MATERIAL AND METHODS

WORK PLAN

Description of Experimental Sites:

Ahmedabad city, one of the biggest textile centers, is the sixth largest city of India, where textile industries, power plants and high vehicular activity contribute varying degrees of pollution in the city. The city is situated on 23.1° North longitude and 72.27° East longitude and comes under arid and semiarid zones of India.

For the present study three areas are chosen:

1. University area is free from industries and has low vehicular activity and hence is selected as "Low Pollution - LP" area i.e. control.

2. Considering the high traffic density, the area at Ashram road, besides income tax cross roads has been selected for automobile exhaust pollution study (High Pollution area, HP₁). At peak hours, frequency of passing vehicles was calculated as 8000 vehicles/hour.

3. Sabarmathi power station, which is located on the western banks of Sabarmathi river is selected as the third area (High Pollution - HP₂).
The coal consumption of Sabarmathi power plant is 4000 tonnes/day to generate 430 MW of electricity and emits 700 tonnes of fly ash.

According to power house authorities, the coal contains 0.5% sulphur, 20-25% ash and the quality is 5000 - 5100 K Cal./Kg. There are 7 stacks of 90m. height from which gaseous and particulate emission occur. Emissions include particulates emission of 150 g/sec., $SO_2$ emission of 35-40 ppm and other gases of 3-4 ppm.

Selection of Plants:

Three plants, *Azadirachta indica* A. Juss., *Ficus glomerata* Roxb. and *Nerium odoratum* Soland., growing commonly in LP area as well as HP1 and HP2 areas were selected for the study. These are dominant species and are distributed prominently throughout Ahmedabad city.

*Azadirachta indica* A. Juss: Belongs to family Meliaceae. It is 10-15 m tall, evergreen tree with compound leaves. Leaflets 7-17 in number, measure 4-6 x 15.2 cm. falcate, ovate-lanceolate and glabrous.

*Ficus glomerata*. Roxb.: Belongs to family Urticaceae. It is a 10-15 m tall evergreen tree. Simple leaves of 5-15 x 2.5 - 6.5 cm size. Leaves are ovate to ovate-oblong or elliptic-lanceolate and glabrous.
*Hemium odorum* Soland: Belongs to family Apocynaceae. 2-3 m tall, evergreen shrub, leaves 5.5 - 20 x 0.7 - 2.5 cm in size, linear-lanceolate or elliptic-lanceolate and hairy beneath.

**Sampling**

Fully expanded mature leaves from the height of 3-4 meters of the tree were collected in polythene bags from all the three areas. This sampling was made at a monthly frequency from November 1986 to October 1987.

With the collected leaf samples, the following studies were carried out every month: Dust fall on leaves, percentage foliar injury, photosynthetic pigments and biochemical parameters.

Trace metal study, leaf epidermal study and histochemical localization were made during winter season.

Based on the prevailing meteorological conditions in different seasons, the degree of pollution changes. Considering this, the experimental period is divided into three seasons (Winter, Summer and Monsoon). Winter includes average of November, December, January and February; summer includes March to June and Monsoon includes July to October months.
EXPERIMENTAL PROCEDURES

pH of leaf and soil samples

Leaf samples were collected from all the three sites of all the three plants. After washing carefully with distilled water, leaf sample of 5 g was crushed in 50 ml deionized water and the pH of the leaf extract was read with a digital pH meter, using a glass electrode.

Soil Analysis

Soil samples at each site were collected from 10 cm. depth. Fly ash also was collected from the deposits of thermal power-house. These were composited, air dried, powdered and stored in polythene bags for later determination of pH.

A 20g soil powder was mixed with 100 ml of deionized water in a 500 ml conical flask. The mixture was shaken for 30 minutes on a horizontal shaker. The suspension was filtered through a Whatman No. 44 filter paper and pH was determined with a digital pH meter, using a glass electrode.

Trace Metals And Major Elements of leaf and soil samples

Leaves of all the three plants were collected separately from different areas. After carefully washing three to four times with distilled water and lastly with double distilled water, all the leaves were dried separately at 70°C for 48
hours and then ground in mortar. The wet digestion modified method, described by Garty and Fuchs (1982) was followed. Powdered 500 mg leaf material was digested in Kjeldhal flask (50 ml capacity) with triple acid i.e. concentrated HNO_3, H_2SO_4 and HClO_4 in the ratio of 10:0.5:2, at low temperature to minimize the loss of metals. The digested material was transparent and colourless. Perchloric acid treatment was given to remove the black residues. The digested material was dissolved in 1 M HNO_3 and final volume was made to 10 ml. For blank 10 ml triple acid was evaporated and residue was dissolved in 1 M HNO_3 and final volume was made to 10 ml. The samples were analysed on Varian Atomic Absorption Spectrophotometer with GTA - 96, Spectra A.A. 20. The reading of blank was deducted from the samples reading. The results of Zn, Fe, Mn, Cu were expressed in concentration in ppm. and results of Na, K, Ca, Mg were expressed in % dry weight.

For determination of Zinc, Iron, Manganese and Copper in all the soil sample, method of Lindsay (1972), was followed. Known amount of soil (10g) was shaken with a 20 ml of extractant (the extractant consists of 0.005M diethylene-triaminepenta acetic acid (DTFA), 0.01M calcium chloride and 0.1 M triethanol-amine buffered at pH 7.3) for two hours and was filtered. The concentrations of zinc, iron, manganese and copper were determined directly on the extracts by atomic absorption spectrophotometry.
For determination of exchangeable cations, Ca, Mg, K and Na in all the soil samples method of Thomas (1962), was followed. A 10 g of air-dried soil was taken (in a 125 ml flask) and 40 ml of 1N Ammonium acetate was added to it. Then it was swirled and kept for 1 hour. The soil solution was transferred to 5.5 cm Buchner funnel filled with No. 42 Whatmann filter paper and connected to a 250 ml suction flask. The extract was poured into a 250 ml volumetric flask and made up the volume by rinsing the suction flask with fresh 1N Ammonium acetate. The concentration of Ca, Mg, K and Na were determined by Varian Atomic Absorption Spectrophotometer with GTA - 95, Spectra A. A. 20.

The instrumental range was established by using triple distilled water as "low standard" and 100 ppm of standard solution of element, for "High standard". The results were expressed in % dry weight.

Following wavelengths were used.

<table>
<thead>
<tr>
<th>Element</th>
<th>Wavelength in Å</th>
</tr>
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<tbody>
<tr>
<td>Na⁺</td>
<td>5890</td>
</tr>
<tr>
<td>K⁺</td>
<td>7665</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>4227</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>2852</td>
</tr>
</tbody>
</table>

Surface Deposit Determination.

The method described by Lal and Ambasht (1980) was followed for the determination of surface dust deposit.
From each plant, ten matured leaves at random were collected in a clean polythene bags so as to avoid loss of dust from the leaf surface. The leaves were transferred to a 500 ml conical flask with 200ml double distilled water. The dislodged particles in collection bags were also removed by washing the bags with double distilled water. This water was also transferred to conical flask and was shaken in a mechanical shaker. The water was filtered through a Whatman filter paper No. 1 and 100 ml of double distilled water was again added to rinse the conical flask and leaves. The water was filtered again with the same filter paper. The filter paper was weighed after drying in an oven set at 60°C to determine the quantity of dust deposit on the leaf. Individual total leaf area and injured part (visible as brown, red or yellow in colour) in square cm. was calculated by tracing out the boundary lines of leaf on graph paper. The surface dust deposit and % foliar injury were calculated as follows:

\[
\text{Surface dust Deposit} = \frac{\text{Total weight of dust (g)}}{\text{Leaf area (cm}^2\text{)}} \times 10000
\]

\[
\text{Percent Injury} = \frac{\text{Injured leaf area (cm}^2\text{)}}{\text{Leaf area (cm}^2\text{)}} \times 100
\]

**EPIDERMAL TRAIT STUDY**

At each site, five fully expanded leaves were collected
from each plant. Following the method of Pande (1985), epidermal peels were separated. By treating 5 x 5 mm leaf strips in equal volumes of hydrogen peroxide, glacial acetic acid and water for 24 hours, upper and lower epidermal peels were obtained. The peels were stained in Safranin and mounted in glycerine for microscopic examination. Three microscopic fields were randomly selected in each peel and the mean of these was treated as one individual observation.

Frequency and size of stomata, epidermal cells and trichomes and stomatal index were measured by selecting five individual observations. The microscopic study was carried out using 40 x objective and 10 x ocular lens for stomata and epidermal cells, 10 x objective and 10 x ocular lens for the frequency and size of trichome.

The stage micrometer was mounted on the stage of the microscope. The scale was one mm, that is divided into 100 equal divisions and each division equal to 10 µ. With the help of Camera lucida (mirror type, Leitz Wetzlar, Germany) graph paper scale was calibrated against the standard micrometer scale. The area of the known square on the graph in the µ² was calculated using high power objective. Number of stomata and epidermal cells were counted in the area of square on the graph paper and following the equation of Wolf et al. (1979), stomatal index and frequency of epidermal cells and stomata were calculated.
Number of Stomata

Stomatal Index (%) \[ \times 1000 \]

Number of Stomata + Number of epidermal cells

Number of Stomata

Frequency of Stomata/mm² = \[ \times 1 \text{ mm}^2 \]

area of square on graph paper (mm²)

The Stomatal pore area was expressed in \( \mu \text{m}^2 \) and stomatal size, viz., length and width of guard cell was expressed as \( \mu \text{m} \).

Slides prepared for stomatal study were also used for determination of epidermal cell size, frequency, epidermal trichome.

Scanning Electron Microscopy

Fully expanded leaf samples were collected from area LP, HP₁ and HP₂. The samples were kept in FAA (Formal aceto alcohol) immediately after plucking from the plants and transported to the laboratory.

To obtain scanning electron micrographs, technique of Chaudhari et al. (1984) was used. Square pieces of approximately 5 mm² were taken from either side of midrib. Each leaf piece was placed between two glass slides and
tied together by rubber bands. The slides were marked with diamond pencil denoting abaxial and adaxial surfaces. Leaf samples were dehydrated in ethanol series of 30, 50, 70, 80, 90% and finally with absolute alcohol. The dehydrated sample was dried in oven at 40°C for two hours and was mounted onto an aluminium specimen stub with adhesive tape. The stub with the material was coated with gold/palladium. Specimens were scanned with Cambridge stereoscan S4-10 scanning electron microscope. Scanning electron micrographs of stomatal frequency, stomatal size, damaged/abnormal stomata, at different magnifications were taken by the camera, attached to the scanning electron microscope.

CHLOROPHYLL CONCENTRATIONS

The chlorophyll was extracted from fresh leaf samples by macerating the tissue in cold 80% aqueous acetone with a pestle and mortar. The homogenate was filtered and the residue was washed twice with 80% acetone. The mixture was centrifuged for 15 minutes. After centrifuging, the supernatant solution was decanted into a measuring cylinder and a known volume was maintained using 80% acetone. The concentration of total chlorophyll, chlorophyll 'a' and chlorophyll 'b' in the extract were then determined by measuring the absorbance at 663 and 645 nm using Systronics 106 Spectrophotometer. The chlorophyll
concentrations were calculated using the formulae derived by Arnon (1949).

\[
\text{Chlorophyll 'a' (mg/g) = 12.7 (A663) - 2.69 (A645) x } \frac{V}{Wt.} \\
\text{Chlorophyll 'b' (mg/g) = 22.9 (A645) - 4.69 (A663) x } \frac{V}{Wt.} \\
\text{Total Chlorophyll (mg/g) = 20.2 (A645) + 8.02 (A663) x } \frac{V}{Wt.}
\]

Where, \( A \) is the absorbance at 645 and 663 nm.

\( V \) is volume (ml) of chlorophyll solution

and \( Wt. \) represents fresh weight (g) of leaves.

**BIOCHEMICAL ESTIMATIONS**

**CARBOHYDRATE METABOLISM**

**Invertase activity**

Invertase activity was assayed following the method of Hatch and Glasziou (1963).

Leaf samples collected from all the three areas were washed thoroughly, first with tap water and then with double distilled water. For the assay, known amount (100 mg) of leaf tissue was macerated in cold double distilled water, centrifuged and made to a known volume (15 ml). The supernatant was used as enzyme aliquot.

Enzyme aliquot (1ml) was taken in a test tube and to it 1 ml of 0.2% sucrose (200 mg sucrose in 100 ml of double
distilled water) and 1 ml of 0.1 M citrate buffer (pH 5.5) was added. This was incubated for 30 minutes at room temperature. The enzyme activity was inactivated by adding 2 ml of 5% perchloric acid. The solution was made to a final volume with double distilled water. The amount of glucose released was determined by 'Anthrone reagent' (McCready et al., 1950). 0.5 ml of the above mixture was taken in a tube and to it was added 1.5 ml double distilled water and 4 ml of Anthrone reagent (200 mg of anthrone was dissolved in 100 ml of 95% chilled H$_2$SO$_4$). The tubes were capped with glass marbles and heated in a water bath at 100°C for 7 minutes and immediately cooled in ice bath. The absorbance was read at 660 nm using red filter on Systronics 106 Spectrophotometer. Regression analysis was carried out by using different concentrations of glucose and was calculated using the regression equation.

$$y = 302.55x - 5.57$$

The results were expressed as mg glucose released/ hour/ g fresh weight

**Sugars**

Sugars were determined by the method of McCready et al. (1950).

Oven-dried plant material was weighed and boiled in 80% ethanol for 4-5 minutes, homogenised with sand and kept overnight in the fridge. After centrifugation, residue was
again extracted with ethanol. The supernatant was used as ethanol extract.

A known volume (10 ml) of ethanol extract was evaporated and the residual sugars were dissolved in a known amount (10 ml) with double distilled water.

From the 10 ml of the extract, 5 ml each was taken for reducing and total sugars. To the extract of total sugars, 5 ml 1N hydrochloric acid (0.75 ml of concentrated hydrochloric acid in 91.25 ml double distilled water) was added and kept in boiling water bath for 20 minutes to hydrolyse non-reducing sugars. It was cooled and neutralised by adding 5 ml of 1N sodium hydroxide.

The rest of the aliquot volume (5 ml) was used to determine the reducing sugars using 'Anthrone reagent'. Solution to be analysed and 'Anthrone reagent' were mixed in 1:2 ratio in ice cold condition and was boiled in a boiling water bath for 7 minutes. The furfural groups of sugar react with anthrone to form a green complex. The absorbance of this coloured complex was read out on Systronics Spectrophotometer 106 at 660 nm. By referring to the regression equation of different concentration of glucose,

\[ x = 302.55y - 5.57 \]

amount of reducing and total sugars were calculated and expressed as \( \mu g/\text{mg dry weight} \).
PROTEIN METABOLISM

Protease activity

The method of Penner and Ashton (1967) was used for the determination of protease activity.

Fresh weighed plant material was ground in ice cold distilled water and centrifuged. The supernatant was collected and a known amount of aliquot (1 ml) was taken and 2 ml of 0.5% casein solution (adjusted to pH 7.0) and 3 ml of 0.1 M phosphate buffer (pH 7.0) was added. This was incubated at room temperature for one hour. Later, the activity was terminated by adding 2 ml of 15% trichloroacetic acid in 2 ml of enzyme reaction mixture. After 20 minutes, the precipitated proteins were discarded by centrifugation. The liberated tyrosine in the supernatant, was estimated using Folin phenol Reagent [Lowry et al., (1951)].

1 ml of the supernatant was taken in a tube and to it 4 ml (0.5N) sodium hydroxide was added. After 30 minutes, 1.2 ml of Folin phenol reagent was added and to stand for 5 minutes for colour development. The extinction value of the reaction mixture was read at 660 nm using red filter on Systronics Spectrophotometer 106. The regression equation $X = 87.96y - 0.161$ was prepared by using known concentrations of tyrosine. Protease activity was expressed as mg tyrosine liberated/hour/gm fresh weight.
Protein

A weighed amount of oven-dried plant material was ground in 80% ethanol and extracted twice. The residue was first washed with cold 5% perchloric acid (to remove sugars and soluble protein fractions), centrifuged and then it was washed with the mixture of ethanol:ether:chloroform in the ratio of 2:1:1: (to remove acid soluble fractions and lipids) and centrifuged. The protein fraction was dissolved in 1N sodium hydroxide and kept for one hour and then centrifuged. The supernatant was made up to a known volume and 5 ml of 'Lowry reagent C' was added (prepared by mixing 50 ml Reagent 'A' which was 2% sodium carbonate in 0.1N sodium hydroxide and 1 ml of Reagent B which is 0.5% copper sulphate in 1% sodium-potassium tartrate) and incubated at room temperature for 30 minutes. The colour was developed by adding 0.5 ml 'Folin- phenol reagent' and incubated for 10 minutes at room temperature. The extinction value was read at 660 nm on Systronics Spectrophotometer 106. The following regression formula was prepared by using known concentrations of Bovine Serum Albumin (Sigma)

\[ X = 378.13 \times y - 8.56 \]

The protein content was expressed \( \mu g /mg \) dry weight.

Amino acids

The content of amino acids was determined following the method of Harding and Maclean (1916).
Oven dried plant material was weighed and boiled in 80% ethanol for 4-5 minutes, homogenised with sand and kept overnight in the fridge. After centrifugation, residue was again extracted with ethanol. The supernatant was used as ethanol extract.

A known (1ml) ethanol extract of the leaf material was taken and 1ml of 10% pyridine and 1 ml of ninhydrin reagent were added to it. The test tubes were capped with marbles and heated in hot water-bath at 100°C for 30 minutes. Later it was cooled and diluted with double distilled water to a final volume of 10 ml. The absorbance of the violet blue colour was read at 570 nm. The following regression formula was prepared using different concentrations of isoleucine as a standard.

\[ X = 88.99y + 4.83 \]

The amino acid content was expressed as μg/mg dry weight.

RNA METABOLISM

RNAse activity

The method described by Peach and Tracy (1964) was employed to assay RNAse activity.

Leaf samples collected from all the three sites were washed thoroughly with double distilled water. Known amount (100 mg) of leaf material was macerated in cold double distilled water, centrifuged and made to a known volume (15 ml). The supernatant was further processed for the enzyme
activity as enzyme aliquot.

The assay system consisted of known (1 ml) volume of enzyme aliquot 1 ml of 0.4% RNA solution (400 mg RNA in 100 ml of 0.1M acetate buffer - pH 5.0) and 1 ml 0.1M acetate buffer. The reaction was carried out for 30 minutes, at room temperature. The enzyme activity was inactivated by adding 1 ml of 0.75% uranyl acetate (750 mg uranyl acetate in 100 ml of 25% perchloric acid). The content was centrifuged for 15 minutes. The amount of ribose released was determined by 

Orcinol reagent (Markham, 1955).

To a known volume (0.5 ml) of reaction mixture, 6 ml of 'Orcinol reagent' (10 ml of 1% orcinol, 1 ml of 10% ferric chloride and 40 ml of concentrated hydrochloric acid) was added and heated in a boiling water bath for 10 minutes. The absorbance was read at 660 nm on Systronics Spectrophotometer 106. The following regression equation was prepared by using known concentrations of Ribose Sugar:

\[ V \times 100 - 32 y + 1.276 \]

The results were expressed as mg ribose released/hour/ g fresh weight.

Ribonucleic acid

The method described by Bonner and Zeevart (1962) was followed for the estimation of ribonucleic acid.

Weighed, oven-dried plant material was crushed in 80% ethanol. After centrifugation, the residue was washed with
5% perchloric acid to remove acid soluble substances, then ethanol:chloroform:ether (2:1:1) and finally with 1M trichloroacetic acid. The residue was dissolved in 0.3 M potassium hydroxide and incubated at 37°C for 42 hours. Later it was centrifuged. The supernatant was adjusted to pH 3.0 and made to known volume. This was used as aliquot. RNA was analysed by the method of Markham (1955).

To a known volume of aliquot 6 ml of 'Orcinol reagent' was added and heated in boiling water bath for 10 minutes.

The absorbance was read at 660 nm on 'Systronics Spectrophotometer' 106. Regression analysis was carried out by using different concentrations of RNA.

\[ y = 229.44x + 3.153 \]

The results were expressed as µg/mg dry weight.

Free Proline

The method described by Bates et al. (1973) was followed to estimate free proline content.

Weighed, oven-dried plant material was extracted in 3% sulphosalicylic acid. After centrifugation 2 ml of aliquot was taken and to that 2 ml of acid ninhydrin reagent and 2 ml of glacial acetic acid were added. (Acid ninhydrin reagent was prepared by dissolving 1.25 g of ninhydrin in 30 ml of glacial acetic acid and 20 ml of 5 M phosphoric acid 76 ml of ortho phosphoric acid in 124 ml double distilled water). The tubes were stoppered and the reaction mixture was allowed to
boil in water bath at 100°C for 1 hour, and the reaction was terminated in ice bath. The reaction mixture was extracted against 6ml toluene with a separating funnel. The absorbance of the toluene extract was read at 520 nm against a toluene blank. The regression formula

\[ X = 61.48y + 0.122 \]

was prepared by using known concentration of proline and proline content was expressed as µg/mg dry weight.

**TOTAL PHENOLS**

Total phenols were determined by the method described by Farks and Kirly (1962).

Oven-dried plant material was weighed and boiled in 80% ethanol for 4-5 minutes, homogenised with sand and kept overnight in the fridge. After centrifugation residue was again extracted with ethanol. The supernatant was used as ethanol extract.

The reaction mixture containing known volume (0.5 ml) of the ethanol extract, 1 ml of 20% sodium carbonate and 0.5 ml 'Folin - Phenol' reagent was heated on a water bath for 10 minutes and cooled. Final volume was made by diluting it with double distilled water. It was filtered using whatman filter paper No.1 to remove the pre-precipitate. The absorbance of the blue coloured filtrate was read at 560 nm. A regression equation was prepared by using different concentrations of gallic acid.
\[ x = 95.47 \ y + 3.00 \]

The phenolic content was expressed as \( \mu g/mg \) dry weight.

**OXIDIZING ENZYMES**

**Peroxidase activity**

George (1953) and Maehly's (1954) method was employed to assay peroxidase activity.

Leaf samples collected from all the three sites were washed thoroughly first with tap water and then with double distilled water. For the assay, known amount (100 mg) of leaf tissue was macerated in cold double distilled water, centrifuged and made to a known volume (15 ml). The supernatant was further processed for the enzyme activity as enzyme aliquot.

The reaction mixture consisted of known volume of enzyme extract, 2 ml of 0.2 M phosphate buffer (pH 7.0) and 2 ml 20 mM guaiacol reagent (0.22 ml guaiacol in 100 ml double distilled water was prepared 24 hours before carrying out the activity) was taken in a cuvette. The cuvette was placed in the spectrophotometer. The wavelength was adjusted to 470 nm and extinction value was noted. 2 drops of 10 mM hydrogen peroxide (0.4 ml hydrogen peroxide (20 volumes) in 9.6 ml double distilled water) was added to the reaction mixture in the cuvette and again the extinction value was noted after 30 seconds. The change in the absorbance of oxidized hydrogen peroxide was used to calculate peroxidase
activity. Peroxidase activity was expressed as O.D. difference/minute/mg fresh weight.

HISTOCHEMICAL STUDIES

Fresh leaf samples (5 x 5 mm) were fixed in Carnoy's fixative (absolute alcohol, chloroform and glacial acetic acid in the ratio of 6:3:1) for three hours at room temperature for studies of insoluble polysaccharides, ribonucleic acid and total proteins.

The fixed material was washed with two changes of 70% alcohol and were proceeded for dehydration through ethanol-tertiary butyl alcohol series (TBA series from 10% to 20% ... 100%). Paraffin infiltration was done in an oven at 60\(^\circ\) C. Paraffin embedding of the material with proper labelling was done in paper embedding boats.

Paraffin embedded blocks were kept in refrigerator at 10\(^\circ\) C. After proper trimming of the blocks, all sections were cut uniformly at 10 \(\mu\). The paraffin ribbons were affixed to the slides with the help of egg albumin.

The staining techniques adopted for localization of insoluble polysaccharides, total protein and RNA are as follows.

**Insoluble polysaccharides**

Periodic acid - Schiff's (PAS) reaction of Hotchkiss (1948) as detailed by Jensen (1962) was followed.
The sections were deparaffinized in xylene and were hydrated through ethanol:water series. The hydrated sections were treated with 0.5% periodic acid (w/v) for 30 minutes at room temperature, after testing for different periods for its colouristic purity. The sections were washed under running tap water for 10 minutes and then placed in Schiff's reagent at room temperature for 10 minutes. Extra stain was washed off in 2% potassium metabisulphite (w/v) for 2 minutes. The slides were again washed in water and dehydrated in ethanol series and mounted in DPX.

Total Proteins:

‘Mercuric bromophenol blue’ method of Mazia et al. (1953) was employed.

The deparaffinated sections were hydrated in ethanol series and were placed in ‘Mercuric Bromophenol Blue’ reagent at room temperature. The sections were washed under running tap water and were placed in 0.5% glacial acetic acid to remove excess stain. Again the sections were washed in tap water for less than a minute. The blue coloured sections were dehydrated, cleaned in xylene and mounted in DPX. Absorbance was measured at 590-620 nm using a red filter.

Ribonucleic acid

The RNA was stained with Pyronin-G reagent as described by Tepper and Gifford (1962).
Deparaffinized and hydrated sections were placed in Pyronin-G dye for seven minutes at room temperature. The slides were rinsed in distilled water and dried with blotting paper. N-butanol was used for dehydration of the sections and to remove excess stain (less than a minute). The slides were air dried and then cleared in xylene for 2 hours and mounted in DPX. Absorbance was measured with green filter (500-570 nm).

Cytophotometry

Since all the histochemical techniques employed in this study are specific and stoichiometric, relative content per cell and concentration of the metabolites per unit area were determined with a cytophotometer.

For cytophotometer, the instrument used to record the absorbance of the stained sections, the necessary light was supplied with a Kohler illumination source equipped with 15W, 6V tungsten filament bulb. The transmission was measured by a light dependent resistor (LDR) connected to an ammeter.

The extinction values and content per cell were calculated by the following formulae (Shah et al., 1975).

\[ E = \log_{10} I_0 - \log_{10} I_s = KCL \]

where,

- \( I_0 \) = absorbance of control section
- \( I_s \) = absorbance of stained section
- \( K \) = extinction coefficient
C = concentration of the chromophore
L = thickness of the section

Content per cell = extinction value \times cell area.

\[
\text{Concentration per unit area} = \frac{\text{extinction value}}{\text{LDR exposed area}}.
\]

Micrometry

Stage micrometer scale was calibrated by sketching it on a graph paper by observing under microscope fitted with a Camera Lucida.

The cell area of the leaf sections were sketched without changing the angle of Camera Lucida.

The cell area of different zones was calculated from the calibrated micrometer value.

**STATISTICAL ANALYSIS:**

Statistical analysis was carried out according to the method given by Sendeoor and Cochrane (1967.) to determine the significance of the data. Student 't' test was applied to LP & HP₁ and LP & HP₂ and the significance of difference is shown in the tables at 5% and 1% levels.