Chapter-III

MATERIALS AND METHODS
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For present study *Moringa oleifera* Lam. (Moringaceae), which produces pollen grains in monads, and *Dregea volubilis* (L.f.) Benth. ex Hk.f. (Asclepiadaceae) and *Dendrobium ovatum* (Willd.) Kranz. (Orchidaceae) are chosen as representatives of pollinia producing plants. Flower buds of *M. oleifera* Lam. were collected from Honavar (U. Kannada). Flower buds of *D. ovatum* were collected from Santeguli (U.Kannada; Western Ghats) and *D. volubilis* from Dharwad. Flower buds were fixed in suitable fixative (see Table-I) during morning hours. Employing standard microtechnique procedures, the fixed floral buds were dehydrated in ethanol-n-butanol series, before paraffin infiltration. Microtome sections of 6μm thickness were obtained. Due care was taken to maintain uniformity in thickness of sections for qualitative assessment of histochemical localization. Sections were deparaffinized in xylol. The deparaffinized sections were processed for the localization of following histochemical substances (also see Table-II).
**TABLE – I**

**Details of fixation and post-fixation procedures**

(Source of chemicals: Hi-Media)

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Biochemical substances under investigation</th>
<th>Fixative</th>
<th>Temperature</th>
<th>Duration of fixation</th>
<th>Post-fixation procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total insoluble polysaccharides</td>
<td>FAA</td>
<td>Room temperature</td>
<td>12 hours</td>
<td>Washed in 70% alcohol &amp; processed for dehydration.</td>
</tr>
<tr>
<td>2</td>
<td>RNA</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>3</td>
<td>Proteins</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>4</td>
<td>Cellulose</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>5</td>
<td>Callose</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>6</td>
<td>Ascorbic acid</td>
<td>Acidified alcoholic AgNO3 (5g AgNO3+34ml distilled water+66ml. of absolute alcohol+5ml. Glacial acetic acid.</td>
<td>0°C-2°C</td>
<td>7 days in darkness</td>
<td>Washed thrice in 50% alcoholic ammonia and processed further.</td>
</tr>
<tr>
<td>7</td>
<td>Lipid</td>
<td>Fresh material section</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Sporopollenin</td>
<td>&quot;</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE - II
Methods employed in the histochemical studies of fixed materials
(Source of chemicals: Hi-Media)

<table>
<thead>
<tr>
<th>Sl.No.</th>
<th>Histochemical substances localized.</th>
<th>Methods adopted</th>
<th>Pretreatment if any</th>
<th>Reaction reagent</th>
<th>Temperature</th>
<th>Duration</th>
<th>Colour indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Insoluble polysaccharides</td>
<td>Periodic acid Schiff’s</td>
<td>0.5% periodic acid for 15 min.</td>
<td>Schiff’s reagents</td>
<td>Room temperature</td>
<td>20 min.</td>
<td>Magenta red</td>
</tr>
<tr>
<td>2.</td>
<td>Starch</td>
<td>IKI</td>
<td>-</td>
<td>IKI</td>
<td>50°C</td>
<td>10 min.</td>
<td>Blue-black Purple</td>
</tr>
<tr>
<td>3.</td>
<td>RNA</td>
<td>a. Azure-B</td>
<td>-</td>
<td>0.025% Azure-B</td>
<td>2 hours</td>
<td>2 hours</td>
<td>Purple</td>
</tr>
<tr>
<td>4.</td>
<td>Proteins</td>
<td>b. Toluidine blue</td>
<td>-</td>
<td>Toluidine blue</td>
<td>room temp</td>
<td>5 min.</td>
<td>purple</td>
</tr>
<tr>
<td>5.</td>
<td>4. Proteins</td>
<td>a. Mercuric bromophenol blue</td>
<td>-</td>
<td>Mercuric bromophenol blue</td>
<td>Room temp.</td>
<td>15 min.</td>
<td>Blue</td>
</tr>
<tr>
<td>6.</td>
<td>b. Amido black</td>
<td>-</td>
<td>0.05% amido black 10B</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td>5. Ascorbic acid</td>
<td>Acidified AgNO₃</td>
<td>-</td>
<td>5% AgNO₃</td>
<td>0°C to 2°C</td>
<td>7 days</td>
<td>black</td>
</tr>
<tr>
<td>8.</td>
<td>Cellulose</td>
<td>Calcofluor white</td>
<td>-</td>
<td>0.01% calcofluor under fluorescence microscope</td>
<td>Room temp.</td>
<td>20 sec</td>
<td>Pale blue</td>
</tr>
<tr>
<td>9.</td>
<td>Callose</td>
<td>Aniline blue</td>
<td>-</td>
<td>0.005% aniline blue under fluorescence microscope</td>
<td>Room temp.</td>
<td>10 min.</td>
<td>Yellowish white</td>
</tr>
<tr>
<td>10.</td>
<td>Lipids</td>
<td>Sudan black B</td>
<td>-</td>
<td>0.7% Sudan black B</td>
<td>Room temp.</td>
<td>10 min.</td>
<td>Blue-black</td>
</tr>
<tr>
<td>11.</td>
<td>Sporopollenin</td>
<td>Spirit-soluble Aniline blue</td>
<td>-</td>
<td>0.005% spirit-soluble aniline blue</td>
<td>Room temp.</td>
<td>4-6 hrs.</td>
<td>Deep blue</td>
</tr>
</tbody>
</table>
1. **Total insoluble polysaccharides: PAS method (Feder and O' Brien, 1968)**

Total insoluble polysaccharides are localized by employing Periodic Acid Schiff's (PAS) method. In this method periodic acid (HIO₄) is used to oxidise 1,2-glycol groups of polysaccharides into aldehyde groups. Aldehyde groups thus formed, react with leucobasic-fuschin of Schiff's reagent to produce a visible magenta-red colour. Periodic Acid Schiff's method is highly recommended for the localization of total insoluble polysaccharides because of the following characteristics.

i. The reaction does not cause the breakage of polysaccharide chains.

ii. It is specific to polysaccharides.

iii. It offers least interference and gives no false localization.

iv. It results in intense and stable colour complex.

### a. Preparation of reagents:

i. **0.5% periodic acid:** 500 mg of periodic acid is dissolved in 100 ml of distilled water.
ii. **Schiff's reagent:** 1 gm of basic fuschin is dissolved in 100ml of 0.15 N HCL, agitated for three hours and then added with 1 gm of sodium metabisulphate and kept overnight. Treatment of this solution with activated charcoal produces straw colour Schiff's reagent.

iii. **Bleach:** 0.5 gm of sodium metabisulphate is dissolved in 95ml of distilled water to which 5 ml of 1 N HCL is added.

### b. Staining procedures:

(i). Deparaffinized and hydrated sections were incubated in 0.5% periodic acid for 15 minutes at room temperature.

(ii). Sections were washed in running water and incubated in Schiff's reagent for 15 minutes at room temperature.

(iii). Stained sections were then washed in distilled water and treated with bleach to remove superfluous stain.

(iv). Sections were then washed in distilled water, dehydrated, cleared in xylol and mounted with DPX.
c. Colour indication:

Polysaccharides stain magenta-red.

Control test for PAS-reaction:

Omitting Periodic acid treatment (Cass and Jensen, 1970):

Hydrated sections were directly incubated in Schiff's reagent without pre-treatment with periodic acid. Known sites, such as cell walls, do not show magenta-red colour.

IKI confirmatory test for starch (Johansen, 1940):

In the present study, this test is used to confirm whether PAS-positive grains are starch grains or not. The accumulation of iodine in the center of the helical starch grain molecule has been considered as the basis for colour formation (Jensen, 1962).
Staining procedure:

Hydrated sections were treated with IKI solution prepared by dissolving 2 gms of potassium iodide in 100 ml of distilled water and 2 gms of iodine was dissolved in this solution.

Colour indication:

Older starch grains stain blue-black and newly formed ones red-violet. Starch grains, are also PAS-positive grains.

2a. RNA: Azure B method (Flax and Himes, 1952):

In this method phosphoric acid is responsible for staining both RNA and DNA with azure B. This technique is based on phenomenon of metachromasia in which certain cell constituents stain differentially from the original colour of the dye. The purple colour of the dye is termed as orthochromatic shade and blue colour is metachromatic shade. With azure B at pH 4.0, RNA exhibits orthochromatic shade, whereas DNA exhibits metachromatic shade. Lignin appears green. Although azure B stains both RNA and DNA, only the localization of RNA is taken into account in the present investigation.
a. Preparation of reagents:

i. Citrate buffer of pH 4.0:

Stock solutions:

A: 0.1 M solution of citric acid (21.01 gms in 1000 ml).

B: 0.1 M solution of sodium citrate (29.41 gms in 1000 ml).

33.0 ml of A + 17.0 ml of B, diluted to a total of 100 ml.

ii. 0.025% azure B stain: 12.5 mg of azure B dissolved in 50 ml of citrate buffer at pH 4.0.

b. Staining procedure:

i. Hydrated sections were incubated in 0.025% azure B stain at 50° C for 2 hours.

ii. Sections were rinsed in distilled water, air-dried, differentiated in butanol, cleared in xylol and mounted with DPX.

c. Colour indication:

RNA stains purple and lignin green.
Control test for RNA:

Ribonuclease extraction method (Pearse, 1960):

Deparaffinized and hydrated sections were incubated for 1 hour at 37°C in 0.1mg/ml solution of ribonuclease in distilled water. After washing in water, the sections were stained with azure B. Sites of RNA do not stain.

Removal of RNA and DNA by 1 N HCL method (Dempsey et al., 1947):

Hydrated sections were immersed in 1 N HCL at 37°C, for 3 hours, rinsed in water and stained with azure B. The sites of RNA and DNA do not stain.

Removal of RNA alone by 10% perchloric acid method (Erickson et al., 1949):

Hydrated sections were immersed in 10% perchloric acid at 2°C for about 18 hours. Then the sections were rinsed in 1% sodium carbonate and again in water before proceeding to azure B staining. Both nucleus and cytoplasm do not stain.
2b. RNA: Toluidine blue method (Chayen et al., 1973).

a. Preparation of reagents:
   i. 0.50 mg toluidine blue is dissolved in 100 ml of 0.05 M citrate phosphate buffer at pH 4.4.

   Stock solutions:
   A: 0.1 M solution of citric acid (19.21 gms in 1000 ml)
   B: 0.2 M solution of disodium phosphate, (53.65 gms of Na₂HPO₄ 7H₂O or Na₂HPO₄, 12H₂O in 1000 ml).
   27.8 ml of A + 22.2 ml of B, diluted to a total of 100 ml.

b. Staining procedure:
   i. Deparaffinized and hydrated sections were immersed in 0.05% toluidine blue for 5 minutes.
   ii. Sections were rinsed in distilled water, air dried, cleared in xylol and mounted with DPX.

c. Colour indication:
   RNA stains purple and DNA blue green.
Control test:

As described for azure B method.

3a. Total proteins: Mercuric bromophenol blue method (Mazia et al., 1953; Bhandari, 1997).

This method is employed for the localization of total proteins for the following advantages.

i. Even the minute quantity of proteins can be localized.

ii. The dye binds itself to the basic proteins even when mercury is present and also with other proteins when mercury is absent.

iii. Amount of dye bound is proportional to the quantity of proteins present.

a. Preparation of reagent:

i. 10 mg of bromophenol blue dissolved in 100 ml of 10% mercuric chloride solution in 95% alcohol.

b. Staining procedure:

i. Deparaffinized sections were brought to absolute alcohol
and incubated for 15 minutes in mercuric bromophenol blue at room temperature.

ii. Sections were rinsed in 0.5% acetic acid for 5-10 minutes and differentiated in tap water until the sections were blue.

iii. Sections were air dried, cleared in butanol and then xylol and mounted with DPX.

c. **Colour indication:**

Sites of proteins stain blue.

**Control test for proteins:**

**Trypsin method (Pearse, 1960):**

Deparaffinized and hydrated sections were incubated for 1 hour at 37°C in 0.05 M phosphate buffer at pH 8.9 containing 1 gm/ml of pure trypsin. After washing the sections in water, sections were dehydrated upto 90% alcohol and stained with mercuric bromophenol blue. The sites of protein do not show blue colour.

a. Preparation of reagent.

i. 50 mg of amido black 10B is dissolved in 100 ml of 7% acetic acid.

b. Staining procedure.

i. Hydrated sections were incubated for 15 minutes in 0.05% amido black 10B at room temperature.

ii. Sections were rinsed in 7% acetic acid for 1 minute, air dried, cleared in xylol and mounted with DPX.

c. Colour indication:

Proteins appear blue.

Control test:

As described for, mercuric bromophenol blue test.

Earlier workers developed methods for localization of ascorbic acid in which the tissues were subjected to staining with aqueous silver nitrate, prior to sectioning. The problem of ascorbic acid localization arose from the solubility of ascorbic acid in aqueous solution. To avoid this, only freeze-dried tissue sections are used. This method prevented the tissue coming in contact with aqueous solution prior to localization. (Jensen and Kavaljian, 1956). Keeping these points in view, Dave et al. (1968) devised a method in which both fixation and staining steps are combined together. This method is known as acidified alcoholic silver nitrate method. According to Dave et al. (1968), at low temperature, silver nitrate is reduced to silver and there is no shifting of ascorbic acid because both fixation and staining are done simultaneously.

a. Preparation of reagent:

i. 5 gms of silver nitrate is dissolved in a mixture of 34 ml distilled water, 66 ml absolute alcohol and 5 ml glacial acetic acid.
b. **Staining procedure:**

i. The flower buds of different sizes were incubated in acidified alcoholic silver nitrate solution for 7 days at 0°C-2°C.

ii. After 7 days, flower buds were washed thrice in 5% liquid ammonia in 50% alcohol for 15 minutes under red light in dark room, dehydrated in alcohol-butanol series, embedded in paraffin and microtomed at 6 µm thickness.

iii. The slides were deparaffinized and mounted with DPX.

c. **Colour indication:**

The sites of ascorbic acid appear as dark coloured fine grains of silver.

**Control test for ascorbic acid:**

Prior to the fixation, as per the procedure mentioned above, the materials were treated with 10% formalin for 4 hours. Known sites of ascorbic acid do not show any colouration.

The compositional changes occurring in the cell wall during growth and differentiation of plant cells can be detected by fluorescence technique.

In the present study changes in the cellulose composition of cell wall are detected by using calcofluor-white as fluorochrome. According to Maeda and Ishida (1967) calcofluor white binds not only cellulose composition and chitin but also to a variety of β-linked polymers. Walls were brilliantly fluorescent while most cytoplasmic components are unstained. Calcofluor white is also known to bind strongly in addition to cellulose, to carboxylated polysaccharide and callose.

a. Preparation of reagent:

i. 10 mg of calcofluor white is dissolved in 100 ml of distilled water.
b. **Staining procedure:**

Deparaffinized sections were stained for 20 seconds in 0.01% aqueous solution of calcofluor white and examined under fluorescent microscope using ultra violet excitation using G. 247nm and 410nm filters.

c. **Colour indication:**

Sites of cellulose fluoresce strongly as yellowish green colour.

**Confirmatory test:**

**Differential extraction of the cell wall constituents**

*(Jensen, 1962):*

Three slides containing hydrated sections were treated with 0.5% ammonium oxalate at 70°C-80°C to remove both the water soluble and pectic substances. One slide is stained with calcofluor white solution.
The remaining two slides were then extracted with 4% NaOH at 25°C to remove the hemi cellulose from the wall. Of these two slides, one stained with calcofluor white solution.

The third slide then treated with 17.5% NaOH at 25°C to remove all non-cellulosic polysaccharides and stained with calcofluor white solution.

All the three slides showed no difference in the fluorescence indicating the specificity of calcofluor white staining.


Callose is a specialized cell wall polysaccharide, chemically being β-1-3 glucan in nature. It is an amorphous and colourless substance, insoluble in water and alcohol, and is soluble in cold concentrated H₂SO₄ and dilute KOH. Formerly aqueous aniline blue staining method was used to localize callose in plants. Currier (1957) and Linskens and Esser (1957) have shown that decolourised aniline blue may also serve as a vital fluorochrome.
Now the method of aniline blue combination with fluorescent microscopy is commonly used.

a. Preparation of reagent:

i. Phosphate buffer of pH 8.3: Stock solution consists of (a) M/15 Sodium phosphate dibasic Na₂HPO₄ (9.47 gms dissolved and diluted to make 1 liter) and (b) M/15 Potassium phosphate monobasic NaH₂PO₄ 2H₂O (9.08 gms dissolved and diluted to make 1 liter). Stock solution (a) and (b) are mixed as indicated below:

<table>
<thead>
<tr>
<th>pH</th>
<th>(a)</th>
<th>(b)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.3</td>
<td>95 ml</td>
<td>5 ml</td>
<td>200 ml</td>
</tr>
</tbody>
</table>

The above mixture will have about 8.47 pH. To reduce pH to 8.3 solution (b) is added drop-wise, until pH meter shows 8.3.

ii. 0.005% aniline blue solution: 5 mg of water-soluble aniline blue is dissolved in phosphate buffer of pH 8.3. In the beginning the solution is blue coloured, after few minutes the solution
becomes decolourised. The decolourised solution is used for staining.

b. Staining procedure:

i. Deparaffinized and hydrated sections were treated with 0.005% decolourized water-soluble aniline blue solution prepared in phosphate buffer of pH 8.3 for 10 minutes.

ii. Sections were observed under the fluorescent microscope using G. 247nm and 410nm filters.

c. Colour indication:

Under fluorescent microscope callose appears as yellowish white fluorescence.

Control test for callose:

The deparaffinized and hydrated sections were treated with cold concentrated H₂SO₄ or dilute KOH for 10 minutes. The sections are washed in water and treated with aniline blue. Known sites of callose do not fluoresce.
7. Test for lipid: Sudan Black B method (Baker, 1947; Gomori, 1952):

Sudan black B method was employed for in situ localization of lipids in fresh material sections.

a. Reagents required:

1. Sudan black B.
2. Ethylene glycol.
3. Glycerine jelly.

b. Preparation of Glycerine jelly:

Gelatin-10gms, distilled water-60ml, glycerol-70ml, phenol crystals-250mg.

Preparation of solutions:

i. Sudan black B: 0.7gms of Sudan black B powder was dissolved in 100ml of ethylene glycol. While dissolving the dye, ethylene glycol should be heated to 90°C. Hot solution was filtered, cooled and stored in freeze.
ii. Differentiating bath: 85ml of ethylene glycol mixed with 15ml of distilled water.

c. Staining procedure:

i. Fresh sections were placed in 50% ethylene glycol, for 5 minutes.

ii. The ethylene glycol was removed and tissue sections were stained with Sudan black B solution for about 10 minutes.

iii. Sections were differentiated in the solution of glycol and water for about 1 minute. During staining as well as during differentiation sections were agitated occasionally.

iv. Sections were washed thrice in distilled water about 2 minutes each.

v. Sections were mounted in glycerine jelly.


d. Colour indication:

The lipids stain blue-black.

Sporopollenin is an unusual biopolymer of extremely high chemical, physical and biological stability. It is derived from carotenoids and carotenoid esters. Sporopollenin forms the basic structure of the exine of pollen. Spirit soluble aniline blue selectively stains both the Ubisch bodies and pollen exine, which are composed of sporopollenin.

a. Preparation of the stain:

0.005% spirit-soluble aniline blue solution in 50% ethanol.

b. Staining procedure:

(i). A fine film of Haupt's adhesive is applied to a clean slide and allowed to air dry for about 3-5 minutes.

(ii). Hand-cut sections of anther were placed in a drop of water on the slide.

(iii). The sections were dried for 10-15 minutes at 30-35°C.

(iv). Sections were stained for 4-6 hours in 0.005% spirit-soluble aniline blue dissolved in 50% ethanol.
(v). Slides were treated in acetone for 10 minutes.

(vi). Sections were dehydrated in 1:1 mixture of acetone and xylene, cleared in xyline and mounted with DPX.

c. Colour indication:

Sporopollenin stains deep blue.

Acetolysis test:

i. Pollinia were pretreated with 70% alcohol.

ii. Centrifuged and alcohol was poured out.

iii. Pollinia were treated with 5ml of glacial acetic acid, centrifuged and decanted.

iv. Pollinia were incubated in acetolysis mixture (1ml of concentrated H$_2$SO$_4$ was added drop by drop to 9ml of acetic anhydride) for 1 minute. Then heated from 70 °C to boiling point in water bath.

v. Stirred with glass rod, centrifuged and decanted waste acetolysis mixture.

vi. 10ml of glacial acetic acid was added, centrifuged and decanted.
vii. Washed repeatedly with distilled water centrifuged and decanted.

viii. Pollinia were mounted in glycerine jelly.

**Colour indication:**

Acetolysed pollen appears brown.

**Photomicrography:**

Photomicrography is made by using Kodolith 36ASA black and white negative films and Nicon 35-mm optical camera. For fluorescent studies, 400 ASA black and white films were used.

In most of the histochemical staining, the intensity of the colour of the reaction product is directly proportional to the quantity of substance present in a cell or a tissue. Since uniformity in thickness of section is maintained, the colour intensity of the reaction product is considered as an indication of the quantity of the substance present and is described using arbitrary terms such as ‘high’, ‘low’, ‘rich’, ‘weak’ etc.