B.Sc. BOTANY LAB MANUAL

3rd Semester

Prepared By
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Botany

MIDNAPORE CITY COLLEGE

CP5: Morphology and Anatomy - Lab

SLIDE PREPARATION OF STEM AND ROOT

Aim: To prepare temporary stained glycerine mounts of transverse sections of stem and root of Dicot and Monocot plants.

Material required: Sharp razor, brush, dropper, needles, watch glass, microscopic slides, cover-slips, safrannin, glycerine and compound microscope.

Technique:

- Take 2-3cm long pieces of the material.
- Hold the material between thumb and first finger of your left hand.
- Hold the razor in the right hand with edge of the blade facing you and handle at right angle to it.
- Dip the top of the material in water.
- Then start cutting transverse sections as fast as possible in a watch glass containing water.
- Select the thinnest section of the material with the help of a delicate brush.
- Take a clean watch glass with water, transfer thin sections of the material.
- Put a few drops of saffranin stain in the watch glass with water.
- Leave it for 3-5 minutes.
- Drain off stain and wash with water if necessary.
- Put the thinnest section in the centre of the slide.
- Put a drop of glycerine over the material.
- Cover it with a coverslip with the help of needle.
- Observe it under a compound microscope after staining and mounting.

Study under the microscope: Focus the slide under lower of microscope and then change to high power if needed

Precautions:

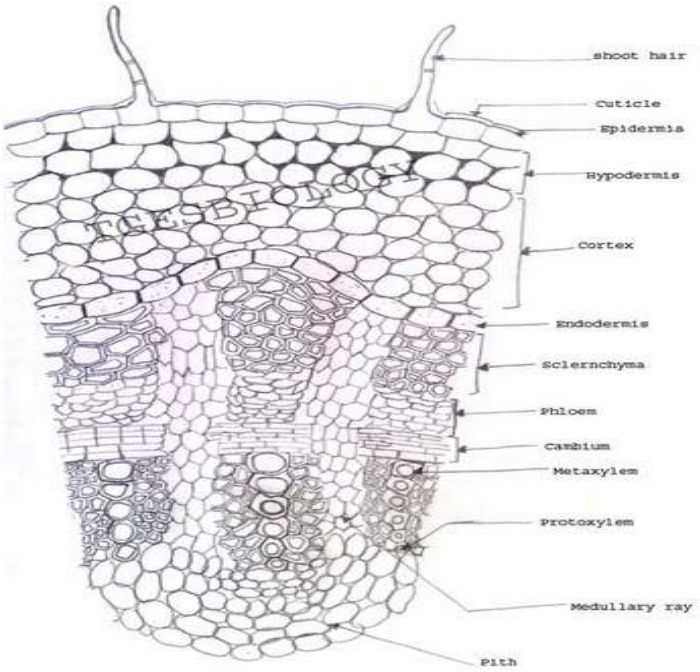
- Safranin is to be used to stain only the lignified tissues, over staining can be removed by washing in water.
- Air bubbles must be avoided in the sections.
- Use only brush to transfer or to handle the sections. Do not use needles for this purpose.
- Discard the incomplete and oblique sections.

A. Temporary slide preparation of Dicot stem

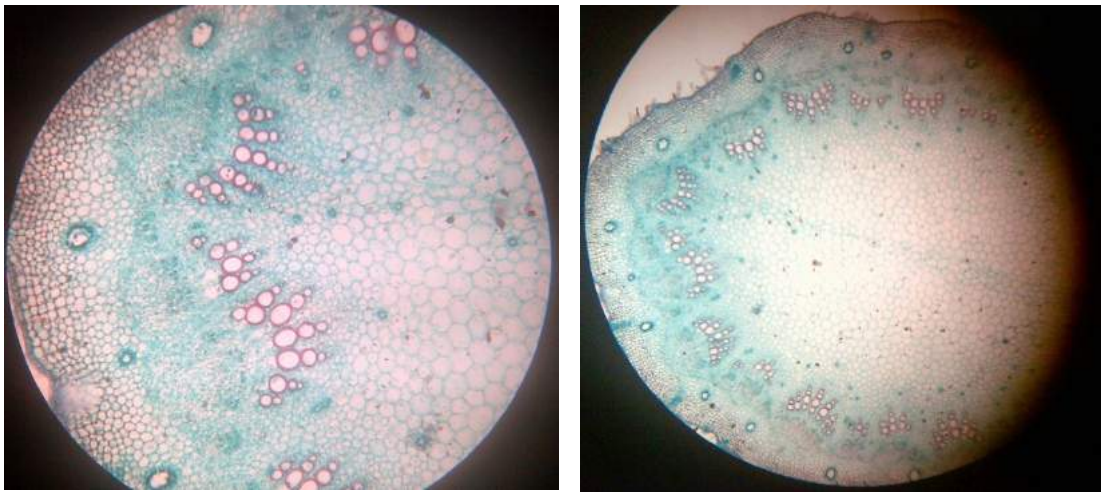
Identifying characteristics-

- Multicellular hair present on the epidermis.
- Hypodermis collenchmatous.
- Xylem endarch (metaxylem towards periphery and protoxylem towards centre)
- Vascular bundles are arranged in a ring.
- Vascular bundles conjoint, collateral and open.(cambium present)

Inference: The given specimen is the section of dicot stem.



T.S of dicot stem (for drawing)



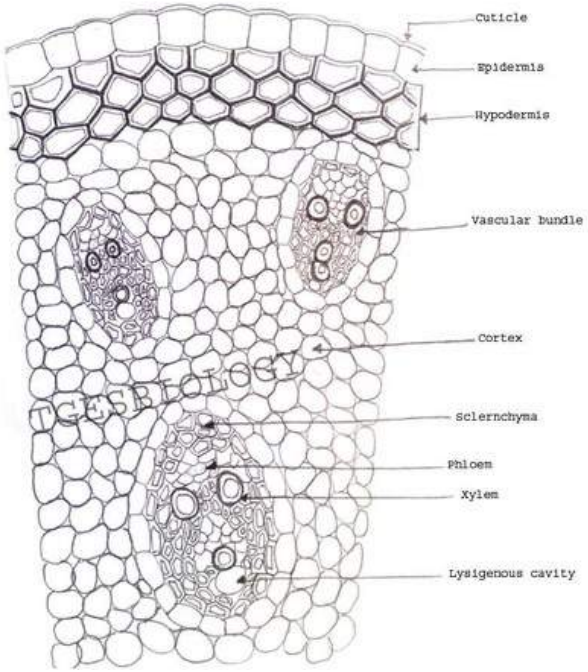
T.S of dicot stem as seen under microscope.

B. Temporary slide preparation of monocot stem

Identifying characteristics-

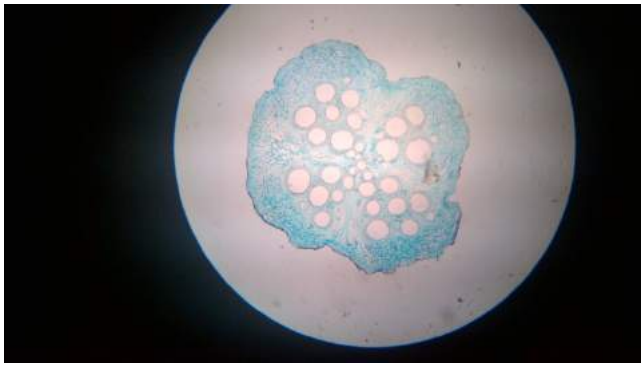
- Hypodermis is sclerenchymatous.
- Cortex is not differentiated into endodermis and pericycle.
- Vascular bundles are scattered in the ground tissue.
- Vascular bundles are conjoint,collateral and closed, i.e cambium is absent.
- Each vascular bundle is surrounded by a bundle sheath.
- Xylem is y-shaped and metaxylem lies towards periphery.

Inference: The given specimen is the section of monocot stem.



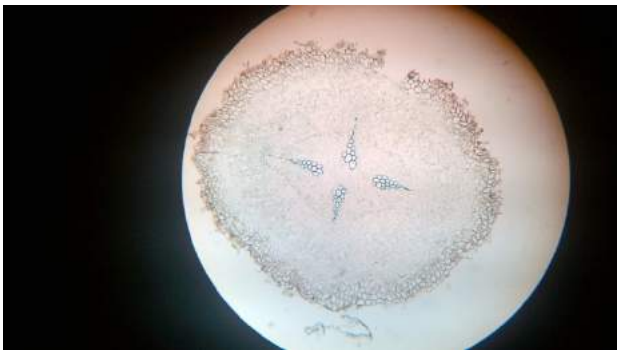
Above:- T.S of monocot stem (for drawing)

below: Section of monocot stem as seen under microscope



Section of dicot root as seen under the microscope

Section of dicot root as seen under the microscope

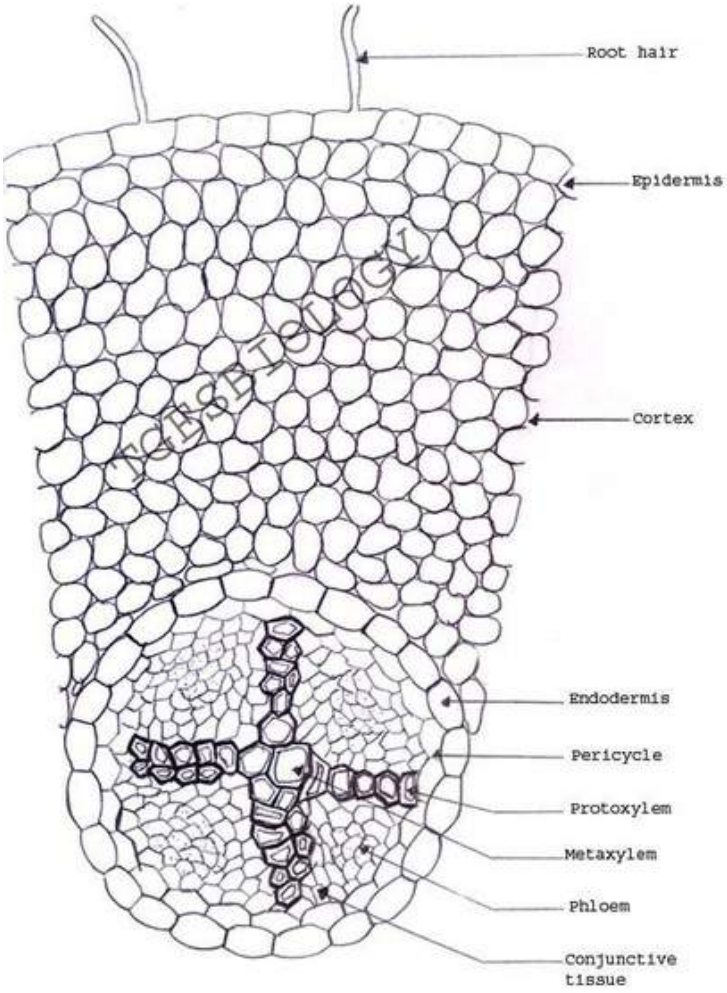


C. Temporary slide preparation of dicot root

Identifying characteristics

- Unicellular hair is present on the epidermis.
- Hypodermis is absent
- Vascular bundles are radial. Xylem and phloem are present on separate radii.
- Xylem and phloem bundles are less than 6.
- Protoxylem lies towards periphery and metaxylem lies towards centre.

Inference: the given specimen is the section of dicot root



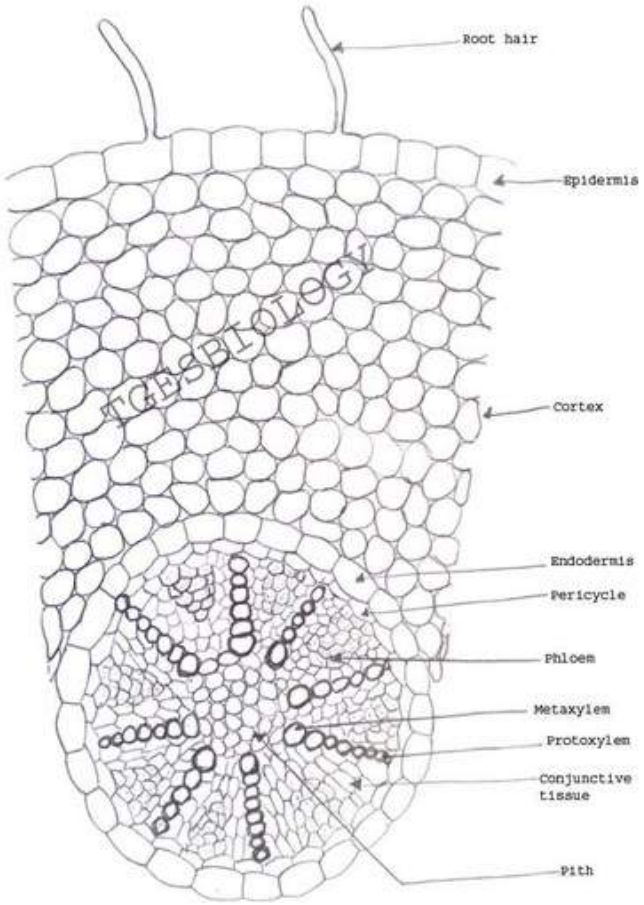
Γ.S of dicot root (for drawing)

D. Temporary slide preparation of monocot root

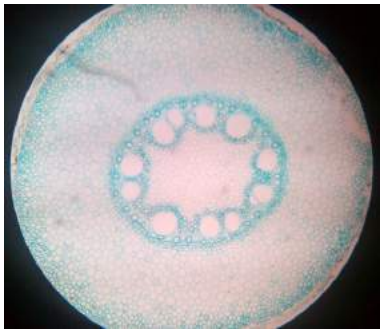
Identifying characteristics

- Unicellular hairs are present on the epidermis.
- Hypodermis is absent
- Vascular bundles are radial. xylem and phloem are present on separate radii.
- Xylem or phloem bundles are more than 5.
- Metaxylem lies towards centre.

Inference: the given specimen is the section of monocot root.



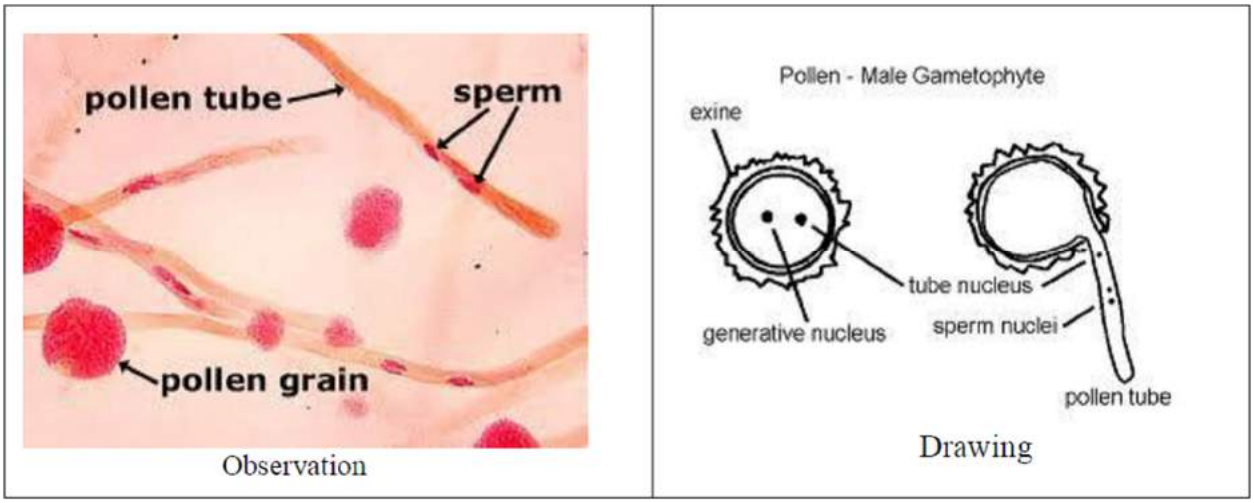
T.S of monocot root (for drawing)



TEMPORARY SLIDE PREPARATION OF GERMINATING POLLEN GRAINS

Aim: To prepare a temporary slide of germinating pollen grains.

Material Required: Petri dish, glass slides, coverslips, microscope, needle, forceps, eye piece graticule, filter paper, pollen culture solution [prepared using boric acid, KNO3, MgSO4, (CaNO3) 2], 40% sucrose solution, flowers with pollen grains(e.g.evergreen/Catharanthus, Hibiscus, Lily etc.).



Procedure:

1. Place a filter paper in a Petri dish, moisten the paper with water and replace the lids. These will form chambers in which your slides of germinating pollen can be kept humid.

2. Take 5 cm3 of pollen culture solution in a test tube and make it up to 10 cm3 with 40% sucrose solution. This now contains 20% sucrose.

3. Take two absolutely clean microscope slides and place a drop of medium in the centre of each slide. Label the slides with the names of the flowers you are investigating.

4. Gently rub the point of a mounted needle over the anthers so that pollen falls onto the drop of a culture medium. Transfer any pollen from the needle by tapping the needle against a pair of forceps. Put a coverslip over it gently.

5. Clean the needle thoroughly and repeat step **4** with other flower.

6. Note the time of adding pollen to the medium and place the slides in the Petri dish. Handle the slides with great care so that the drops of pollen medium remain in the centre of the slides.

7. Use a microscope, with x100 magnification and an eyepiece graticule, to observe the slides over the next 15-30 minutes for signs of pollen germination. Between quick observations, turn the microscope lamp off and return the slide to the Petri dish.

Observation:

- Pollen grain are seen with the pollen tubes.
- Some of the pollen grains are non-viable as they are not germinating.

Observation Table:

Result:
The viable pollen grains germinate in proper nutrient medium and show variable length of the pollen tubes.

Precautions

- Dust few pollen grains on the slide to avoid overlapping.
- Label the slides properly for different flowers.
- There should not be any air bubble under the coverslip.

Apical meristem of root, shoot and vascular cambium

Meristems are centers of cell division and growth. In animals, **totipotent** stem cells, which can differentiate into any tissue type are only found early in development; however, plants contains such embryonic tissues throughout their lives.

Apical meristems are located on the very ends of shoots (**shoot apical meristem**; SAM; Figure 18.2.118.2.1) and roots (**root apical meristem**; RAM; Figure 18.2.218.2.2). They produce three types **primary meristems**: the protoderm, ground meristem, and procambium. The **protoderm** gives rise to epidermis, which surrounds the plant. The **ground meristem** gives rise to ground tissue, a group of tissues with generalized functions such as photosynthesis, storage, and support. Finally, the **procambium** gives rise to the vascular tissue, which functions in transport. The three primary meristems first appear in the embryo proper, with the protoderm on the outside, the procambium in the center, and the ground meristem in between them.

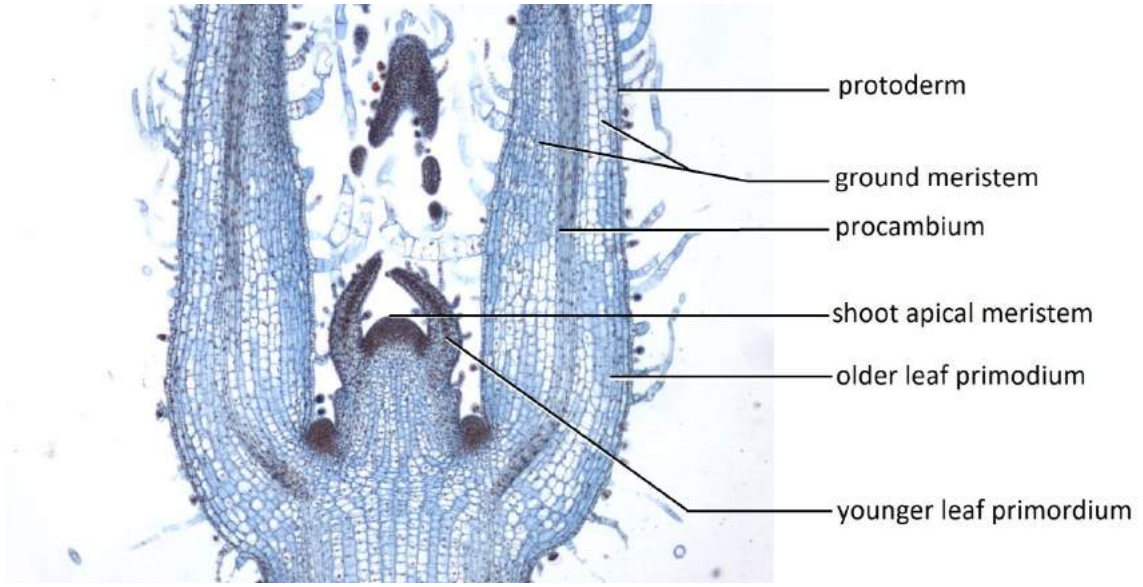


Figure 18.2.118.2.1: A longitudinal section of the shoot apex of *Coleus* includes the **shoot apical meristem** and the developing leaves (leaf primoridia) that surround it. The three **primary meristems** are visible in the leaf primordia. The **protoderm** surrounds the leaf primoridum, the **procambium** appears as a line running through the center, and the ground meristem fills the rest of the leaf primodium. Image labeled from Maria Morrow (CC-BY-NC).

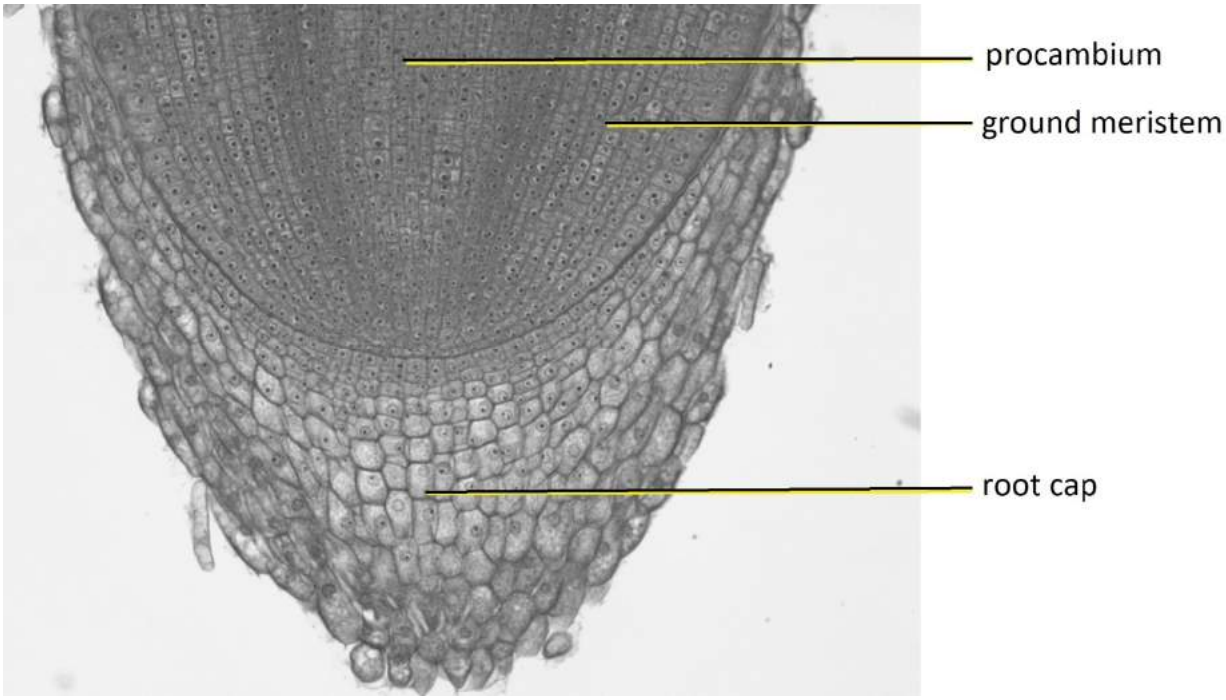


Figure 18.2.218.2.2: A longitudinal section of the root tip of corn (*Zea mays*). The large, lighter cells at the bottom form the protective root cap. The remaining visible portion of the root tip (above the root cap) is the **root apical meristem** (zone of cell division). It gives rise to the central **procambium** and the **ground meristem**, which is external to the procambium. The third primary meristem (**protoderm**) is not visible here. It would be the outer layer of the root tip, just above the current view. Image labeled from Maria Morrow (CC-BY-NC).

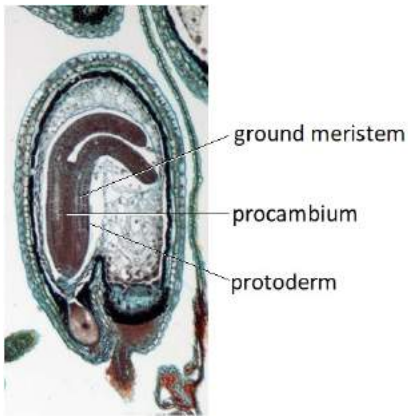
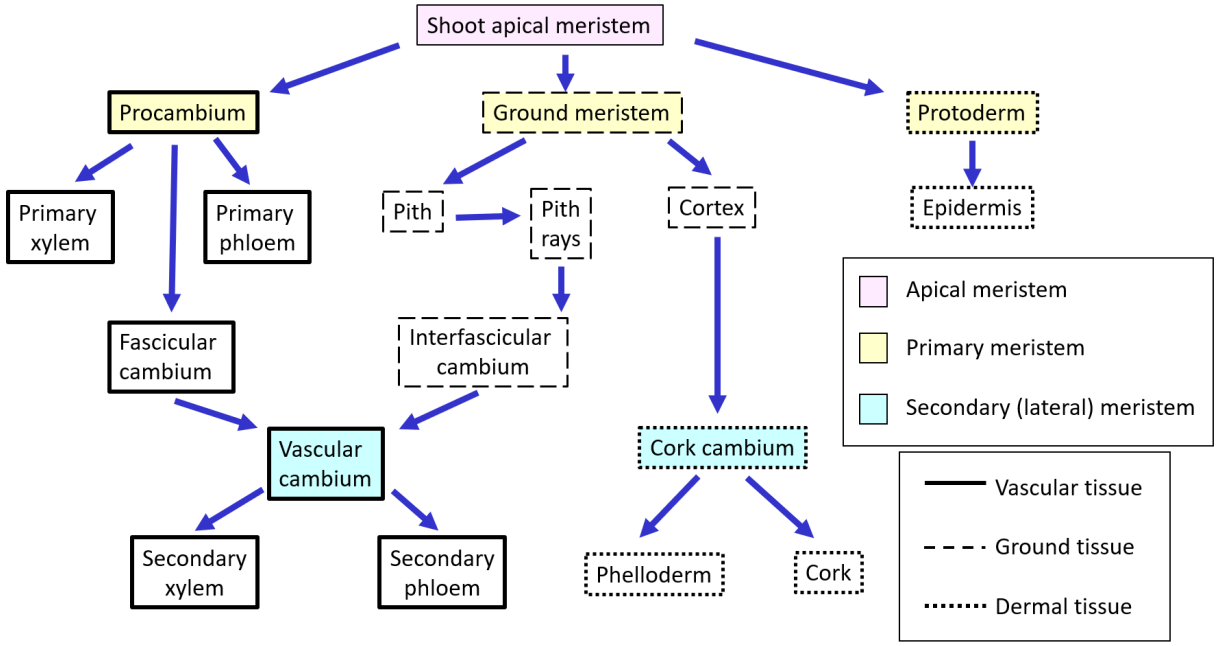


Figure 18.2.318.2.3: The torpedo stage of the *Capsella busra-pastoris* embryo proper showing the three primary meristems. Image labeled from Wisewire (CC-BY).

Secondary meristems (lateral meristems) result in secondary growth, a woody increase in girth. These include the vascular cambium and cork cambium. The **vascular cambium** arises from from the procambium and pericycle in roots. In stems, it arises from procambium cells of the vascular bundles (fascicular cambium) and parenchyma cells between vascular bundles (interfascicular cambium). The vascular cambium gives rise to secondary phloem (part of the bark) and secondary xylem (wood; Figures 18.2.4−518.2.4−5).



The **cork cambium** arises from the pericycle in roots and the parenchyma cells of the cortex in stems, both of which arise from the ground meristem. The cork cambium produces periderm, secondary dermal tissue that is also a component of bark. (See Roots and Secondary Stem for more details; Figures 18.2.4−518.2.4−5).)

Figure 18.2.418.2.4: Tissue development in stems. The **shoot apical mersitem** produces the three primary meristems: **procambium**, **ground meristem**, and **protoderm**. The **secondary mersitems** (lateral meristems) are the **vascular cambium** and **cork cambium**. The vascular cambium arises from the procambial cells of the fascicular cambium and the interfascicular cambium, which ultimately arose from the ground meristem. The ground meristem also produces the cortex, which generates the cork cambium. Image by Melissa Ha based on

diagram from Maria Morrow (CC-BY).

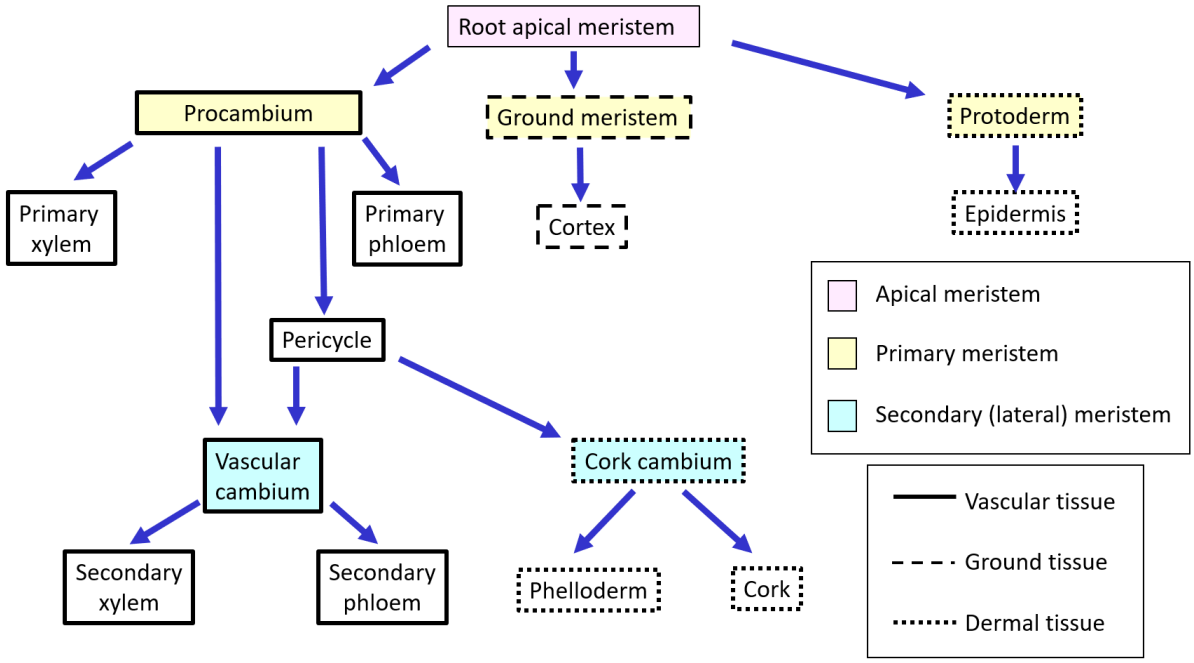


Figure 18.2.518.2.5: Tissue development in roots. The **root apical mersitem** produces the three primary meristems: **procambium**, **ground meristem**, and **protoderm**. The **secondary mersitems** (lateral meristems) are the **vascular cambium** and **cork cambium**. The vascular cambium arises from the procambium and pericycle. The pericycle also produces the cork cambium. Image by Melissa Ha (CC-BY-NC).

Other meristems include **intercalary meristems** which elongate stems from the "middle" (in between nodes) and **marginal meristems**, which are located along leaf edges and are responsible for leaf development.

Distribution and types of parenchyma, collenchyma and sclerenchyma

The tissue which provides support and mechanical strength to a plant or a growing organ in a plant is known as a mechanical tissue.

• Haberlandt (1914) called the mechanical tissue as stereome.

• Plant organs are to withstand various strains like stretching due to presence of large fruits, bending due to natural calamities like high wind and passing animals and downpour, heavy snow etc.

• The stems are compressed due to presence of large number of branches and leaves at their top; branches are to withstand bending as they lie either in oblique or horizontal position; the fruit stalks tend to be extended due to weight of fruits; the roots are also subjected to extension when the stem bends due to high wind. In high wind the leaves lacerate. Thus, to withstand these strains the mechanical tissue is developed in various parts of plants. Both the ground tissues- COLLENCHYMA (living) and SCLERENCHYMA (dead) constitute the

mechanical tissue of a plant. Collenchyma (Gk. Colla-glue; enchyma – an infusion) Structure-

• Collenchyma is a simple, living mechanical tissue.

• Collenchyma cells are living which retains protoplasm even at maturity.

• The cells are elongated and appear polygonal in cross section.

• The cell wall is unevenly thickened. It contains more of hemicellulose and pectin besides cellulose. No lignin is deposited.

• These thickening materials get deposited at the corners or at the tangential walls of collenchyma cells.

• It provides mechanical support and elasticity to the growing parts of the plant.

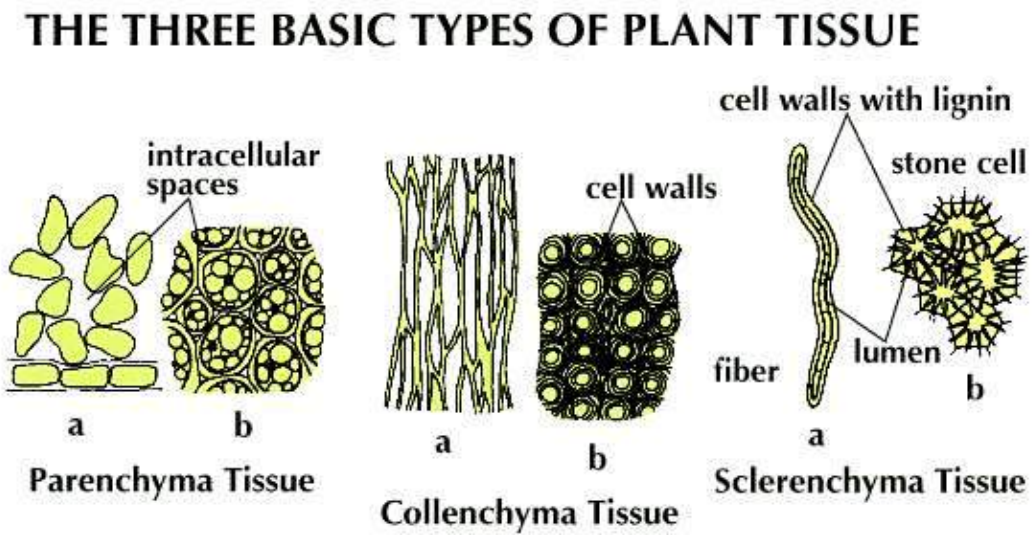
• Elasticity is provided by collenchyma because of presence of hydrated pectin in their cell walls. • Collenchyma consists of narrow cells.

• Unlike parenchyma cells they possess thick primary cell wall. 2

• It has only a few small chloroplast or none. Tannin maybe present in collenchyma.

• Collenchyma mainly provides mechanical strength to the growing plant parts.

• Collenchyma generally occurs in hypodermis of dicot stem. It is absent in the roots and also occurs in petioles and pedicels.



• Based on pattern of pectinisation of the cell wall collenchyma can be:

1. Angular collenchyma It is the most common type of collenchyma with irregular arrangement and thickening at the angles where cells meets. Example: Hypodermis of Datura and Nicotiana

2. Lacunar collenchyma The collenchyma cells are irregularly arranged. Cell wall has thickening on the walls bordering intercellular spaces. Example: Hypodermis of Ipomoea

3. Lamellar collenchyma The collenchyma cells are arranged compactly in layers (rows). The cell wall thickening is at tangential walls. These thickening appear as successsive tangential layers. Example: Hypodermis of Helianthus

4. Annular Collenchyma: Duchaigne (1955) reported another type called Annular collenchyma in petiole of Nerium. The lumen is more or less circular in shape. DistributionCollenchyma is one of the important mechanical cells of the growing organs and the mature organs of herbaceous plants. In stems it usually occurs just beneath the epidermis e.g. Cucurbita. In Tilia stem it is separated from epidermis by parenchyma cells that may be one or two layered. It occurs as a continuous cylinder in Sambucus, Helianthus etc. It is present as individual bundle in Cucurbita etc. Collenchyma cells are very conspicuous below the ridges in the petioles and stems with projecting ribs (e.g. Chenopodium).

3 Collenchyma may occur as bundle cap (e.g. Apium graveolens). It may form a sheath around the entire vascular bundle of many plants. In leaves collenchyma occurs in the petiole and blade. In the leaf of Sambucus it occurs below the phloem of large vascular bundles and forms a prominent cap under the phloem. These caps are formed in large vascular bundles that protrude on the underside of the leaf as ribs. Collenchyma cells also occur along the margins of a leaf thus making it tough and resistant to tearing. The rinds of some fruit also gain mechanical rigidity from collenchyma, e.g. Vitis, Cassia etc

Function:

• They provide support mainly to primary plant body.

• Collenchyma confers flexibility to various parts of the plant like petiole and stem, allowing for easy bending without breakage.

• They permit the growth and elongation of plant part.

• They possess chloroplasts and thus can photosynthesize.

• The living cells of collenchyma store food.

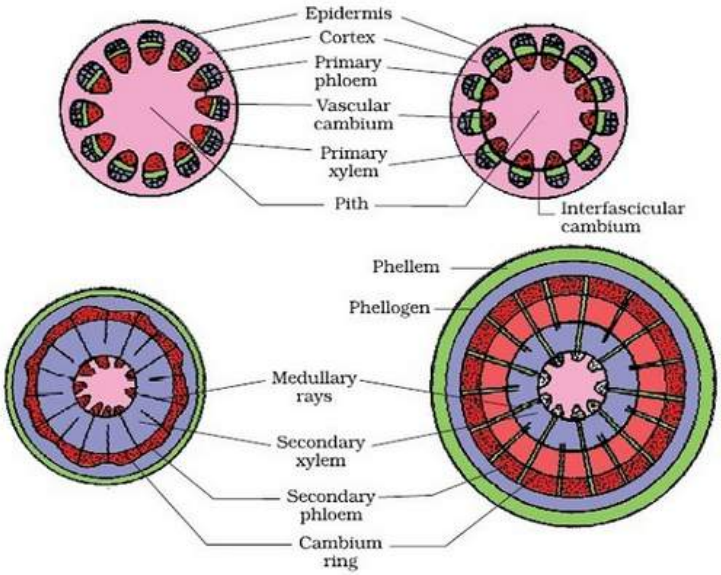
• Collenchyma present in leaves also prevents them from tearing.

Secondary growth in dicot stem

Secondary growth

- Meristem is responsible for the development of primary plant body.
- Primary growth increases length of the plant as well as lateral appendages.

- However, secondary Grier increases thickness or girth of the plant by the formation of secondary tissues.
- There secondary tissues are formed by the two types of lateral meristem i.e. vascular cambium and cork cambium (phellogen).
- Secondary growth occurs in stem and root of dicots and gymnosperms.
- However, it is absent in stem and root of monocot and completely absent in leaf.
- A process of formation of secondary tissues due to activity of vascular cambium and cork cambium for increasing thickness or girth or diameter of plant is termed as secondary growth.
- On the basis of the activities of vascular cambium and cork cambium, the process of secondary growth can be discussed under the following headings:
  - Activity of the vascular cambium
  - Activity of the cork-cambium



Secondary growth in stellar region due to activity of the vascular cambium

i. Formation of cambium ring:

- In vascular bundles of a dicot stem, the cambium is present in between the xylem and phloem. It is known as intrafascicular cambium.
- During secondary growth, some cells of medullary rays become active and show meristematic activity which form a strip of cambium in between vascular bundles called inter-fascicular cambium.
- Both the intra-fascicular and inter-fascicular cambium unite together to form a complete ring called the cambium ring.
- The activity of the cambium ring gives rise to secondary growth.

ii. Formation of the secondary tissues:

- The cambium ring acts as a meristem which divides.
- The cambium layer consists of a single layer of cells.
- These cells divide in a direction parallel with epidermis.
- A cambial cell divides into two daughter cells, one of which remains meristematic and other differentiates into secondary vascular tissue.
- The cell formed towards inner side develops into secondary xylem.
- Likewise, the cell formed towards outer side develops into secondary phloem.

- Normally, more secondary xylem cells are formed towards the center due to which cambium ring moves towards the periphery.

- Due to the formation of secondary xylem and secondary phloem, the primary xylem and primary phloem which were initially closed, moves towards inner and outer side respectively.
- As a result, they become separated apart.
- The layers of secondary tissues gradually added to the inner and outer side of the cambium continuously throughout the life of the plant.

iii. Formation of secondary medullary rays:

- Certain cells of the cambium instead of forming secondary xylem and phloem for some narrow bands of living parenchyma cells.
- These form two or three layers of thick radical rows of cells passing through the secondary xylem and secondary phloem and are called secondary medullary rays.
- These provide the radial conduction of food from the phloem, and water and mineral salts from the xylem.

iv. Formation of annual rings:

- The activity of cambium is affected by variations in temperature.
- In moderate climate, the cambium becomes more active in the spring and forms greater number of vessels with wider cavities, whereas in winter it becomes less active and forms narrower and smaller vessels.
- The wood formed in the spring is known as spring wood or early wood and that formed in the dry summer or cold winter is autumn wood or late wood.
- These two kinds of wood appear together as a concentric ring known as the annual ring or growth ring, as seen in transection of the stem and successive annual rings are formed year after year by the activity of the cambium.
- The growth of the successive years appears in the form of concentric or annual rings, each annual ring representing the one year's growth.
- The age of the plant thus, can be approximately determined by counting the number of annual rings.

v. Formation of heart wood and sap wood:

- In the old trees, where sufficient amount of secondary growth has taken place, the secondary wood of inner side lose the power of conduction.
- Their cells get filled with tannins, resins, gums, essential oils which makes the plant part hard and darker called the heart wood or duramen.
- The heart wood ceases the function of conducting tissue and simply provides mechanical support to the stem.
- However, the outer region of secondary wood, which consists of younger living xylem cells, remains yellow in colour called the sap wood or laburnum.
- It functions as the conducting tissue and also as the food storage tissue.

Secondary growth in extra stellar region due to activity of cork-cambium:

- The marked increase in diameter or thickness of stem brought about by the secondary thickening exerts a great pressure on the outer tissues.
- This results in the rupture of the cortex and epidermis, the outer cortical cells become meristematic and begins to divide. This is known as cork cambium or phellogen.
- The cork cambium divides to form secondary tissue on both the sides i.e. internal and external but its activity is more on the outer side than on the inner side.
- The cells formed on the outer side constitutes the phellem or cork and those on the inner side form secondary cortex or phelloderm.
- The phellogen, phellem and phelloderm together are called periderm.

C7P: Genetics

Demonstration on pretreatment, fixation, staining and squash and smear preparation.

The following points highlight the six main steps in chromosome methodology.

The steps are:

1. Pretreatment

2. Fixation

3. Hydrolysis

4. Processing

5. Staining

6. Mounting.

Step # 1. Pretreatment:

Pretreatment is an important step in the course of preparations of plant tissue for studying the chromosome number and their morphology in order to establish the karyotype of a species.

It is carried out for:

(i) Causing mitotic block and metaphase arrest through destruction of spindle fibre

(ii) Bringing about scattering of chromosomes by removing the binding force

(iii) Clarification of constriction regions through differential hydration in chromosome segments

(iv) Influencing chromosome condensation. In soma special cases this is done also for

(v) Clear ring the cytoplasm by removing the heavy contents to bring about the transparency of cytoplasmic background

(vi) To achieve rapid penetration of the fixative by removing undesirable deposits from the surface of the tissue

(vii) Separation of middle lamella causing softening of the tissue

The most important aspect of pretreatment is to fulfill the first two requirements which exert a direct effect on the chromosome, and the underlying principle is the viscosity change of the cytoplasm. The spindle formation depends on the viscosity balance between cytoplasmic and spindle constituents.

Therefore, a change in cytoplasmic viscosity brings about destruction of the spindle mechanism making the chromosomes free from any binding force within the cell.

Viscosity change of the cytoplasm also affects the chromosomes, which undergo differential hydration in its segments making the primary and the secondary constriction regions conspicuous and the satellite gap greatly exaggerated. Spindle inhibition also results in high frequency of metaphase stage.

Pretreating Agents:

A number of chemicals are used for the purpose of pretreatment, the most common being colchicine. These chemicals are not universally applicable to all plant materials. Generally, a particular group of plants gives better results in a particular chemical. Suitable pretreating agent must be worked out through several trials (Table 21.1).

Table 21.1: Some common pretreatment chemicals

Chemical	Effective concentration	Period of treatment	Temperature
1. Aesculine [Extracted from Aesculus hippocastanum]	Half saturated to saturated	30 min – 24 hr	4°C – 16°C
2. α Bromonaphthalene	Saturated	15 min – 4 hr	10°C – 16°C
3. Colchicine [Extracted from root of Colchicum autumnale, C. luteum, Gloriosa superba	0.05% – 1%	1 hr – 4 hr	8°C – 16°C
4. Coumarin	Saturated	3 hr – 6 hr	Both cold and room temp
5. p-Dichlorobenzene (pDB)	Saturated	3 hr – 5 hr	12°C – 16°C
6. 8-hydroxyquinoline	0.002M	3 hr – 4 hr	12°C – 16°C

In some special cases, pretreatment Is done for clearing the cytoplasm from its heavy contents and softening of tissue, brought about by acid treatment (NHCI), alkali treatment (NaOH), and enzyme treatment (pectinase, cytase, clarase, cellulose).

Removal of secretory and excretory deposits from the surface of the tissue or cell wall is achieved by the application of hydrofluoric acid, chloroform and also some other chemicals.

Step # 2. Fixation:

Fixation is the most critical step in chromosome study. It is the process by which tissues and their components are fixed selectively at a particular stage thereby respective divisional stages are arrested. The purpose of fixation is to kill the tissue instantaneously (lethality) without pycnosis, i.e., not causing any distortion of the components to be studied.

Fixation in chromosome study brings about blocking of cell divisions and enables the preservation of the structural integrity of nucleic acid and protein of the chromosomes.

A truly effective fixative should fulfill the following conditions: (i) Rapid penetration to cause immediate killing of the tissue (ii) Coagulation of the protein component and consequent precipitation causing a marked change in the refractive index of the chromosomes

(iii) Checking denaturation of protein, consequent to the death of cells. Due to lethality the medium becomes acidic which causes enzymes to act in the reverse direction, with the breakdown of complex protein molecule into simpler amino acids (IV) Checking bacterial action with the onset of lethality, thus preventing tissue decomposition

(v) Precipitating the chromatin matter to render the chromosome visible

(vi) Increase the basophilic of the chromosomes, helping in the adherence of the acidic stain

Fixatives:

Since all the above-mentioned requirements are rarely exhibited by a single chemical, a suitable fixative is, therefore, a combination of several chemicals. Some common fixatives are presented in the Table 21.2. Even with the best fixative, the chemical changes undergone by the nuclear bodies in the entire process cannot be ignored.

This can be eliminated by freeze-drying method of fixation which involves rapid cooling of the tissue at low temperature followed by extraction of water in vacuum.

Based on ingredients, fixatives may be metallic (chromic acid, osmic acid) or non-metallic (ethanol, chloroform, acetic acid). All metallic fixatives require thorough washing with water after fixation. The most commonly used fixative is Carnoy's fluid I and the tissue is kept in cold for overnight. For rapid fixation tissue can be treated with acetic-ethanol (1:2) for 1-2 hours at cold.

Ethanol precipitates nucleic acid, causes irreversible denaturation of protein and due to its dehydrating property causes undesirable hardening effect. Acetic acid also precipitates nucleic acid and when kept for prolonged period it dissolves histones.

However, in scheduled time, it does not cause any distortion to nucleoprotein but causes excessive swelling of chromosome segments. Therefore, acetic acid is used in combination with alcohol to resist hardening of tissues.

Table 21.2: Some common fixing mixtures

Fixing mixture	Constituents	
1. Carnoy's fluid I	Glacial acetic acid	1 part
	Ethanol	3 parts
2. Carnoy's fluid II	Glacial acetic acid	1 part
	Chloroform	3 parts
	Ethanol	6 parts
3. Navashin's fluid	Solution A (1 part)	
	Chromic anhydride	1.5 g
	Glacial acetic acid	10 ml
	Distilled water	90 ml
	Solution B (1 part)	
	Formalin	40 ml
	Distilled water	60 ml
4. Newcomer's fluid	Isopropyl alcohol	6 parts
	Propionic acid	3 parts
	Petroleum ether	1 part
	Acetone	1 part
	Dioxane	1 part
5. Lillie's AAF/FAA	Formalin	2 parts
	Glacial acetic acid	1 part
	Absolute ethanol	17 parts

N.B. : The nature of chemical, duration and temperature will vary according to the material

Carnoy's fluid II yields good result with materials containing fatty and waxy substances as chloroform is a good solvent for them. Carnoy's fluid II, as well as, propionic-ethanol are used for material with oil contents like Lens, as they clear the cytoplasmic background.

Step # 3. Hydrolysis:

The step hydrolysis is required in chromosome preparation to bring about the softening of tissue by dissolving the pectic substances of middle lamella causing separation of cells, also called maceration. In squash preparation, this maceration will help to get an uniform, monolayer of cells with application of slight pressure.

In acetic-orcein staining schedule, hydrolysis and staining are simultaneously achieved by warming with a mixture of 2% acetic-orcein and IN HCl (9:1).

In chromosome preparation from callus tissue, maceration is done with 5N HCI at room temperature for 1 hour. Enzymes like pectinase, cellulose, clarase, etc. are also used for maceration in chromosome preparation.

In Feulgen staining, hydrolysis has another important objective, to liberate the aldehyde group involved in the Schiff's reaction. In this case hydrolysis is done prior to staining with 1N HCI at58-60°C for 10-12 min.

Step # 4. Processing:

Processing of tissue is required for chromosome study after suitable fixation or after staining.

Different schedules employed are:

(a) Block preparation and microtomy;

(b) Squash preparation and

(c) Smear preparation.

(a) Block Preparation and Microtomy:

For getting serial sections, instead of squashes and smears, tissues are dehydrated and embedded in a suitable medium (paraffin) which supports the material on all sides and allows sections of the material to be cut without distortion. It is useful to get the sequence of stages of growth of the tissue and divisional stages of meiosis in an anther or mitotic divisions in the somatic cells.

The procedure involves the following operations:

Washing:

After fixation the tissue is thoroughly washed in running water for 24 hours (if fixed in metallic fixative) to remove all traces of the fixing chemical.

Dehydration:

The tissue is passed through successive grades of ethanol 30%, 50%, 70%, 80%, 90%, 95% and absolute ethanol keeping for 1 hr. in each, except overnight treatment in 70% and absolute ethanol.

Clearing:

As paraffin does not mix with many dehydrating agents, an intermediate medium (ante-medium) is used. After dehydration with ethanol the tissue is passed through a series of ethanol-chloroform grades 3:1, 1:1 and 1:3, being kept for 1 hr. in each. Finally it is kept in pure chloroform for 10-30 min.

Infiltration:

Small chips of paraffin of low melting point are added to the chloroform containing the tissue and kept at 37°C on a hot plate for 48 hr. with tight corking. The mouth of the container is then opened for 24 hr. Then it is transferred to a hot bath of 45°C and kept overnight.

The tissue is finally transferred to a hot bath of 55-60°C. As the paraffin melts, it is changed with molten embedding paraffin and two more successive changes are given with the same intervals of 30 min and the final change in molten paraffin being given only when no trace of the smell of chloroform is left.

Embedding:

After infiltration of the tissue with paraffin, embedding is done by pouring the molten paraffin with the tissue into a suitable receptacle (paper tray), arranging the tissue in a proper manner with heated needles. After partial solidification in air, transfer of the tray to ice-cold water is done.

Section cutting:

Rotary microtomes are commonly used for section cutting. A block is trimmed to a suitable size with the material at the centre and it is attached to a block holder with the help of a heated scalpel. The holder is fitted to the microtome with the edge of the razor barely touches the face of the block.

Sections are cut generally of 14μ thick with uniform strokes. The ribbon is transferred to a sheet of paper and cut into short equal segments.

A minute quantity of Mayer's adhesive (albumin 50 ml + glycerin 50 ml + sodium salicylate 1 ml) is smeared on a clean grease-free slide and flooded with water. Generally 3 or 4 strips of the ribbon are transferred to the slide and warmed on a hot plate to stretch out the strips. The slide is then tilted to drain off the excess water and is kept on hot plate for 3 hr. to overnight for drying.

Removal of embedding material:

The sections are passed through xylol for removing the paraffin and gradually bring down to the medium in which the stain is dissolved. Atypical grade of chemicals for this purpose is : Xylol, I, II and III – 1 hr. each; Ethanol (1:1) – 1 hr; Absolute ethanol – 30 min; 90%, 80%, 70%, 50% and 30% ethanol – 30 min each; and Distilled water – 10 min.

Staining:

Then the sections are stained with crystal violet (vide staining).

(b) Squash Preparation:

This method has great advantage over sectioning method because one can carry out observation on separated single cells and the entire process is rapid. Squashing is recommended for studying chromo-somes in somatic tissue (root tip, leaf tip).

After fixation, NHCI treatment is carried out for dissolution of the pectic salts of the middle lamella so that monolayer of separated individual cells can be obtained from a compact mass of cells. Softening is carried out prior to staining or along with staining. Following staining, the materials are teased with the needle in the mounting medium (45% acetic acid) and covered with a square coverslip.

Then the tissue is finally squashed by applying uniform pressure over the coverslip through a piece of blotting paper and properly sealed. The pressure should be hard enough to loosen the cell mass-to form a single-layered squash. Moreover, the cells have to be flattened, so that the chromosomes are well scattered.

The best way to do it is by placing the slide on a blotting paper, the blotting paper is then folded so that the slide remains within it, then it is tapped with the blunt end of a needle and excess fluid is blotted off. The slide is moved slightly to the dry side of the blotting paper and further pressure is applied. The preparation can be kept in temporary condition for 1-2 weeks if it is perfectly sealed.

(c) Smear Preparation:

Smearing is recommended for studying chromosomes in meiotic cells (pollen mother cells). In smears, the cells are directly spread over a slide prior to fixation and no treatment is necessary to secure cell separation. The procedure involves squeezing out of fluid from anther on to a clean, grease-free, dry slide, quickly spreading it with the aid of a scalpel and immediately inverted it in a tray containing fixative.

The procedure may be modified by directly smearing the anther with a scalpel in a drop of acetic-carmine solution (vide staining) which serves the double purpose of fixing and staining. Finally, it is covered with a cover-slip and gentle pressure (as the PMCs are fragile) is applied for the spreading of cells.

Excess stain is soaked with a blotting paper and is sealed with paraffin. This temporary preparation can be kept in cool place for 1-2 weeks.

Step # 5. Staining:

Staining is done to make the cell constituents visible under the microscope helping in the study of their structure and behaviour. The colour of the chromosomes as well as of different cell constituents is usually secured through the use of different stains or dyes. Staining may be vital or non-vital.

In case of vital staining, water soluble non-toxic dyes (methylene blue) are applied to the living tissue so that the latter can be studied without being killed. In non-vital staining, the colouration of the chromosomes and other cell constituents in the killed tissue is caused by certain chemicals which are insoluble in chromosome substance.

The stains are usually termed acidic, basic or amphoteric on the basis of their chemical nature and behaviour. Most of the acidic dyes are salts of potassium or sodium, whereas basic dyes are mostly available as chlorides or sulphates. In acidic dye, the balance of the charge on the dye ion is negative and they react with basic (acidophilous) substances.

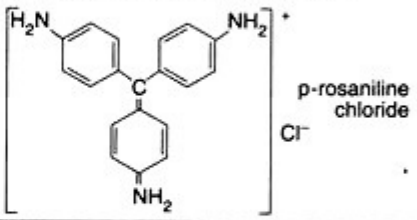
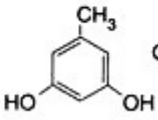
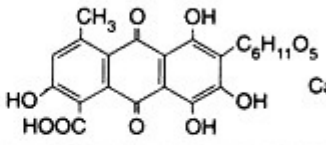
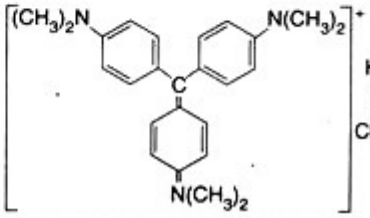
Aniline blue, lacmoid are examples of acidic dye and colour the cytoplasm which is predominantly basis. In basic dye, the ion charge is positive and it stains acidic substance (basophilic).Carmine orcein are basic dyes and stains chromatin and chromosome which is strongly acidic.

The process of staining is principally due to physical adsorption and/ chemical reaction. The colour of the dye is due to certain chemical configurations in itself, known as chromophores; and the adherence of the dye to the cellular constituents is due to auxochromes, other chemical configurations in itself.

The best example of a chromophoric group is the quinonoid ring and the auxochromes are mostly amino or hydroxyl groups. The adherence of a dye may be accelerated through the process of mordanting by the use of metallic salts (ferric chloride, ferric acetate) which form compounds with the dye and attach the dye to the cellular constituents.

Of all the different stains employed for the study of chromosomes, a few important ones are given in Table 21.3.

Table 21.3: Some common stains

Stains	Source and composition
1. Fuchsin: It is magenta red in colour and used as colourless feulgen solution or fuchsin sulphurous acid. On controlled hydrolysis with N HCl, the unmasked aldehyde group of deoxyribose sugur undergoes schiff's reaction with fuchsin sulphurous acid to give typical magenta colour.	The commercially obtained basic fuchsin is a mixture of three compounds – p-rosaniline chloride, basic magenta and new magenta.  p-rosaniline chloride
2. Orcein: It is deep purple coloured dye and used as acetic-orcein.	It is prepared from the action of H_2O_2 and NH_3 on parent substance orcinol which is obtained from lichens — Rocella tinctoria and Lecanora parella.  Orcinol
3. Carmine: It is crimson-coloured dye and used as acetic-carmine.	It is prepared by adding compounds of aluminium or calcium to colchineal, the dried females of Coccus cacti. The active principle of carmine is due to carminic acid which is obtained by extracting conchineal with boiling water followed by treatment with lead acetate and decomposition of lead carminate with sulphurous acid.  Carminic acid
4. Crystal violet: It is a bluish violet dye and used as aqueous solution followed by mordanting in iodine which makes the colour bluish-black.	Crystal violet is hexamethyl p-rosaniline chloride which forms large crystal with nine molecules of water.  Hexa-methyl p-rosaniline chloride

Preparation of Common Stains and Staining Procedure:

A. Feulgen Stain (fuchsin sulphurous acid):

Principle:

The principle underlying the preparation of Feulgen solution (Schiff's reagent) is the conversion of β-rosaniline chloride to leucosulphinic acid by sulphurous acid which is obtained through the action of HCI on potassium metabisulphitfe. The excess of SO_2 liberated by this action undergoes reaction with leucosulphinic acid to produce bi-N-aminosulphinic acid, popularly known as Schiff's reagent.

Requirements:

Basic fuchsin = 0.5 g

NHCI = 10 ml

Potassium metabisulphite = 0.5 g

Activated charcoal = 0.5 g

Distilled water = 100 ml

Preparation:

In 100 ml boiling distilled water 0.5 g basic fuchsin is dissolved. The solution is allowed to cool at 58°C. It is then filtered and the filtrate is further cooled down to 26°C. 10 ml of NHCI and 0.5 g potassium metabisulphite are added to it. The solution is kept in a dark container, properly sealed and stored in a cool dark chamber for 24 hours.

The magenta coloured solution turns straw coloured and is ready for use. If the solution remains otherwise coloured, charcoal powder is added to it, thoroughly shaked and kept overnight in cold temperature. The solution is then filtered and ready for use.

Precaution:

The stain should always be kept in a dark container, with proper sealing under cool temperature and away from light.

Staining procedure:

1. The fixed tissue is hydrolyzed in normal HCI at 56-60°C for 10-12 min.

2. The tissue is washed with distilled water giving 3 changes to wash out excess HCI.

3. The tissue is then stained in Feulgen stain (Schiff's reagent) for 30-45 min at 10-12°C under dark condition. The colour develops within a short time and the chromosomes take up magenta colour against a clear cytoplasmic background.

Chemical basis of Feulgen reaction:

It is a DNA specific stain. By hydrolysis with NHCI, the purine containing fraction of DNA is separated from the sugar and the breakdown of glycofuranosidic linkage occurs, unmasking the -CHO

group of the deoxyribose sugar. The reactive -CHO then enters into combination with fuchsin sulphurous acid to yield the typical magenta colour.

B. Acetic-orcein and Acetic-carmine stain:

Requirements:

Orcein or = 2 g (for 2% soln.)

Carmine = 1 g (for 1 % soln.)

Glacial acetic acid = 45 ml

Distilled water = 55 ml

Preparation:

Distilled water is added to glacial acetic acid to make 45% acetic acid solution. The solution is heated in a conical flask to boiling. The dye is gradually added to the boiling solution, stirring with a glass rod. The solution is boiled at simmering point for 10 min, cooled down to room temperature, filtered and stored in a bottle with a glass stopper.

Precaution:

The mouth of the flask should be kept covered while the solution is being heated.

Staining procedure:

(a) Squash preparation (somatic tissue)

1. The fixed tissue is first kept in 45% acetic acid for 10 min.

2. Then it is transferred to a mixture of 2% aceticorcein stain and NHCI (9:1).

3. The mixture is gently heated over the flame for 5-10 seconds, taking care that the liquid does not boil.

4. It is kept at room temperature for 45 min – 1 hr. to attain perfect staining of chromosomes.

(b) Smear preparation (meiotic tissue):

The tissue is stained directly in 1% acetic- carmine stain on a slide and the slide is heated slightly over a flame. The use of a scalpel during smearing aids in the addition of iron which acts as a mordant through the formation of iron acetate.

C. Crystal violet stain:

Requirements: Crystal violet = 1 g

Distilled water = 100 ml

Preparation:

1g dye is dissolved in 100 ml boiling water with constant stirring. The solution is cooled, filtered and allowed to mature for a week before use.

Staining procedure:

1. The fixed tissue (smears, sections) is stained in 1% crystal violet solution for 30 min.

2. The excess dye is washed off by rinsing in distilled water.

3. Then the slides are processed through 1% iodine and 1% potassium iodide mixture in 80% ethanoi for 45 sec to obtain proper colour of chromosome.

Step # 6. Mounting (Permanent Slide Preparation):

Mounting of tissue (section, squash, smear) in a suitable medium (Table 21.4), after staining, is necessary to avoid drying up of tissue and not to render it opaque. The mounting media should have a refractive index closer to glass to avoid refraction. It should harden quickly in contact with air and should check de-staining.

The chief aims of mounting are:

(i) To render the tissue transparent;

(ii) To increase the visibility of tissue under microscope;

(iii) To hold it with the protecting cover-slip firmly in place; and

(iv) To preserve it for a long period.

Table 21.4: Some commonly used mounting media

Media	Refractive indices	Advantages	Drawbacks
1. Canada balsam An oleoresin from Abies balsamia	1.53	Perfect mounting medium, transparent, thick, with air forms a hard medium, and stable.	It becomes dark and acidic with time by slowly oxidizing xylol and causes stains to fade.
2. Euparal It is a mixture of camsal, sandarac, eucalyptol and paraldehyde	1.48	It does not dry too rapidly, soluble in butanol, ethanol, in addition to xylol; dehydration schedule can be shortened.	It has a tendency to become quickly clouded.

A. Mounting of Sections and Smears after Crystal Violet Staining

1. For dehydration, the slide is passed through absolute ethanol, II, ill keeping in each for 2 sec.

2. Differentiation is done by passing the slide through clove oil I for 2-5 min (observation under microscope is required for satisfactory staining) and then transferred to clove oil II and kept for 10-15 min.

3. For clearing, the slide is kept in xylol I, II and III for 1 hr. in each.

4. Finally, It is mounted in Canada balsam under a cover-slip and the slide is allowed to dry overnight on a hot plate (35-45°C).

B. Mounting of Squashes/Smears after Aceticorcein, Acetic-carmine and Feulgen staining:

(a) Acetic-alcohol schedule:

1. The paraffin seal of temporary preparations is carefully removed with blade after 1-2 days and inverted in a covered petridish containing glacial acetic acid – ethanol (1:1) mixture till the cover-glass is detached.

2. Both the slide and cover-glass with materials are transferred to ethanol and kept for 10 min.

3. These are then passed through ethanol-xylol (1:1) mixture, xylol I and xylol II, keeping in each for 10 min.

4. The slide and the cover-slip are mounted separately in Canada balsam (two slides will be prepared) and allowed to dry overnight on a hot plate.

(b) Butanol schedule:

1. The paraffin seal of temporary preparations is carefully removed with blade and inverted in a covered petridish containing glacial acetic acid – ethanol (1:1) mixture till the cover-glass is detached.

2. Both the slide and cover-glass with material are transferred to ethanol: n- butyl alcohol (1: 1) for 5 min.

3. Both the slide and cover-glass with material are passed through n-butyl alcohol I and n-butyl alcohol II, keeping 20-30 min in each.

4. The slide and the cover-glass are mounted separately in euparal (two slides will be prepared) and dried on a hot plate for overnight.

Study of different stages of Mitosis from temporary acetocarmine squash preparation of root-tip cells of onion *(Allium cepa).*

INTRODUCTION:

The reason for using onion root tips for viewing mitosis –

The roots are easy to grow in large numbers, the cells at the tip of the roots are highly dividing and thus many cells will be in stages of mitosis, the chromosomes can be stained to make them more easily observable.

There are three cellular regions near the tip of an onion tip –

i. The **root cap** contains cells that cover and protect the underlying growth region.
ii. The **region of cell division** (or meristem), where cells are actively dividing but not increasing significantly insize.
iii. In the **region of cell elongation**, cells are increasing in size but notdividing.

REQUIREMENTS:

CHEMICALS:

1. 0.1 N HCl (1ml conc. HCl added in 119ml Distilledwater).
2. 70 % and 90 %ethanol.
3. Acetic-alcohol fixative (1: 3 acetomethanolfixative).

   Glacialaceticacid = 1 part

   Ethyl/ methyl alcohol = 3parts
4. 2 % acetocarminestain
5. 45 % acetic acid.

MATERIALS:

Slide, Cover slip, Watch glass, squashing needle, spirit lamp, blotting paper, Onion root tips.

PROCEDURE:

Fixation of Root tips :

1. Freshly cut onion root tips (1 cm long) are fixed in acetometanol fixative for overnight in a specimentube.
2. Then, 90 % ethanol is added after decanting the fixative. Finally 70 % ethanol added after decanting.
   The root tips can be stored in 70 % ethanol for a long period of time in tightly closed container at 40 ^{0}C.

Staining and making squash preparation:

1. Root tips are treated with 0.1%N HCl for 1 min. This will soften the cellwall.
2. Root tips are rinsed in water and transferred to acetocarmine stain for 30mins.
3. A drop of 45 % acetic acid is taken on a slide having one root tip and left for 1-2 min. If acetic acid drop becomes coloured, it is decanted and a fresh drop isadded.
4. A cover glass is placed on the root tip and squashed it using a rubber-end pencil under the folds of a blotting paper.
5. Then edges of the cover glass is sealed with molten was or with nail polish immediately to prevent drying of acetic acid film and entry of airbubbles.
6. Now the slide is observed undermicroscope.

OBSERVATION:

The following stages of mitosis are observed under microscope.

Interphase: Interphase is also called the resting phase. It is the longest phase.

1. Replication of DNA takes place during thisphase.
2. The chromosomes are thinlycoiled.
3. Presence of nucleolus and nuclearmembrane.

Prophase :

Metaphase:

Anaphase:

1. The chromatin appears as a network of fine threads. If the cell is in early stage of prophase, then the chromatin fibres (chromosomes) are very thin, while the cells at late prophase show comparatively thicker chromatin fibres.
2. Spindle formation isinitiated.
3. The nuclear membrane and nucleolus start disappearing at the laterstage.
4. Disappearance of the nucleolus and nuclearmembrane.
5. Chromosomes are at their maximum condensed state with two chromatids joined at theircentromere.
6. Spindle formationcomplete.
7. The chromosomes align in the equatorial position of the spindle and form the equatorial pate that is at right angle to the spindleaxis.
8. The centromeres are arranged exactly at the equatorialplate.
9. The centromere of the chromosomes divides and the two chromatids of each pair separate.
10. Each chromatid now represents a separate chromosome and it starts moving towards the oppositepoles.
11. The daughter chromosomes assume ‘V’ or ‘J’ shapes or depending upon the position of centromere inthem.

Telophase :

Cytokinesis :

1. Chromosomes reach the opposite poles and look like a mass ofchromatin.
2. The new nuclear membrane starts to reappear around each set of chromosomes.
3. The nucleolus getsreorganized.

In plants a cell plate is formed in the middle after telophase, finally dividing the cells into two.

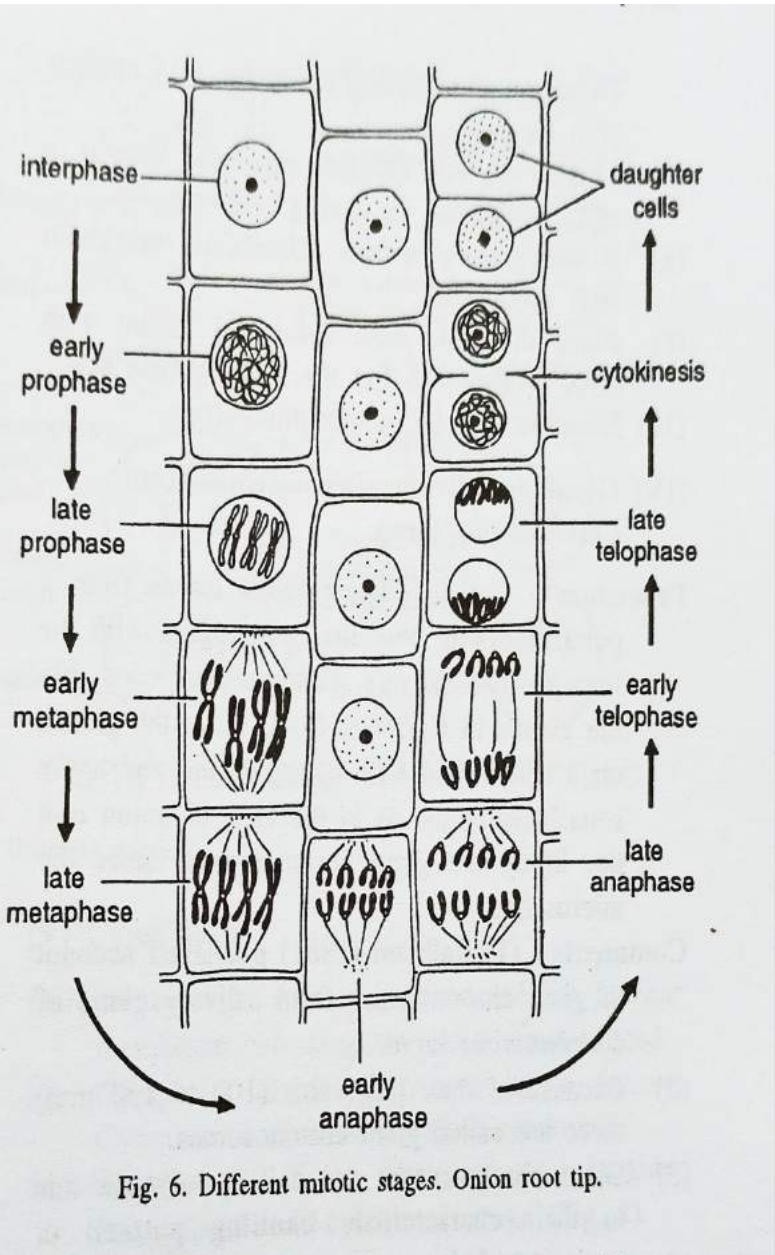


Fig. 6. Different mitotic stages. Onion root tip.

Diagram for practical copy

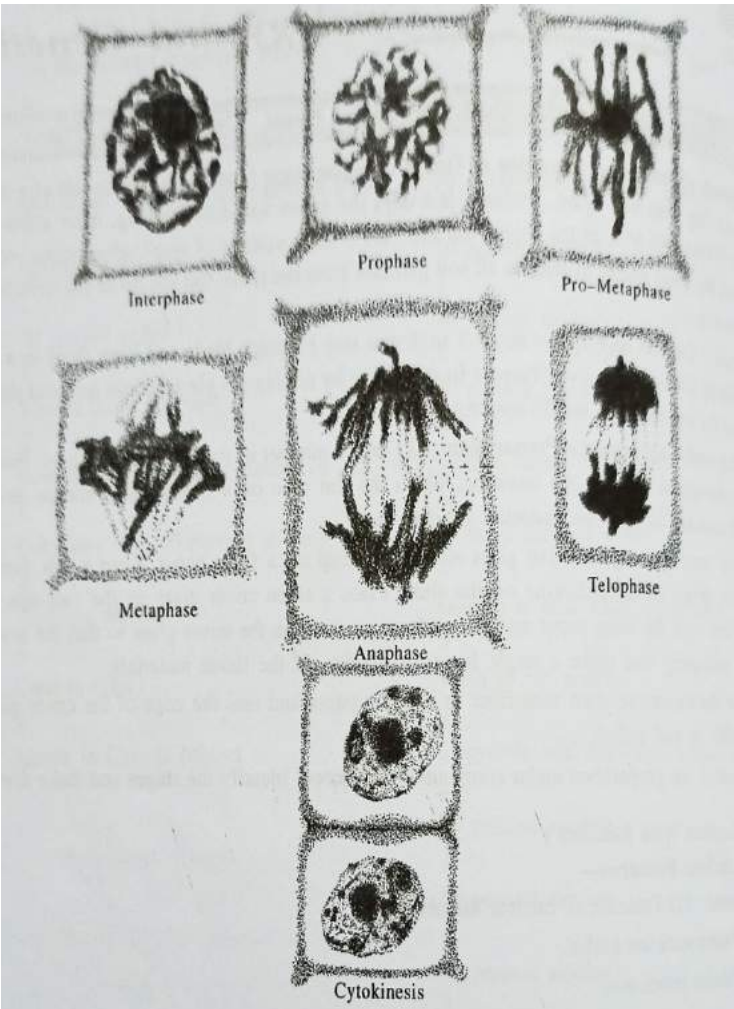
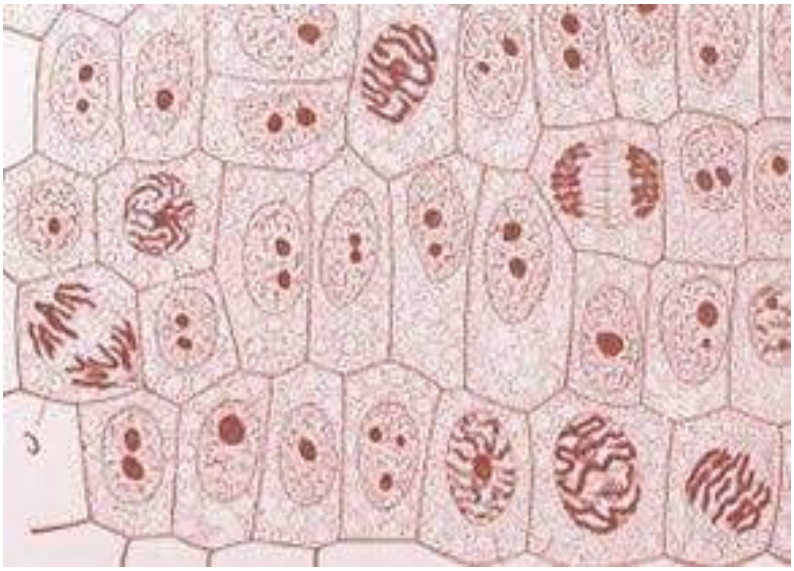
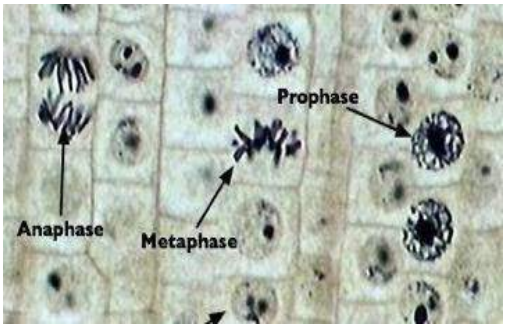
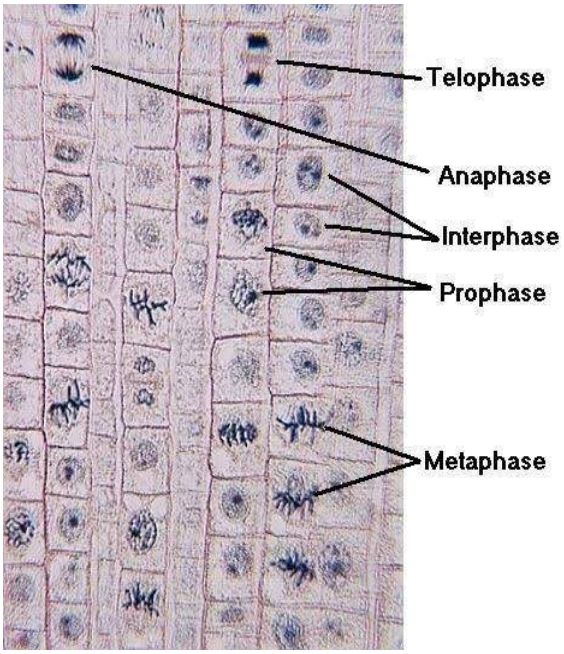


Fig. 13.1: Stages of mitotic cell division prepared from the onion root tip




Prophase
Anaphase
Metaphase


Telophase
Anaphase
Interphase
Prophase
Metaphase