PRACTICAL-III
(DBOTL21)
(MSC BOTONY)

ACHARYA NAGARJUNA UNIVERSITY

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PRACTICAL III

DEVELOPMENTAL BIOLOGY OF ANGIOSPERMS AND ETHNOBOTANY, MICROBIOLOGY, MYCOLOGY AND PLANT DISEASE

List of Experiments :

Embryology : (Through the study from slides)

1. Demonstration through slides structures and development of anther, pollen and male and female gametophytes
2. Types of Ovules
3. Endosperm and Embryo
4. Development of Fruit and Seeds

Anatomy :

5. A Critical Study on various types of tissues
6. Tissue systems in the primary and secondary plant bodies
7. Nodal Anatomy
8. Anomalous Secondary growth in Boerhavia
9. Leptadenia, Amaranthus, Nyctanthus
10. Dracaena and beet root
11. Wood Maceration
12. Wood Anatomy

Ethanobotany :

Taxonomic Study of important medicinal plants
Anatomical studies of some important medicinal plants
Tissue culture studies of medicinal plants.
Screening of medicinal plant extracts for antimicrobial activity

Microbiology, Mycology and Plant Diseases

List of experiments

1. Bacterial Gram staining
2. Bacterial spore staining
3. Negative staining
4. Measurement of bacterial cell
5. Preparation of culture media
6. Isolation and enumeration of bacteria from soil
7. Isolation and enumeration of fungi from soil
8. Demonstration of antibiotic sensitivity
9. Observation of symptoms caused by plant viruses
10. Microscopic observation of fungal slides (permanent slides)
11. Anatomical studies on leaves infected with fungal pathogens
12. Effect of fungicides on fungal spore germination
13. Phytotoxic effect of fungal culture filtrate

M.Sc., Botany (Final)

PRACTICAL - 3 : EMBRYOLOGY

C O N T E N T S

1. T.S. OF TETRASPORNAGIATE ANTHER

A typical anther is four sporangiate. It has a column of sterile tissue, called the connective, on either side of which is an anther lobe. Each lobe has two microsporangia separated by a stripe of sterile tissue. In each lobe some hypodermal cells become more prominent than the others because of their larger size, slight radial elongation and conspicuous nuclei. These cells constitute the archesporium.

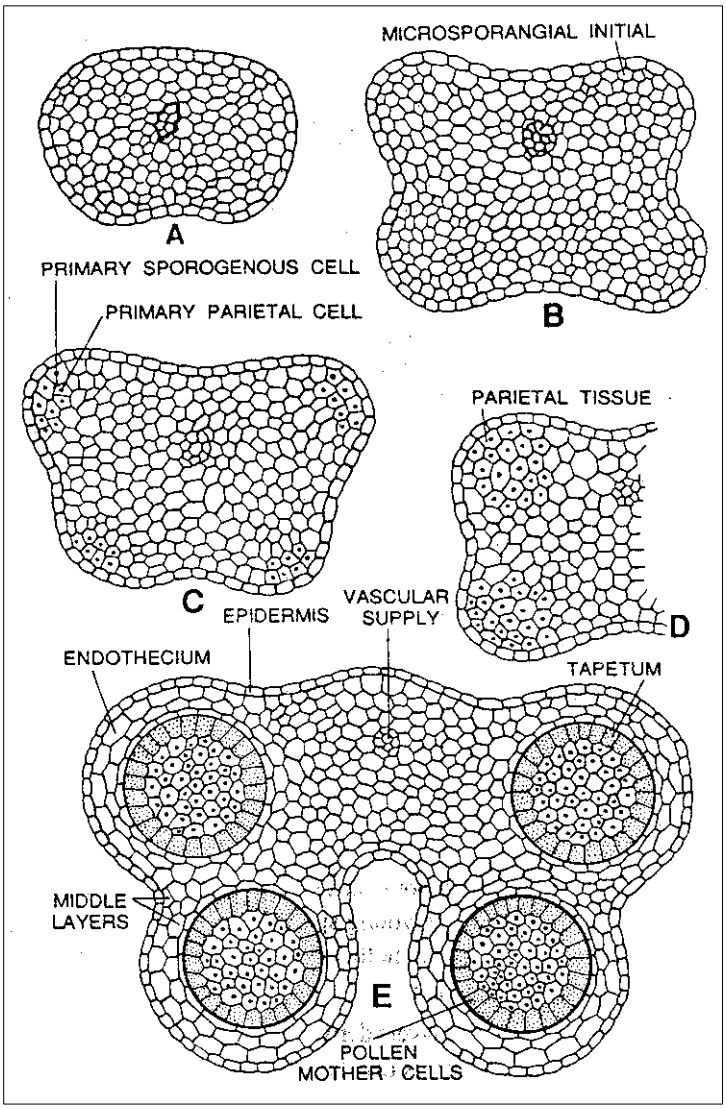

Fig. 1. Various stages of the development of microsporangium (pollen sac) upto the formation of pollen mother cells

2. ANTHER WALL

The mature anther wall comprises an epidermis, followed, on the inside by a layer of endothecium. 2 or 3 middle layers and a single-layered tapetum.

Epidermis: The outermost layer is called as the epidermis. It is continuous, greatly stretched and flattened. Epidermis performs its usual protective function.

Endothecium:

(1) The cells of endothecium are radially elongated. This layer attains its maximum development when anther is ready to dehisce for the discharge of pollen.

(2) Cells of this layer develops bands of callose which arise from the inner tangential walls (rarely from the radial walls) and run outward and upward ending near the outer wall of each cell.

(3) The presence of callose bands, differential expansion of the outer and inner tangential walls, and the hygroscope nature of the endothecial cells help in the dehiscence of anthers at maturity.

Middle Layers:

(1) The cells of middle layers are generally ephemeral and as a rule, become flattened and crushed by early meiosis in the pollen mother cell.

(2) In many species, the cells of the middle layers are storage centres for starch and other reserves which get mobilized during later development of pollen.

Tapetum:

This is the inner most layer of anther wall and attains its maximum development at the tetrad stage of microsporogenesis. It completely surrounds the sporogenous tissue and is of considerable physiological importance because all the food material to the sporogenous tissue must pass through it. Typically, the tapetum is composed of a single layer of cells characterised by the presence of dense cytoplasm and prominent nuclei.

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3. MICROSPORE TETRADS

After the meiotic division, a wall is formed separating the two nuclei. This leads to the formation of a dyad. Then the two cells of the dyad undergoes second meiotic division callose wall are formed in the same way resulting into a tetrad.

Mostly, the four spores within a tetrad are completely isolated from one another and from the spores in other tetrads of the locule. Usually, the arrangement of microspores in a tetrad is tetrahedral or isobilateral. However, decussate, linear and T-shaped tetrads are also found.

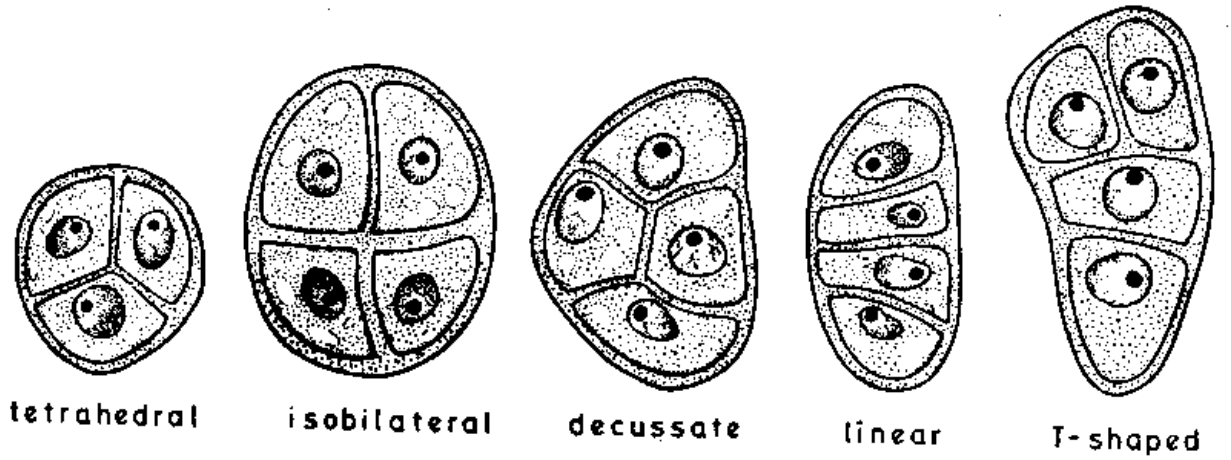

Fig. 2. Types of microspore tetrads in *Aristlochia*

4. MICROSPORE (pollen grains)

Microspores particularly after their release from the tetrads are usually referred to as pollen grains. During gametogenesis, the pollen nucleus divides to forma generative cell and vegetative cell. The former undergoes another mitosis to form two sperm (malegamets).

Pollen grain Young

5. FORMATION OF VEGETATIVE AND GENERATIVE CELLS

1. The first division of a pollen grain results into two unequal cells.
2. The larger one is the vegetative cell, which eventually forms the pollen tube.
3. The other one is the generative cell, which produces the sperms by another mitosis.
4. The generative cell is initially attached to the wall of the pollen grain but later comes to lie freely in the cytoplasm of the vegetative cell.

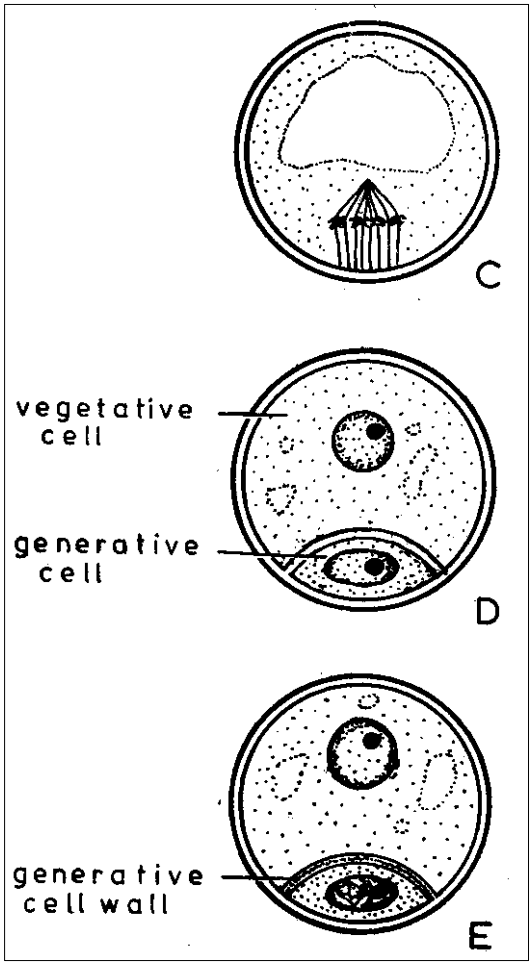

Fig. 3. Pollen mitosis; note the asymmetric spindle. **D.** Two-celled-pollen soon after pollen mitosis. **E.** Generative cell wall has appeared in between the plasma membranes of the vegetative cell and the generative cell

6. FORMATION OF SPERMS

1. Sperms are formed by a mitotic division in the generative cell.

2. This may take place while the pollen grains are confined to the anther (Beta, Hordeum) or it may take place after the release of pollen.

3. In the former condition the pollen are shed at the 3-celled stage and the latter at the 2-celled stage.

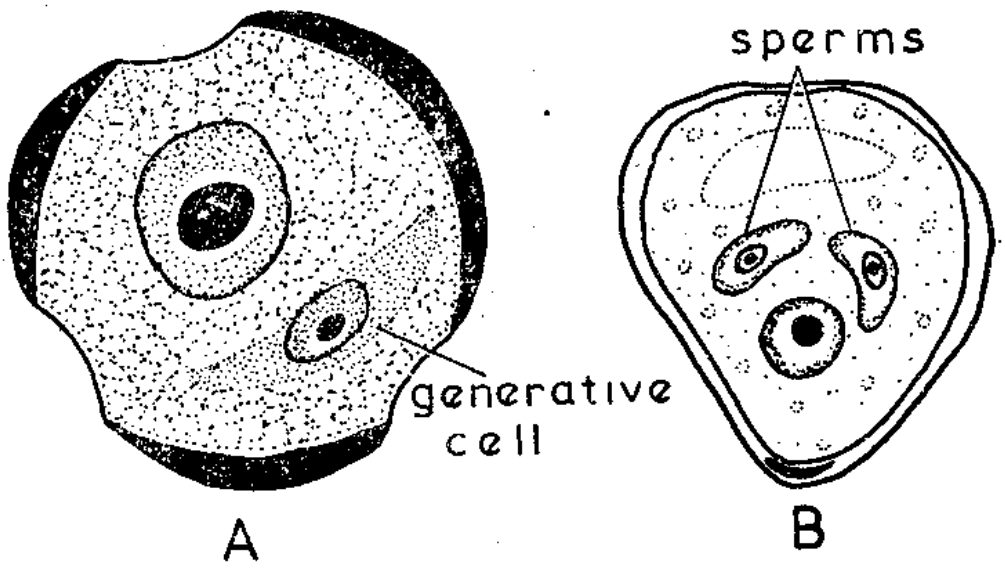

Fig. 4. Pollen at shedding. **A.** Two-celled pollen of Klugia. **B**. Three-celled pollen of Cyperus.

7. TYPES OF OVULES

On the basis of the relative position of the micropyle, chalaza and funiculus the mature ovules can be classified as six types.

1. **Orthotropous or straight ovule:** In this type, the ovule is erect or straight so that the funicle, chalaza and micropyle lie in one and the same vertical line, as in members of Polygonaceae (e.g. Polygonum, Rumex etc.) and Piperaceae (e.g. *Piper nigrum*, *Piper betle*) (Fig. 5A).

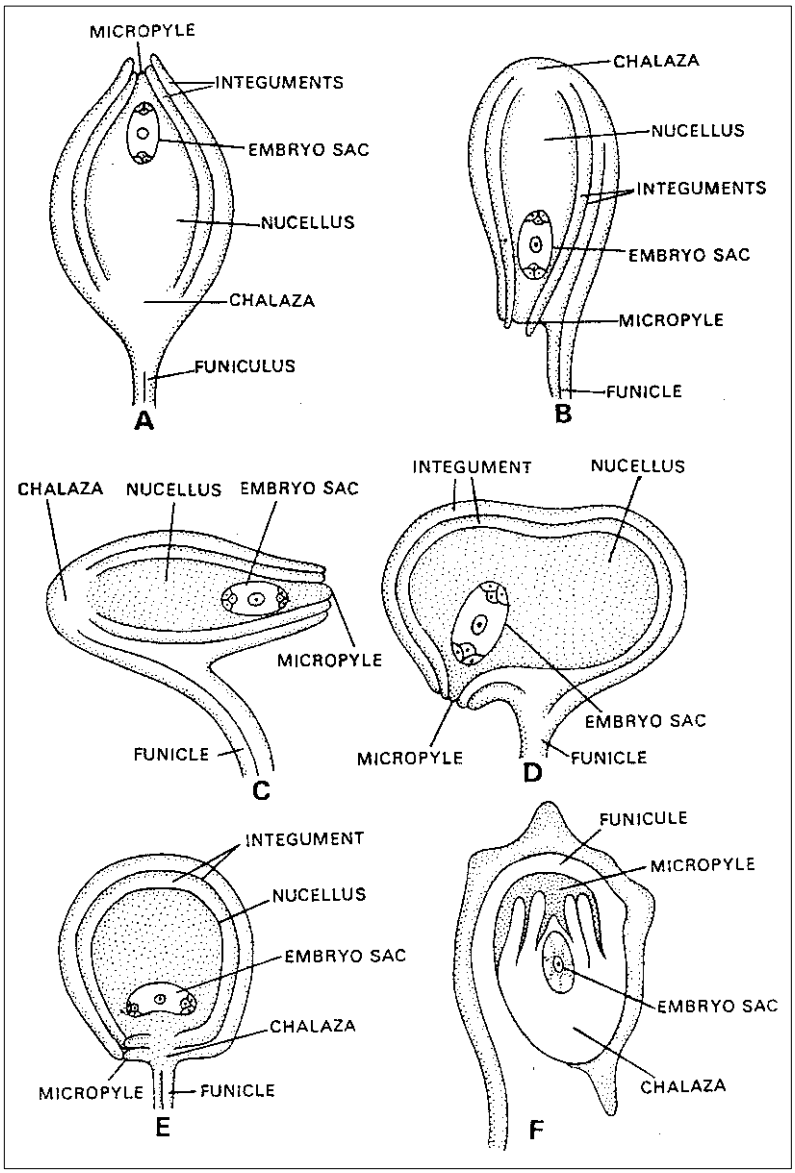

Fig. 5. Different forms of the ovule in longitudinal section. **A**, orthotropous; **B,** anatropous; **C,** hemianatropous; **D,** campylotropous; **E,** amphitropous; **F,** circinotropous.

2. **Anatropous or inverted:** In this type, the ovule bends along the funicle so that the micropyle lies close to the hilum. The chalaza lies at the other end. This is the commonest type of ovule found both indicotes and monocots (Fig. 5B).

3. **Amphitropous or transverse:** In this type, the ovule is placed transversely at a right angle to its stalk or funicle, as found in Lemna (Fig. 5C).

4. **Hemitropous or Hemianatropous:** In this type, the body of the ovule is straight but twisted in such a way that it is placed transversely at right angle, and so the chalazal micropyle line is at right angle to the funiculus. It is found in *Ranunculus* (Fig. 5D)

5. **Campylotropous or curved:** In this type, the transverse ovule is bent round like a horse-shoe so that the micropyle and the chalaza do not lie in the same straight line, as in Capparis, Cruciferae, Mirabilis, Jalapa etc. (Fig. 5E).

6. **Circinotropous:** In this type, the nucellus and the axis remain in the same line in the beginning but due to rapid growth on one side, the ovule gets inverted. This curvature continues and thus the ovule turns completely and once again the micropyle faces upwards. This type is found in *Opuntia* and *Plumbago* (Fig. 5F).

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8. L.S. OF A TYPICAL OVULE OF ANGIOSPERM

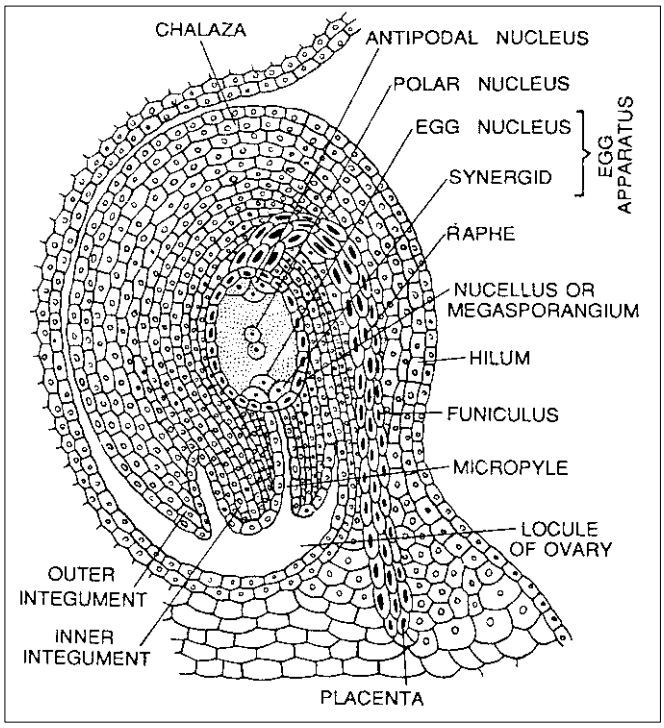

Fig. 6. Ovule, L.S. of a typical ovule of angiosperm

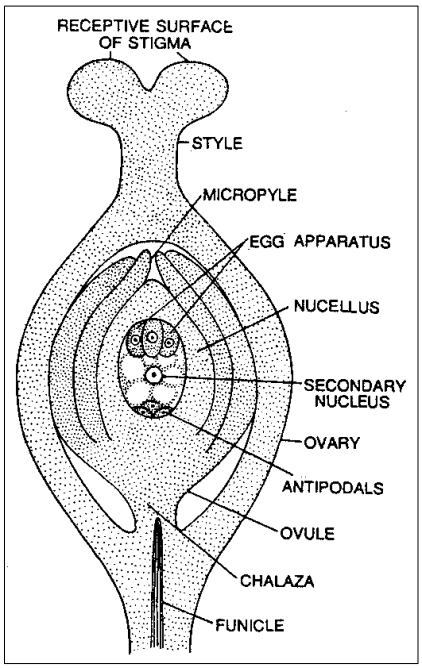

Fig. 7. Pistil in longitudinal section showing different parts of mature ovule attached to a basal placenta.

Generally, the ovule consists of a nucellus, enveloped by one or two integuments and is found attached to the placenta, on the inner wall of the ovary, by a stalk known as the funicle. The part of the funicle that runs parallel with the nucellus is called the "raphe". An ovule ready for fertilization consists of nucellus enveloped by one or two integuments, leaving a small opening at the apical end. This opening called micropyle, is the main passage for the entry of the pollen tube into the ovule. The chalaza is the region where the integuments are inserted. In fact, funicle, raphe, chalaza and nucellus forms a continuous tissue.

9. TYPES OF NUCELLUS

Nucellus represents the wall of megasporangium. Each ovule has only one nucellus. As an abnormality, however, twin nucelli may occur in a common fold of integuments.

1. **Tenuinucellate:** In Sympetalae the archesporial cell directly functions as the megaspore mother cell so that the sporogenous cell is also hypodermal. Such ovules, where the sporogenous cell is hypodermal, and the nucellee tissue around it remains single layered are called tenuinucellate.

2. **Crassinucellate:** The hypodermal archesporial cell divides transversely, cutting an outer parietal cell and an inner sporogenous cell. The parietal cell may either remain undivided or undergo a few periclinal and anticlinal divisions. So that the sporogenous cell becomes embedded in the massive nucellus where, the sporogenous cell becomes sub-hypodermal, either due to the formation of parietal cells or due to divisions in the nucellar epidermis, or both, are called crassinucellate.

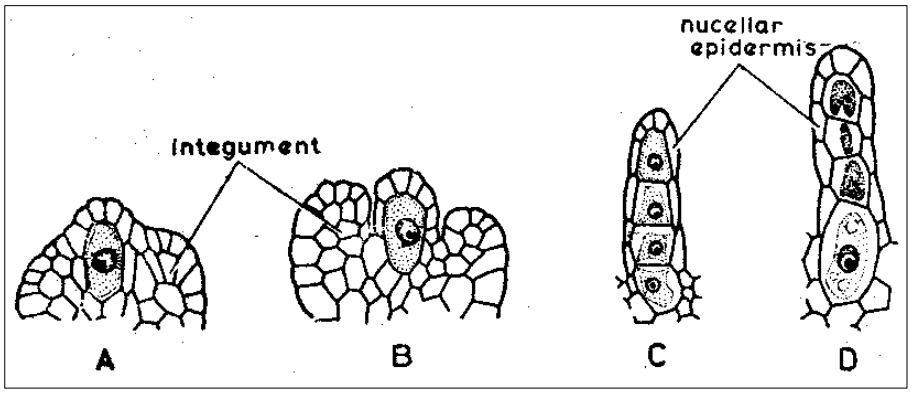

Fig. 8. Megasporogenesis in tenuinucellate ovule of *Elytraia acaulis*. Integument is not drawn in *C* and *D*. *A, B*. Megaspore mother cell stage. *C*. Tetrad stage. *D*. The functional, chalazal megaspore has enlarged while the other three megaspores have degenerated.

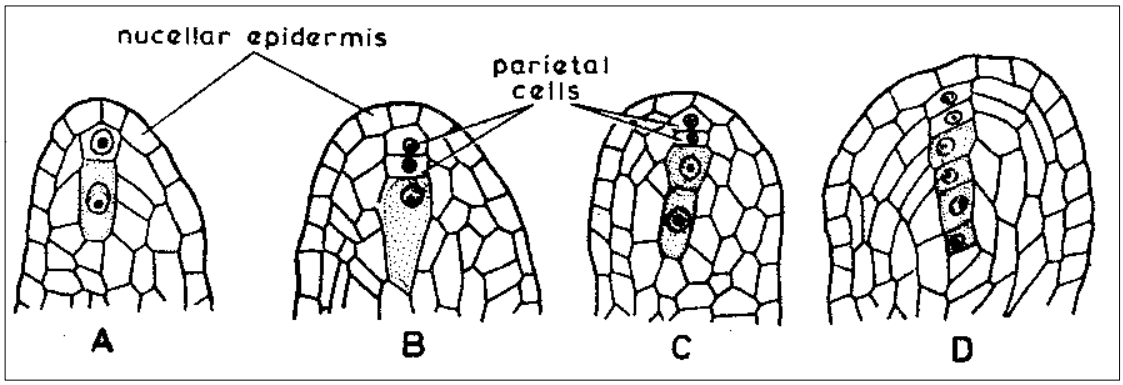

Fig. 9. Megasporogenesis in crassinucellate ovules of *Myrlophvllum intermedium*. Stippled, nucleate cells are megaspore mother cells and their derivatives whereas unstippled, nucleate cells are the primary parietal cell and its derivatives. *A.* After the division of the archesporial cell, forming primary parietal cell and primary sporogenous cell. *B.* The primary parietal cell has divided periclinally whereas the primary sporogenous cell has simply enlarged. *C.* Dyad stage. *D.* Tetrad stage.

antipodal cells, whereas the fourth one act as the lower polar nucleus. Eventually, the latter comes to lie close to the upper polar nucleus. This mode of embryo sac development occurs in the majority of flowering plants and is designated as the Polygonum type.

10. MEGASPORE MOTHER CELL

1. A single hypodermal cell in the nucellus functions as the archesporium.
2. It becomes more prominent than its surrounding cells because of its larger size, denser cytoplasm and larger nucleus.
3. In tenvinucellate and pseudo-crassinucellate ovules the archesporial cell directly functions as megaspore mother cell.
4. Whereas in crassinucellate ovules it divides periclinally, cutting an outer primary parietal cell and an inner primary sporogenous cell.
5. The latter functions as megaspore mother cell.
6. Megaspore mother cell also called megasporocyte, undergoes meiosis to form four haploid megaspores.
7. In this, only one is functions and forms the female gametophyte.

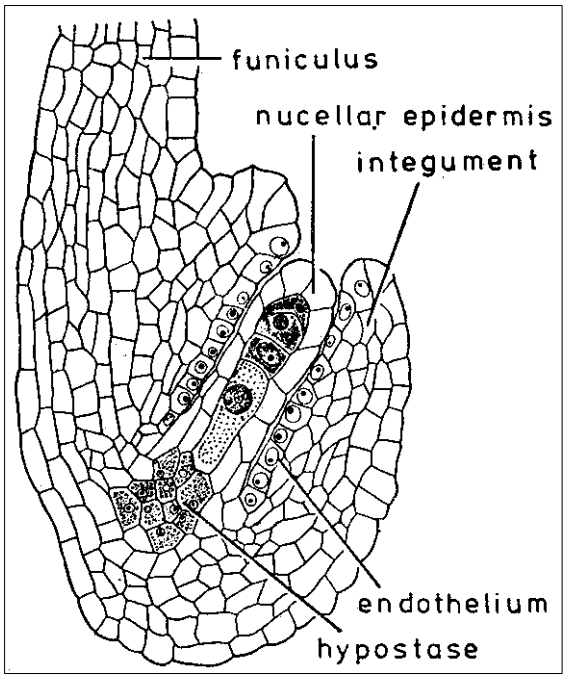

Fig. 10. *Bupleurum tenue*. Longitudinal section of ovule at the tetrad stage, showing hypostase and young endothelium. The three non-functional megaspores are on way to degeneration.

11. POLYGONUM TYPE OF EMBRYO SAC DEVELOPMENT

The chief characteristic of monosporic embryo sac is that it is derived from only one megaspore, as in the Polygonum type. The embryo sac is formed by the chalazal megaspore in the tetrad and is eight nucleate. The mature embryo sac comprises an egg apparatus, three antipodal cells, and a binucleate central cell.

The development of embryo sac begins with elongation of the functional megaspore, which is usually the chalazal one of the tetrad. The elongation is largely in the micropyle chalaza axis. Initially, the megaspore cytoplasm is non-vacuolate but later small vacuoles appear which may fuse together to form a large vacuole. The spindle of the first nuclear division in the megaspore is oriented along the long axis of the cell. Wall formation does not follow the nuclear division. A large central vacuole now appears between the two daughter nuclei and as it expands, the nuclei are pushed toward opposite poles of the cell (Fig. 11A). Both nuclei divide twice, forming four nuclei at each pole (Fig. 11B, C). At this stage, all the 8 nuclei are present in a common cytoplasm (Fig. 11C, D). After the last nuclear division, the cell undergoes appreciable elongation, assuming a sac like appearance. This is followed by the cellular organization of the embryo sac (Fig. 10E). Of the four nuclei at the micropylar end of the sac, three organise into egg apparatus and the fourth one is left free in the cytoplasm of the central cell of the upper polar nucleus. Similarly, three nuclei of the chalazal and three forms antipodal cells, whereas the fourth one act as lower polar nucleus. Eventually, the latter comes to lie close to the upper polar nucleus. This mode of embryosac formation occurs in the majority of flowering plants and id designated as the polygonum type.

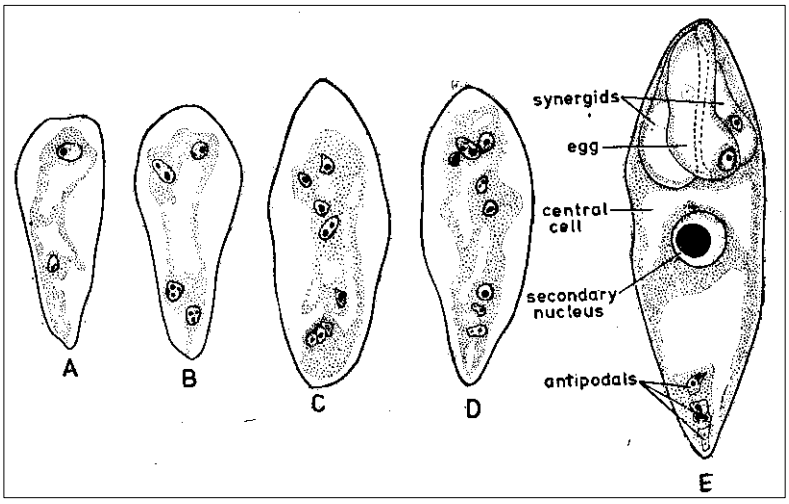

Fig. 11. Stages in megagametogenesis in *Morina longifolia*. **A.** Megaspore after first post-meiotic mitosis. **B.** 4-nucleate stage. **C.** 8-nucleate stage. **D.** Older 8-nucleate stage, showing 3+2+3 distribution of the nuclei. **E.** Mature embryo sac; it comprises 3-celled egg apparatus, 3 antipodal cells, and a large central cell with a secondary nucleus.

12. MATURE EMBRYO SAC OR ORGANISED EMBRYO SAC

The female gametophyte, also called embryosac, is mostly a 7-celled structure. There is a large central cell with two polar nuclei, which later fuse to form a secondary nucleus. The micropylar end of the central cell is occupied by the egg apparatus, comprising an egg cell and two synergids, and at its chalazal end three antipodal cells are present. Cells of the egg apparatus and the antipodals cells are uninucleate and haploid where as the central cell is binucleate or diploid.

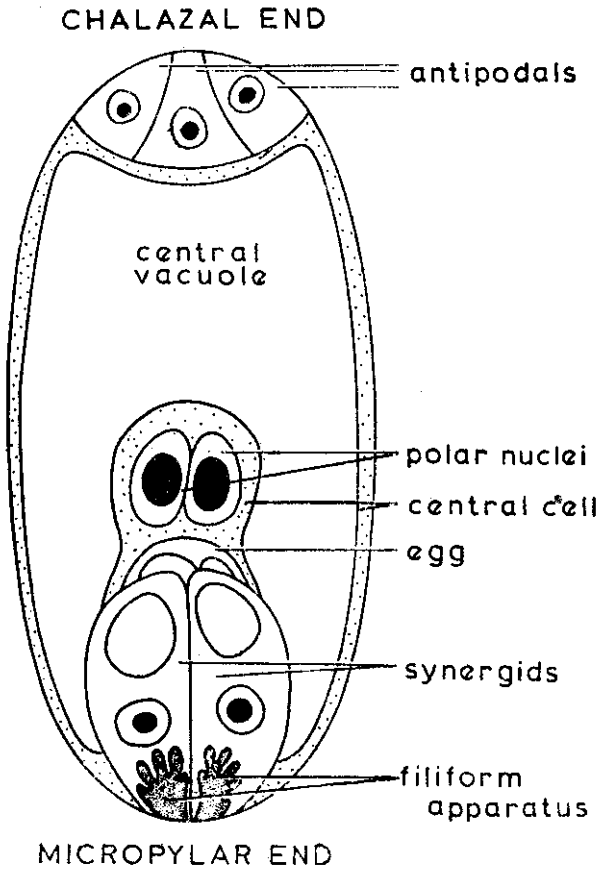

Fig. 12 Diagram of an organised embryo sac

13.ENDOSPERM

Endosperm is the most common nutritive tissue for the developing embryos in angiosperms. After double fertilization the fusion product of polars and the second male gamete is termed primary endosperm nucleus.

The only angiosperms which do not form endosperm are the members of the families Orchidaceae, Podostemaceae and Trapaceae. Where present, the endosperm may either be consumed by the developing embryo, so that the seeds are non-0endospermous (Pea, beans) or it may persist in mature seeds and continue to support the growth of embryo during seed germination. Common examples of endospermous seeds are cereals castor-bean and coconut. Endosperm forms the edible part of cereals and coconut and it is the source of commercial castor oil in castor-bean.

Depending on its mode of development, the endosperm may be:

(a) Nuclear

(b) Cellular

(c) Helobial.

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14. NUCLEAR ENDOSPERM

In this type of endosperm, the division of the primary endosperm nucleus and a few subsequent nuclear divisions are not accompanied by wall formation. This results in a condition where the central cell of the embryo sac has formed a few to several thousand nuclei freely suspended in its sap. Such a condition of endosperm may persist until it is consumed by the developing embryo (Limnanthus, Oxyspora) or it may become cellular at a later stage. When latter is the case, which is more common, the wall formation is mostly centripetal, i.e., from the periphery toward the centre.

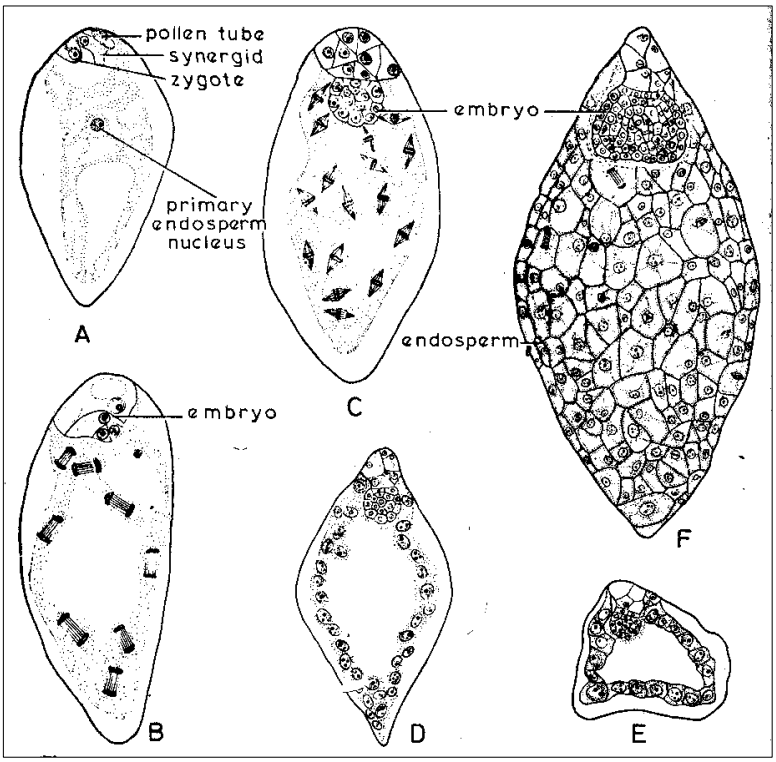

Fig. 13 Nuclear endosperm in *Acalypha indica*. ***A.*** Embryo sac after fertilization, the primary endosperm nucleus and the zygote have not yet divided. ***B, C.*** Embryo sac showing synchronous divisions of the endosperm nuclei. ***D***. The endosperm nuclei have moved to the periphery. ***E***. The peripheral part of the embryo sac has become cellular. ***F.*** Completely cellular endosperm.

15. CELLULAR ENDOSPERM

The cellular endosperm is characterized by the absence of free-nuclear stage. The division of the primary endosperm nucleus and a few subsequent nuclear divisions are followed, regularly, by wall formation. The occurrence of haustoria is a common feature of this type of endosperm, it is more varied than that in the nuclear endosperm. The haustoria may be micropylar or chalazal. Occasionally, both types of haustoria are present in the same plant.

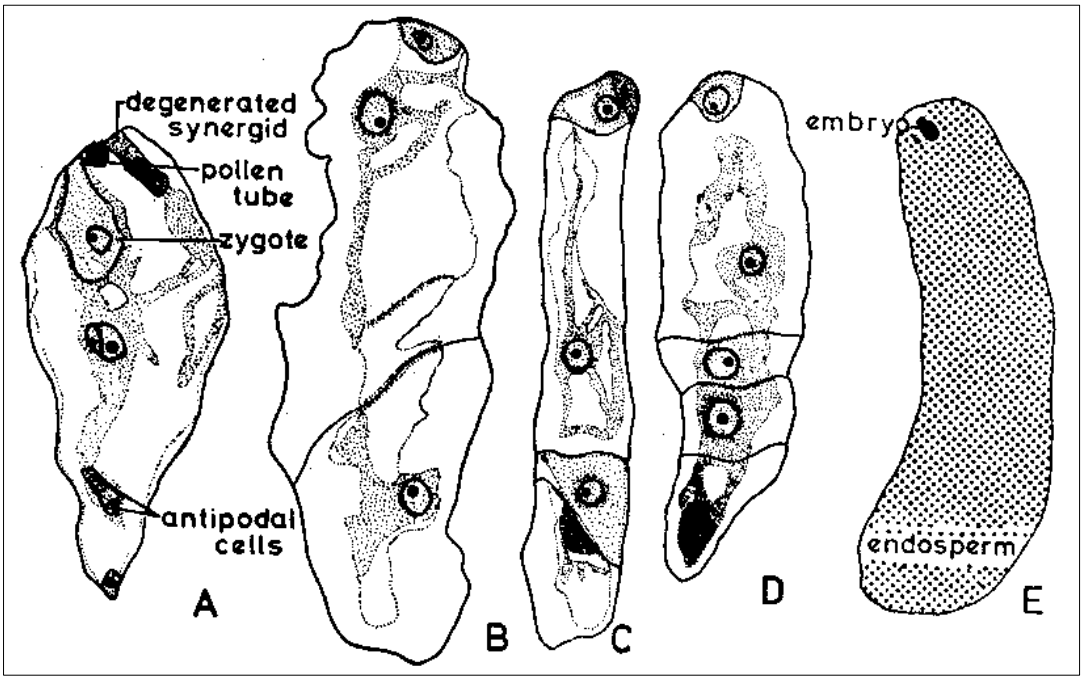

Fig. 14. Cellular endosperm in *Drimys winteri*. ***A.*** Embryo sac after fertilization. ***B-D***. Two-celled, 3-celled, and 4-celled endosperm, respectively. ***E***. Older embryo sac completely filled with cellular endosperm.

16. HELOBIAL ENDOSPERM

This type of endosperm is restricted largely to the monocotyledons. The primary endosperm nucleus moves to the chalazal end of the embryo sac where it divides forming a large micropylar chamber and a small chalazal chamber. In the micropylar chamber, as a rule, free nuclear divisions and cell formation, if any, start at a much later stage. In the chalazal chamber the nucleus either remains undivided or divided only a few times. If later is the situation, the divisions are usually free-nuclear. However, sometimes, it may become cellular.

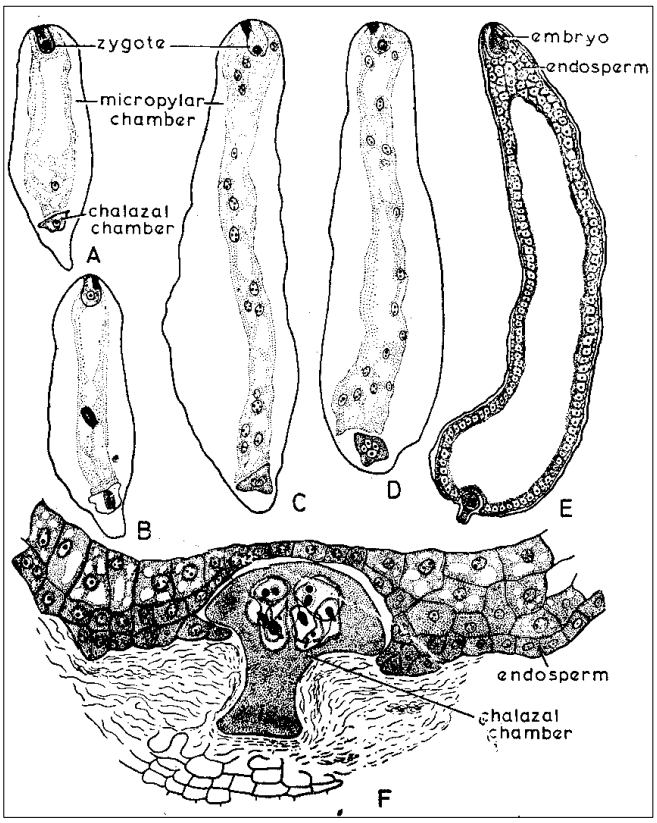

Fig. 15. Helobial endosperm in *Asphodelus tenuifolius*. **A, B,** Two-celled endosperm; note the large micropylar chamber and the small chalazal chamber, **C, D**. Later stage; showing multinucleate micropylar chamber and 4-nucleate chalazal chamber. The zygote has not yet divided. **E**. Peripheral region of the micropylar chamber has become cellular. **F.** Magnified view of the lower part of the seed showing free-nuclear chalazal chamber.

17. EMBRYO

1. The fertilized egg is called zygote. Following a predetermined mode of development (embryogeny) it gives rise to an embryo, which has the potentiality to form a complete plant.

2. A typical dicotyledonous embryo as seen in median longitudinal section shown in Figure 16.

3. It comprises an embryonal axis with two cotyledons attached to it laterally.

4. The portion of embryonal axis above the level of cotyledons is called epicotyl, and the portion below the level of cotyledons is known as hypocotyl.

5. The epicotyl terminates into plumule (embryonic shoot), and the hypocotyl at its lower end bears radicle (embryonic root).

6. The embryo of a monocotyledon differs from that of a dicotyledon mainly in having only one cotyledon.

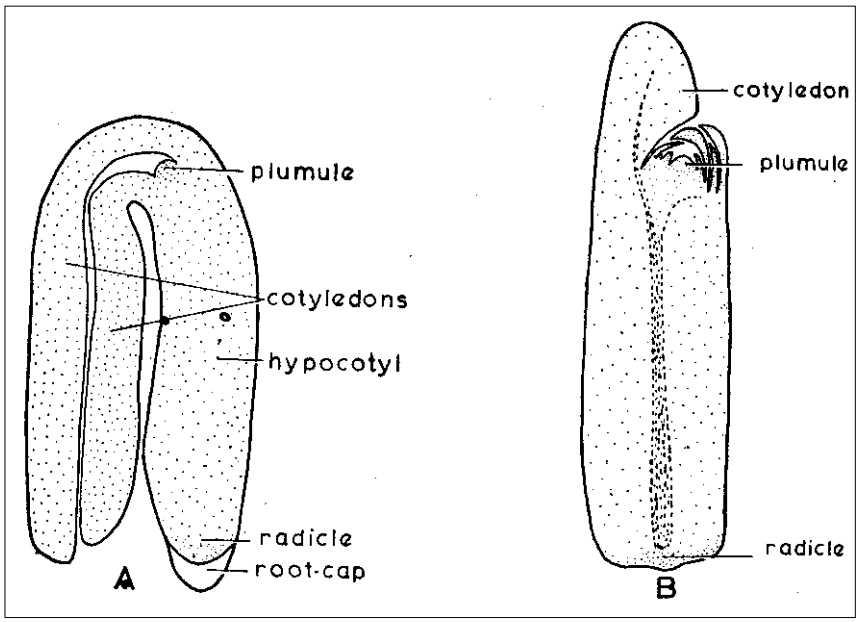

Fig. 16. Median longitudinal section of: **A**, Mature dicotyledonous embryo of Capsella bursa-postoris, and **B**. Mature monocotyledonous embryo of Hydrilla verticillata.

18. DEVELOPMENT OF A DICOTYLEDONOUS EMBRYO

To illustrate complete development of a dicotyledonous embryo the work of Bhandari and Asnani (1968) on *Ceratocephalus falcatus* (Ranunculaceae) is described. In this species, the embryogeny is of Onagrad type.

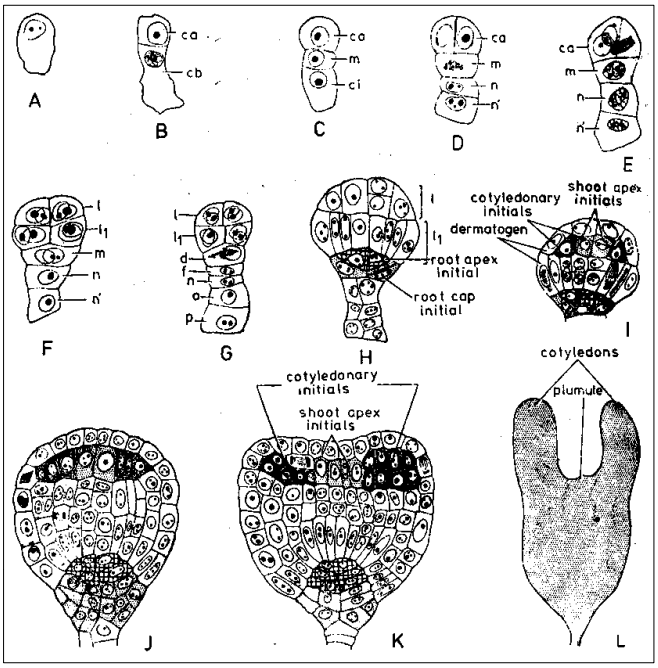

Fig. 17. Development of embryo in *Ceratocephalus falcatus*

Ceratocephalus falcatus: The zygote (Fig. 17A) divides transversely forming a small apical cell (ca) and a large basal cell (cb) (Fig. 17B). Cell cb divides transversely forming two superposed cells ci and m (Fig. 17C), and cell ca undergoes a vertical division giving rise to two Juxtaposed cells (Fig. 17D). Thus, a T-shaped, 4-celled proembryo is formed. Of the two daughter cells of **cb**, cell **ci** divides transversely giving rise to n and n′ (Fig. 17D). These two cells further divide forming a linear row of 3 or 4-celled suspensor. Cell **m** and its derivatives divide by a vertical division to form 4-6 cells. Oblique periclinal divisions in each of these cells result in an inner set of cells (th initials of root apex) and an outer set of cells (the initials of root cap (Fig. 17H). In the mean time, the daughter cells of the apical cell divide by another vertical division at right angles to the first division (Fig. 17 E), forming a quadrant q. A transverse division of the quadrant results in an octant arranged in two tiers (l, l_1) of four cells each (Fig. 17F, G). Vertical divisions in tiers land l_1 give rise to a globular proembryo (Fig. 17H). Periclinal divisions in the peripheral cells of the globular proembryo demarcate a single-layered dermatogen, the future epidermis (Fig. 17 I). Cells of the tier l differentiate the initials of plumule and the two cotyledons (Fig. 17 I-K). Growth in the cotyledonary zones is much faster than that in the plumular zone. As a result, the mature embryo the plumule is enclosed at the base of the two cotyledons (Fig. 17 L).

19. EMBRYOGENY IN MONOCOTYLEDONS

The development of embryo upto the octant stage is almost similar in monocotyledons and dicotyledons. The differences appear later. the main difference between the mature embryos of monocotyledons and dicotyledons is in the number of cotyledons. The single cotyledons in monocotyledons has been regarded by many authors as a terminal structure. Wardlaw (1955) has remarked; "In the dicotyledonous embryo the plumule is typically distal and is situated symmetrically between two equivalent cotyledons; in the monocotyledonous embryo the shoot apex occupies a lateral position in the somewhat cylindrical embryo and the cotyledon is terminal".

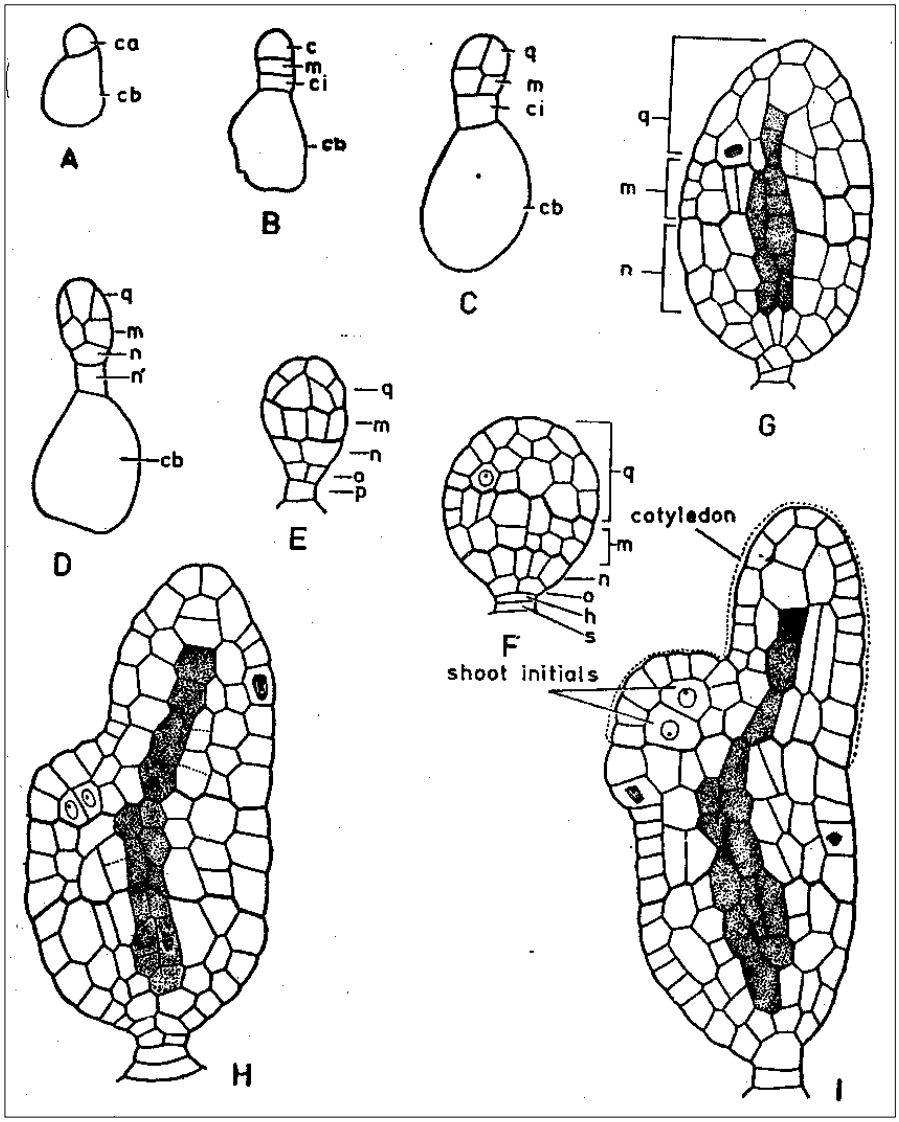

Fig. 18. Development of embryo in *Najas lacerata*

In *Najas lacerate* transverse division of the zygote results in a large basal cell (cb) and a small apical cell (ca) (Fig. 18A). The basal cell, without dividing even once, enlarges to form a single-celled haustorium (Fig. 18A-D). Thus, the entire embryo is derived from the apical cell. It (ca) divides transversely into two cells, **c** and **d**. Of these, the cell **d** once again divides transversely. In this way, the linear proembryo of four cells (c, m, ci, cb) is formed (Fig. 18B). Two vertical divisions at right angles to each other in the two distal cells (c, m) lead to the formation of two superposed tiers (q, m) of four cells each. In the mean time, cell **ci** divides transversely to give rise to **n** and **n′.** Whereas cell **n** divides vertically **n′** undergoes transverse division giving rise to two cells (Fig. 18 E).

The quadrant **q** divides by a periclinal division cutting a four-celled dematogen surrounding the four axial cells. At the eight celled stage of ties q (4 axial cells and four circumaxial cells) three of the axial cells divides faster than the fourth one. This disturbs the symmetry of the pro-embryo, and its top becomes notched (Fig. 18H). The rapidly growing portion of the tier q forms the single-cotyledon (Fig. 18 I) and slow growing tissue derived from the fourth axial cell, gives rise to the initials of epicotyl.

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20. POLYEMBRYONY

1. Polyembryony has been defined as the occurrence of more than one embryo in a seed.

2. The first case of Polyembryony was reported in certain orange-seeds by Antoni van Leeuwenhock in 1719.

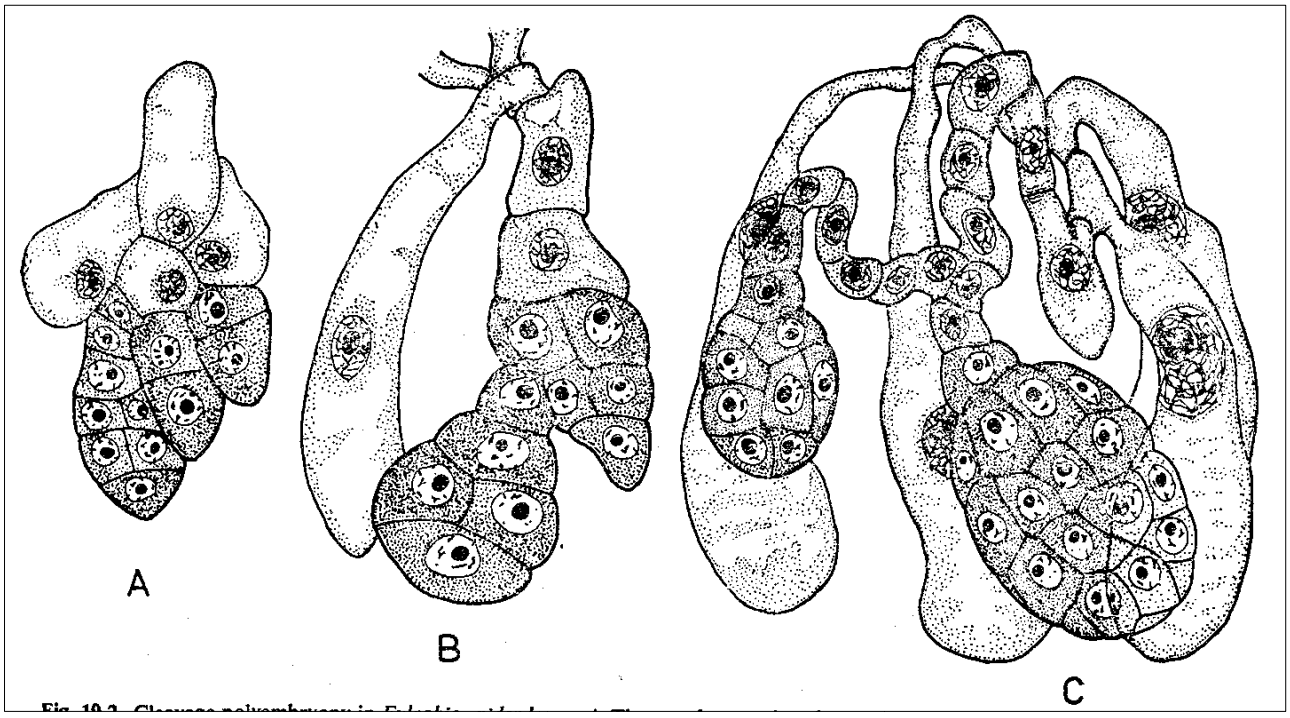

Fig. 19. Cleavage polyembryony in *Eulophia epidendraea*. **A.** Three embryos arisen by proliferation of the zygote. **B.** Budding of the embryo. **C.** Two embryos arisen by splitting of a single embryo. Large, vacuolated cells belong to the suspensor

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M.Sc. BOTANY (Final) **Practicals**

ANATOMY

I. Various types of Tissues and Tissue Systems

Fixed or fresh plant parts are collected (locally available plants) and take thin sections (freehand sections). Stain section with 1% safranin (1 g safranin in 100 ml of 70% ethyl alcohol). Destain sections in acid water (3% HCl) if excess stain prevails.

Primary Plant body

Entire plant body is derived from the shoot and root apices. Meristematic tissue is located in shoot and root apices. The meristematic cells undergo divisions and derivatives of these cells are differentiated into permanent tissues.

Permanent tissues may be classified into three main groups: (1) Simple (parenchyma, collenchma and sclerenchyma), (2) Complex (xylem and phloem), (3) Secretory tissues.

Tissue systems are of three types found in the primary plant body. These are as follows:

(1) Epidermal tissue system, i.e., epidermis with stomata, trichomes etc.
(2) Ground tissue system, i.e. ground tissue with cortex, pericycle, medullary rays, pith etc.
(3) Vascular tissue system, e.g. vascular bundles with xylem and phloem.

(1) Parenchyma

(i) Parenchyma is the most common tissue which is morphologically and physiologically simple and unspecialized.
(ii) Parenchyma is found in the cortex, pith, pericycle and mesophyll of leaf.
(iii) Parenchymatous cells are thin-walled, compactly arranged (palisade tissue of leaf) or with small intercellular spaces. Numerous, larger intercellular spaces (parenchyma) are found in aquatic plants such as *Eichhornia*, *Hydrilla*, *Potamogeton* (Fig. 1.1) etc. Aerenchyma in aquatic plants provide buoyancy to float easily in or on the water.
(iv) Storage parenchyma in potata contain ergastic substances like starch grains and protein granules.
(v) Parenchymatous cells covered with cuticle on leaf epidermis are protective in nature which prevent transpiration and also provide mechanical strength to plant organ.

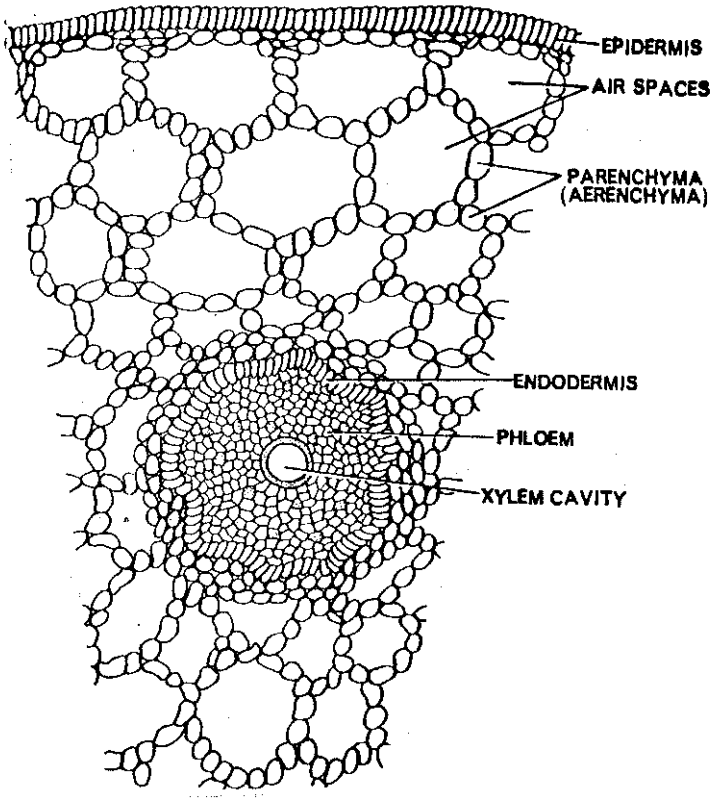

Fig. 1.1 Transection of *Potamogeton* stem (hydrophyte)

(2) Collenchyma

(i) Collenchyma is a living tissue composed of more or less elongated cells with thick primary non-lignified walls. On the basis of thickenings on cell wall and arrangement of cells, the following three types are recognized.

(ii) **Angular collenchyma:** The thickenings are found at the corners or angles of the cells, e.g. stems of *Cucurbita* (Fig. 1.2 B), *Datura, Lycopersicon* etc. This parenchyma is without intercellular spaces.

(iii) **Lacunar or tubular parenchyma:** This parenchyma is with larger intercellular spaces. The thickenings are restricted to the walls of the regions bordering air spaces, e.g. *Salvia, Malva,* aerial roots of *Monstera, Lactuca* (Fig. 1.2 C) etc.

(iv) **Plate or lamellar parenchyma:** The thickenings occur chiefly on the tangential walls and lesser in the radial walls. As a result, cells appear like plates, bands or lamellae, e.g. *Raphanus, Sambucus* (Fig. 1.2 A) etc.

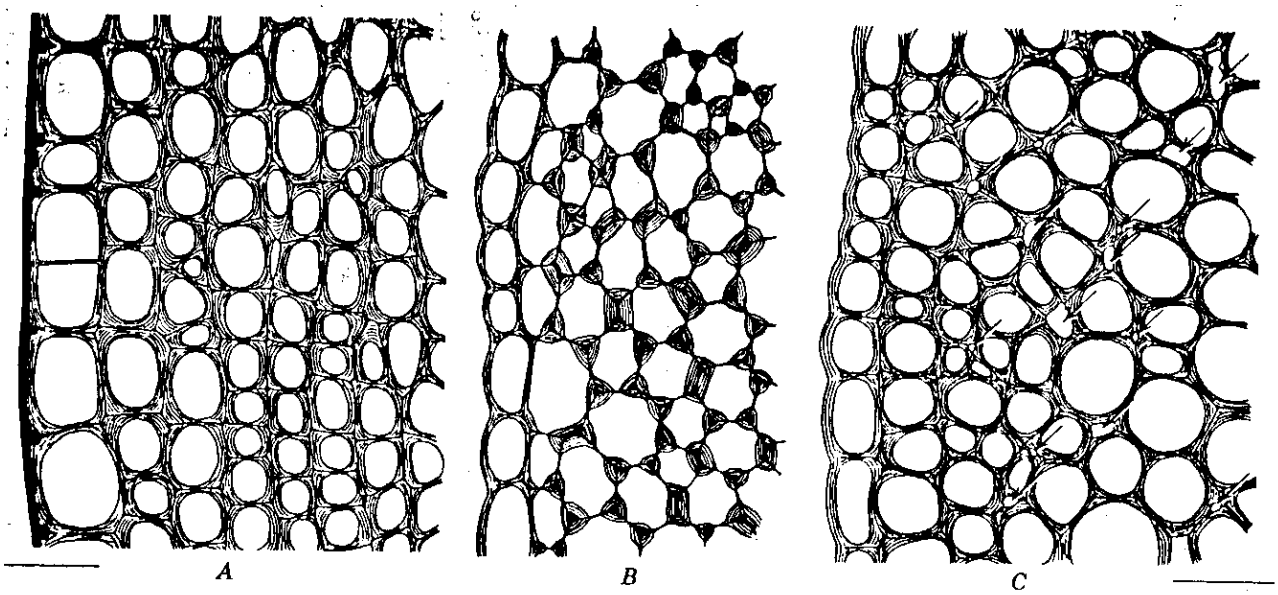

Fig. 1.2 Collenchyma in stem transections. **A**. Lamellar parenchyma in *Sambucus*, **B**. angular collenchyma in *Cucurbita*, **C.** Lacunar Parenchyma in *Lactuca*.

(3) Sclerenchyma

(i) Sclerenchyma is a dead tissue with lignified thickenings on the cell walls. It provides mechanical strength to the plant body. Sclerenchymatous cells show much variation in form, structure, origin and development, and are classed into two groups namely, **fibres** and **sclereids**.

(ii) **Fibres:** These are classified into two groups, the xylary fibres (intra-xylary) and extra-xylary fibres.

(iii) Xylary fibres are also known as wood fibres. Some of the xylary fibres are **libriform fibres** and **fibre tracheids**. The libriform fibres are characterized by very thick secondary wall and simple pits, while fibre tracheid by relatively thin walls and bordered pits.

(iv) Cortical fibres (found in cortex), peri-vascular fibres (periphery of the vascular cylinder) and phloic fibres (phloem fibres; Fig. 1.3) are come under the category of extra-xylary fibres.

(v) **Sclereids (sclerotic cells):** Greater deposition of lignin is found, as a result lumen becomes narrow in these cells. Five categories of sclerieds are given below:

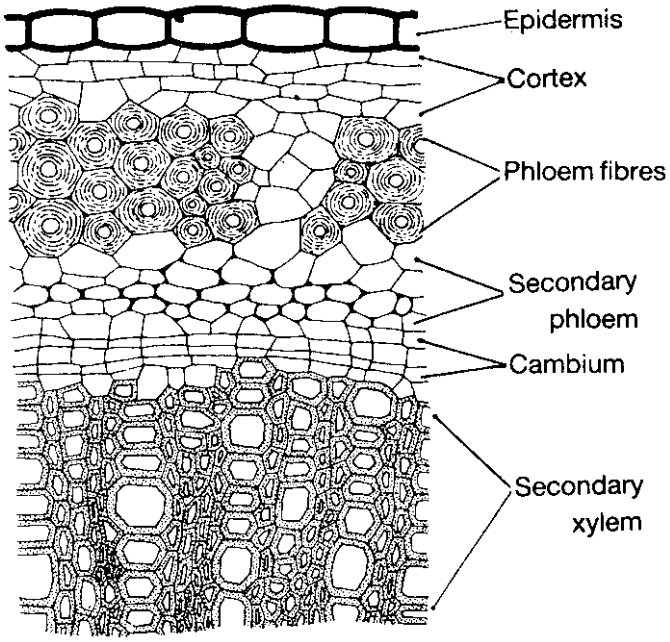

Fig. 1.3 Transection of stem of *Linum usitatissimum* (flax) showing phloem fibres.

(vi) Brachysclereids (stone cells) are roughly isodiametric found in cortex, pith and phloem stems and flesh of fruits, e.g. *Pyrus* (Fig. 1.4A).

(vii) Macrosclereids are elongated columnar ones, commonly form palisade like epidermal layer, e.g. seed coats of legumes (Fig.1.4 D).

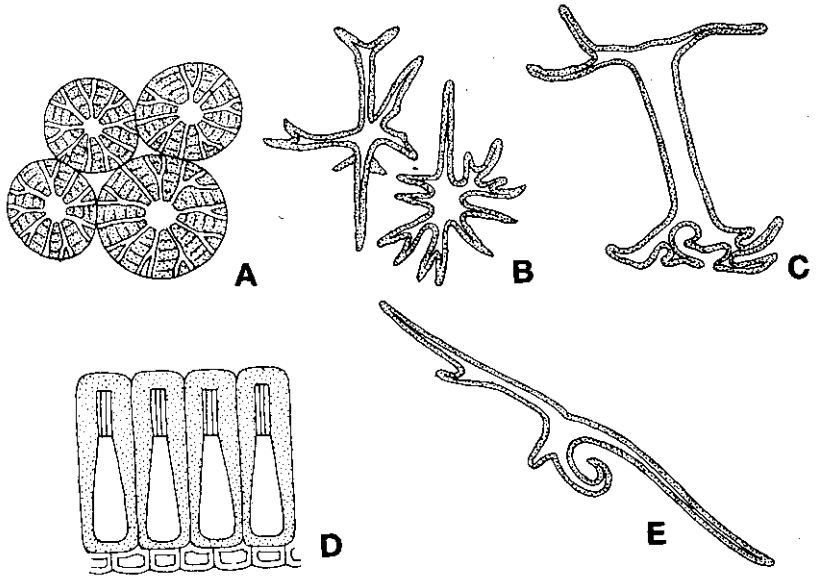

Fig.1.4 Different types of sclereids.
A. rachysclereids, **B**. astrosclereids, **C**. osteosclereid, **D**. macrosclereids

(viii) Osteosclereids are bone or barrel-shaped with dilated ends. Commonly found in leaves and seed coats of monocots (Fig. 1.4 C).

(ix) Astrosclereids are star-shaped (stellate type) found in leaves of *Nymphoea* and, stem and leaves of *Trochodendron* (Fig. 1.4 B).

(x) Trichosclereids are hair-like branched and unbranched ones found in hydrophytes and also aerial roots of *Monstera* (Fig. 1.4 E).

(4) Xylem

Xylem is a complex tissue, consisting of tracheids, vessels, xylem fibres and xylem parenchyma. Xylem involves in conduction of water and provides mechanical strength to the plant body.

Tracheids:

(i) Tracheid is an elongated structure with tapering ends. It has got hard, thick and lignified walls and a large lumen. The tracheids are considered as primitive and found in the ancient vascular plants. These are the only elements found in the fossil seed plants.

(ii) A tracheid differs from xylem fibres in having: (i) the cell wall comparatively less lignified, (ii) large number of pits, (iii) bordered pits and (iv) a very large lumen.

(iii) Lignified secondary walls are highly characteristic and these are deposited in different pattern such as annular (ring type, Fig. 1.5A), spiral (Fig. 1.5D), scalariform (ladder type, Fig. 1.5E), reticulate and pitted (Fig. 1.5 F).

(iv) Generally tracheids with annular and spiral thickenings are found in the early formed primary xylem whereas scalariform and pitted tracheids in later formed primary xylem and also in secondary xylem.

(v) **Vessels or trachea:** Vessel members are joined one end to another end and form the vessel.

(vi) It is long, cylindrical, tube-like structure with lignified walls and a wide lumen.

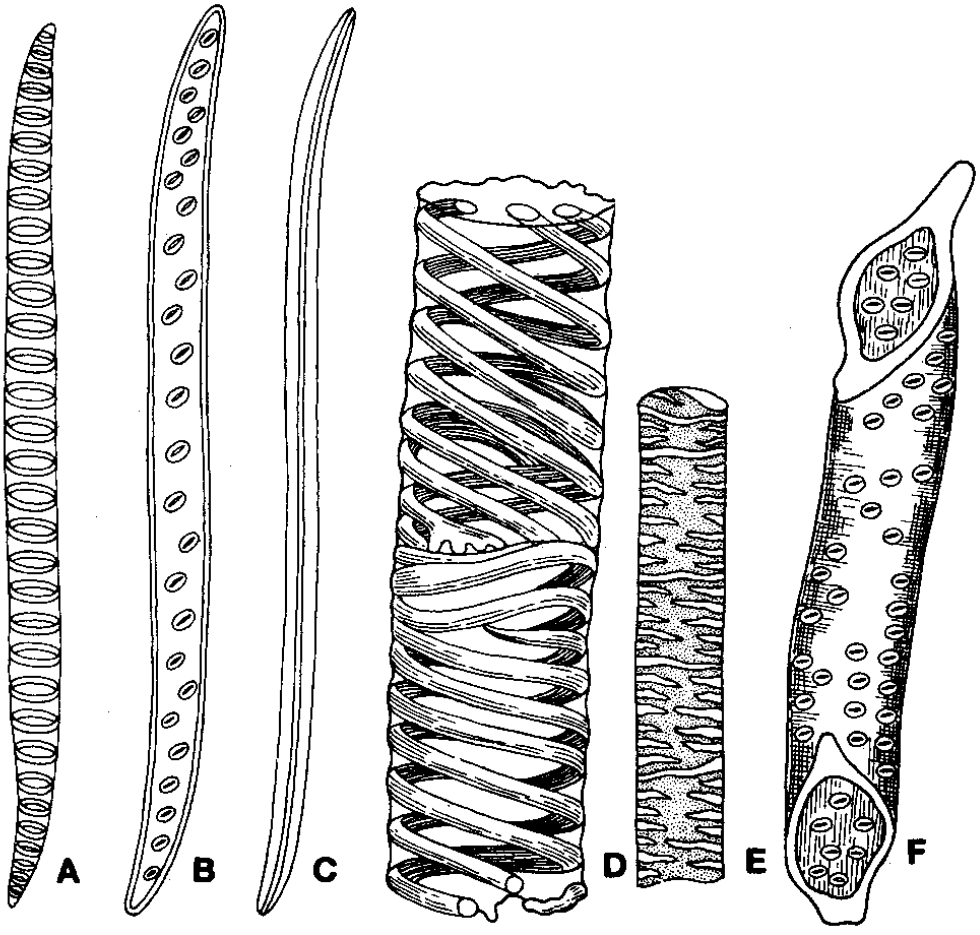

Fig. 1.5 Different types of tracheids.
A. annular, **B** and **C**. tracheidal fibres, **D**. spiral, **E**. scalariform and **F**. pitted.

(vii) The characteristic feature of vessel members is that they have **perforation plates** (end walls) (Fig. 1.6).

(viii) If perforation plate containing one large pore, it is called **simple perforation plate**; if there are several pores, it is known as **compound (multiple) perforation plate**.

(ix) In compound perforation plates, pores are arranged in several patterns (Fig. 1.6). When the pores are arranged in ladder-like manner, it is called *scalariform* (Fig. 1.6 A,B). In some cases, perforations are circular and grouped together, the perforation plate is called *foraminate* type.

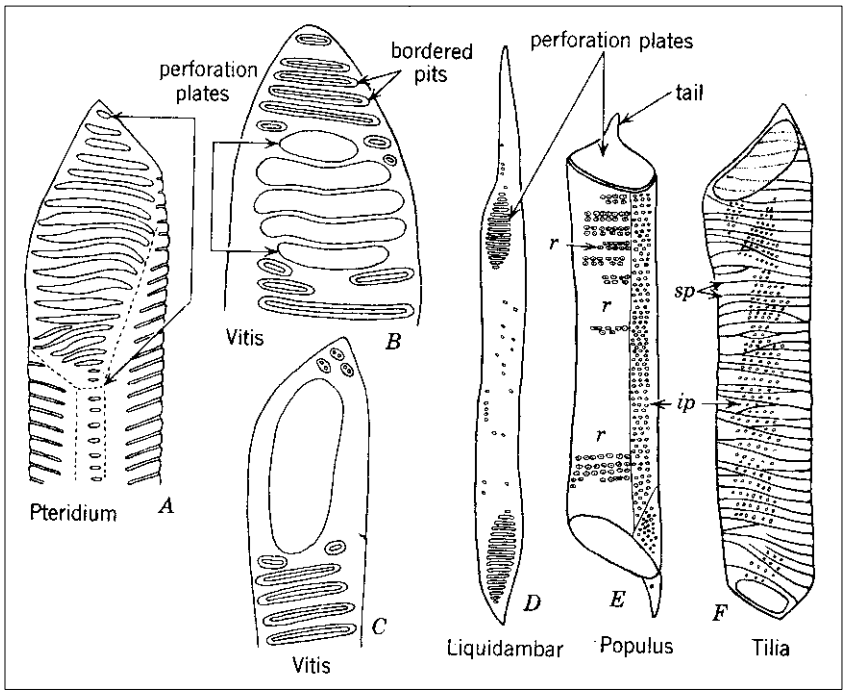

Fig. 1.6 **A-C.** End walls of vessel member showing various types of perforation plates. **A**. and **B**. scalariform type, **C**. simple type, **D-F**. Complete vessel members, **D.** scalariform perforation plates, **E.** simple perforation plates, **F**. simple perforation plates, (*ip*- intervessel pitting; *r*- ray contact areas; *sp*- spiral thickenings).

(5) Phloem

Phloem is a complex tissue, consisting of sieve elements, companion cells, phloem fibres and phloem parenchyma. Phloem can be detected by staining with aniline blue or resorcin blue (lacmoid).

Characteristic features

(i) Sieve elements include two types of cells, the less specialized **sieve cells** and the more specialized **sieve-tube members**.

(ii) The longitudinal series of sieve-tube members constitute the sieve tube.

(iii) The most characteristic features of sieve elements are the sieve areas (Fig. 1.7) on their walls and disappearance of nucleus in their protoplasts.

(iv) Sieve-tube members have got highly specialized sieve areas and these are localized in the form of sieve plates.

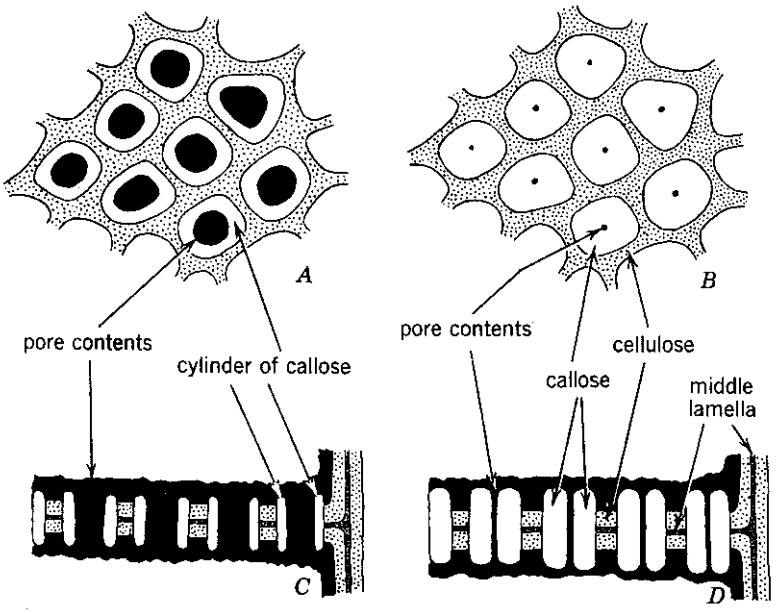

Fig. 1.7 A-D. Sieve areas in angiosperm sieve tube.
A, B. surface views, **C, D**. sectional views. **A, C**. illustrate younger sieve areas; **B, D.** older sieve areas

Secondary Plant body

Locally available dicot stems (thick and mature) are collected and study the secondary structures. Secondary plant body is formed by the activity of lateral meristems such as vascular cambium (Fig. 1.8) and phellogen. By the activity of these meristems, the girth of the stem is increasing and giving rise to secondary plant body.

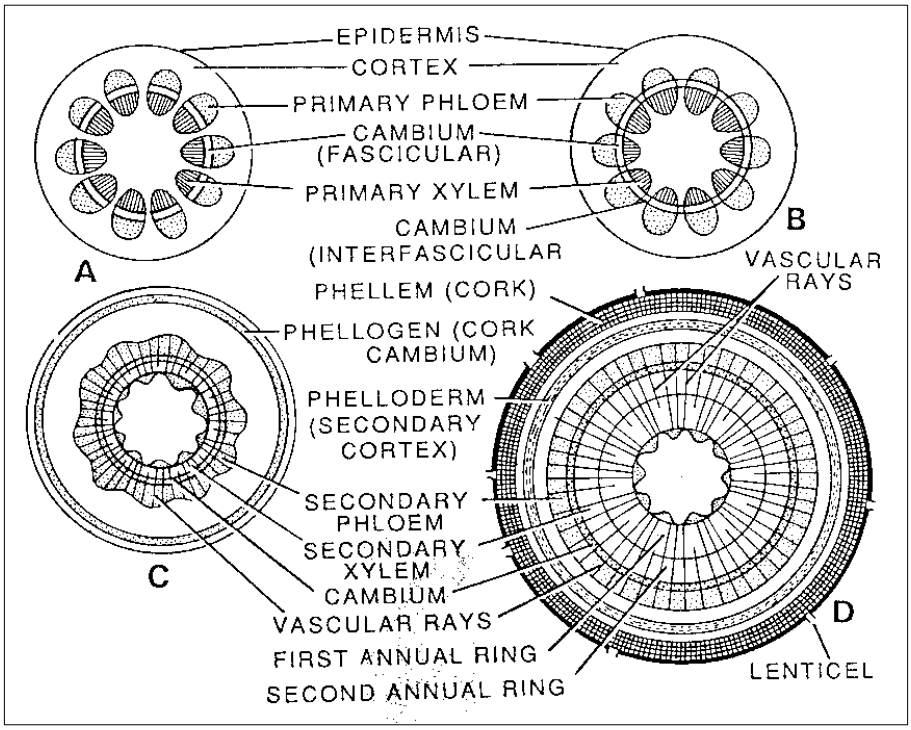

Fig. 1.8 Secondary growth in dicot system

Vascular cambium cuts off secondary phloem towards outer side and secondary xylem towards innerside. Secondary xylem formed during spring and also of autumn constitute together to form annual ring or growth ring. During secondary growth periderm is formed by the activity of phellogen.

Secondary growth in stem of *Peristrophe bicalyculata*

(i) The stem is angular in outline in transection and shows six distinct ridges and furrows.

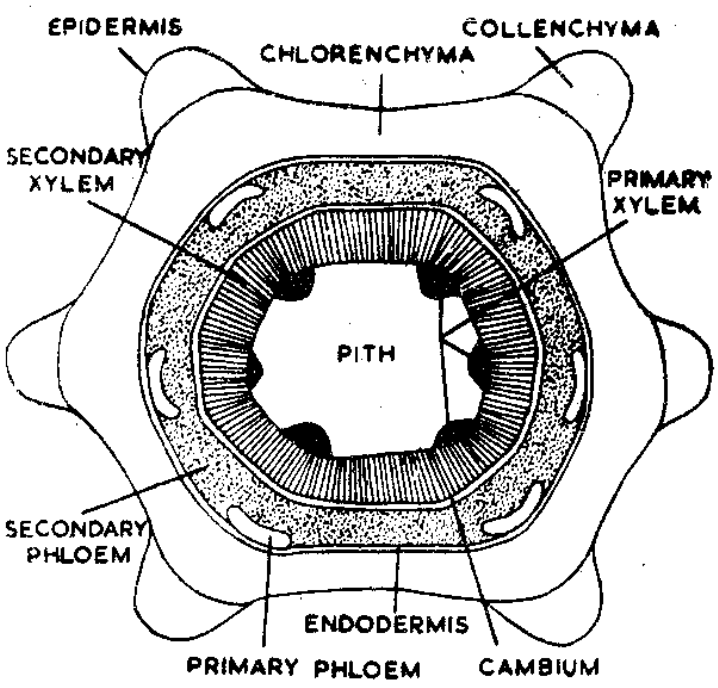

Fig. 1.9 Outline diagram of transection of stem of *Peristrophe bicalyculata*.

(ii) Epidermis is single layered with thin-walled parenchymatous cells.

(iii) Cortex is collenchymatous in ridges, whereas in furrows it is chlorenchymatous.

(iv) Endodermal cells are barrel-shaped, with casparian strips on their radial walls.

(v) In the primary stem, six vascular bundles are present corresponding to ridges; these are conjoint, collateral, open and endarch.

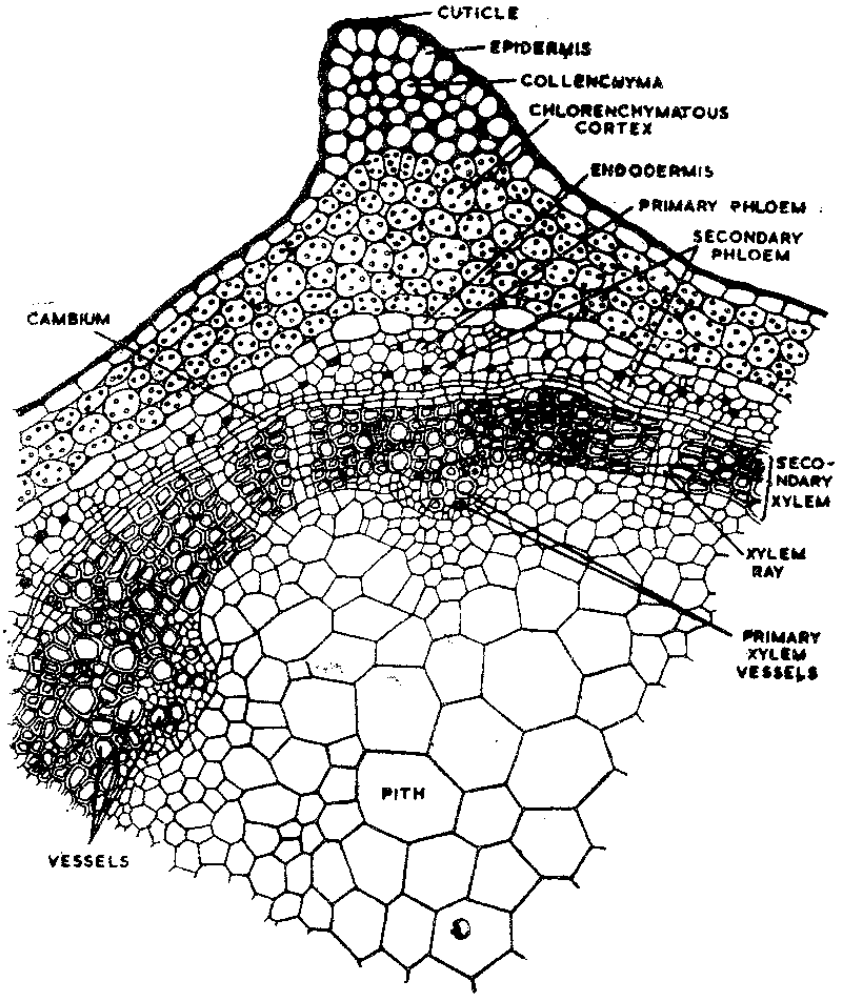

Fig. 1.10 Transection of stem of *Peristrophe bicalyculata* – a cellular detains

(vi) During secondary growth, vascular cambium cuts off the secondary phloem towards outside and secondary xylem towards inside. Secondary xylem is interrupted by narrow rays, that may be uniseriate or biseriate.

(vii) Pith is made up of thin-walled polygonal cells.

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Dr. S.M. KHASIM

II. NODAL ANATOMY

To study the structure of the node, take series of sections from internode to the above node and stain them with 10% phloroglucinol and 5% conc. nitric acid. Xylem takes dye slowly and stains dark orange red.

In angiosperms, stem possesses nodes and internodes. The place where leaf traces or branch traces arise is referred to as **node**. The structure of vascular cylinder shows variations in the regions of nodes and internodes.

Leaf traces – In the nodal region, a part of the vascular cylinder enters in the leaf is known as **leaf trace** (vascular supply to the leaf). A leaf trace may also be defined as a vascular bundle that connects the vascular bundle of the leaf with that of the stem. The vascular supply that goes to branch at the nodal region, is known as **branch trace**.

Leaf gaps – In higher plants such as ferns, gymnosperms and angiosperms, there is a discontinuity of vascular cylinder of stem just above the diverging leaf traces. This break up region of vascular cylinder made up of parenchyma is known as **leaf gap** (nodal lacuna). Leaf traces are always accompanied by leaf gaps. However, in Lycopsida leaf gaps are absent and these nodes are known as **Cladosiphonic nodes**. Branch traces are also accompanied by branch gaps.

In some ferns, such as *Pteridium* and *Pteris*, the leaves are so crowded that the gaps formed at the successive nodes overlap one another, as a result actual vascular cylinder becomes dissected. Presence of branch gaps further complicates the structure.

In general, at the internodal region solid vascular cylinder is present whereas at the node it becomes dissected due to presence of leaf gaps and branch gaps.

Types of nodes

The arrangement of leaf traces and their complexes varies in different groups of plants and is related to phyllotaxis. There are four basic types of nodes found in dicotyledons. These are as follows:

(i) **Unilocunar** node with a single leaf gap and a single leaf trace, e.g. *Spiraea* (Fig. 2.1A), and it is found in the opposite and whorled phyllotaxis.

(ii) **Unilocunar two trace** is found in opposite leaves, e.g., *Clerodendron*, *Veronica* etc.

(iii) **Trilocunar node** with a three leaf gaps and three leaf traces, e.g., *Salix* (Fig. 2.1B), *Brassica* (Fig. 2.1C), *Leptadenia* etc.

(iv) **Multilocunar node** with several leaf gaps and leaf traces associated with a single leaf, e.g. *Rumex* (Fig. 2.1D), *Ricinus* etc.

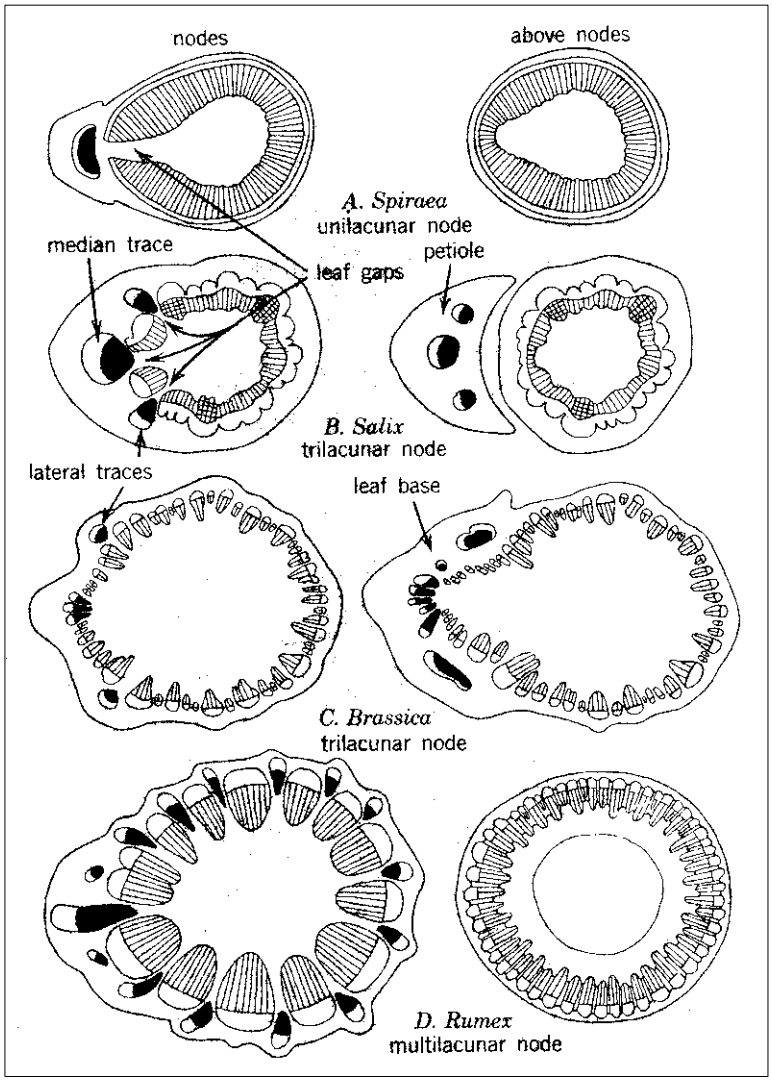

Fig. 2.1 A-D. Transection of stems of dictoyledons showing different types of nodes

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Dr. S.M. KHASIM

III. ANOMALOUS SECONDARY GROWTH

The process of secondary growth that gives rise to the secondary plant body may be termed the **common type of secondary growth**. However, in many angiosperms there are deviations from this type of secondary growth. Such deviated pattern of secondary growth is described as **anomalous secondary growth**. With the result of the combinations of unusual structure, certain anomalous and extremely complex structures are formed. These complex structures are referred to as **anomalies**.

The anomalies can be listed as follows:

(i) Anomalies in Primary Plant body

A. Occurrence of scattered vascular bundles in dicotyledons, e.g. members of Nymphaeaceae, Papaveraceae and Piperaceae.

B. Vascular bundles arranged in a ring in monocotyledons, e.g. *Hordeum, Oryza, Triticum* etc.
C. Presence of medullary bundles, e.g., *Amaranthus, Boerhavia, Bongainvillaea* etc.

D. Occurrence of cortical bundles, e.g., *Nyctanthes, Casuarina*, members of Rutaceae etc.

E. Presence of intraxylary phloem (bicollateral vascular bundles), e.g. members of Apocyanaceae, Cucrbitaceae and Solanaceae.

(ii) Anomalies in secondary plant body

A. Abnormal behaviour of the normal cambium e.g., *Aristolochia, Bignonia* etc.
B. Abnormal behaviour of abnormal cambium.
   (a) Formation of exstrastelar cambium from the pericycle, e.g. *Achyranthes, Amaranthus, Chenopodium* etc.
   (b) Formation of accessory cambia from the cortex, e.g. *Boerhavia, Bougainvillaea* etc.
   (c) Formation of interxylary phloem (included phloem), e.g. *Combretum, Leptadenia, Strychnos* etc.

(iii) Anomalies in monocotyledons, e.g. *Dracaena, Musa* etc.

***Boerhavia* stem**

In the stems of *Boerhavia* and other members of Nyctaginaceae (*Bougainvillaea, Mirabilis* etc.) several cambia arise successively in a contrifugal direction. Each cambium cuts off xylem and conjunctive tissue inside, and phloem and conjunctive tissue outside. In this way, there is

continuous increment of secondary vascular tissues arranged in concentric rings; as a result diameter of stem is increased.

Characteristic features

(1) Secondary xylem and phloem are formed as usually by the activity of vascular cambium (Fig. 3.1).
(2) After the formation of secondary tissues, the cambium ceases its activity and a new cambium ring arises from the parenchyma cells, that is outside the phloem.
(3) This first accessory cambium behaves similarly to the vascular cambium, cutting off secondary xylem alternating with lignified conjunctive tissue on the inner side and secondary phloem opposite to secondary xylem and parenchyma on outer side.
(4) (4) In this way, many accessory cambia are formed, resulting into continuous increment of secondary vascular tissues (Fig.3.2).

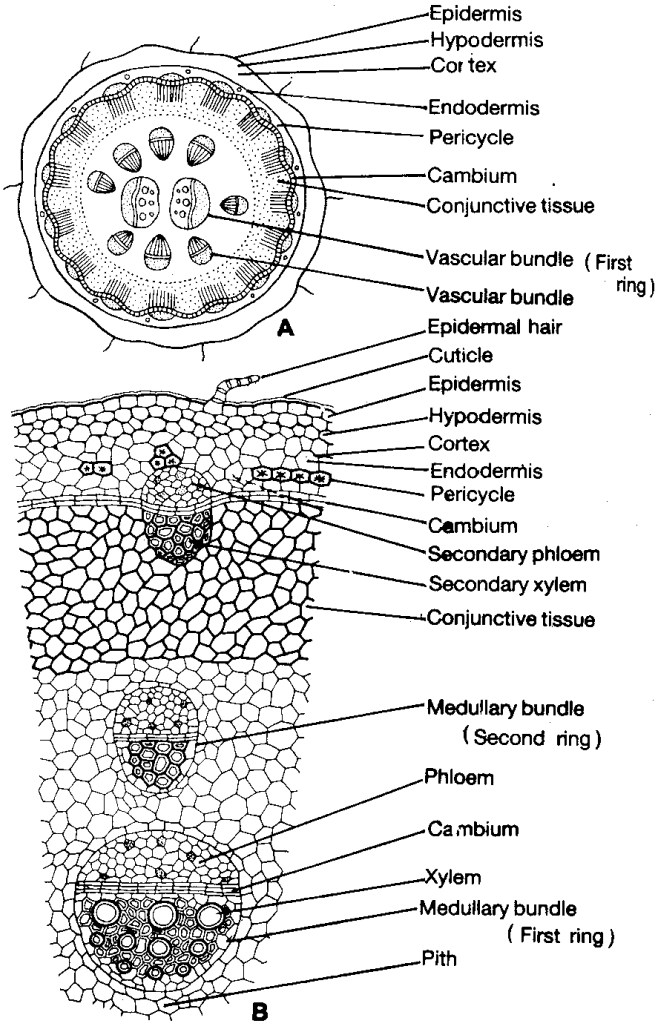

Fig. 3.1 **A**-**B**. Transection of stem of *Boerhavia diffusa*. **A**. outline diagram, **B**. cellular details

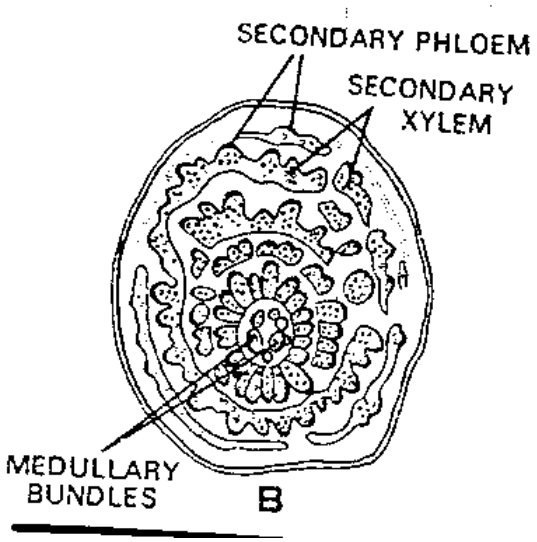

Fig. 3.2 Transection of stem of *B. diffusa* showing continuous increment of secondary tissues

(3) *Nyctanthes* stem

(1) A layer of epidermis with cuticle.

(2) Hypodermis is collenchymatous.

(3) Well developed cortex made up of parenchymatous tissue.

(4) The characteristic feature of *Nyctanthes*, is presence of four cortical vascular bundles at four prominent ridges. These are inversely oriented (Fig. 3.3). This feature is considered as anomalous character.

(5) Normal cambium cuts of secondary phloem towards outerside and secondary xylem inside.

(4) *Leptadenia* stem

(1) In *Leptadenia* and other species of *Combretum, Entada* and *Salvadora*, certain small segments of the cambium behave abnormally and cut only the phloem towards innerside, instead of xylem, for a short period of time.

(2) Later, the entire cambium regains its activity and cuts off secondary xylem towards inside and secondary phloem outside in its normal way.

(3) Due to above process, the abnormally formed phloem is pushed into the secondary xylem and appear as islands remain included in the secondary xylem, hence it is known as **interxylary** or **included phloem** (Fig. 3.4).

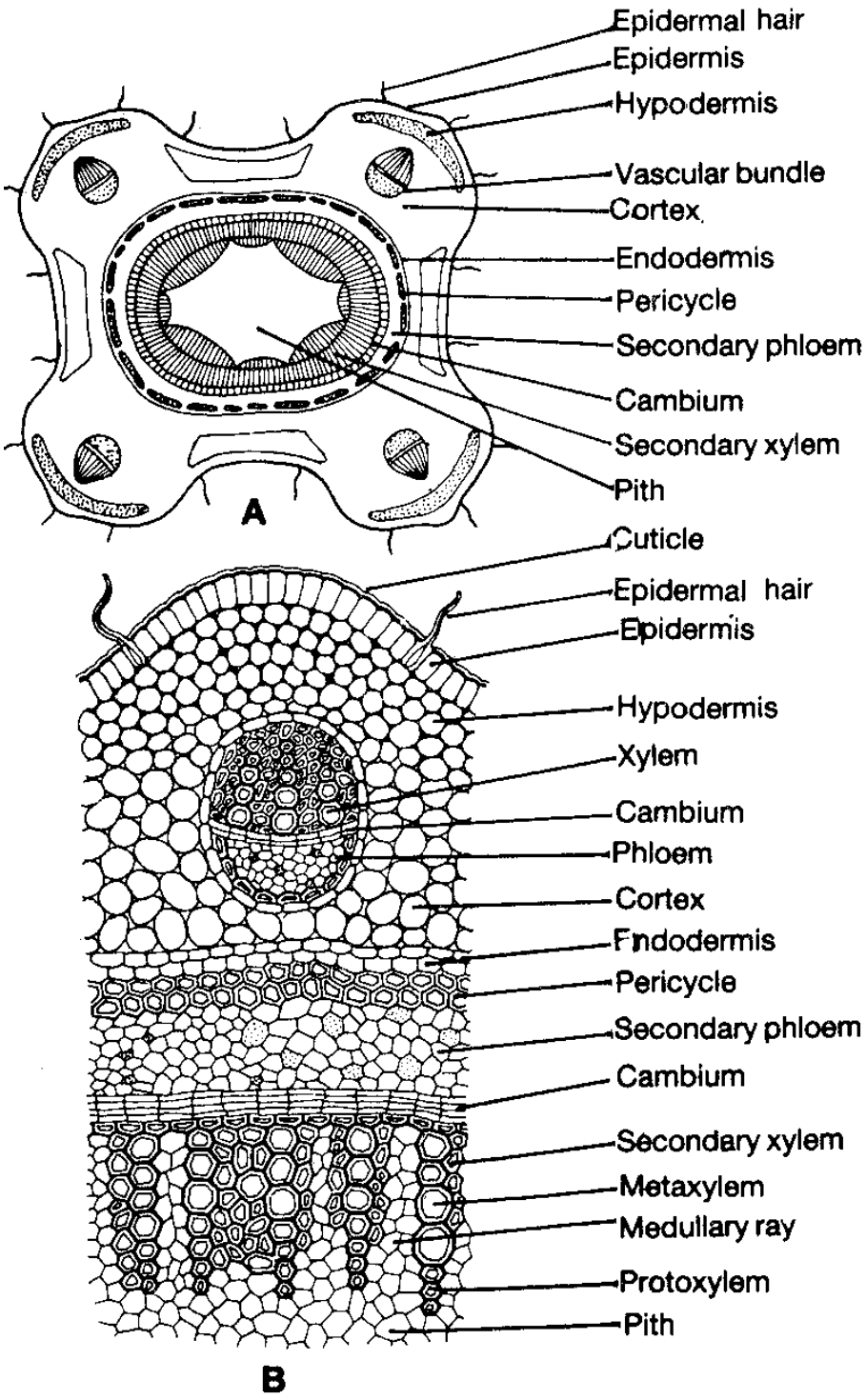

Fig. 3.3 Transection of stem of *Nyctanthes*. **A**. outline diagramme, **B**. Cellular details.

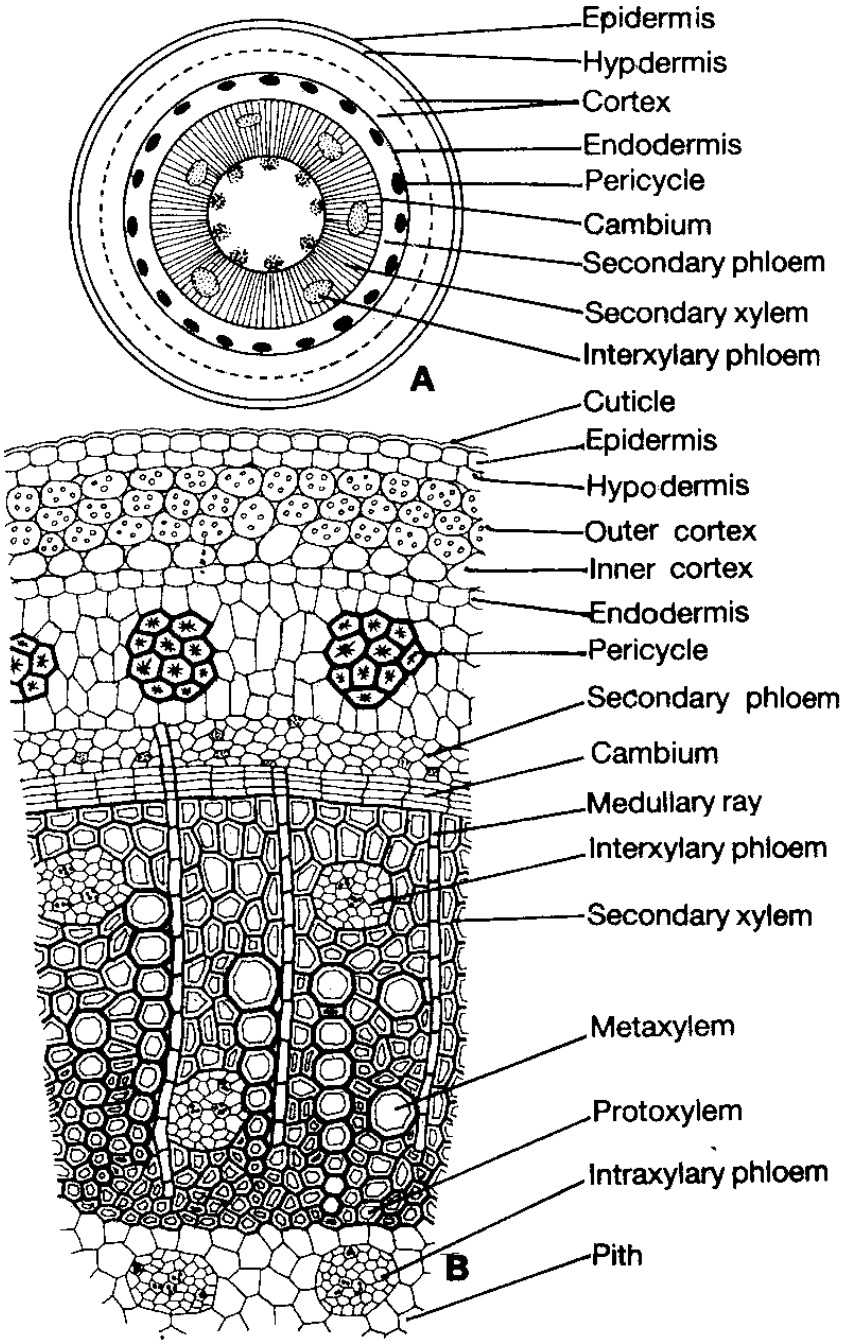

Fig. 3.4. Transection of stem of *Leptadenia* showing interxylary phloem. **A.** Outline diagram. **B**. Cellular details of the sector

V. *Dracena* stem (monocot; Fam: Liliaceae)

Young stem of *Dracaena*

(1) A single layer of epidermis.

(2) Presence of sclerenchymatous hypodermis.

(3) Collateral, and closed vascular bundles are sparsely distributed through out the parenchymatous ground tissue region.

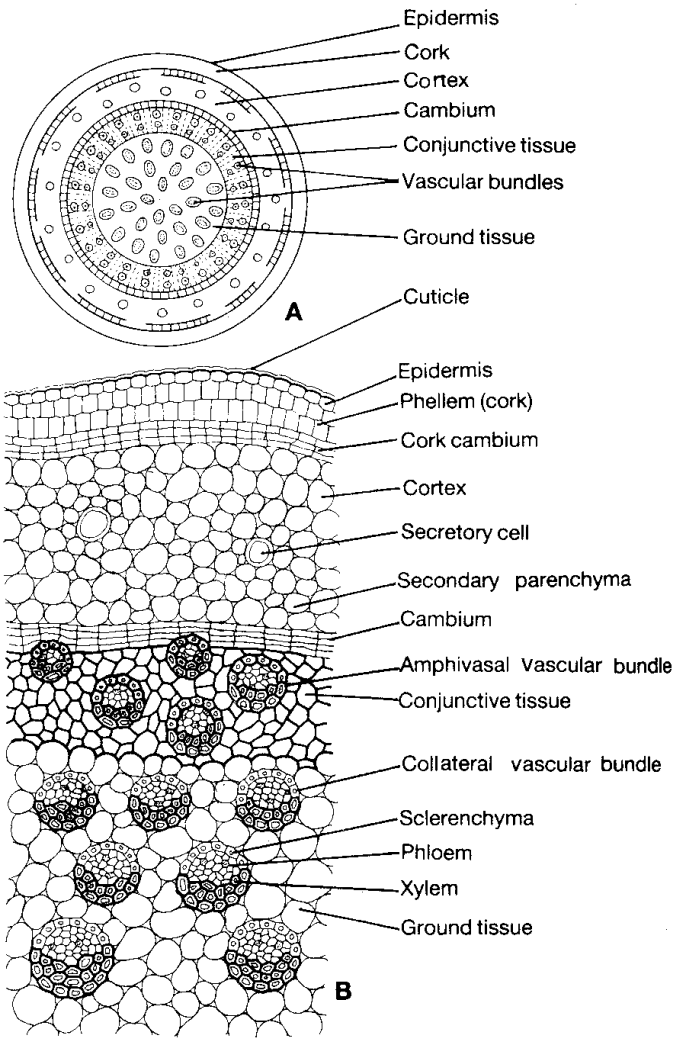

Fig. 3.5 **A-B**. Transection f stem of *Dracaena*. **A**. Outline diagram, **B**. Cellular details of a sector

Mature stem

(4) Secondary growth initiated by the formation of a special meristematic zone developed in the inner cortical region. The cortical cells lying external to the vascular bundle develop to form a multilayered secondary Cambium.

(5) The secondarily formed Cambium behaves abnormally and cuts off a few cells on the outer side but larger number of cells towards the inner side. The cells cut off outerside become parenchymatous, whereas cells towards innerside are differentiated into vascular bundles and also conjunctive tissue.

(6) Numerous secondarily formed vascular bundles are very closely distributed in secondary stem. These vascular bundles may be amphivasal in nature (central mass of phloem surrounded by xylem).

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Dr. S.M. KHASIM

IV. WOOD MACERATION AND WOOD ANATOMY

(1) Wood Maceration with Jeffrey's method

Locally available dicot woods are collected and study the tracheary elements.

(i) Take small silvers of wood (about 300 μm thick) and evacuate till free of air.

(ii) Macerate in 1:1 mixture of 10% aqueous chromium trioxide and 10% aqueous nitric acid for one to several days at room temperature. This process may be hastened by heating at 60^{o}C for secondary wood.

(iii) Gently crush the tissue with a glass rod.

(iv) If cells are not separated, then change the fluid once or twice and repeat step (ii).

(v) Wash thoroughly with water to remove acids by using hand centrifuge.

(vi) Stain with 1% solution of cationic dyes (e.g. safranin, crystal violet), rinse in water and dehydrate, finally mount in a resinous mountant. By observing under microscope, study various tracheary elements (tracheids, vessel members) and fibres.

(2) Wood Anatomy

The angiosperm wood (hard wood) commonly refers to secondary xylem of dicotyledons. Angiosperm wood is generally more complex than the gymnosperm wood, since its elements are more varied in size, kind, form and arrangement. The most complex dicotyledonous woods, such as oak, may contain vessel members, tracheids, fibre-tracheids, libriform fibres, axial parenchyma and ray parenchyma. Because of the complexity of structure of dicotyledonous woods many characters may be used in their identification, such as presence or absence of vessels and their distribution pattern, types of perforation plate in vessels, distribution of axial parenchyma, types of rays, presence of storied or non-storied structure etc.

Distribution of Vessels – The arrangement of vessel in dicotyledonous woods show two main patterns, these are **diffuse-porous** and **ring-porous**. In diffuse-porous woods, the vessels are essentially equal in diameter and are uniformly distributed through out the growth ring (Fig. 4.1A), e.g. *Acer, Betula, Liriodendron, Eucalyptus, Populus* etc., whereas in ring-porous woods, the vessels of unequal diameter with largest vessels localized in the early wood are present, e.g. *Fraxinus, Robinia, Quercus* etc. The vessels in ring-porous wood are longer than that of in the diffuse-porous wood.

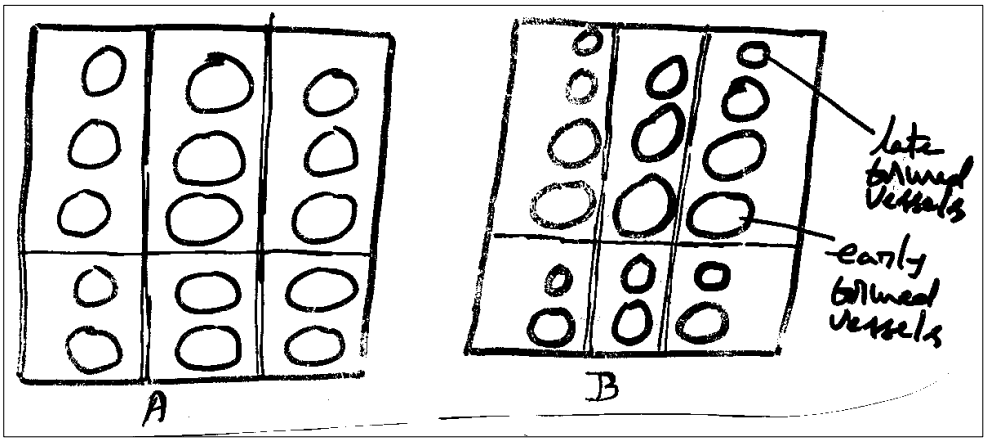

Fig. 4.1. **A, B**. Dicotyledonous woods.
A. Diffuse porous wood, **B.** Ring porous wood

Storied and non-storied woods – These woods are formed on the basis of arrangement of fusiform initials present in the cambium. **Storied wood** is derived from storied cambium, in which fusiform initials (140-250 μ in length) arranged in horizontal rows (Fig. 4.2A), e.g. *Tamarix*, *Robinia* etc., whereas non-storied wood from non-storied cambium where fusiform initials (320-6,200 μ in length) partially overlap one another (Fig. 4.2B), e.g. *Fraxinus*.

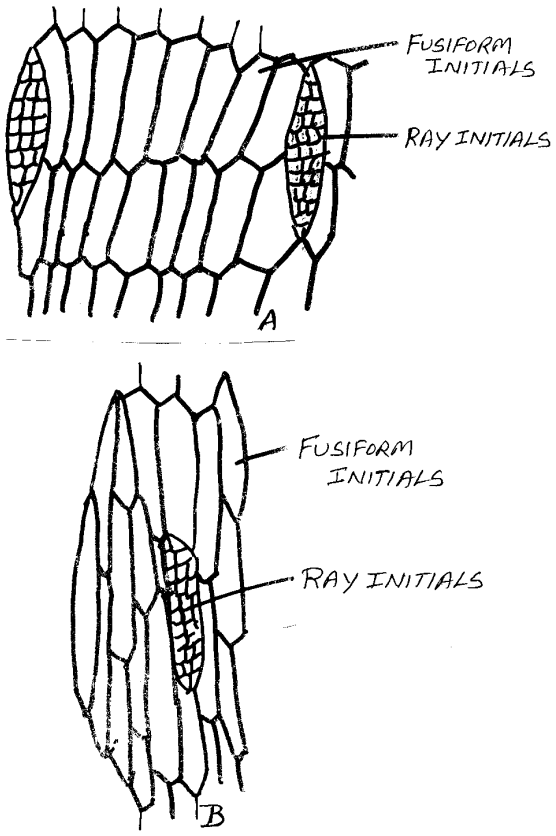

Fig. 4.2 Cambia. **A**. Storied fusiform initials, **B**. Non-storied fusiform initials

***Cassia fistula* stem**

(i) During secondary growth, vascular cambium, in its normal way, cuts off secondary phloem towards outer side and secondary xylem towards inner side.

(ii) Secondary xylem formed during spring has larger number of vessels with wide lumen, whereas autumn wood has smaller number of vessels with narrow lumen. This type of wood is known as ring-porous wood (Fig. 4.3). Other examples for ring-porous are *Dalbergia sissoo, Tamarix dioica* etc.

(iii) In transection of stem, both spring and autumn woods appear in the form of distinct concentric circles known as annual rings.

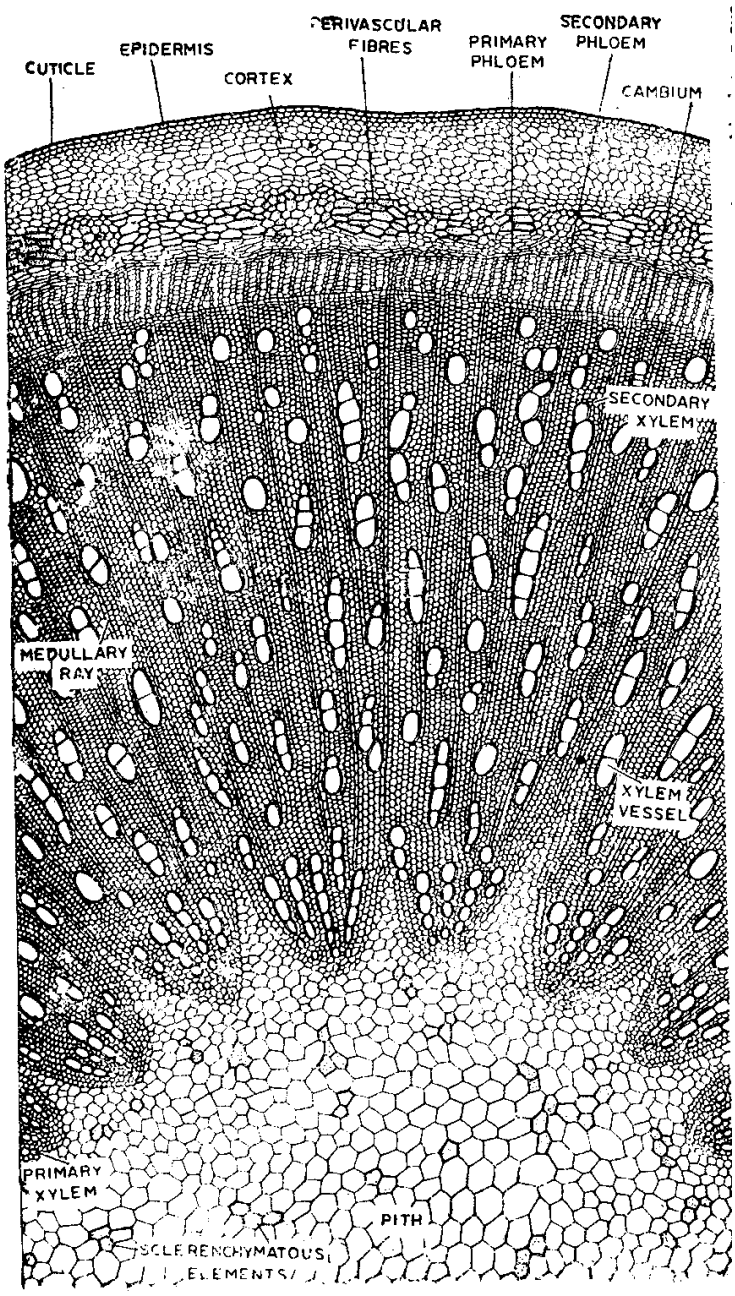

Fig. 4.3 Transection of stem of *Cassia fistula* showing ring-porous wood.

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Dr. S.M. KHASIM

M.Sc., Botany (Final) **PRACTICAL**

ETHNOBOTANY

I. TAXONOMY OF MEDICINAL PLANTS

1. *Azadirachta indica*

Family : Meliaceae
Trade Name : Margosa
Telugu : Vepa

Habit: Members of the family are mostly trees, rarely shrubs

Leaves: Leaves are usually compound, unipinnate (Azadirachta) or bicompound (Melia) alternate, exstipulate, not gland dotted. Leaflets are asymmetrical not serrate. Reticulate venation.

Inflorescence: Usually terminal or axillary, mostly a panicle.

Flowers: Flowers are bracteate, complete, regular, bisexual flowers, pentamerous, hypogynous. A tubular, annular disc is present between the stamens and pistil.

Calyx: It consists of four or five sepals, free, valvate or imbricate

Corolla: 4 or 5 petals, Aestivation valvate or imbricate.

Androecium: 8-10 stamens, twice to the number of petals. Mostly the filaments unite together (monodelphous) to form a short or long tube. Teeth are seen at the margin of the staminal tube. Anthers are inserted at the tip of the tube opposite or alternating with the teeth.

Gynoecium: 2-5 carpellary syncarpous superior ovary. The locules are equal to carpels. Ovules are anatropous, mostly two in each locules on axile placentation.

Fruit: Drupe

Seed: Dicotyledonous endospermic.

Medicinal value: The Bark of Melia azedirech contains many alkaloids and are used in medicines. The bark and leaves as well as neem oil obtained from Azadirachta are of medicinal value.

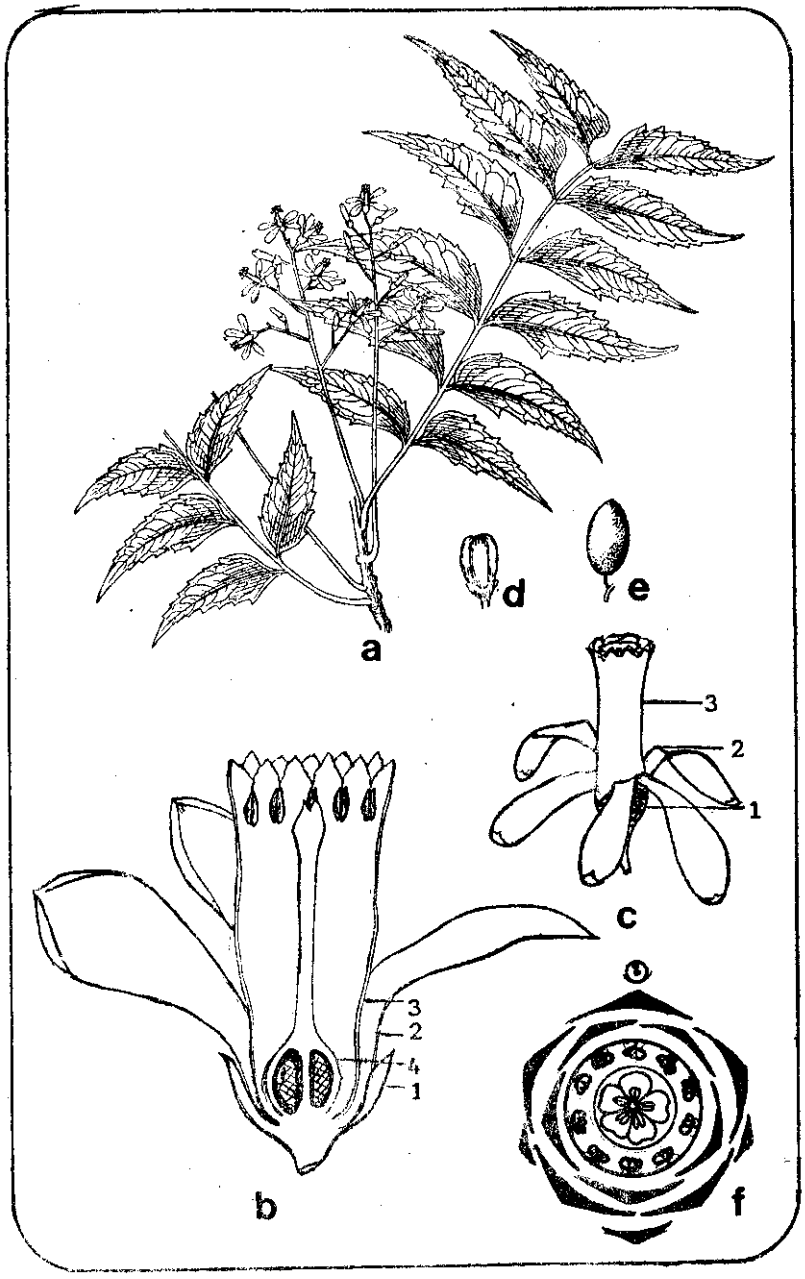

Fig. *Azadirachta indica*. a (Twig b) L.S. flower, 1. sepal, 2. petal, 3. staminal tube

2. *Centella asiatica* (Linn)

(Syn. *Hydrocotyle asiatica* Linn).
Verna. Brahmi. Eng. Asiatic pennywort

Habit: Prostrate herbs, rooting at nodes.

Root: Adventitious roots.

Stem: Prostrate, herbaceous, weak, cylindrical, stolon leaves and roots at nodes.

Leaves: Simple, arising in groups from nodes, orbicular or reniform, crenate and often lobed, glabrous, shining, petiolate, long petioled, stipule, adnate to petioles.

Inflorescence: Racemose, simple umbels, 3-6 flowers in each group.

Flower: Pedicellate, small pedicels, bracteate, bracts small, ovate, embracing the flowers, hermaphrodite, actino morphic, regular complete, epigynous.

Calyx: 5 sepals, minute, connate, valvate.

Corolla: 5 petals, polypetalous valvate.

Amdroecium: 5 stamens, polyandrous, alternate to petals, filament short, antherdithecous introse.

Gynoecium: 2 carpels, syncarpous, ovary inferior, 2-celled, disk 2-lobed, vittae present in ovary wall, axile placentation, one ovule in eachlocale.

Fruit: Cremocarp, laterally compressed.

Floral formula: Br $\oplus$ ♂♀ $K_{(5)}C_5A_5G_{(\underline{2})}$

Economic value of the family: The family is very important from the economic point of view. The fruits of some plants are used as condiments whereas the other plants are of medicinal value.

***Coriandrum sativums*:** Verna. Dhania. Eng. Coriander. As aromatic hub, native of the Mediterranean region, now cultivated chiefly in Madhya Pradesh, Maharashtra, Karnataka, Bihar and Uttara Pradesh for its fruits and leaves, which are used as condiment and spice. The fruits are also used as stimulant, carminative, stomachie and tonic.

Centello asiatica: A creeping herb, used as an antidote against cholera and also to cure madness. The plants are used as remedy of certain forms of leprosy.

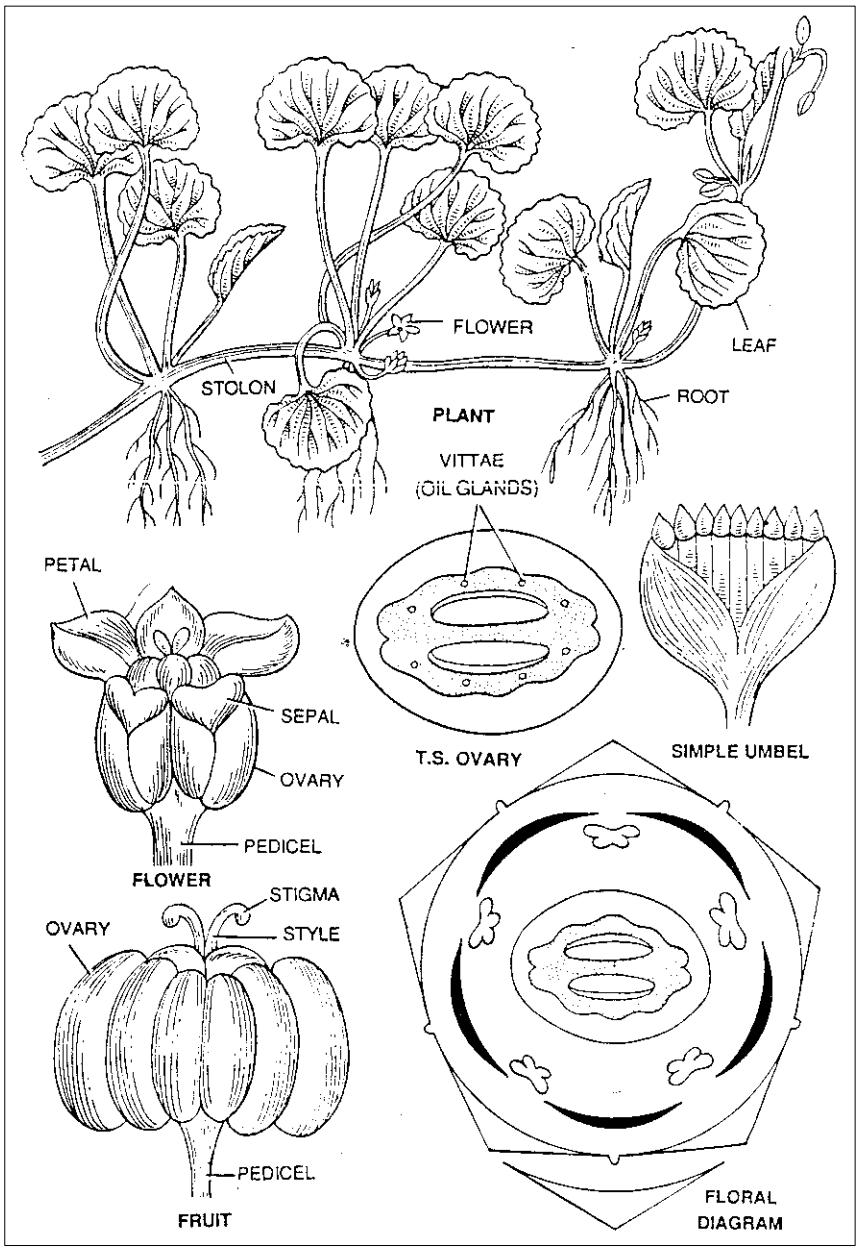

Fig. Umbelliferae (Apiaceae). *Centella asiatica* (Linn), Urb. (Syn. *Hydrocotyle asiatica* L.); Eng. Asiatic pennywort; Verna. **Brahmi.**

3. *Ocimum sanctum*

***Ocinum sanctum*:** Belongs to the family Labiatae or Lamiaceae.

Habit: A perennial herb with typical aromatic smell.

Stem: Erect, branched, quadrangulars, somewhat woody, solid, branches covered with soft hairs.

Leaf: Simple opposite, short petioled, erslipulate, ovate, serrate, acute, gland dotted, unicostate reticulate venation.

Inflorescence: Verticillaster, 6-10 followed whorls present, emitting five smell.

Flower: Pedicellate, bracteate, bracts small and caducous, hermaphrodite, zygomorphic complete, purple, hypogenous.

Calyx: 5 sepals, gamosepalous, bilabiate, petaloid (purple coloured), posterior lip broad and boat shaped, anterior lip with 4 small lobes possessing mucronate teeth, gland dotted, imbricate aestivation inferior.

Corolla: 5 petals, gamopetalous, bilabiate, corolla tube short, upper lip four lobed, lower lip large, imbricate aestivatio, inferior.

Androecium: 4 stamens, polyandrous, didynamous, epipetalous, fifth postrior stamen completely suppressed, anthers bicelled introse, dorsifixed.

Gynoecium: 2 carpels (bicarpellary) syncarpous, ovary bilocular in early stage but becomes interlocular in later stage, ovary superior, four chambered axile placentation, single ovule in each loculus, gynobasic style (i.e. it arises from the base of the ovary) stigma bifid.

Fruit: Schizocarpie, carcerulus 4 nutlets developed.

Floral formula: % ♂♀ $K_{(1+4)}C_{(4+1)}A_{2+2}G_{(\underline{2})}$

Economic importance of the Plant

***Ocimum sanctum*:** Eng. Holybasil

Verna: Tulasi

This is a small shrub and treated as holy plant by Hindus. Usually grown in the courtyards of Hindus and by the side of temples. This plant possesses several medicinal properties. The juice of the leaves is used as a stimulant and as a remedy for bronchities. The seeds are used as a remedy for urino-genital troubles. The plant is used as an antidote for snakebite.

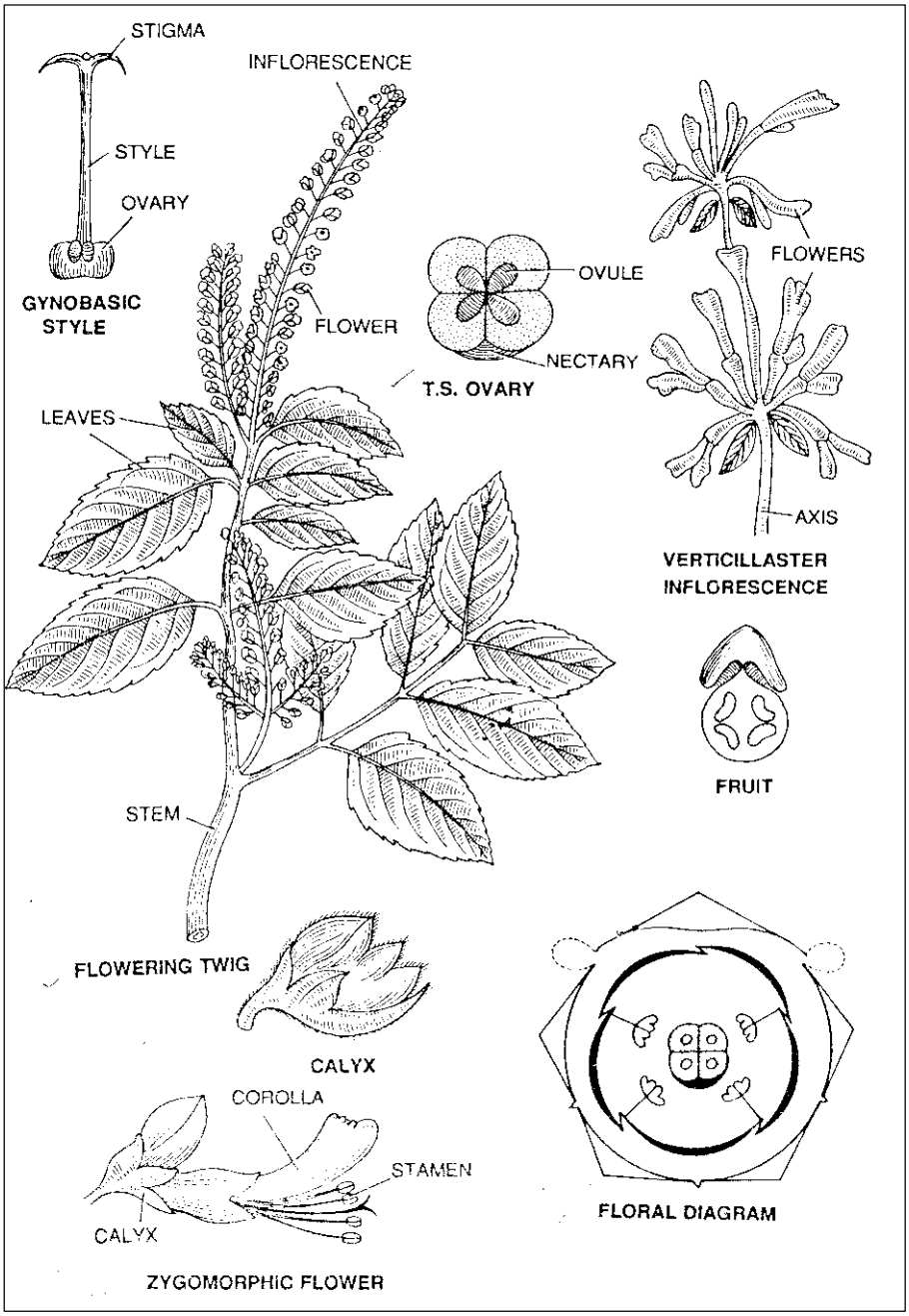

Fig. Labiatae-*Ocimum sanctum* Linn. (Verna tulsi)

4. *Achyranthes aspera* Linn.

Family: Amaranthaceae

Trade Name: Prickly chaff flower plant

Telugu: Uttareni

Habit: Perennial herbs

Root: Tap and branched

Stem: Erect, herbaceous, quadrangular, branched, solid, green pubescent.

Leaf: Cauline and ramal, opposite, exstipulate, simple, sub-sessile ovate, entire, acute unicustate reticulate, rough, coriaceous hairy.

Inflorescence: Racemose, spike

Flower: Bracteate, bracteolate, susile bracts and bracteoles, spinous and persistant, hermphrodite, actinomorphic, regular, complete pentamerous, hypogynous, cyclic.

Perianth: 5 tepals, polyphyllous, dry, membranous, white or coloured hairy.

Androecium: 2 bicarpellary, syncarpous ovary unilocular, superior, single basal ovule, style short, stigma bifid.

Fruit: Dry, Utricle

Floral formula: Br Brl $\oplus$ ♂♀ $P_5A_5G_{(2)}$

Medicinal Value of this plant: Whole plant is used for medicine. Asthma, Haematinic, Leprosy, Skin diseases, Renal and Vesical Calculi, Cardiac disorders, Anaemia, General debility, Conjuctinitis, Corns, cuts and wounds, Dog bite, Toothache.

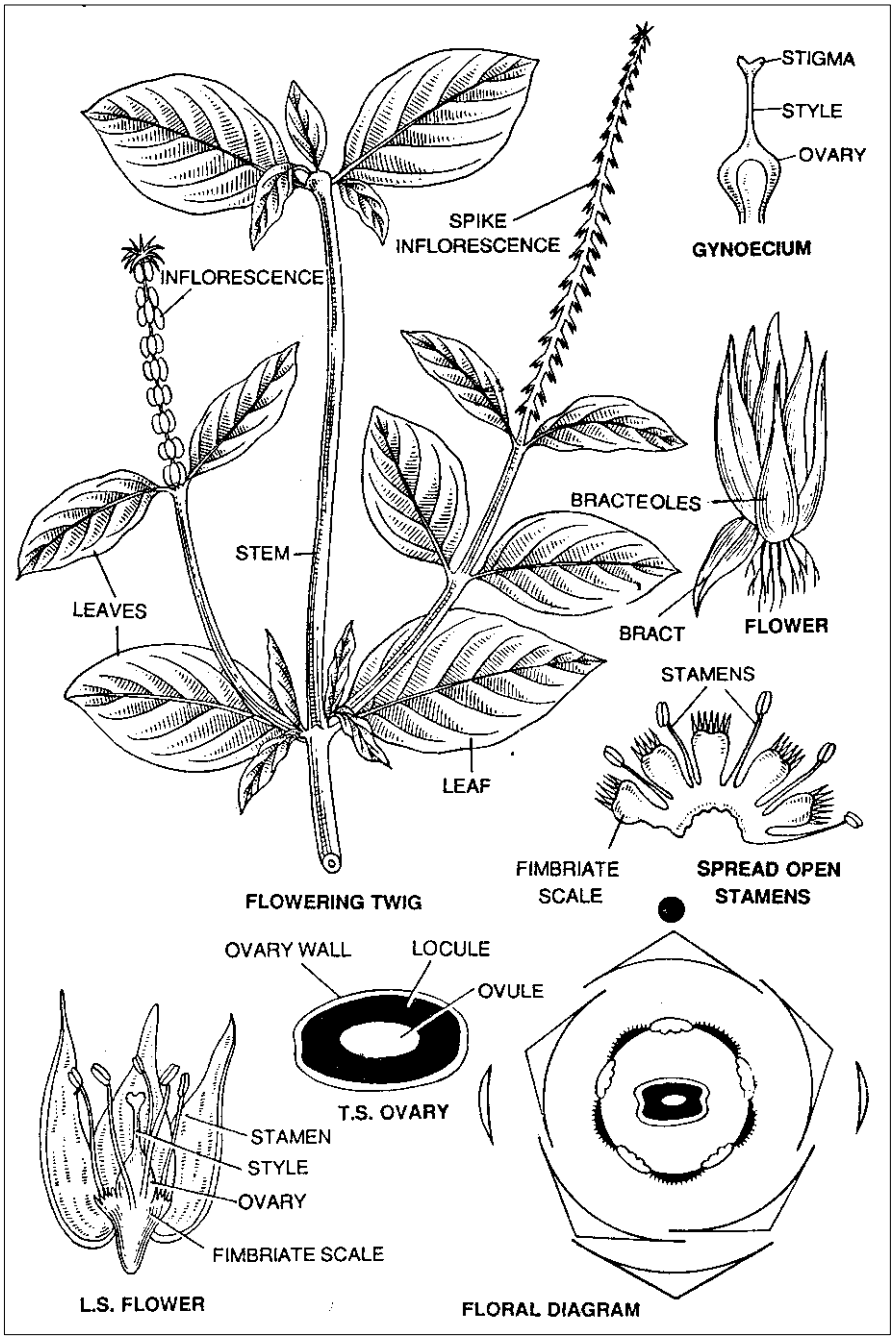

Fig. Amaranthaceae. *Achyranthes aspera* Linn.; Verna, **Chirchita, latzira, apmarg.**

5. *Allium cepa* Linn

Family: Amaryllidaceae

Trade Name: Onion

Telugu: Ulligadda, Erragadda.

Habit: Annual herb, grown for edible bulbs

Root: Adventitious, fibrous

Stem: Underground bulb, the bulb bears outer scaly and inner fleshy leaves.

Leaves: Radical, cylindrical, fistular sheathing.

Inflorescence: Racemose, umbellate, actinomorphic, regular, hermaphrodite, small, white and hypogynous.

Perianth: Six perianth, segments in two whorls of three each, three outer and three inner, free but united at base, valvate.

Androecium: Six stamens, filaments slender, broad at base, free-anthers bicelled, basifixed and introrse.

Gynoecium: Three, tricarpellary, syncarpous ovary, superior, trilocular, two ovules in each locule placentation axile, style short, filiform, stigma short.

Fruit: Small capsule.

Seed: Endospermic with straight embryo

Floral formula: $\oplus \, ♂♀ \, P_{(3+3)}A_{3+3}G_{(3)}$

Medicinal value: Antiperiodic, paralysis, Antibacterial, Emmengogue, Diceretic Asthma, Bronchitis, Epilepscy Tumours, Leucoderma, Skin diseases, Bleeding piles, Bleeding gume, High blood pressure, Scorpion sting.

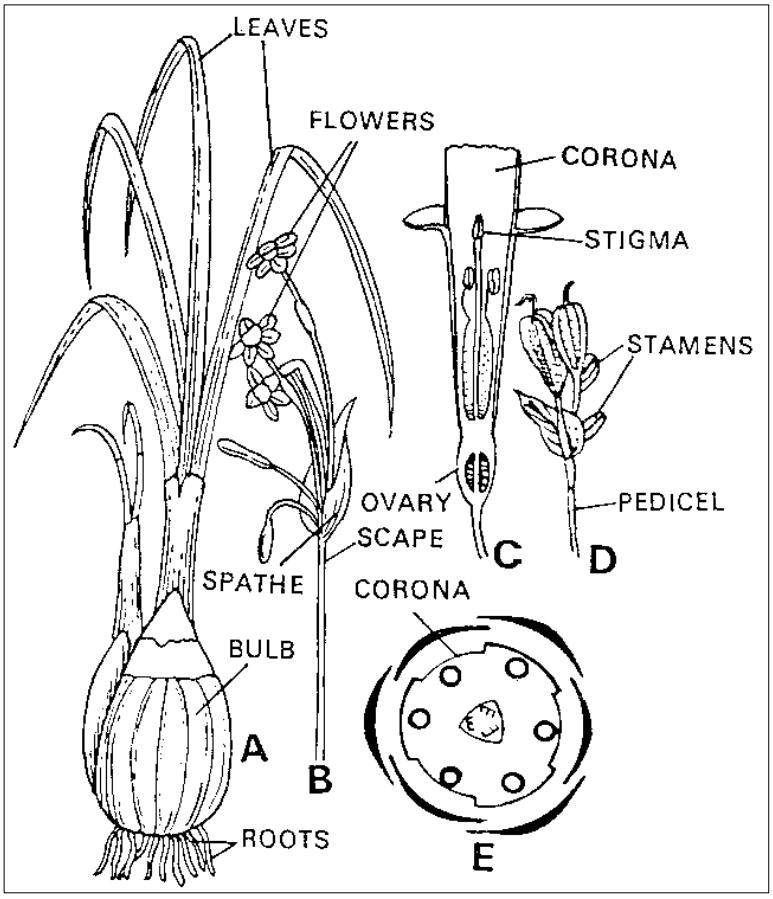

Fig. Amaryllidaceae-*Narcissus*; (Verna-Nargis); **A,** entire plant with bulb and leaves; **B**, inflorescence; **C**, flower in vertical section; **D**, Fruiting stage; **E**, floral diagram.

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Mrs. J. VIJAYAMMA

M.Sc. BOTANY (Final) **Practicals**

ETHNOBOTANY

II. ANATOMICAL STUDIES OF SOME IMPORTANT MEDICINAL PLANTS

In recent years indigenous system of medicine particularly Ayurveda has been attracted by modern scientist in finding out solutions for many challenging diseases. The scientific investigation of indigenous drugs was started by early workers like Roxburgh, Dymock, Chopra and others at School of Tropical Medicine, Calcutta in late 19^{th} century. There are about 500,000 Ayurvedic practitioners in south-east Asia besides some 800 Ayurvedic pharmacies. Unfortunately, drugs have been adulterated by manufacturers during their formulations. Need for authentic drug with predictable quality, standard efficacy and effective action continues to be the epicentre of rational therapeutic uses of any living system.

Some of the standardization techniques have been adopted to detect the adulteration of drug. Botanist can study the morphology and anatomy of medicinal plants and this data can be compared with commercial samples of drugs. It is one of the methods for standardization of herbal drugs.

Use of any drug largely designated by morphologists based on the plant part which is under use. They can be named and classified as

1. Radix/root
2. Tubera/tubers
3. Rhizoma/rhizome
4. Rhizoma and radix/rhizome and root
5. Cortex/bark
6. Lignum/Wood
7. Folia leaf
8. Flores/flower
9. Fructus/fruits
10. Semen/seed
11. Herba/herb
12. Amylum/starches, etc.

For standardisation of any drug, it is mandatory to study the morphology and anatomy apart from physical examination.

Stains and other materials

Safranin 1% (in 70% ethy alcohol
Acid water (3% HCl)
Iodine-Potassium iodide solution (2 g potassium iodide in 100 ml of water and add 1 gr iodine).
Drug samples
Locally available medicinal plants

Locally available medicinal plants, such as *Datura, Sida cordifolia, Tribulus terrestris, Phyllanthes niruri, Tinospora cordifolia, Ipomoea paniculata, Andrographis paniculata* etc., are collected and study the following features:

1. Dermal characters (epidermis, periderm etc.)
2. Surface features (stomata, trichomes etc.)
3. Shape, size and colour
4. Fundamental tissue
5. Vascular tissue

For Leafy drugs

(a) Epidermal study

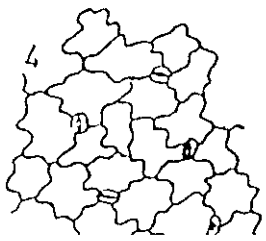
(i) Simple epidermal cells, e.g. *Datura*
(ii) Beaded epidermal cells, e.g. *Digitalis*

(b) Trichomes

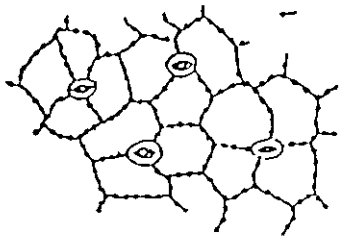
(i) Unicellular (foot & body) – *Senna*
(ii) Unicellular lignified – Tea
(iii) Multicellular – *Datura*
(iv) Branched – *Verbascum, Anisochilous carnosus*
(v) Glandular multicellular and uniseriate – *Digitalis, Datura*
(vi) T-shaped – *Hiptage benghalensis*
(vii) Stellate – *Solanum trilobatum, S. torrum*

(c) Stomata

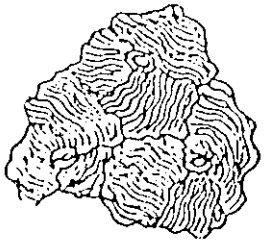
(i) Anomocytic – *Eucalyptus, Solanum, Anisomeles*
(ii) Paracytic – *Senna, Cajanus cajan*
(iii) Diacytic – *Andrographis paniculata*



Anomocytic type
Digitalis purpurea

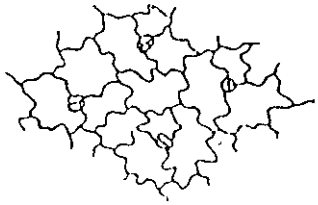


Anomocytic beaded epidermal cells
Digitalis lanata

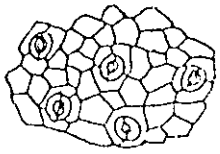


Cruciferous type
Atropa belladonna

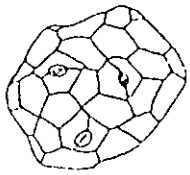
Amisocytic type
Hyoscyamus niger



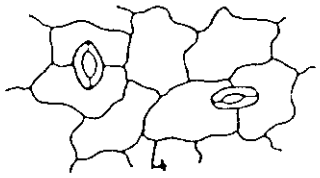
Datura stramonium



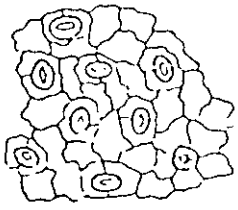
Camella sinensis



Rubiaceous (Paracytic)
Cassia angustifolia **(vahl.)**



Caryophyllaceous (Diacytic)
Mentha piperata



(Diacytic) Caryophyllaceous wavy margins
Adhatoda vasica

Fig. 2.1 LEAF STOMATA

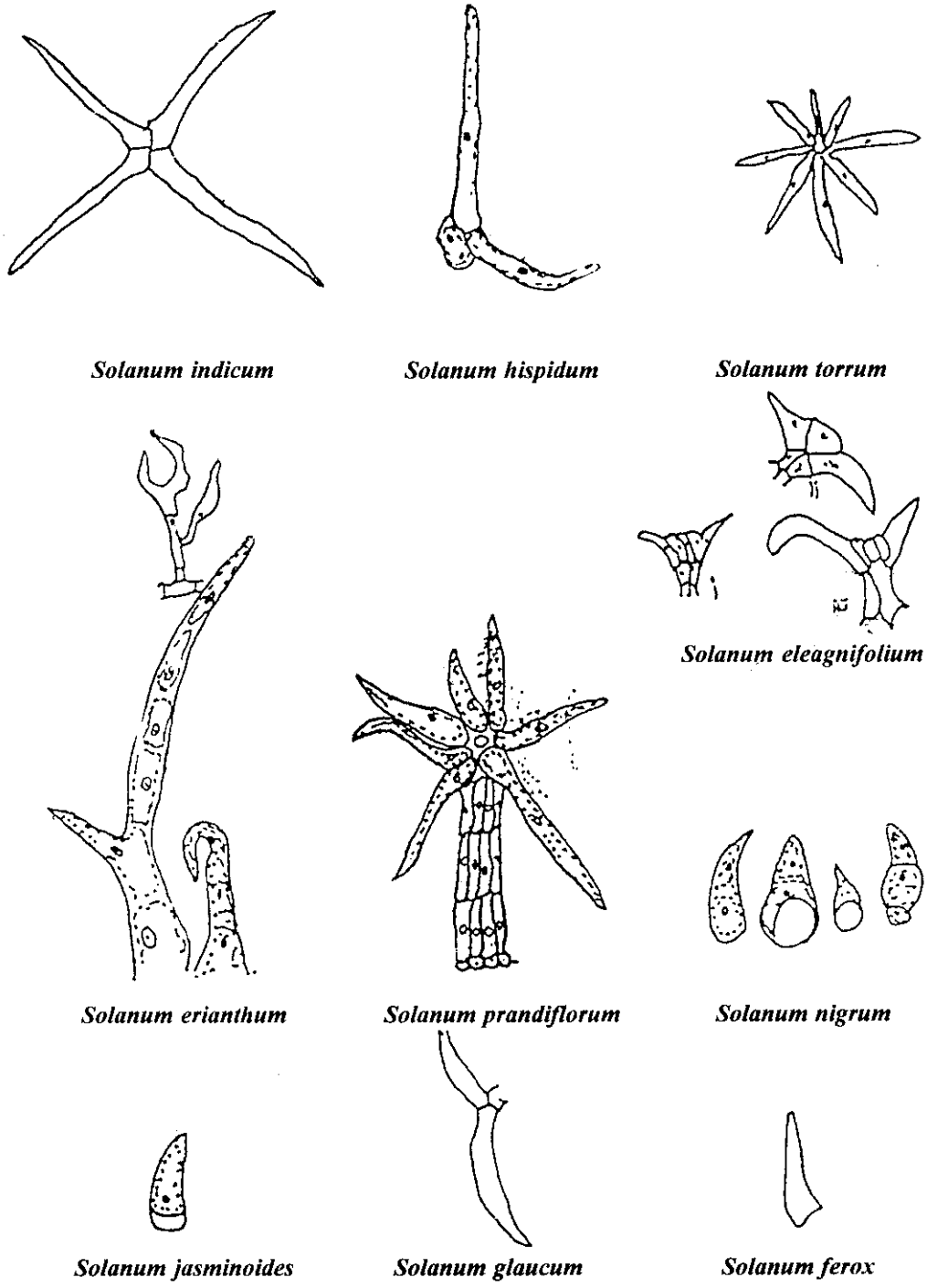


Fig. 2.2 TRICHOMES

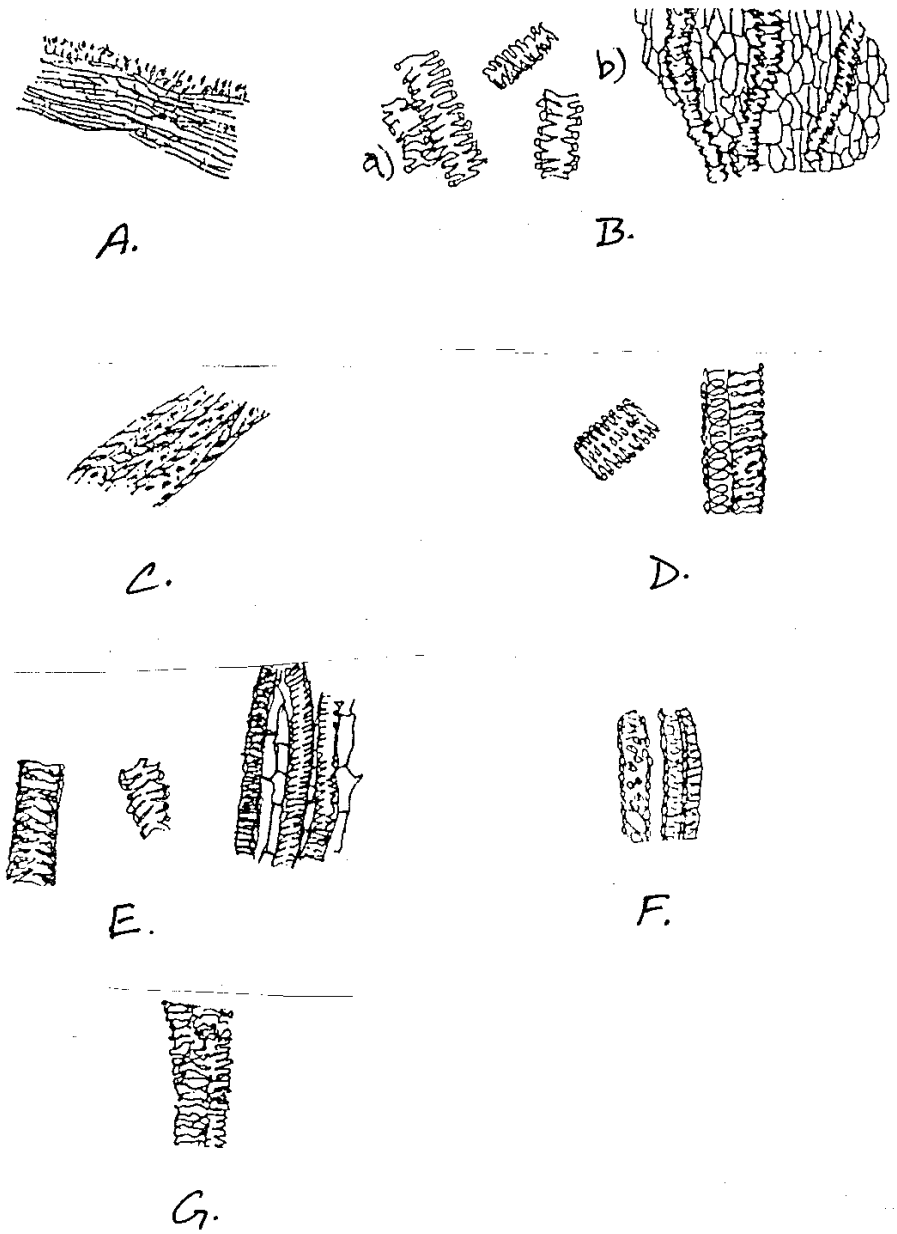


Fig. 2.3 **A-G**. Root and rhizome vessels. **A.** *Atropa belladonna* root showing vessels and fibres interlocked with each other to form a spindle structure; **B.** *Gentiana lutea* root showing well developed xylem vessels with reticulate thickenings (a), characteristic pattern of thickenings of vascular element in root (b); **C.** *Rauwolfia serpentina* root showing few long vessels with oblique end walls and perforations; **D.** *Curcuma longa* rhizome showing well developed, wide vessels with reticulate and spiral thickenings; **E.** *Rheum palmatum* rhizome showing wide vessels, reticulately thickened and do not possess lignin thickenings; **F.** *Valeriana wallichii* rhizome showing vessels with bordered pits, scalariform and spiral thickenings; **G**. *podophyllum emodi* rhizome.

(d) Cystolith

Calcium carbonate – *Andrographis paniculata* (dissolves in HCl and acetic acid)

(e) Crystals in cluster – *Datura*

(f) Acicular crystals – *Andrographis paniculata*

(g) Microsphenoidal crystals – *Belladonna, Solanum trilobatum.*

Starch grains

Many roots, rhizomes and stem bark contain reserve food material in the form of starch grains. These can be easily identified by staining with iodine and can be used as a tool of standardisation. These starch grains differ from each other in their shape, size, hilum structure or by virtue of their being simple or compound in nature (Table 2.1) and serve as an important tool for identification.

Table 2.1 Starches

No.	Botanical Name	Structure	Simple/Compound	Hilum
		Root		
1.	*Aconitum* sp.	Round	Simple 3-30μ and compound 2-6 compound	Slit like
2.	*Atropa belladonna* L.	Spherical/round	Simple and compound 2-4 component	Indistinct
3.	*Gentiana lutea* L.	Small	Simple	
4.	*Cephaelis ipecacuanha*	Polygonal to round	Simple and compound up to 15μ	Prominent
5.	*Glycyrrhiza glabra* L.	Oval to elongated	Simple and compound minute	
6.	*Rauwolfia serpentina*	Large round oval	Simple more and compound	Star or split
		Rhizome		
1	*Curcuma longa* L.	Big rounded pasty masses with yellowish tinge		
2	*Rheum palmatum* L.	spheroidal angular	Simple and compound 2-5 component	Central hilum

3	*Zingiber officinale*	Ovoid or sack shaped	Simple 5-60μ	Eccentric
4	*Podophyllum emodi*	Spherical to ovoid	Simple and compound 3-8 component	
5	*Valeriana wallichi* DC	Rounded	Simple/rarely compound 2-4 component	Indistinct as a cleft
Bark Rhizome				
1	*Cinnamomium cassia*	Rounded up to 20 microns	Simple and compound 2-5 component	Slit like
2	*Cinnamomium zeylanicum*	Rounded up to 10 microns	Simple and compound 2-3 component	Distinct
3	*Cinchona calisaya*	Minute 3-10 microns	Simple and compound 2-5 component	

Detailed gross anatomy of locally available medicianal plants, such as *Leptodenia, Aristolochia, Strychnos, Calotropis, Nyctanthes* etc., can be studied.

(1) Anatomy of *Calotropis procera* (Asclepiadaceae) stem

C. procera shows many medicinal properties. Its stem anatomical features are given below.

(i) Single layer of epidermis made up of radially elongated cells with thick cuticle is present.
(ii) Cortex is composed of thin-walled, loosely arranged parenchyma cells. The cortical cells may possess chloroplasts.
(iii) Endodermis is made up of barrel-shaped cells possess starch grains.
(iv) Pericycle is discontinuous with sclerenchymatous patches.
(v) Vascular region possesses discrete vascular bundles which are conjoint, collateral, endarch and open.
(vi) In secondary structure, secondary phloem is located towards outer side and secondary xylem towards innerside. The secondary phloem is present next to the pericycle and forms a continuous cylinder consisting of sieve-tubes, companion cells, and phloem parenchyma. The primary phloem gets crushed during the secondary growth. The secondary xylem also form continuous cylinder, consisting of vessels, tracheids and xylem parenchyma.
(vii) The internal phloem patches are found next to the primary xylem.
(viii) Pith is composed of thin-walled polygonal cells. Numerous latex vessels are found in the pith region.

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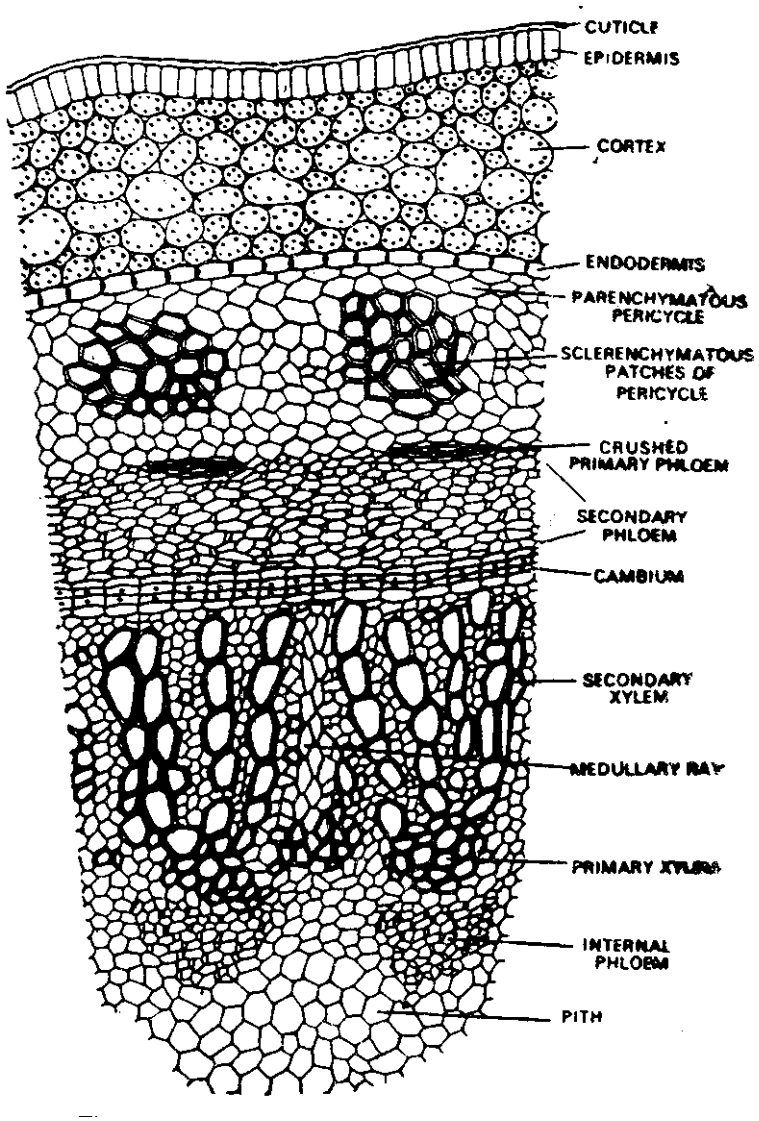

Fig. 2.4 T.S. stem of *Calotropis procera*. Note the groups of internal phloem.

III. TISSUE CULTURE STUDIES OF MEDICINAL PLANTS

Nutrient medium

The Principal contents of medium are inorganic nutrients (macro and micro nutrients), carbon source, organic supplements, growth regulators and gelling agent. The nutritional composition varies with respect to various cell, organ and protoplast culture systems. Some tissues grow on simple medium containing only inorganic salts and utilize carbon source (sugar), but most others, it is essential to supplement the medium with vitamins, amino acids and growth substances. The appropriate composition of the medium largely determines the success of the culture system. Generally MS medium and its modifications are widely used for all culture systems.

(1) Seed Culture

Seeds of some locally available medicinal plants are collected, e.g. *Achyranthes aspera, Ageratum conyzoides, Berberis asiatica, Blumea, Cassia, Catharanthes roseus, Hyptis sauveolens, Merremia, Nyctanthes arbor-tristis, Ocimum* and *Phyllanthes* etc.

Important equipment essential for tissue culture Laboratory

Autoclave
Laminar Airflow hood
Electronic balances
Tabletop centrifuge
Freezer (-20^{o}C)
Magnetic stirrer
Ovens
Rotary shakers
Water distillation unit
Microscopes
Micropipettes
Agarose gel electrophoresis apparatus

Reagents and other material

Culture tubes/conical flasks containing media
Sterile petridishes
Beakers
Sieves
Muslin cloth/cheese cloth/nylon pouch
Scalpels and forcepts

Sterilants, such as $HgCl_2$, sodium hypochlorite, ethanol
Sterile filter papers
Plastic or earthen pots.

(i) Wash the seeds with tap water. If the seeds are highly contaminated, then they can be taken in a muslin cloth pouch and tied to tap in such a way that water passes through the pouch for one hour to wash off the adhering spores and other pollutants.

(ii) Take the seeds and submerge them in 70% alcohol for 30-60 sec. Later decant the alcohol.

(iii) Surface sterilize the seeds by transferring them to 20-40% sodium hypochlorite solution for 15-20 min (or in 0.01 – 0.1% $HgCl_2$). Rinse the seeds with sterile distilled water.

(iv) Place 2-3 seeds per culture vessel on the surface of MS agar medium with growth regulators.

(v) Incubate the cultures at 25^{o}C under 16 h photoperiod with ~ 1000 lux light intensity for 1-2 weeks.

(vi) Observe regularly for germination of seeds. If needed transfer the individual plantlets to half MS medium (half MS medium refer to the half or ¼ concentration of inorganic nutrients only).

Plant establishment and hardening

In vitro derived plants have got varied physiological and morphological features when compared to plants growing in natural conditions. *In vitro* developed plants are adapted to conditions of high humidity, optimal nutrient supply, low light intensity and a good supply of sucrose. They have thinner cuticular wax on leaf surface. Reduced levels of chlorophyll is found in these plants. Gradual expose to natural conditions leads to progressive physiological and morphological adaptation and it is referred to as 'hardening'.

The relative humidity *in vitro* may be reduced by loosening the vessel and increasing light intensity during the weeks preceding transplanting. It activates chlorophyll synthesis and photosynthetic activity.

(i) Gently take out the well-rooted plantlet from culture vessel without damage. Carefully rinse the roots with lukewarm water to remove the agar attached to them.

(ii) Plant the regenerants in small plastic pots with sterile soil mix comprising sand: farmyard manure: soil (1:1:1 proportion) or peat : vermiculite : sand (1:1:1). Make sure that the soil is moist with water. Now plant is covered with plastic wrap (form a tent on the regenerant). Plants do not need to be watered for the first few days.

(iii) Place the pots in humid chambers under diffused light at 25^{o}C. Open the plastic wrappers to allow air exchange briefly every day. After one week, expose the plants to air for one hour. After another week, increase gradually the expose to air for several hours per day. After 2-3 weeks, remove the wraps completely, so as to adjacent with natural conditions. Fungicide or pesticide should not be sprayed during acclimatization. If any infected plants are found, they should be remove quickly.

(2) Micropropagation of *Aegle marmelos* (Rutaceae)

A. marmelos have got many medicinal properties such as astringent, digestive and diarrhoea. The active principle in this plant is 'marmelosin' which acts as a laxative and dimetic.

Reagents and other materials

Teepol solution 1%
Mercuric chloride 0.1%
MS medium
α-naphthalene acetic acid (NAA)
2,4-dichlorophenoxy acetic acid (2,4-D)
Benzylaminopure (BAP)
Kinetin (KIN)
Agar

Protocol

Plant material : *Aegle marmelos*

(i) Young shoots from the crown of mature tree are collected.

(ii) Washed in 1% teepol solution and surface-sterilized with 0.1% mercuric chloride for few minutes.

(iii) Wash with sterile distilled water repeatedly to remove surfactant.

(iv) The internodes with axillary buds (stem segments) are cut into 1 cm long pieces and these can be used as explants.

(v) Callus initiation is achieved by planting the stem segment on MS medium (supplemented with 2 mg/l KIN and 5 mg/l NAA). Callus is compact, globular and yellowish-green in colour.

(vi) Callus is subculture in the same medium; callus develops many shoot apices (embryoids).

(vii) The callus with embryoids are transferred to medium supplemented with only BAP (1 mg/l). Multiple shoot development is achieved within 10-15 days of culture.

(3) Secondary metabolite production in a cell suspension culture

Plants are the valuable source of secondary metabolites which may not useful to the plants but useful to the mankind. Some of the secondary metabolites are the antimicrobial substances, pharmaceuticals, fragrances, flavours, natural sweetners etc. Cell suspension cultures are established from which secondary metabolites can be harvested continuously.

Secondary metabolites production can be obtained by two-stage culture system. In a two-stage culture system, the first stage involves growing the cells on a standard growth medium and the second stage involves transferring these cells into a production medium suitable for synthesis of secondary metabolites. One-stage culture system may also be adopted, in which both growth and production steps are combined together.

Serpentine production in *Catharanthus roseus* cell suspension culture

Protocol

Plant material : *Catharanthus roseus*

Reagents and other material

Haemocytometer
Hartley filter funnel
Liquid nitrogen
Miracloth/cheese cloth
Nylon mesh filter (100 μm)
Mortar and pestle
Scalpel, forceps
Separating funnel
Sterile distilled water
Sterile petri dishes/slides
Sterilized sieve
Thin layer chromatography plates and associated accessories
Wide mouth Erlenmeyer flasks containing media
Sterilants such as alcohol or $HgCl_2$ or sodium hypochlorite
Nutrition medium reagents – MS medium, Gamborg's B_5 basic salts and vitamins.
Growth regulators – 2,4-D, IAA, KIN, BAP.
Counting of cells – Chromium trioxide

Viability – Fluorescein diacetate (FDA test), acetone; TTC test – KH_2PO_4, 2,3,5 triphenyl tetrtazolium chloride, alcohol.
Acetone
Ammonia
Cerric ammonium sulphate
Chloroform ($CHCl_3$)
Alcohol
Ethylacetate
Fluorescent indicator (Merck 5714)
HEPES buffer – N-2 hydroxy ethylpiperazine – N′-2 ethane sulphonic acid
(or)
MES buffer – 2-(N-morpholino) ethane sulphonic acid
Methanol
Na_2SO_4
NH_4OH
Phosphoric acid
Silica gel G
Standard alkaloids (serpentine hydrogen tartrate, vinblastine sulphate, ajmalicine, vindoline, catharanthine)

Other equipments

Centrifuge
Fluorescent microscope
Low speed centrifuge
Microscope
Orbital shaker
Sonicator
Soxhlet apparatus
Vacuum evaporator

Media

Growth medium: B_5 + 1.0 mg/l 2,4-D + 0.1 mg/l kinetin + 2% sucrose

Production medium: MS + 1.0 mg/l IAA + 0.1 mg/l BAP, + 5% sucrose + 10 mM MES (or) HEPES.

(1) Initiate Callus cultures from *C. roseus* on a B5 medium supplemented with 1.0 mg/l 2,4-D and 0.1 mg/l kinetin by taking a suitable explant.
(2) Make cell suspension culture from the callus, with the same medium used for callus induction.
(3) Characterize the cell suspension culture with respect to age, biomass yield, cell viability, mitotic index etc.

(A) Counting of cells

The cell number in suspension cultures may be counted directly in a haemocytometer. This can be performed in a following manner:

(i) Take 2 ml of cell suspension into a sterile test tube.
(ii) Add 4 ml of 8% aqueous chromium thioxide and mix well.
(iii) Heat the solution at 70^{o}C for 2-15 min.
(iv) After cooling, shake cultures vigorously; if any clumps are there, that will be broken and cells are separated.
(v) Pipette 100 µl of the solution and place on the haemocytometer. Now count the number of cells in each square with the help of compound microscope.

No. of cells / ml = Average count of cells per square x dilution factor x 10^3
(each large square represents an area of 1 mm square and a depth of 0.1 mm, i.e. a volume of 0.1 mm^3)

(B) Cell viability

Cell viability can be observed by microscopic examination for protoplasmic streaming and the presence of an intact nucleus. Cell viability may also be assessed by using vital dyes, such as fluorescein diacetate (FDA), phenosafranine (stains dead cells only) etc. The 2,3,5-two phenyl tetrazoliumchloride (TTC) is also used to measure the respiratory efficiency of cells as a measure of viability.

(4) Grow a culture of *C. roseus* cells maintained on a 14-day cycle by inoculation of 20 ml culture to 100 ml of fresh Gamborg's B_5 medium and put on a shaker with 150 rpm at 25^{o}C.

(5) Later transfer the cells to a production medium. This should be carried out when cultures are between 4 and 7 days in a growth medium in order to obtain a good alkaloid yield.

(6) Filter 100 ml of cell suspension by using a sterile Hartley filter funnel fitted with a 100 µm nylon mesh filter.

(7) Wash the cells with 100 ml, of production medium and allow the excess to drain off.
Production medium: MS + 5% sucrose + 1.0 mg/l IAA + 0.1 mg/l BAP + MES or HEPES (100mM)

(8) Take 5 g of wet filtered cells using a sterile spatula and transfer to 100 ml production medium in a 250 ml flask.

(9) Cultures are incubated at 25^{o}C on a shaker at 150 rpm with light intensity of 5 W/m^2.

(10) Take samples from time to time (0, 7, 14, 21 days after subculture) and estimate the cell viability and culture biomass. Packed cell volume method can be used to determine the culture biomass.

(11) **Product analysis:** Freeze drying of samples has been adopted for alkaloid extraction.

(i) take 10 g of fresh cells in a 250 ml round-bottom flask and add distilled water (50-70 ml) to obtain a fluid slurry.

(ii) Freeze samples in liquid nitrogen by constant rotation of the flask in liquid nitrogen until the sample becomes solid.

(iii) Freeze dry samples under a vacuum for 24-48 h, depending upon sample size.

(iv) Take out the dried cells from flask, powder in a mortar and pestle, and store in capped vial at below 4^oC.

(v) Place 50-150 mg dry weight of powdered cells in a soxhlet thimble (12 x 50 mm). Plug lightly with cotton wool.

(vi) Take 50 ml of methanol in 100 ml round-bottom flask.

(vii) Extract the sample in an apparatus for 2 h.

Solvent extraction of alkaloids from crude methanol extract:

(i) Reduce crude methanol extract to dryness on a rotary vacuum evaporator at 60^oC.
(ii) Resuspend in chloroform: water mixture (1:1). Three washes can be given in this mixture.
(iii) Adjust the pH to 10 of aqueous phase with NH_4OH.
(iv) add 60 ml chloroform, shake for 5 min in a separating funnel to separate phases.
(v) Decant the lower chloroform phase and repeat step (iv) twice.
(vi) Pool all the chloroform phase and add 60 ml aqueous NH_4OH (pH 10.0).
(vii) Dry the chloroform phase over anhydrous Na_2SO_4.
(viii) Evaporate the chloroform to dryness and resuspend in a minimum volume of methanol.

Separation of alkaloids and quantification:

Alkaloids can be separated from the extracts by TLC (thin layer chromatography) or paper chromatography or HPLC. A protocol of TLC separation is given below:

(i) Resuspend the alkaloid fraction in a minimum volume of methanol and spot 3 to 4 times at intervals starting 2 cm from the bottom on TLC plates coated with silica gel G containing a fluorescent indicator (Merck 5714).

(ii) Also spot 5 µl of a 1mg/l methanolic solution of standard alkaloids (serpentine hydrogen tartrate, ajmalicine, vinblastine sulphate, vindoline and catharanthine).

(iii) When dry, develop plates in the following solvent systems with 100 ml solvents:
S1 – ethyl acetate : ethanol (3:1)
S2 – Chloroform : acetone : ammonia (5:4:1)

(iv) After development (45-60 min), remove the plates, mark the solvent fronts and air dry it.

(v) Examine the plates under white light and, long and short wavelength UV light (366 and 254 mm). Mark the coloured and fluorescent spots.

(vi) Spray the plates with cerric ammonium sulphate (1 g in 100 ml 80% phosphoric acid). Note the colours immediately after spraying.

(vii) Examine the plates after 24 h for any colour changes and also examine under UV light. The following table shows the chromatographic properties of these alkaloids.

Table shows TLC properties of some common *Catharanthus* alkaloids

Alkaloid	Rf (x100) in		Colour in UV light (366 nm)	Colour with cerric ammonium sulphate
	S_1	S_2		
Serpentine	2-5	31-33	intense blue	None
Ajmalicine	80-81	68-70	Apple green	Yellow/green
Catharanthine	70-71	44-46	green	Yellow/blue
Vindoline	72-73	67-71	None (quenches at 254 nm)	Purple
Vinblastine	38-39	65-66	None (quenches at 254 nm)	Lavender

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Experiment No. 4

SCREENING OF MEDICINAL PLANT EXTRACT FOR ANTIMICROBIAL ACTIVITY

Aim: To demonstrate the anti-microbial activity of medicinal plants by disc diffusion method.

Requirements: Fresh aqueous leaf extract of any plant with medicinal value, Nutrient agar medium plates, Inoculation chamber, Forceps, Whatman filter paper discs.

Procedure:

1. Preparation of bacterial suspension: Take the bacterial culture of *Bacillus subtilis*, from pure slant culture with the help of a inoculation loop and mix it with sterile distilled water in a test tube in an inoculation chamber. Mix the contents thoroughly until a suspension was formed.
2. Preparation of medium seeded plates:

- Prepare the nutrient agar medium as per the following composition

Peptone	– 5.0 g
Beef extract	– 3.0 g
NaCl	– 5.0 g
Agar agar	– 20.0 g
Distilled water	– 1000 ml

pH – 7.0

- Sterilize the medium by autoclaving at 121^{o}C temperature and 15 lbs pressure for 15 minutes.
- Cool the medium to room temperature and add sufficient amount of the prepared bacterial suspension to it. Mix the contents thoroughly.
- Disperse 20 ml aliquots of the seeded medium into each petri plate and allow them to solidify.

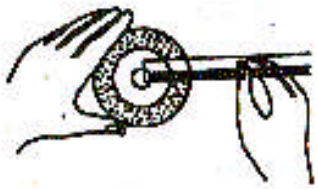
3. Prepare 5% aqueous extract of leaf from the medicinally important plant.
4. Soak the whatman filter paper discs in the extract.
5. Place the soaked disc at the center of the each seeded plate with the help of sterilized forceps and gently press it towards the medium surface.
6. Incubate the plates in inverted position for 24 hours at 37^{o}C temperature.
7. Observe the plates after incubation, for the formation of clear zone around the disc.
8. Measure the radius (r) of the clear zone and calculate the area of clear zone by using the formula πr^2, if necessary.

soaking of filter paper disc in plant leaf extract

1)

placeing the soaked filter paper disc on to a seeded medium plate

2)



measurement of clear zone formed around the filter paper disc.

3)

Result : Presence of a clear zone surrounding the filter paper disc, indicate the anti-microbial activity of the plant extract.

M.Sc. Botany II year Practical Manual

List of experiments

1. Bacterial Gram staining
2. Bacterial spore staining
3. Negative staining
4. Measurement of bacterial cell
5. Preparation of culture media
6. Isolation and enumeration of bacteria from soil
7. Isolation and enumeration of fungi from soil
8. Demonstration of antibiotic sensitivity
9. Observation of symptoms caused by plant viruses
10. Microscopic observation of fungal slides (permanent slides)
11. Anatomical studies on leaves infected with fungal pathogens
12. Effect of fungicides on fungal spore germination
13. Phytotoxic effect of fungal culture filtrate

Experiment 1 : BACTERIAL GRAM STAINING

Aim: To characterize the bacterial types – positive and negative, by Gram staining technique.

Principle: Bacteria respond differentially to grams staining because of the difference in thickness of the peptidoglycan layer in the cell wall. Gram positive bacteria, due to thick peptidoglycan layer retain the crystal violet dye and appear purple while gram negative bacteria loose the dye due to thin peptidoglycan layer and retain the pink color of the counter stain safranin.

Requirements: One day old bacterial cultures of gram positive (*Bacillus subtilis)* and gram negative (*Escherichia coli)* , Crystal violet stain, Gram's iodine solution, Ethyl alcohol, Safranin, Glass slides, Spirit lamp, Microscope etc.

Procedure:

1. Prepare the bacterial suspension by taking sufficient amount of bacterial culture and mixing in appropriate amount of distilled water in a test tube.
2. Place a small drop of suspension on a clean dry glass slide and prepare a smear by gently warming the slide over a spirit lamp.
3. Flood the smear with crystal violet stain for 30 seconds.
4. Wash the smear with distilled water to remove the excess of dye.
5. Now flood the smear with Gram's iodine solution for 30 seconds.
6. Wash the smear with 95% ethyl alcohol slowly until the smear become colorless.
7. Then wash the slides with distilled water.
8. Add the counter stain, safranin to the smear for 30 seconds.
9. Then, repeat the washing with distilled water and dry the slide by gently blotting with a blotting paper.
10. Observe the slide through compound microscope under oil-immersion objective, after placing a drop of immersion oil on the smear.

Result: Bacteria that appear purple (cells of *B. subtilis*) are confirmed as Gram-positive and those appear pink (cells of *E. coli)* are considered as Gram-negative.

Experiment No. 2
BACTERIAL SPORE STAINING

Aim: To stain and observe the endospores produced by bacteria.

Principle: Bacterial endospores, when stained with special type of dyes like Malachite green, appear like green refractile bodies in side the cell.

Requirements: Old culture (3 days old) of spore forming bacteria e.g. *Bacillus cereus* or *Bacillus subtilis*, *Clostridium*, and certain species of *Sarcina*, 5% malachite green (5g in 100ml of dis. water), 0.5% safranin (500mg in 100ml of dis. water), Glass slides, compound microscope etc.

Procedure:

1. Prepare the bacterial suspension by taking sufficient amount of bacterial culture and mixing in appropriate amount of distilled water in a test tube.
2. Place a small drop of suspension on a clean dry glass slide and prepare a smear by gently warming the slide over the flame of a spirit lamp .
3. Flood the smear with malachite green dye.
4. Heat the slide intermittently on spirit lamp flame for 10 minutes by adding excess stain to the smear every time.
5. Wash the slides slowly under running tap water, by keeping the slide in slant position.
6. Stain the slide with the counter-stain, safranin and keep it for 30 seconds.
7. Wash the smear with distilled water and blot-dry the slide with a blotting paper.
8. Observe the slide through compound microscope under oil-immersion objective, after placing a drop of immersion oil on the smear.

Result: Vegetative part of the cell takes the counter-stain and appears red in color. The elliptical or spherical or oval spores inside the cell take malachite green stain and appear like green refractile bodies.

Experiment No.3

NEGATIVE STAINING OF BACTERIA

Aim: To observe the bacteria by staining with negative stains like nigrosin.

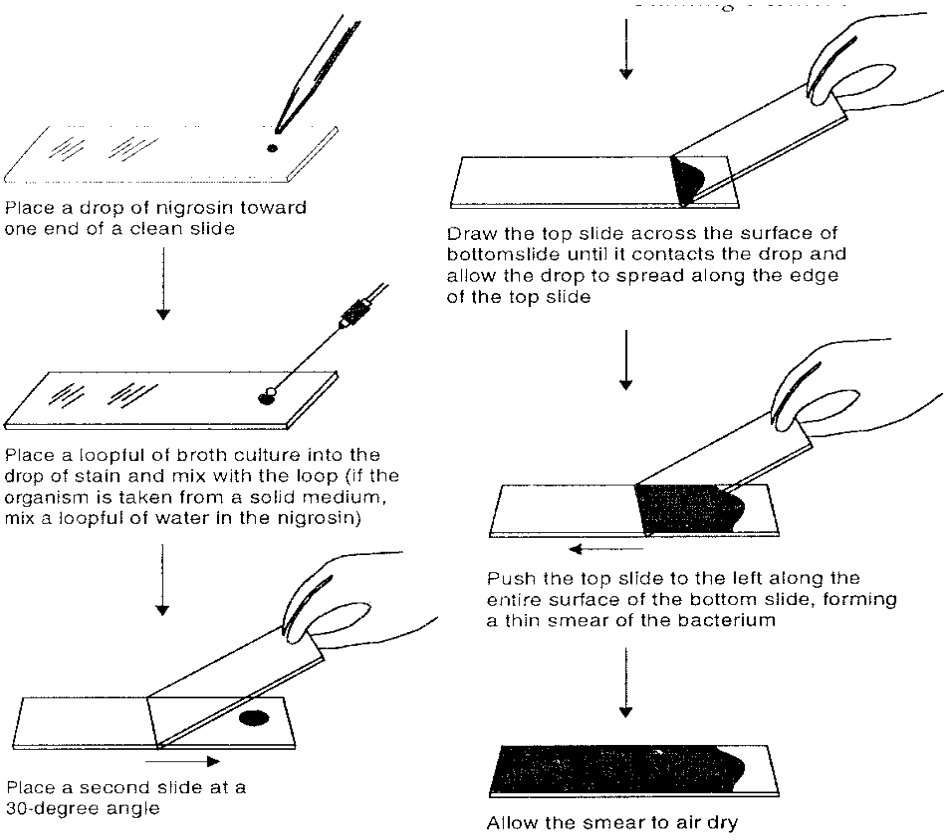
Requirements: One day old bacterial culture of *Bacillus subtilis*, Nigrosin solution, Clean glass slides, Compound Microscope, etc.

Principle: Negative stains, which are acidic and negatively charged, cannot stain the bacterial cell, as every bacterial cell carries a negative charge on its cell surface. Due to repulsion between the similar charges, the stain do not penetrate into the cell, but will gets deposited or accumulated, only around the cell surface.

Procedure:

1. Prepare the bacterial suspension by taking sufficient amount of bacterial culture and mixing in appropriate amount of distilled water in a test tube.
2. Take one drop of nigrosin and place it towards one end of a clean glass slide (as shown in the figure).
3. With the help of a sterile inoculating loop, take a loopful of bacterial suspension and gently mix it with the drop of stain on the slide.
4. Take another clean slide, place it against the drop of suspended organism at an angle of 30^{o} (as shown in the figure) and allow the droplet to spread across the edge of the top slide.
5. Spread the mixture of the stained inoculum into a thin and wide smear by pushing the top slide to the left along the entire surface of the bottom slide.
6. Air-dry the slide and observe the slide through compound microscope under oil-immersion objective, after placing a drop of immersion oil on the smear.

Result: Bacterial cells appear transparent or colorless against a blue-black background.



Procedure for negative staining

Experiment No. 4

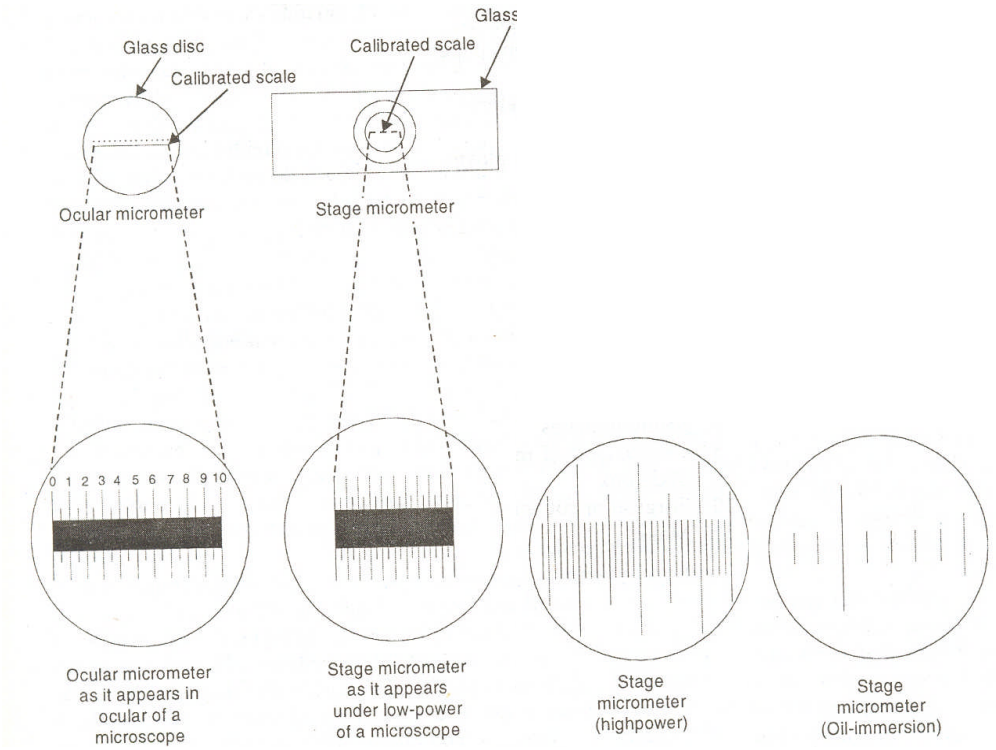
MEASURMENT OF BACTERIAL CELL SIZE

Aim: To measure the size of the bacterial cell, by micrometry, using ocular micrometer.

Requirements: Bacterial slide after gram staining, (slide prepared in the previous expt. No.1 can be used), Ocular micrometer, Stage micrometer, Compound Microscope, etc.

Ocular micrometer is a round glass disc in which 1 cm scale is divided in to 100 divisions. Thus each division is equivalent to 0.01 mm i.e., 10 µm.

Stage micrometer is a special glass slide in which 1 mm scale was divided in to 100 divisions.



Stage micrometer as it appear under high power and oil-immersion objectives

Procedure:

I STEP : <u>Calibration of ocular micrometer</u> (for different objectives .viz., low-power, high-power and oil-immersion) of a microscope by the procedure given below –

(a) Place the ocular micrometer disc in side the eyepiece by carefully unscrewing lens lid.

(b) Place the stage micrometer on the microscope stage and center it over the illumination source.

(c) By observing through low power (10 x) objective lens, bring its scale to the center of the microscopic field area i.e., the number of divisions on either side of the central division must be equal.

(d) Turn the ocular lens until the divisions of the ocular micrometer coincide with those of the stage micrometer.

(e) Select any two ocular divisions, which coincide with that of stage divisions (preferably separated apart).

(f) Count the number of divisions between them in both ocular and stage micrometers.

(g) Take at least five such counts by selecting two new sets of divisions every time and average them.

(h) Repeat the same with high-power (40x) and oil-immersion (100x) objectives.

i) Calculation of length of individual ocular division while observing through low power (10 X) objective :

Length of one ocular micrometer is calculated by using the formula

$$\text{One division of ocular micrometer (in } \mu m) = \frac{\text{No. Of stage micrometer divisions (between the coincided sets)}}{\text{No. of ocular micrometer divisions (between the coincided sets)}} \times 10$$

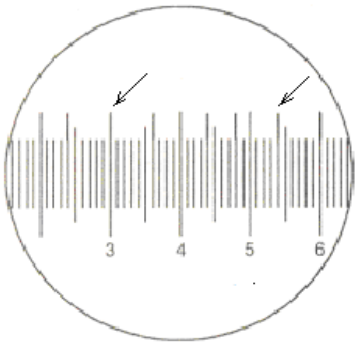
where 10 is the original length of the individual division (10 μm) in a ocular micrometer.

For e.g. If 23 ocular divisions and 28 stage divisions are recorded between the coinciding set of divisions,

$$\text{Then one ocular division is} = \frac{\text{28 (no. of stage divisions)}}{\text{23 (no. of ocular divisions)}} \times 10 = 12\ \mu m$$

(because original length of single division in a ocular micrometer is equal to 10 μm)

ii) Similarly, calculate the length of ocular division using high power and oil immersion objectives.



Calibration of ocular micrometer. (in oil immersion) Ocular micrometer super imposed (below graduations which are numbered) over stage micrometer above (dark colored graduations). Count the number of OM divisions coinciding with SM divisions; twenty four divisions on the OM coinciding with four graduations of SM. Calibrate and multiply each OM division with the calibration factor

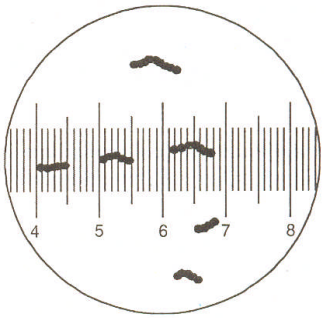
As given in the above figure,

Each ocular division = $4 \div \mathbf{24 \times 10} = \mathbf{1.6}$ μm
(in oil immersion)

STEP II Measurement of bacterial cell size :

a) After calibration, retain the ocular in the eyepiece and replace the stage micrometer with the bacterial slide for measuring the cell size.
b) Measure the size of the bacterial cell by counting the no. of ocular divisions within the boundaries of the cell wall.
c) Take at least 10 readings each for length and width of cells (for rod shaped cells) or diameter of the cells (for round cells) and average them.
d) The size of the cell is determined by multiplying the number of ocular micrometer divisions occupied with calibration factor, for the objective used.

Length of the bacterial cell = no . of ocular divisions occupied x calibration factor for one ocular division
Breadth of the bacterial cell = no . of ocular divisions occupied x calibration factor for one ocular division
The size of rod shaped bacterial cell = length x breadth
The size of round cells = diameter



Measurement of microorganisms with the calibrated OM.
Cont the number of divisions on the OM which the different cells occupy.
Each filament occupies 5-7 divisions on the OM.
Multiply OM divisions with the calibration factor

7. Observation

S.No	No. of ocular divisions occupied by bacterial cell (bacillus type)		Bacterial cell (cocci type)
	Length	Breadth	Diameter
01			
02			
03			
04			
05			
06			
07			
08			
09			
10			

Result: The length and width of rod shaped cells and diameter of rounded cells (in μm) can be measured.

Experiment No. 5

PREPARATION OF CULTURE MEDIA

Aim : To prepare the synthetic culture media for the culturing of bacteria and fungi.

Requirements : Sterilized petri plates, conical flasks, measuring cylinder, autoclave (or) pressure cooker, cotton, spirit lamp, chemicals and distilled water.

Note : One of the commonly used synthetic and solid media for culturing of bacteria is Nutrient agar and for that of fungi is Czapek Dox agar.

Preparation of Nutrient agar medium :

Procedure :

1. Weigh the following chemicals in the given proportions

Peptone	- 5.0 g
Beef extract	- 3.0 g
Agar agar	- 20.0 g
Distilled water	- 1000.0 ml
pH	- 7.0

2. Dissolve all the chemicals, except agar, in distilled water, in a conical flask, by thorough mixing.
3. Then add agar (do not shake the contents of the flask after adding agar, to prevent the agar to adhere to the walls) and plug the flask with cotton.
4. Sterilize the medium by autoclaving at 121 ^{0}C and 15 lb pressure for 15 minutes, in an autoclave or pressure cooker.
5. After sterilization, cool the medium to the room temperature. Now the medium is ready for dispersion in to sterilized petri plates for isolation of bacteria.
6. To isolate the bacteria, prepare the media plates by dispersing about 20 ml of the medium, when it is about to solidify, in to each of the sterilized petri plate, in front of the flame, in an inoculation chamber.
7. Allow the medium in the plates to solidify. Store them in an inverted position, to avoid contamination.
8. These media plates are ready to use for isolation of bacteria.

Preparation of Czapek Dox agar medium :

Procedure :

1. Weigh the following chemicals in the given proportions

Sodium Nitrate	- 0.2 g
Magnesium sulphate	- 0.5 g
Potassium dihydrogen ortho phosphate	- 1.0 g
Potassium chloride	- 0.5 g
Ferrous sulphate	- traces
Sucrose	- 30.0 g
Agar agar	- 20.0 g
Distilled water	- 1000 ml
pH –5.0	

9. Dissolve all the chemicals, except agar, in distilled water, in a conical flask by mixing the contents thoroughly.
10. Then add agar (do not shake the contents of the flask after adding agar, to prevent the agar to adhere to the walls) and plug the flask with cotton. Sterilize the medium by autoclaving at 121 ^{0}C and 15 lb pressure for 15 minutes, in an autoclave or pressure cooker.
11. After sterilization cool the medium to the room temperature. Now the medium is ready for dispersion in to sterilized petri plates for isolation of Fungi.
12. To isolate the fungi, add 5% of streptomycin sulfate (antibiotic) to the medium when it is about to solidify and mix the contents thoroughly.
13. Then prepare the media plates by dispersing about 20 ml of the medium, in to each of the sterilized petri plate, in front of the flame, in an inoculation chamber.
14. Allow the medium in the plates to solidify. Store them in an inverted position, to avoid contamination.
15. These media plates are ready to use for isolation of fungi.

Experiment No. 6

ISOLATION AND ENUMERATION OF BACTERIA FROM SOIL

Aim : To isolate and enumerate the bacteria present in soil by dilution plate method

Principle : At ultimate dilution , individual colonies are developed from a individual cells.

Requirements : Sterilized petridishes, pipettes, test tubes, conical flasks, measuring cylinders, cotton, sterile distilled water, etc.,

Procedure :

STEP I : Preparation of media plates :

1. Prepare Walkimoto Agar medium by using the following composition.

Calcium Nitrate	– 1.0 g
Ferrous sulphate	– 1.0 g
Disodium hydrogen orthophosphate	– 2.0 g
Bacterial Peptone	– 5.0 g
Sucrose	– 15.0 g
Distilled water	– 1000 ml
pH	– 7.0

2. Sterilize the medium by autoclaving at 121 ^{o}C and 15 lb pressure for 15 minutes.
3. After sterilization, cool the medium to room temperature.
4. Then disperse about 20 ml of the medium into each of the sterilized petri dishes, in front of the flame, in an inoculation chamber or laminar flow to avoid contamination. Allow the plates to solidify.

STEP II : Preparation of serial dilution of the soil :

1. Take one gram of soil sample in a 250 ml conical flask and add 100 ml of sterile distilled water, in an inoculation chamber, that gives 10^{-2} (1:100) dilution.
2. From 10^{-2} dilution, take one ml and dilute to 10 ml by adding 9 ml of sterile dis. Water, in a sterile test tube, which gives 10^{-3} (1: 1000) dilution.
3. Likewise, prepare the serial dilutions up to 10^{-6} (1:1000000) dilution in separate sterilized test tubes.

STEP III: Plating :

1. Inoculate a media plate with 0.5 ml of soil suspension from 10^{-5} dilution and spread the inoculum by using sterile bent glass rod, in front of spirit lamp flame, in an inoculation chamber.
2. Similarly inoculate another media plate from 10^{-6} dilution and spread the inoculum.
3. Maintain three replicates for each dilution. Incubate the plates at room temperature for 24 hours.
4. After incubation, observe the plates for bacterial colonies. Count the number of colonies per plate, tabulate the results and comment on the result.

Dilutions	Replicates	No. of colonies per plate	No. of colonies per plate (av. of 3 replicates)
10^{-5}	1		
	2		
	3		
10^{-6}	1		
	2		
	3		

Observation : Number of bacterial colonies decreased with increase in dilution.

Experiment No. 7

ISOLATION AND ENUMERATION OF FUNGI FROM SOIL

Aim : To isolate and enumerate the fungi present in soil by dilution plate method

Principle : At ultimate dilution , individual colonies are developed from a individual cells.

Requirements : Sterilized petri dishes, pipettes, test tubes, conical flasks, measuring cylinders, cotton, sterile distilled water, etc.,

Procedure :

STEP I : Preparation of media plates :

1. Prepare Czapek-Dox medium by using the following composition.

Sodium Nitrate	- 0.2 g
Magnesium sulphate	- 0.5 g
Potassium dihydrogen ortho phosphate	-1.0 g
Potassium chloride	- 0.5 g
Ferrous sulphate	- traces
Sucrose	- 30.0 g
Agar agar	- 20.0 g
Distilled water	- 1000ml
PH –4.5	

5. Sterilize the medium by autoclaving at 121 ^{o}C and 15 lb pressure for 15 minutes.
6. After sterilization, cool the medium to room temperature (when the medium was about to solidify) and add 5% streptomycin and 5% rose bengal, and mix the contents thoroughly.
7. Then disperse about 20 ml of the medium into each of the sterilized petri dishes, in front of the flame, in an inoculation chamber or laminar flow to avoid contamination. Allow the plates to solidify.

STEP II : Preparation of serial dilution of the soil :

1. Take one gram of soil sample in a 250 ml conical flask and add 100 ml of sterile distilled water, in an inoculation chamber, that gives 10^{-2} (1:100) dilution.

2. From 10^{-2} dilution, take one ml and dilute to 10 ml by adding 9 ml of sterile dis. Water, in a sterile test tube, which gives 10^{-3} (1: 1000) dilution.
3. Likewise, prepare the serial dilutions up to 10^{-4} (1:10000) dilution in separate sterilized test tubes.

STEP III: Plating :

1. Inoculate a media plate with 0.5 ml of soil suspension from 10^{-3} dilution and spread the inoculum by using sterile bent glass rod, in front of spirit lamp flame, in an inoculation chamber.
2. Similarly inoculate another media plate from 10^{-4} dilution and spread the inoculum.
3. Maintain three replicates for each dilution. Incubate the plates at room temperature of 2-3 days. After incubation, observe the plates for fungal colonies.
4. Count the number of colonies per plate, tabulate the results and comment on the result.

Dilutions	Replicates	No. of colonies per plate	No. of colonies per plate (av. of 3 replicates)
10^{-3}	1		
	2		
	3		
10^{-4}	1		
	2		
	3		

Observation : Number of fungal colonies decreased with increase in dilution.

Experiment No. 8

DEMONSTRATION OF ANTIBIOTIC SENSITIVITY

Aim: To demonstrate the anti-microbial activity of antibiotics – tetracycline and penicillin by disc diffusion method.

Requirements: Fresh bacterial culture (*Bacillus subtilis),* Petri plates, Nutrient agar medium, Inoculation chamber, Forceps, selected antibiotic discs.

Procedure:

1. Preparation of bacterial suspension: Take the bacteria from pure slant culture with the help of a inoculation loop and mix it with suitable amount of sterile distilled water, in a test tube, in an inoculation chamber. Mix the contents thoroughly until a suspension was formed. (Take any antibiotic sensitive strains of bacteria, for better results. E.g. *Bacillus subtilis*).
2. Preparation of medium for seeded plates : Prepare the nutrient agar medium as per the following composition

Peptone	– 5.0 g
Beef extract	– 3.0 g
NaCl	– 5.0 g
Agar agar	– 20.0 g
Distilled water	– 1000 ml
	pH – 7.0

Sterilize the medium by autoclaving at 121^{o}C temperature and 15 lbs pressure for 15 minutes.

3. Cool the medium to room temperature and add sufficient amount of the prepared bacterial suspension to it. Mix the contents thoroughly.
4. Disperse 20 ml aliquots of the inoculated medium into each petri plate.
5. Allow the plates to solidify.
6. After solidification, place the selected antibiotic disc at the center of the plate with the help of sterilized forceps and gently press it towards the medium surface.
7. Incubate the plates in inverted position for 24 hours at 37^{o}C temperature.
8. Observe the plates after incubation, for the formation of clear zone around the disc.

9. Measure the radius (r) of the clear zone and calculate the area of clear zone by using the formula πr^2 and compare the zones produced by the two types of antibiotic discs used.

Experiment No. 9

OBSERVATION OF SYMPTOMS CAUSED BY PLANT VIRUSES

Aim: To observe and note the important symptoms of plants infected with disease causing viruses.

Sugarcane streak mosaic disease

Genus : Tritimo virus of Potyviridae

The elongated yellowish stripes alternate with the normal green portions on the leaf gives a mosaic appearance.

In severe infections, the chlorotic area may be greater than the healthy.

The mosaic symptoms are more clearly apparent on the young leaves than on older ones.

Similar symptoms are seen on the leaf sheaths and stalks, and in highly susceptible varieties yellow stripes are seen even on the canes.

The entire plant is stunted and becomes chlorotic, and easily identified from a distance.

Transmission through sets and also sap transmitted

Tobacco mosaic disease

Genus : Tobacco Mosaic Virus

Disease is caused by *Nicotiana Virus I*

First symptoms are light discoloration along the veins of the youngest leaves.

Leaves develop a characteristic light and dark green pattern, the dark green areas usually associated with the veins i.e., mosaic symptoms with interveinal depletion

Severely infected leaves show blistered appearance and much distorted with leaf margins folded down, and finally resulted in the reduction of leaf size.

Mosaic symptoms are prominent in young and middle aged leaves.

Flower breaking symptoms, orange and white discoloration are also common

Tungro disease of Rice

Genus : Rice tungro spherical waikavirus (RTSV) and Rice tungro bacilliform badnavirus (RTBV)

This disease is the result of mixed infection by two viruses.

Stunting of plant and discoloration of leaves are characteristic of the disease.

Discoloration ranges from various shades of yellow to orange and rusty blotches spreading downwards from the leaf tip.

Young leaves how a mottled appearance and slightly twisted, whereas older leaves appear rusty colored.

In some susceptible varieties, infection delays flowering.

In highly susceptible varieties, if infection takes place at early stages the plants may die before flowering.

The virus is transmitted by green leaf hoppers.

Ground nut mosaic disease

Genus : Peanut green mosaic virus

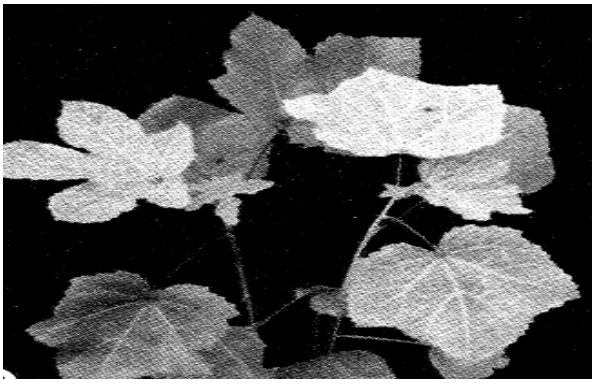
Solid chlorotic spotting

Ring spots consisting of central dark green areas surrounded by chlorotic rings.

Some leaves show alternate dark green and chlorotic rings

Infected plants show distorted leaves and stunted growth

Sap and aphid transmitted



Vein banding of Bhendi

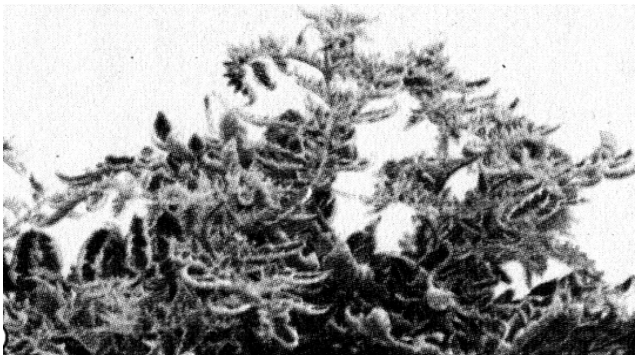
Genus : Yellow vein mosaic of okhra

Characterized by yellowing of entire network of veins in leaf blade with thick green inter veinal regions.

In severe cases young leaves turn yellow and reduce in size, plants show stunted growth.

Due to infection, flowering of the plant is restricted; fruits if formed are small, rough, look pale coloured and harder.

Caused by Gemini virus and transmitted by aphids.



Tomato yellow leaf curl

Genus : Tomato yellow leaf curl virus (TYLCV)

Most devastating and wide spread diseases of tomato

Infected plants remain stunted

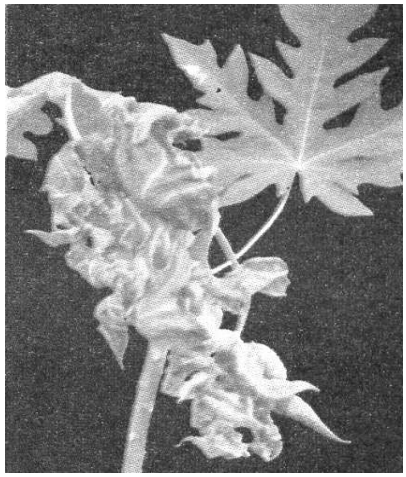
The shoots and leaves become smaller and assume an erect position

Leaves are severely chlorotic and usually rolled upwards and inwards.

The leaves become deformed and chlorotic

There is drop of flowers, fruits fail to set.

Transmitted by white fly



Leaf curl of papaya

Genus : Papaya leaf curl virus

Characterized by severe curling, crinkling and deformation of leaves. Mostly young leaves are affected.

Other symptoms like vein-clearing, reduced size, inward rolling of leaves and thickening of veins are also common.

Twisting of petioles may occur.

Diseased leaves become leathery and brittle, plants become stunted, fruit yield reduced, defoliation often results.

Transmittion is either by grafting or by means of whitefly.

Rosette disease of Ground nut

Genus : Groundnut rosette virus

Characterized by a clumping together of the foliage.

Reduction in the size of leaf blade occurs.

Bunching and erect appearance of the plant occur.

Virus is transmitted through grafting and by *Aphis craccivora*.

Experiment No. 10

MICROSCOPIC OBSERVATION OF FUNGAL SLIDES

Aim: To study the characteristic features of some important fungi, by observing the permanent slides.

Requirements: Permanent slides of various fungi, microscopes etc.

Description of some common fungi:

1. *Stemonitis*

2. *Plasmodiophora*

3. *Synchytrium*

4. *Phytophthora*

5. *Albugo*

6. *Mucor*

7. Ascocarps - Cleistothecium

Perithecium

Apothecium

8. Agaricus Gill

9. *Polyporous*

10. *Puccinia*

11. *Alternaria*

Stemonitis

Division : Myxomycota
Class : Myxomycetes
Sub-class : Stemonitomycetidae
Order : Stemonitales
Family : Stemonitaceae

It is an myxomycetous fungus commonly found on moist, decaying wood.

It is an example of true slime molds with vegetative stage represented by plasmodium.

The plasmodium is multi nucleated, branched and appear like network.

Sporangium is a fan shaped structure developed on a stalk.

Sporangium consists of central columella and a network formed by numerous threads radiating from columella to form capillitium

Numerous spores are present within the network of capillitium.

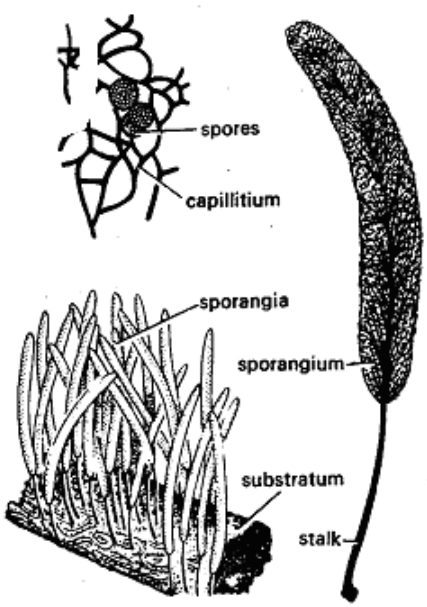


Figure showing the nature of the fungus, structure of sporangium and spore

Plasmodiophora

Division: Myxomycota
Class : Plasmodiophoromycetes
Order : Plasmodiophorales
Family : Plasmodioharaceae

Plasmodiophora brassicae causes club-root of crucifers

Infected root show hypertrophy and appear with club shaped swellings

The multinucleate plasmodium inside the infected cell, later transforms into a sporangium producing numerous resting spores.

The infected cells with mass of resting spores appear dark in colour.

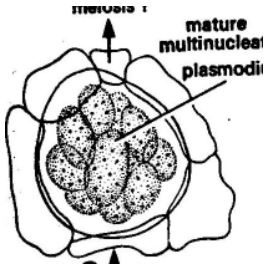


Figure showing the plasmodia present inside the host cells

Synchytrium

Division : Eumycota
Sub- Division : Mastigomycotina
Class : Chytridiomycetes
Order : Chytridiales
Family : Synchytriaceae

Synchytrium endobioticum causes black wort or wort disease of potato.

The thallus is holocarpic present inside the cell as spherical sac like structure

Numerous resting spores are developed inside the infected cell.

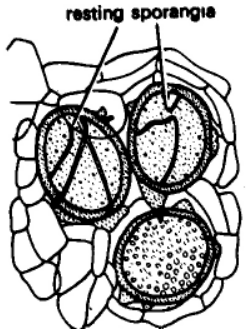


Figure showing resting spore inside the host tissue

Phytophthora

Division : Eumycota
Sub- Division : Mastigomycotina
Class : Oomycetes
Order : Peranosporales
Family : Pythiaceae

Phytophthora infastans causes late blight of potato

Long, branched sporangiophores are produced from stomata in the infected leaf

The sporangia are spherical, oval, lemon or pear shaped, hyaline to yellow in colour

Characteristically each sporangia are papillated.

The male sex organ antheridia are attached either laterally or appear as collar above the base of female sex organ, oogonium.

Oospore, the fertilized egg formed inside the oogonium, appears like big, dark and thick walled spore inside the host cells.

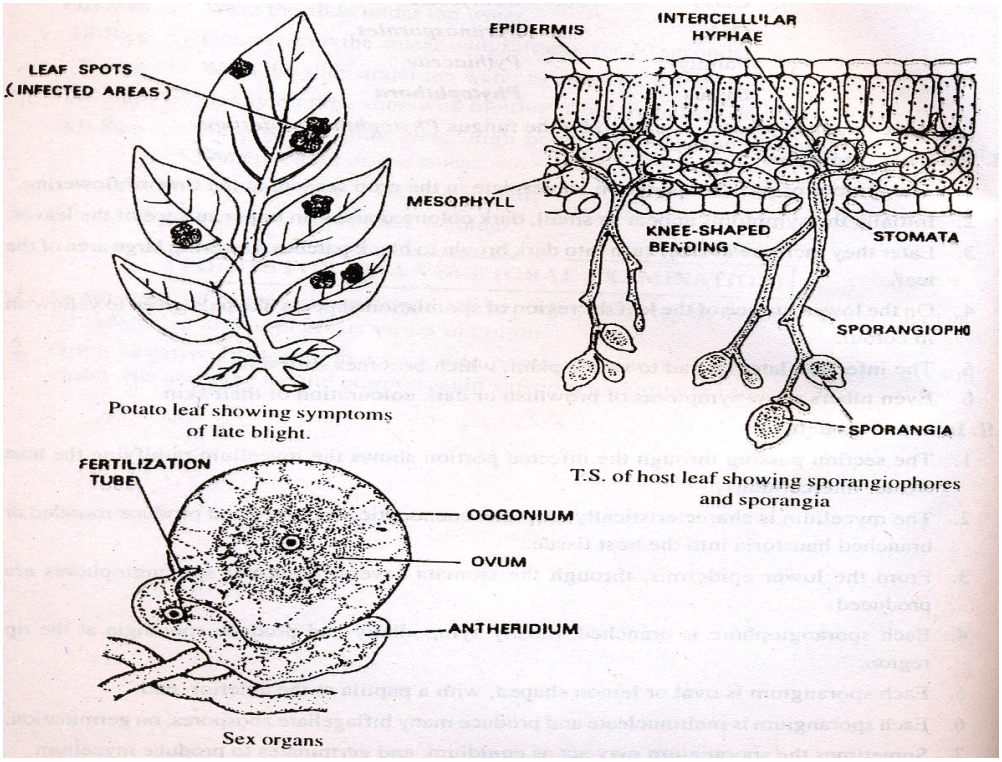


Figure showing the symptoms on leaf, structure of conidiophores and conidia and sex organs

Albugo or Cystopus

Division : Eumycota
Sub-Division : Mastigomycotina
Class : Oomycetes
Order : Peranosporales
Family : Albuginaceae

It is an obligate parasite on plants, producing white blisters or white rust on all parts except roots

On leaves symptoms appear like white shiny pustules

Vegetative hyphae produce knob shaped haustoria in to the host cell.

Like rust pustules, the conidial sorus rupture the epidermis and exposed to outside

Conidia are produced basipetally on short club shaped conidiophores.

Each sporangium or conidium is small, spherical, hyaline and multi nucleate

The male sex organ antheridia and female sex organ, oogonium are produced side by side, at the tip of the hyphae.

Oospore, the fertilized egg formed inside the oogonium, appears like big, dark and thick walled spore inside the host cells.

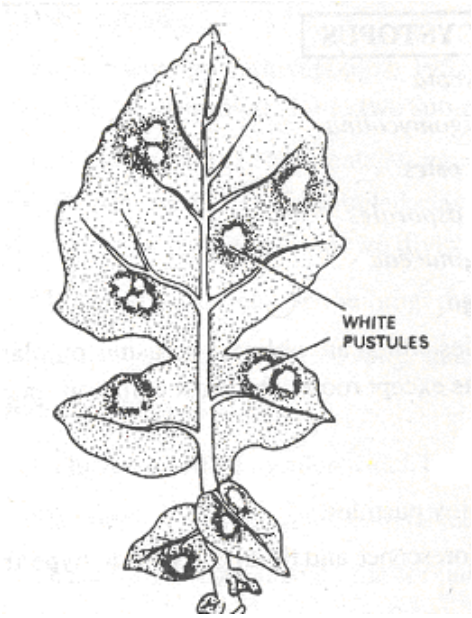


Figure showing the symptoms on the leaf

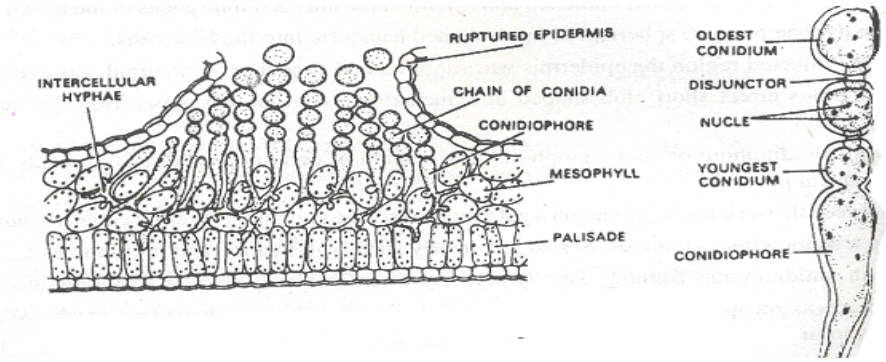


Figure showing the structure of conidiophores and conidia

Mucor

Division : Eumycota

Sub- Division : Zygomycotina
Class : Zygomycetes
Order : Mucorales
Family : Mucoraceae

Thallus is made up of hyphae which are white, branched, coenocytic and usually with tapering ends. Do not differentiated into stolons and node as in case of *Rhizopus*.

Sporangia are developed singly at the terminal ends of sporangiophores

Each sporangium is spherical in shape with a dome shaped septum that encloses the columella.

Large numbers of non-motile, single celled aplanospores are produced in the spore sac, space between the columella and the outer sporangial wall.

During sexual reproduction, dark coloured, black thick walled and warty zygospore is produced after isogamy at the pint of contact between two compatible hyphae.

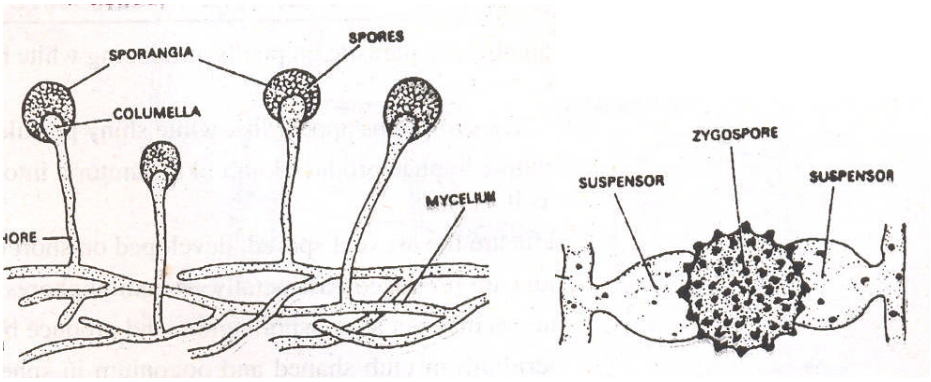


Figure showing the structure of sporangiophore, sporangium and zygospore

FRUIT BODIES OF ASCOMYCOTINA

a) Cleistothecium

A cleistothecium is a globose, completely closed fruit body with no special opening to the outside. The asci are scattered randomly inside the ascocarp as in *Eurotium* or arising in tufts from the basal region of the ascocarp as in *Erysiphae.*

The asci are usually globose, broadly oval or pear shaped and are nearly sessile.

b) Perithecium

This if a flask shaped structure opening by a pore or ostiole. There is a well defined wall

Asci are released through a pore. Pore may be lined with hair-like structures called paraphysis.

From the inner wall of the perithecium, asci develop. E.g. members of sphaeriales and Pyrenomycetes

c) Apothecium :

This is a wide, open saucer-shaped fruit body. The asci are present in a hymeninm. The asci are freely exposed at maturity. Examples – Pezizales

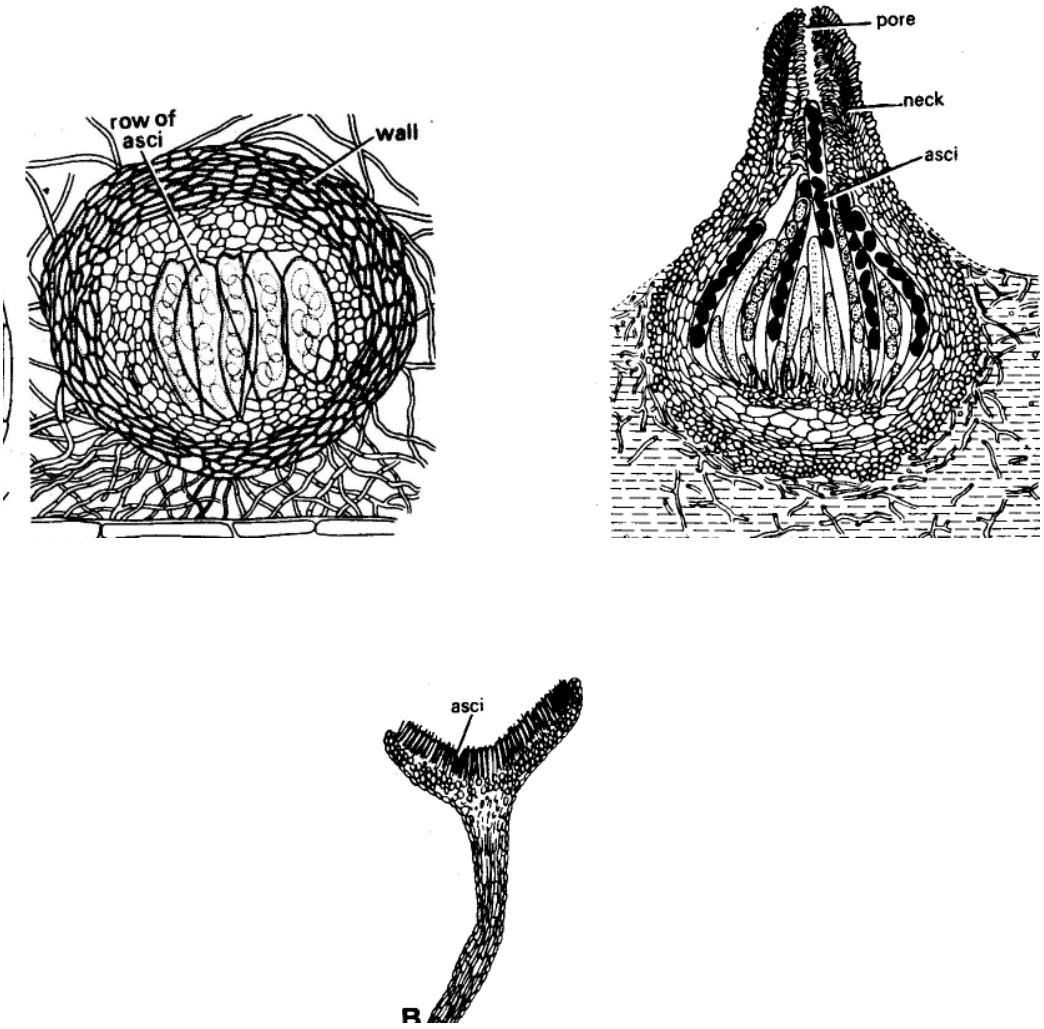


Fig. Types of fruit bodies : Cleistothecium, Perithecium, and Apothecium

Agaricus

Division : Eumycota

Sub- Division : Basidiomycotina
Class : Hymenomycetes
Order : Agaricales
Family : Agaricaceae

A mature basidocarp is an umbrella shaped structure with a central rigid stalk or stipe, bearing a more or less circular cap or pileus.

On the lower side of the pileus, there are numerous radially arranged gills or lamellae

Internally, gill composed of three regions viz., trama, subhymenium and hymenium.

A central group of longitudinally running threads termed as Trama

The hymenium consists of paliside like layer of mature basidia, In between the trama and hymenium, the ends of the hyphae of trama constitute a layer called subhymenium

Each basidium is barrel shaped bearing 4 sterigma at the tip with a dingle kidney shaped basidiospore on each sterigma.

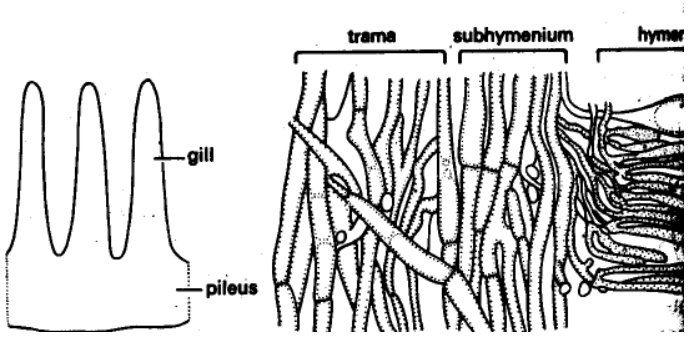


Fig. *Agaricus*, part of fruit body and L.S. of the gill

Polyporus

Division : Eumycota

Sub- Division : Basidiomycotina
Class : Hymenomycetes
Order : Aphyllophorales
Family : Polyporaceae

The mature fruit bodies are generally leathery, annual, appearing as fan-shaped brackets, sessile or stalked.

On the lower surface of the fruit body there are minute pores which are round, angular or irregular in shape.

These are the openings of numerous vertically placed tubes which run parallel to each other within the sporocorp.

Cross section of the tube shows the presence of hymenium, comprises of a layer of hyphae from which arise, at right angles, the fertile clavate single celled basidia.

Sterile structures called cystidia are produced interspersed between basidia. This layer lyning the pores and composed of basidia and cystidia is the hymenium.

Each basidium is barrel shaped bearing 4 sterigma at the tip with a dingle kidney shaped basidiospore on each sterigma.

Fig. L.S. of the fruit body

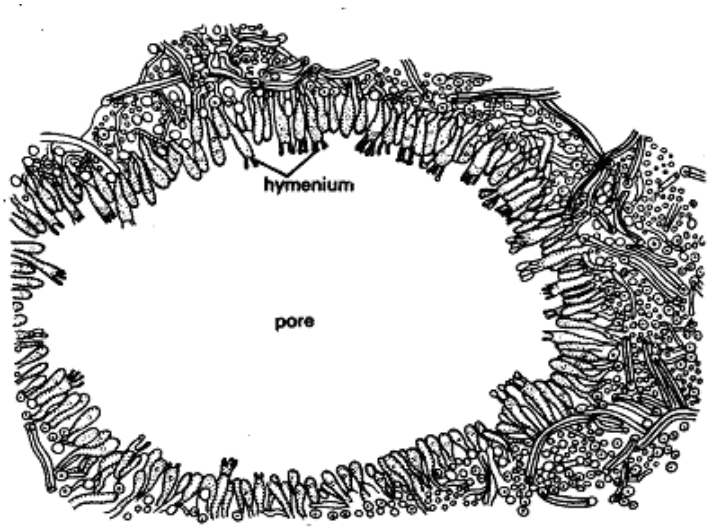


Figure showing structure of a pore

Puccinia

Division : Eumycota

Sub- Division : Basidiomycotina
Class : Teliomycetes
Order : Uredinales
Family : Pucciniaceae

Puccinia graminis* var *tritici attacks the wheat plants and causes black or stem rust

The uredospores are exposed to the outside by rupturing the epidermis in the region of pustule.

Each uredosori comprises of numerous uredospores interspersed with sterile paraphysis

Each uredospore is oval or round, hyaline or brown, binucleate, thick walled with or without attenuations and short stalks.

Each teleutosori comprises of numerous bi-celled, teleutospores.

Each teleutospore is dark brown in colour, elongated, thick and smooth walled with long stalk.

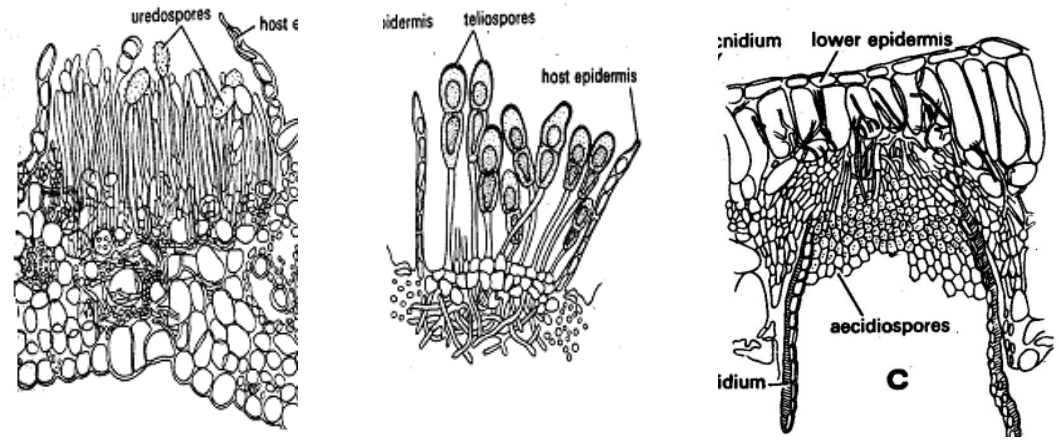


Figure showing the structure of Uredosori, Teleuto sori and Aecial cup

Alternaria

Division -- Deuteromycotina

Class -- Hyphomycetes
Order -- Moniliales
Family -- Dematiaceae

Hyphae are branched, septate and light brown in color.

Conidiophores are dark brown, branched, simple, short or elongated, bearing a simple or branched chain of conidia at the tip region.

Conidia are borne in acropetalous succession in long chains.

Each conidium is dark brown, pyriform with a long beak and possess transverse and longitudinal septa and are called as muriform conidia or dictyospores.

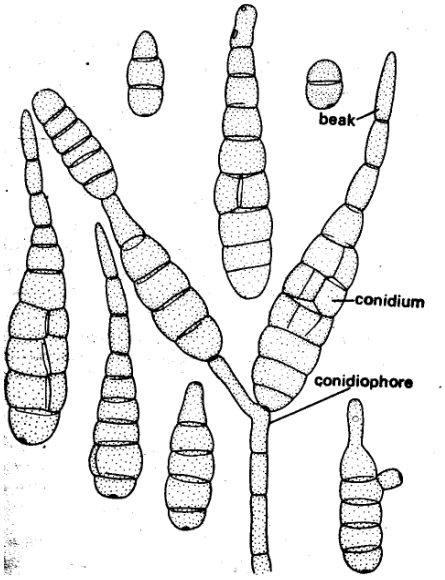


Figure showing conidiophores and conidia

Experiment No. 11

ANATOMICAL STUDIES ON LEAVES INFECTED WITH FUNGAL PATHOGENS

Aim : To study the anatomical features of leaves infected with some important pathogenic fungi. For example –

a) Tikka leaf spot disease of ground nut caused by *Cercosporidium personatum*

b) Leaf spot disease of chilli caused by *Cercospora capsicii*

c) Groundnut rust disease caused by *Puccinia arachidis*

Requirements : Infected leaf material (collected from field and preserved in FAA solution), pith material , Sharp edged blade, needle , brush, watch glass, microscope, slides, cover slips, cotton blue stain, lactophenol/ lactoglycerol, etc.,

Note : Collect the diseased leaves from the field in paper bags and bring to the laboratory for study. First, observe the external symptoms like shape, size, colour, structure and pattern of distribution of symptoms on the leaf. Then, it is also essential to observe the fungal structures like spores and conidiophores/ sporangiophores by scrapping the infected region using any sharp devise, on to a slide and observe under microscope.

Procedure:

- Take the infected leaf and cut into 1 cm^2 bits covering the infected portion.
- Place this bit in the vertical slit made in the pith material.
- Take very thin hand made sections of the pith passing thorough the infected leaf
- Place them in a watch glass containing water, and select the thinnest section possible
- Transfer it on to a clean glass slide using a camlin brush, stain it with cotton blue, remove the excess of stain by blotting.
- Then, mount the section by gently placing a cover-slip over the section after adding a drop of lactoglycerol on it.

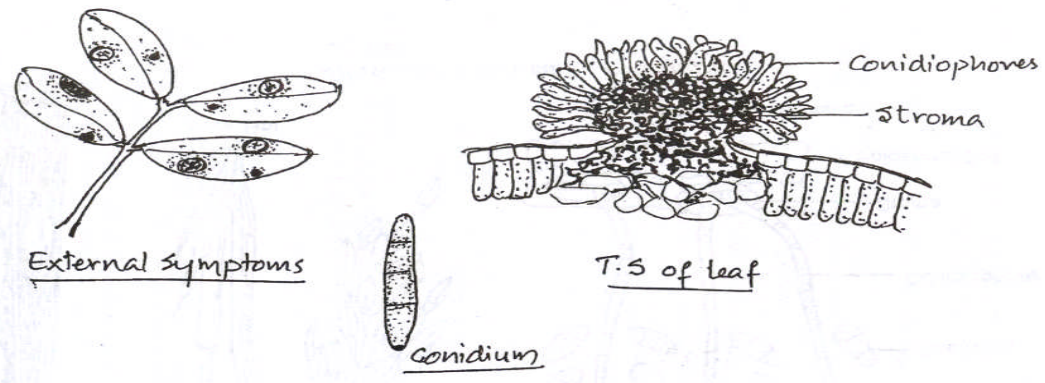
- Observe the prepared slide under compound microscope first under low power objective, then under high power objective.
- Draw a neat and labeled diagram of the leaf section from the slide including the structures like internal mycelium, structure of stroma, conidiophores and conidia.

Observations:

a) Tikka leaf spot disease of ground nut caused by *Cercosporidium personatum*

External symptoms : Leaf spots on upper surface of the leaf are very dark blackish, brown or irregular 1-6 mm in diam., with a yellow halo. On the lower surface of the leaf spot are carbon black in colour.

Internal structures : Transverse section of leaf shows, pinhead shaped stroma present inside the host tissue and extended beyond the leaf surface. It is formed by mass of hypha and measures about 40-65 um in diam. Numerous conidiophores arise in clusters from stroma. Each conidiophore is pale olivaceous in colour, geniculate , 1-3 septate, and measure 25-54 µm long and 5-9 µm broad. Conidia are obclavate or cylindrical, 1-7 septate, pale olivaceous, measuring 18-60 µm in length, 6-10 µm in width with prominent scar at base.



Tikka leaf spot disease of groundnut caused by *Cercosporidium personatum*.

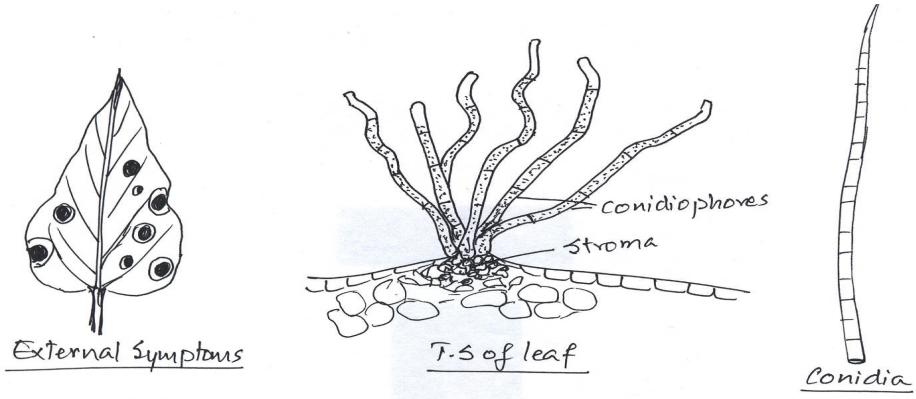
Figure showing the external symptoms & T.S. of leaf showing stroma with conidiophores and conidia

2) Leaf spot disease of chilli caused by *Cercospora capsici*

External Symptoms : Leaf spots are dark brown in colour, round or irregular in shape, measuring 0.5 –1 cm, in diam., irregularly distributed on the leaf. Each leaf spot appears like target spot with concentric rings of dark colour with or without yellow halo.

Internal Structures :

In the infected portion, section of the leaf shows very small stroma produced on both surfaces of the leaf. Conidiophores emerged from stroma are much divergent, 4-12 in number per stroma. Each conidiophore is long, reddish brown, branched or unbranched, septate, geniculate, measuring 45-75 μm long, 2-5 μm width. Conidia are hyaline, acicular, 6-25 septate, measuring 90-220 μm long and 1-3 μm in width.



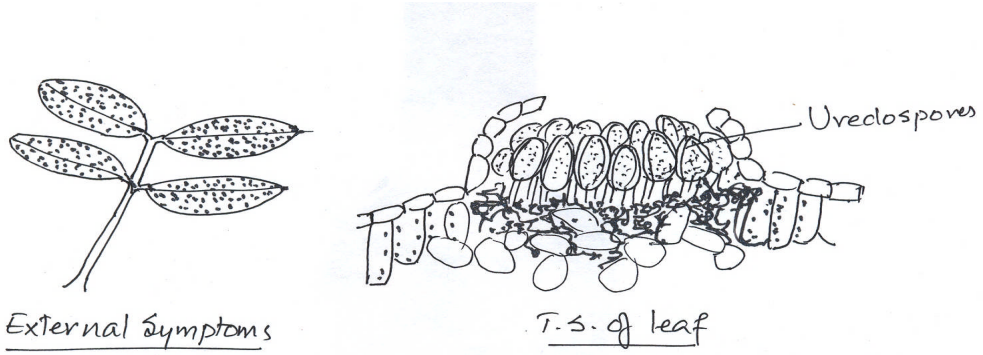
Leaf spot disease of chilli caused by *Cercospora capsici.* Figure showing the external symptoms & T.S. of leaf showing stroma with conidiophores and conidia.

3) Rust disease of ground nut caused by *Puccinia arachidis*

External Symptoms : On leaves the disease appear like pustules distributed on the entire lamina giving the rusty appearance.

Internal structures :

Section passing through the disease portion shows many uredospores from the sori exposed to out side by rupturing the epidermal layer. Each uredospore is reddish brown, round to oval, thin walled, sessile with attenuations and measuring 10-35 μm in diam.



Ground nut rust caused by *Puccinia arachidis.* Figure showing the external symptoms & T.S. of leaf showing uredospores

Experiment No. 12

EFFECT OF FUNGICIDES ON FUNGAL SPORE GERMINATION

Aim: to study the effect of contact and systemic fungicides on germination of fungal spores.

Requirements : Fungicides – Bavistin, Dithane –M 45 ; moist chambers, slides ; leaves infected with fungal pathogens. E.g. *Alternaria* leaf spots, *Cercospora* leaf spots etc.,

Preparation of fungicide concentrations : Depending on the effective concentration (EC) of the fungicide, 10, 20, 30, 40, and 50 ppm solutions were prepared using distilled water.

Procedure :

- Collect the infected leaves from the field and brought to the laboratory
- Wash them gently under tap water, and blot under blotting papers
- Place them in a moist chamber for 24 hours
- Collect the spores from infected regions of leaves using a brush, in to a watch glass
- Take a slide, place a drop of 10 ppm fungicide concentration and transfer some spores on to it.
- Similarly, prepare the slides for all dilutions and prepare a slide with distilled water to act as control, incubate all, in moist chambers for 24 hours.
- After incubation, remove the slides, make them air-dry, stain with cotton blue, and observe under microscope for germination of the spores.
- Observe nearly, 100 spores for the study of percentage of germination
- Count the number of germ tubes per spore (for nearly 25 spores and take the average).
- Measure the length of germ tube produced from apical cell of spore (for nearly 25 spores and take the average).
- Tabulate the results and comment of them
- Repeat the same procedure using different fungicides

Fungicide Concentration	Av. no . of germ tubes per spore	Av. length of germ tube (μm)
Control (dis.water)		
Bavistin		
10		
20		
30		
40		
50		
Dithane – M45		
10		
20		
30		
40		
50		

Result : By comparing the values of control with different concentrations, the effective concentration of the fungicide that has maximum effect on spore germination is identified. Of the types of fungicides tested the effective fungicide is identified.

Experiment No. 13

PHYTOTOXIC EFFECT OF FUNGAL CULTURE FILTRATE

Aim : To study the phytotoxic effect of fungal culture filtrate on seedling growth.

Requirements : Culture filtrates of pathogenic species of *Alternaria, Fusarium, Curvularia* , seedlings of wheat or rice, conical flasks,

Procedure : Prepare Czepak dox broth medium by using following composition

Sodium Nitrate	- 0.2 g
Magnesium sulphate	- 0.5 g
Potassium dihydrogen ortho phosphate	-1.0 g
Potassium chloride	- 0.5 g
Ferrous sulphate	- traces
Sucrose	- 30.0 g
Distilled water	- 1000ml

pH –4.5

Transfer 100 ml of medium in to each of three conical flasks, and sterilize them by autoclaving at 121 ^{0}C and 15 lb pressure for 15 minutes. After sterilization, cool the medium to room temperature. Inoculate the flasks with spores of *Alternaria, Fusarium* and *Curvularia* from pure cultures, using a inoculation needle, in front of the flame in an inoculation chamber. Incubate the flasks at room temperature, for one week (till thick mats of fungal growth was observed). After incubation, remove the fungal mats and filter the culture filtrates of the 3 flasks in to another set of 3 conical flasks separately, in an inoculation chamber.

Experiment set up : Label the above three flasks as per the type of the fungus cultured. Maintain a conical flask with 100 ml of distilled water to serve as control. Place a healthy seedling in to each of the 4 conical flasks, such that the root system was submerged in the culture filtrate and aerial parts are exposed to out side through neck of the conical flask using a thin cotton plug. Place the flasks under normal conditions of growth for the seedling. Observe the seedlings for wilting or any changes after 2-3 days.

Observations : Seedling in the control show normal growth, seedling in *Fusarium* culture show wilting, due to exudates; that of *Alternaria* show slight wilting symptoms, due to production of toxins; that of *Curvularia* show very little effect on seedlings.

Dr. M. Raghu Ram

PRACTICAL – II : CELL BIOLOGY, MOLECULAR BIOLOGY AND PLANT BIOTECHNOLOGY

List of Experiments:

1. **The Study of Structure and Functions of Cell Organelles (diagrammatic sketeches)**
   a) Lysosomes
   b) Mitochondria
   c) Chloroplast
   d) Microtubules
2. **Molecular Biology Assignments:**
   a) DNA Structure, Replication and Repair.
   b) Bacterial Genetics
   c) Gene Expression
   d) Potein Synthesis and Genetic Code.
   e) Gene Regulation.

Plant Biotechnology

3. Sterilization techniques
4. Preparation of MS Medium
5. Callus Culture from seedling explants
6. Cloning Vectors.
7. The Study of features of TL plasmid
8. Analysis of proteins by Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
9. Southern Blotting
10. Polymerase Chain Reaction
11. Synthesis of r-DNA molecule
12. Gene Cloning and Genetic Engineering

M.Sc. Botany (Final) Practical-II

CELL BIOLOGY, MOLECULAR BIOLOGY AND PLANT BIOTECHNOLOGY

1. The Study of Structure and functions of cell organelles with diagrammatic sketches

(a) Lysosomes

Every eukaryotic cell has a group of cytoplasmic organelles, the lysosomes (Fig. 1). Their main function is intracellular or extracellular digestion. Lysosomes show greater polymorphism. The primary lysosomes are dense particles of 0.4 μm, formed from small golgi vesicles and are not involved in the digestive processes. The formation of primary lysosome can be blocked by puromycin.

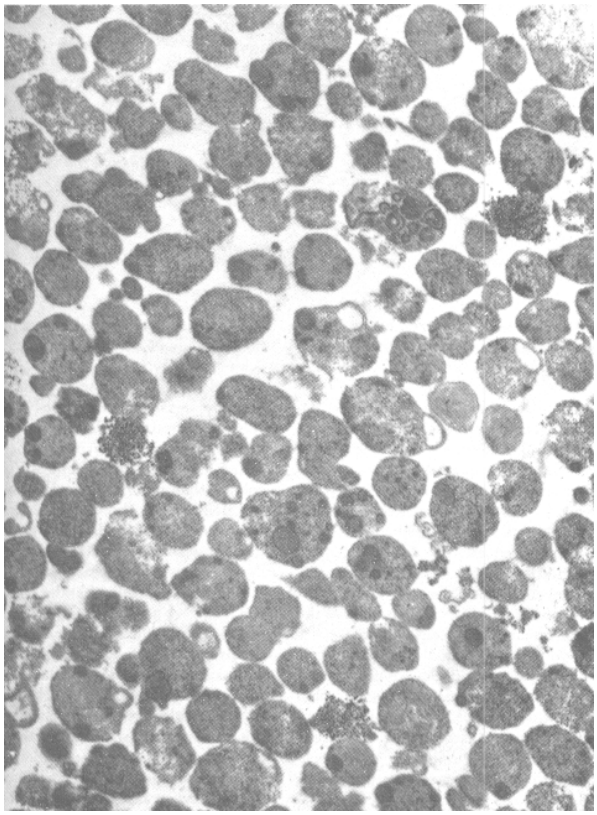


Fig. 1. Lysosomes isolated by differential centrifugation from rat liver, showing the dense particles and the variety of other dense material contained within the single membrane of the lysosome. x60,000 (Courtesy of C. De Duve).

Secondary lysosomes (digestive vacuoles) result from the association of primary lysosomes with vacuoles containing phagocytized material. The engulfed material is digested by the lysosomal enzymes. At present, 50 lysosomal hydrolases are known. The digested material is incorporated into the cell to be used again in metablic pathways. If digestion is incomplete residual bodies are formed. In amoeba and other protozoa, the residual bodies are eliminated by defecation.

Lysosomes regularly engulf bits of cytosol (mitochondrion or protein of ER) which is degraded by a mechanism called microautophagy. This is a mechanism by which the cell can achieve the degradation of its own constituents without irreparable damage. Autophagy is common during the period of rapid change (metamorphosis in insects and amphibians).

The lysosomal enzymes are secreted into ER lumen, they are glycosylated and are associated with golgi complex. Newly synthesized enzymes removed from golgi cisternae and delivered to GEBL (Golgi-Endoplasmic Lysosome) region and to lysosomes by a vesicular carrier. They contian high levels of degrading enzymes which catalyze the break down of proteins, nucleic acids, lipids and carbohydrates. In Eukaryotic cells, lysosomal enzymes breakdown old organelles, making room for new organelles, e.g. mitochondria are replaced in some tissues every 10 days.

In addition to breaking down organelles and other structures within cells, lysosomes eliminate other cells, the cell has engulfed in a process called phogocytosis (Fig. 2). When a white blood cell, phagocytizes a passing pathogen, lysosomes fuse with the resulting food vesicle releasing their enzymes into the vesicle and degrades the material within.

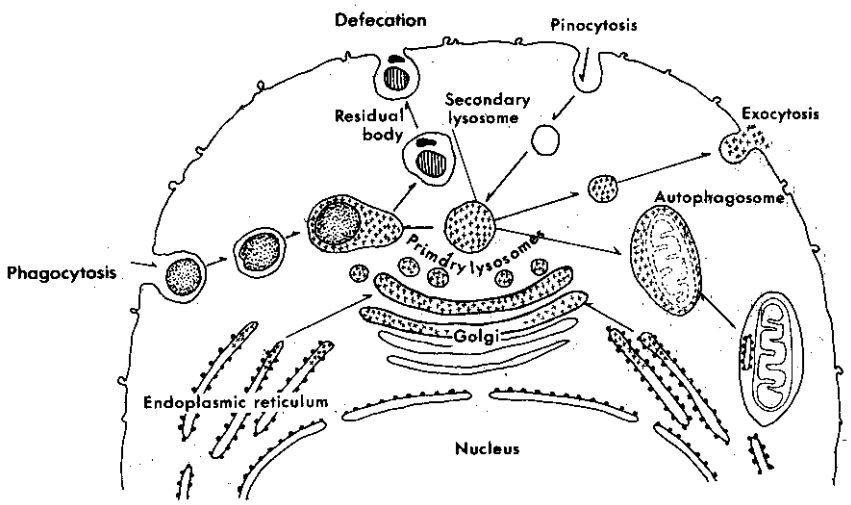


Fig. 2. Diagram representing the dynamic aspects of the lysosome system. Observe the relationships between the processes of phagocytosis, pinocytosis, exocytosis, and autophagy.

Residual bodies are formed if digestion is incomplete. In some cells like *Amoeba* and other protozoa, these residual bodies are eliminated by defecation.

Autophagic vacuoles are formed by the fusion of primary lysosomes and engulfing of degenerated intracellular organelles. Autophagic vacuoles cause the digestion of endogenous material. The products of digestion pass through the membrane of lysosome and are incorporated into the cell.

Functions: During starvation, lysosomes mobilise food from the storage organ. They take part in intracellular digestion. During metamorphosis, embryonic organs are digested (tail in tadpole of amphibians) by lysosomes. Genetic diseases result from the absence of even a single lysosomal hydrolase enzyme, e.g. bones are deformed due to accumulation of glycosaminoglycon in lysosomes. Arthritic joints may be caused by malfunctioning of lysosomes. They remove old organelles and control secretory activity of the endocrine glands. Lysosomes are important in medicine as they are involved in many diseases and syndromes. Acute release of lysosomal enzymes cause myocardial infarct. In pathological conditions, rheumatoid arthritis, silicosis and gout, release of lysosomal enzymes are found from macrophages. Lysosomes of leukocytes and macrophages are essential to the defense of the organism against bacteria and viruses. There are about 20 congential diseases called storage diseases due to accumulation of glycogen and glycolipids in lysosomes. Lysosomes in plant cells are involved in seed germination.

Mitochondria

They are present in the cytoplasm of eukaryotic cells. Mitochondria are called as chondriosomes and the power house of the cell as most of the energy is generated by oxidation of food materials. The number of mitochondria per cell varies from 50 to several thousands depending upon the type of organism. Plant cells have fewer number of mitochondria in comparison to animal cells.

They are 1 μm to 10 μm in length and 0.2 μm to 1.0 μm in width, made up of two unit membranes (Fig. 3). Outer membrane is smooth $60A^o$ to $75A^o$ thick. The inner membrane goes into folds called cristae. Cristae extend into mitochondrial matrix. It is $50\text{-}70A^o$ thickness. The space between the two membranes is intermembrane space. On the external surface of the outer membrane and on internal surface of inner membrane are found minute structure called microbodies. These microbodies on the outer surface are called subunits of Parson and inner surface are knob like structures called oxysomes. Each oxysome consists of head, stalk and base.

The cavity of mitochondria is occupied by dense fluid material called mitochondrial matrix. It is the site of Kreb's cycle. The matrix contains mitochondrial DNA, similar to bacterial DNA.

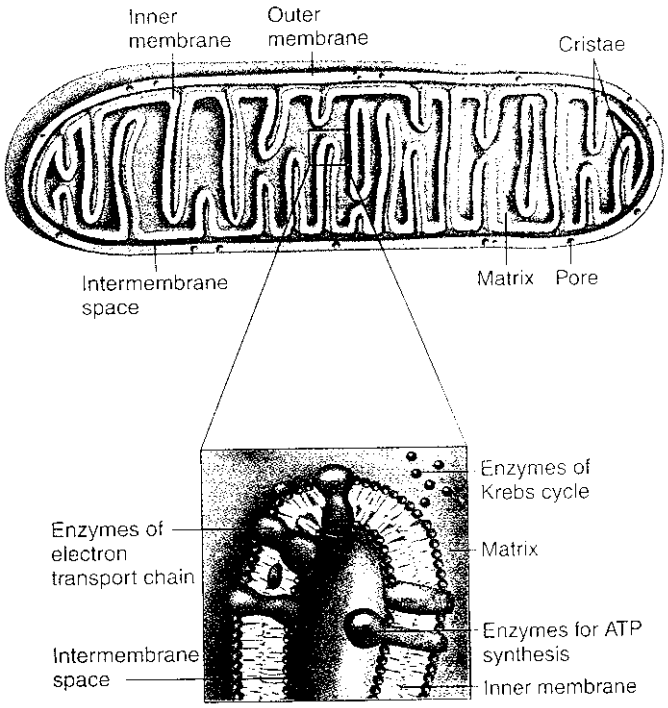


Fig. 3. Anatomy of a mitochondrion. The organization and structure of a single mitochondrion is shown. Core regions that are entirely enclosed within an inner membrane are known as the matrix. The matrix contains the mitochondrial DNA and enzymes of the Krebs cycle. Individual inner membrane foldings are called cristae. A single crista is magnified to show how enzymes of the electron transport chain carry out oxidative phosphorylation between the matrix and the intermembrane space.

Mitochondrial genome -- Mt DNA is mostly circular double stranded with a few exceptions. Numerous mitochondrial DNAs have been sequenced. It has 16s and 12s rRNA. Each haploid yeast cell has 20 mt DNA molecules. Huge variation exists in number of mitochondria and mt DNA molecules. The replication of mtDNA molecule can occur through out the cell cycle.

The size and gene content of mtDNA varies from organism to organism (Table 1and 2).

Table 1
Mitochondrial DNA sizes

Organism	Size (kb)
Plasmodium	6
Yeast	75
Drosophila	18
Pea	110
Human	16.5

Table 2

Comparison of Some Functions Encoded in mtDNA

Organism	Oxidative phosphorylation Genes	tRNAs	Genome Size (kb)
Yeast	7	25	75
Marchantia (liverwort)	14	29	186.0
Human	13	22	16.5

Like size and gene contents, the shape of mtDNAs varies mtDNAs of most species are circular, but mtDNAs of ciliated protozoans *Tetrahymena* and *Paramecium,* the alga *chlamydomonas* and the yeast are linear. The single cell eukaryotes contain a single mitochondrion.

A comparison of mitochondrial genome organization in humans, yeast and liverwort illustrates the details of mtDNA diversity (Fig. 4). 16.5 kb human mitochondrial genome carries 37 genes. In addition to 13 polypeptide encoding genes, there are 22 tRNA genes, the remaining 2 genes code for small rRNAs. There are no introns in human mtDNA.

Yeast Mt DNA is four times larger than human. Long introns A-T rich sequence spaces separate the genes in yeast mt DNA, 25% of yeast mitochondrial genome constitute introns. mtDNA of *Marchantia polymorpha* was the first plant mt DNA to be sequenced. It has many genes than non-plant mtDNAs. Most mitochondrial genome contains 16 genes for ribosomal proteins and 29 genes for unknown function.

Functions: Major part of ATP is synthesized in mitochondria. They provide raw materials for synthesis of chlorophyll, cytochrome, alkaloids and steroids. They are the sites of amino acid synthesis and fatty acid synthesis.

Although mitochondria in different eukaryotic organisms play similar role in the conversion of food to energy, evolution has produced mtDNAs with an astonishing diversity in the content and organization of their genes. Mitochondrial evolution has led to some remarkable variations on the basic mechanisms of gene expression.

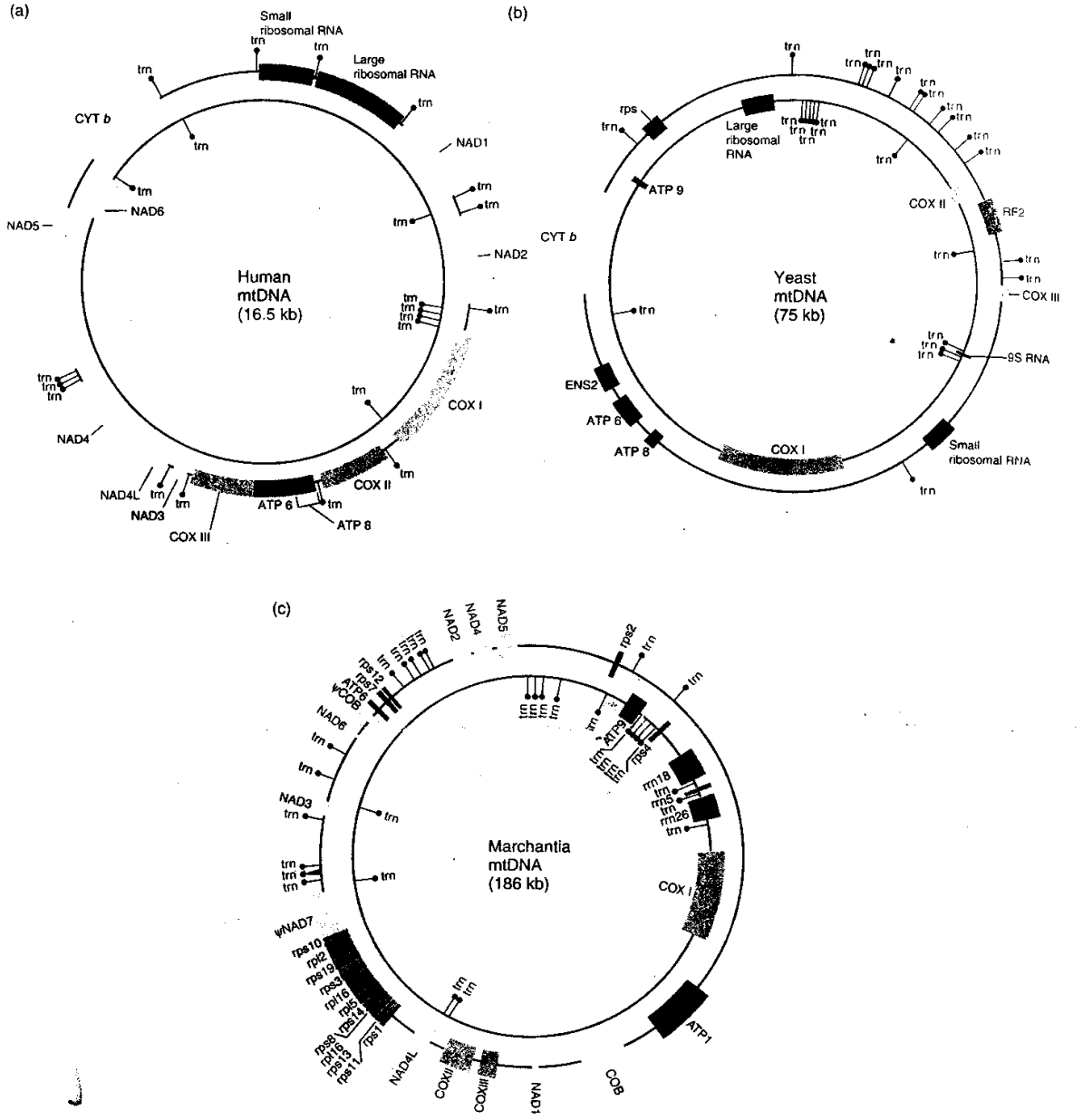


Fig. 4. Mitochondrial genomes of three species. Comparison of mitochondrial genomes of humans, baker's yeast, and liverwort. Human mtDNA is 16.5 kb long, while that of baker's yeast is 75 kb long, and liverwort is 121 kb long. Important differences illustrated in the diagrams are the presence of long intergenic sequences in yeast mtDNA and their absence from human mtDNA and differences in the numbers of genes in the mt genomes from these three organisms. The color scheme allows you to quickly see differences in numbers of genes for various functions in these three species. The following color scheme is used green, genes coding for cytochrome oxidase proteins, red, genes coding for ATPase subunit proteins; yellow, genes coding for NADH complex proteins; tan, genes coding for cytochrome complex proteins; purple, genes coding for ribosomal proteins of ribosomal RNAs, tRNA genes (tm) are indicated by black ball and stick. Genes shown on the outer and inner circles are transcribed in opposite directions.

Chloroplast

Chloroplasts are distributed through out the cytoplasm of green cells of plants. They are present in mesophyll and palisade cells of angiosperms, gymnosperms, pteridophytes and green cells of lower plants.

Chloroplasts may be spherical, oval or disc shaped (Fig. 1). They are 4-6 μm in diameter and 1 μm in thickness. Chloroplast is enclosed in two smooth membranes separated from one another by internal spaice or periplastidial space. The internal structure of a chloroplast can be differentiated into grana and stroma. The inner membrane surrounds a large central ground substance, colloidal in nature called stroma. The lamellar system with sac like green bodies are called grana.

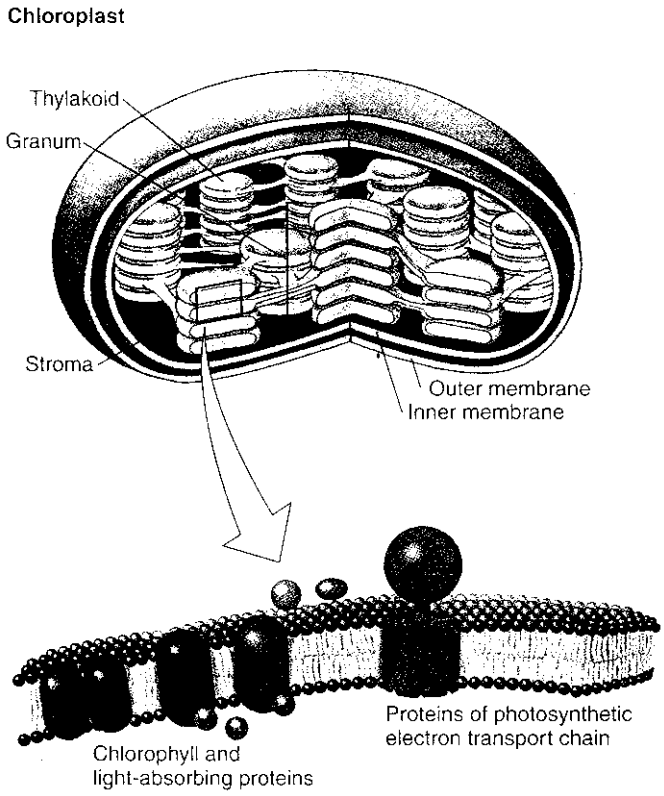


Fig. 5. Anatomy of a Chloroplast. Like a mitochondrion, a chloroplast also has an outer membrane and an inner membrane, but the inner membrane is not folded. The space found within the inner membrane-containing the chloroplast DNA and photosynthetic enzymes-is called a stroma. The stroma contains thylakoid membranes surrounding the thylakoid lumes. The magnified portion of a thylakoid membrane indicates the presence of photosynthetic enzymes.

The number of grana vary from 10-100 in each chloroplast. Granum consists of 10 or more thylakoids, like stack of coins arranged one above the other. The space enclosed by thylakoid is lumen. The thylakoid membranes connecting one granum with the other are called intergranal lamella.

In thylakoids, chlorophyll, carotonoid molecules are present. They are sites of light reaction of photosynthesis. Stroma is the site of dark reaction with necessary enzymes. The products of light reaction, ATP, NADPH, H^+ diffuse into stroma from grana. Ribosomes, DNA, RNA are also found. Due to the presence of these nuclear materials they are self replicating and are semiautonomous in nature.

The chloroplast DNA varies in size from 120-217 kb (Table 3). It is circular, like genes of yeast mtDNA and shows introns.

Table 3

Chloroplast DNA sizes

Organism	**Size (kb)**
Chlamydomonas reinhardtii	196
Marchantia (liverwort)	121
Nicotiana tabacum (tobacco)	156
Oryza sativa (rice)	135

The liverwort chloroplast genome is sequenced (Fig. 6). It contains 92 protein encoding genes and 36 genes for tRNAs and rRNAs. The similarity with bacterial genome is, cpDNA genes are organied in clusters that resemble bacterial operons. cpDNA encodes proteins that carry out photsynthetic electron transport and other aspects of photosynthesis, RNA polymerase and chloroplast gene expression. Inhibitors of bacterial translation, such as chloramphenicol and streptomycin inhibit translation in chloroplasts as they do in mitochondria.

Because of morphological and molecular likenesses, it is probable that mitochondria and chloroplasts started out as free-living bacteria that merged with the ancestors of modern eukaryotic cells to form cellular community. It is now accepted as **endosymbiont theory**.

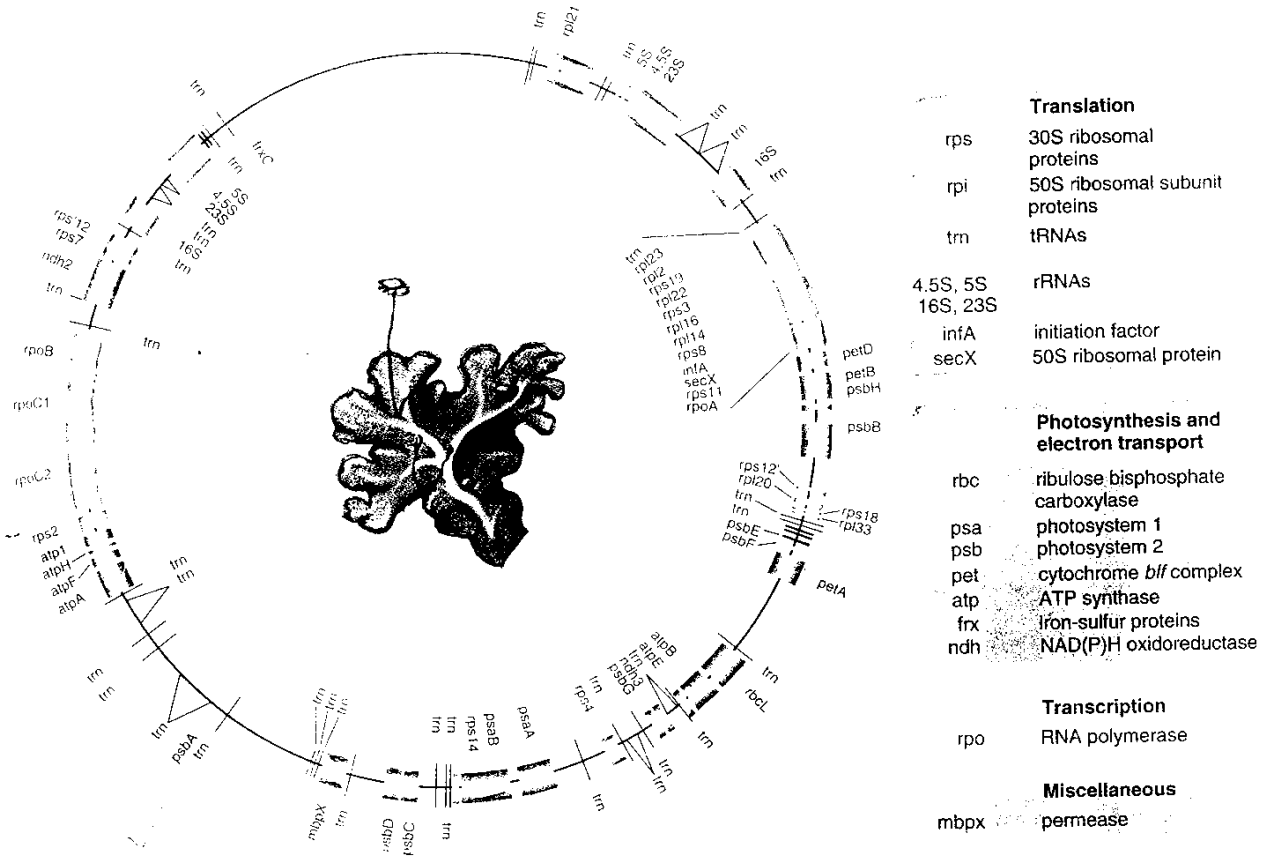


Fig. 6. Chloroplast genome of the liverwort, *M. polymorpha*. The relative locations and symbols of some of the 128 genes are indicated. Genes are color-coded according to general function.

Microtubules

Microtubules are universally present in the cytoplasm of eukaryotic cells and are characterized by their tubular appearance in different cell types. The first observation was made by Robertis and Franchi, 1953. The stability of different microtubules varies. Cytoplasmic and spindle microtubules are rather labile, whereas those of cilia and flagella are resistant to various treatments.

Cytoplasmic microtubules are uniform in size and are straight (Fig. 7). They are 25 nm in diameter and several micrometers in length. The wall of microtubule consists of linear filamentous structures about 5 nm in diameter. Although they show the same morphological characteristics, they differ in other properties, e.g. microtubes of cilia and flagella are much more resistant to various treatments, cytoplasmic microtubules disappear if stored at 0^{o}C after treatment with colchicine.

Fig. 7. Electron micrograph of the pancreas of a chick embryo showing cytoplasmic microtubules, and the replication of centrioles; *c*, the two centrioles; *dc*, daughter centrioles; *cl*, cilium; *m*. microtubules. X50,000 (Courtesy of J. Andre).

Microtubules are composed of protein subunits. Tubulin is the principal protein of cilia and flagella. Two different monomers A and A are present in flagella. The isolation of tubulin has permitted specific antibodies against this protein to be produced. These antibodies may be used as immunofluroscent probes for localizing microtubules in the cytoplasm of a wide variety of cultured cells (Fig. 8).

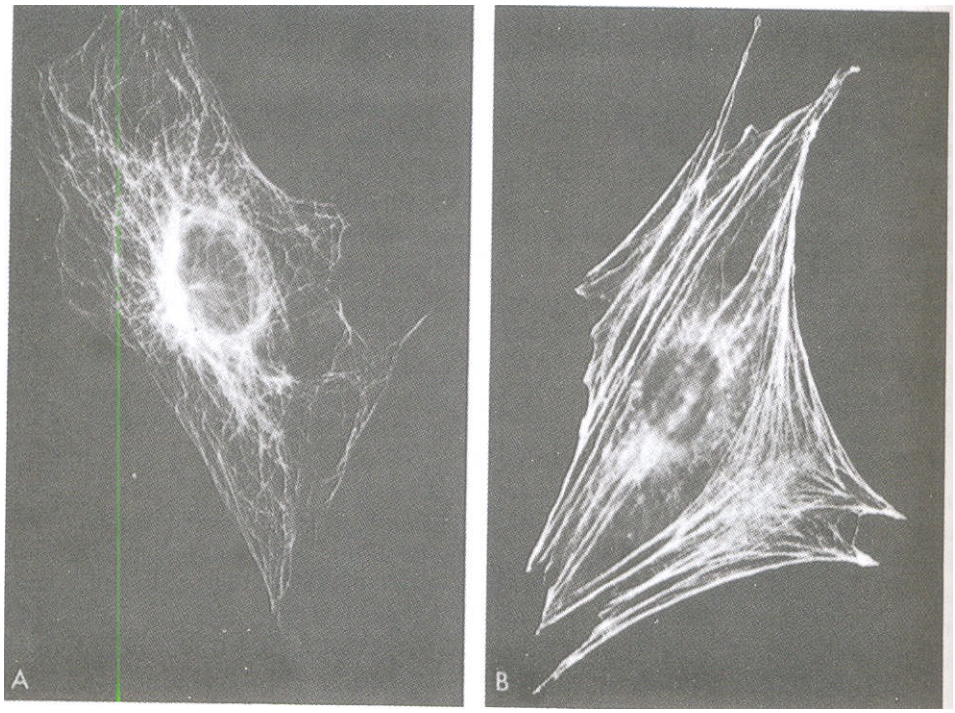


Fig. 8. Cultured 373 cells stained by immunofluorescence with antibodies against tubulin (A) and actin (B). x600. (Courtesy of M. Osborn and K. Weber)

FUNCTIONS

Mechanical Function: The shape of some cell processes has been correlated to the orientation and distribution of microtubules. They determine the shaping of the cell.

Morphogenesis: They shape the cell during cell differentiation.

Cellular polarity and motility: The determination of intrinsic polarity of certain cells is related to the microtubules. The directional gliding of the cell can be replaced by a random movement.

Circulation and Transport: They are involved in the transport of macromolecules in the cells interior.

Thus several functions related to the primitive forms of cell motility have been attributed to microtubules. They play mechanical function, shape of the cell, cell differentiation in nerve cells, spermiogenesis. The polarity and directional gliding of cultured cells depend on microtubules. They play role in the contraction of the spindle movement of chromosomes and centrioles, ciliary and flagellar motion etc.

2. MOLECULAR BIOLOGY ASSIGNMENTS

(a) DNA Structure, Replication and Repair

1. Which bases in nucleic acids are purines and which are pyrimidines?

Ans: Purines: adenine, guanine. Pyrimidines: cytosine, thymine, uracil.

2. In a nucleic acid which carbon atoms are connected by a phosphodiester group?

Ans: The 3' and 5' carbon atoms.

3. How many hydrogen bonds are there in an AT and a GC base pair?

Ans: AT, 2; GC, 3.

4. What chemical groups are at the end of a single polynucleotide strand?

Ans: A 5′-phosphate and a 3′-hydroxyl group.

5. In what sense are the two strands of DNA antiparallel?

Ans: The sugars have opposite orientation in the two strands; thus, at one end of a double-stranded DNA molecule one strand terminates with a 3′-OH group and the other strand with a 5′-P group.

6. Analysis of a DNA sample from a bacterium indicates that 18% of the bases are A What fraction is C?

Ans: If 18% is A, 18% is also T, for a total of 36% for A + T. Therefore, G + C will be 64%, and C will be half that, or 32%.

7. One of the complementary strands of two DNA molecules is given below. Which DNA molecule would have the lower temperature for strand separation? Why?
1. AGTTGCGACCATGATCTG 2. ATTGGCCCCGAATATCTG

Ans: Molecule 1, because of the long tract of GC pairs in molecule 2.

8. ^{15}N-labeled DNA from phage T4 is mixed with T4 DNA of normal density. The solution is then heat-denatured and renatured. The resulting DNA is analyzed by centrifugation in a CsCl density gradient. How many bands will be observed and what will their relative proportions be?

Ans: Pairing will occur randomly. Thus, there are three bands, in the ratios 1 $^{15}N^{15}N$: 2 $^{14}N^{15}N$: 1 $^{14}N^{14}N$.

9. The sequence 5′ – AGTCTGACT-3′ in DNA is equivalent to which sequence in RNA?

A. 5′-AGUCUGACU-3′
B. 5′-UGTCTGUTC-3′
C. 5′-UCAGUCUGA-3′
D. 5′-AGUCAGACU-3′

Ans:- A

10. Which of the following correctly describes A-DNA?

A. a right-handed antiparallel double helix with 10 bp/turn and base lying perpendicular to the helix aixs.
B. a right-handed antiparallel double helix with 12 bp/turn.
C. a right-handed antiparallel double helix with 11 bp/turn and bases tilted with respect to the helix axis.
D. a globular structure formed by short intramolecular helices formed in a single stranded nucleic acid.

Ans:- C

11. Denaturation of double stranded DNA involves…

A. breakage into short double-stranded fragments.
B. separation into single strands.
C. hydrolysis of the DNA backbone.
D. cleavage of the bases from the sugar-phosphate backbone.

Ans:- B

12. Which of the following is common to both *E. coli* and eukaryotic chromosomes?

A. the DNA is circular.
B. the DNA is packaged into nucleosomes.
C. the DNA is contained in the nucleus.
D. the DNA negatively supercoiled.

Ans:- D

13. In what region of the interphase chromosome does transcription take place?

A. the telomere
B. the centromere
C. euchromatin
D. heterochromatin

Ans:- C

14. What can be concluded about the nucleic acids in the table below?

Molecule %U	%A	%T	%G	%C	
1	28	28	22	22	0
2	31	0	31	17	21
3	15	15	35	35	0

Ans:- We must first look to see if U or T is present, for this determines whether the molecule is RNA or DNA, respectively. Molecule 2 is RNA. Now we look at base composition. In double stranded molecules, A bands with T (or U) and G bonds with C. This relationship holds for molecules 1 and 3, so they are double stranded, and molecule 2 is single stranded. Finally, the melting temperature increases with the amount of G-C, so the melting temperature of 3 is greater than that of 1.

15. Which of the following is an example of highly repetitive DNA.

A. Alu element
B. histone gene cluster
C. DNA minisatellites
D. dispersed repetitive DNA

Ans:- C

16. In which mode of replication does a parental circle generate a circle with a linear branch?

Ans:- Rolling circle replication.

17. Name three enzymatic activities of DNA polymerase I.

Ans:- Polymerasing activity, 5′-3′ exonuclease, 3′-5′ exonuclease.

18. In what direction does a DNA polymerase move along a template strand?

Ans:- From 3′ end to 5′ end

19. What are the precursors for DNA synthesis?

Ans:- Deoxynucleoside triphosphates

20. Which enzymatic activity of pol I and of Pol III is responsible for proof reading?

Ans:- 3′ $\rightarrow$ 5′ exonuclease activity

21. How do organisms solve the problem that all DNA polymerases move in the same direction along a template strand, yet double stranded DNA is antiparallel?

Ans:- One strand is copied from 3′ to 5′ end and other strand is copied in the direction opposite to that of the movement of the replication fork by synthesis in short pieces.

22. Which strand, leading or lagging, is usually separated from the replication fork by a gap?

Ans:- Lagging strand.

23. What is the fundamental difference between the initiation of replication and of rolling circle replication?

Ans:- Rolling circle replication is initiated by single strand break 8 replication does not need such a break.

24. If UV-irradiated phage are incubated in a buffer or exposed to light prior to plating, how will the efficiency of plaque b formation change?

Ans:- It will be unaffected, since phage particles do not contain any repair enzymes.

25. Which repair system cleaves thymine dimmer's?

Ans:- Photoreactivation.

26. Which enzymes are required for excision repair in *E. coli*?

Ans:- Uvr endonuclease, polymerase I and DNA ligase.

(b) Bacterial Genetics

1. How does an Hfr cell differ from an F^+ cell?

Ans: In an Hfr cell F is integrated into the chromosome.

2. Is there only one or several possible sites of integration of F in the chromosome?

Ans: Many. Each integration site produces a particular Hfr cell line.

3. Distinguish F^+ and Hfr transfer with respect to the amount of genetic material (DNA) transferred and the intactness of the transferred unit.

Ans: An Hfr cell can transfer more DNA, but usually only a fragment of the chromosome is transferred, An F^+ or F′ cell generally transfers the intact plasmid.

4. In a time-of-entry experiment, recombination frequency is determined for a particular gene at various times. How are these frequencies used to determine the time of entry of that gene?

Ans: The values are extrapolated back to the time axis, the time corresponding to a frequency of zero is the time of entry.

5. Genes *p*, *f*, and *q* have times of entry of 7, 11, and 19 minutes, respectively. What is the gene order and what are the map distances, in time units, with respect to the transfer origin?

Ans: The order is p f q and the map distances are origin-7-*p*-4-*f*-8-*q*

6. How are F′ plasmids produced?

Ans: Aberrant excision of F from an Hfr cell, such that adjacent bacterial genes are contained in the circular plasmid.

7. Could a single stranded DNA molecule with base sequence 5′-GATTGCCGGCAATC-3′ fold back on itself to form a hairpin.

Ans: Yes

8. It has been demonstrated experimentally that most highly repetitive DNA sequences in the chromosomes of eukaryotes are not transcribed. What does this indicate about the function of highly repetitive DNA?

Ans:- Highly repetitive DNA is junck DNA, not expressed.

9. How does a geneticist, doing interrupted matting experiments, know that the locus for the drug-sensitivity allele, used to eliminate the Hfr bacteria after conjugation, has crossed in the F-strain?

Ans:- When the drug sensitivity locus pass into the F-strain there will be general decline in the recovery of recombinants after selection because both Hfr and F-members of a conjugation event can be killed.

10. Three Hfr strains of E. coli (P4x, KL 98, and Ra-2) are mated individually with an auxotrophic F-strain, using interrupted mating techniques. Using these data construct a map of the *E. coli* chromosome, including distances in minutes.

Approximate Time of Entry

Donor Loci	Hfr P4X	Hfr KL98	Hfr Ra-2
Gal+	11	67	70
Thr+	94	50	87
Xyl+	73	29	8
Lac+	2	58	79
His+	38	94	43
Ilv+	77	33	4
Argg+	62	18	19

11. How many different petri plates and selective media are needed?

Ans:- Lac - Gal, 9 mts; gal - his, 27 mts; his - arg G, 24 mts; argG – xyl, 11 mts, xyl – ilu 4 mts; ilu – thr, 12 mts; thr – lac, 8 mts.

For every interruption, a complete medium plate and one each of the seven selected plates are needed.

12. A mating between his^+, leu^+, thr^+, pro^+, str sensitive cells (Hfr) and his-leu-thr-pro-, str resistance cells (F-) is allowed to continue for 25 minutes, the mating is stopped and the genotypes of the recombinants determined. The results appear at the top the next column. What is the first gene to enter and what is the probable gene order?

Ans: The leu-pro his. We see the cells that are thr^+ are most frequent. The chance of conjugation being interrupted increases with the length of time for mating. Therefore, genes far from origin of

transfer appear less frequently. Since we see no his^+ and we stopped the mating at 25 mts, his must be after 25 minutes on the map.

c. Gene Expression

1. If the following sequence of bases represents the start of a gene, what is the sequence of the transcribed RNA, what is its polarity, and what is the polarity of the DNA?

GCTACGGATTGCTG
CGATGCCTAACGAC

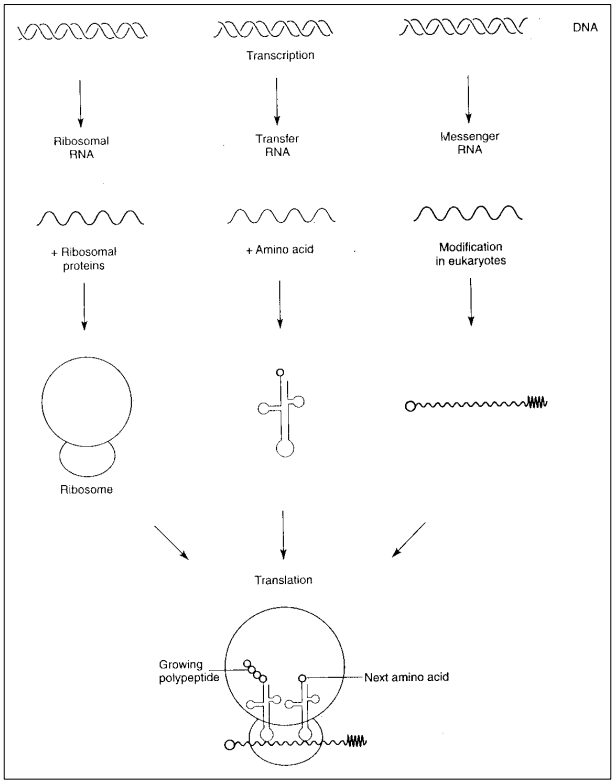
Ans:- Begin by writing the complementary strand to each DNA strand: CGAUGCCUAACGAC for the top and GCUACGGAUUGCUG for the bottom. Now look for the start codon, AUG. It is present only in the RNA made from the top strand, so the top strand must be transcribed. The polarity of the start codon is 5′-AUG-3′. Since transcription occurs 5′-3′, and since nucleic acids are antiparallel, the left end of the top strand is the 3′-end.

2. What are the transcription start and stop signals in eukaryotes and prokaryotes? How are they recognized? Can a transcriptional unit include more than one transnational unit (gene)?

Ans:- The transcription start signal is the promoter, recognized by the sigma factor of RNA polymerase transcription stop signals are terminators, some of which require the factors, olycistronic transcripts are common n prokaryotes.

3. Diagram the relationship of the three types of RNA at a complementarily?

Ans:-



Relationship among the three types of RNA—ribosomal, transfer, and messenger—during protein synthesis. All three types are found together at the ribosome during protein synthesis.

4. In what ways does the transcriptional process differ in eukaryotes and prokaryotes?

Ans:- Although, the overall process is similar in prokaryotes and eukaryotes, differences exist in number of different RNA polymerases and transcription factors in eukaryotes; exact DNA sequences recognized; polycistronic nature of prokaryotic DNA; and post-transcriptional process in eukaryotes.

5. What would be the effect on the final protein product if an intervening sequence were removed with an extra base? One base too few?

Ans:- Removing one base too many or few would result in a shift in the reading frame during translation. Thus radically alter the protein product.

6. What is heterogeneous nuclear in RNA? Small nuclear ribonucleoproteins?

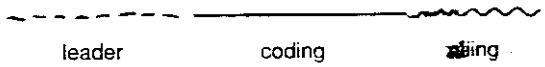
Ans:- Heterogenous nuclear mRNAs are eukaryotic transcripts before post-transcriptional modifications. Small nuclear ribonucleoproteins are the components of the eukaryotic mRNA splicing apparatus.

7. What is a stem-loop structure? An inverted repeat? A tandem repeat? Draw a section of DNA double helix with an inverted repeat of seven base pairs.

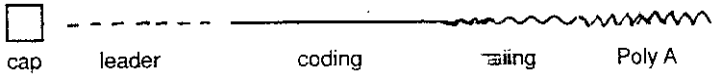
Ans:- When a single strand of DNA or RNA forms a short interval double helical part, an inverted report is read outward on both strands of helix from central point. A tandem repeat is a segment of nucleic acid repeated consecutively.

5′TCCGGTCCGGTCCGG3′
3′AGGCCAGGCCAGGCC5′

8. Draw a typical mature mRNA molecule of prokaryote and eukaryote. Label all regions.



Leader prokaryote coding trailing



Cap Eukaryote Poly A

Both molecules will have 5′ and 3′ ascending regions, the leader and trailing sequences, resperacy. Eukaryotic mRNA has a 5′ cap and a 3′ poly A tail.

9. For the RNA sequence below, determine the sequence of both strands of the DNA from which it was transcribed. Indicate the 5′ and 3′ ends of the DNA with an arrow, which strand was transcribed.

5′-CCAUCCAUGACACCCUUGCUAACGC-3′

d. Protein synthesis and Genetic code

1. Synthetic mRNA is made from a mixture of ribonucleotides supplied to a cell free system in the relative proportion of 3U : 2G : 1A.

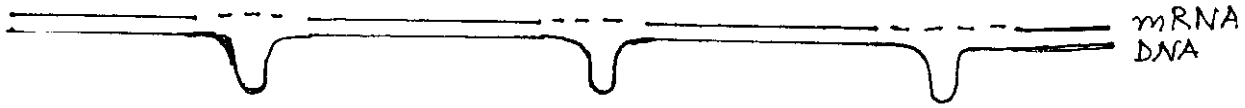
What fraction of the resulting triplets would be: a) UGA b) uuu

Since 6 is the total proportion

Ans: $UGA = \frac{3}{6} \times \frac{2}{6} \times \frac{1}{6} = \frac{6}{216}$

$UUU = \frac{3}{6} \times \frac{3}{6} \times \frac{3}{6} = \frac{27}{216}$

In the drawing of gene below, solid lines (_____) represent coding regions and dashed lines (- - - - - - -) represent introns. Draw what an RNA-DNA hybrid would look like if cytoplasmic mRNA is hybridized to a nuclear DNA.



3. What amino acids could replace methionine by a one base mutation?

Ans:- The code for methionine (internal as well as initiation) is AUG. If the A is replaced, we would get UUG (Leu), CUG (Leu), and GUG (Val); if the U is replaced we would get AAG (LYS)

< ACG (THr), and AGG (Arg); and if the Gis replaced, we would get AUA (Ile), AUU (Ile), and AUC (Ile). Hence, one base change in the codon for methionine could result in six different amino acids.

4. If an artificial mRNA contains two parts uracil to one of cytosine, name the amino acids.
Ans:

12 6 6 3
--- phenylalanine, ---- serine; ---- leucine; ---- proline
27 27 27 27

5. What amino acids could replace methionine by a one-base mutation?

Ans: The codon for methionine (internal as well as initiation) is AUG. If the A is replaced, we would get UUG (Leu), CUG (Leu), and GUG (Val); if the U is replaced we would get AAG (Lys), ACG (Thr) and AGG (Arg); and if the G is replaced, we would get AUA (Ile), AUU (Ile), and AUC (Ile). Hence, a one-base change in the codon for methionine could result in six different amino acids.

6. How do prokaryotic and eukaryotic ribosomes recognize the 5′ end of mRNAs? Could eukaryotic mRNAs be polycistronic?

Ans: In prokaryotes, the Shine-Dalgarno sequence is used for mRNA-ribosomal alignment. In eukaryotes, the scanning hypothesis describes the need for a 5′ cap and scanning behavior. Because a 5′ cap is needed for ribosomal alignment, only one polypeptide can be synthesized per mRNA (there is only one site of ribosomal attachment per eukaryotic mRNA).

7. How many single-base deletions are required to restore the reading frame of an mRNA? Give an example.

Ans: Three.

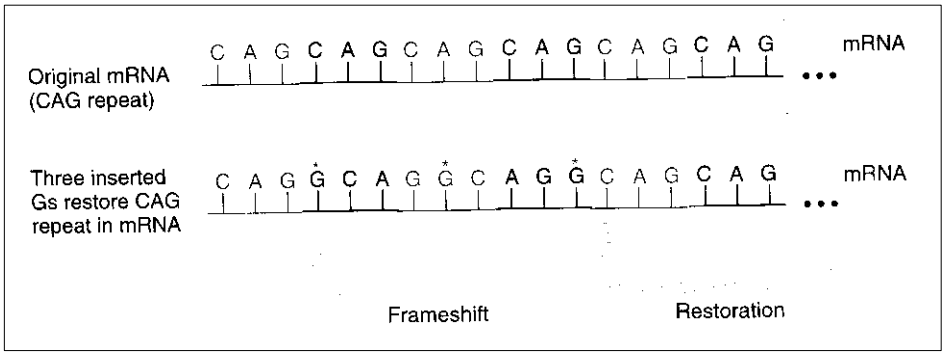


Fig. 1. The coding frame of CAG repeats is first shifted and then restored by three additions (insertions), *Asterisks* (*) indicate insertion.

8. A "nonsense mutation" is one in which a codon for an amino acid changes to one for chain termination. Give an example. What are its consequences?

Ans: 5′-UUA-3′ → 5′-UAA-3′. The consequence is that the growing polypeptide will be terminated at an improper point, probably producing a nonfunctioning enzyme or protein.

9. What are the consequences of having a prokaryotic initiation tRNA recognized by an internal methionine codon?

Ans: There would be blockage of further protein synthesis because of the N-terminal formyl group that prevents a peptide bond. The growing peptide would be stopped at that point.

10. What is the role of EF-Ts in elongation? EF-Tu? What are their eukaryotic equivalents?

Ans: EF-Tu brings a tRNA to the A site at the ribosome. EF-Ts is involved in recharging EF-Tu (see Fig. 10). The eukaryotic equivalent is eEF1.

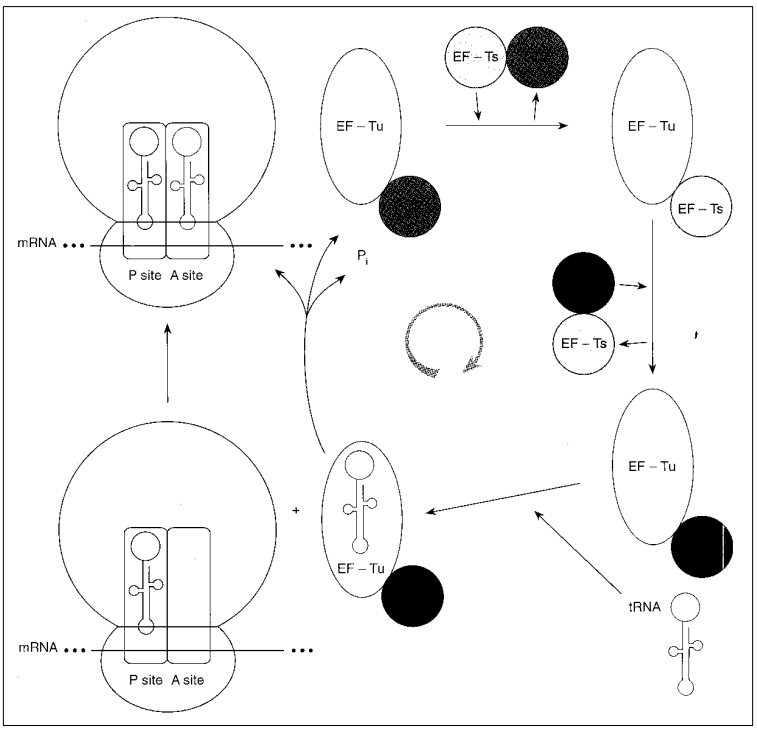


Fig. 2. The EF-Ts/EF-Tu cycle. EF-Ts and EF-Tu are required for the attachment of a tRNA to the A site of the ribosome. At **top center**, we have EF-Tu attached to a GDP. The GDP is then displaced by EF-Ts, which in turn is displaced by GTP. A tRNA attaches and is brought to the ribosome. If the codon-anticodon fit is correct, the tRNA attaches at the A site, with the help of the hydrolysis of GTP to GDP + P_i. The EF-Tu is now back where we started.

11. What are the roles of RF1 and rF2 in chain termination? What are their eukaryotic equivalents?

Ans: RF1 and RF2 recognize nonsense codons. Their eukaryotic equivalent is cRF.

12. What is a signal peptide? What role does it play in eukaryotes? What is its fate?

Ans: A signal peptide is a sequence of amino acids at the amino-terminal end of a protein that signals that the protein should enter a membrane (see Fig. 11.24). The situation in eukaryotes is more complex because there are so many different membrane-bound organelles, each having their own membrane-specific requirements. Signal peptides are usually cleaved off the protein after the protein enters or passes through the membrane.

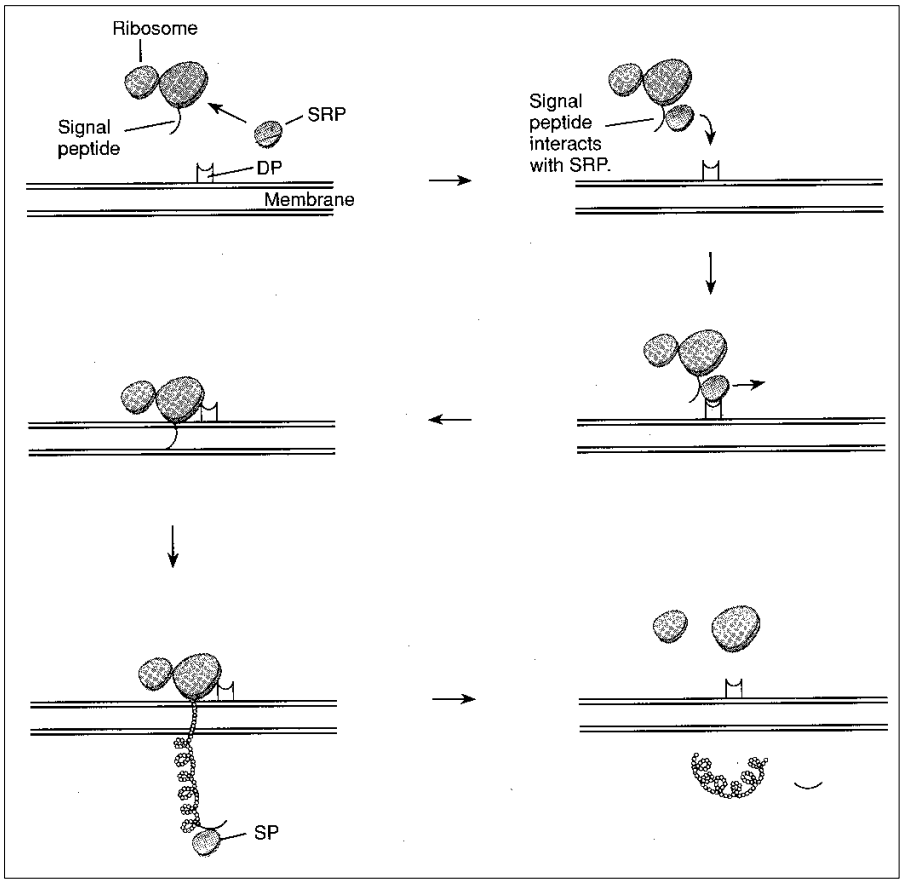


Fig. 3. The signal hypothesis. A signal peptide is recognized by a signal recognition particle (SRP) that draws the ribosome to a docking protein (DP) on the membrane. The peptide synthesized then passes directly into and through the membrane. A signal peptidase (SP) on the other side of the membrane

removes the signal peptide, which has completed its function. When translation is completed, the ribosome drops free.

13. A protein has leucine at a particular position. If the codon for leucine is CUC, how many different amino acids will appear as a result of single base substitutions?

Ans: 6. Begin with CUC, then list all possible codons in which the first C is changed; then list all possible codons in which only the U is changed. Finally, list all codons that result in a change of the second C.

List 1	**List 2**	**List 3**
AUC : *ile*	CAC : *his*	CUG
UUC : *phe*	CGC : *arg*	CUA all *leu*
GUC: *val*	CCC: *pro*	CUU

14. A transcribed DNA strand has the following sequence:

3′-TACTAACTTACGCTCGCCTCA-5′

a. What is the sequence of RNA made from this strand?
b. What is the sequence of amino acids made by the RNA?

Ans: a. 5′-AUG AUU GAA UGC GAG CGG AGU-3′
b. N-met-ile-glu-cys-glu-arg-ser

First determine the sequence of the RNA complementary to the given DNA strand. Don't forget about polarity; as the strand is written, the 5′ end of the RNA will be on the left. Blocking off successive groups of three bases allows the determination of the codons. Use the code to determine the amino acid sequence.

15. Which of the following list of feature correctly apply to the genetic code?

A. Triplet, degenerate, nearly universal, comma-less, non-overlapping.
B. Triplet, universal, comma-less, degenerate, non-overlapping.
C. Overlapping, triplet, comma-less, degenerate, nearly universal.
D. Overlapping, comma-less, non-degenerate, nearly universal.

Ans:- A

16. Which of the following statements about tRNAs is false?

A. most tRNAs are about residues long and have CCA as residues 74, 75 and 76.
B. Many tRNAs contain the modified nucleosides pseudouridine, dihydrouridine, ribothymidine and inosine.

C. tRNAs have a common L-shaped tertiary structure with three nucleotides at one end able to base pair with an anticodon on a messenger RNA molecule.
D. tRNAs have a common coverleaf secondary structure containing three single stranded loops called the D-, T-, and anticodon loop.

Ans:- C

17. Which three statement are true? The aminoacyl tRNA synthetase reaction …

A. joins AMP to the 3′-end of the tRNA.
B. is a two step reaction.
C. joins any amino acid to the 2′ - or 3′ hydroxyl of the ribose of residue A 76.
D. is highly specific because the synthetases use identify element in the tRNA as to distinguish.
E. join AMP to the amino acid to produce an intermediate.
F. releases Ppi in the second step.

Ans:- B, D and E.

18. Which one of the following statement correctly describes initiation of protein synthesis in *E. coli*.?

A. the initiator tRNA binds to the shine- Dalgarno sequence.
B. three initiation factors are involved and IF2 binds to GTP.
C. the intermediate containing IF1, IF2, IF3, initiator tRNA and mRNA is called the 30s initiation complex.
D. binding of the 50s sub-unit releases IF1, IF2, IF3, GMP and Ppi.
E. the initiation process is complete when and empty p site.

Ans:- B.

19. Coli release factor 1 (RF1) recognizes which codons?
A. UAA only
B. UAG only
C. UGA only
D. UGA and UAA.
E. UAG and UAA.
F. UAG and UGA.

Ans:- E.

20. Assume that an average polypeptide contains 400 amino acid residues. How many base pairs will be required to code for 50,000 polypeptides.

Ans: 60×10^6 (400 x 3 x 50000)

21. If a DNA-RNA hybrid were made, would the strands be parallel or antiparallel?

Ans: Antiparallel.

22. Define coding strand and antisense strand.

Ans: A coding strand is a segment of a DNA strand that is copied by RNA polymerase. An antisense strand is a DNA strand that is complementary to a coding strand.

23. Write down the two RNA sequences which could conceivably result from complete transcription of the following DNA duplex.

5′ A G C T G C A A T G 3′
3′ T C G A C G T T A C 5′

Indicate the 5′ and 3′ ends of each transcript.

Ans: PPP-5′-AGCUGCAAUG-3′ and PPP-5′-CAUUGCAGCU-3′

24. What parts of a mRNA molecule are not translated?

Ans: Leaders, spacers, and the unnamed region following the last stop codon of an mRNA are untranslated regions.

25. How many codons could be contained in a four-letter code?

Ans: $4^4 = 256$

26. What are the three stop codons?

Ans: UAA, UAG, UGA.

27. What is the principal start codon and to what amino acid does it correspond?

Ans: AUG; methionine.

28. Which of the following properties are essential for the function of a tRNA molecule: (1) recognition of a codon; (2) recognition of an anticodon; (3) ability to distinguish one amino acid from another; (4) recognition of DNA molecules?

Ans: 1.

29. Which chain-termination codon could be formed by a single base change from UCG, UUG, and UAU?

Ans: UAG.

30. Ribonuclease contains 124 amino acids. What is the least number of nucleotides you would expect to find in the gene encoding the protein? The first amino acid is not methionine.

Ans: (3 x 124) + 3 (termination) + 3 (initiation) = 378.

31. With what polarity is mRNA read?

Ans: The 5′ end is read first.

32. What is the direction of synthesis of a polypeptide chain?

Ans: The amino end is made first.

30. Which site, A or P, can never be occupied unless the other site is also occupied?

Ans: The A site.

33. A polysome has a 3′ and 5′ end. At which end will the polypeptide chain attached to the ribosome be longer?

Ans: The 5′ end, since ribosomes move along the mRNA from the 5′ end to the 3′ end.

(e) Gene Regulation

1. Would synthesis of an enzyme that is needed continually be regulated?

Ans:- Not always especially it needed in large quantities.

2. What is the biochemical action of an inducer?

Ans:- It binds to a repressor.

3. Which enzymes of the lac operon are regulated by the repressor.

Ans:- B Galaetosidase, (Laczz product) lactose permease lac Y produce and transacetylase C (lac A product)

4. What term describes a gene that is expressed continually, even though its transcription may be autoregulated?

Ans:- It is constitutive.

5. Is the lac repressor itself made constitutively or is it induced?

Ans:- Constitutive.

6. Is it necessary for a repressor gene to be adjacent to the operator?

Ans:- No, repressor is a diffusible protein.

7. Is it necessary for the operator to be very near the promoter?

Ans:- Yes, since binding of the repressor to the operator interferes with binding of RNA polymerase to the promoter.

8. When glucose is present, is the concentration of cyclic AMP high or low?

Ans:- Low.

9. Does the binding of cAMP-CRP to DNA affect the beinding of a repressor in any way?

Ans:- No

10. Which one of the following statement about catabolic-regulated operons is false?

A. cAMP receptor protein (CARP) and catabolite activator protein (CAP) are different names for the same protein.
B. When glucose is present in the cell cAMP levels fall.
C. CRP binds to cAMP and as a result activates transcription.
D. CRPbinds to DNA in the absence of cAMP.
E. CRP can bend DNA resulting in activation of transcription.

Ans: A

11. Which two of the following statement about transcription factors are true?

A. the helix turn helix domain is a transcriptional activation domain.

B. dimerization of transcription factors occurs through the basic domain.
C. leucine zippers bind to DNA.
D. it is often possible to get functional transcription factors when DNA binding domains and activation domain from separate transcription factors are fused together.
E. The same domain of a transcription factors can act both as a repressor and as an activation domain.

Ans:- D and E.

PLANT BIOTECHNOLOGY
3. STERILIZATION TECHNIQUES

Principle

The culture medium, especially when it contains sugar, will also support the growth of micro-organisms like bacteria, fungi etc. so if they come in contact with medium either in cellular form or in spore form, the microorganisms grow faster then the higher plant tissue due to their brief life cycle and will kill the tissue. The micro-organisms may come from glass vials, instruments, nutrient medium used for culture and even from plant material itself. Therefore the surface of plant tissue and all non-living articles including nutrient medium should be sterilized.

Procedure

(i) **Sterilization of Non-living Articles:-** The routine sterilization procedure of non-living articles such as nutrient medium, glass gods, distilled water, instruments (wrapped with brown paper) is by autoclaving under steam at a pressure of 15 1b/in^2 and a temperature of 120^{o}C for 15 minutes.

Thermolabile compounds are often added in the medium and such medium is sterilized either at room temperature or in cold by passing through bacterial filter.

An alternative method of sterilizing glass goods and instruments is by heating in an oven at 150^{o}C for 3-4 hrs.

(i) **Sterilization of Plant Material:-** Plant material which si to be cultured, should be surface sterilized to remove the surface borne micro-organisms. This procedure is done in front of a laminar air flow or inside the inoculation chamber before the plant material is inoculated on to the culture medium.

(1) Thoroughly washed plant material or explant in tap water is immersed in 5% u/v solution of liquid detergent such as Tween for 10-15 minutes. Then wash the material thoroughly in tap water and finally in distilled water. This step can be

done in the general laboratory. Subsequent steps are done infront of a laminar air flow or the presterilized inoculation chamber.

(2) Dip the explants in 70% ethylalcohol for 60 seconds.
(3) Immediately transfer the material into an autoclaved jaw bottle and pour 0.1% mercuric chloride ($HgCl_2$) 5-10% sodium hypochlorite (u/v) solution. Keep them for 10-15 minutes. During that period, the bottle is frequently swirled for shaking so that all surfaces of plant material come equally in contact with sterilant.

(4) After 10-15 minutes, decant the sterilant and wash the explants thoroughly with several canges of autoclaved distilled water to remove all traces of sterilant.

(5) Then the explants are ready for culture.

PREPARATION OF MS STOCK SOLUTION

Aim

To prepare stock solutions for media preparation.

Principle

As it is not possible to weigh and mix all the constituents just before the preparation of medium. It is time consuming and a tedious job. If 100 ml or 200 ml medium is to be prepared then it is very difficult to weigh some constituents that are used in very small quantity for one litre medium. So it is convenient to prepare the concentrated stock solutions of macro salts, micro-salts, vitamins, amino acids, hormones etc.

Procedure

Macro salts should be made at ten/twenty times of (x 10@ x 20) their final concentration in the medium while micro-nutrients can be prepared at thousand times (x1,000) of final strength. While making the stock solutions it is advisable to dissolve each constituent completely before adding another otherwise precipitation of salts may occur.

Stock solution of Macro-salts (x 20)

To make 1,000 ml of this stock solution, dissolve the salts one after another in 800 ml of double distilled water (DDH_2O) and then make up the volume. The solution is filtered and can be stored in refrigerator (10-16^{O}C) for a long period until the solution is totally used.

Stock solution of Micro-salts (x 1,000)

The minor elements are weighed and dissolved in 100ml double distilled water and prepare a stock solution of 1000 x concentration.

Stock solution of Iron-EDTA (x200)

Dissolve at first 745 mg of Na_2EDTA in 75 ml boiling distilled water then add gradually 557 mg of $FeSO_47H_2O$. Keep on a magnetic stirrer for at least 1hr in hot condition until the colour of the solution changes to golden yellow. Finally make the volume to 100 ml and store in refrigerator (5^oC). This solution specifically must be stored in an amber coloured bottle.

1. MS – Major :

Constituents	Amount mg/l	Amount (gm) to be taken for stock (x20)	Final volume of stock (ml)
NH_4NO_3	1650	33	1000
KNO_3	1900	38	
$CaCl_2$, $2H_2O$	440	8.8	
KH_2PO_4	170	3.4	
$MgSO_4$ $7H_2O$	370	7.4	

2. Stock solution of Micro salts (x1000)

Constituents	Amount mg/l	Amount (x100) to be taken for stock solution	Final volume of stock (ml)
H_3BO_3	6.2	620	100
$Na_2MoO_42H_2O$	0.25	25	
$CaCl_2$, $6H_2O$	0.025	2.5	
$CuSO_45H_2O$	0.025	2.5	
$ZnSO_472O$	8.6	860	
$MnSO_44H_2O$	22.3	2230	

3. Stock Solution of MS vitamins (x1000)

Constituents	Amount mg/l	Amount (x50) to be taken for stock solution	Final volume (ml)
Thiamine HCl	0.1	5	50
Nicotinic acid	0.5	25	
Pyridorine HCL	0.5	25	

4.

Hormone	Required amount for stock solution (mg)	Amount of solvent required to dissolve 1N NaoH	Amount of DD4	Final Cane mg/ml
Auxins	10	1ml	9ml	0.5
2,4-dichloropheroxy acetic acid (2,4-D)	10	1ml	9ml	0.5
Indole acetic acid (IAA)	10	1ml	9ml	0.5
α-Naphthalene acetic acid (NAA)	10	1ml	9ml	0.5
Cytokinins Kinetin (6-furfuryl amino purire)	10	1ml	9ml	0.5
BAP (6-Benzl-amino-purine)	10	1ml	9ml	0.5

Stock solution of MS vitamins : (x1,000)

A 1,000 x stock solution of vitamin were prepared by dissolving Thiamine HCL, Nicotinic acid & Pyridonine HCl in 50 ml of distilled water.

Stock solution of Glycine (x 1,000)

Dissolve 40mg glycine in 20ml of DDH_2O store at O^OC for 15 days.

Stock solution of kI (x 1,000)

Dissolve 83mg of kI in 100ml of DDH_2O. Store in refrigerator ($10 – 16^O$).

Stock solution of Meso-Inositol (x 500)

Dissolve 1gm meso-inositol in 20ml DDH_2O. Store at O^OC for 15 days.

Stock solution of Hormones

Auxins and cytokinins are not directly dissolved in water. So they are first made soluble in water miscible solvents and then water is added to get the final volume.

4. PREPARATION OF MS MEDIUM

Aim : To prepare 1000ml MS medium.

Principle

To vivo plant cells, tissues and organs get their appropriate nutrient and growth requirements from the intact plant body for their organized growth and development. Isolated cell, tissues and organs also need nutrients for their *in vitro* growth and development. So nutrients are supplied artificially according to the medium formulated. The main objective of medium preparation is to culture the cell, tissue and organ *in vitro*.

Procedure

Media should be prepared with care and the following procedure is recommended.

To make 1 litre of MS medium –

(i) Dissolve 30 gms cane sugar in 200ml DDH_2O.

(ii) Take DDH_2O in another flask and add in sequence the appropriate amount of stock solution as follows :

Stock solution of macro salts	50 ml
Stock solution of micro salts	1 ml
Stock solution of kI	1 ml
Stock solution of FE-EDTA	5 ml
Stock solution of MS vit	1 ml
Stock solution of Glycine	1 ml
Stock solution of meso-inositol	2 ml

Desired concentration of auxin and/or cytobinin are added from stock solution according to the formula.

$$\frac{\text{Desired concentration}}{\text{Stock concentration}} = \text{amount (ml) of stock solution to be taken for 1 litre medium}$$

If the quantity of the medium is less than 1 litre, then hormones are added using another formula.

$$\frac{\text{Required concentration} \times \text{volume of medium}}{\text{Stock concentration} \times 1{,}000} = \text{Amount (ml) of stock solution to be added}$$

(iii) Pour filtered sucrose solution and salt, vitamins, amino acid, hormone solution mixture into a one litre measuring cylinder. Make the final volume to one litre with DDH_2O. Shake well to mix up uniformly.

(iv) Adjust the pH of the liquid medium 5.6–5.8 with the aid of 0.1 (N) HCl as 0.1 (N) NaOH. This operation is done using the pH meter.

(v) Add 8% agar to the liquid medium to make solid medium. Heat to $60^{O}C$ to dissolve the agar completely.

(vi) Dispense the culture medium into culture tube (20 ml/tube) wide mouth conical flask (25-40 ml/flask). Insert non-absorbent cotton plug wrapped with gauge cloth. Cover the plug with the help of brown paper and rubber band.

(vii) Medium is finally sterilized by autoclaving.

SEED CULTURE

Aim: To culture given seed for the production of seedlings.

Principle: Surface sterilized seeds are used to develop healthy and disease free seedlings *in vitro*.

Requirements: Surface sterilized seeds, MS medium, forcep's, filter paper, petriplates, sterile DDW.

PROCEDURE

Surface sterilization of seeds

Seeds are washed by submerging in water with few drops of liquid detergent between for 5 minutes. The seeds are rinsed under running tap water to remove detergent. They are transferred into Laminar airflow hood where 70% Ethanol is added for 2 min. The seeds are thoroughly washed with sterile distilled water. Then add 0.1% $HgCl_2$ and treat the seeds for 10 min. Wash the seeds repeatedly with sterile DDW.

Inoculation

With the help of sterile forceps, seeds are inoculated on to the test tubes with autoclaved MS media. The culture is maintained in the culture room at $25 \pm 2^{O}C$.

Result

Seedlings with healthy cotyledenay leaves were observed with in 7 days.

5. CALLUS CULTURE FROM SEEDLING EXPLANTS

Aim: To induce callus formation in seedling explants

Principle

Callus tissue is good source of genetic or karyotypic variability, so it may be possible to regenerate a plant from genetically variable cells of the callus tissue.

Procedure:

(i) Aseptic preparation of plant material.

(ii) Selection of suitable nutrient medium supplemented with appropriate ratio of auxins and cytokinins (or) appropriate auxins.

(iii) Incubation of culture under controlled physical condition.

The *in vitro* grown seedling is taken as an plant material. It is cut aseptically with sterile blade under the laminar flow into small segments. The explants are finally transferred asceptically on a suitable nutrient medium solidified with agar. For healthy callus growth usually both an auxin and a cytokinin are required.

Hormonal concentration in the medium (Indole 3 – Acetic Acid).

Auxin – IAA – 1.5 mg/lit

Cytokinin – BAP – 2 mg/lit

Incubation

Culture tubes with small segments of leaves, internodes, nodes, cotyledons are incubated at 25 $\pm$ 2^{o}C for 15 days.

Result

Proliferating cell mass (or) callus is observed in the culture tubes.

6. CLONING VECTORS
(PBR 322, cosmid and yeast artificial chromosome)

One of the most important elements in r-DNA technology is the vector. There are different types of cloning vectors for use with different types of host cells. There are plasmid vectors, cosmids, phages, phagemids, yeast artificial chromosome, transposons etc. Any vector should have the following features.

1. It must contain a replicon that enables it to replicate in host cells.

2. It should have several marker genes, to differentiate the transformed cells from non-transformed cells.

3. It should have unique cleavage site for the insertion of foreign DNA into the marker gene.

4. For the expression of cloned DNA, the vector should contain suitable control elements such as promoters, terminators etc.

PBR 322

This is one of the artificial cloning vector, widely used cloning vector (Bolivar and Rodriguez, 1977a, b). It is a plasmid vector with 4.36 kb kb double stranded DNA (Fig. 12). It contains Col EI origin of replication. It contains two antibiotic resistance genes, one is ampicillin resistance (amp^R) gene coding for β-lactamase and the other is tetracycline-resistance gene (tet^R). The plasmid contains 20 unique recognition sites for restriction enzymes. Cloning of a DNA fragment into any of these sites results in the insertional inactivation of either one of the antibiotic resistance markers.

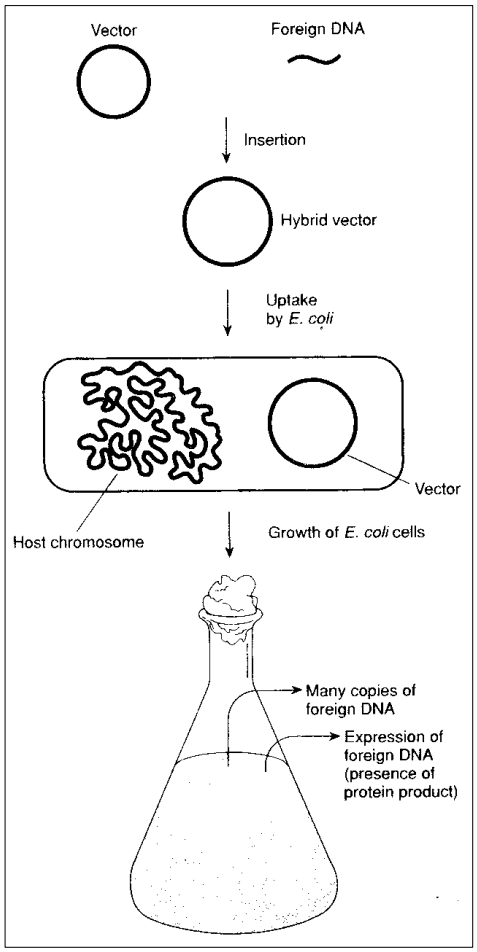


Fig. 12. Overview of recombinant DNA techniques. A hybrid vector is created, containing an insert of foreign DNA. The vector is then inserted into a host organism. Replication of the host results in many copies of the foreign DNA and, if the gene is expressed, quantities of the gene product. (All DNA shown is double-stranded).

Three types of cells are obtained:

1. Cells that have not been transformed and so contain no plasmid molecules and will be amp^S tet^S.

2. Cells that have been transformed with PBR 322 but without inserted DNA, they are amp^R, tet^R. These are transformed cells.

3. Cells that contain a recombinant DNA molecule i.e., DNA fragment has been inserted into the PBR 322 at tet^R gene cluster. These cells lose tetracycline resistance, since the fragment has inserted in the middle of tetracycline resistance gene cluster. There are recombinants amp^R tet^S. Colonies that do not grow on tetracycline agar are recombinants, whereas both transformants and recombinant will grow on ampicillin agar. By comparing the replica plates, recombinant can be picked up from agar ampicillin plates.

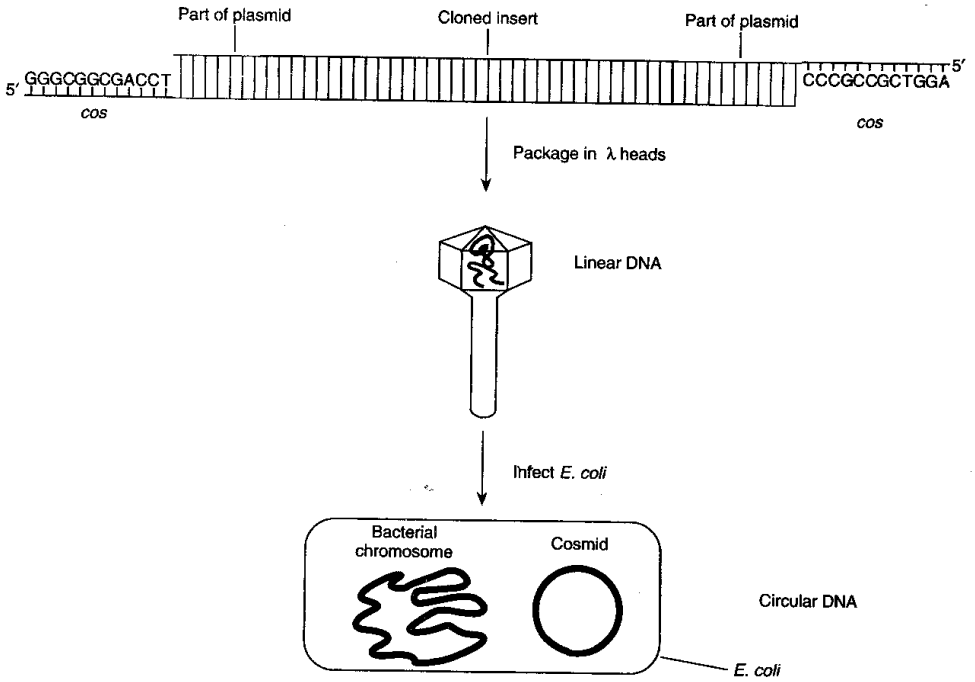


Fig. 2. A cosmid is a plasmid with *cos* sites that can be transferred into bacteria within phage lambda heads, a very efficient method of infection. The *cos* sites are single-stranded; they renamed to a *circle* when inside the host.

Cosmids: Cosmids are plasmid vectors (Fig. 2.) that contains a bacteriophage lamda *cos* site, that directs insertion of DNA into phage particles. The development of cosmid vector is based on the observation that-200 bp DNA sequence called *cos* is required for DNA packaging into phage particle. For successful packaging of DNA, there must be two COS sequences separated by 38-52

kb cosmid cloning vectors with DNA inserts of 30-45 kb can be packaged into λ phage particles. λ phage head can hold upto to 45 kb of DNA. Following absorption of these phage particles on *E. coli* host cells, the cosmid vector circularizes via the cohesive ends and replicates as a plasmid. Cosmid vectors possess an origin of replication, a selectable genetic marker and suitable cloning sites. The advantages of cosmids are that relatively large size of insert DNA (upto 45 kb) can be cloned. Therefore, cosmids are ideal vectors for genome mapping.

Yeast artificial chromosome: The development of yeast artificial chromosomes (YAC) by David Burke *et al*., (1987) extended the cloning range to several thousand bases (1000-2000 kb). A typical YAC consists of centrome elements (CEN4) for chromosome segregation during cell division, two telomere sequences (TEL) for chromosome stability, an origin of replication and one or more growth selectable markers (URA3) (Fig. 3). Normal eukaryotic chromosomes are linear. In yeast, the sequence elements required for circular plasmid vectors to be replicated and stage maintained in an extrachromosomal state have been identified as Autonomously replicating sequences (ARS). The most commonly used YAC for genome mapping of higher eukaryotes is PYAC4. The left and right arms have yeast chromosomal elements to enable replication in a yeast host, as well as markers TRP and 4RA, which permits selection for transformants. The two arms can be ligated to ECOR I ends of a large fragment of genome DNA insert to create a recombinant chromosome. This can be maintained as a linear chromosome in yeast, and is referred to as a yeast artificial chromosome. Yeast transformants containing recombinant YAC molecules can be identified by red/white color selection.

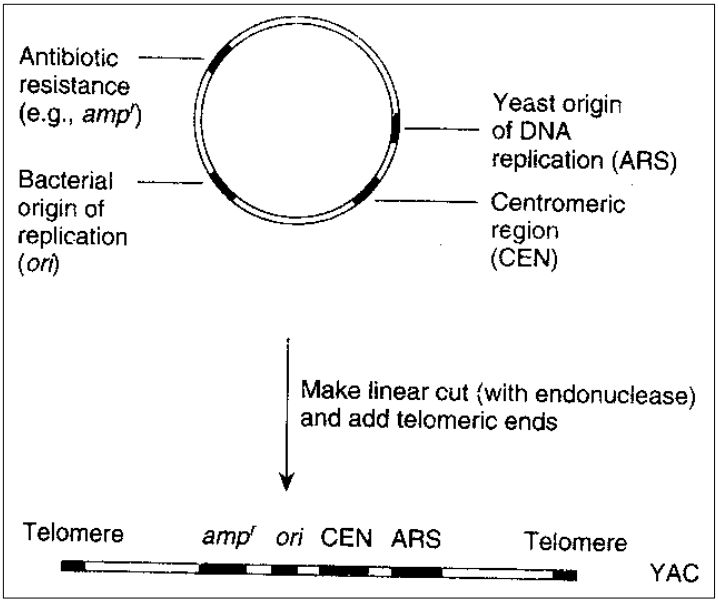


Fig. 3. *Escherichia coli* plasmid pBR322 modified for use in yeast. This plasmid survives and replicates in both yeast and *E. coli* because it contains the origin of replication for both, as well as a yeast centromeric region (CEN). When it is made linear and telomeres are added, the yeast artificial chromosome (YAC) becomes suitable for cloning large pieces of DNA.

Transformed yeast containing recombinant YAC molecules – Red colony.

Non-transformed yeast – white colony.

The advantage of this vector is, it can accommodate large inserts of DNA.

(7) The study of features of Ti plasmid – schematic representation

Ti Plasmid: Gene transfer methods are grouped as vector mediated or direct gene transfer. Vector mediated gene transfer is the transfer of genetic information between plants and transfer from other organisms to plants using vectors. Plant gene vectors being exploited for transfer of genes are plasmids of *Agrobacteria.*

Agrobacterium tumefaciens in gram negative soil bacterium, which transfers Ti plasmid into the host cells, where it becomes integrates into the plant DNA. The Ti plasmids are large, circular DNA molecules upto 200 kb in length. The T-DNA carries genes for opine synthesis (nos or ocs) and phytohormone biosynthesis and oncogenic (onc) region which cause crown gall tumour formation (Fig. 1).

This Ti plasmid have major regions for virulence, origin of replication, conjugation, oncogenicity and catabolism of opines (Fig. 2). Ti DNA is bordered by 25-bp highly conserved direct repeats of DNA. Any DNA sequence located between the borders is transferred to the plant. Oncogenes, necessary for tumor production, can be deleted, disarming the T-DNA. In this disarmed plasmids, foreign genes can be inserted (Fig. 3).

Virulence genes: They are necessary for T-DNA transfer. They are present in 8 operons vir A – vir H. They are silent, till they are induced by plant factors. Wounding is necessary for synthesis of these compounds e.g. acetosyringone and β-hydroxy acetosyringone. Dicot species produce these phenolic compounds, while monocots do not. In such cases, these compounds are added in the medium to induce vir genes. Besides the products of certain bacterial chromosome genes are also required, as they help in the attachment of **Agrobacteria** to host plant cells.

Agrobacterium plasmids are disarmed by deleting T-DNA encoded oncogenes and replacing them with foreign genes of interest. They are co-integrate vectors. The binary vector system consists of the autonomously replicating plasmids within *A. tumefacieus*, a shuttle vector that contains genes of interest between T-DNA border and helper Ti plasmid that provides vir gene products to facilitate transfer into plant cells. The helper Ti plasmids provide necessary vir gene products for transferring the T-DNA to the host plant cell. Most recently developed plant transformation vectors are binary vectors.

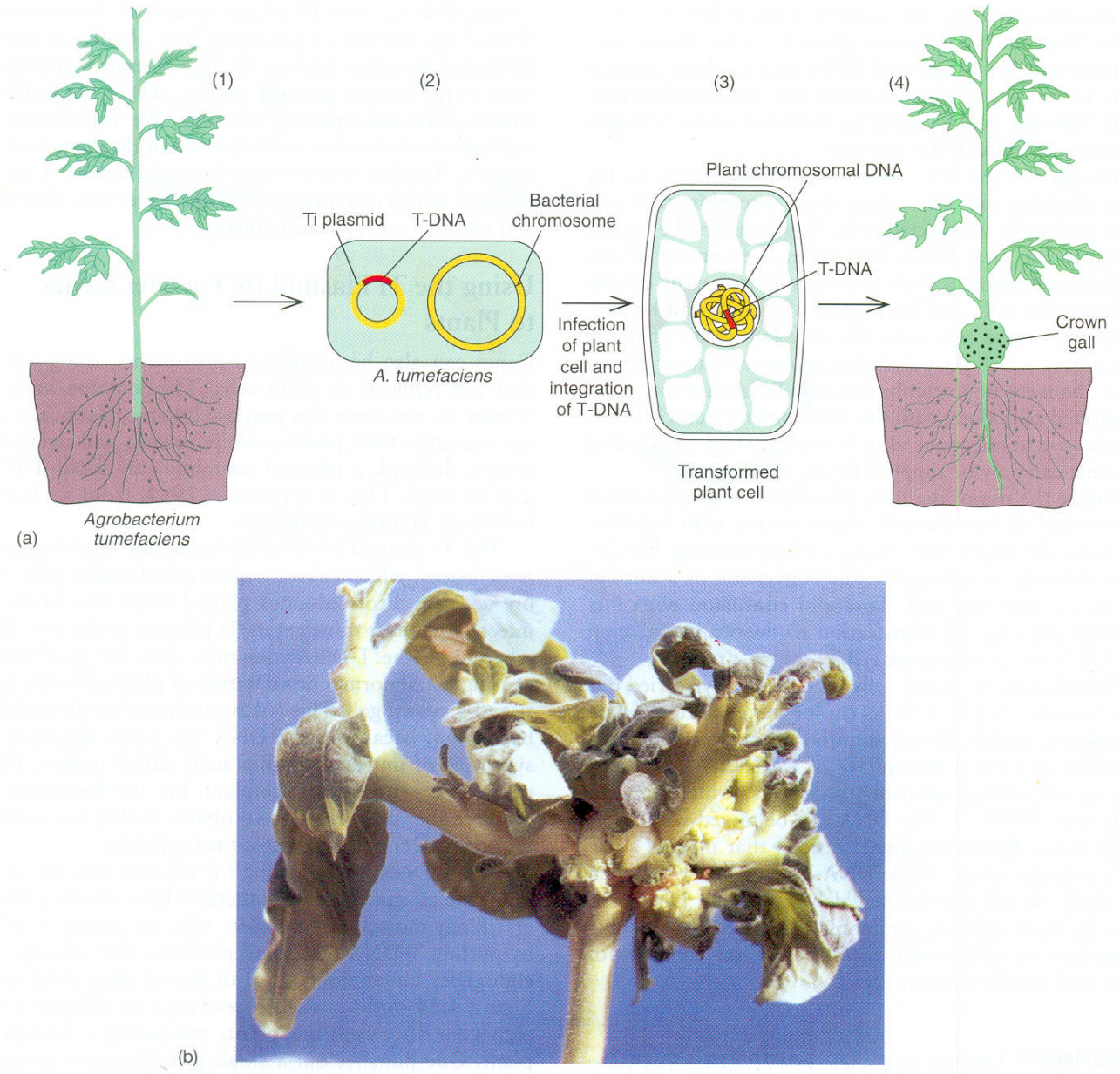


Fig. 15. Genetic organisation of Ti plasmid.

Ti plasmid has three important regions: (1) T-region with genes for hormone and opine biosynthesis and flanked by 24 bp direct repeat sequences is transferred to the plant cells; (2) Virulence region codes for proteins involved in T-DNA transfer; (3) Opine catabolism region codes for proteins involved in the uptake and metabolism of opines.

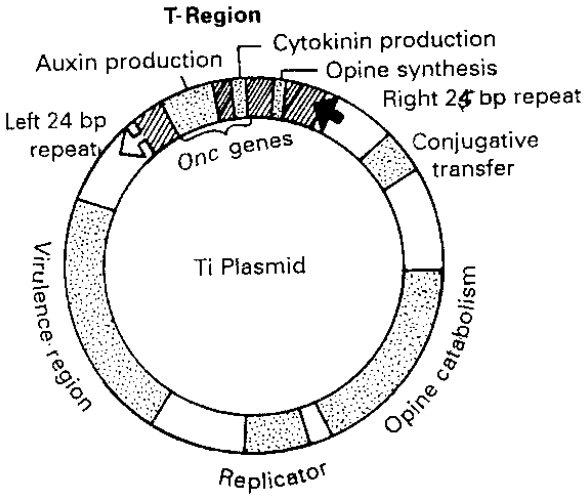


Fig. 2. Genetic organisation of Ti plasmid.

Ti plasmid has three important regions: (1) T-region with genes for hormone and opine biosynthesis and flanked by 24 bp direct repeat sequences is transferred to the plant cells; (2) Virulence region codes for proteins involved in T-DNA transfer; (3) Opine catabolism region codes for proteins involved in the uptake and metabolism of opines.

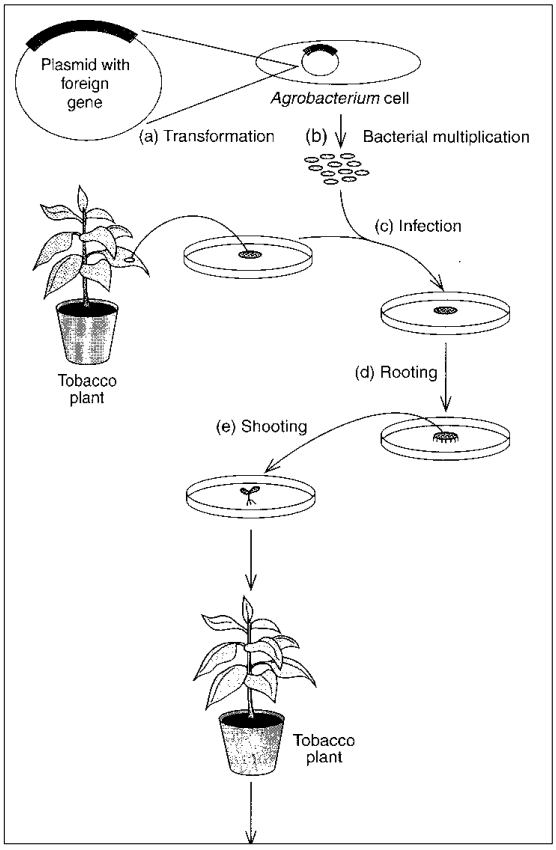


Fig. 3. **Using a T-DNA plasmid to introduce a gene into tobacco plants. (a)** A plasmid is constructed with a foreign gene (red) under the control of the mannopine synthetase promoter (blue). This plasmid is used to transform Agrobacterium cells, **(b)** The transformed bacterial cells divide repeatedly, **(c)** A disk of tobacco leaf tissue is removed and incubated in nutrient medium, along with the transformed *Agrobacterium* cells. These cells infect the tobacco tissue, transferring the plasmid bearing the cloned foreign gene, which integrates into the plant genome. **(d)** The disk of tobacco tissue sends out roots into the surrounding medium. **(e)** One of these roots is transplanted to another kind of medium, where it forms a shoot. This plantlet grows into a transgenic tobacco plant that can be tested for expression of the transplanted gene. (Source: (a) M Dell-Chilton, "A Vector for introducing New Genes into Plants:" Copyright (c) 1983 Scientific American, Inc. Reprinted by permission).

8. ANALYSIS OF PROTEINS BY SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Electrophoresis initially described by Arne Tiselius (1937) is the process by which charged particles move through a media in the presence of an electric field at a given pH. Many biological molecules possess ionisable groups at any given pH, they exist in solution as cations (+) or anions (-) under the influence of an electric field, these charged particles will migrate either to cathode or anode depending on their net charge. This phenomenon is known as electrophoretic mobility. It is technique that separates changed molecules (DNA, RNA and Protein) on the basis of migration in a appropriate matrix, subject to electron field.

Instrumentation: A wide range of Electrophoretic equipment is available. It consists of two items i.e., electrophoretic tank and a power supply. Vertical or horizontal electrophoretic tanks are commercially available. A well designed tank includes a cover to prevent evaporation of the buffer. A stabilized voltage supply is necessary (Fig. 1.).

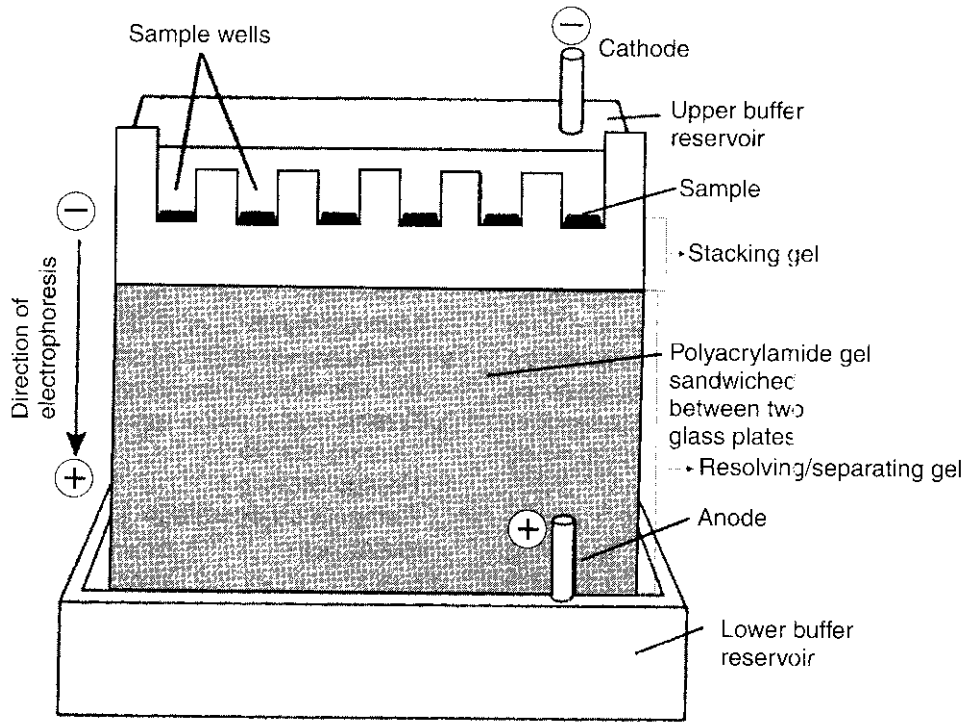


Fig. 1. Native polyacrylamide gel electrophoresis

Polyacrylamide gel: Polyacrylamide shows many advntages over starch gel because of its synthetic nature and pore size. The gel is formed by the polymerization of two nomomers, acrylamide and a cross linking agent N_2N_1 methylene-bis-acrylamide. Pore size in the gel can be

changed by changing the concentration of both polyacrylamide and bis-acrylamide. Polyacrylamide gel electrophoresis (PAGE) is performed either in cylindrical glass tubes or in flat beds.

Electrophoresis of Proteins: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used method for analysis of protein mixtures qualitatively. Samples to be run on SDS-PAGE are boiled for 5 minutes in sample buffer containing P-mercaptoethanol and SDS. The mercaptoethanol reduces disulphide bonds thus denatures the protein. An ionisable tracking dye, bromophenol blue is mixed with the sample buffer. Once the sample is loaded, current is passed through the gel. This forces all the ionic species to migrate. The negatively changed complexes continue to move towards anode. After the dye has reached to the bottom of the gel the current is turned off and gel is removed from between the glass plates and shaken in an appropriate stain sol for a few hours and washed in destain solution. The destain solution removes unbond dye from the gel. Stained proteins are observed as clear blue bands on destaining, the RF value for each band was calculated using the following formula:

$$RF = \frac{\text{Distance of the band from origin}}{\text{Distance of the dye from origin}}$$

A typical gel lake 1 – 1½ hr to prepare and set, 8 hrs to run at 30 mA and 2-3 hrs staining time. Vertical slab gels are run since it allows upto 20 different samples to be loaded. The molecular man of a protein can be determined by comparing the mobility with those of standard proteins of known molecular mass.

Equipment and Materials

Equipment

1. Slab gel electrophoresis apparatus
2. Glass plates (14 x 16 x 0.3 cm)
3. Plexiglass spacers, 1 cm wide and 1 mm thick
4. Plexiglass comb with 10 or 12 teeth
5. Power supply
6. Clamps
7. Vacuum pump
8. Gadient mixer
9. Microsyringe
10. Staining boxes

SOLUTIONS

Solution A: 29.2% (w/v) acrylamide, (w/v), N_2N_1-methylene bisacrylamide, in distilled water, stored at 4^oC in dark bottles.

Solution B: 1.5 M Tris-HCl buffer, pH 8.8, containing 0.4% SDS (w/v) (Tris 18.15 g, 0.4 g SDS, distilled water to 100 ml, adjust pH to 8.8 with 6 N HCl).

Solution C: 10% (w/v) Ammonium persulphate, freshly prepared.

Solution D: 0.5 M Tris-HCl containing 0.4% SDS (w/v), pH 6.8 (6.05 g Tris, 0.4 g SDS, distilled water to 100 ml adjust pH to 6.8 with 6 N HCl).

TEMED

Electrophoresis Buffer: 0.025 M.Tris, 0.192 M glycine, 0.1% SDS, pH 8.3 (3.0 g Tris, 14.4 g glycine, 1.0 g SDS, distilled water to 1000 ml, pH 8.3).

Sample Solubilizing Buffer: 62 mM Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol and 5% b-mercaptoethanol. Heat sample for 3-5 min in boiling water.

Standard Molecular Weight Markers: Kits are commercially available.

Fixing Solution: 50% (v/v) methanol, 10% (v/v) acetic acid in distilled water.

Staining Solution: 0.2% (w/v) Coomassie Blue R-250 in the fixing solution. Filter the solution immediately before use. It can be reused 8 to 10 times.

Destsaining Solution: 5% (v/v) methanol, 7% (v/v) acetic acid in distilled water.

Experimental Procedure:

1. Clean the glass plates by soaking overnight in a detergent, wash thoroughly with water, and then with ethanol.

2. Grease three plexiglass spacers (both sides) and place them along two side edges and along the bottom edge of the dry plate.

3. Assemble the two glass plates and the three spacers tightly with 6 clamps.

4. Place the plates vertically on a flat surface.

5. Connect the gradient mixer to the two glass plates by inserting the teflon tube between the two glass plates at the top edge.

6. Prepare the polyacralamide gradient gels (seperating gel) by mixing the mixtures as follows:

Acrylamide (%)	**5**	**10**	**15**
Solution A (ml)	1.5	3	4.5
Solution B (ml)	3	3	3
Distilled water (ml)	4.5	25	25
Solution C (µl)	25	25	25
TEMED (µl)	5	5	5

Note: Solutions should be deaerated before adding TEMED.

7. Pour the high concentration mixture (9.0 ml) (e.g. 15%) into the mixing chamber of the gradient mixer (the chamber mearest the out-let) and the low concentration (e.g. 10%) mixture into the other chamber. Start the stirrer and open the valve between these two chambers.
8. Pump the gradient into the casting apparatus using a flow rate sufficient to complete delivery well within the polymerization time, but not too fast. Ten minutes should be enough to fill the system.

9. When all the gradient has been delivered, add about 3 ml of distilled water to overlay the polymerized mixture.

10. Without disturbing the casting apparatus, disconnect the teflon tube and gradient mixer and rinse them out immediately to remove any traces of unpolymerized gel mixture.

11. Allow the gradient gel slab to polymerize completely without disturbance for at least 1-2 hours.

12. Pour out liquid and unpolymerized gel or remove by using microsyringe with long needle.

13. About 30 min before starting electrophoresis, the stacking gel of 4.6% acrylamide is prepared by mixing:

Solution A ..	1.5 ml
Solution D ..	2.5 ml
Distilled Water	6.0 ml
Solution C ..	25 µl
TEMED ..	5 µl

Note: Deaerate the mixture before adding TEMED. Polymerization will occur within 30 min.

14. Pour stacking gel and insert comb.

15. Allow stacking gel to polymerize, suck off the unpolymerized gel mixture and remove bottom clamps and bottom spacer.

16. Remove comb carefully by sliding vertically upwards.

17. Suck out unpolymerized gel again.

18. Rinse with distilled water 2 times.

19. Place slab gel plates in the electrophoresis apparatus, connect leads and add electrophoresis buffer to lower chamber.

20. Prepare samples by diluting (1:2) in sample solubilizing buffer, and place in boiling water bath for 2 min. Typically load 20-50 μl sample, containing approx. 10,000 cpm of radioactivity or 10-30 μg protein.

21. Load 10ml of samples by using micropipettes.

22. Load 10 μl molecular weight standard (5 μg total protein)

23. Fill upper chamber with SDS electrophoresis buffer and add 2 drops of 0.1% Bromophenol blue.

24. Use a syringe with bent needle to remove bubbles from under gel between the glass plates.

25. Electrophoresis the gradient gel at constant 150 V until the tracking dye has reached 0.5 cm from the bottom of the gel (approx. 3-5 h total time).

26. After completion, open plates by running a large spatula along edges to free gel from spacers.

27. Fix, stain and destain gel as follows:

- Incubate slab gel in 3-5 volumes of staining solution in staining boxes for a few hours at room temperature.

- Remove stain by placing gel in first destain (fixing solution) for a few hours. The gel will shrink due to dehydration.

- Place gel in second destain (5% methanol – 7% acetic acid) overnight with several changes. This will revert the gel to previous size.

28. Dry on gel dryer for preservation or for autoradiography.

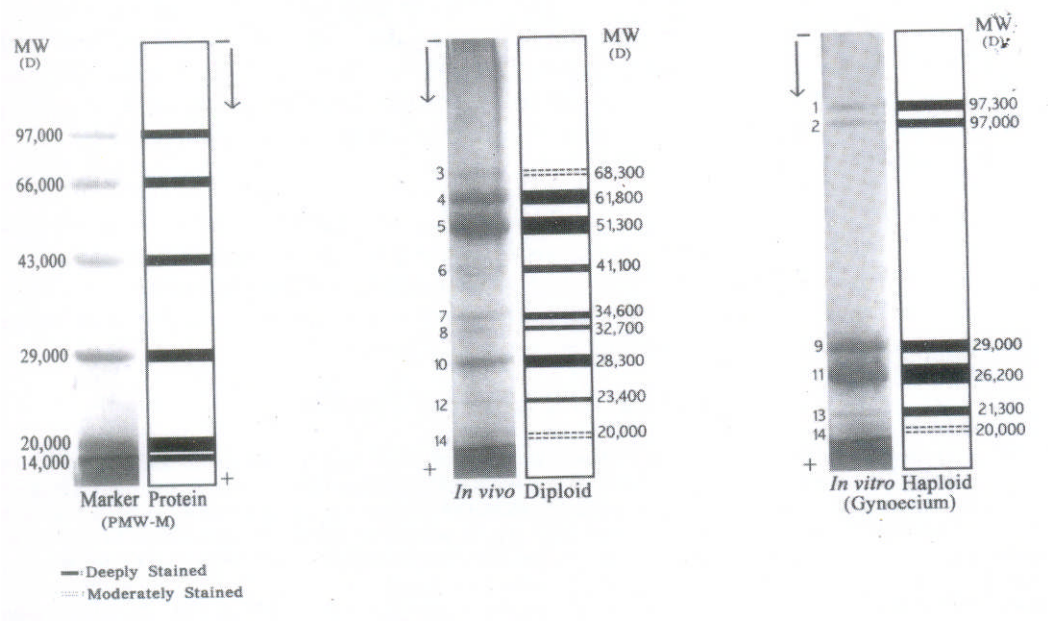


Fig. 2. Electrophoretogram of protein patterns of diploid and gynogenic haploid plants of mulberry var S_{54} analysed using standard protein marker PMW-M (Arrows indicate migration of bands towards anode). (Courtesy of Gayatri *et al*., 2002)

The analysis of the electrophoretic data revealed that the haploid plants are characterized by limited number of protein bands as compared to those of the diploid plant. Further, deeply stained and slow moving bands, 4 and 5 are specific to diploid plant and fast moving bands, 9 and 11 are specific to haploid plant. However, bands, 1, 2 and 13 are purely specific to haploid plant and are absent in the diploid plant.

9. SOUTHERN BLOTTING

When DNA segments are generated by endonuclease digestion, a desired gene must be located. We look for the gene either before or after it is cloned. In a DNA digest, there are many fragments. To proceed further, we have to transfer the electrophoresed fragments to another medium for probing. Nitrocellulore filters are excellent for hybridization, because DNA fragments bind to these members. The transfer procedure, devised by E.M. Southern is called **Southern Blotting**.

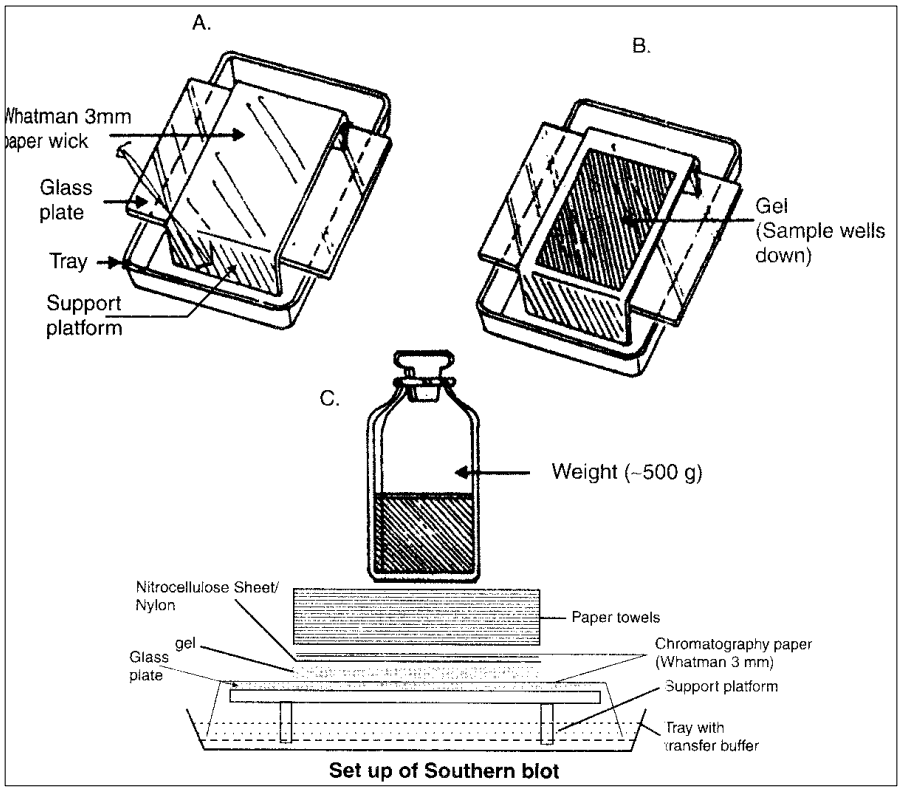


Fig. 20. Different stages in set up of a Southern blot. A-Whatman 3 MM paper wick draped over glass plate in a tray. B-Gel is placed over paper wick. C-Diagrammatic representation of different components in a Southern pyramid for blotting.

It is a method of transferring electrophoresed DNA fragments from the gel onto a nitrocellulose membrane, and subjecting it to hybridization by using a specific probe.

Steps involved in Southern Blotting

1. The given DNA sample is digested with restriction enzymes.

2. The resultant fragments are run on agarose or polyacrylamide gel. Agarose gel was used to separate DNA fragments of 20 kb, Polyacrylamide is preferred to separate smaller fragments, Pulse field gel electrophoresis was used to separate 1000-2000 kb.

3. The fragments are separated based on their size under electric field. Smaller fragments run faster than larger once.

4. The gel is stained with ethidium bromide so that the fragments flouresce in presence of uv light.

5. Marker DNA fragments are also run for comparison.

Blotting procedure

1. The separated DNA fragments are treated with alkali and the DNA is denatured.
2. The DNA fragments are now transferred to a nitrocellulose filter by placing the gel on top of a buffer then lying the nitrocellulose filter membrane on the top of gel

Order:

Weight
↑
Stack of dry filter papers
↑
Nitrocellulose membrane
↑
Gel

3. A stack of dry filter papers are placed on the membrane and a weight is placed over it.
4. The DNA becomes trapped to the nitrocellulose membrane as the buffer passed to dry filter papers through capillary action.

5. The nitrocellose membrane is baked in oven at 80^{o}C in vacuum to fix the DNA.
6. The Radio labbled probe is used to identity the specific sequence containing DNA.
7. Then the membrane is washed to remove the unhydridized probe.
8. Then x-rays are passed on to the membrane to identity where probe hybridized to single struded DNA of specific sequence, through autoradiography.

10. POLYMERASE CHAIN REACTION

The polymerase chain reaction (PCR) developed into a technique in 1985 by kary Mullis is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA (fig.1.). The number of applications of PCR include direct cloning from genomic DNA or cDNA, *in vitro* mutagenesis and engineering of DNA etc. The PCR technique basically involves three nucleic acid segments. The segment of double stranded DNA to be amplified and two single stranded oligonucleotide primers flanking this segment. Additionally there is a protein component (Taq DNA polymerase), appropriate deoxyribo-nucleoside triphosphates (dNTPs), a buffer and salts. Taq DNA polymerase is a thermostable enzyme isolated from Thermus aquaticus growing in hot springs. This enzyme does not lose its activity at high temperatures.

The primer is added in vast excess compared to the DNA to be amplified. They hybridize to opposite strands of DNA< so that synthesis by DNA polymerase extends areas the segment of DNA between them. Unlimited supply of amplified DNA is obtained by repeating the reaction which is made possible by regular denaturation of freshly synthesised double stranded DNA molecules by heating it to 90-98^{o}C. AT this high temperature, the two strands separate and the mixture with two primers recognising the two strands and bordering the sequence to be amplified is cooled to 40-60^{o}C. This allows the primer to bind to their complementary strands through renaturation.

Material required:

1. Sterile water
2. 100 mM MgCl2
3. 10x amplification buffer
4. 2 mM dNTP mix
5. 50 uM Oligunucleotide primers 1 and 2 (50 pmol/µl in sterile water: store at –20^{o}C)
6. 15 µg/ml template DNA
7. 2.5 µ/µl Taq DNA polymerase
8. Automated thermal cycler

1. Prepare a cocktail by mixing
   80 µl 10x amplificates buffer with MgCl2 to a final concentration of 2 mM.
   80 µl 2 mM dNTP mix
   8 µl 50 uM Oligonucleotide
   8 µl 50 mM Oligonucleotide 2
   544 µl 15 µg/ml template DNA.

2. Add 1 µl of 2.5 U/µl Taq DNA polymerase to each tube

3. Heat samples for 90 seconds at 94^{o}C (in either a water bath or a automated thermal cycler). This is the denaturation step.

4. Incubate the samples 2 mins. at 42^{o}C. This is the annealing step of the cycle.

5. Incubate the samples 3 min. at 72^{o}C. This is synthesis or extension step of the cycle.

6. Repeat steps 3 to 5 for another 29 cycles. The number of cycles necessary depends on both the efficiency of the reaction and the amount of template DNA in the reaction.

7. Electrophoresis 10 µl from each sample on a 1.4% agarose gel and stain with ethidium bromide.

Precautions: For applications that amplify rare templates, reagent purity is the most important parameter and avoiding contamination in every step is critical.

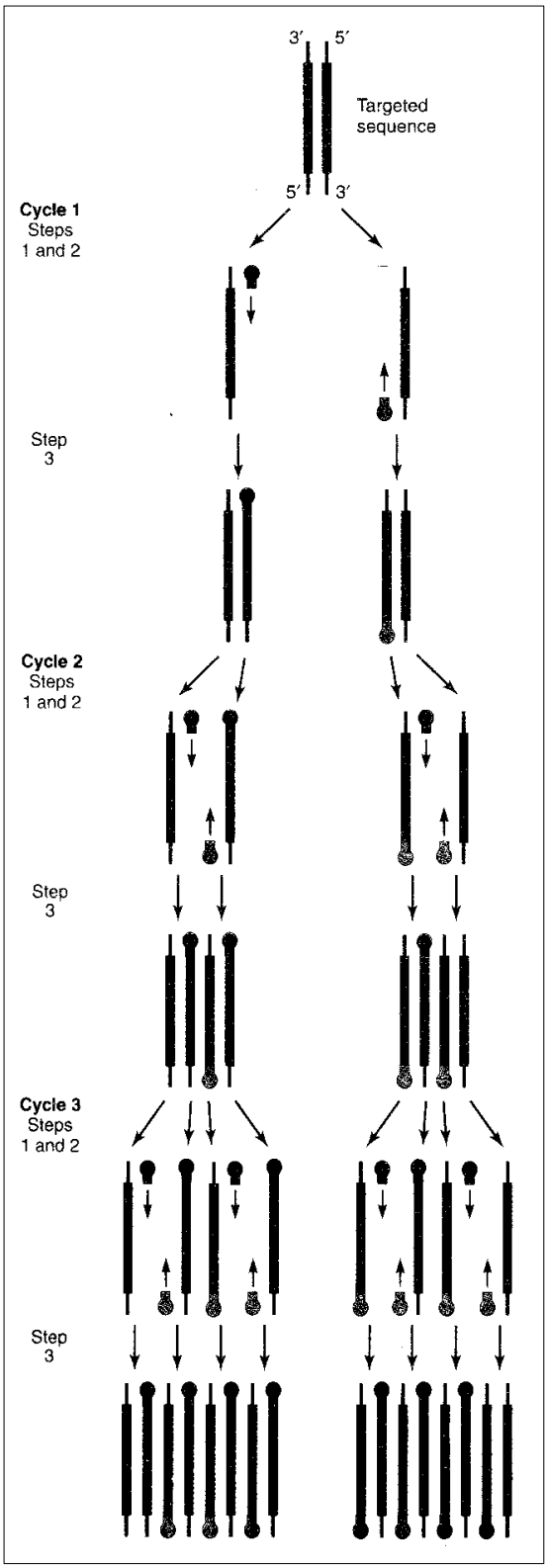


Fig. 1. Polymerase chain reaction. DNA is denatured, (step 1), primer oligonucleotides that are complementary to end sequences on the two strands anneal (step 2), and DNA replication takes place (step 3). Each step in the cycle is controlled by temperature changes. The targeted sequence is shown as *red* on one stand *blue* on the other. Primers are shown as either *green* or *yellow* lollipops. A green primer begins the copying of the red strand into a complementary blue strand; a yellow primer begins the copying of a blue strand into a complementary red stand. In three cycles, one double-stranded region of DNA becomes eight. The process requires the addition of primers, deoxynucleotide triphosphates, and DNA polymerase, as well as changing temperature cycles.

11. SYNTHESIS OF r-DNA MOLECULE

Restriction endonuclease enzymes cut DNA molecules at specific sites called palindromes. They are 4-8 base pair sequences with inverted repeats. Different enzymes recognize different but specific sequences. The first enzyme was isolated from *Haemophilus influenzae* in 1970. Now many types of restriction endonucleases have been isolated from a wide variety of bacteria (Fig.1)

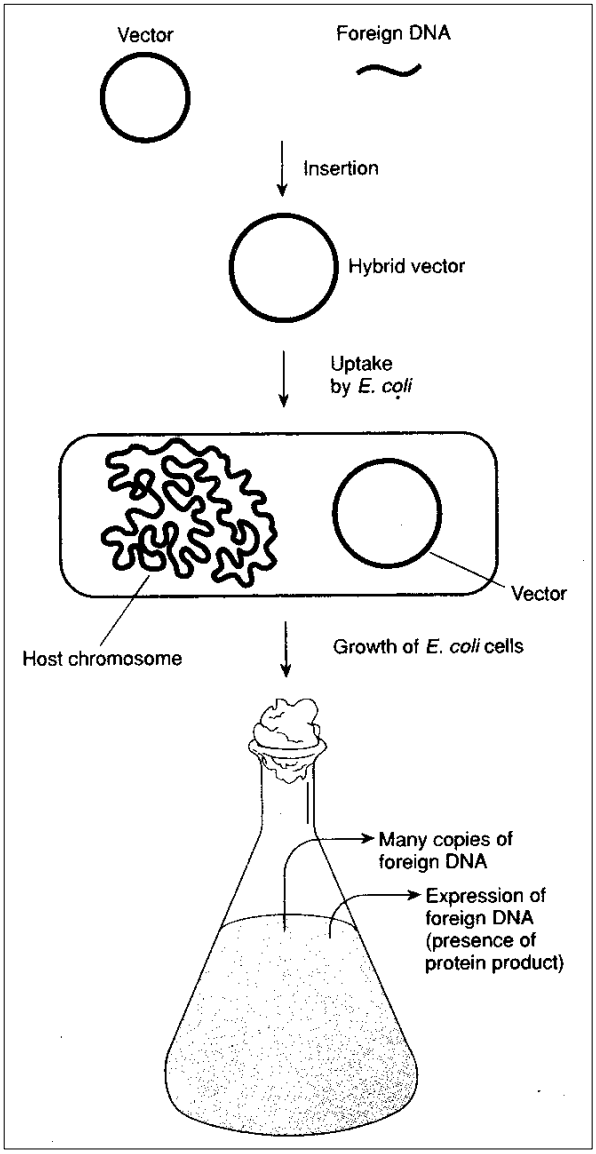


Fig. 1. Overview of recombinant DNA techniques. A hybrid vector is created, containing an insert of foreign DNA. The vector is then inserted into a host organism. Replication of the host results in many copies of the foreign DNA and, if the gene is expressed, quantities of the gene product. (All DNA shown is double-stranded).

The enzymes cut DNA resulting staggered cuts and blunt end cuts. (overhang) (fig 2).

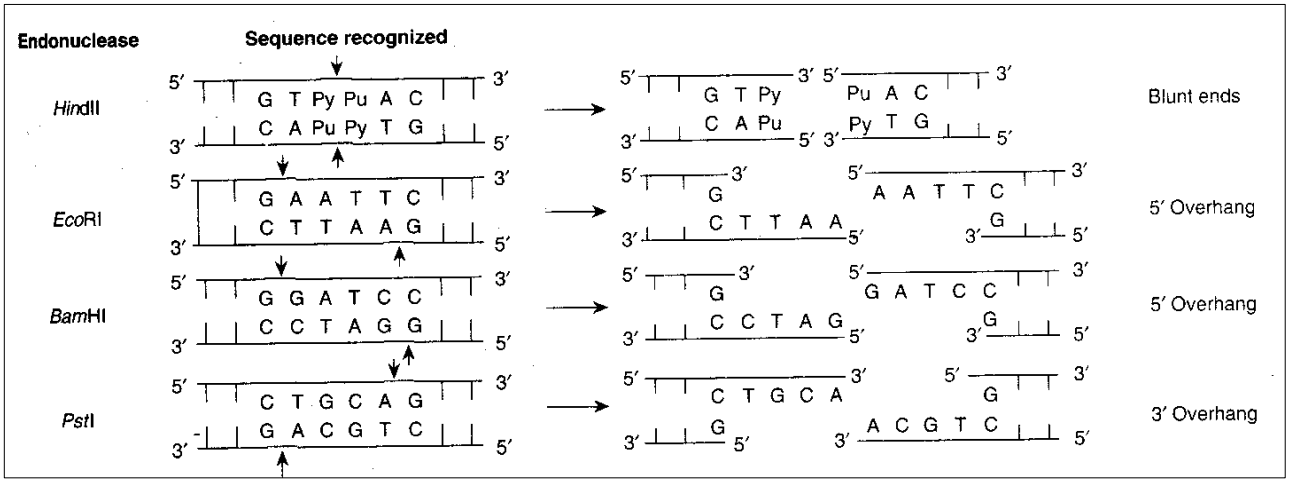


Fig. 2. Sequences cleaved by various type II restriction endonucleases. Py is any pyrimidine and Pu is any purine. *Arrows* denote places where endonucleases cleave the DNA. In 1971, K. Danna and D. Nathans showed that a restriction endonuclease would consistently cut DNA into pieces of the same size. The precision and repeatability of enzyme action made enzymes useful for further research. Not all restriction endonucleases make staggered cuts with 3' and 5' overhangs; some produce blunt ends.

The number of Type II enzyme cleavage sites in DNA molecule depends on the size of DNA, its base composition and GC content of the recognition sequences. Different DNA modifying enzymes are involved during the synthesis of r-DNA molecule.

- DNA ligase from *E. coli* and bacteriophage T_4 seals single stranded nicks.
- Bacteriophage T_4 polynucleotide kinase catalyzes the transfer of phosphate of ATP to a 5′ terminus of DNA or RNA.
- Alkaline phosphatase removes 5′ phosphate group from DNA, thus modifies the termini of DNA.
- DNA polymerase-I directs the synthesis of complementary nucleic acids using single stranded DNA as a template since *in vitro* enzymatic synthesis of DNA is important in genetic manipulation experiments.
- Terminal transferase purified from calf thymus can add oligodeoxynucleotide tails to the 3′ ends of DNA duplexes. Thus, it adds d(A) sequence to 3′ ends of one population and oligod (T) to 3′ ends of another population of DNA. The two types of DNA molecules can anneal to form a dimeric circle.

If suitable enzymes are not available for manipulation of the DNA, the cleavage sites can be added as linker or adaptor molecules (fig 3 & 4). The oligonucleotide dCCGAATTCGC will self associate to give a duplex structure containing ECORI recognition sequence.

Adaptor molecules are chemically synthesized DNA molecules within performed cohesive ends with a blunt ended foreign DNA, synthetic adaptor molecule is ligated. The adaptor is used in 5′ hydroxyl form to prevent self-polymerization. The foreign DNA plus ligated adaptor is phosphorylated at 5′ terminus and ligated to the vector, cut with a particular enzyme.

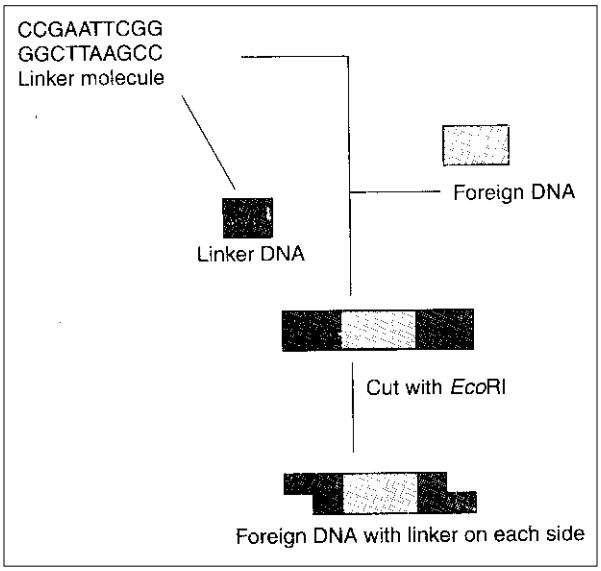


Fig. 3. The use of linker for construction of DNA fragments with cohesive termini.

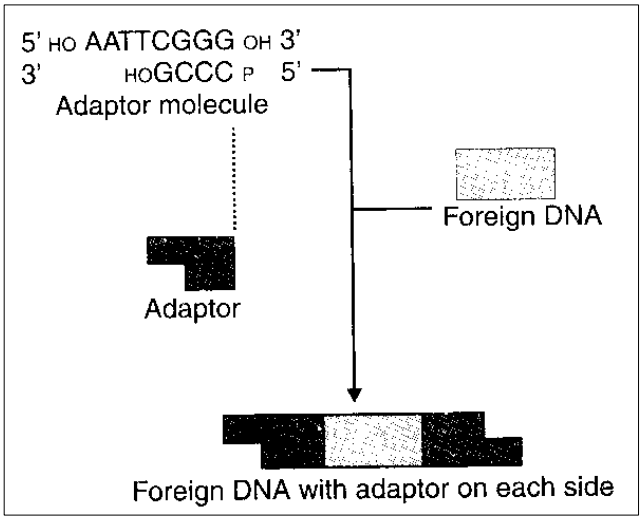


Fig. 4. The use of adaptors for construction of DNA fragments with cohesive termini.

12. GENE CLONING AND GENETIC ENGINEERING

Gene Cloning

Insertion of a fragment of DNA, carrying a gene, into a cloning vector and subsequent propagation of the recombinant DNA molecule in a host organism is called as gene cloning (Fig. 1).

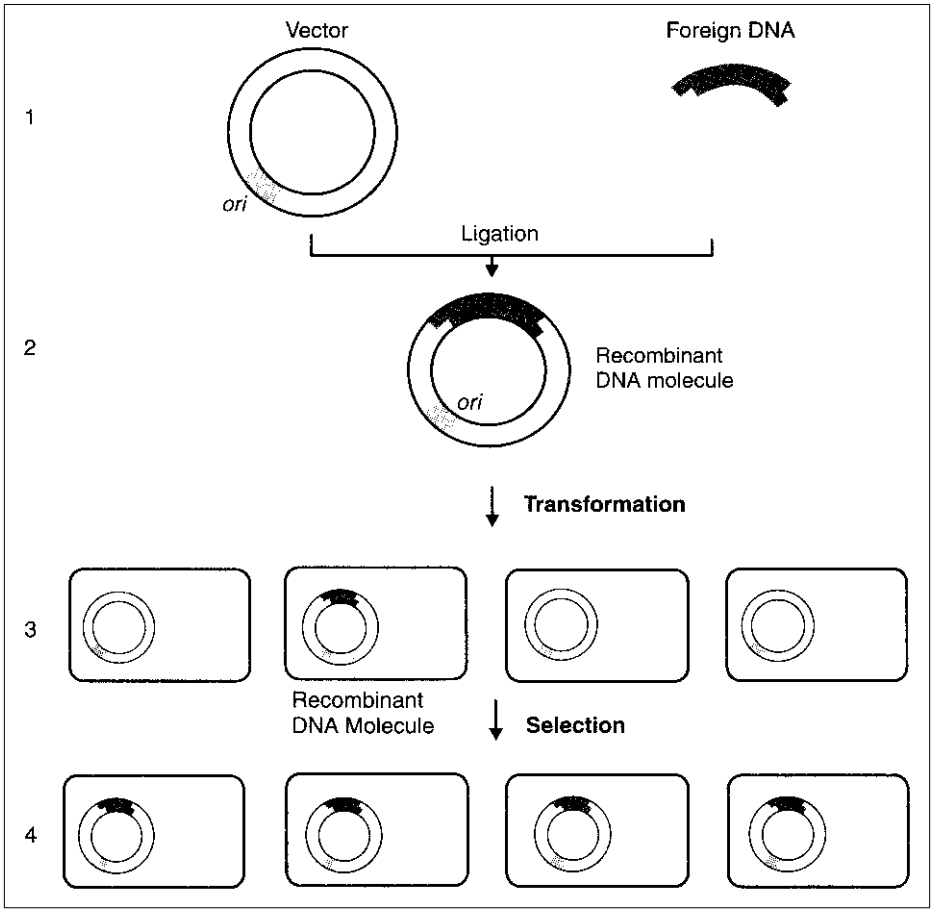


Fig. 1. Fundamental steps of a cloning experiment. The cloning experiment involves (1) the production and preparation of vector and foreign (passenger) DNA, (2) the ligation of vector and passenger DNA so as to construct a recombinant DNA molecule, (3) the transformation of suitable host cells, and (4) the selection of those cells which have acquired recombinant DNA molecules.

The basis steps in a gene cloning experiment are:

1. A fragment of DNA, containing the gene to be cloned, is inserted into a circular DNA molecule called a vector, to produce a chimaera or recombinant DNA molecule.
2. The vector acts as a vehicle that transports the gene into a host cell.

3. Within the host cell the vector multiples, producing numerous identical copies not only of itself but also of the gene that it carries.

4. When the host cell divides, copies of the recombinant DNA molecule are passed to the progeny and further vector replication takes place.

5. After a large number of cell divisions, a colony or clone of identical host cells is produced. Each cell in the clone contains one or more copies of the recombinant DNA molecule: the gene carried by the recombinant molecule is now said to be cloned.

CLONING STRATEGIES

Any DNA cloning procedure has four essential parts.

1. A method for generating DNA fragment.

This can be achieved by restriction endonuclease digestion or mechanical shearing or duplex cDNA synthesis or by direct chemical synthesis.

2. Reactions which join foreign DNA to the vector.

This can be done by homopolymer tailing or ligation of cohesive termini produced by restriction endonuclease or by blunt end ligation or by linker molecules.

3. A method of introducing the artificial recombinant into a host cell in which it can replicate:

   This is done either by transfection with recombinant phage DNA or transformation with recombinant plasmid DNA or by *in vitro* packaging into phage coat, transduction with recombinant phage or cosmid.

4. A method of selecting or screening for a clone of recipient cells that has acquired the recombinant.

Screening

Once a genomic library or cDNA library is available, screening of the libraries for isolation of specific gene sequence is done.

This can be done by

(a) Genetic methods
(b) Immuno chemical methods
(c) Nucleic acid hybridization methods
(d) Hybrid released translation (HRT) and hybrid arrested translation (HART).

Once you get the desired clone or gene, the gene is then characterized by DNA sequencing.

Genetic Engineering in Plants

To introduce a desired gene or set of genes by conventional methods requires a sexual cross between two lines and repeated back-crossing. This is restricted to plants that can hybridize only. Recombinant DNA techniques promise to overcome these limitations by enabling plant geneticists to identify and clone specific genes for desirable traits.

Genetic engineering aims of isolating DNA fragments and recombining them. The term recombinant DNA is used to refer to composite DNA molecules, that result from the physical combination of DNA segments derived from different sources (see synthesis of r-DNA). Gene cloning or genetic engineering is essentially the insertion of a piece of DNA into a cell, in such a way that inserted DNA is replicated. It can be performed by either direct gene transfers or vector mediated. Therefore genetic engineering is manipulation of genes in the laboratory to produce useful plants, animals or products. It is completely dependent on r-DNA technology as mentioned earlier. Thus genetic engineering is the application of recombinant DNA methods to confer new traits on organising by introducing new genes into their cells (See Synthesis of r-DNA molecule).

Through genetic engineering Insect, virus, disease, herbicide resistant plants, plants for quality like nutritional status, improved storage, plants for production of chemicals and pharmaceuticals can be produced. Genetic engineering of Bt gene is shown in the development of transgenic plants resistant to insects (Fig. 2)

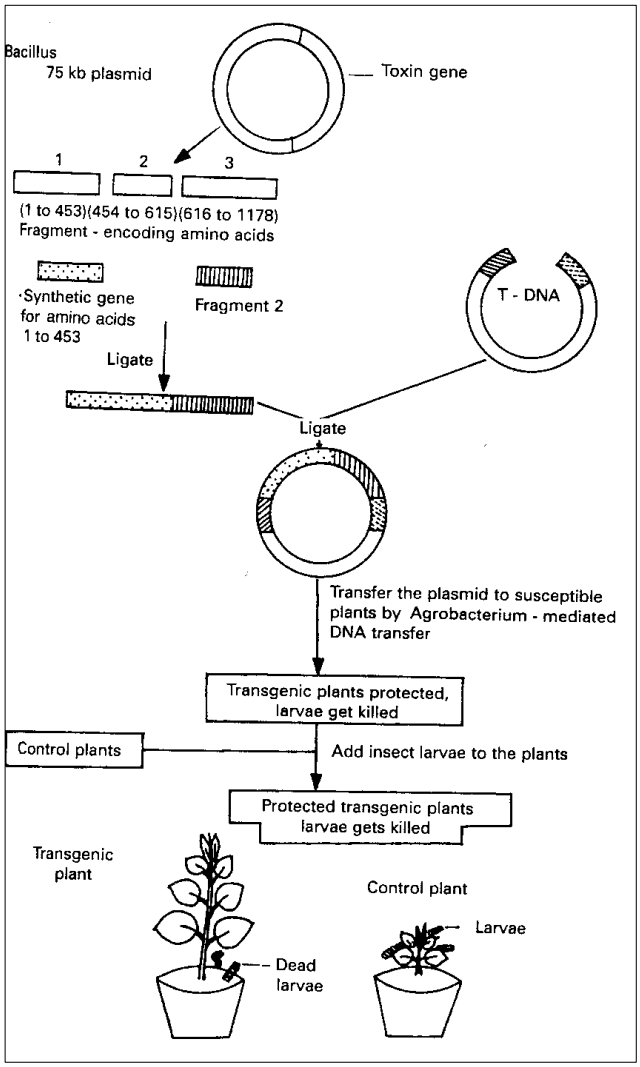


Fig.2. Expression of *Bacillus thuringiensis* toxin gene in transgenic plants to protect them against insect damage.

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