MATERIALS AND METHODS

The current investigation was carried out with following varieties of soybean (Glycine max L), raised from pure line seeds obtained from Punjab Agricultural University, Ludhiana.

(1) Glycine max L. var. PB1
(2) Glycine max L. var. Bragg
(3) Glycine max L. var. J53.NE

Sowing:

Healthy pure line seeds of each variety were selected for uniformity of size, shape and colour. These were then, sown in earthenware pots (20 cm dia) containing 2:1 mixture of garden soil and farmyard manure with 15 seeds in each pot. After germination when plants had established, these were thinned to leave 5 uniform and healthy plants in each pot, which were watered adequately daily. No hoagland's solution was needed as plants were green and seemed to derive nutrients from nodulated roots.

Photoperiodic treatment

For seasonal studies, the seeds were sown under SD, ND and LD conditions. For short day photoperiods, natural day-length was curtailed by screening the plants with thick canvas black sheets with proper aeration, daily from 5:00 p.m.
to 9:00 a.m. For long day photoperiods, plants were kept under continuous illumination. This was done by keeping them under natural sunlight for 8 hr from 9:00 a.m. to 5:00 p.m. and artificial illumination afterwards by incandescent lamps fitted in domes. For other experiments plants were grown under continuous illumination and when plants had attained the proper growth stage, these were subjected to different photoperiodic and chemical treatments as detailed separately in each experiment.

**Chemical treatment**

**Preparation of solutions:** Plants were treated with GA₃, SA, βN, caffeic acid and catechol in different experiments. The stock solutions of these containing 100 mg/l were prepared by dissolving exactly weighed quantities of each chemical in 1 ml of ethanol and making the total volume to one litre with distilled water in a volumetric flask. The solutions were stored in amber coloured bottles and kept in a refrigerator till used. These were suitably diluted when needed.

**Method of application:** Plants were transferred to well-lit shade and were treated with 0.3 ml of the solution dispensed through tiny cotton wads kept on apices. The treatment was given at periodic intervals.

**Morphological observations**

The following observations were recorded:

**Height of the main axis:** The height of the plants
taken as the distance between the surface of the soil to the base of the topmost expanded leaf was measured at weekly intervals.

**Number of leaves:** The total number of leaves produced on the main axis was counted at weekly intervals. Since the first pairs of leaves are simple and opposite, and upper ones are alternate, the number of leaves does not denote the number of nodes.

**Size of leaflets and length of petioles:** The length and breadth of the largest leaf was measured and also the length of the individual petiole of each plant was taken at periodic intervals.

**Height and leaf number of branches:** Length of individual branches and the number of leaves borne by them was also recorded at termination of the experiment.

**Vegetative and reproductive periods:** Daily observations were made of the number, position and dates of emergence of both vegetative and reproductive buds and also for the opening of the latter into flowers. The mean period elapsing from the date of sowing to the date of appearance of the first floral bud was taken as 'vegetative period'. Calculation were also made of the total number of buds and flowers produced and the first node to produce floral buds and flowers.

Data were also taken of the time taken to initiate
Visual observations

In addition to above, visual observations such as the general appearance of plant, colour of the leaves, leaf fall, thickness of plants were also noted.

Microtomy

The technique used for cutting microtomic sections of various samples taken, was essentially the same as given by Johnson (1950) with minor adjustments. The details are as follows:

Fixation: The plant material was fixed in FAA (45 ml rectified + 45 ml of distilled water + 5 ml formalin + 5 ml acetic acid, glacial) for at least 4 days after which it was transferred to 70% alcohol, till sectioned. Before cutting sections, hairs were removed from the plant with the help of needles and forceps, as this plant contains abundant hairs or bristles.

Dehydration: The samples were dehydrated by passing them through tertiary butyl alcohol grades. The concentration of butyl alcohol along with the other components used in each grade and the time for which the samples were kept in each are as follows:
<table>
<thead>
<tr>
<th>Grade</th>
<th>Water</th>
<th>Rectified</th>
<th>TBA</th>
<th>Absolute alcohol</th>
<th>Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>40</td>
<td>10</td>
<td>0</td>
<td>1½</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>50</td>
<td>20</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>15</td>
<td>50</td>
<td>35</td>
<td>0</td>
<td>1½</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>45</td>
<td>55</td>
<td>0</td>
<td>1½</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
<td>75</td>
<td>25</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

In fourth grade, a pinch of eosin crystal was added, so that the material gets a light colour which helps in locating the material in the wax.

**Penetration:** A few drops of molten paraffin wax (m.p. 60-62°C) were added to the tube containing the sample in 100% tertiary butyl alcohol. After 15 min half the mixture of the tube was poured off and molten wax was added to bring the level to the original. This procedure was repeated 6 to 7 times, each at an interval of 20 min, till the smell of tertiary butyl alcohol disappeared.

**Block making:** Small paper trays were prepared for making the blocks of wax containing the material. Small quantity of glycerine was pasted on the inner surface to prevent the sticking of the wax with the paper. Some molten wax was poured into the tray and then the samples were placed in it and arranged in a proper fashion with the help of warm forceps. The molten wax was allowed to solidify and then the wax containing the material was
trimmed to make a rectangular block.

Sectioning: Vertical longitudinal sections 8 μm thick, were cut with the help of a microtome and the ribbons were mounted on a clean glass slide with uniformly spread gelatin adhesive and were stretched using 3-4% formalin water on a hot plate. After drying them overnight, dewaxing and staining was done as follows:

Dewaxing and staining: The slides were kept in pure xylol for 4-6 hrs till wax was completely removed. These were then passed through xylol and alcohol grades in the following order, keeping in each xylol grade for 3 min and in each alcohol grade for 2 min.

Pure xylol, 75% xylol (in absolute alcohol), 50% xylol, 25% xylol, Absolute alcohol, 95% alcohol, 75%, 50%, 25%, saffaranine (16-17 hrs), 25% alcohol, 50%, 75%, 95%, 100%, 50% clove oil (in absolute alcohol), pure clove oil, fast green, clove oil, xylol (2-3 hrs) and finally mounting in canada balsam.

The slides were microscopically examined, microphotographs were prepared and studied.

Biochemical observations

Plant samples were separated into stem and leaves which were then pooled together. Three such replicates were taken for each determination.
Protein content: The total protein content was determined by the method described by Lowry et al. (1951).

Preparation of the sample: The plant tissue was crushed in acetone and was freed of pigments by repeated washings with acetone. The tissue was then dehydrated by a 1:1 mixture of acetone and ether and dried by keeping in the air at room temperature for 24 hr. The whitish powder, thus obtained, was used for protein estimation.

Reagents used: For estimation of proteins, following reagents were used:

(A) 2% sodium carbonate in 0.1N sodium hydroxide,
(B) 0.5% copper sulphate in 1% sodium citrate,
(C) 1 ml of reagent B mixed with 50 ml of reagent A,
(D) Folin-Giocalteu reagent which was prepared as follows:

100 gm of sodium tungstate (AR) and 25 gm sodium molybdate (AR) were dissolved in 700 ml of distilled water. To this were added 50 ml of orthophosphoric acid (sp.gr.1.75) and 100 ml conc hydrochloric acid. The reaction mixture was refluxed for 10 hr in a water bath, using an air-condenser. 150 gm lithium sulphate, 50 ml distilled water and a few drops of bromine water were added and the solution boiled for another 15 min, cooled and the volume made up to one litre in a volumetric flask. The reagent was stored in a coloured bottle in the refrigerator.

Estimation: Took 5 mg of the powder and added to it
1 ml of reagent C and then 0.1 ml of reagent D after 10 min. The solution was stirred and the optical density of the blue colour that developed was measured with the help of a Bausch and Lomb spectronic 20 photocolorimeter at 660 nm after 30 min.

**Calculations:** The protein content (mg/gm dry wt) was then calculated from the standard curve prepared by using different concentrations of bovine albumin.

**Peroxidase activity**

Peroxidase activity was determined by the method described by Mitra et al. (1970).

**Extraction:** The leaves and stems of the plants were washed and homogenized in a glass pestle and mortar with a pinch of acid washed sand at 4°C and the crude enzyme was extracted in 0.067 M phosphate buffer,

\[
\text{sodium dihydrogen orthophosphate} \quad 1.04 \text{ gm} \\
\text{disodium hydrogen orthophosphate} \quad 1.19 \text{ gm} \\
\text{pH} \quad 7.0
\]
mixed in the ratio 1:1. The homogenate was centrifuged at 2,000 rpm for 2-3 min to remove the debris. The supernatant was again centrifuged at 15,000 rpm for 5 min and stored at 0°C till used for experimentation.

**Estimation:** To ensure uniformity and accuracy of methodology, the enzyme extracts were first equalized with respect to their protein content. To this was added 1 ml of \( \text{H}_2\text{O}_2 \) (1%) and 1 ml benzidine solution (1.5% benzidine in
optical density was determined at 660 nm after the maximum blue colour had developed. The activity was expressed as units of peroxidase/mg protein content.

RNA content

The RNA content was estimated by the method described by Mezbaum (1939):

**Extraction:** Two ml of 3 N perchloric acid (PCA) previously cooled at 4°C was added to 5 mg pigment free material and kept for 30 min. The material was centrifuged at 2,000 rpm for 10 min and the supernatant collected. The sediment was once more extracted with PCA and the pooled supernatant used for estimation of RNA, while the residue was stored for the extraction of DNA.

**Estimation:** Two ml orcinol (1% orcinol and 0.5% FeCl₃ in conc HC1) was added to 1 ml of the supernatant and the tubes boiled for 10 min on a water bath. The optical density of the green solution was noted in a photocolorimeter at 610 nm. The RNA content was calculated using ribose as standard and expressed as mg/gm dry weight.

DNA

**Extraction:** To the residue of RNA, was added 2 ml of 3N PCA and kept in a water bath at 60°C for 1/2 hr and in this way extracted twice. The material was centrifuged at 2,000 rpm for 10 min and the supernatent collected. The DNA content was
Estimated by the method of Burton (1956).

**Estimation:** Two ml of DNA reagent (1 gm diphenyl-lamine + 1.5 ml conc sulphuric acid (AR) + 0.5 ml of CH₃CHO diluted 50 times and the total volume made to 100 ml with glacial acetic acid) was added to 2 ml of the supernatent and the tubes boiled for 30 min on a water bath. The optical density of the bluish-green solution was noted at 610 nm and the DNA content was calculated using deoxyribose as standard.

**Polyacrylamide gel electrophoretic studies of water soluble proteins and peroxidases**

Electrophoresis was carried out on 10% polyacrylamide gels at 4°C by the method of Omstein (1964), Davis (1964) and Brewbaker et al. (1968) with some modifications. 9.0 cm long tubes were used having 7.0 cm gel and 2.0 cm space for loading the extract.

**Preparation of gels:** The following stock solutions were prepared in distilled water and stored in refrigerator at 15°C till used:

**Solution A**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 N HCL</td>
<td>48 ml</td>
</tr>
<tr>
<td>Tris</td>
<td>36.6 gm</td>
</tr>
<tr>
<td>B-dimethylaminopropionitrile</td>
<td>0.23 ml</td>
</tr>
<tr>
<td>Water to make up</td>
<td>100 ml</td>
</tr>
<tr>
<td>pH</td>
<td>8.9</td>
</tr>
</tbody>
</table>
Solution B

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide</td>
<td>28 gm</td>
</tr>
<tr>
<td>Bis-acrylamide</td>
<td>0.735 gm</td>
</tr>
<tr>
<td>Water to make up</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Solution C

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Riboflavin</td>
<td>4 mg</td>
</tr>
<tr>
<td>Water to make up</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

Solution D

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium persulphate</td>
<td>0.14 gm</td>
</tr>
<tr>
<td>Water to make up</td>
<td>100 ml</td>
</tr>
</tbody>
</table>

The above solutions were allowed to warm up to room temperature before use and mixed in the following ratio:

Solution A 3 parts
B 9 parts
C 6 parts
D 6 parts
Water 3 parts

The mixture was poured into glass tubes (0.5 cm x 9.0 cm) fitted with rubber stoppers. The top portions of the tubes were layered gently with distilled water to eliminate any meniscus and to obtain a flat smooth gel surface. The tubes were placed before a fluorescent tube for 15 min to allow photopolymerization. When the gels were set, these were removed from the stoppers and were stored in distilled water at 15°C till used.
Electrophoretic apparatus: Two plastic chambers were placed one above the other, so that the base of the upper fitted into the rim of the lower chamber. Eight gel tubes were fitted in the base of the upper chamber, with the help of bored rubber stoppers in such a manner that they dipped in the buffer solution (0.20 M boric acid and 0.025 M LiOH) of the lower chamber. Two platinum electrodes were provided, cathode in the upper and anode in the lower chamber. The electrodes were connected to an electrophoretic power supply unit (Model Toshniwal, CM 01/02 Sr. No. 126). A constant current of 5 mA/tube was passed through the electrode chambers during the electrophoretic run.

Electrophoretic run: Each gel tube was fed with a calculated quantity of the extracted material (1,000, 2,000 or 5,000 µg protein equivalents as required) with the help of a pipette. The material was gently layered with 2-3 drops of 60% urea or sucrose solution. The upper chamber was also filled with the tank buffer (as in lower chamber) with the help of a pipette. A few drops of marker dye (Bromophenol 2%) were added to mark the solvent front. The current was passed for 3-4 hrs, till the dye reached the lower limit of the gel tubes.

Removal of gels: The gels were then removed from their respective tubes by rimming them with the help of a clean
needle that was inserted and moved slowly in between the gel and the tube. During the process, the tubes were kept in a trough filled with ice cold water to avoid entry of air bubbles which hinder the process.

**Staining for proteins:** Properly labelled test tubes were half filled with T.C.A. (10%) and the gels were suspended in it for 15 min. TCA was then replaced by the staining solution (coomisie blue 2% in 7% glacial acetic acid) and the gels were left in it for 8-10 hrs.

**Destaining:** The gels were then kept in the destaining solution (7% acetic acid) to remove the excessive dye. The destainer was changed after every one hr, for about 15 days till the bands became clear and distinct.

**Staining for peroxidase isoenzymes:** The gels were suspended in 1:1 benzidine (1.5% in 25% acetic acid) and H₂O₂ (1%) solution and incubated at 30°C for few min. The blue bands that turned brown with time, indicated the position of peroxidase bands. The Rf values were recorded immediately.

**Calculations:** The Rf values were calculated by inserting the gels in a 10 ml measuring cylinder and noting the position of the solvent front, as well as the bands, against an fluorescent tube, and calculated as follows:

\[ Rf = \frac{\text{Distance travelled by the band}}{\text{Distance travelled by solvent front}} \]
Figs. 1-2: Mean maximum and minimum temperatures and day-lengths during the period of experimentation (April, 1975 - March, 1976).