Material & methods
Seeds of cytoplasmic male sterile 234-B and its nuclear isogenic male fertile, sunflower (Helianthus annuus L.) were obtained from oil seed section, University of Agriculture, Dharwad. Seedlings were raised in the botanical garden of Kittel Science College, Dharwad. Initiation of flowering was observed in two and half months-old plants (Plate I A, B).

**A Fixed material studies** (Table I and II)

Flower buds, from both the lines, at successive developmental stages were fixed in a suitable fixative (Table I). The fixed flower buds were dehydrated in ethanol series and n-butanol, before paraffin infiltration. Microtome sections of 5-6 μm thickness were obtained. Due care was taken to maintain uniformity in the 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.

1. **Total insoluble polysaccharides : PAS method** (Hotchkiss, 1948 in Jensen, 1962)

Localization of total insoluble polysaccharides was
## TABLE I

Details of fixation and post-fixation procedures

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Biochemical substances under investigation</th>
<th>Fixative</th>
<th>Temperature of fixation</th>
<th>Duration of fixation</th>
<th>Post-fixation procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Insoluble polysaccharides</td>
<td>Carnoy's</td>
<td>room temp.</td>
<td>12 hours</td>
<td>washed in 70% alcohol &amp; processed further</td>
</tr>
<tr>
<td>2.</td>
<td>DNA</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>3.</td>
<td>RNA</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>4.</td>
<td>Proteins</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>5.</td>
<td>Cellulose</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>6.</td>
<td>Callose</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
<td>&quot;</td>
</tr>
<tr>
<td>7.</td>
<td>Histones</td>
<td>NBF</td>
<td>&quot;</td>
<td>18 hours</td>
<td>washed in running water overnight, dehydrated &amp; processed further</td>
</tr>
<tr>
<td>8.</td>
<td>Ascorbic acid</td>
<td>acidified alcoholic AgNO₃</td>
<td>0°C to 2°C</td>
<td>7 days in darkness</td>
<td>washed thrice in 50% alcoholic ammonia &amp; processed further</td>
</tr>
<tr>
<td>SL No</td>
<td>Histochemical substances localized</td>
<td>Methods adopted</td>
<td>Pretreatment if any</td>
<td>Reaction reagent</td>
<td>Temperature</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------</td>
<td>----------------</td>
<td>---------------------</td>
<td>------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>1</td>
<td>Insoluble polysaccharides</td>
<td>Periodic acid-Schiff's</td>
<td>0.5% periodic acid for 15 minutes</td>
<td>Schiff's reagent</td>
<td>room temp.</td>
</tr>
<tr>
<td>2</td>
<td>Starch</td>
<td>IKI</td>
<td>--</td>
<td>IKI</td>
<td>--</td>
</tr>
<tr>
<td>3</td>
<td>DNA</td>
<td>Feulgen</td>
<td>1NHC1 for 13 min at 60°C</td>
<td>Schiff's reagent</td>
<td>--</td>
</tr>
<tr>
<td>4</td>
<td>RNA</td>
<td>a. Azure-B</td>
<td>--</td>
<td>0.025% azure-B</td>
<td>50°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b Toluidine</td>
<td>--</td>
<td>0.05% toluidine blue</td>
<td>room temp</td>
</tr>
<tr>
<td>5</td>
<td>Proteins</td>
<td>a. Mercuric bromophenol blue</td>
<td>--</td>
<td>Mercuric bromophenol blue</td>
<td>room temp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>b. Amido black 10B</td>
<td>--</td>
<td>0.05% amido black 10B</td>
<td>room temp</td>
</tr>
<tr>
<td>6</td>
<td>Histones</td>
<td>Ammoniacal AgNO₃</td>
<td>10% NBF for 1 hour</td>
<td>ammoniacal AgNO₃</td>
<td>room temp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Ascorbic acid</td>
<td>Acidified AgNO₃</td>
<td>--</td>
<td>5% AgNO₃</td>
<td>0°C to 2°C</td>
</tr>
<tr>
<td>8</td>
<td>Cellulose</td>
<td>Calciofluor white</td>
<td>--</td>
<td>0.01% calciofluor under fluorescence microscope</td>
<td>room temp</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>
</tr>
</tbody>
</table>
made by employing Periodic Acid Schiff's (PAS) method. This reaction is based on the oxidation of 1, 2-glycol groups of polysaccharides into aldehyde groups with periodic acid (HIO₄). Aldehyde groups 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.

1) The reaction does not cause the breakage of polysaccharide chains.
2) It is specific to polysaccharides.
3) It offers least interference and gives no false localization.
4) It results in a colour complex which is intense as well as stable.

Staining procedure

1) Hydrated sections were treated with 0.5% periodic acid for 15 minutes at room temperature.
2) Sections were washed in running water and incubated in Schiff's reagent for 15 minutes at room temperature. (Schiff's reagent is prepared by dissolving 1 gm of basic fuschin in 100 ml of 0.15 N HCl,
agitated for 3 hours and then added with 1 gm of sodium metabisulphite and kept over night. Treatment of this solution with activated charcoal produces straw colour Schiff's reagent.)

iii) Stained sections were washed in distilled water and treated with bleach to remove superfluous stain (bleach is prepared by dissolving 0.5 gms of sodium metabisulphite in 95 ml of distilled water to which 5 ml of 1 N HCl is added).

iv) Sections were then washed in distilled water, dehydrated, cleared in xylol and mounted with DPX.

Colour indication

Polysaccharides stain magenta-red (Plate II A, B).

Control test for PAS reaction

Avoiding pre-treatment with periodic acid (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 were starch grains or not. The accumulation of iodine in the centre 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 and 2 gms of iodine in 100 ml of distilled water.

Colour indication

Older starch grains stain blue-black and newly formed ones red-violet.

2. DNA : Feulgen method (Gomori, 1952)

This method is based on Schiff's reaction for staining aldehydes, released from deoxyribose sugar, after the removal of purines at the level of purine-deoxyribose glucosidic bonds of DNA by hydrolysing in 1 N HCl.
Staining procedure

1) Hydrated sections were hydrolysed in 1 N HCl at 60°C for 13 minutes.

ii) Sections are rinsed in water and incubated with Schiff's reagent at room temperature for 30 minutes.

iii) After rinsing in water sections are treated with bleach and again rinsed in water, dehydrated, cleared in xylol and mounted with DPX.

Colour indication

DNA appears magenta-red (Plate II C, D).

3a. 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 differently from the original colour of the dye. The purple colour of the dye is termed as orthochromatic shade and blue colour is called as 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.

Staining procedure

1) Hydrated sections were incubated in 0.025% azure B stain (12.5 mg azure B dissolved in 50 ml of citrate buffer at pH 4.0) at 50°C for 2 hours.

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

Colour indication

RNA stains purple and lignin green (Plate III A,B,C).

Control test for RNA

Ribonuclease extraction method (Pearse, 1960) : Deparaffinized and hydrated sections were incubated for 1 hour at 37°C in 0.1 mg/ml solution of ribonuclease in distilled water. After washing in water, the sections are 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 and DNA by hot perchloric acid method (Erickson et al., 1949): Hydrated sections were treated with 5% perchloric acid at 60°C for 30 minutes, rinsed in 1% sodium carbonate solution for 2 minutes, then in distilled 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 at 2°C for about 18 hours. Then the sections were rinsed in 1% sodium carbonate and again in water before subjected to azure B staining procedure. Both nucleus and cytoplasm do not stain.

3b. RNA: Toluidine blue method (Chayen et al., 1973)

Staining procedure

i) Hydrated sections were immersed in 0.05% toluidine blue for 5 minutes (0.50 mg toluidine blue dissolved
in 100 ml of 0.05 M citrate phosphate buffer at pH 4.4).

11) Sections were rinsed in distilled water, air dried, cleared in xylol and mounted with DPX.

Colour indication

RNA stains purple and DNA blue-green.

4a. Total proteins: Mercuric bromophenol blue method (Mazia et al., 1953)

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

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) The amount of dye bound is proportional to the quantity of proteins present.

Staining procedure

1) Deparaffinized sections were brought to the absolute
alcohol and incubated for 15 minutes in mercuric bromophenol blue at room temperature (10 mg of bromophenol blue dissolved in 100 ml of 10% mercuric chloride solution in 95% alcohol).

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

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

Colour indication

Sites of proteins stain blue (Plate IV A, B).

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 8.9 pH containing 1 mg/ml of pure trypsin. After washing the sections in water, sections were dehydrated up to 90% alcohol and stained with mercuric bromophenol blue. The sites of protein do not show blue colour.

**Staining procedure**

1) Hydrated sections were incubated for 15 minutes in 0.05% amido black 10B at room temperature (stain is prepared by dissolving 50 mg amido black 10B in 100 ml of 7% acetic acid).

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

**Colour indication**

Proteins appear blue.

5. Histones: Ammoniacal silver nitrate method (Black and Ansley, 1964)

Flower buds were fixed in Neutral Buffer Formalin (NBF) at pH 7.0 for 18 hours, washed in running water overnight, dehydrated, and finally embedded in paraffin. Microtome sections of 5-6 μm thickness were obtained.
Staining procedure

1) Deparaffinized and hydrated sections were pre-treated in 10% NBF for 1 hour.

11) Sections were repeatedly washed in distilled water and incubated in dark for 45 seconds at room temperature in the standard ammoniacal silver nitrate reagent (the reagent is prepared with utmost care by adding 10% aqueous AgNO₃ solution dropwise to 2 ml of liquid ammonia until the first visible turbidity appeared).

111) Sections were then repeatedly washed in distilled water and developed in 3% formalin for 2 minutes.

iv) Finally the sections were dehydrated and mounted with DPX.

Colour indication

The sites of histone appear black (Plate IV C).

Control test for histone

Trypsin method (Pearse, 1960): Hydrated sections were incubated for 1 hour at 37°C in 0.05 M phosphate buffer at pH 8.9 containing 1 mg/ml of pure trypsin. Sections were washed in water, pre-treated in NBF and stained with
ammoniacal silver nitrate solution. Sites of histone do not stain.

6. Ascorbic acid: Acidified alcoholic silver nitrate method (Dave et al., 1968)

Earlier workers developed methods for the localization of ascorbic acid in which the tissues are subjected to staining with aqueous silver nitrate, prior to sectioning. The problem of ascorbic acid localization arises not from the final reaction, but from the solubility of ascorbic acid in aqueous solution. This means that, only freeze dried tissue sections should be used to avoid solubility of ascorbic acid. In this way great care has to be taken to prevent 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 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.
Staining procedure

i) The flower buds at successive growth phases were treated with acidified alcoholic silver nitrate (prepared by dissolving 5 gms of silver nitrate in a mixture of 34 ml distilled water, 66 ml of absolute alcohol and 5 ml of glacial acetic acid) 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 5-6 μm thickness.

iii) The slides were deparaffinized and mounted with DPX.

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.
7. **Cellulose : Calcofluor white method** (Hughes and McCully, 1975; O'Brien and McCully, 1981)

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 walls are detected by using calcofluor as a fluorochrome. According to Maeda and Ishida (1967) calcofluor white binds not only to cellulose and chitin but also to a variety of other β-linked polymers. But Johnson et al. (1974) questioned the specificity of calcofluor white binding to chitin. Hughes and McCully (1975) used calcofluor white to stain walls of higher plants. Walls were brilliantly fluorescent while most cytoplasmic components are unstained. Calcofluor is also known to bind strongly to cellulose, carboxylated polysaccharides and callose.

**Staining procedure**

Deparaffinized sections were stained for 20 seconds in 0.01% aqueous solution of calcofluor and examined under fluorescence microscope using ultra violet light excitation.
Colour indication

Sites of cellulose fluoresce strongly with pale blue colour (Plate V A, B, C).

Confirmatory test

Differential extraction of the cell wall constituents (Jensen, 1962): Three slides containing hydrated sections were treated with 0.5% ammonium oxalate at 70-80°C to remove both the water-soluble and the pectic substances. One slide is stained with calcofluor solution.

The remaining two slides were then extracted with 4% NaOH at 25°C to remove the hemicellulose from the wall. Of these two slides, one is stained with calcofluor solution.

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

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

Callose is a specialized cell wall polysaccharides, 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. Until recently 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 in combination with fluorescence microscopy is commonly used.

**Staining procedure**

1) Hydrated sections were treated with 0.005% decolourised water soluble aniline blue solution prepared in phosphate buffer of pH 8.2-8.5 for 10 minutes.

11) Sections were observed under the fluorescence microscope.

**Colour indication**

Under fluorescence microscope callose appear as a yellowish white fluorescence (Plate VI A, B, C, & D).
B. Fresh material studies (Table III)

Histo-enzymology is relatively a recent approach which helps to correlate physiological aspects with anatomical and developmental patterns. In histo-enzymological studies what is localized is not enzyme itself but its reaction product produced by the action of the enzyme on the substrate contained in the reagents employed. Since the use of fixatives will lead to the loss of the activity of the enzyme, fresh, unfixed material is used to retain the enzyme activity in the tissues to the normal level. The use of fresh material possesses several practical difficulties such as sectioning, leaching or diffusion of enzyme etc. Further, the clarity of histo-enzymological preparations are not comparable to fixed plant materials (Hall et al., 1977).

Fresh material studies with respect to localization of enzyme activities were conducted with either unfixed frozen sections or free hand sections wherever satisfactory frozen sections could not be obtained.

Procedure followed to obtain frozen sections by cryostat

i. Isolated disc florets were treated with 10% Dimethyl Sulfoxide (DMSO) for 12 hours.
Methods employed in the histo-enzymological studies of fresh materials

<table>
<thead>
<tr>
<th>Sn No</th>
<th>Enzyme activity localized</th>
<th>Source of the procedure</th>
<th>Pre-treatment</th>
<th>Substrate in reagent</th>
<th>Temperature</th>
<th>Duration of incubation</th>
<th>Colour of reaction product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Esterases</td>
<td>Gomori 1952</td>
<td>10% DMSO for 12 hr. embedded in 15% gelatin</td>
<td>α-naphthol acetate</td>
<td>room temp.</td>
<td>10 min</td>
<td>purple red-brown</td>
</tr>
<tr>
<td>2</td>
<td>Lipase</td>
<td>Bancroft 1975</td>
<td>&quot;</td>
<td>Tween</td>
<td>&quot;</td>
<td>3 hrs</td>
<td>brownish black</td>
</tr>
<tr>
<td>3</td>
<td>Acid phosphatase</td>
<td>Gomori 1952</td>
<td>&quot;</td>
<td>Sodium β-glycero-phosphate</td>
<td>35°C</td>
<td>30 min.</td>
<td>black</td>
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<tr>
<td>4</td>
<td>Alkaline phosphatase</td>
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<td>&quot;</td>
<td>&quot;</td>
<td>37°C</td>
<td>&quot;</td>
<td>&quot;</td>
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<td>5</td>
<td>Adenosine tri-phosphatase</td>
<td>Wachstein et al. 1960</td>
<td>&quot;</td>
<td>Adenosine triphosphate</td>
<td>30°C</td>
<td>&quot;</td>
<td>&quot;</td>
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<td>6</td>
<td>Glucose-6-phosphate</td>
<td>Jensen 1962</td>
<td>&quot;</td>
<td>Glucose-6-phosphate</td>
<td>35°C</td>
<td>&quot;</td>
<td>&quot;</td>
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<tr>
<td>7</td>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>Bancroft 1975</td>
<td>&quot;</td>
<td>&quot;</td>
<td>20 min.</td>
<td>blue-violet</td>
<td></td>
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<td>8</td>
<td>Malate dehydrogenase</td>
<td>Lehmann 1973</td>
<td>&quot;</td>
<td>Sodium malate</td>
<td>&quot;</td>
<td>15 min.</td>
<td>&quot;</td>
</tr>
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<td>9</td>
<td>Succinic dehydrogenase</td>
<td>Bancroft 1975</td>
<td>&quot;</td>
<td>Sodium succinate</td>
<td>room temp.</td>
<td>30 min.</td>
<td>purple</td>
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<tr>
<td>10</td>
<td>Cytochrome oxidase</td>
<td>Burstone 1959</td>
<td>&quot;</td>
<td>Nadi-reagent</td>
<td>&quot;</td>
<td>15 min.</td>
<td>bluish-brown</td>
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<tr>
<td>11</td>
<td>Peroxidase</td>
<td>Jensen 1962</td>
<td>&quot;</td>
<td>Benzidine</td>
<td>30°C</td>
<td>5 min.</td>
<td>blue turns brown</td>
</tr>
</tbody>
</table>
11. Flower buds were embedded in solidified 15% antifreeze gelatin medium (15 gms of gelatin dissolved in 100 ml of 0.8% DMSO and solidified at 10°C).

111. Using cryostat, gelatin block containing the flower bud is cut at 12 μm thickness.

iv. The frozen sections were taken directly on the slide and subjected to the following histo-enzymological tests.

1. Non-specific esterases: α-naphthol acetate method (Gomori, 1952)

Esterases are capable of hydrolysing esters. If substrate is a simple ester such as α-naphthol acetate, the hydrolysing enzyme is called non-specific esterases.

Preparation of incubating medium

5 mg of α-naphthol acetate was dissolved in 0.1 ml acetone to which 0.2 M phosphate buffer at pH 7.4 is added. To this solution 30 mg of fast blue B was added, filtered and used immediately.
Staining procedure

Sections were placed in the incubating medium for 10 minutes at room temperature. The incubated sections were mildly washed in water for 3 minutes and then mounted in glycerine jelly.

Colour indication

The reaction product of the enzyme activity stains purple-red to brown colour (Plate VII A).

Control test

The sections were incubated in the solution lacking the substrate α-naphthol acetate.

2. Lipase: Tween method (Bancroft, 1975)

A large number of substrates have been employed for this method. Gomori (1952) recommended as best for most purposes either Tween 60, the stearic ester of a polymannitol, or product 81, the stearic ester of a polyglycol. To demonstrate his 'true lipase' Gomori (1952) recommended Tween 80, the oleic ester of polymannitol.
Preparation of incubating medium

2 ml of 10% CaCl$_2$, 2 ml of 5% Tween 60 and 40 ml of distilled water were added to the 5 ml of 0.5 M tris buffer (hydroxy methyl amino methane buffer) at pH 7.2-7.4.

Staining procedure

Frozen sections were incubated in the incubating medium for 3 hours at room temperature. The incubated sections were washed in distilled water and treated with 1% lead nitrate for 15 minutes. After washing in distilled water, the sections were immersed in 1% ammonium sulphide for 1-2 minutes. Sections were again washed in distilled water and mounted in glycerine jelly.

Colour indication

A brownish-black deposit indicates the presence of lipase.

Control test

Sections were treated with incubating medium not containing the substrate.
3. **Acid phosphatase: Metal salt method (Gomori, 1952)**

Acid phosphatase catalyzes a variety of the biochemical reactions. It regulates the plant cell metabolism through inorganic phosphorus level. It is shown that carbohydrate metabolism of plants is indirectly controlled by availability of inorganic phosphate which in turn is regulated by acid phosphatase level.

**Preparation of incubating medium**

Incubating medium was prepared by mixing 10 ml of 0.5 M acetate buffer at pH 5.0, 30 mg of sodium β-glycerophosphate and 10 mg of lead nitrate.

**Staining procedure**

Sections were incubated for 30 minutes at 35°C in reaction mixture. After incubation sections were rinsed in distilled water twice for 5 minutes each and then transferred to freshly prepared 1% ammonium sulphide solution for 5 minutes. Finally sections were washed in distilled water and mounted in glycerine jelly.
Colour indication

The reaction product in the tissue sections appears brown-black (Plate VII B).

Control test

The reaction mixture without sodium β-glycerophosphate was used.

4. Alkaline phosphatase: Metal salt method (Gomori, 1952)

Alkaline phosphatases are also hydrolytic enzymes responsible for the breakdown of phosphomonoesters. These phosphomonoesters are not specific in respect of the alcohol radical which is attached to the phosphoric acid group of the substrate and they will thus hydrolyse a wide variety of organic phosphates. The phosphomonoesterases which work on the alkaline side of neutrality and particularly at pH 9.0 and above are called alkaline phosphatases. They are concerned with the transfer of phosphate from one alcohol to another. For detection of alkaline phosphatase activity, the metal cations used are usually calcium or cobalt.
Preparation of incubating medium

The incubating medium contained 10 ml distilled water, 50 mg sodium barbiturate, 80 mg calcium chloride (CaCl$_2$·6H$_2$O), 60 mg sodium $\beta$-glycerophosphate and 10 mg magnesium sulphate (MgSO$_4$·7H$_2$O).

Staining procedure

Sections were incubated in the reaction medium at 37°C for 30 minutes. Then sections were treated successively with distilled water for 2 minutes, 2% cobalt nitrate for 5 minutes, again distilled water for 2 minutes and 2% ammonium sulphide for 5 minutes. Finally the sections were washed in distilled water twice for 2 minutes each, and mounted in glycerine jelly.

Colour indication

The reaction product in the tissue sections appears brown-black (Plate VII C).

Control test

The sections were incubated in the above incubating medium omitting sodium $\beta$-glycerophosphate.
5. Adenosine triphosphatase: Lead method (Wachstein et al., 1960)

Preparation of incubating medium

Reaction mixture was composed of 45 ml of 0.05 M, pH 7.0 tris maleate buffer, 25 mg of adenosine triphosphate (disodium salt), 50 mg of lead nitrate and 5 ml of magnesium nitrate (2.5%).

Staining procedure

Sections were incubated in the incubating medium for 30 minutes at 30°C. After incubation sections were rinsed repeatedly in distilled water and then placed in 0.5% ammonium sulphide for 1 minute. Sections were washed in distilled water for 2 minutes and then mounted in glycerine jelly.

Colour indication

Sites of adenosine triphosphatase activity is indicated by brownish-black deposits of lead (Plate VIII A).
Control test

Either heat-inactivated sections or incubating medium without adenosine triphosphate was used.

6. Glucose-6-phosphatase: Lead method (Modified from Jensen, 1962)

Preparation of incubating medium

Incubating medium was prepared by mixing 20 ml of 0.1 M, pH 6.5 acetate buffer, 13 mg of glucose-6-phosphate (monosodium salt) and 1 ml of 0.1 M lead nitrate.

Staining procedure

Unfixed sections were incubated for 30 minutes in the incubating medium at 35°C. Then they were washed several times in distilled water and immersed in 0.5% (V/V) ammonium sulphide solution for 1 minute. Then the sections were washed in distilled water and mounted.

Colour indication

Sites of glucose-6-phosphatase activity were indicated by brown-black precipitate of lead sulphide.
Control test

Heat-inactivated sections were used.

7. Glucose-6-phosphate dehydrogenase : NBT method
(Bancroft, 1975)

It is wide spread enzyme in plants and animals. It is one of the important enzymes of the hexose monophosphate shunt (pentose shunt) and play significant role in the regulation of sugar metabolism. It depends on this enzyme whether glucose shall undergo glycolysis or to be utilized via the pentose shunt. The pentose shunt has the following physiological significance.

1. Pentose formed can be utilized for the synthesis of ribonucleic acids (Lojda et al., 1979).

ii. By the direct oxidation of glucose, NADPH is produced which is utilized in the biosynthesis of many important metabolites such as fatty acids, lipids etc. (Lojda et al., 1979).

iii. The pentose phosphate path way is also capable of generating erythrose, a tetrose that is used in the biosynthesis of complex pentose compounds (cited in Noggle and Fritz, 1977).
Preparation of incubating medium

The reaction mixture was prepared by mixing 0.9 ml of stock NBT solution (10 mg of Nitro-BT dissolved in 2.5 ml of distilled water to which 2.5 ml of 0.2 M tris buffer pH 7.4 was added. To this, 1.0 ml of 0.05 M magnesium chloride and 3 ml of distilled water was added. The pH was adjusted to 7.0 using stock buffer) and 0.1 ml of glucose-6-phosphate stock substrate solution (304 mg of glucose-6-phosphate dissolved in 0.8 ml of distilled water to which 0.06 ml of 1 N HCl was added. pH was adjusted to 7.1). To this 2 mg of NADP was added and dissolved.

Staining procedure

Freshly cut frozen or hand cut sections were covered with one or two drops of incubating medium for 20 minutes at 35°C. After incubation the sections were washed in water and mounted.

Colour indication

Sites of glucose-6-phosphate dehydrogenase activity exhibit blue-violet colour (Plate VIII B).
Control test

Either heat-inactivated sections were incubated in normal incubating medium or fresh sections were incubated in incubating medium without substrate.


It is suggested that different isozymes of malate dehydrogenase are localized in different cellular compartments, such as mitochondria, peroxisomes, cytoplasm and even chloroplast (see Malik and Singh, 1980). The enzyme is concerned with the respiratory process and carboxylation phenomena, especially with the reaction catalysed by PEP-carboxylase. The oxaloacetate produced by the carboxylase enzyme can be reduced to malate by malate dehydrogenase.

Preparation of incubating medium

Incubating medium contained 10 ml of 0.5 M, pH 7.6 triethanolamine buffer, 16 mg of EDTA disodium salt, 36 mg of sodium malate (Na₃ salt), 10 mg of NAD, 13 mg of nitro-BT and 0.10 ml of phenazine methosulphate (PMS 15 mg/ml).
Staining procedure

After the sections are incubated in the reaction mixture for 15 minutes at 35°C, they were washed in distilled water and mounted in glycerine jelly.

Colour indication

Sites of malate dehydrogenase activity are indicated by blue-violet colour (Plate VIII C; Plate IX A).

Control test

Sections were incubated in the reaction mixture minus sodium malate. Heat-inactivated sections also fail to produce blue-violet colour at the sites of enzyme activity.


Succinic dehydrogenase is a part of Kreb's cycle (tricarboxylic acid cycle). It is bound to mitochondria and it contains flavin adenine-dinucleotide. Succinic dehydrogenase catalyses the reversible oxidation of succinate to fumarate when phenazine methosulphate is used as an electron acceptor.
Preparation of incubating medium

To 0.9 ml of stock NBT solution (as described for glucose-6-phosphate dehydrogenase) 0.1 ml of stock succinate substrate solution (6.75 gm of sodium succinate dissolved in 8 ml of distilled water to which 0.05 ml of 1 N HCl was added. The pH was adjusted to 7.1 with 1 N HCl) was added.

Staining procedure

Freshly cut frozen sections or hand cut sections were covered with one or two drops of incubating medium and incubated for 30 minutes at room temperature. The sections were washed in water and mounted in glycerine jelly.

Colour indication

Sites of succinic dehydrogenase activity exhibit purple formazan deposits.

Control test

Control tests were conducted either on heat-inactivated sections incubated in normal incubating medium, or incubating medium without sodium succinate was used for fresh sections.
10. Cytochrome oxidase : Metal chelation method
(Burstone, 1959)

This enzyme has been demonstrated for many years and is considered to be the characteristic of the mitochondrial membrane to which it is relatively firmly bound. It is highly active in cells containing large number of mitochondria. Thus, high activity of this enzyme can be taken as parameter to assess the degree of oxidative metabolism (Lojda et al., 1979). Cytochrome oxidase is responsible for the termination of respiratory chain. This enzyme actually catalyzes the transfer of electrons from substrate to molecular oxygen, yielding water and hydrogen peroxide as the end products. Cytochrome oxidase oxidizes the NAD+ reagent, a mixture of α-naphthol and N-phenyle-p-phenylenediamine. The reaction product is then cleared with cobalt ions.

Preparation of incubating medium

Solution 1 : 10 mg of α-naphthol and 10 mg of N-phenyle-p-phenylenediamine were dissolved in 0.5 ml of absolute alcohol. To this 35 ml of distilled water and 15 ml of 0.2 M tris buffer of pH 7.4 were added.
Solution 2: 500 mg of cobalt acetate was dissolved in 5 ml of formaldehyde and 45 ml of distilled water.

Staining procedure

Frozen or free hand cut sections were incubated in solution 1 for 15 minutes at room temperature. The incubating solution was carefully drained off.

Then the sections were treated with solution 2 for one hour, washed with distilled water and mounted in glycerene jelly.

Colour indication

Sites of cytochrome oxidase exhibit bluish-brown colouration (Plate IX B).

Control test

Either heat inactivated sections or reaction mixture without substrate were used.

Peroxidases are haemoproteins that catalyze the oxidation of various substances by means of hydrogen peroxidase. These reactions depend on the oxidation of benzidine by the peroxide-peroxidase system to a blue or brown product. The intermediate product, benzidine blue, is unstable and is oxidized to brown product without enzymic intervention.

It is proposed that peroxidases have the ability to oxidise IAA (Siegel and Glaston, 1955; Stonier et al., 1970), decomposition of hydrogen peroxidase (see Zeffren and Hall, 1973), hydroxylation of proline in the wall (Ridge and Osborne, 1970), lignification (Hepler et al., 1972; Harkin and Obst, 1973; Fielding and Hall, 1978) and ion translocation (De Jong, 1966a).

Preparation of incubating medium

The reaction mixture consisted of 5 ml of saturated benzidine, 5 ml of 0.2% hydrogen peroxide and 1 ml of 5% ammonium chloride.
Staining procedure

Sections were incubated in the reaction mixture for 5 minutes at 30°C, rinsed in distilled water and mounted in glycerine jelly.

Colour indication

The sites of enzyme activity indicate blue which after sometime turns to brown (Plate IX C).

Control test

The incubation was done in reaction mixture, lacking benzidine, or heat inactivated tissues were incubated in the normal incubating medium.

C. Photomicrography

Photomicrography of the selected sections are made by using ORWO MA-8 black and white negative films and 'Mirax Laborec' 35 mm optical camera. To indicate the colour of the reaction product, sample colour photographs were taken using Kodak Gold 100 ASA colour negative films. For fluorescent studies, 400 ASA colour and 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 the substance present in a cell or a tissue. Since uniformity in thickness of the sections 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.

D. Tissue culture studies

A small portion of the young capitulum of CMS 234-B sunflower was cut and used for the tissue culture studies. The remaining large portion of the capitulum was left intact on the plant and allowed to develop fully to confirm that the flowers of the capitulum used as explants really belonged to the cytoplasmic male sterile line (Plate X A, B, C).

From the isolated part of the capitulum, disc florets were isolated and washed repeatedly with distilled water. Then the flower buds were treated with 0.1% mercuric chloride solution for about 10-15 minutes and washed thrice with sterilized distilled water in order to make the flower buds free from the traces of sterilent. These disc florets were
categorized into:

(1) flowers containing young anther primordia with a homogenous mass of tissue,
(2) flowers containing anthers at sporogenous stage, and
(3) flowers containing anthers at callose-bound meiocytes stage.

All the 3 categories of flowers were cultured separately in Murashige and Skoog's medium (1962). One litre of nutrient medium contained:

<table>
<thead>
<tr>
<th></th>
<th>Macro elements</th>
<th>mg/litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.</td>
<td>Ammonium nitrate, NH₄NO₃</td>
<td>… 1650</td>
</tr>
<tr>
<td></td>
<td>Potassium nitrate, KNO₃</td>
<td>… 1900</td>
</tr>
<tr>
<td></td>
<td>Calcium chloride, CaCl₂.2H₂O</td>
<td>… 440</td>
</tr>
<tr>
<td></td>
<td>Magnesium sulphate, MgSO₄.7H₂O</td>
<td>… 370</td>
</tr>
<tr>
<td></td>
<td>Potassium dihydrogen orthophosphate, KH₂PO₄</td>
<td>170</td>
</tr>
<tr>
<td>ii.</td>
<td>Micro elements</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Potassium iodide, KI</td>
<td>… 0.83</td>
</tr>
<tr>
<td></td>
<td>Boric acid H₃BO₃</td>
<td>… 6.20</td>
</tr>
<tr>
<td></td>
<td>Mangnous sulphate, MnSO₄.7H₂O</td>
<td>… 22.30</td>
</tr>
<tr>
<td></td>
<td>Zinc sulphate, ZnSO₄.7H₂O</td>
<td>… 8.60</td>
</tr>
<tr>
<td></td>
<td>Sodium molybdate, Na₂MoO₄.2H₂O</td>
<td>… 0.025</td>
</tr>
</tbody>
</table>
Copper sulphate, CuSO$_4$.5H$_2$O ... 0.025
Cobalt chloride, CoCl$_2$.6H$_2$O ... 0.025

### iii. Iron source
Ferrous sulphate, FeSO$_4$.7H$_2$O ... 27.85
Ethylene diamine tetraacetic acid, Na$_2$EDTA disodium salt ... 37.25

### iv. Amino acid
Glycine $H_2NCH_2COOH$ ... 2.00

### v. Vitamins
Meso-inositol $C_6H_{12}O_6$ ... 100
Nicotinic acid, $C_6H_{5}NO_2$ ... 0.5
Pyridoxine-HCl, $C_6H_{12}ClN_3$ ... 0.5
Thiamine-HCl, $C_{12}H_{16}Cl_2N_4O_5$ ... 0.1

### vi. Carbohydrate
Sucrose ... 20000

The following supplements were added to the medium.

Coconut milk ... 10-15%
6-benzyl adenine ... 5 mg/litre
These cultures were maintained at 25 ± 2°C and 55-60% relative humidity under diffused light (intensity 150-200 lux) for 10 hours daily.

The *in vitro* growing flower buds were fixed in FAA, once in 8 days, dehydrated in alcohol-butanol series, infiltrated with paraffin and microtome sections were obtained. The anther sections showing successive developmental stages were stained with PAS method and photomicrographed.