MATERIALS AND METHODS
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The design of the laboratory experiments and plan of work are as follows:

The plant selected for the present investigation to study/understand the incompatibility and pollen-pistil interaction is, Consolea rubescens Lemaire, belongs to the family Cactaceae. The flower buds and flowers were collected from the botanical garden of Gujarat University, Ahmedabad. Following six different developmental stages were marked for the study.

STAGE - I: Early floral bud. Transformed vegetative apex to reproductive apex. Floral buds with small, pale greenish tepals covering all floral parts.

STAGE - II: Medium floral bud. Many pinkish yellow tepals covering the floral parts.

STAGE - III: Late floral bud. Pink coloured tepals exposed and loosely arranged.

STAGE - IV: Early anthesis. The fleshy pinkish tepals of the outer whorl emerged and anthers were exposed.
STAGE - V: At the time of anthesis. Fleshy perianth was exposed fully and the stigmatic tip exposed with protruding style.

STAGE - VI: Late anthesis. The stigma becomes dry, brown and the flower at senescent stage.

The above developmental stages were determined based upon the size and age (time before and after anthesis) of the flower buds/flowers.

Various studies were undertaken to understand,

(i) the anatomy of stigma and style;
(ii) the nature of stigmatic secretion;
(iii) the chemical nature of pollen and pistil during pollination;
(iv) the alterations of metabolites during the various stages of pollen and pistil development;
(v) the relation between the metabolites of pollen with that of the pistil; and
(vi) to find out the location (site) where the incompatibility lies.
The following techniques were used for the above study:

I. Microscopy (light, electron (SEM) and fluorescence);
II. Biochemical estimations (Table - III);
III. Histochemistry, cytochemistry and micrometry (Table - IV);
IV. Electrophoresis (proteins);
V. Atomic absorption spectroscopy (mineral elements in pollen);
VI. Chromatography (amino acids);
VII. Pollen viability tests;
VIII. Pollen germination (Table - V) and pollen leachates studies using pH meter;
IX. Cytophotometry (Quantification of metabolites-in situ);
X. Statistical analysis.

I. MICROSCOPY:

a. LIGHT MICROSCOPY:

It was used to study the anatomy of the style and stigma. The flowers of all developmental stages (I - VI) were collected and the pistil and the anthers were separated immediately and fixed in FAA for 24
hours and preserved in 70 % alcohol after repeatedly washing with 70 % alcohol. (FAA formalin : acetic acid : alcohol was prepared by mixing 90 ml. of 70 % alcohol, 5 ml. of glacial acetic acid and 5 ml. of formaldehyde 40 %). Materials were dehydrated through ethanol - xylene series infiltrated and embedded in paraffin and cut uniformly at 10µ thickness using a rotary microtome (E. Leitz Wetzler, Germany). Deparaffinized sections were stained with various dyes (Table - IV) and photomicrographs were taken using Leica Camera (DBP), Ernst Leitz GMBH Wetzler, Germany, fixed with E. Leitz Wetzler compound microscope. Konica 135/36 colour films and Kodak CP 135/36 colour films were used to record the images.

b. SCANNING ELECTRON MICROSCOPY :

The materials used for Scanning electron microscopy (SEM) were dehydrated by passing through the graded ethanol series. The dehydrated materials were mounted on the stubs with double side adhesive tape. Metal coating was given using Gold - Palladium (Au - Pd) at 10 nm thickness. These stubs with samples were examined under Scanning electron microscope (Cambridge Stereoscan S4 - 10). The desired pollen grains and stigma portions were
BLOCK DIAGRAM OF SCANNING ELECTRON MICROSCOPE

F  - Filament
A  - Anode
C.L. - Condenser lens
M.C. - Magnification control
O.L. - Objective lens
P&AC - Photomultiplier and Amplification Circuit
S.C. - Scan Generator
C  - Collector
S.P. - Specimen
S.C - Scanning coils

Fig.1
photographed using Indu 120 films (125 ASA/22 DN, Black and white). The block diagram of SEM is given in Figure - 1.

c. FLUORESCENCE MICROSCOPY:

To study the presence of callose and cuticle on the pistil and pollen fluorescence microscopic study was employed using

(i) aniline blue and

(ii) auromine O respectively.

Fresh sections were also stained with rhodamine B for lipids at the time of stigma receptivity. For these fluorescence microscopic study stained sections were observed under Carl-Zeiss epi-fluorescence microscope fitted with HBO-50 mercury lamp using filters of various wavelengths and types as under:

<table>
<thead>
<tr>
<th>Excitation filters - G</th>
<th>Barrier filters - BP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long pass filters - LP</td>
<td></td>
</tr>
<tr>
<td>1. G 365</td>
<td>BP 436</td>
</tr>
<tr>
<td>FT 365</td>
<td>FT 460</td>
</tr>
<tr>
<td>LP 420</td>
<td>LP 470</td>
</tr>
</tbody>
</table>
(i) LOCALIZATION OF CALLOSE:

ANILINE BLUE METHOD:

(Currier, 1957; Smith and McCully, 1978 a,b)

Aniline blue is a fluorochrome most widely used in plant tissues, which in low concentrations imparts a yellow fluorescence to various wall features, particularly sieve plates, new cell walls, cell plates and all areas considered to contain the β-1, 3-glucan known as callose (Smith and McCully, 1978 b). The exact nature of the fluorochrome and its reaction
with cell walls has not been established. However, Smith and McCully (1978b) have shown that the dye binds to isolated samples of a variety of P-1,3 and 1,4 - linked glucans. The purified fluorochrome binds strongly to all regions of primary cell walls and also to lignified walls as well as regions known to contain a high proportion of callose. Crude aniline blue also binds weakly to all cell walls but the intense fluorescence in callose rich areas may be due to more ready penetration of the fluorochrome into these regions than into other regions of the wall. The apparent specificity for callose may also be due to interference with the fluorochrome by other components of the crude dye which binds to cell wall region not containing callose. Fluorochrome binding to callose is unaffected by prior periodic acid - Schiff's reagent treatment.

The staining procedure is as follows:

1. Paraffin embedded material was used.
2. Sections were deparaffinized in xylene and hydrated through ethanol - distilled water series.
3. Hydrated sections were stained in 1 % aqueous aniline blue for one minute.
4. Sections were cleared repeatedly in distilled water.

5. They were mounted in glycerine and observed under fluorescence microscope.

The callose containing sites appeared as fluorescent white.

(ii) CUTICLE:

AUROMINE O METHOD:

(Heslop - Harrison, 1977; Malti and Shivanna, 1984)

Cutin in the cuticle was studied employing the fluorescent stain auromine O (Romall chemicals) as suggested by Heslop - Harrison (1977).

The staining procedure is as follows:

1. Sections were deparaffinized in xylene and hydrated through ethanol - distilled water series.

2. Lipids were removed by treating the hydrated sections in chloroform for five minutes.

3. Sections were stained in 0.01 % aqueous auromine O for 10 - 15 minutes.
4. Sections were cleaned in distilled water, mounted in glycerine and observed under fluorescent microscope. Cuticle showed bright fluorescence.

For control, material was pretreated with ether-methanol (1:1 v/v) before staining in auromine O to remove the cuticle.

(iii) TOTAL LIPIDS:

RHODAMINE B METHOD:

(Gahan, 1984)

Papillar secretion present at the time of pollination was tested for total lipid content using 1% aqueous rhodamine B solution.

STAINING PROCEDURE:

Fresh stigmatic heads were harvested and thin hand sections were taken. Staining was done in 1% aqueous rhodamine B solution for 30 minutes. Mounted in 1:1 glycerine-distilled water and examined under UV light fluorescence microscope.
II. BIOCHEMICAL ESTIMATIONS:

A. METABOLITES:

Changes in the concentration of various metabolites viz., protein, free amino acids, RNA, DNA, total sugars, reducing sugars and phenols were estimated in pollen and stigma during all the six developmental stages by employing the standard biochemical methods (Table - III).

PROCEDURES:

1. PROTEIN:

(Lowry et al., 1951)

Weighed plant material (dried anthers and stigma) were crushed in 80% ethanol and centrifuged. The residue was washed first in 5% perchloric acid (to remove sugars and soluble nitrogen fractions) and secondly in a mixture of ethanol : ether : chloroform (2 : 1 : 1) to remove acid soluble fractions and lipids. Then the residue was suspended in 1M trichloroacetic acid (cold), centrifuged and the residue was dissolved in 1N NaOH and kept for one hour at room temperature. After centrifuging the mixture, the supernatant was collected and made up to a known volume and used as aliquot. To a known volume of aliquot 5 ml. of Lowry reagent C (prepared by mixing
<table>
<thead>
<tr>
<th>Sr. Metabolite No.</th>
<th>Reagents used</th>
<th>Final colour of the product</th>
<th>Wave length used to measure optical density (nm)</th>
<th>Architect of the technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>3. DNA</td>
<td>Diphenylamine reagent</td>
<td>Blue</td>
<td>660</td>
<td>Burton, K. (1956)</td>
</tr>
<tr>
<td>5. Reducing sugars and total sugars</td>
<td>Nelson-Somogyi reagent</td>
<td>Greenish yellow</td>
<td>540</td>
<td>Warton and McCarty (1972)</td>
</tr>
<tr>
<td>7. Protease</td>
<td>Folin reagent</td>
<td>Blue</td>
<td>660</td>
<td>Penner, D. and Ashton, F.M. (1951)</td>
</tr>
<tr>
<td>8. Peroxidase</td>
<td>Quaicol-H$_2$O$_2$ reagent</td>
<td>Yellowish brown</td>
<td>470</td>
<td>George (1953) and Maehly (1954)</td>
</tr>
</tbody>
</table>
50 ml. of reagent A - 2 % sodium carbonate in 0.1 N NaOH and 1 ml. of reagent B - 0.5 % copper sulphate in 1 % sodium potassium tartarate) was added and incubated for 30 minutes at room temperature. In control, the aliquot was replaced by distilled water. The characteristic blue colour was developed by adding 0.5 ml. of 1N Folin-phenol reagent to the mixture and incubating it for 10 minutes at room temperature (Lowry et al., 1951) Optical Density (O.D) was read at 660 nm on a Spectrophotometer (Systronics - 105, India). Protein content was calculated by using the following formula prepared by using Bovin serum albumin as standard protein and the results are expressed in µg protein/mg dry weight.

Concentration (X) = 505.25 x O.D. (Y) - 5.08.

2. FREE AMINO ACIDS:

Free aminoacid content of the pollen and stigma samples were determined by employing Lee and Takahashi's (1966) method. 0.5 ml. of the ethanol extract of the samples was mixed with ninhydryn reagent (a mixture of 1 % ninhydrin in 0.5 M citrate buffer of pH 5.5 in a ratio of 5 : 12 : 2) in a test tube. The tube was then stoppered and heated in a boiling waterbath for 12 minutes to develop a violet
blue colour to the mixture. It was then cooled, diluted to a known volume with distilled water and O.D. was read at 570 nm. In control sets, the ethanol extract of plant material was replaced by 1 ml. of distilled water. The following regression formula prepared by using glycine as standard, was used for calculating the free aminoacid content and the results were expressed as μg. free aminoacid/mg. dry weight.

\[ X = 413.42Y + 19.23 \]

Where \( X \) = Concentration and \( Y \) = Optical density

3. DNA:

DNA was estimated by the method of Burton (1956).

Weighed plant material was crushed in 80% ethanol and extracted twice. The residue was washed first with cold 5% perchloric acid and secondly with a mixture of ethanol, ether and chloroform (2:1:1 v/v) to remove acid soluble fractions and lipids. The residue was then mixed with 1M cold trichloro acetic acid and incubated for 42 hours in freeze and centrifuged. The residue was then mixed with 10 ml. of 1M cold perchloric acid, stirred well for 10 minutes at 90°C, cooled and centrifuged. The supernatant was used as aliquot.
To a known volume of aliquot (2 ml.) 4 ml. of diphenyl amine reagent was added and the content was incubated for 18 hrs. at 28 - 30°C. The optical density of the blue coloured solution was read in a Spectrophotometer - 103 (Systronics, India) at 660 nm. The following regression formula was used and the results were expressed as μg. DNA/mg. dry weight.

\[
X = \frac{6.676}{Y - 2.1884} - \frac{0.002}{0.002}
\]

Where \(X\) = Concentration and \(Y\) = Optical density

**DIPHENYLAMINE REAGENT:**

6 gm. of pure diphenylamine (BDH, India) was dissolved in 460 ml. of glacial acetic acid and 6 ml. of Con. \(H_2SO_4\). For each 20 ml. of reagent 0.1 ml. of acetaldehyde was added, which was prepared by mixing 1 ml. acetaldehyde and 9 ml. of distilled water.

4. **RNA:**

RNA was extracted following the procedure of Bonner and Zeevaart(1962).
Weighed plant material was crushed and extracted with 80% ethanol to remove all the sugars and the residue was washed with 5% perchloric acid to remove acid soluble substances. Perchloric acid and phospholipids were removed with two changes of ethanol : ether : chloroform (2 : 1 : 1) mixture. The residue was dissolved in 0.3 M potassium hydroxide and incubated at 37°C for 42 hours and centrifuged. The supernatant was adjusted to pH 3.0 and made to a known volume. This was used as aliquot. RNA was estimated by orcinol method. To a known volume of aliquot 5 ml. of reagent was added and the stoppered tubes were heated for 10 minutes on a boiling water bath. In control the aliquot was replaced by distilled water. After cooling, the O.D was read at 660 nm.

RNA content was calculated using the following regression formula,

\[ X = 96.05 \times Y - 0.35 \]

Where \( X = \) Concentration and \( Y = \) Optical density expressed as \( \mu g. \) RNA/mg. dry weight
ORCINOL REAGENT:

Orcinol reagent was prepared by mixing 1 % orcinol, concentrated hydrochloric acid and 10 % ferric chloride in the ratio 10 : 40 : 1.

5. REDUCING SUGARS:

Reducing sugar content of the samples were estimated by employing Nelson-Somogyi reaction (Warton and McCarty, 1972).

Ethanol extract of the sample was kept in boiling water bath for 4 to 5 minutes to evaporate the alcoholic fraction and made to a known volume (20 ml.) with distilled water. From this 10 ml. was taken in a test tube and added 1 ml. 25 % lead acetate followed by 1 ml. of 25 % sodium carbonate. The volume was then made up to 20 ml. with distilled water and filtered. 1 ml. of the filtrate (aliquot) was mixed with 1 ml. of Nelson-Somogyi reagent (prepared by mixing A* and B** in 50 : 2 ratio). The test tubes were stoppered and heated at 100°C on a water bath for 20 minutes. It was then cooled rapidly; added 1 ml. of arsenomolybdate***, and mixed thoroughly by shaking the tubes for 5 minutes to dissolve the red precipitate. Final volume of the
resultant greenish yellow coloured solution was made upto 25 ml. with distilled water and the optical density was recorded at 540 nm. The following regression equation was prepared by taking different concentration of glucose as standard to calculate and expressed the concentration of reducing sugar in μg/mg dry weight.

Concentration (X) = 426.67 x Optical density (Y) - 15.25

* Solution 'A' was prepared by dissolving 6.25 gm. sodium carbonate; 6.25 gm. sodium potassium tartarate; 5 gm. sodium hydrogen carbonate and 50 gm. sodium sulphate in 250 ml. of distilled water.

** Solution B was prepared by mixing 15 gm. of copper sulphate in 100 ml. of distilled water and few drops of concentrated sulphuric acid was added into it.

*** Arsenomolybdate reagent was prepared by mixing 12.5 gm. ammonium molybdate in 200 ml. of distilled water; 10.5 ml. of concentrated sulphuric acid and 1.5 gm. of sodium arsenate in
12.5 ml. distilled water and made the volume to 250 ml. with distilled water. This solution was kept in dark for 24 hours before use.

6. TOTAL SUGARS :

(Warton and McCarty, 1972)

Plant material was boiled in 80 % ethanol, macerated and centrifuged. Alcoholic fraction of the supernatant was evaporated and made up to a known volume (20 ml.) with distilled water. To 10 ml. of the above extract 3 ml. of 1N HCl. was added and kept in boiling waterbath for 20 minutes to hydrolyse non-reducing sugars. It was cooled and neutralised by adding 3 ml. of 1N NaOH solution. To the mixture 1 ml. of 25 % lead acetate solution and 1 ml. of 25 % sodium carbonate solution were added and the volume was made up to 20 ml. It was filtered and then used as aliquot for total sugars. The content of sugar was estimated by employing Nelson-Somogyi reaction as described for reducing sugars and expressed as μg total sugar/mg. dry weight.

7. TOTAL PHENOLS :

Total phenol content of the pollen and stigma was determined in all the stages selected employing the
method of Farkas and Kiraly (1962). The reaction system containing 1 ml. of ethanol extract and 0.5 ml. of Folin-phenol reagent and 1 ml. of 20% sodium carbonate was heated at 100°C on a waterbath for 10 minutes. The blue coloured mixture obtained was then cooled and diluted to a known volume with distilled water and its optical density was recorded at 660 nm. In control, 1 ml. of ethanol extract was replaced by 1 ml. of distilled water. Phenol content was expressed as gallic acid equivalents. The following regression formula prepared with different concentrations of gallic acid, was used for calculating the phenol content and expressed as μg phenols/mg. dry weight.

\[ X = 96.05 \times Y + 10.3 \]

Where \( X \) = Concentration
\( Y \) = Optical density

B. ENZYMES:

Activities of enzymes Viz., protease, invertase and peroxidase were assayed in all selected stages by employing the standard analytical methods (Table - III).
Preparation of enzymic aliquot:

Pollen and stigma of all selected stages were macerated in ice-cold distilled water at 4 - 5°C and centrifuged in a refrigerator centrifuge (Remi, India) at 5°C. The supernatant was collected, made up to a known volume with ice-cold distilled water and used as enzyme source.

(i) PROTEASE ACTIVITY:

The enzyme activity was assayed by the method of Penner and Ashton (1967). 1 ml. of enzyme aliquot was added to 3 ml. of phosphate buffer (pH 7) and 2 ml. of 0.5 % casein solution (pH was adjusted to 7) and the reaction mixture was incubated at 30°C for 1 hour. The enzyme activity was stopped by adding 2 ml. of the reaction mixture to test tube containing 2 ml. of 15 % trichloro acetic acid. After 20 minutes the contents were centrifuged and the supernatant was used to determine the amount of tyrosine liberated from casein using Folin reagent (Lowry et al., 1951). 2 ml. of the reaction mixture was mixed with 4 ml. of 0.5 N NaOH, to this 1.2 ml. of Folin reagent was added and allowed to stand for 20 minutes to develop blue colour. The optical density of the colour was
read at 660 nm against the blank, which contained all solutions except reaction mixture, which was replaced by 2 ml. of distilled water. The enzyme activity was calculated and expressed in terms of μg tyrosine liberated/hour/mg fresh weight using the following regression formula prepared by using tyrosine as standard.

\[ X = 224.07 Y - 0.40 \]

Where

\( X \) = Concentration
\( Y \) = Optical density

(ii) PEROXIDASE ACTIVITY:

Peroxidase activity was determined by the method of Maehly (1954) and George (1953). The reaction mixture was prepared by mixing 2 ml. phosphate buffer (pH 7); 1 ml. of 20 mM aqueous guaicol (in 100 ml. of distilled water) and 1 ml. of enzyme extract. Control was made by preparing the reaction mixture in the above manner except that the enzyme aliquot was replaced by 1ml. of distilled water. The reaction mixture was placed in the cuvette of a Spectrocolorimeter (Systronics - 103, India) and read the initial reading at 470 nm. A drop of \( \text{H}_2\text{O}_2 \) (0.4 ml. of 20 vol. \( \text{H}_2\text{O}_2 + 9.6 \) ml. distilled water) was
then added to the reaction mixture with a glass rod and the optical density was read after 30 seconds at the same wave length (470 nm). The enzyme activity was calculated and expressed in terms of optical density developed / minute / gm. fresh weight.

(iii) INVERTASE ACTIVITY :

Invertase activity was determined by the method of Hatch and Glasziou (1963). To 1 ml. of enzyme aliquot, 1 ml. of acetate buffer (pH 4.8) and 1 ml. of 0.25 % sucrose were added. This was incubated for 1 hour at room temperature and the enzyme activity was stopped by adding 2 ml. of 5 % perchloric acid. The volume was then made up to 10 ml. 1 ml. of the above mixture was taken and estimated the amount of reducing sugar by employing the Nelson - Somogyi reaction (described under the method for reducing sugars). The amount of reducing sugar released by invertase activity was calculated and expressed as μg. glucose released / hour / mg. fresh weight.

III. HISTOCHEMISTRY :

(i) Histochemical studies were undertaken to localize and quantify the metabolites in situ. The various
sites (stigma papillae, transmitting tissue, canal lining cells, sub-stigmatic region) on the pistil and the pollen at various stages of development were stained for proteins, nucleic acids, polysaccharides and lipids (Table - IV).

The detailed procedures are given below:

1. PROTEINS:

(Mazia *et al.*, 1953)

Mercuric bromophenol blue method was used for the quantification of insoluble proteins, as all the soluble ones are lost during fixation. It is stoichiometric and highly specific (Berlyn and Miksche, 1976; Gabe, 1976). The staining schedule is as follows:

1. FAA fixed material was used.
2. Sections were deparaffinized in xylene and hydrated through ethanol : double distilled water series.
3. Sections were hydrated and stained in mercuric bromophenol blue for 15 minutes at room temperature.
4. The sections were kept in cold tap water for 2 minutes.
<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Metabolite</th>
<th>Staining reagent</th>
<th>Colour of the dye-metabolite complex</th>
<th>Wave length (nm) and filter used</th>
<th>Architect of the technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.</td>
<td>Insoluble polysaccharides</td>
<td>Periodic acid Schiff's reagent</td>
<td>Megenta</td>
<td>500-570 green</td>
<td>Hotchkiss, R.D. (1948)</td>
</tr>
</tbody>
</table>
5. The excess stain was removed in 0.5% glacial acetic acid.

6. Slides were washed under tap water which leads to the development of blue colour.

7. Dehydrated through ethanol series, cleared in xylene and mounted in DPX. Bluish positive reaction is appeared in the cytoplasm and nucleus, indicate the sites of distribution of protein.

8. The dye absorbance was measured in 610 nm. using orange filter.

MERCURIC BROMOPHENOL BLUE REAGENT:

50 mgs. bromophenol blue (BDH, Poole, England) and one gram of mercuric chloride were dissolved in 100 ml. of 2% acetic acid. Control preparations were made by deaminating (50 ml. 10% TCA + 50 ml. 10% sodium nitrite for 20 minutes at room temperature) the hydrated sections (Jensen, 1962).

2. HISTONES:

ALKALINE FAST GREEN METHOD:

(Alfert and Geschwind, 1953)

This method was first introduced and employed for connective tissues in animals using the blue-green
anionic triphenyl methane dye and also as a counterstain to methods that produce strong blue, purples and reds. It is soluble in water and considerably less in alcohol. Fast green FCF should contain at least 85% by weight of the anhydrous dye.

Histones have a high content of basic amino acids (lysine and arginine) and have isoelectric points that are more acid than other proteins. Generally, when a tissue is at pH 8.0, most proteins are near to or above their isoelectric points, except the histones, which will be below their isoelectric point. Alfert and Geschwind (1953) produced a method in which fast green FCF was applied at pH 8.0, when in the absence of nucleic acids only histones will possess groups capable of binding the dye. This method was shown to be specific for basic proteins. The colour developed will be directly proportional to the amount of histone and could be assessed cytophotometrically.

FAA fixed material was used. Paraffin embedded materials were sectioned at 10 μ and used for histone localization.

1. Sections were deparaffinized and hydrated through ethanol - distilled water series.
2. Slides were placed in 15% TCA solution in boiling water bath for 15 minutes to extract the nucleic acids.

3. Sections were cleaned in 70% alcohol which was repeated three times.

4. The slides were placed in 0.1% aqueous solution of fast green FCF at pH 8.9 for 30 minutes (pH was adjusted using a minimum quantity of NaOH solution).

5. Slides were washed in distilled water for 5 minutes.

6. The slides were dehydrated directly using 95% ethyl alcohol and mounted in DPX.

The basic proteins (histones) appeared green. Histones are normally found in the nuclei and chromosomes, which are the only cell part that stained in this test.

CONTROL:

DEAMINATION AND ACETYLATION:

After TCA treatment, the slides were dehydrated and brought to absolute alcohol then treated with 1% glacial acetic acid so that the -COOH group will be blocked.
3. TOTAL NUCLEIC ACIDS:

(Gabe, 1976)

Gallocyanin – chrome alum specifically stains total nucleic acids (Gabe, 1976; Brown and Scholtz, 1979). But the chemical significance is not known. However, the structures containing basophilia take on a bluish black colour. There is a stoichiometric relationship between the stain intensity and the amount of nucleic acids (Brown and Scholtz, 1979). Dye binding in nuclei after treatment with ribonuclease is attributable to DNA (Kiefer, 1970). Thus, the amount of total nucleic acids can be obtained. The staining schedule is as follows:

1. Carnoy’s fixed material was used.
2. Sections were deparaffinized, hydrated by passing through ethanol: distilled water series.
3. Sections were incubated overnight in gallocyanin-chrome alum (pH 1.6) at 40°C.
4. Slides were rinsed in acid water (pH 1.6) to remove excess stain.
5. Sections were dehydrated and cleared in xylene and mounted in DPX.

Cytoplasm and nucleus showed bluish black colour and absorbance at 575 nm was measured to obtain total nucleic acid content.
GALLOCYANIN - CHROME ALUM REAGENT:
(Pearse, 1972)

600 mg. of gallocyanin (Burr Ltd., England, B.N. 17279) was dissolved in 200 ml. of double distilled water by shaking for 1 minute, filtered and the filtrate was discarded. The extracted residue from the filter paper was dissolved in freshly prepared 200 ml. of 5% chrome alum (chromic potassium sulfate BDH, India) in a boiling water bath for 30 minutes. The pH of the dye was adjusted to 1.6 with 1N HCl. The final volume was made to 200 ml. with double distilled water.

4. RNA:
(Tepper and Gifford, 1962)

Histochemical detection of RNA was achieved by using pyronin-β. The specificity of pyronin to localize RNA is in question (Kurnick, 1955), because in animal tissue pyronin binds with DNA when it is not competitively inhibited methyl green. But Tepper and Gifford (1962) demonstrated the specificity of pyronin reaction to RNA in plant tissue by enzyme digestions and extractions of RNA from the tissue. Furthermore, many workers (Corson and Gifford, 1969; Heslop - Harrison and Heslop - Harrison, 1970; Molder and
Owens (1973; Patel et al., 1978) showed the use of pyronin reaction to detect RNA in plant tissues. Histochemical analysis of RNA has its own limitations in quantitative terms, because some types of RNA (mRNA and sRNA) are lost during fixation and dehydration. The RNA available to the dye is rRNA. Since rRNA constitutes 80-90% of the total cellular RNA (Busch and Smetana, 1970) the staining reaction is accessible to quantitative terms.

The staining schedule is as follows:

1. Carnoy's fixed material was used.
2. Sections were deparaffinized and hydrated through downgrade series of ethanol: distilled water.
3. Sections were placed in pyronin -6 dye for 7 minutes at room temperature.
4. Slides were blotted with blotting paper after giving a brief rinse with distilled water and dehydrated through a n-butanol (BDH - India).
5. Slides were cleared in xylene and mounted in DPX.
6. The dye absorbance was measured at 500 - 570 nm. Control slides were prepared by extracting RNA from the hydrated sections with 1N perchloric acid at 4°C for 12 hrs. (Jensen, 1962).
PREPARATION OF THE REAGENT:

Pyronin reagent was prepared by dissolving 2 gms. of pyronin - G (Fluka - Swiss, B. No. 565 238) in 100 ml. of double distilled water and equal volume of acetate buffer (pH 3.5) was added to it. The dye impurities were extracted by repeated washing with chloroform until the lower layer in the separating funnel becomes colourless (Tepper and Gifford, 1962).

5. INSOLUBLE POLYSACCHARIDES:

(Hotchkiss, 1948)

Localization of insoluble polysaccharide was carried out by periodic acid - Schiff's reaction (PAS) of Hotchkiss (1948) as detailed by Jensen (1962). For light microscopic study PAS reaction is effective (Chao, 1977a) and it is accessible for cytophotometry after removing starch from the tissue (Jona and Foa, 1977). It gives intensive colour. Periodic acid oxidation is a critical step where 1,2 glycol linkage releases the free aldehyde groups. If the oxidation prolongs long time other complex molecules like polyphenols and proteins also mimic the reaction giving false PAS positive staining (Barka and Anderson, 1965). PAS reaction yielded yellow colour where proteins reacted and red colour with polyphenols.
(Considine and Knox, 1979; Ling-Lee et al., 1977). However, success on the method depends on a large extent on the colouristic purity of the stained chromophore (magenta colour).

The method to localize polysaccharides is as follows:

1. Carnoy’s fixed material was used (fixative was prepared by mixing absolute alcohol and acetic acid in 3:1 ratio).

2. Sections were deparaffinized in xylene and hydrated by passing through ethanol:distilled water series.

3. Hydrated sections were kept in 0.5% periodic acid (Riedel, Germany) for 10 minutes at room temperature after trying for different period for its colouristic purity.

4. Slides were washed thoroughly under running tap water for 5 minutes.

5. Sections were washed and kept in Schiff’s reagent for 10 minutes at room temperature.

6. Excess stain was removed by keeping the slides in 2% sodium metabisulphite (BDH, India) for two minutes.

7. Slides were washed in running tap water for five minutes.
8. The sections were dehydrated in ethanol, cleared in xylene and mounted in DPX.

The characteristic magenta colour indicates the sites of polysaccharides.

SCHIFF'S REAGENT:

Schiff's reagent was prepared by adopting the method of Leuchtenberger (1958). One gram of basic fuchsin (Harleco, U.S.A.C.I. 42510) was dissolved in 200 ml. of boiling double distilled water for 5 minutes. The solution was cooled to 50°C, and before being filtered through Whatman No. 1 filter paper, 20 ml. of 1N HCl was added and the solution was further cooled to room temperature (25°C). At this temperature 2 gms. of potassium metabisulphite was added and stored in a tightly stoppered bottle overnight. The solution appeared straw yellow colour. To this 0.5 gms. of activated charcoal was added, shaken rapidly for one minute, filtered through coarse filter paper and stored in a stoppered amber coloured bottle at 5°C. The reagent thus prepared was a colourless liquid (pH 2.5).
In case of control preparations, oxidation by periodic acid was omitted and strictly followed the remaining steps in the above staining schedule (Erickson et al., 1949).

LIPID :

SUDAN BLACK B METHOD :

(Bayliss - High, 1982; Bancroft and Cook, 1984).

Sudan black B can dissolve in hydrophobic lipids and behave like a cationic dye and bind to the hydrophilic phosphate-ester groups of phospholipids, in which it is more soluble than the other Sudan dyes. Adams (1965) points out that the staining of lipids is affected by the solvent used for the lysochrome. Thus phospholipids are more likely to be stained from a solution in 70% than from one in absolute alcohol. Lysochromes can only dissolve in lipids at temperatures above the melting points of the latter. This is a point of some importance, since lipids in which all the fatty acid chains are saturated melt above 60°C. Unsaturated lipids are mostly liquid at room temperature. However, it is possible to stain lipids with Sudan black B.
The following steps describe the staining procedure:

1. Sections were deparaffinized in xylene.
2. Slides were rinsed in 70% ethanol.
3. Sections were stained in saturated solution of Sudan black in 70% ethanol for 15 minutes (solution was filtered before use).
4. Excess stain was removed by washing the slides in 70% ethanol (two to three changes).
5. Sections were dehydrated and mounted in DPX.

The triglycerides and phospholipids stained blue-black.

(ii) CYTOCHEMICAL METHODS:

Cytochemical staining was followed for the localization of esterase and peroxidase activity on the stigmatic receptive region and staining was also done to study the nature of stigma secretion at the time of pollination (Table - IV). The methods are as follows:
ESTERASE ACTIVITY:

\( \alpha \)-NAPHTHYL ACETATE - FAST BLUE B METHOD:

(Nachlas and Seligman, 1949; Pearse, 1972).

Esterases are enzymes which are capable of hydrolysing esters. Within this group there are many types of esterases, acting upon a number of substrates. If the substrate is a simple ester such as \( \alpha \)-naphthyl acetate, the hydrolysing enzyme is called non-specific esterase. The \( \alpha \)-naphthyl acetate method for non-specific esterase will probably demonstrate all types of esterase activity. It is normally carried out at a pH of 7.4. It is an azo-dye simultaneous coupling method which was first described by Nachlas and Seligman (1949). The method can be carried out by using \( \alpha \)-naphthyl acetate as a substrate employing the diazonium salt fast blue B as a coupling agent.

INCUBATION MEDIUM:

The incubation medium contains \( \alpha \)-naphthyl acetate dissolved in acetone and buffered to pH 7.4 with phosphate buffer. The diazonium salt, fast blue B, is included in the medium as a coupler. The esterase activity in the section splits the \( \alpha \)-
naphthyl acetate releasing α- naphthol. This combines rapidly with the fast blue B salt to produce an insoluble azo-dye at the sites of enzyme activity.

The procedure is as follows:

1. Fresh sections were taken and kept in the incubation medium for 15 minutes at room temperature.
2. Sections were washed in running tap water for 2 minutes after incubation.
3. The materials were again placed in fast blue B solution for 20 minutes.
4. The sections were washed in running tap water for 30 minutes and mounted in glycerine.

Control was prepared by omitting the substrate (α- naphthyl acetate).

PREPARATION OF THE MEDIUM:

10 mg. of α- naphthyl acetate was dissolved in 0.25 ml. of acetone and 20 ml. of 0.01 M phosphate buffer of pH 7.4. The mixture was shaken thoroughly until most of the initial cloudiness disappear.
100 mg. of fast blue B salt was added into the above mixture and the solution was filtered, and used immediately.

2. PEROXIDASE:

P - PHENYLENE DIAMINE METHOD:

(Raa, 1973; Malik and Singh, 1980).

P - Phenylenediamine is a hydrogen donor, which is used along with hydrogen peroxide to localize peroxidase enzyme in the stigma.

The procedure is as follows:

1. Fresh stigmas were harvested and thin hand sections were taken.

2. Sections were incubated in the reaction mixture containing 5 ml. of 0.5 % P -Phenylenediamine and 5 ml. of 0.5 % hydrogen peroxide for five minutes at room temperature.

3. Sections were washed in distilled water and mounted in glycerine.

4. Brownish red products were formed at the sites of enzyme activity.

Control: Heat-killed sections were used as control.
3. DETECTION OF PAPILLAR SECRETION:

(Cresti et al., 1986)

Alcian blue was used to localize the stigmatic papillar secretion. Fresh stigmas were taken and placed in 0.5% solution of alcian blue for 15 minutes. To remove the extra stain, they were washed in distilled water. Mounted in glycerine and observed under light microscope.

(iii) MICROMETRY:

Micrometry was done to measure the cell area of pollen, papillae, stylar canal cells and transmitting tissue region with the help of Camera lucida (E. Leitz Wetzler, Germany) and a stage micrometer (E. Leitz Wetzler, Germany). Stage micrometer divisions were sketched on a graph paper by observing under a microscope fitted with Camera lucida to calibrate the graph paper divisions to microns. The outline figure of the pollen, papillae, canal lining cells and transmitting tissue were also sketched in a similar way without changing the angle of Camera lucida or magnification. The cell areas were calculated from the calibrated graph paper divisions by counting the number of graphical divisions occupied by each cell.
This cell area was employed to calculate the actual content of metabolite in a particular cell or area, after taking reading in the Cytophotometer. The results are expressed in content per cell and concentration per unit area.

IV. QUALITATIVE STUDY OF SOLUBLE PROTEINS BY POLYACRYLAMIDE GEL ELECTROPHORESIS:

Electrophoresis in polyacrylamide gels in the presence of anionic detergent sodium dodecyl sulfate (SDS), have proven to be useful tool for the separation of protein subunits and the determination of their molecular weights. SDS (Sodium Dodecyl Sulfate, NaSO₃ - O - C₁₂ H₂₅) is an anionic detergent that has a polar sulfate group linked to non-polar aliphatic chain. It reacts with proteins before electrophoresis. Protein-SDS complexes are soluble and under the influence of an electrical field, it will migrate through a polyacrylamide gel towards the anode, generally at a rate inversely proportional to the logarithm of their molecular weight. This relationship may be used to determine the molecular weight of an unknown protein when run together with proteins of known molecular weight.
The procedure followed here is a modification of the method of Laemmli (1970). Qualitative study of water soluble proteins were carried out in all the six developmental stages of pollen and pistil selected for this study. The suitable gel for the best separation of proteins was found as 10%.

PREPARATION OF REAGENTS:

A. SEPARATING GEL BUFFER:

36.3 gms. of Tris and 0.23 ml. N1, N, N1, N1-tetramethylethylenediamine were dissolved in 90 ml. of double distilled water. The pH was adjusted to 8.9 at 25°C with concentrated HCl and made the volume to 100 ml. with double distilled water.

B. STACKING GEL BUFFER:

5.98 gms. of Tris and 0.46 ml. of N1, N, N1, N1-Tetramethylethylenediamine were dissolved in double distilled water. The pH was adjusted to 6.7 at 25°C with concentrated HCl and diluted to 100 ml. with double distilled water.
C. SEPARATING GEL SOLUTION:

28 gms. of acrylamide and 0.74 gms. N, N\textsuperscript{1} - methylenebisacrylamide were dissolved in double distilled water and diluted to 100 ml. Insoluble material remained in the solution was removed by filtration.

D. STACKING GEL SOLUTION:

10 gms. of acrylamide and 2.5 gms. methylenebisacrylamide were dissolved in double distilled water. Insoluble material was removed by filtration.

E. SDS SOLUTION:

0.21 gms. of sodium dodecyl sulfate was dissolved in double distilled water and diluted to 100 ml.

F. 2X SAMPLE BUFFER:

1.51 gms. Tris and 20 ml. of glycerol were dissolved in 35 ml. of double distilled water and the pH was adjusted to 6.75 with concentrated HCl. 4 gms. sodium dodecyl sulfate; 10 ml. of 2 - mercaptoethanol
and 0.002 gms. bromophenol blue were added to the above solution. Final volume was made upto 100 ml. with double distilled water.

6. ELECTRODE BUFFER:

6.05 gms. Tris; 28.8 gms. glycine and 2 gms. sodium dodecyl sulfate were dissolved in double distilled water and final volume was made upto 2 litres. The pH was adjusted to 8.3 with HCl.

H. FIXATIVE SOLUTION:

Fixative solution was prepared by combining 400 ml. methanol; 70 ml. glacial acetic acid and 530 ml. double distilled water.

I. STAINING REAGENT:

1.25 gms. Coomassie brilliant blue R was dissolved in 500 ml. of fixative solution (solution - H).

PREPARATION OF ELECTROPHORESIS GELS:

The electrophoresis glass tubes were tightly
closed at one end with flat topped rubber stoppers. Standing upright on these stoppers the tubes were filled with 1.6 ml. of 10% separating gel prepared by mixing the following:

3 ml. separating gel buffer (A)
8.6 ml. separating gel solution (C)
11.5 ml. SDS solution (E)
17 mg. ammonium persulphate
0.9 ml. distilled water

Before the gel hardens 0.05 ml. of distilled water was layered on the top of each gel solution without disturbing the surface. After the polymerization of the gels, the water layer was removed and rinsed the tops of the gels with 0.25 ml. of stacking gel prepared by mixing solutions B, D, E and distilled water (containing 8 mg. of ammonium persulphate, freshly prepared) in the ratio of 1 : 2 : 4 : 1. Again the top of the gels were layered with 0.05 ml. of distilled water before the gels hardens without disturbing the surface. After polymerization the water layer was removed and the gels were used for electrophoresis.
PREPARATION OF MOLECULAR WEIGHT MARKERS:

The following five known molecular weight proteins were ran as described above and their relative mobility (Rf) values were calculated.

<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Product No.</th>
<th>Product (mg./vial)</th>
<th>Relative mobility (Rf)</th>
<th>Molecular weight (in daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>L 4385</td>
<td>L - Lactalbumin</td>
<td>0.86</td>
<td>14,200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Bovine milk)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>C 5024</td>
<td>Carbonic anhydrase</td>
<td>0.533</td>
<td>29,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Bovine erythrocytes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>A 8529</td>
<td>Albumin</td>
<td>0.38</td>
<td>45,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Chicken egg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td>A 8654</td>
<td>Albumin</td>
<td>0.2</td>
<td>66,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Bovine serum)</td>
<td></td>
<td>(monomer)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1,32,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(dimer)</td>
</tr>
<tr>
<td>5.</td>
<td>U 7752</td>
<td>Urease</td>
<td>0.083</td>
<td>2,72,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Jack bean)</td>
<td></td>
<td>(trimer)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5,45,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(hexamer)</td>
</tr>
</tbody>
</table>

*Procured from Sigma*
Reconstituted each of the protein standard vial with 1 ml. of 50 mM NaCl., 1mM sodium phosphate of pH 7 - except the vial containing urease, which was dissolved in 5 ml. of double distilled water. This was diluted with an equal volume of sample buffer before use.

PREPARATION OF SAMPLE:

Weighed plant material was macerated in 2X sample buffer (solution - F) diluted 1: 1 with double distilled water and filtered. This was heated at 100 °C for 1.5 minute prior to electrophoresis and filtered. The filtrate was used as protein sample for electrophoretic separation.

ELECTROPHORESIS:

After the gels were polymerized the lower rubber gaps were removed and the tubes were placed carefully in the electrophoresis chamber. A fixed volume (0.02 ml.) of the sample was loaded in each tube and a drop of marker dye was added above the sample. The remaining space in each glass tube was filled with electrode buffer. The tubes were attached to the anode buffer compartment, which is then filled with
electrode buffer. A hanging drop of buffer was attached to the bottom of each tube. The anode compartment supported on a stand was placed, so that the tubes were immersed about 1/4 inch into the buffer contained in the cathode compartment. To prevent mixing, the density of the sample solution was increased with sucrose. Electrophoresis was carried out by applying constant current at one milliampere/gel until the marker dye was completely passed through the stacking gel and 2 milliampere/gel until the dye was one centimeter from the anodic end of the gel.

When electrophoresis was completed, the gels were immediately removed from the glass tubes by gently squirting water with a 22-gauge syringe between the gel and the glass tube wall. The gels were immersed in the fixative solution for at least seven hours. After fixing, the gels were stained in staining reagent (I) for six hours. The stained gels were removed and destained with several changes of solution H. Repeated this process until the gels become clear with only protein bands.

The relative mobility (Rf) of each band appeared in the gel was calculated by using the following formula,
Distance of protein migration

\[
\text{Relative mobility (Rf)} = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}
\]

By comparing the Rf values of the standard with samples, the approximate molecular weight of each protein was determined. The relative percentage of each protein separated on the gel was measured by scanning the gels with the help of Beckman's Spectrophotometer (model R-112, USA).

V. ATOMIC ABSORPTION SPECTROPHOTOMETRY:

The qualitative and quantitative estimation of trace (Cd, Cr, Cu, Fe, Mn, Ni, In, Pb and Na) and major elements in the pollen were performed on an atomic absorption spectrophotometer by the technique given in Perkin Elmer (373) manual.

500 mg of dry powdered pollen grain was digested in Kjeldhal flask with triple acid (concentrated nitric, sulphuric and perchloric acids in the ratio of 10:4:1) at low temperature to minimise the loss of metals. The digested material should be transparent and colourless. When necessary, perchloric acid treatment was given to remove the black residue. This digested material was dissolved in 0.1 M nitric acid and final volume was made to 10 ml.
For blank, 10 ml of triple acid was evaporated and residue was dissolved in 0.1 M nitric acid and final volume was made to 10 ml.

The samples were analysed on "Atomic Absorption Spectrophotometer" (Perkin Elmer 373). The reading of blank was deducted from the sample's reading. The results were expressed as µg/g dry weight.

The following wavelengths were used:

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Metal</th>
<th>Wavelength (nm)</th>
<th>Slit (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Al</td>
<td>309.3</td>
<td>0.7</td>
</tr>
<tr>
<td>2.</td>
<td>Ca</td>
<td>429.7</td>
<td>0.7</td>
</tr>
<tr>
<td>3.</td>
<td>Cd</td>
<td>228.8</td>
<td>0.7</td>
</tr>
<tr>
<td>4.</td>
<td>Cr</td>
<td>357.9</td>
<td>0.7</td>
</tr>
<tr>
<td>5.</td>
<td>Cu</td>
<td>324.8</td>
<td>0.7</td>
</tr>
<tr>
<td>6.</td>
<td>Fe</td>
<td>248.3</td>
<td>0.2</td>
</tr>
<tr>
<td>7.</td>
<td>K</td>
<td>766.5</td>
<td>2.0</td>
</tr>
<tr>
<td>8.</td>
<td>Mg</td>
<td>285.2</td>
<td>0.7</td>
</tr>
<tr>
<td>9.</td>
<td>Mn</td>
<td>279.5</td>
<td>0.2</td>
</tr>
<tr>
<td>10.</td>
<td>Na</td>
<td>589.6</td>
<td>0.7</td>
</tr>
<tr>
<td>11.</td>
<td>Ni</td>
<td>232.0</td>
<td>0.2</td>
</tr>
<tr>
<td>12.</td>
<td>Pb</td>
<td>283.3</td>
<td>0.7</td>
</tr>
<tr>
<td>13.</td>
<td>Zn</td>
<td>213.9</td>
<td>0.7</td>
</tr>
</tbody>
</table>
VI. CHROMATOGRAPHY:

Paper chromatography - ascending method was followed for the identification of aminoacids in stigma and pollen. The plant extract was prepared by crushing the pollen and the stigma separately in 80% ethanol and centrifuged. Saturated solution of standard aminoacids in 80% ethanol were also prepared. The solvent was prepared by adding n-butanol, glacial acetic acid and distilled water in 12:3:5 ratio. Whatman no:1 paper was used as a stationary phase.

PROCEDURE:

Leaving 2 cm. space from the margin of the paper, the sample and different aminoacids (standards) were spotted in a straight line maintaining sufficient distance between them. The chromatogram was placed in a thick rectangular glass jar containing solvent and left for 8 hours. Then it was removed, dried and sprayed with 0.2% ninhydrin in n-butanol. Chromatogram was kept in Oven at 85°C for 10 minutes. The aminoacids present in the sample was identified by comparing the coloured spots of the sample with the standard aminoacids spotted on the paper.
VII. POLLEN VIABILITY TEST:

Alexander's stain (1969)

The viability of the pollen grains was tested using the method proposed by Alexander (1969). The differential staining of aborted and non-aborted pollen was used to find out the percent viability before, at the time and after anthesis.

Medium:

The staining medium was prepared by adding the various constituents in the order given below, shaking after addition and stored in a coloured bottle.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>95 % alcohol</td>
<td>10 ml.</td>
</tr>
<tr>
<td>Malachite green</td>
<td>10 mg. (1 ml. of 1 % solution in 95 % alcohol)</td>
</tr>
<tr>
<td>Distilled water</td>
<td>50 ml.</td>
</tr>
<tr>
<td>Glycerol</td>
<td>25 ml.</td>
</tr>
<tr>
<td>Phenol</td>
<td>5 gm.</td>
</tr>
<tr>
<td>Chloral hydrate</td>
<td>5 gm.</td>
</tr>
<tr>
<td>Acid Fuchsin</td>
<td>50 mg. (5 ml. of 1 % solution in distilled water)</td>
</tr>
<tr>
<td>Orange G</td>
<td>5 mg. (0.5 ml. of 1 % solution in distilled water)</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>1 - 4 ml.</td>
</tr>
</tbody>
</table>
The amount of glacial acetic acid to be added to the mixture depends upon the thickness of the pollen walls. The differentiation of aborted and non-aborted pollen was found to depend upon the concentration of the dyes, thickness of the pollen walls and pH of the staining solution.

The staining was done by mounting the pollen grains directly on the slide in a drop of stain and covered with a cover slip and the slides were warmed slightly. The aborted and non-aborted pollen grains were differentiated by the green and red to deep red colour respectively. The percentage of viable pollen grains was calculated.

VIII. POLLEN GERMINATION AND POLLEN LEACHATE STUDIES:

Germination medium was prepared by using various concentration of sucrose, boric acid and calcium nitrate. This basic medium was supplemented with various concentration of growth hormones as given in Table - V. Snake venum was also used to induce the germination of pollen in the laboratory (Table - V).

The effect of pollen leachate on the pH of germination medium was studied. The release of
<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Date</th>
<th>Incubation medium</th>
<th>Incubation time</th>
<th>Temperature</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>02.09.1989</td>
<td>Sucrose (10-60 %)</td>
<td>12-48 hrs.</td>
<td>24-26°C</td>
<td>No germination</td>
</tr>
<tr>
<td>2.</td>
<td>28.11.1989</td>
<td>Sucrose (10-30 %) with boric acid (0.01 %)</td>
<td>12-48 hrs.</td>
<td>21-25°C</td>
<td>No germination</td>
</tr>
<tr>
<td>3.</td>
<td>30.11.1989</td>
<td>Sucrose (40-60 %) with 0.01 % boric acid</td>
<td>12-48 hrs.</td>
<td>20-24°C</td>
<td>No germination</td>
</tr>
<tr>
<td>4.</td>
<td>16.12.1989</td>
<td>Sucrose (10-60 %)</td>
<td>12-48 hrs.</td>
<td>17-20°C</td>
<td>No germination</td>
</tr>
<tr>
<td>5.</td>
<td>16.12.1989</td>
<td>Sucrose (10-60 %) + 0.01 % boric acid and 0.03 % calcium nitrate</td>
<td>12-48 hrs.</td>
<td>17-20°C</td>
<td>No germination</td>
</tr>
<tr>
<td>7.</td>
<td>17.12.1989</td>
<td>Germination medium at different pH (3-10)</td>
<td>12-48 hrs.</td>
<td>17-20°C</td>
<td>No germination</td>
</tr>
<tr>
<td>9.</td>
<td>17.12.1989</td>
<td>Sucrose (10-60 %) with snake venum</td>
<td>12-48 hrs.</td>
<td>17-20°C</td>
<td>No germination</td>
</tr>
</tbody>
</table>
aminoacids and protein into the medium was estimated using standard methods (Table - III). The germination medium was adjusted to various pH (3 - 10) using a minimum of HCl or NaOH. 50 mg. of anthers were added to each of the above medium. The change in the pH of the medium was recorded periodically up to 30 min. with the help of a pH meter. After 30 minutes the pollen grains were filtered and the filtrate was used for the estimation of aminoacids and proteins released from the pollen grains into the medium.

PREPARATION OF THE GERMINATION MEDIUM:

It was prepared by mixing sucrose (100 mg./litre), boric acid (100 mg./litre) and calcium nitrate (300 mg/litre) in distilled water (Gauch and Dugger, 1954; Brewbaker and Kwack, 1963) and 10 ml. was taken for the study. The initial pH of the medium was 6.8 and it was adjusted to acidic (3-6) and alkaline (8-10) by using 0.1N HCl and 0.1N NaOH respectively.

IX. CYTOPHOTOMETRY:

The Cytophotometer used in this piece of work was designed in our laboratory (Shah et al., 1975). This
is a Pollister microspectrophotometer type (1952). It follows Lambert-Beer's laws of light absorption. The main components in the Cytophotometer (Fig. 2) are Kohler illumination (15 W 6 V bulb fitted with a Leitz Wetzel microscope), photometric device (microammeter) and a light dependent resistor (LDR) (Phillips, Holland). Microscope and LDR are kept inside a dark chamber. Kohler bulb receives current from the main supply through a series of stabilizers to resist against the fluctuations in the current supply. Over the Kohler illumination, narrow band (wave length) filters were kept to get the light of required wave length. Thus filtered light passes through the stained preparation (slide on the platform of the microscope). A part of the light being absorbed by the chromophore and the transmitted light passes through the objective of the microscope and reflected by the mirror on to the LDR kept parallel to it.

**WORKING OF LDR:**

On applying current to one end of the LDR, it does not allow the current to pass through it, when it is in complete darkness. But, when a beam of light falls on LDR, it loosens resistance according to the
intensity of the light to which it is exposed. Thus there exists a positive correlation between the intensity of light and the decrease in the resistance of LDR (Phillips - Data Hand Book, 1970). These differences between the flow of current are recorded by a microammeter connected to the other end of the LDR. If more current is flowing (implies more loss of the resistance of LDR) microammeter records more reading and less current records less reading (because of a gain in the resistance of LDR). Stain intensity is related with ammeter readings because of the interdependence of all these parameters as shown below:

\[
\text{Stain intensity} \propto \text{light absorbance}
\]

\[
\text{Stain intensity} = \frac{1}{\text{transmitted light}}
\]

Transmitted light \(\propto\) current passed through LDR. Current released by LDR \(\propto\) ammeter reading.

On adjusting the control preparation (control slide) on to the LDR, more light will fall on the LDR because of more transmittance of filtered light through the slide. Thus higher reading will be recorded on the ammeter. Reverse is the case with stained preparation.
WORKING OF THE INSTRUMENT:

1. Instrument kept on working by putting on the main switch. Allowed to stabilize the current for 15 minutes.

2. Slide was kept on the microscope and adjusted to required cell/nucleus on the LDR. (Camera lucida was used to calculate the area of the cell/nucleus).

3. The mirror image of the required region was kept on the LDR and the ammeter reading was noted.

4. Blank portion of the slide was projected on the LDR, and again ammeter reading was noted.

The logarithmic difference between the readings of blank and stained region will give the extinction value (Pollister et al., 1969).

Extinction value = Log of blank reading (Io) - Log of stained reading (Is)

Content or total dye binding is the product of extinction value and corresponding area/volume.

Content = extinction value x area / volume
The number of readings measured for a parameter range from 15 to 20 (both stained and blank readings) in a section. Thus 2 - 3 slides were exposed for one parameter. Cells near edges of the sections were omitted since they are more prone to damage in preparation of the tissue. Those cells which have come completely in the selected section thickness were only subjected for measurements. In nuclear measurements to avoid light scatter from cytoplasm, LDR was covered with a diaphragm having apertures of different diameters. Thus the exposing surface of LDR was always kept smaller than the dimensions of the image falling over it. In a particular set of experiment exposed surface of the LDR was kept constant. The instrument was checked for its performance and sensitivity in a manner suggested by Pollister et al., (1969) and Ruthman (1970).

The results of two such experiment are as follows:

1. Lambert's law was checked by measuring the extinction values (dye absorbance) of the stained material at different section thickness. A linear relationship was found.

2. Beer's law was checked by measuring the extinction values of fast green dye of different
concentrations. A linear relationship was obtained. In a series of publications by Shah and his school (1975, 1978, 1980) discussed the results obtained by this Cytophotometer.

The assembly of Cytophotometer is given in figure . In this the LDR was kept in a dark chamber and the image of the tissue was projected on to a LDR with the help of a mirror. There are some chances of experimental error because of stray light while working with the instrument. So the LDR is kept always in a completely closed wooden box. The circuit diagram of the Cytophotometer is depicted in the figure 3. (Shah et al., 1975; Bhatt et al., 1983).

Photographs of the stained preparations were made using Photomicroscope — I with planapochromatic lenses, Carl — Zeiss, Germany. Konica colour negatives of 35 mm (36 exposures) were used to record the images. A strict control of the time of exposure for both negatives and positives was maintained to permit the visual qualitative comparison of the stain intensity.
CIRCUIT DIAGRAM OF CYTOPHOTOMETER

MIRROR  DARK CHAMBER  MAINS

SLIDE  LDR  TRANSFORMER

FILTER  TPS  STABILIZER

KOHLER ILLUMINATION

MICROSCOPE CHAMBER

LDR: LIGHT DEPENDANT RESISTOR  TPS: TRANSISTORISED POWER SUPPLY  A: AMMETER
X. STATISTICAL ANALYSIS OF OBSERVATIONS:

Statistical analysis of the observations was done using analysis of variance (ANOVA) and Karl Pearson's correlation coefficient as suggested by Sokal and Rohlf (1980). This was used to study the significance and variations between the various metabolites in pollen and pistil in the selected stages of development and also within the stages (I - VI) of pollen as well as pistil.