MATERIAL AND METHODS

The present studies were undertaken as per the objectives stated earlier and were accomplished in the following experiments:

Experiment I: Assessment of relative sensitivity of the Desi and Kabuli genotypes to chilling stress (5-20°C) at the germination and seedling stage in comparison to the controlled condition (Room temperature, 25-28°C).

Experiment II: Comparative evaluation of Desi and Kabuli genotypes under contrasting temperature conditions and effects on Reproductive Biology.

Experiment III: Investigating the Role of cryoprotectants, (Polyamines- Spermine and Putrescine), compatible solutes (Glycine Betaine and Proline) and growth Hormone (Abscisic Acid) at the Reproductive stage.

Experiment IV: Probing the biochemical basis of cold sensitivity under contrasting conditions at various stages in both the genotypes.

3.1. EXPERIMENT I

Chickpea (*Cicer arietinum* L.; GPF-2 (Desi), L-550 (Kabuli) genotypes were procured from Panjab Agricultural University, Ludhiana. Only healthy seeds were selected for the experimental work. The seeds were surface sterilized by 0.1% mercuric chloride for two minutes and
washed thoroughly with distilled water. The genotypes were raised under the range of low temperatures (5-20°C) and at room temperature (25 ± 3°C) in distilled water (Control) to assess for germination and seedling growth. These were followed for early vegetative growth under control conditions to find out the relative sensitivity of various stages to chilling stress. Involvement of various cryoprotectants (ABA, polyamines, praline and Glycine betaine) were examined in these studies by using certain traits of chilling injury like membrane damage, mitochondrial stability (Triphenyl tetrazolium chloride reduction activity), pigment changes (reduction or accumulation). Observations were recorded on daily and weekly basis. The subsequent growth and other parameters were recorded by keeping the seeds at different temperatures 5°C, 10°C, 15°C, 20°C and at room temperature i.e. (25 ± 3°C; control). The seeds were grown in distilled water (control) as well as in various concentrations of cryoprotectants (Polyamines, Proline and Glycine betaine); 1μM, 2.5μM, 5μM and 10μM and ABA; 0.25μM, 0.50μM, 0.75μM and 1μM. Some preliminary tests were performed to observe the concentrations imparting beneficial and protective effects in both the genotypes at seedling stage. Preliminary parameters were as follows:-

- Time to initiate germination
- Days to 50% germination
- Days to 100% germination
- Total germination
- Root growth
- Shoot growth
- Electrolyte leakage (of hypocotyls)
- TTC test (of hypocotyls)
- Chlorophyll content
Material and methods

- Carotenoid content
- Fresh weight/Dry weight

This experiment provided us with the information about the relative sensitivity of early growth stages to chilling conditions. A general picture about the effectiveness of various concentrations of cryoprotectants provided us with the valuable results.

3.1.1. GROWTH

Growth rate (cm/day) for roots and shoots was calculated as follows:

\[
\ln L_2 - \ln L_1 / T_2 - T_1
\]

\( \ln = \) Natural log of initial (L1) and final (L2) length
\( T_1 = \) Initial time and
\( T_2 = \) Final time

3.1.2. STRESS INJURY AS AN ELECTROLYTE LEAKAGE

Electrolyte leakage was measured as described by Premachandra et al. (1990) using hypocotyls (0.1g) at all the temperature and concentration ranges in both the genotypes at seedling stage. Samples were washed thrice with deionised water to remove surface adhered electrolytes. Hypocotyl discs were placed in closed vials containing 10ml of deionised water and incubated at 40°C for 30 min. After the expiry of period, their electrical conductivity was recorded by conductivity meter (C₁) subsequently. The same samples were placed in boiling water bath at 100°C for 10 min. and their electrical conductivity was also recorded (C₂). The EL was defined as follows:

\[
\text{EL} \% = \left( \frac{L_1}{L_2} \right) \times 100.
\]
3.1.3. CHLOROPHYLL AND CAROTENOID CONTENT

Chlorophyll content as Chl. a and Chl. b was measured at seedling developmental stage. The chlorophyll was extracted with 80% acetone repeatedly to ensure complete extraction and the extract filtered using Whatman filter paper. The absorbance of the supernatant was read at 645nm, 665nm along with 440nm for carotenoid content against 80% acetone solvent (Arnon, 1949). The amount of chlorophyll and carotenoids present (mg/gm FW) in the extract was calculated according to the following equations stated below:-

\[
\text{Chl a.} = 12.9 \left( \text{Abs}_{665} \right) - 2.69 \left( \text{Abs}_{645} \right) \frac{V}{1000 \times W}
\]

\[
\text{Chl b.} = 22.9 \left( \text{Abs}_{645} \right) - 4.68 \left( \text{Abs}_{663} \right) \frac{V}{1000 \times W}
\]

\[
\text{Total chlorophyll} = 20.2 \left( \text{Abs}_{645} \right) + 8.02 \left( \text{Abs}_{663} \right) \frac{V}{1000 \times W}
\]

\[
\text{Carotenoids} = 4.69 \left( \text{Abs}_{440} \right) - 0.268 \left( 20.2 \text{Abs}_{645} + 8.02 \text{Abs}_{663} \right) \frac{V}{1000 \times W}
\]

Where

Abs = Absorbance in nm.

V = The final volume of the chlorophyll extract.

W = Fresh weight in grams of the tissue used.

3.1.4. TTC TEST (TRIPHENYL TETRAZOLIUM CHLORIDE REDUCTION ASSAY)

This method was based on that of Steponkus and Lanphear
Material and methods

(1967) except for the use of citrate which was added to the solution. The sample used to perform the test were hypocotyl of both the genotypes. The discs were washed thrice with deionised water and blotted with filter paper. For each treatment 4 replicates were used two replicates were incubated for 2 min in 1 ml of distilled water at 95°C (Heated discs). All the 4 replicates (Heated and Non-heated) were then placed in glass tubes containing 4 ml of 0.1M \(K_2HPO_4-KH_2PO_4\) buffer (pH 7) with 0.5% (w/v) TTC. The discs were incubated for 20 hours at 28°C in darkness. The TTC solution was drained. The discs were washed twice and 2 ml of ethanol was added. The tubes were kept in a water bath at 95°C until complete evaporation of the ethanol. 4 ml of ethanol was added again and tubes were vigorously shaken. The absorbance due to the formazon was recorded against the ethanol.

Reagents

(i) Phosphate Buffer : 0.1M pH 7
(ii) TTC : 0.5%
(iii) Ethanol : 100%

3.2. EXPERIMENT II

A precise data on temperature and other weather parameters was recorded for characterization of stress environment (Fig. 2.1). The seeds were inculcated with \textit{Rhizobium} culture and sowing was done in the last week of October in earthen pots. The current experiment was conducted with the following sets of observations. The plants were raised in earthen pots under field (FD-Plants) and glass house conditions (GH-Plants). To examine the low temperature impacts at flowering stage, plants were shifted to field conditions from glass house (GH-FD Plants) when they initiated around 25% flowering (field-
transferred plants). The plants were irrigated as and when required. A selective number of plants were tagged for observations on floral biology and pod set. Data were recorded on the following aspects.

3.2.1. GROWTH STUDIES

Following observations were carried out on both genotypes under above mentioned 3 conditions (Glass house, field-grown and Field-transferred plants) with 9 replicates (3 plants/pot).

3.2.1.1. Growth and Phenology

- Days to emergence
- Number of branches
- Height at 60-days interval

After 60-days the following observations were recorded under all the three conditions:

- Days to initiation of flowering
- Days to pod formation.
- Days to pod maturity

After maturity the plants were uprooted for the following observations:-

- Root length
- Shoot length
- No. of primary branches

3.2.1.2. Flower observations per plant

- Total number of flowers produced per plant.
- Total number of Abscised/retained flowers per plant.
- Number of flowers converted into pod (Pod set).
3.2.1.3 Pod observation per plant
- Total number of pods formed per plant
- Total number of abscised/retained pods per plant

3.2.1.4 Yield parameters
- Root weight (grams)
- Shoot weight (grams)
- Total no. of pods (including 1-, 2-, 3- and empty pods)
- Total pod weight (grams)
- Total seed weight (grams)
- Pod dimensions (millimeters)

The main emphasis was given on reproductive stage to record the variation in flower formation, floral biology, flower functioning and reproductive biology during the coldest period of season. The parameters studied were:

The plants were observed for following in different months:

3.2.2. FLOWER DEVELOPMENT
3.2.2.1. Morphology
- Size and shape of flowers
- Pigmentation changes (qualitative)
- Abnormal flower development (qualitative)

3.2.3. FLORAL BIOLOGY

The comparison of gamete performance was made on the basis of following parameters in both the genotypes under all the growth situations.
3.2.3.1. Pollen functioning

(a) Pollen viability

Pollen viability (%) was tested on pollen grains with 0.5% acetocarmine or Alexander triple stain (ATS) solution (Alexander, 1969).

(b) Pollen load

The no. of flowers with fully dehiscent anthers and pollens on stigma were counted and pollen-load on stigma was scored on 1-5 scale (1-low and 5-high) (Srinivasan et al., 1999).

(c) Pollen Germination percentage (in vitro)

Pollen germination was assessed (Brewbaker and Kwack, 1963) after incubating pollens in a medium (pH 6.5) containing.

i. 10% Sucrose
ii. 100 ppm Boric acid
iii. 300 ppm Calcium nitrate
iv. 200 ppm Magnesium sulphate and
v. 100 ppm Potassium nitrate

Incubation was done at 25 ± 2°C for overnight (dark) in medium. The in vitro germination process was stopped after incubation by adding a drop of acetocarmine to the medium pollen was counted as germinated when pollen tubes were at least to the diameter of the pollen grain. The percentage germination was determined on the basis of at least 100 pollen grains per replicate.

(d) Pollen germination (in vivo) and fate of pollen tube growth

Fluorescence microscopy was done to assess the pollen germination on stigma and to trace the pollen tube path in style and ovary. Flowers were collected at one to three days after anthesis and fixed in acetic alcohol (1:3) for 24 hr. and then transferred to 8N NaOH.
for 6 hr at 60°C for clearing purpose. Complete gynoecium part was transferred to aniline blue (0.1%) and kept overnight. Finally they are mounted on a slide in a 1 : 1 (aniline blue : 10% glycerin) solution. The stained gynoecia were then observed under fluorescence photomicrograph microscope (Nikon, Japan) (Dumas and Knox, 1983).

(e) Pollen morphology

Scanning electron microscopy was carried out to observe any structural changes on pollen. On the day of anthesis, fresh flowers were collected early in the morning from unstressed and stressed plants. Anthers were collected and teased on a metallic stub. Remaining extra debris were removed and these stubs were gold plated and scanned under scanning electron microscope (Postek et al., 1980).

3.2.3.2. Stigma Receptivity Test

Esterase test using α-naphthyl acetate as substrate in the azo-coupling reaction with fast blue B as modified by Mattson et al. (1974) was used for detecting stigma receptivity. Stigmas were removed one day before flower opening, immersed in the working solution, at 37°C for 15 min. A positive test was indicated with purple red to brown colour. Staining intensity was scored on a 1-5 scale.

Working solution

(i) α-naphthyl acetate - 1 ml
(ii) Phosphate buffer 0.1 M, pH 7.0 - 99 ml
(iii) Sucrose (as osmoticum) - 10-15%
(iv) Fast Blue B - 100 mg

Control : Working solution without substrate (α-naphthyl acetate)

3.2.3.3 Ovule viability test (TTC test)

This method was based on that of Steponkus and Lanphear
Material and methods

(1967) except for the use of citrate which was added to the solution. The sample used to perform the test were ovules taken out from the flowers (3-days after anthesis). The ovules were taken in 4 replicates with 2 ovules/replicates. All the replicates were then placed in small glass tubes containing 3 ml of 0.1M K$_2$HPO$_4$-KH$_2$PO$_4$ buffer (pH 7) with 0.5% (w/v) TTC. The ovules were incubated for 20 hr at 28°C in darkness. The TTC solution was drained. The ovules were washed twice and 2 ml of ethanol was added. The tubes were kept in a water bath at 95°C until complete evaporation of the ethanol. 4 ml of ethanol was added again and tubes were vigorously shaken. The absorbance due to the formazan was recorded against the ethanol.

Reagents

(i) Phosphate Buffer : 0.1M pH 7
(ii) TTC : 0.5%
(iii) Ethanol : 100%

3.2.4. ANATOMICAL CHANGES (MICROSPOROGENESIS AND MEGASPOROGENESIS)

The main emphasis of these investigations was to study the post-pollination and fertilization events in unstressed and stressed conditions.

Buds and flowers (from the days of anthesis to 3 days after an anthesis) were collected especially in the morning hours. The plant material was fixed in F.A.A. (Formalin : Acetic acid : Alcohol) (1:1:18) for 24 hours and then transferred to long term storage in 70% alcohol. The stored material was subjected to dehydration and infiltration in tertiary butyl alcohol (TBA) series, erythrosine solution was an additive for distinction during section cutting.
Material and methods

TBA Series

<table>
<thead>
<tr>
<th>Grade</th>
<th>Ethyl alcohol 95%</th>
<th>Absolute Ethyl alcohol</th>
<th>TBA</th>
<th>Distilled water</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 ml</td>
<td>-</td>
<td>10 ml</td>
<td>40 ml</td>
</tr>
<tr>
<td>2</td>
<td>50 ml</td>
<td>--</td>
<td>20 ml</td>
<td>30 ml</td>
</tr>
<tr>
<td>3</td>
<td>50 ml</td>
<td>--</td>
<td>35 ml</td>
<td>15 ml</td>
</tr>
<tr>
<td>4</td>
<td>50 ml</td>
<td>--</td>
<td>50 ml</td>
<td>--</td>
</tr>
<tr>
<td>5</td>
<td>--</td>
<td>25 ml</td>
<td>75 ml</td>
<td>--</td>
</tr>
<tr>
<td>6</td>
<td>--</td>
<td>--</td>
<td>100 ml</td>
<td>--</td>
</tr>
</tbody>
</table>

After passing through TBA series, wax infiltration was done by adding molten paraffin wax to TBA final grade repeatedly till the TBA got gradually and completely replaced by molten wax. This process was handled at 60°C depending upon the melting point of paraffin wax. The replacements are assessed by the odour of the volatile TBA. Blocks of wax were made with metal angles and the material placed within proper orientation. Section cutting of these blocks fixed on a wooden block was done with spencer rotary microtome (American optical company). Sections (7-10μ thickness) were sliced and the wax ribbons so formed were fixed onto the slide by Haupt’s adhesive (boiling 10g gelatin in 100ml distilled water, cooled and 2.0gm phenol crystals added). The slides were subsequently gently heated for even and uniform stretching of material by adding 4% formalin.

Staining

The material was dewaxed when passed through xylene alcohol series. The sections were stained with safranin-fast green as follows (Johansen, 1940).

The dewaxed slides were passed through absolute alcohol grade
Material and methods

of 95%, 90%, 80%, 70%, 50% serially for 5 min each and stained in safranine (1% in 50% alcohol) for 4-6 hours and passed through 70%, 95% and absolute alcohol for 1 min. each before placing in clove oil. Basic stain was done in fast green [0.5 to 1% in clove oil (stock solution) further diluted to 10%] for 4-5 min. and rinsed in clove oil. The slides were cleared in xylene and rectified for 3-5 min. Finally double stained material was mounted on DPX and ovendried for 2-3 days. Photomicrographs were made after studying the slides under light microscope (olympus) under different magnifications 4x, 10x, 20x and 40x.

3.3. EXPERIMENT III

In this experiment, the plants of both the genotypes were subjected to following conditions:

1. Glass house (GH) grown (controls; warm conditions)
2. Field-transferred (GH-FD) plants at flowering stage
3. Field-growing (FD) plants

These plants were subjected to treatments with polyamines, compatible solutes and ABA. Each chemical was applied as 100µM concentration except ABA with 1µM concentration (along with tween 20) in the first week of January and the second spraying was done at the 15 day interval over the same plants. 9 Plants (3 pots/treatment) were tagged to observe the flower and yield traits. The plants were examined by using certain traits of chilling injury like membrane damage, mitochondrial stability, pigment changes (reduction or accumulation). Observations were recorded on weekly and monthly basis.

3.3.1. CHILLING INJURY WAS EXAMINED AFTER FIRST AND SECOND SPRAYING BY THE FOLLOWING TRAITS

i. Electrolyte Leakage (As stated earlier)
Material and methods

ii. TTC test (as stated earlier)

iii. Chlorophyll content (as stated earlier)

iv. Carotenoid content (as stated earlier)

v. Relative leaf water content (RLWC) (as explained later)

3.3.2 DURING THE FLOWERING AND POD STAGE, THE FOLLOWING OBSERVATIONS WERE TAKEN

i. Total flowers/plant

ii. Retained flowers/plant

iii. Aborted flowers/plant

iv. Total pods/plant

v. Retained pods/plant

vi. Aborted pods/plant

3.3.3 AFTER THE MATURATION OF PODS AND CROP MATURITY, THE PLANTS WERE UPROOTED AND WERE EXAMINED FOR FOLLOWING OBSERVATIONS

i. Shoot length (cms)

ii. Root length (cms)

iii. Plant height (cms)

iv. Shoot weight (g)

v. Root weight (g)

vi. Primary branches

vii. Total pods/plant

viii. Total pod weight
Material and methods

ix. Seed yield/plant (g)

x. One seeded pods/plant

xi. Two seeded pods/plant

xii. Three seeded pods/plant

xiii. Infertile pods/plant

xiv. Pod length (mm)

xv. Pod width (mm)

3.4. EXPERIMENT IV

Both the genotypes were selected for characterization of cold injury at bud, reproductive pod initiation, pod development and pod maturation stage for specific traits/ markers at sub cellular, cellular and whole plant level with particular emphasis on :

i) Flowering

ii) Pod and seed development

Observation were taken on :

3.4.1. Growth and yield (As described in Experiment III)

3.4.2. Analysis of various enzymes associated with carbohydrate metabolism, nitrogen metabolism, oxidative molecules antioxidants, sugars and cryoprotective solutes were examined in leaves at flowering stage and at pod development stage in glass house grown -plants, field-transferred plants and field-growing plants. At flowering stage the anthocyanin content was also examined in flowers in both the genotypes.

3.4.2.1. Stress injury as Electrolyte leakage (As described earlier)
3.4.2.2. TTC test (As described earlier)
3.4.2.3. Chlorophyll content (As described earlier)
3.4.2.4. Carotenoid content (As described earlier)
3.4.2.5. Relative leaf water content (%)
3.4.2.6. Anthocyanin content
3.4.2.7. Carbohydrate metabolism
3.4.2.8. Nitrogen metabolism
3.4.2.9. Oxidative molecules
3.4.2.10. Antioxidants

3.4.2.5. Relative leaf water content (%)

The relative leaf water content was measured at every stage during the period of stress according to Weatherley (1950). Leaf tissues (0.1g) were excised from the plant and their fresh weight was recorded. The leaves were immersed in distilled water for 2 hr. in petridish. The leaves were taken out and surface dried with blotters and weight (turgid weight) was recorded. Thereafter the leaves were oven dried for 24 hr. at 110°C in oven and weighed again (dry weight). The relative leaf water content was calculated as follows:

\[
\text{Relative leaf water content (RLWC \%)} = \frac{\text{Fresh wt} - \text{Dry wt}}{\text{Turgid wt} - \text{Dry wt}} \times 100
\]

3.4.2.6 Anthocyanin content

The tissues were homogenized in 5 ml. of methanol containing 1N HCl (9:1/Methanol : HCl) maintained at 4°C for 4 hr (Christe et al., 1994). The particulates were removed by centrifugation of the homogenate at 10,000 g for 30 min. The absorbance of the clear supernatant was read at 530 nm and expressed as per gram of tissue as described by Kho et al. (1977).

\[
A_{\text{anth}} = A_{530} - 2.2 A_{645}
\]
3.4.2.7. Carbohydrate Metabolism

The endogenous level of certain biomolecules associated with carbohydrate metabolism such as sugars, reducing sugars, starch were determined. The activity of certain enzymes associated carbohydrate metabolism i.e. like α-amylase, β-amylase, invertase and sucrose synthase was also assayed by following methods: The analysis was conducted in leaves at two stages-flowering and pod development. The samples were analysed immediately fresh for enzymes or dried in oven at 80°C for dry matter analysis.

3.4.2.7.1. Total sugars

Extraction

The oven dried plant material was homogenized in hot ethanol (80%) and centrifuged at 2000 rpm for 10 min. Supernatant was clearly decanted off. Three ml of ethanol (80%) was added to the residue and recentrifuged. The extraction was repeated twice to ensure the complete recovery of sugars. The residue was kept for further analysis of starch. The supernatant was pooled and evaporated to dryness in chinadish on a boiling water both. The residue was eluted with 5ml of 20% ethanol and subjected to analysis for total sugars reducing sugars and amino acids.

Analysis: Total Sugars were estimated according to the method described by Yemm and Willis (1954).

(i) Anthrone reagent: Anthrone reagent (0.2%) was prepared, in 95% chilled sulphuric acid. The reagent was allowed to stand for at least 30 min. with occasional stirring to make it clear. It was freshly prepared every time and used within 12 hrs.

(ii) Standard: Glucose solution in distilled water (10 mg/100 mg)
**Material and methods**

**Estimation:** To 4 ml of chilled anthrone reagent, 1 ml of ethanol extract was added. Tubes were shaken gently to mix the solution. These were then covered with glass marbles and immediately placed in boiling water bath for 10 min and then cooled in ice bath. The absorbance of blue green coloured solution was read at 625 nm in spectrophotometer (Chemito, Japan) against blank containing 80% ethanol. The concentration of total sugars (mg/g DW) was calculated from standard curve plated with known concentrations of glucose.

**3.4.2.7.2. Reducing Sugars**

The reducing sugars were estimated as per method of Sumner (1935).

**Reagents**

(i) **DNSA Reagent:** 1.25 g of dinitro-salicylic acid (DNSA) was dissolved in 25 ml distilled water. 2 g sodium hydroxide was added and dissolved. 37 g of sodium potassium tartrate was added thereafter to make the final volume 125 ml with distilled water.

(ii) **Standard:** Glucose solution in distilled water (10 mg/100 mg).

**Estimation:** To 1 ml of DNSA reagent, ethanol extract (1 ml), prepared as above was added. The reaction mixture was boiled for 12 minutes. 2 ml of distilled water was added and absorbance was recorded at 560 nm against a blank containing 80% ethanol in place of ethanol extract. The concentration of reducing sugars (mg/g DW) was calculated from a standard curve plotted with known concentration of glucose.

**3.4.2.7.3. Starch**

Starch content was measured by acid hydrolysis method given by McCreddy *et al.* (1950).
Reagents
i) Ethanol: 80%
ii) Perchloric acid: 52%
iii) Anthrone reagent: Dissolved 0.2 gm of anthrone in 100 ml cold 95% H₂SO₄ (sulphuric acid) and stored the reagent at 0°C but not for more than 2 days.
iv) Standard: Glucose solution in distilled water (10 mg/100 ml).

Extraction: The residue of ethanol extract (used in above estimations) washed with 80% ethanol to remove all the traces of soluble sugars.

To the residue, 5 ml of distilled water and 6.5 ml of 52% perchloric acid was added to extract the starch by placing the samples at 0°C for 20 minutes. The mixture was centrifuged and retained the extract. The process was repeated 3-4 times using fresh perchloric acid and diluted to final volume 100 ml.

Estimation: To 0.5 ml of diluted extract, 4.5 ml of distilled water was added followed by addition of 10 ml of cold anthrone sulfuric acid reagent in ice bath. The sample mixture was heated at 100°C for 8 min. and cooled rapidly to room temperature. The absorbance was measured at 630 nm. The final content of starch was calculated from a standard curve plotted with known concentration of glucose.

3.4.2.7.4. α-amylase

The enzyme activity was assayed by the method described by Shuster and Gifford (1962).

Reagents
i) Starch Solution: Dissolved 150 mg potato starch, 600 mg potassium dihydrogen phosphate (KH₂PO₄) and 20 ml of anhydrous. CaCl₂ and 100 ml of distilled water, boiled for one
Material and methods

minute, cooled, filtered and used as substrate. Starch substrate was prepared fresh every time.

ii) **Iodine solution**: It was prepared by dissolving 254 mg I₂ and 4 gms of KI (potassium iodine) in one litre of distilled water.

iii) **Extraction buffer**: 0.1 M phosphate buffer pH 7.0.

**Extraction**: Fresh plant material was homogenized in ice cold extraction buffer (0.1 M phosphate buffer pH 7.0). Centrifuged at 4°C at 10,000 rpm and supernatant was treated as enzyme extract.

**Estimation**: One ml of starch substrate was added to 0.5 ml of enzyme extract. At zero time 0.2 ml of aliquot was removed from this and added 3 ml of KI. The absorbance was recorded at 620nm. Then the reaction mixture left was incubated at 25°C. Then after every 30 min. removed the aliquot and repeated the colour developing process (violet blue). Blank was run simultaneously without having substrate. In control the enzyme extract was substituted with 0.5 ml of distilled water. The enzyme activity was expressed in terms of decrease in OD at 620 nm per unit-time (min.).

3.4.2.7.5. β-amylase

The enzyme activity was assayed described by Shuster and Gifford (1962). The reaction mixture was prepared by adding following constituents.

**Reagents**

i) **Starch solution**: 0.2% starch solution was prepared in 0.067 M phosphate buffer pH 6.0.

ii) **Di-nitro salicylic acid**: It was prepared by mixing 2.5 g of DNSA with 150 ml distilled water containing 4.0 g of sodium hydroxide, 75 g sodium potassium tartrate and total volume was made to 250 ml.
iii) **Standard**: Glucose solution

**Extraction**: Homogenization of fresh plant material was done in ice cold 0.067 M phosphate buffer pH 6.0. Centrifuged at 4°C at 10,000 rpm and supernatant treated as enzyme extract.

**Estimation**: Reaction mixture containing 0.2 ml enzyme extract and 1.0 ml of freshly prepared starch solution was incubated at 30°C for 1 hr. Reaction was terminated by adding 1 ml of DNSA. After that, tubes were kept in boiling water for 10 min. and then cooled at room temperature. Two ml of distilled water was added to each tube and absorbance was recorded at 560 nm. Control for every reaction mixture was run along with to check the level of endogenous sugar in time where reaction was terminated by adding 1 ml DNSA reagent just before incubation. The standard curve prepared by using known concentrations of glucose.

3.4.2.7.6. Sucrose Synthase

The activity of this enzyme was assayed by the method described by Hawker et al. (1976).

**Extraction**: The plant material was extracted in 0.1 M tris-HCl buffer, pH 7.6 containing 0.3 M mannitol, 0.01 M MgCl₂ and 0.2 M EDTA. The crude enzyme extract was dialysed against 0.1M tris-HCl buffer (pH 7.6) for 24 hr. to remove soluble sugars from the enzyme extract.

**Composition of reaction mixture**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.015M UDPG</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>0.05 M fructose</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>0.2 M Tris-HCl buffer, pH 8.2 containing</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>0.025 M MgSO₄</td>
<td></td>
</tr>
<tr>
<td>Enzyme extract</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>Total volume</td>
<td>0.5 ml</td>
</tr>
</tbody>
</table>
Material and methods

Estimation: The reaction mixture was incubated at 37°C for 30 min. and the reaction was stopped by heating the contents in the boiling water both for 10 min. and then cooled. Residual fructose (phosphate) was destroyed by adding 0.5ml 6% KOH and heating the content in the boiling water bath for 20 min. After cooling the contents, sucrose (phosphate) formed was estimated by the method of Roe (1934).

Free fructose present in the sugar extract is destroyed by boiling in alkaline solution. The fructose present in the sucrose is released by acid hydrolysis and the sucrose is thus estimated as sucrosyl fructose.

Reagents

(i) Resorcinol solution: 1% resorcinol solution in glacial acetic acid.
(ii) KOH: 6% KOH solution in distilled water
(iii) Hydrochloric acid: 30%

Estimation: After destroying the fructose content, samples were cooled to room temperature, 1 ml of 1% resorcinol solution and 3 ml of 30% HCl were added. The samples were incubated/heated at 80°C for 10 min. and intensity of the pink colour developed was read at 490 nm. The enzyme activity was expressed as sucrose synthesized min⁻¹ from the standard graph prepared by using sucrose standard (40-280 ml⁻¹).

3.4.2.7.7. Invertase

The activity of invertase was assayed according to Hawker and Hatch (1965) and Nygaard (1977).

Reagents

i) Sucrose solution: 0.4 M sucrose solution in 0.2 M sodium acetate buffer (pH 4.8).
ii) Di-nitrosalicylic acid: as prepared for β-amylase estimation.
Material and methods

Extraction: Plant material was homogenized in chilled sodium acetate buffer (0.2 M pH 4.8) containing polyvinyl pyrrolidone) centrifuged at 10,000g at 4°C and supernatant was used as enzyme extract.

Estimation

Reaction mixture was prepared by adding 0.6 ml of 0.2 M acetate buffer pH 4.8, 0.3 ml of 0.4 M sucrose solution in 0.1 ml of enzyme extract. In control tubes, sucrose was added only when enzyme preparation has been inactivated by boiling for 5 min. After incubation at 30°C for 30 min. 1 ml of 3-5 ml di-nitro-salicylic acid reagent (DNSA) was added to reaction mixture. Thereafter tubes were placed in boiling water bath for 10 min. and then cooled to room temperature. The entire samples were diluted to 5 ml and absorbance was recorded at 560 nm. The enzyme activity was expressed as glucose and fructose h⁻¹ g⁻¹ protein.

3.4.2.8. Nitrogen Metabolism

The enzymes associated with nitrogen metabolism and content of free amino acid was quantified following; these methods.

3.4.2.8.1. Nitrate Reductase

The nitrate reductase activity was measured by the method described by Hewitt (1974).

Reagents

i) Phosphate buffer: 0.1 M pH 7.2.
ii) NADH : 1.5 mM
iii) Potassium nitrate : 0.1 M
iv) Zinc acetate : 1 M
v) Sulphanilamide : 1% in 1.5 N HC1
(vi) NEDD : 0.2%
Material and methods

Extraction: Fresh plant material was extracted with phosphate buffer 0.1M pH 7.2) centrifuged at low temperature (10,000 rpm) and supernatant was treated as enzyme extract.

Estimation: 1 ml phosphate buffer, 0.5 ml NADH, 0.5 ml distilled water and 1 ml enzyme extract was mixed and equilibrated the tubes to 25°C and then initiated the enzyme reaction by addition of 0.5 ml potassium nitrate. After incubating for 30 min. the reaction was terminated by addition of 0.8 ml zinc acetate. After centrifugation 3 ml of supernatant was retained to this 2 ml colour developing reagent (1% Sulphanilamide in 1.5 N HCL and 0.2% NEDD, mix in equal volume) was added. After 10 min. the absorbance was recorded at 540 nm. For keeping the enzyme extract inactivated by boiling. The activity was calculated against standard curve plotted with known concentrations of KNO₂ (mg/g FW).

3.4.2.8.2 Protease

The proteolytic activity was estimated according to the method of Basha and Beevers (1975) and Salmia et al. (1978).

Reagents

(i) Phosphate buffer: 0.1 M (pH 6.0).

(ii) Substrate solution: Casein dissolved in 0.1 M phosphate buffer with pH 6.0.

(iii) Trichloro acetic acid: 40%.

(iv) Folin-phenol reagent

Estimation: 100 mg of plant tissue was homogenized in 5ml of distilled water/phosphate Buffer (0.1 M pH 6.0) and then centrifuged at low temperature (4°C) at high speed (10,000 rpm) in cold centrifuge for 15 min. and the supernatant was used as enzyme extract.
Material and methods

**Estimation:** In 0.5 ml of casein solution (1% in 0.1 M phosphate buffer pH 6.0), 0.1 ml of enzyme extract was added and mixture was incubated at 45°C for 1 hr. The reaction was terminated by addition 0.1 ml TCA (40%). It was centrifuged and TCA soluble components were retained. To this 3 ml of folin-phenol reagent was added. The amino acid content was measured after reaction with Folin-phenol reagent. The absorbance was recorded at 620nm. For keeping the control the reaction was terminated at zero time by addition of TCA. The enzyme activity was expressed in terms of μg amino acids released per unit time.

3.4.2.8.3. Free Amino acids

The estimation of free amino acid content was done by method of Lee and Takahashi (1966).

**Reagents**

i) **Ninhydrin reagent**: Ninhydrin reagent was prepared by mixing solutions A, B & C given below in the ratio of 5:12:2 and pH adjusted to 6.0 with sodium citrate.

ii) **Solution A**: 1% ninhydrin in 0.5 M citrate buffer pH (5.5)

iii) **Solution B**: Pure glycerol

iv) **Solution C**: 0.5 M citrate buffer pH (5.5)

v) **Standard**: Glycine solution in distilled water (10 mg/100 ml)

**Extraction:** Ethanol extract prepared for sugars was also used as extract to estimate free amino acids.

**Estimation:** Ninhydrin reagent (3.8 ml) was added to 1 ml of ethanol extract and the contents were shaken vigorously. The mixture was heated in boiling water bath for 12 min. and cooled in running tap water to room temperature. The absorbance of the coloured solution was read at 570 nm against a blank containing 80% ethanol. The
concentration of free amino acid (mg/g DW) was calculated from a standard curve plotted with known concentration of glycine.

3.4.2.9. Oxidative Molecules

Among oxidative molecules, the level of lipid peroxides (as Malondialdehyde) and H$_2$O$_2$ was measured spectrophotometrically.

3.4.2.9.1. Malondialdehyde (MDA)

The level of lipid peroxidation was measured in terms of melondialdehyde (MDA) content, a product of lipid peroxidation by the method described by Heath and Packer (1968).

Reagents

i) 0.1% TCA

ii) 0.5% TBA (thiobarbituric acid) in 20% TCA (trichloro acetic acid)

**Extraction:** Plant tissue was homogenized in 0.1% TCA trichloroacetic acid. The homogenate was centrifuged at 15,000 g for 5 min. Supernatant was treated as extract.

**Estimation:** To 1 ml aliquot of the supernatant, 4 ml of 0.5% of TBA in 20% TCA was added. The mixture was heated at 95°C for 30 min. and then quickly cooled in ice bath. After centrifugation at 10,000 g for 10 min. the absorbance of the supernatant was recorded at 532 nm. MDA content was calculated by its extinction coefficient of 155 mM-1 cm-1 expressed as per gram of fresh weight.

3.4.2.9.2. Hydrogen peroxide (H$_2$O$_2$)

It was estimated by method of Mukherji and Chaudhari (1983).

Reagents

i) **Acetone:** 80%
ii) **Titanium reagent**: 1 gm Titanium dioxide and 10 gm of potassium sulphate mixed and digested with 150 ml of concentrated H$_2$SO$_4$ for 2 hr. on a hot plate. Digested mixture was cooled and diluted to 1.5 cm$^3$ with distilled water and used as titanium reagent.

iii) H$_2$SO$_4$ : 1M

iv) **Ammonia solution**

v) **Standard**: Pure H$_2$O$_2$

**Extraction**: 0.5 gm of plant material was homogenized in 5 ml chilled acetone (80%) and filtered with Whatman filter paper and kept as extract.

**Estimation**: To the above 5 ml of extract, added 4 ml of titanium reagent followed by the addition of 5 ml of ammonia solution. The mixture was centrifuged at 10000 g and supernatant was discarded. The residue was dissolved with 1 M H$_2$SO$_4$ and O.D. was read at 410 nm. Calculations were made with standard curve plotted with pure H$_2$O$_2$.

### 3.4.2.10. Antioxidants

Among the activity of antioxidants, level of ascorbic acid, superoxide dismutase, ascorbate peroxidase and catalase was measured by following methods:

#### 3.4.2.10.1. Ascorbic Acid

The estimation of ascorbic acid was done according to the method of Mukherji and Chaudhari (1983).

**Reagents**

i) **Trichloroacetic acid** : 6%

ii) **Di-nitro-phenylhydrazine**: 2% in acidic medium H$_2$SO$_4$

iii) **Thiouria**: 10% in 70% ethanol
Material and methods

iv) **Sulphuric acid**: 80%

v) **Standard**: ascorbic acid

**Extraction**: Plant tissue was homogenized in 6% TCA and the homogenate was centrifuged at 8000 rpm. The supernatant was used as extract for estimation.

**Estimation**: To 4 ml of extract, 2 ml of 2% DNPH was added followed by 1 drop of 10% Thiourea. Mixture was boiled for 15 mm. in water bath and cooled at room temperature, 5 ml of chilled H₂SO₄ was added at 0°C. The absorbance was recorded at 530 nm and ascorbic acid content (mg/g FW) was calculated from a standard curve plotted with known concentration of ascorbic acid.

3.4.2.10.2. Superoxide dismutase (SOD)

The activity of SOD was assayed following the method of Dhindsa et al. (1981).

**Extraction**: The fresh plant material was homogenized in 50 mM chilled/ice cold phosphate buffer pH 7.0, 0.25% triton X-100 (m/v) and 1% polyvinylpyrrolidone and centrifuged at 10,000 rpm for 10 min at 4°C and supernatant was treated as enzyme extract.

Reagents

i) **50 mM Potassium phosphate buffer**: 2.4 ml

ii) **50 mM sodium bicarbonate**: 0.1 ml

iii) **13 mM methionine**: 0.1 ml

iv) **25 mM NBT (Nitroblue tetrazolium)**: 0.1 ml

v) **0.1 mM EDTA**: 0.1 ml

vi) **2 mM Riboflavin**: 0.1 ml

vii) **Enzyme extract**: 0.1 ml

(50 mM phosphate buffer pH 7.8)
Material and methods

**Estimation:** The reaction mixture (3 ml) contained 13 mM methionine, 25 mM NBT, 0.1 mM EDTA, 50 mM sodium bicarbonate, 50 mM phosphate buffer pH 7.8 and 0.1 ml of enzyme extract. The reaction was started by addition of 2 mM riboflavin and exposing to 15 W fluorescent light for 10 min. The absorbance was read at 560 nm and the total SOD activity of the samples was assayed by measuring its ability to inhibit the photochemical reduction of nitro-blue-tetrazolium (NBT). 1 unit of SOD activity was defined as the amount of enzyme, which causes 50% inhibition of the photochemical reduction of NBT.

3.4.2.10.3. Ascorbate peroxidase (APX)

APX activity was determined by following oxidation of ascorbate as a decrease in absorbance at 290 nm (Nakano and Asada, 1981). Ascorbate (2 mM) was added to extraction medium to prevent the inactivation of enzyme.

**Reagents**

- **i)** 50 mM phosphate buffer (pH 7.0) : 2.6 ml
- **ii)** 0.5 mM Ascorbic acid : 0.1 ml
- **iii)** 0.1 mM EDTA : 0.1 ml
- **iv)** 0.1 mM H$_2$O$_2$ : 0.1 ml
- **v)** Enzyme extract : 0.1 ml

**Extraction:** Plant material was homogenized in ice cold. 50 mM phosphate buffer, centrifuged at 10,000 rpm at 4°C and supernatant was kept for assay.

**Estimation:** The assay was carried out at 20°C in 3 ml of reaction mixture containing 50 mM Phosphate buffer pH 7.0, 0.1 mM EDTA, 0.5 mM ascorbic acid, and enzyme extract. The change in A$_{290}$ was recorded at 30 seconds intervals after addition of H$_2$O$_2$. The rate constant was calculated using the extinction coefficient of 2.8 mM$^{-1}$ cm$^{-1}$. 

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3.4.2.10.4. Catalase

Catalase activity was determined by the method of Teranishi et al. (1974).

Reagents

i) 50 mM phosphate buffer (pH 7.0) : 2.7 ml

ii) 20 mM H$_2$O$_2$ : 0.2 ml

iii) Enzyme extract : 0.1 ml

iv) Titanium reagent : 2.0 ml

v) Titanium reagent : (as prepared for H$_2$O$_2$ estimation)

Estimation: The reaction mixture (3 ml) containing 50 mM phosphate buffer (pH 7.0), 20 mM H$_2$O$_2$, 0.1 ml of enzyme extract. The reaction was stopped by adding 2 ml of titanium reagent. It was centrifuged at 10,000 rpm to 10 min. The absorbance was read at 410 nm. The catalase activity was measured using the extinction coefficient 40mM-1 cm$^{-1}$ and expressed as $\mu$ Mol H$_2$O$_2$ reduced/sec/g FW.

3.4.2.10.5. Proline

Proline was estimated using the method of Bates et al. (1973).

Reagents

i) Acid Ninhydrin: 1.2g ninhydrin in 30 ml glacial acetic acid and 20ml 6M orthophosphonic acid.

ii) Sulfosalicylic acid : 3%

iii) Toluene

iv) Standard : Proline solution (10 mg/100 ml).

Extraction: Fresh material of plant was homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged to 10,000 rpm. Supernatant was estimated for proline.
**Material and methods**

**Estimation:** The reaction mixture consisting of 2 ml supernatant, 2 ml acid ninhydrin and 2 ml of glacial acetic acid was boiled at 100°C for 1 hr. After termination of the reaction in ice bath, the reaction mixture was extracted with 4 ml toluene and the absorbance was read at 520 nm. The amount of proline was calculated from the standard curve plotted with known concentrations of proline.

The samples were replicated and statistically analyzed for standard errors.