. Catalase

Catalase activity was measured according to Chance and Machly (1955). Enzyme extract (20 µl) was added to 3 ml of H_2O_2- phosphate buffer (0.16 ml of H_2O_2 to 100 ml of phosphate buffer, pH 7.0) and the breakdown of H_2O_2 was measured at 240 nm in a spectrophotometer. An equivalent amount of buffer containing H_2O_2 was used as reference. The enzyme activity was expressed as enzyme units mg protein^{-1} where one enzyme unit was defined as a change of 0.01 absorbance min^{-1} caused by the enzyme aliquot.

3.10.4. Superoxide dismutase

Superoxide dismutase activity was assayed by monitoring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) according to the method of Dhindsa *et al.*, (1981) with some modification. Each 3 ml of the assay mixture constituted of 0.1 ml enzyme extract, 1.5 ml phosphate buffer (0.1 M, pH 7.8), 0.1 ml Na_2CO_3 (1.5 M), 0.1 ml NBT (2.25 mM), 0.2 ml methionine (200 mM), 0.1 ml EDTA (3 mM), 0.1 ml riboflavin (60 µM) and 0.8 ml of distilled water. The reaction tubes containing enzyme samples were illuminated with 15 W fluorescent lamp for 10 min. The other set of tubes lacking enzymes were also illuminated and served as control. A non-irradiated complete reaction mixture served as blank. The absorbances of samples were measured at 560 nm and 1 unit of activity was defined as the amount of enzyme required to inhibit 50% of the NBT reduction rate in the controls containing no enzymes.

3.10.5. Glutathione reductase

Glutathione reductase activity was determined by the oxidation of NADPH at 340 nm with extinction coefficient of 6.2 mMcm^{-1} as described by Lee and Lee

(2000).The reaction mixture consisted of 100 mM potassium phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH, 0.5 mM glutathione (oxidized form, GSSG) with an appropriate volume of enzyme extract. The reaction was initiated by the addition of NADPH at 25°C. Enzyme activity was finally expressed as μM min^{-1} mg $protein^{-1}$.

3.11. Isozymes analysis by polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) using 8% resolving gel and 5% stacking gel in Tris-glycine buffer (pH 8.3) was performed for isozyme analysis of different enzymes following the method of Davis (1964). The various solutions required for the analysis were prepared as follows:

Preparation of the stock solution

Solution A: Acrylamide stock solution (Resolving gel)

For the preparation of acrylamide stock solution for resolving gel 28 g of acrylamide and 0.74 g of N' N' methelene bisacrylamide was dissolved in 100 ml of warm distilled water. The stock solution was filtered with Whatman No. 1 filter paper and stored at 4°C in dark bottle.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10 g of acrylamide and 2.5 g of bis- acrylamide was dissolved in 100 ml of warm distilled water. The stock solution was filtered and stored at 4°C in dark bottle.

Solution C: Tris- HCl (Resolving gel)

36.6 g of Tris base was mixed with distilled water and 0.25 ml of TEMED was added. The pH was adjusted to 8.9 with conc. HCL. The volume of the solution was made up to 100 ml with distilled water. The solution was then stored at 4°C for further use.

Solution D: Tris- HCl (Stacking gel)

5.98 g of Tris base was mixed with distilled water and 0.46 ml of TEMED and the pH was adjusted to 6.7 with conc. HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4°C for further use.

Solution E: Ammoniumpersulphate solution (APS)

Fresh soution of APS was prepared by dissolving 0.15 g of APS in 10 ml of distilled water.

Solution F: Riboflavin solution

Fresh solution of Riboflavin was prepared by dissolving 0.4 mg of riboflavin in 10 ml distilled water. The solution was kept in dark bottle to protect from light.

Solution G: Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6 g of Tris base and 2.9 g Glycine in 1 L of distilled water.

Preparation of gel

For the polyacrylamide gel electrophoresis of peroxidase isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5 mm thick spacers were placed between the glass plates on three sides and these were sealed with high high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5 % resolving gel was prepared by mixing solution A: C: E: distilled water in the ratio of 1:1: 4:1 by pasture pipette leaving sufficient space for (comb + 1 cm) the stacking gel. This resolving gel was immediately over layered with water and kept for polymerization for 2 hrs. After polymerization of the resolving gel was complete, over layer was poured off and washed with water to remove any unpolymerized acrylamide. The stacking gel solution was prepared by mixing solutions B: D: F: distilled water in the ratio of 2:1:1:4.

Stacking gel solution was poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 min in strong sunlight. After polymerization of the

stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoresis apparatus. Tris-Glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

Sample Preparation

Sample (32 µl) was prepared by mixing the sample enzyme (20 µl) with gel loading dye (40 % sucrose and 1 % bromophenol blue in distilled water) in cyclomixer in ice. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe.

Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of 3-4 hrs at 4°C until the dye font reached the bottom of the gel.

Fixing and Staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained using suitable staining dye.

3.11.1. Peroxidase

Extract for peroxidase isozyme analysis was prepared by grinding 1 g of plant tissue in liquid nitrogen in pre-chilled mortar and pestle and finally extracting in 0.1 M Sodium phosphate buffer (pH 7.0) as described by Davis (1964). Peroxidase isozyme pattern was estimated by staining the gel in Benzidine dye in acetic acid-water mixture consisting of Benzidine (2.08 g), Acetic acid (18 ml), 3 % H_2O_2 (100 ml) for 5 min followed by 15 min incubation in 0.1mM H_2O_2 with gentle shaking (Reddy and Gasber.1973). The reaction was finally stopped with 7% acetic acid after

the appearance of clear blue coloured bands. Analysis of isozyme was done immediately.

3.11.2. Catalase

Extract for isozyme analysis was prepared by crushing 1 g of leaf tissue in a pre-chilled mortar and pestle in 0.1 M potassium phosphate buffer (pH 7.0) on ice as described by Woodbury *et al.* (1971) and immediately used for the isozyme analysis.

After electrophoresis the gel was soaked in 3.3 mM H_2O_2 for about 20 min. The gel was then rinsed in water and incubated in a freshly mixed solution consisting equal volumes of 1% potassium ferricyanide and 1% ferric chloride for about 20 min. Analysis of the isozymes was done immediately and R_m values for different isozymes were calculated.

3.12. Extraction and quantification of non-enzymatic antioxidants

3.12.1. Ascorbate

Quantification of ascorbate was done following the method of Mukherjee and Choudhuri (1983). The seedlings were homogenized in a cold mortar placed on ice using 10 ml of 6% trichloroacetic acid. To 4.0 ml of the extract, 2.0 ml of 2% dinitrophenylhydrazine (in acidic medium) and 1 drop of 10% thiourea (in 70% ethanol) were added. The mixture was then kept in a boiling water bath for 15 min and after cooling at room temperature 5 ml of 80% (v/v) H_2SO_4 was added to the mixture at 0°C. The absorbance at 530 nm was recorded. The concentration of ascorbate was calculated from a standard curve plotted with known concentration of ascorbic acid.

3.12.2. Carotenoids

Carotenoids were extracted and estimated according to the method given by Lichtenthaler (1987). 1g of the plant sample was homogenized in methanol for the extraction of carotenoids. After extraction the O.D. value was observed at 480 nm in a UV-VIS spectrophotometer and the carotenoid content was calculated using the following standard formula.

$A_{480} - (0.114 \times A_{663}) - 0.638\ (A_{645})\ \mu g\ g^{-1}$ fresh weight

3.13. Determination of peroxidation of membrane lipids

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content as described by Dhindsa *et al.*(1981).1 ml of supernatant of leaf extracts was mixed with 4mL of 20% (v/v) trichloroacetic acid containing 0.5% (v/v) thiobarbituric acid. The mixture was heated at 100°C for 30 min, quickly cooled, and then centrifuged at 10000 g for 10 min. The absorbance of the supernatant was read at 532 nm and 600 nm. The concentration of MDA was calculated by means of an extinction coefficient of 155 $mM^{-1}cm^{-1}$ (Heath and Packer 1968).

3.14. Determination of cell membrane thermostability

Membrane thermostability was tested by cell membrane stability (CMS) test with the pinnules obtained from seedlings following the method of Martineau *et al.* (1979).Leaf tissues were washed with 3-4 changes of distilled water and placed in test tubes (150×25 mm) containing 2 ml of pre-heated (to the treatment temperature) water. Tubes were covered with plastic wrap and placed in a water bath at the desired temperature for 15 min, while the control tubes were kept at 25°C. After cooling to room temperature, distilled water was added to make up the final volume of 10 ml. Samples were incubated at 10°C for 16 hrs and conductivity measured with a conductance meter (EI Model 181 E). The tubes were covered with aluminum foil and autoclaved at 120°C for 15 min. to release all electrolytes. After cooling tubes to 25°C. the contents were mixed and final conductance measured. The injury was determined as follows:

$$\text{Relative Injury [RI] (\%)} = \{1-[1-(T_1/T_2)] / [1-(C_1/C_2)]\} \times 100$$

where T and C refer to the conductance in treatment and control tubes and subscripts 1 and 2 refer to readings before and after autoclaving respectively.

3.15. Extraction and estimation of chlorophyll

Chlorophyll was extracted according to the method of Harbone (1973) by homogenizing 1 g of leaf sample in 80% acetone and filtering through Whatman No.1 filter paper 80% acetone was repeatedly added from the top till the residue

became colourless. The filtrate was collected and the total volume was made up to 25 ml. The chlorophyll content was estimated by observing the O.D. values at 645 nm and 663 nm respectively in a UV-VIS spectrophotometer (DIGISPEC-200GL) and calculation was done using the following formulae (Arnon,1949).

Total chlorophyll: $(20.2\ A_{645} + 8.02\ A_{663})$ mg g^{-1} fresh weight

Chlorophyll a: $(12.7\ A_{663} - 2.69\ A_{645})$ mg g^{-1} fresh weight

Chlorophyll b: $(22.9\ A_{645} - 4.68\ A_{663})$ mg g^{-1} fresh weight

3.16. Determination of Hill activity

3.16.1. Extraction

1 g of fresh weight of pinnules kept in a tray filled with ice water were macerated by mortar and pestle with 5 ml of chilled suspension medium (pH 7.6) consisting of 20 ml (1 M) Sorbitol, 15 ml (0.1 M) K_2HPO_4, 12 ml (25 mM) sodium EDTA, 12 ml (10 m M) $NaHCO_3$ and 1 ml distilled water (Trebst, 1972). Extract was filtered through muslin cloth in an ice-cold beaker. After centrifuging the filtrate at 3000 rpm for 10 min, the supernatant was decanted and pellet was resuspended in 3 ml suspension medium and stored at 10°C.

3.16.2. Assay of chloroplast activity by Hill reation

2.8 ml of assay medium and 0.1 ml of chloroplast suspension was taken into cuvette of spectrophotometer. 0.1 ml of DCPIP (2,6, dichlorophenol indophenol) dye was added. The cuvette was inverted once for mixing. Absorbance was determined immediately after mixing in dark at 600 nm representing the starting point. Next the cuvette was exposed to light source for 5 min. The contents were mixed by inverting the tube once and absorbance was taken. Hill activity was calculated in terms of μM of DCPIP reduced per minute per mg of chloroplast (μM of DCPIP min^{-1} mg^{-1} chloroplast) following the method of Trebst (1972).

3.17. Extraction and estimation of free proline

3.17.1. Extraction

For the extraction of free proline the method of Bates *et al.*(1973) was followed. 1 g of plant tissue was crushed with 5ml of 3% sulfosalicylic acid in mortar with pestle.The slurry was filtered through Whatman No.1 filter paper at room temperature in dark condition. The supernatant was collected and stored at 4°C for further analysis.

3.17.2. Estimation

Proline content of the extract was estimated as described by Bates *et al.* (1973) with some modification. To 1ml of extract, 3 ml of distilled water and 1 ml of Ninhydrin solution (2 g in 50 ml of acetone and water mixture) were added. Then the mixtures were kept on a boiling water bath for 15 min. After cooling, the reaction mixture was poured in a separating funnel and 5 ml of toluene was added and mixed vigorously. Lower colored layer was taken and O.D.values were measured at 520 nm.Quantification was done from a standard curve of proline.

3.18. Extraction and estimation of total and reducing sugar

3.18.1. Extraction

For extraction of sugar, method of Harbone (1973) was followed. Fresh tissues were crushed with 95% ethanol and filtered. Then the alcoholic fractions of the filtrate were evaporated off on a boiling water bath. The aqueous fractions were centrifuged in a table centrifuge and the supernatants were collected. Finally the volume was made up with distilled water.

3.18.2. Estimation

Estimation of total sugar was done following Anthrone's method as described by Plummer (1978). To 1 ml of test solution 4 ml of Anthrone's reagent was added and mixed thoroughly. Mixtures were placed in a boiling water bath for

10 min. The reaction mixture was then cooled under running tap water. Absorbance was measured at 570 nm in a colorimeter. Total sugar content was then calculated from the standard curve of dextrose solution

Somogyi's method as described by Plummer (1978) was followed for the estimation of reducing sugar. 1ml alkaline Cu-tartrate solution (prepared by dissolving 4 g $CuSO_4 .5H_2O$, 24 g of anhydrous Na_2CO_3, 16 g of sodium potassium tartrate and 180 g of anhydrous Na_2SO_4 in distilled water and making up the final volume to 1L) was added to 1 ml of test solution. Reaction mixture was then allowed to boil in a water bath for 15 min. The mixture was then cooled under running tap water and to each reaction mixture 1ml of Nelson's arseno molybdate reagent was added. Reaction mixtures were diluted to 5 ml by adding 2 ml more distilled water and O.D. values were measured in a colorimeter at 540 nm. The concentration of reducing sugar was determined by plotting the O.D. values on the standard curve of dextrose solution.

3.19. Extraction of phenolic compounds from seedlings and HPLC analysis

Phenolic compounds from freshly harvested plant tissues of different genotypes were extracted in ethanol-water following the method of Sarma *et al.* (2002).1 g of tissue was macerated using a pestle and mortar followed by suspension of finely crushed samples in 5 ml of ethanol-water (80:20 v/v). Samples were collected in screw-capped tubes and the suspension was subjected to ultrasonication (Sonics Vibra cell) at 60% duty cycles for 15 min at 40°C followed by centrifugation at 7500 rpm. for 15 min. The clear greenish supernatant was then subjected to charcoal treatment to remove pigments in each sample and transferred to glass tubes after filtering through Whatman filter paer No.1. The residue was re-extracted twice and the supernatant was pooled prior to evaporation under vacuum. Dried samples were re-suspended in 1 ml HPLC grade methanol by vortexing and stored at 4°C for further analysis.

High performance liquid chromatography (HPLC) of the samples were performed according to Sarma *et al.* (2002). The HPLC system (Shimadzu Advanced VP Binary Gradient) used for sample analysis was equipped with C-18 hypersil column. Reverse phase chromatographic analysis was carried out using this C-18

reverse phase HPLC column at 25°C under isocratic conditions where the concentration of mobile phase was constant throughout the run. Running conditions included an injection volume of 20 µl, mobile phase methanol- 0.4% acetic acid (80:20 v/v), flow rate 1ml min^{-1}, attenuation 0.02 and detection at 290 nm. Samples were filtered through a membrane filter (pore size 0.45 µm) prior to injection in the sample loop. Phenolic compounds and salicylic acid present in the samples were identified by comparing retention time (Rt) of standards and coinjection.

3.20. *In vitro* callus formation

For *in vitro* callus formation, seeds were washed with teepole and were surface sterilized with 0.1% mercuric chloride for 10 min and soaked overnight in sterilized distilled water. Seeds were cultured asceptically (Plate V) on germination medium having sucrose (2.0%) and agar (0.8%) as suggested by Batra *et al.* (2004).

Ten-day old *in vitro* grown seedlings were used for excision of explants i.e. shoot tip, cotyledonary node, internode and hypocotyls for callus initiation (Plate VI). A number of medium having MS salts (Murashige and Skoog, 1962) supplemented with 30 g/L sucrose, 8 g/L agar and B_5 vitamins (Gamborg *et al.*, 1968) with varying concentrations and combinations of cytokinins, auxins and growth regulators (NAA+BAP, NAA+Kinetin and IAA+BAP) were used for initiation of callus from different explants. MS media used were supplemented with 1 mg/L and 1.5 mg/L of NAA, 0.5 mg/L of BAP and 1.5 and 2.0 mg/L of kinetin. The pH of the medium was adjusted to 5.8 and autoclaved at 15 psi for 15 min. The cultures were incubated under 70 µE $m^{-2}s^{-1}$ incandescent light in a photoperiod cycle of 16 hrs light/8 hrs dark at 25 ± 1°C (Roy *et al.*, 2001). The callus was subcultured three times at 15 days interval in MS medium with different combinations of growth regulators for regeneration.

3.21. Treatment of calli

Calli (20 days old) raised from different plant explants were subjected to two different pre-treatments followed by lethal temperature treatment of 40°C for 2 hrs. First pre-treatment involved heat-acclimation treatment of 35°C for 2 hrs followed

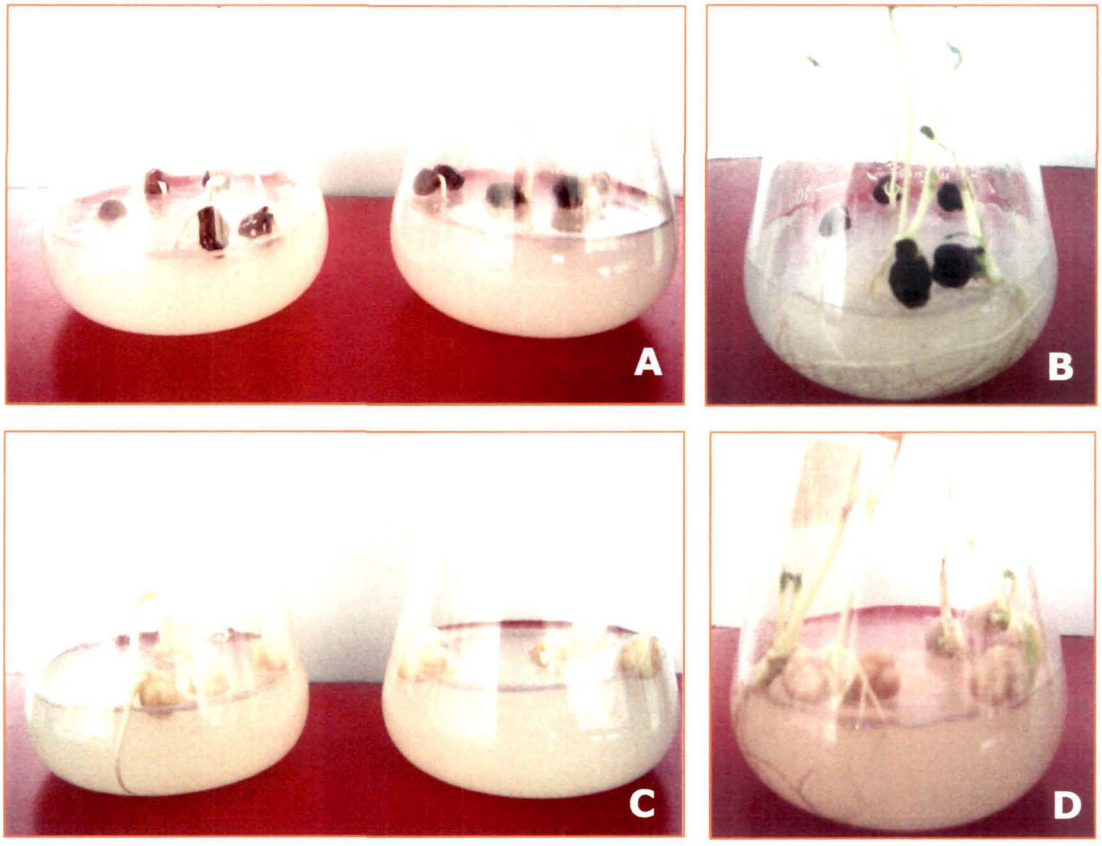


Plate V (A-D) : Aseptic germination of chickpea seeds of two different genotypes for callus culture **A & B:** ICC C37 (Kranthi). **C& D:** ICC 7344

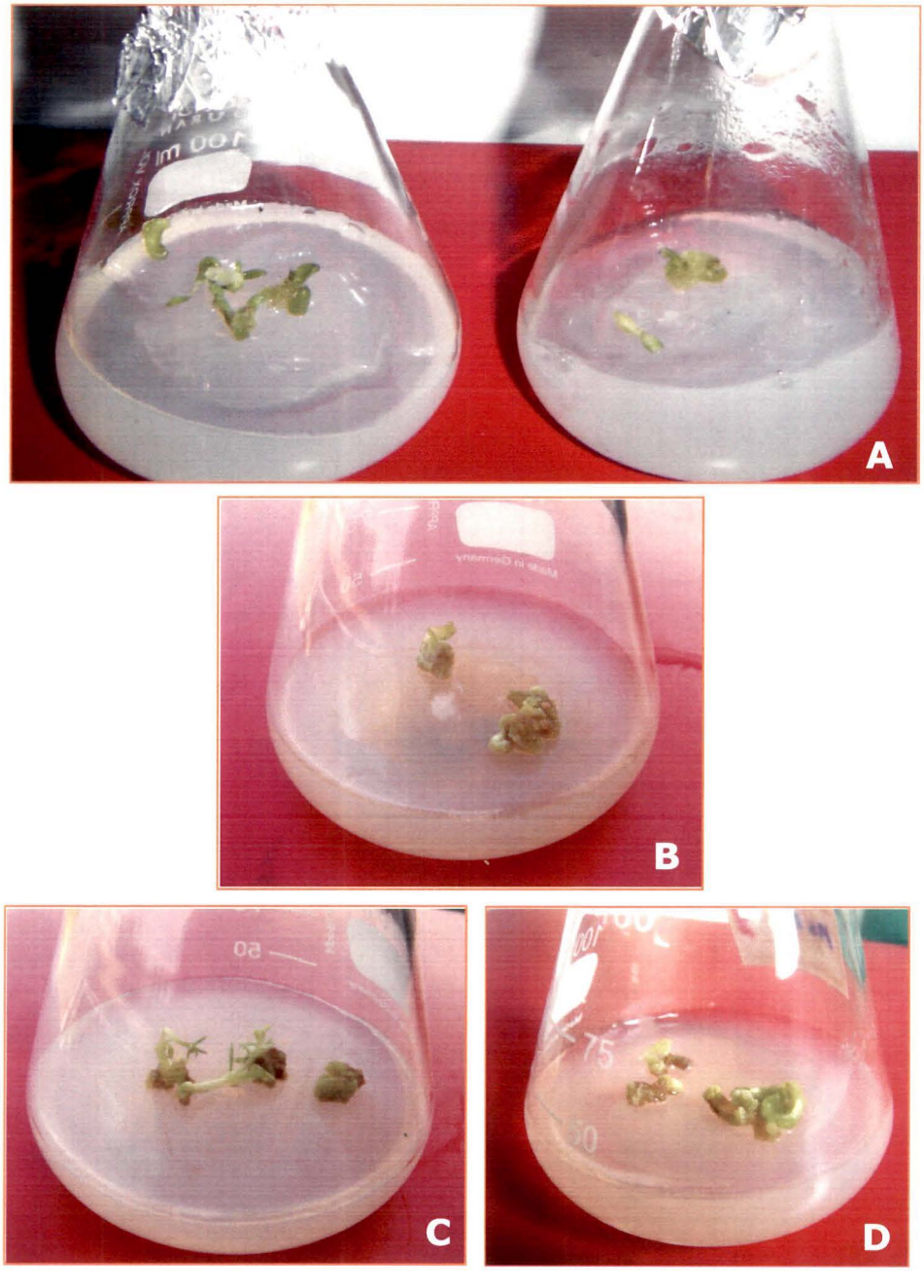


Plate VI (A-D): Induction of callus formation from different plant parts. **A:** shoot tip; **B & D:** internode and **C:** shoot tip with a portion of leaf

by challenge with a lethal temperature for the same duration. The second pre-treatment involved growing of calli in MS media supplemented with 10^{-5} and 10^{-6} M salicylic acid (SA) and subjecting the calli to lethal temperature treatment. The calli subjected to two different pre-treatments following exposure to lethal temperature were returned to normal conditions and allowed to grow for 60 days. The changes in growth of the calli following lethal temperature treatment were recorded regularly till 60 days at 10 days time interval by checking the weights of calli. Experiments were carried out in multiple sets and calli once used were discarded after weighing.