3. MATERIAL AND METHODS

The present work was undertaken in three different experiments as follows:

3.1 To study the effect of normal and late sowing conditions on the growth, phenology, reproductive biology and yield of Lentil genotypes under field conditions.

The seeds of three genotypes of Lentil LL699, LL931 and LL1122 (released cultivars of P.A.U., Ludhiana) were inoculated with *Rhizobium* sp. compatible with Lentil and grown in the pots following completely randomized block design. Pots were filled with soil+sand+farm yard manure (3:1:1) and phosphorous (7mg/Kg of soil) in protected enclosures at two different sowing dates, to find out any differences in their temperature sensitivity. To impose heat stress, the plants were raised four months later than the normal-sown plants. Thus, the normal sowing was done in the first week of October whereas the second or late sowing was done in the second week of February. Five seeds were planted in each pot initially and after emergence, the plants were thinned to 3 per pot. The plants were well irrigated to keep the soil at full field capacity to rule out any effects of water stress. Data for temperature was recorded daily and is presented in Fig. 4.1.1. The plants were analyzed for phenology, floral biology and yield. The samples for damage to leaves, floral biology, and biochemistry were collected from normal-sown plants when the average temperatures were 23.5/11.5°C (max/min) and from late-sown plants, when the temperatures ranged between 38.3/25.5 °C (max/min.) for at least 7 days.
3.1.1. **Growth and Phenological observations**

A selective number of plants were tagged for observation on number of flowers and pod set. Data were recorded on the following aspects:

**Observations:**

(i) Total no. of flowers  
(ii) Number of aborted flowers  
(iii) Total no of pods produced during the growing period of the crop  
(iv) Number of aborted pods  
(v) Days to flowering  
(vi) Days to end of flowering  
(vi) Days to podding  
(vii) Days to end of podding

3.1.2. **Floral biology**

The main focus was given to reproductive stage to notice the variation in floral biology during period of growth. Three pots of each genotype were marked separately for the evaluation of various floral biology attributes under different temperature conditions i.e. cold, control, and heat stress experiments. Cold stress experiment was done at 8-9°C whereas heat stress experiment was conducted at 38-40°C for four days. The control plants were kept at temp. of 23-25°C at the same time. On the fifth day after exposure to stressful temperatures, the flowers were collected from these plants and assessed for pollen viability (viable and non viable pollen), pollen germination percentage (**in vitro** and **in vivo**), pollen load, stigma receptivity and ovule viability.
(i) **Pollen viability**

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

(ii) **Pollen Germination Percentage (in vitro)**

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

i. 0.01g of boric acid

ii. 0.01g of calcium nitrate

iii. 1.75g of sucrose

iv. 0.03g of agar

Make 0.01g of calcium nitrate and 0.01g of boric acid in 100 ml of distilled water. Then take 5ml of above solution and add 1.75g of sucrose and 0.03g agar.

Pollens grains from anthers were dusted on the surfaces of germination medium containing sucrose and solution of calcium nitrate and boric acid. Keep them for 3-4 hrs at 37°C.

The *in vitro* germination process was stopped after incubation by adding a drop of acetocarmine to the medium, pollens were counted as germinated when pollen tubes were at least equal to the diameter of the pollen grain. The percentage germination was determined on the basis of at least 100 pollen grains per replicate.
(iii) **Pollen Load**

The number 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).

(iv) **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 were mounted on a slide in a 1:1 (aniline blue: 10% glycerin) solution. The stained gynoecium was then observed under fluorescence photomicrograph microscope (Nikon, Japan) (Dumas and Knox, 1983).

(v) **Stigma Receptivity Test**

Esterase test using α-napthyl 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-napthyl 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)

(vi) Ovule viability test

Ovule viability was assessed using TTC test. Fertilized flowers were taken and ovules were carefully removed from the ovary. The ovules were placed on a clean glass slide in a drop of TTC solution (0.5% in 1% sucrose solution). Cover slip was gently placed and the slide was then placed in a petri-dish with moist filter paper layer followed by covering it with black paper or placed in dark and incubated at room temperature. At the end of incubation period (variable) slide was observed under microscope and the viability was measured in terms of intensity of red color developed due to the accumulation of formazon, particularly in the center as the red color on sides could be possibly due to higher oxygen availability. The ovule viability was scored on a 1 to 5 scale (1 being the lowest and 5 being the highest).

3.1.3. Yield data

After maturation of pods and crop, the plants were uprooted, oven dried at 80°C for 48 hours and then subjected to following observations:

(i) Biomass
(ii) Pod number
(iii) Filled and unfilled pods
(iv) Total no. of seeds
(v) Harvest index
3.2. To study the effect of normal and late sowing conditions on the metabolic studies (physiology and biochemistry) of Lentil genotypes under field conditions.

The same genotypes were again raised in the field in the following growing season in a manner similar to the previous year. All the genotypes were then analyzed for various leaf injury and biochemical parameters following standard methods. For biochemical estimation, leaf samples were collected from 3rd and 4th leaf from the top at three different stages – twice from normal sown plants i.e. first collected in the month of January for cold-stress observations when the temperatures were below 20/10°C (12.2/4.6°C; max/min), then in the month of March for control observations, when the temperatures were above 20/10°C (23.5/11.5°C; max/min) while for heat stress observations, the samples were collected from late-sown plants in April, when the temperatures were above 32/20 °C (38.3/25.5°C; max/min), for at least 7 days.

3.2.1 Physiological tests (Stress injury parameters)

(i) Electrolyte Leakage (EL)

Electrolyte leakage was used to assess permeability of cell membrane as described by Lutts et al. (1996). Leaf segments were detached, washed with deionized water, placed in closed vials containing 10 ml of deionized water and incubated over night at 25°C. Electrical conductivity of the bathing solution (C₁) was determined after 24 hrs. Samples were then put in boiling water bath for 10-15 min and a last conductivity reading (C₂) was obtained upon equilibration at 25°C. The electrolyte leakage was defined as follows:-
Material and Methods

EL% = \( \frac{C_1}{C_2} \times 100 \)

**Units:** m mhos/ g fw

(ii) **Relative Water Content (RWC)**

The RWC was assayed according to Weatherley (1950). Leaf tissues (100mg) were cut from the seedlings and their fresh weight was recorded. The leaves were immersed in distilled water for 2 hrs. 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 hours at 110°C in oven and weighed again (dry weight). The relative leaf water content was calculated as follows:-

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

**Units:** %

(iii) **Chlorophyll (Chl.) Content**

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

\[
\text{Chl a.} = 12.9 (\text{Abs}_{663}) - 2.69 (\text{Abs}_{645}) \frac{V}{1000 \times W}
\]
Chl b. = \( \frac{22.9 \text{ (Abs}_{645}) - 4.68 \text{ (Abs}_{663})}{1000 \times W} \)

Total Chl. = Chl. a + Chl. b  

**Units:** mg/g fw

(iv) **2,3,5 Triphenyl Tetrazolium Chloride (TTC) reduction assay**

Triphenyltetrazolium chloride (TTC) reduction assay was detected by the method of Steponkus and Lanphear (1967).

**Reagents**

i) 500 mg TTC  

ii) 0.1M PO\(_4\) buffer pH=7

iii) 95% alcohol

**Procedure**

One hundred mg plant material was washed with distilled water and then added 5ml of distilled water in test tube. Incubated them at 85°C for 2 min or at 80°C for 15 min. Another set was made which was not heated and acted as control. After that water was taken out from them and then added 4ml TTC solution. Incubated the test tubes for 20 hrs under dark conditions at room temperature. The colourless TTC was reduced to red triphenyl formazan as a result of the dehydrogenase activity in living cells. Rinsed tissue with distilled water. Added 3ml of rectified alcohol and kept them for 2 hr and finally recorded the OD at 520nm.

**Units:** \( A_{530} / g \text{ fw} \)
(v) Chlorophyll fluorescence (Leaf photosynthetic function)

The photochemical efficiency was measured as chlorophyll fluorescence using the dark-adapted test of the modulated chlorophyll fluorometer OS1-FL (Opti-Sciences, Tyngsboro, MA, USA). With this system, chlorophyll fluorescence is excited by a 660nm solid-state light source with filters blocking radiation longer than 690nm. The average intensity of this modulated light was adjusted from 0 to 1 mE. Detection was in the 700–750nm range using a PIN silicon photodiode with appropriate filtering to remove extraneous light. The clamps of the instrument were installed on the leaves to keep them in the dark and to stop the light reaction of photosynthesis for 45min. After this, the clamps were attached to the optic fiber of the device and the valves of the clamps were opened. After starting the device, the 695nm modulated light was radiated through the optic fibre towards the leaf. Subsequently, the Fv/Fm ratio was recorded.

**Units:** Fv/Fm ratio

(vi) Stomatal resistance

Stomatal resistance of fully-expanded leaves was measured (as s/m) using a portable leaf porometer (Steady State Diffusion Porometer; model SCI; Decagon Devices, Pullman, WA, USA).

**Units:** s/m

(vii) Leaf surface temperature

The leaf surface temperature was measured as °C by IR Thermometer, Food Service, Probe and IR manufactured by Oakton Instruments, Vernon Hills, IL, USA

**Units:** degree Celsius (°C)
3.2.2 Biochemistry

3.2.2.1 Carbohydrate Metabolism

The endogenous levels of certain biomolecules associated with carbohydrate metabolism such as sugars, reducing sugars, starch were determined. The activity of various enzymes associated with carbohydrate metabolism i.e. like α-amylase, β-amylase, invertase, sucrose synthase etc. was also measured by following methods:

(i) 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 (KH$_2$PO$_4$), 20 mg of anhydrous. CaCl$_2$ in 100 ml of distilled water, boiled for one minute, cooled, filtered and used as substrate. Starch substrate was prepared fresh every time.

ii) Iodine solution: It was prepared by dissolving 254 mg I$_2$ and 4 g 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.
**Material and Methods**

**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 it 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 aliquot was removed 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.).

**Units:** μg min /mg protein

**(ii) β-amylase**

The enzyme activity was assayed following the method 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 (DNSA): It was prepared by mixing 2.5 g of DNSA with 150 ml distilled water containing 4.0 g of sodium hydroxide, 7.5 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 having 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 placed 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 was prepared by using known concentrations of glucose.

Units: μg/ min /mg protein

(iii) Invertase

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

Reagents

i) Sucrose solution: 0.4 M sucrose solution in 0.2 M sodium acetate buffer (pH 4.8).

ii) Di-nitrosalicylic acid (DNSA): as prepared for β-amylase estimation.

Extraction

Plant material was homogenized in chilled sodium acetate buffer (0.2 M pH 4.8) centrifuged at 10,000 rpm at 4°C and supernatant was used as an enzyme extract.
Material and Methods

**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 had been inactivated by boiling for 5 min. After incubation at 30°C for 30 min. 1 ml of di-nitro-salicylic acid reagent (DNSA) was added to the 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.

**Units:** μg/min /mg protein

(iv) **Sucrose Synthase**

The activity of this enzyme was measured 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

**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 0.025 M MgSO₄</td>
<td>0.1 ml</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 bath 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 sucrlosyl 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 activity of the enzyme was expressed as sucrose synthesized from the standard graph prepared by using sucrose standard (40-280 ml⁻¹).

Units: µg/min/mg protein
(v) **Sucrose-phosphate-synthase**

Sucrose-phosphate-synthase activity was analyzed following the similar procedure as in sucrose synthase given by Hawker et al. (1976) except that fructose-6-phosphate was used in it instead of fructose. Rest of the analysis was carried out in exactly similar manner.

**Units:** μg/ min /mg protein

(vi) **Starch phosphorylase**

Starch phosphorylase activity was determined by the method given by Fiske and Subbarow (1925).

**Reagents**

(i) 0.2M Tris maleate buffer (pH 6.5) containing 1mM NaF (sodium fluoride): 1.25g maleic acid and 2.42 g Tris buffer dissolved in 100ml distilled water.

(ii) Ammonium molybdate reagent: 1.5 g Ammonium molybdate dissolved in 30.3 mil conc. HCl and the final volume was made to 100ml with distilled water.

(iii) Fiske and Subbarow reagent: Prepared by well dissolving 1.45 g sodium metabisulphite, 0.50mg sodium sulfite, 0.25mg 1-amino-2-napthol-2-sulphonic acid in 5ml distilled water and then making the final volume to 10ml with distilled water.

(iv) 0.05M glucose-1-phosphate

(v) 5% TCA
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Extraction

Plant material was homogenized in chilled Tris maleate buffer (0.2 M pH 6.5) centrifuged at 10,000 rpm at 4°C and supernatant was used as an enzyme extract.

Estimation

Enzyme extract (0.2ml) was added to 0.6ml Tris-maleate buffer followed by 0.2ml 0.005M glucose-1-phosphate. Then after was 1hr incubation at 30°C, 0.5ml cold 5% TCA was added into each test tube resulting in the formation of some precipitates. The mixture was then centrifuged and supernatant was retained. In another set of test tubes, 0.5 ml supernatant, 3.3ml distilled water and 1ml ammonium molybdate was added into each. The tubes were then well shaken for 5 minutes followed by the addition of 0.2ml Fiske and Subbarow reagent into each. The test tubes were then again incubated at 30°C for 15 minutes resulting in the appearance of blue color which was spectrophotometrically read at 660nm.

Units: nmol Pi/min/mg protein

(vii) Starch

The content of starch was measured by acid hydrolysis method given by Mc Creddy 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% H$_2$SO$_4$ (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 sulphuric 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 read at 630 nm. The final content of starch was calculated from a standard curve plotted with known concentration of glucose.

**Units:** mg/g dw

(viii) **Total Sugars**

Total sugars were estimated according to the method described by Yemm and Willis (1954).
Reagents

(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)

Extraction

Ethanol extract prepared for reducing sugars was also used as extract to estimate total sugars.

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 measured at 625 nm against blank containing 80% ethanol. The concentration of total sugars (mg/gdw) was calculated from standard curve plotted with known concentrations of glucose.

(ix) 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. 2g 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).

**Extraction**

The oven dried plant material was homogenized in 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. For complete recovery of sugars the extraction was repeated twice. The residue was kept for further analysis of starch. The supernatant was pooled and evaporated to dryness in a chinadish on boiling water bath. The residue was eluted with 5ml of 20% ethanol and subjected to analysis for reducing sugars, total sugars and amino acids.

**Estimation**

To one 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.

**Units:** mg/g dw

**3.2.2.2 Nitrogen Metabolism**

The enzymes associated with nitrogen metabolism and content of free amino acid was quantified following these methods:
(i) **Nitrate Reductase**

The nitrate reductase activity was assayed 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 HCl
(vi) NEDD (Napthyl ethylene diamine dihydrochloric acid): 0.2%

**Extraction**

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

**Estimation**

Phosphate buffer 1ml, NADH 0.5 ml, distilled water 0.5 ml and 1 ml enzyme extract was mixed and equilibrated in the tubes to 25°C and then initiated the enzyme reaction by addition of 0.5 ml potassium nitrate. After incubating for 30 minutes, the reaction was terminated by addition of 0.8 ml zinc acetate. After centrifugation 3 ml of supernatant was retained to this 2 ml color developing sulphanilamide reagent (1% sulphanilamide in 1.5 N HCl and 0.2% NEDD, mixed in equal volume) was added. After 10 min. the absorbance was read at 540 nm. The activity
was calculated against standard curve plotted with known concentrations of KNO₂.

**Units:** μg nitrate reduced/min/mg protein

(ii) **Protease**

The proteolytic activity was measured 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 (TCA): 40%.

(iv) Folin-phenol ciocalteu's reagent

**Extraction**

100 mg of plant material 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.

**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 of 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 measured at 620nm. For keeping the control the reaction was stopped at zero time by addition of TCA.

**Units:** μg amino acids/h/mg protein.

(iii) **Soluble Proteins**

The soluble protein content was estimated by the method devised by Lowry et al. (1951).

**Reagents**

i) Reagent A: 2% Na₂CO₃ in 0.1 N NaOH.

ii) Reagent B: 0.5% CuSO₄.5H₂O in 1% Sodium potassium tartarate.

iii) Reagent C: 50 ml of reagent A + 1 ml of reagent B (freshly prepared).

iv) Reagent D: Folin-phenol ciocalteu's reagent (1:1 ratio) (1N).

**Extraction**

100 mg of plant material was crushed in 0.1M phosphate buffer pH 7.0 and centrifuged at 3000 rpm for 10 min. To the supernatant 5.0 ml TCA (15%) was added and kept at 4°C for 24 hr. The mixture was centrifuged to separate the precipitates at 3000 rpm for 10 min. The supernatant was discarded and the precipitates were dissolved with 0.1 NaOH (1 ml) and kept for 18 hr for complete dissolution and treated as extract.

**Estimation**

Five ml of reagent C was added to 1 ml of extract, allowed it to stand for 10 min, 0.5 ml of reagent D was added and kept for 30
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min for colour development. The absorbance was read at 570 nm. The total protein content (mg/g dw) was expressed using standard curve plotted with Bovine serum albumin.

Units: mg/ g dw

(iv) 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 minutes 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 (µg/g DW) was calculated from a standard curve plotted with known concentration of glycine.

**Units:** µg/ g dw

### 3.2.2.3 Oxidative metabolism

The oxidative stress was measured as malondialdehyde (indicator of lipid peroxidation) and hydrogen peroxide content in the leaf tissues. Also various enzymatic and non-enzymatic anti-oxidants were measured for all three genotypes subjected to different temperature conditions.

#### 3.2.2.3.1 Oxidative Molecules

Among oxidative molecules, the level of lipid peroxides (as Malondialdehyde) and H₂O₂ was measured spectrophotometrically.

**i) Malondialdehyde (MDA)**

The level of lipid peroxidation was estimated in terms of malondialdehyde (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. The homogenate was centrifuged at 15,000 rpm for 5 min. Supernatant was treated as extract.

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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 rpm for 10 min. the absorbance of the supernatant was read at 532 nm. MDA content was calculated by its extinction coefficient of 155 mM-1 cm-1.

Units: μmol/g dw

(i) Hydrogen peroxide (H₂O₂)

It was estimated by the 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 sulphuric acid (H₂SO₄) for 2 hrs on a hot plate. Digested mixture was cooled and diluted to 1.5 cm³ with distilled water and used as titanium reagent.

iii) Sulphuric acid (H₂SO₄): 1M

iv) Ammonia solution

v) Standard: Pure H₂O₂

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 rpm and supernatant was discarded. The residue was dissolved with 1 M H$_2$SO$_4$ and O.D. was recorded at 410 nm. The extinction coefficient of H$_2$O$_2$ is 0.28 μmol$^{-1}$cm$^{-1}$. Calculations were made with standard curve plotted with pure H$_2$O$_2$.

**Units:** μ mol/g dw

**3.2.2.3.1 Antioxidants**

Among the activity of antioxidants, level of superoxide dismutase, catalase, ascorbate peroxidase, ascorbic acid, glutathione reductase, glutathione was assayed by following methods:

(i) **Superoxide dismutase (SOD)**

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

**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

Extraction

The fresh plant material was homogenized in 50 mM chilled/ice cold phosphate buffer pH 7.0 and centrifuged at 10,000 rpm for 10 min at 4°C and supernatant was treated as enzyme extract.

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 exposed to 15 W fluorescent light for 10 min. The absorbance was recorded 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). One unit of SOD activity was defined as the amount of enzyme, which causes 50% inhibition of the photochemical reduction of NBT.

Units: Units /mg protein

(ii) Catalase (CAT)

Catalase activity was estimated by the method of Teranishi et al. (1974) with some modifications.

Reagents

i) 50 mM phosphate buffer (pH 7.0): 2.7 ml
ii) 200 mM H$_2$O$_2$: 0.2 ml
iii) Enzyme extract: 0.1 ml

Estimation

The reaction mixture (3 ml) containing 50 mM phosphate buffer (pH 7.0), 200 mM H$_2$O$_2$ and 0.1 ml of enzyme extract. The reaction was initiated by addition of 200 mM H$_2$O$_2$. The decrease in absorbance was recorded at 410 nm for 3 minutes. The
catalase activity was measured using the extinction coefficient 40 mM-1 cm-1.

**Units:** mmol H₂O₂ decomposed /mg protein

(iii) **Ascorbate peroxidase (APO)**

APO 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 H₂O₂: 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 reaction 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 A₂₉₀ was recorded at 30 seconds intervals after addition of H₂O₂. The rate constant was calculated using the extinction coefficient of 2.8 mM⁻¹ cm⁻¹.

**Units:** m mol oxidized donor / min / mg protein
(v) **Glutathione reductase**

Glutathione reductase was measured by the method of (Mavis and Stellwagen, 1968).

**Reagents**

i) 100 mM Potassium Phosphate buffer with 3.4 mM EDTA, pH 7.6 at 25°C.

ii) 30 mM Gluthathione substrate solution (GSSG) oxidized. Prepared 5 ml in deionized water using glutathione oxidized form, disodium salt.

iii) 0.8 mM β-NADP, reduced form solution. Prepared 5 ml in cold reagent (i) using β-NADP, tetrasodium salt.

iv) 1%(w/v) Bovin Serum Albumin (BSA). Prepared 100 ml in reagent (i) using BSA.

v) Glutathione reductase enzyme solution (prepared a solution immediately before use containing 0.30-0.60 unit/ml of glutathione reagent in cold reagent (iv).

**Procedure**

The following reagents (in milliliters) were taken into suitable cuvettes

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Deionized water</td>
<td>0.65</td>
<td>0.65</td>
</tr>
<tr>
<td>b) Reagent i (buffer)</td>
<td>1.50</td>
<td>1.50</td>
</tr>
<tr>
<td>c) Reagent ii (GSSG)</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>d) Reagent iii (β-NADP)</td>
<td>0.35</td>
<td>0.35</td>
</tr>
<tr>
<td>e) Reagent iv (BSA)</td>
<td>0.30</td>
<td>0.40</td>
</tr>
<tr>
<td>f) Reagent v (Enzyme solution)</td>
<td>0.10</td>
<td>-</td>
</tr>
</tbody>
</table>

The contents were immediately mixed by inversion and the decrease OD read at 340 nm for approximately 3 minutes.

**Units:** n mol oxidized donor/ min/ mg protein.
(vii) **Ascorbic Acid (ASC)**

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 $\text{H}_2\text{SO}_4$

iii) Thiourea: 10% in 70% ethanol

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 an 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 min in water bath and then cooled at room temperature, 5 ml of chilled $\text{H}_2\text{SO}_4$ was added at 0°C. The absorbance was read at 530 nm and ascorbic acid content (mg/g dw) was calculated from a standard curve plotted with known concentration of ascorbic acid.

**Units:** n mol/g dw

(vi) **Glutathione**

The enzyme glutathione was estimated according to the method of Griffith, 1980
**Material and Methods**

**Reagents**

(i) 2% metaphosphoric acid  
(ii) 10% sodium citrate  
(iii) 0.3 mM NADPH  
(iv) 6 mM DTNB (5,5'-Dithio-bis(2-nitrobenzoic acid))  
(v) 50 μl glutathione reductase

**Procedure**

The glutathione content was measured by using fresh leaf tissue that was homogenised in 2 ml of metaphosphoric acid and centrifuged at 17,000 g for 10 min. The aliquots of the supernatant were neutralized by adding 0.6 ml of 10% sodium citrate to 0.9ml of the extract. A total volume of 1 ml of assay containing 700 μl NADPH (0.3 mM), 100 μl DTNB (6 mM), 100μl distilled water and 100 μl of extract was prepared and stabilized at 25°C for 3-4 min. Later 10 μl of glutathione reductase was added and the absorbance was recorded at 412nm. Glutathione was calculated from a standard graph as described by Griffith (1980).

**Units:** n mol oxidized donor/min/mg protein

**3.2.2.4 Osmolytes**

The content of two very important osmolytes proline and trehalose was also measured under different temperature conditions for all three Lentil genotype

**(i) Proline Content**

Proline was described 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: 4ml

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

Extraction

Fresh material of plant tissue was homogenized in 3% aqueous sulfosalicylic acid and the homogenate was centrifuged at 10,000 rpm. Supernatant was estimated for proline.

Estimation

The reaction mixture containing 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 recorded at 520 nm. The amount of proline was calculated from the standard curve plotted with known concentrations of proline.

Units: µmol/g dw

(ii) Trehalose

Trehalose was estimated as per method of Travelyan and Harrison (1956) and Brin (1966)

Reagents

i) 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.

ii) Trichloro acetic acid (TCA) : 0.5 M

**Extraction**

The oven dried plant material was homogenized in ethanol (80%) and centrifuged at 5000 rpm for 10 min. The supernatant was then assayed according to Anthrone method of Brin (1966)

**Estimation**

The supernatant (0.1ml) mixed with 2ml of TCA to prepare the reaction mixture. 1ml of the above reaction mixture taken out and to it was added 4ml of Anthrone Reagent. Appearance of yellowish green colour indicated the presence of trehalose. The absorbance of the above solution was read at 620 nm in spectrophotometer (Chemito, Japan) against blank containing 80% ethanol. The concentration of total trehalose was calculated from standard curve plotted with known concentrations of glucose.

**Units**: (μmol/g DW)

3.3 To study the effects of different temperatures and role of various molecules such as Proline (Pro), Glycine betaine (GB) and Jasmonic Acid (JA) in varying concentrations under lab conditions

Seeds of Lentil were surface sterilized with 0.1% HgCl₂ (mercuric chloride) for 2 min. and then washed with distilled water at least two times. Sterilized seeds were raised in 15cm petri-dishes having distilled water moistened filter paper at room temperature for one week. One week old seedlings were then shifted to varying
temperatures, ranging from 10-35°C (with an interval of 5°C) and maintained there in distilled water (for control) and in various concentrations of proline (2.5 and 5μM), glycine betaine (2.5 and 5μM) and jasmonic acid (25 and 50μM). The seedlings were allowed to grow under these conditions for next six days. Experiments were terminated on the 7th day of treatment and seedlings (13 days old) were analyzed for growth, stress injury and oxidative damage and antioxidants parameters.

3.3.1 Growth parameters

(i) Root growth
(ii) Shoot growth

3.3.2 Stress injury (detail of each parameter listed above)

(i) Relative leaf water content
(ii) Electrolyte conductivity
(iii) 2,3,5 Triphenyl tetrazolium chloride (TTC) reduction assay
(iv) Chlorophyll content

3.3.3 Oxidative molecules (detail of each parameter listed above)

(i) Malondialdehyde (MDA)
(ii) Hydrogen peroxide (H₂O₂)

3.3.4 Antioxidants (detail of each parameter listed above)

(i) Superoxide dismutase
(ii) Catalase
(iii) Ascorbate peroxidase
(iv) Glutathione reductase
(v) Ascorbic acid
(vi) Glutathione
(vii) Proline

The observations were taken in 3 replications for each variable and subjected to ANOVA using AGRISTAT software. LSD values were worked out (P<0.05) and indicated in the tables and figures.