Chapter 1

Study of the Structure of Cell Organelles through photomicrographs

EXPERIMENT NO. 1

Aim of the experiment: To study plant cell and its organelles with the help of electron micrographs or photomicrographs.

Theory: An **electron microscope** is a microscope that uses a beam of accelerated electrons as a source of illumination. As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher resolving power than light microscopes and can reveal the structure of smaller objects. A transmission electron microscope can achieve better than 50 pm (picometer) resolution and magnifications of up to about 10,000,000 x whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000x.

Fig. 1.1. A Modern Transmission Electron Microscope

Transmission electron microscopes use electrostatic and electromagnetic lenses to control the electron beam and focus it to form an image. These electron optical lenses are analogous to the glass lenses of an optical light microscope.

Electron microscopes are used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, electron microscopes are often used for quality control and failure analysis. Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the image.

Ernst Ruska and **Max Knoll** invented the first prototype of electron microscope in 1931. Ruska built an electron microscope in 1933 that exceeded the resolution attainable

with an optical (light) microscope. The first commercial electron microscope was produced by the company, Siemens in 1938. Now there are different types of electron microscopes like Transmission Electron Microscope (*TEM*), Scanning Electron Microscope (*SEM*), Reflection Electron Microscope (*REM*), Scanning Transmission Electron Microscope (*STEM*). With the modern high resolution TEM, an object is magnified above *50 million times*. Biological bulk samples upto some centimeters in size can be well imaged through SEM which gives three-dimensional shape of the sample. A variety of SEM called environmental scanning electron microscope (*ESEM*), can produce images of sufficient good quality and resolution with the samples being wet or contained in low vacuum or gas.

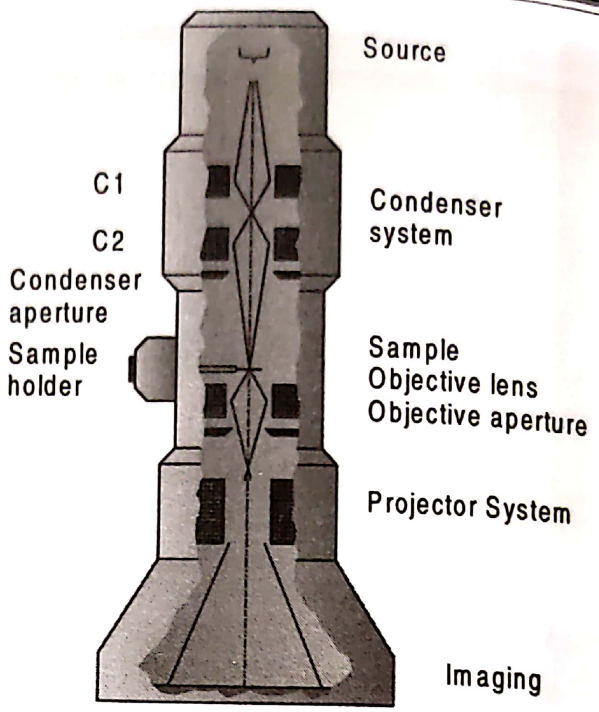


Fig. 1.2. Diagram of a Transmission Electron Microscope (VS, vertical section)

Procedure: The electron micrograph of an object is prepared by using an electron microscope. Materials to be viewed under an electron microscope requires processing to produce a suitable sample. The technique required varies depending on the specimen and the analysis required.

Observation: The electron micrographs of plant cell and its cell organelles are presented below (Fig. 1.3 to Fig. 1.12) and important points in each case are to be written.

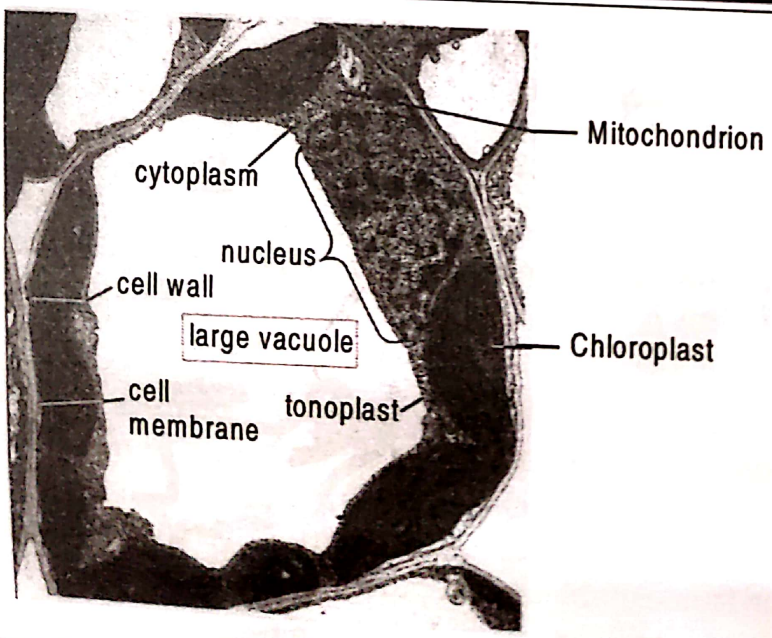


Fig. 1.3. Electron micrograph of a plant cell (X12000)

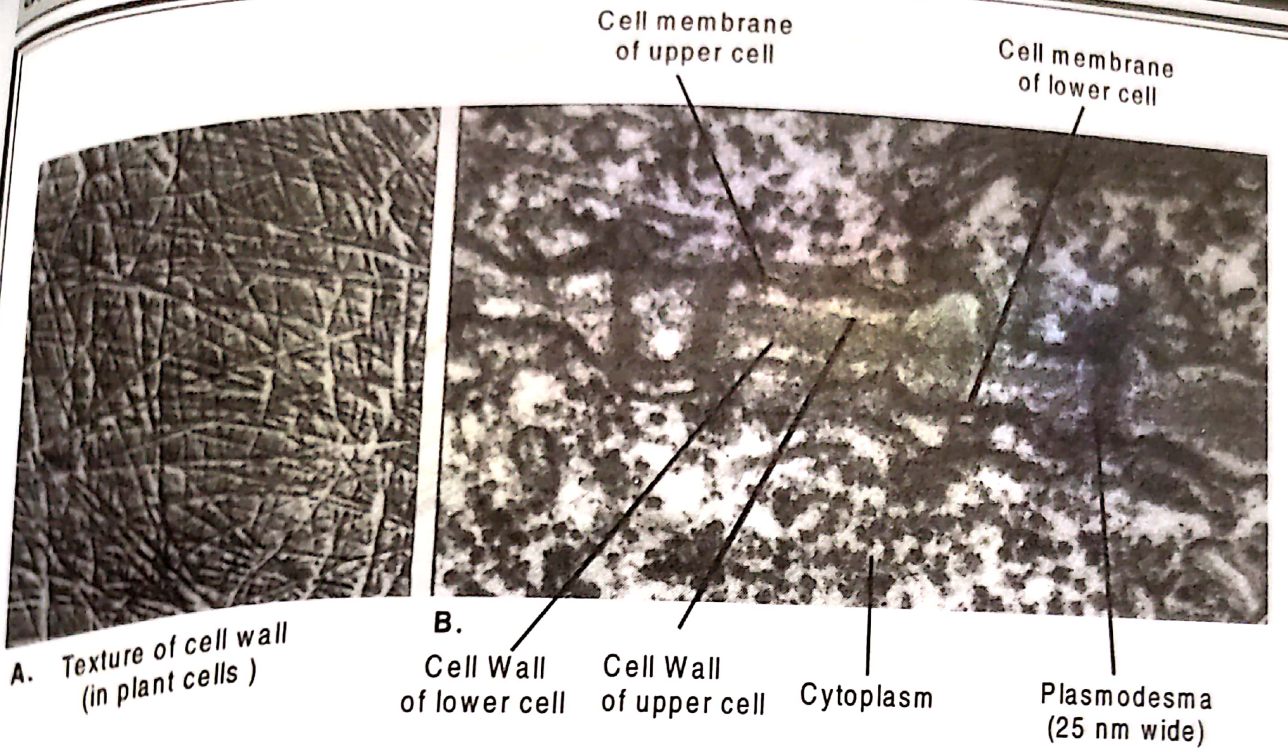


Fig. 1.4. Electron micrograph of cell wall : A : Texture of plant cell wall, B : Details of cell wall

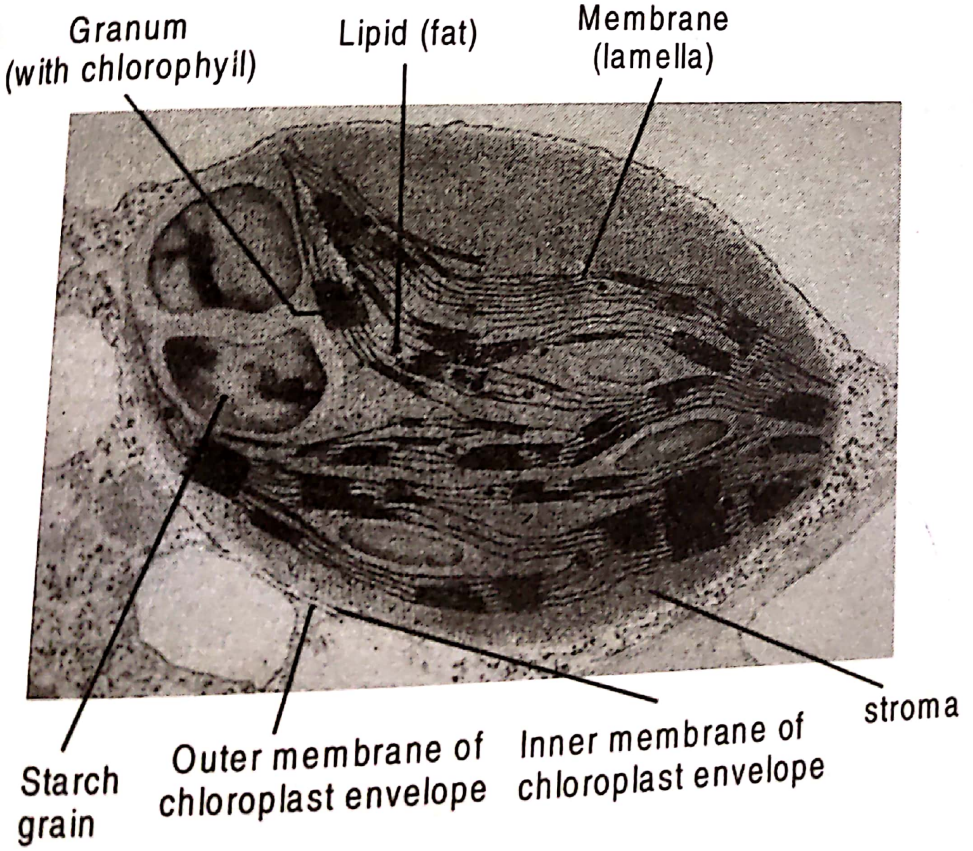


Fig. 1.5. Electron micrograph of a chloroplast (X 8000)

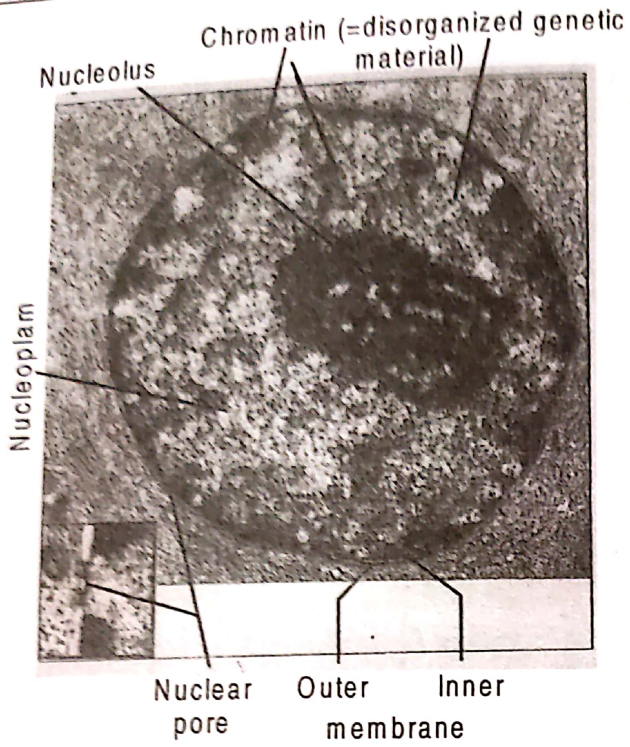
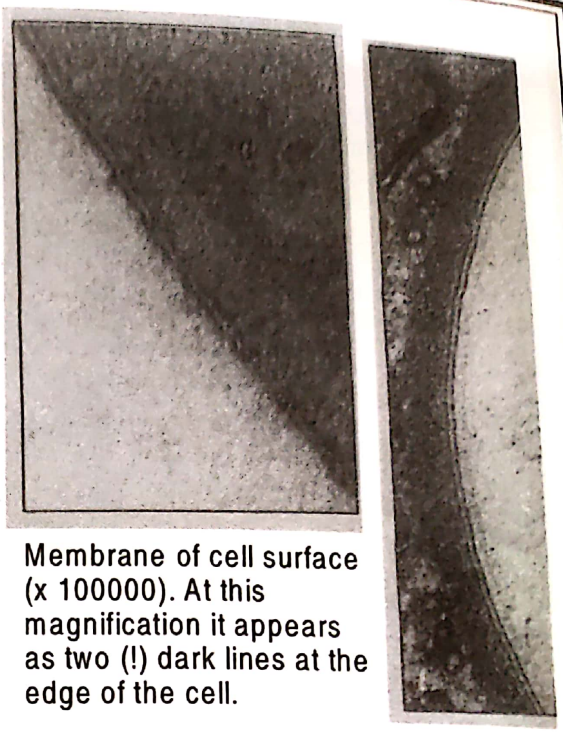


Fig. 1.6. Electron micrograph of a nucleus



Membrane of cell surface (x 100000). At this magnification it appears as two (!) dark lines at the edge of the cell.

Fig. 1.7. Electron micrograph of cell membrane

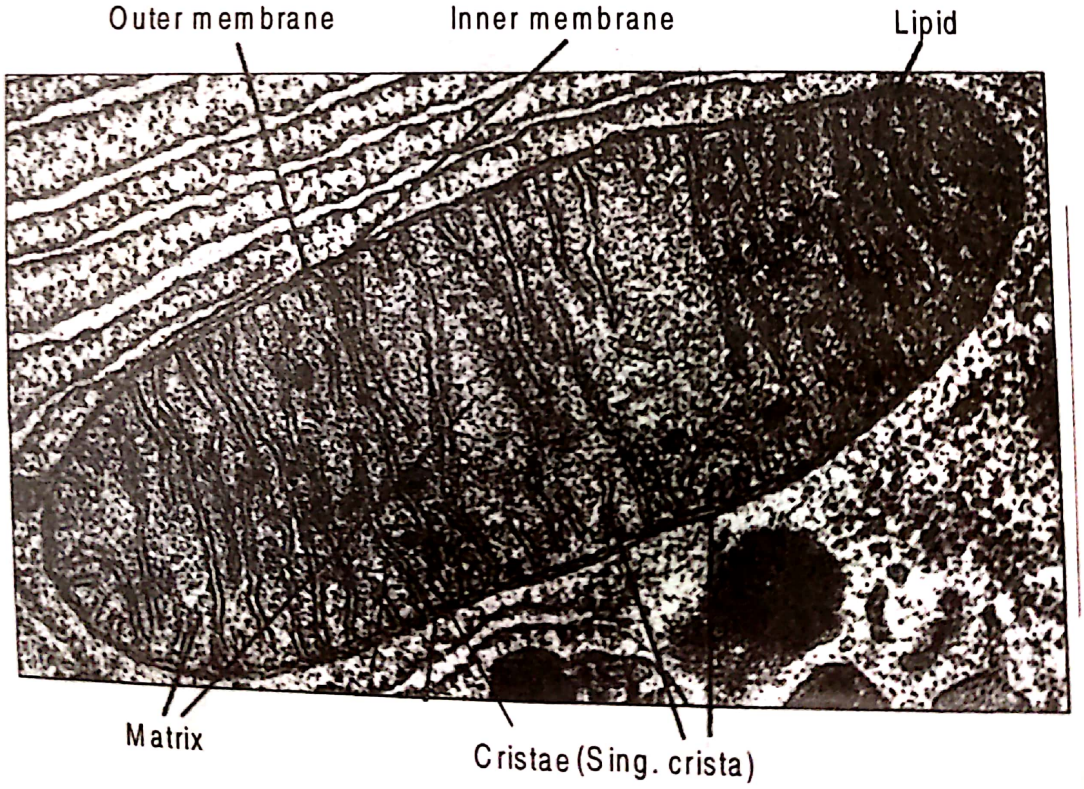
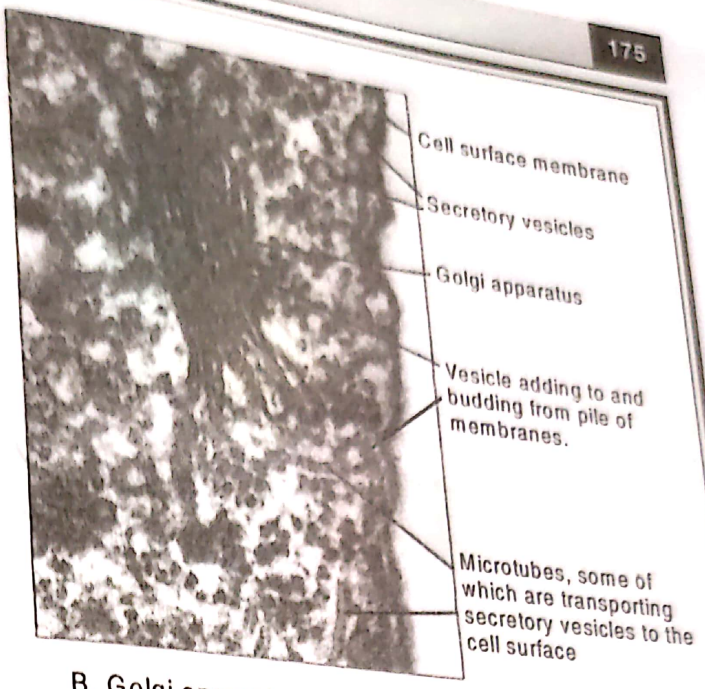


Fig. 1.8. Electron micrograph of a mitochondrion (X 40000)

A. Electron micrograph of Golgi body (apparatus)



B. Golgi apparatus (x 72500)

Fig. 1.9. Electron micrograph of a Golgi body (A, B)

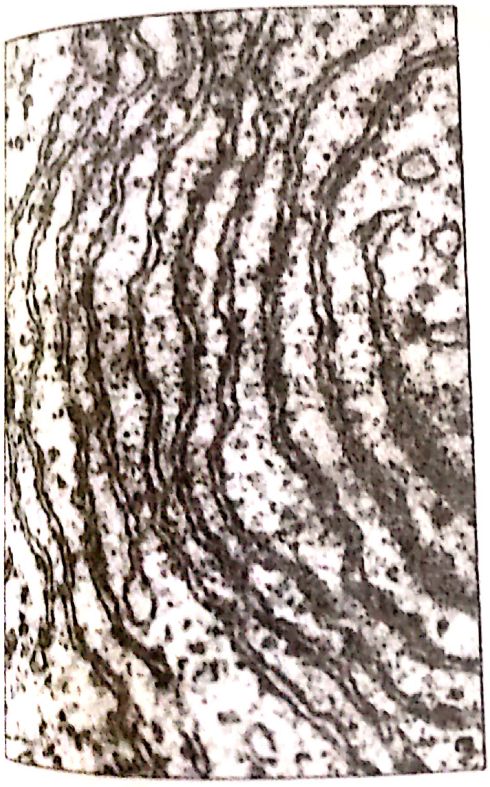


Fig. 1.10. Electron micrograph of endoplasmic reticulum (X 55000)

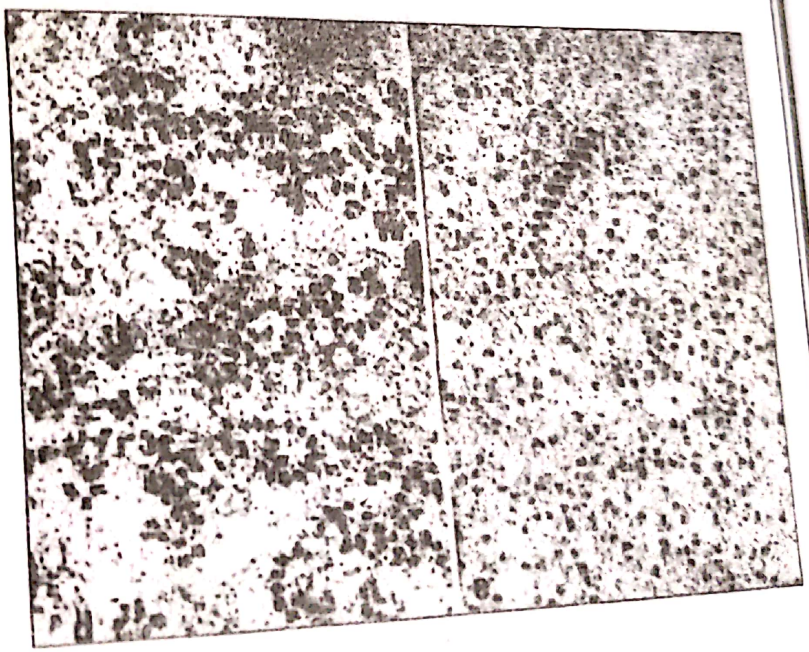


Fig. 1.11. Electron micrograph of free ribosomes

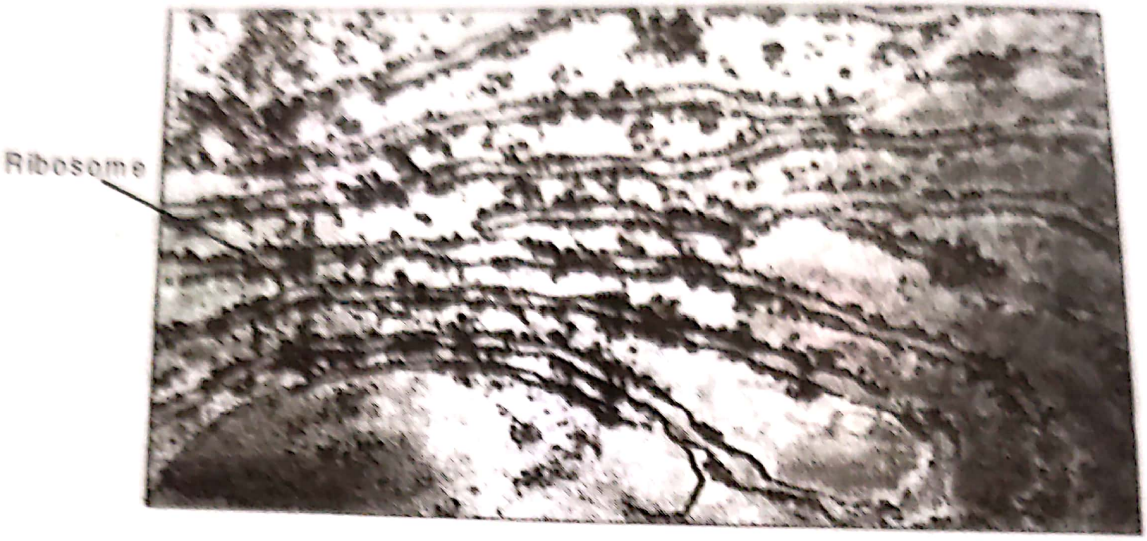


Fig. 1.12. Electron micrograph of "rough" endoplasmic reticulum (ER + bound ribosomes)

CHAPTER 2

Study of Structure of plant cell through temporary mounts

Plant cell was first discovered by **Robert Hooke** (1665) and the term 'cell' was also coined by him. Cell theory was put forth by **Schleiden** and **Schwann** (1839). A cell is the basic structural and functional unit of all living organism. It is also defined as a unit of biological activity delimited by a semipermeable membrane and capable of self reproduction in a medium free of other living systems. A plant cell, unlike the animal cell, has a cell wall.

General structure of a plant cell (Fig. 2.1)

A plant cell has the following structures.

1. Outer cell wall which may be round, oval, rectangular or hexagonal.
2. Below the cell wall, plasma membrane is present which is semi-permeable.
3. Generally each cell has a centrally placed nucleus. But is a mature cell where the vacuole is large, the nucleus is present towards one side.
4. Inside the cell, cytoplasm is present which contains organelles like plastids, mitochondria, endoplasmic reticulum, Golgi bodies, ribosomes etc.
5. The characteristic green colour of plant cell is due to the presence of many choroplasts, containing chlorophyll pigments.

EXPERIMENT NO. 2

Aim of the experiment : To study the plant cell of outer epidermal peel of *Rhoeo discolor* or *Tradescantia*/Onion through temporary mounts.

Requirements : Compound microscope, microslide, cover-slip, needle, distilled water, watch glass, glycenine, ethyl alcohol, safranin solution etc.

Supplied specimen : Leaves of *Rhoeo discolor* or *Tradescantia*/onion.

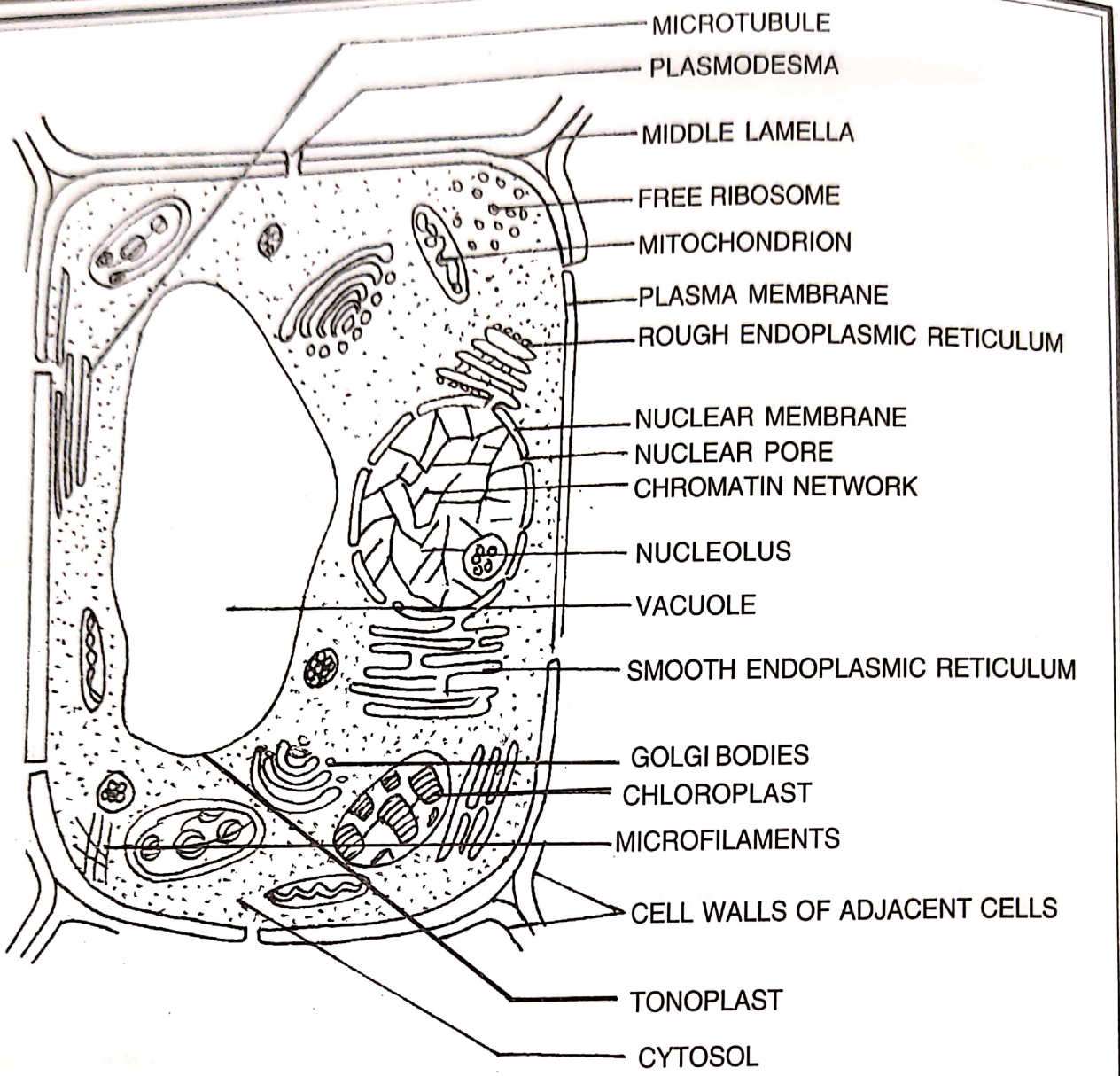


Fig. 2.1 Ultrastructure of a typical Plant Cell

PROCEDURE

A. For *Rhoeo/Tradescantia* Leaf

Fresh leaves of *Rhoeo discolor* or *Tradescantia* are taken and broken at the middle so as to obtain the violet peels of the lower epidermis. These peels are kept in watch glasses. Then a peel of the leaf is kept on microslide at the centre with 2-3 drops of distilled water. A clean cover-slip is put carefully and then examined under a compound microscope first with low power and then with high power objective (Fig. 2.3).

B. For Onion Leaf

A piece of fleshy scale leaf of onion (*Allium cepa*) from the onion bulb is taken and its thin white epidermal peel is removed carefully. A piece of the peel is spread over a clean

Fig. 2.2. *Rhoeo discor* plant

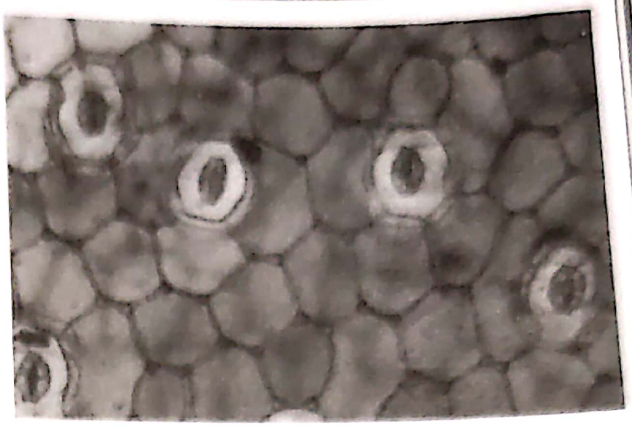
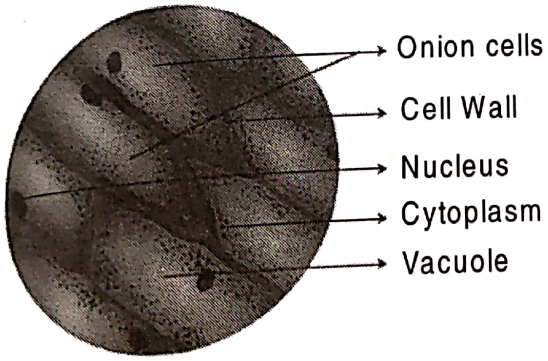
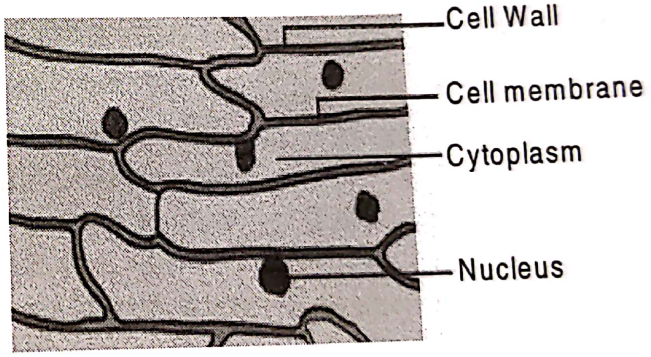


Fig. 2.3. Cells of epidermal peel of *Rhoeo discolor* (Stomata are seen)

micro-slide at the centre and a drop of dilute safranin solution is added. It is kept for one to two minutes and then washed with 50% ethanol carefully. Then 1-2 drops of dilute glycerine (30%) is poured over it and a clean coverslip is put with the help of a needle. If any excess glycerine is present outside the coverslip, then it is soaked by a blotting paper and examined under a compound microscope (Fig. 2.4).



Cells of onion peel
(A)



Epidermal cells of onion peel
(B)

Fig. 2.4. Cell of Onion Peel (A and B)

Observation : Under low power magnification, many rectangular cells of comparatively smaller size are visualized. But under high power magnification few cells of larger size are seen. The cell walls are thick and clearly seen and rectangular is shape. The protoplasm is violet in colour. A round nucleus in seen more or less at the centre. The nucleus is stained red due to safranin is case of onion leaf and crinum leaf peels. Stomata are seen in *Rhoeo*/leaf.

Conclusion : Each plant cell has cell wall, ctyoplasm and nucleus. The plant cell structure is clearly seen in case of epidermal peel of *Rhoeo*/*Tradescantia* and onion. In

case of *Rhoeo* stomata are also seen in the leaf peels. Each stoma is guarded by a pair of kidney-shaped guard cells.

Precautions:

1. The peels should be taken carefully from the lower side of the leaf of *Rhoeo*/ *Tradescantia*.
2. The leaves should be freshly plucked.
3. Stomata should be carefully studied in the peels of *Rhoeo* taken from the lower surface of the leaf which contains more stomata.
4. Glasswares like slide, watch glass should be cleaned and dried before use.

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CHAPTER 3

Study of various stages of mitosis using Cytological preparation of Onion root tips

EXPERIMENT NO. 3

Aim of the experiment : To study the different stages of mitosis of onion root tip by **squashing technique,** using aceto-carmine or aceto-orcein solution.

Requirements : 1% Acetocarmine or acetoorcein solution, HCl, watch glass, measuring cylinder, Petri dishes, slide, coverslip, spirit lamp, squashing needle, compound microscope.

Plant material : Onion (*Allium cepa*) root tip

Theory : Mitosis is a process of cell division which occurs in the somatic or vegetative cells of the plant body and also in the animals. In this type of cell division, each cell divides equally into two indentical cells, hence it is also known as *equational cell division*. The quality and quantity of chromosomes remain the same in the daughter cells in comparison to the mother cell. Mitosis has two phases-(*a*) **Karyokinesis,** where the nucleus is divided into two, (*b*) **Cytokinesis,** where the cytoplasm is divided by the formation of cell plate to form two cells.

Karyokinesis is divided into four phases i.e. (1) Prophase, (2) Metaphase, (3) Anaphase and (4) Telophase. In onion root tip the duration of mitosis is about 1.5 hours and in each cell $2n = 16$, *i.e.* in each diploid cell 16 chromosomes are present.

Procedure : A previously fixed onion root tip (approximately 1 cm in length) is taken and is kept in 1 N HCl (1 ml. conc. HCl + 11 ml. distilled water) in a watch glass for 5 minutes and then gently heated with the help of a spirit lamp for some time (4-5 minutes) so that the middle lamella of the cells are dissolved due to acid hydrolysis. This facilitates the cells of the root tip to be smooth and to spread easily. Now the root tip is taken and kept at the centre of a microslide and a drop of acetocarmine or aceto-orcein solution (1%) is added. Then it is gently pressed with the help of a squashing needle (rusting of this needle helps staining the chromosome more deep) once or twice. Then a

coverslip is placed over it carefully and warmed a little over a spirit lamp. Then it is vertically pressed gently by thumb by keeping a piece of blotting paper over the coverslip so that the cells are spread out. Now it is observed through the low power objective and then high power objective of a compound microscope to see the different stages of mitosis.

Observation :

Prophase : (Fig. 3.1 A & B)

(*a*) The chromatin reticulum, is organized into distinct thread-like **chromosomes** which are coiled.

(*b*) Each chromosome has two **chromatids,** present closely together longitudinally and are attached together at a point known as **centromere.**

(*c*) Presence of cell wall, nuclear membrane and nucleolus in the early stage of prophase.

(*d*) In the late stage of prophase, the nuclear membrane and nucleolus gradually dissappear.

Metaphase (Fig. 3.1C)

(*a*) Absence of nuclear membrane and nucleolus.

(*b*) The chromosomes become more clear and more condensed.

(*c*) The chromosomes are arranged along the middle line of the cell known as **equatorial plate.**

(*d*) The arms of the chromosome face towards opposite poles or to one pole and their centromeres lie on the equatorial plate.

(*e*) **Spindle fibres** or tractile fibres have developed from opposite poles and are attached at the point of centromere of each chromosome.

Anaphase (Fig. 3.1D)

(*a*) Absence of nuclear membrane and nucleolus.

(*b*) Each chromosome splits at the point of centromere so that each chromatid is covereted into a new chromosome.

(*c*) The spindle fibres (made up of **tubulin protein**) are contracted towards the poles so that the two chromatids move to opposite poles.

(*d*) The new moving chromosome, look like 'V' or 'L' or J or I-shaped depending upon the presence of centromere in metacentric, submetacentric, acro-centric or telo-centric chromosomes respectively. But in onion only 'V' and 'L'-shaped chromosomes are found as it contains only metacentric and submetacentric chromosomes.

(*e*) The arms face towards the equatorial plate and the centromeres face towards the poles.

(*f*) On careful observation, 16 chromosomes are seen in each set separating towards the pole.

Telophase (Fig. 3.1E)

(*a*) Two sets of chromosomes, one in each pole, are present within the compartment of the mother cell wall.

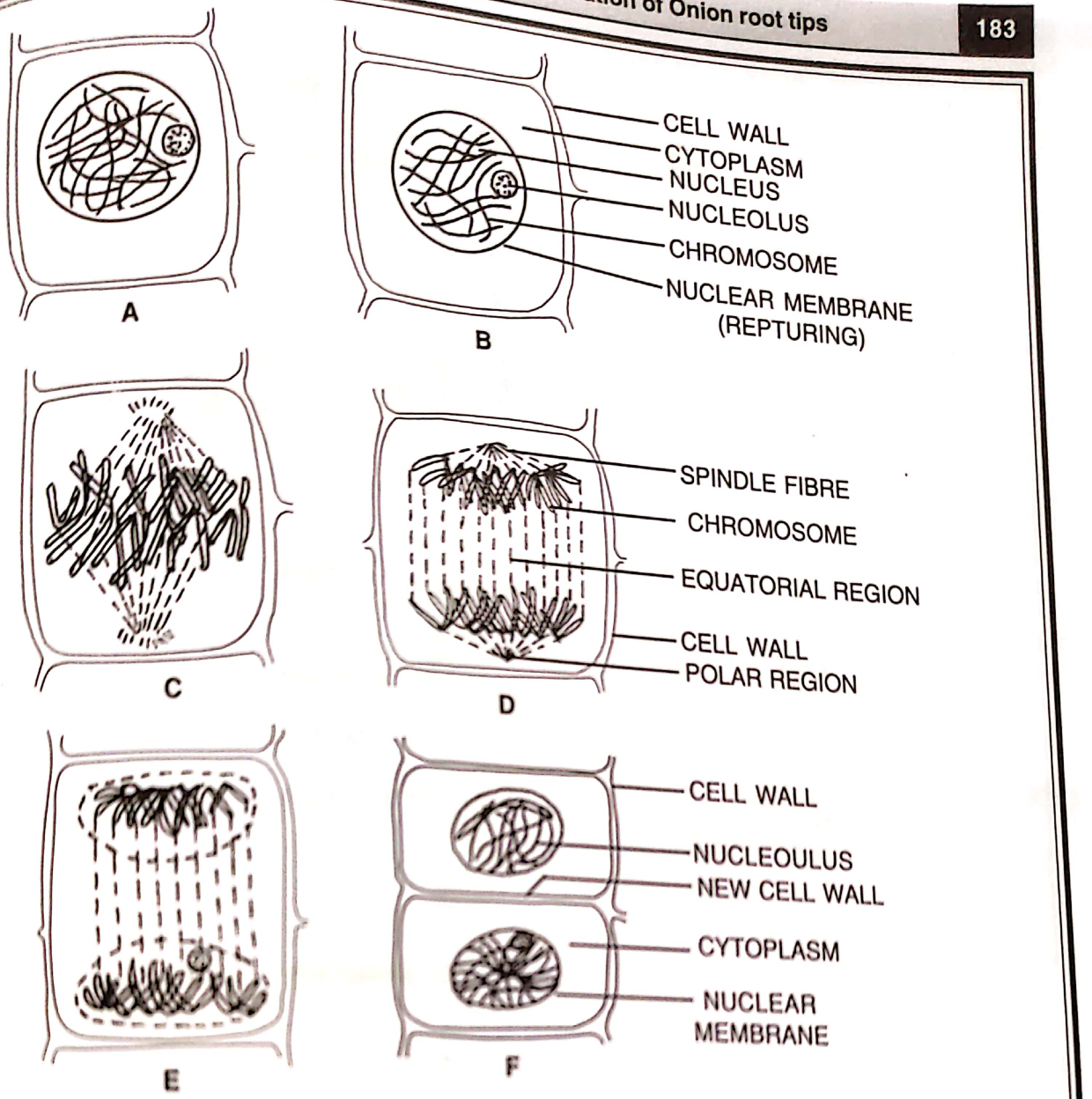

Fig. 3.1. Different Stages of mitosis

(A) EARLY PROPHASE, (B) LATE PROPHASE, (C) METAPHASE, (D) ANAPHASE, (E) TELOPHASE, (F) CYTOKINESIS

(*b*) The chromosome in each pole are gradually become thinner, less distinct and more coiled.

(*c*) Appearance of nuclear membrane and nucleolus in each polar region.

(*d*) Spindle fibres dissappear gradually, but it help in cell plate formation.

Cytokinesis : (Fig. 3.1F)

(*a*) Formation of cell plate at the equitorial plane.

(*b*) Cell plate is formed from phragmoplast which in turn is produced from Golgi bodies.

(*c*) Calcium and magnesium pectates are deposited so that the cell plate is solidified.

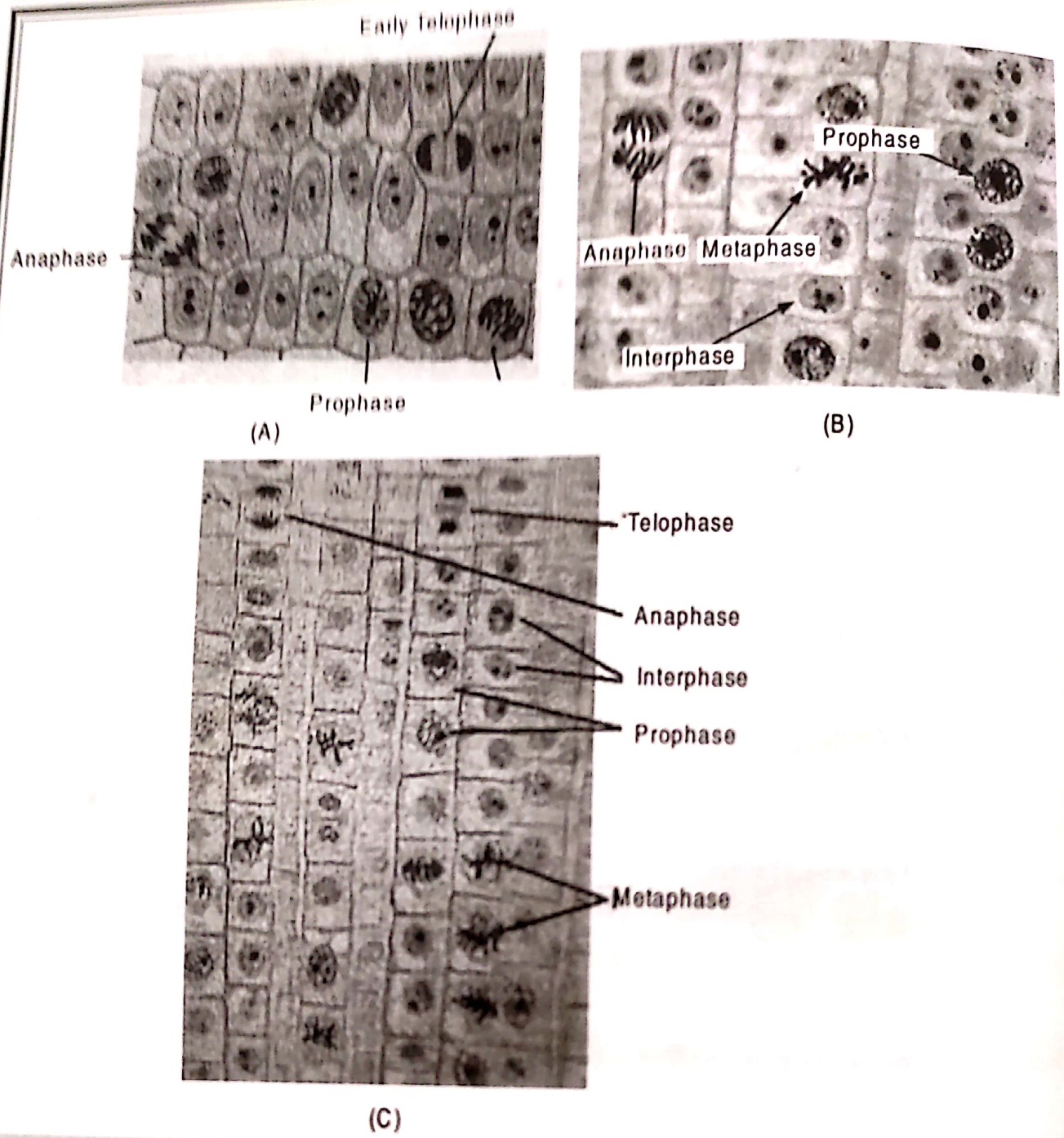

Fig. 3.2. Different stages of mitosis of onion root tip seen under microscope (A, B and C)

(*d*) Due to cell plate formation the mother cell divides into two daughter cells.

(*e*) In some plant cells, cytokinesis may occur by cleavage of the cytoplasm which starts from the periphery.

Conclusion : From the above observation, it is concluded that different stages of mitosis can be studied by using acetocarmine or aceto-orcein solution which stain the chromosome darker bluish-red colour.

N.B. Fixation of root tip – Some healthy onion bulbs are selected and their dry roots are cut. The bulbs are kept on coupling jars full of water in such a way that their

lower portion just touches the water. After 2-3 days, new roots would appear. On the day of fixing healthy roots are cut (approx 1 cm. long from the tip) at 8.00 a.m. (process of cell division is maximum), and then are kept in aceto alcohol (acetic acid and ethyl alcohol in 1 : 3) for 24 hours. Then these are transferred to 70% ethyl alcohol for preservation. These preserved root tips are used for squashing technique.

Preparation of 1% acetocarmine stain : Carmine is a basic dye, reddish purple in colour. It is obtained from the female scale insect *Coccus cacti* which lives on the cactus *Opuntia coccineclifera*. From the dried bodies of these females carminic acid is obtained. The dye carmine is formed by mixing an alum with the carminic acid.

1.0 gm of carmine powder is dissolved gradually in 100 ml of boiling 45% acetic acid. The solution is heated for 15-20 minutes carefully keeping it as simmering point. Then the solution is cooled down to room temperature and then filtered. Now 1% acetocarmine solution is prepared. Staining can be intensified by adding 5 ml ferric chloride ($FeCl_2$, $6H_2O$) solution. But the total volume should be 100 ml.

Preparation of 1% aceto-orcein solution : 55 ml of boiling glacial acetic acid is poured over 1.0 g orcein powder. Cool the solution and add 45 ml of distilled water and filter. Prepare fresh before use.

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CHAPTER 4

Study of DNA packaging through Photomicrographs

EXPERIMENT NO. 4

Aim of the experiment : To study DNA packaging through photomicrographs.

Requirements : Photomicrograph of DNA packaging.

Theory : DNA (deoxyribose nucleic acid) is a double helical structure and is the bearer of hereditary characters. It is the genetic material of all higher plants and animals. The thread like structures of DNA are ultimately packaged in he chromosomes in the nucleus of eukaryotes. During interphase chromosomes are present as long fine thread-like

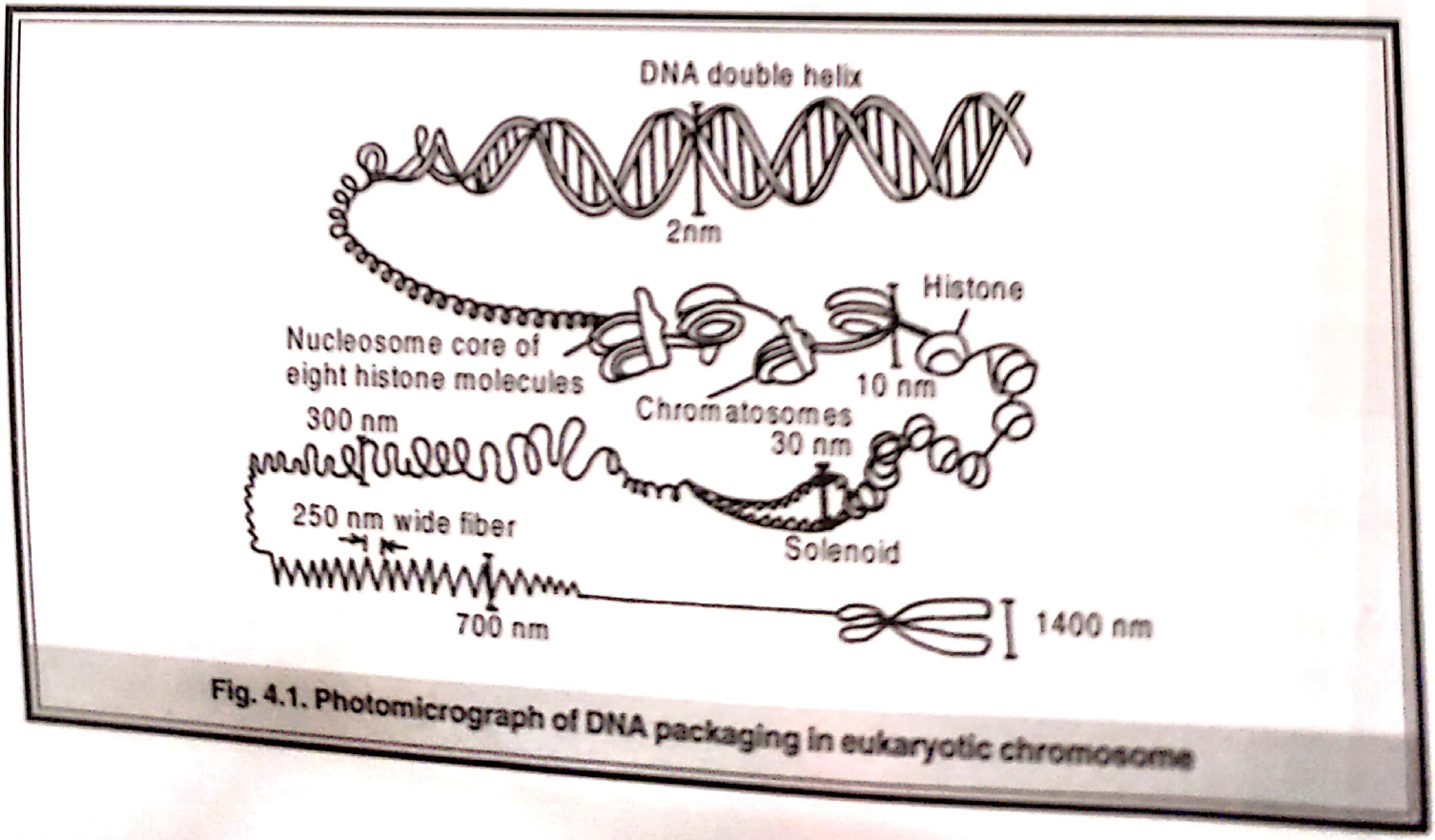

Fig. 4.1. Photomicrograph of DNA packaging in eukaryotic chromosome

filamentous structures known as chromatids which contain DNA-histone protein complexes called **nucleosomes** (euchromatin). Nucleosomes are bead like structures on a string having diameter of 10 nm whereas DNA molecules have 2nm diameter. The nucleosomes are further coiled and compacted to produce 30 nm **solenoid** fibres forming the **heterochromatin**. There is further higher level of DNA packaging of the 30 nm solenoid fibres into chromosomes during mitosis and meiosis. The chromatids are of 700 nm in diameter while metaphase chromosomes have 1400 nm diameter.

Observation : The photomicrograph is to be studied. The levels of packaging of DNA upto chromosome is in the following sequence, gradually towards more complexities.

1. DNA double helix with 2 nm (20Å) width.
2. Nucleosomes with core of 8 histone proteins having width of 10 nm.
3. 30 nm Solenoid fibres
4. 700 nm fibre of chromatid
5. 1400 nm fibre of metaphase chromosome

Conclusion: The supplied photomicrograph is identified to be various levels of DNA packaging starting from 2 nm DNA fibre upto 1400 nm metaphase chromosome having two chromatids each with 700 nm width.

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CHAPTER 5

Study of effect of temperature and Organic solvents on permeability of cell membrane

EXPERIMENT NO. 5

Aim of the experiment : To study the effect of temperature and organic solvents (methanol, acetone, formaldehyde) on the permeability of cell membrane.

Requirement : Cork borer, scalpel, test tubes, pipette, test tube stand, beaker, spirit lamp, ice, methanol, acetone, ethanol, distilled water.

Plant material : Fresh beet (*Beta vulgaris*) root.

Theory : Plant cell membrane has different rates of permeability. Leaching of pigments is a physical phenomenon, where the pigments like chlorophyll, xanthophyll, anthocyanin, carotene etc. of the plant cells come out due to increased cell permeability. Cell permeability may be increased if cell membrane is expanded or ruptured or damaged. It may be caused when the plant or plant parts are subjected to high temperature in aqueous medium or organic solvents like ethanol, methanol, ethanol, benzene or acetone etc.

Different pigments produce different colours and depending upon the depth of the colour, the degree of leaching is known. The colours of different pigments are as follows: chlorophyll- green, xanthophyll- yellow, anthocyanin- purple, α (alpha)- carotene-violet, β (beta)-carotene-red, γ (gamma)-carotene-dark red, lycopene (of tomato)-red. Carrot contains all the three types of carotenes out of which the amount of beta-carotene is the highest (85%).

The pigment **carotene** was first isolated by **Wackenroder** (1831) from carrot.

Procedure. A fresh beet root is taken and washed thoroughly with water. A cylindrical piece is taken at the middle portion of the root through the help of a cork borer and cut into small slices of almost equal thickness by the scalpel. The slices are then washed thoroughly by water so that the traces of pigments are removed. Now six test tubes marked 1 to 6 are taken and filled with 10 ml of distilled water (control), hot water, ice-cold water, methanol (methyl alcohol), acetone and ethanol respectively. In each test tube one slice of the beet root is put and kept for 15 minutes. Then the test tubes are shaken and the intensity of the colour, if any, is noted.

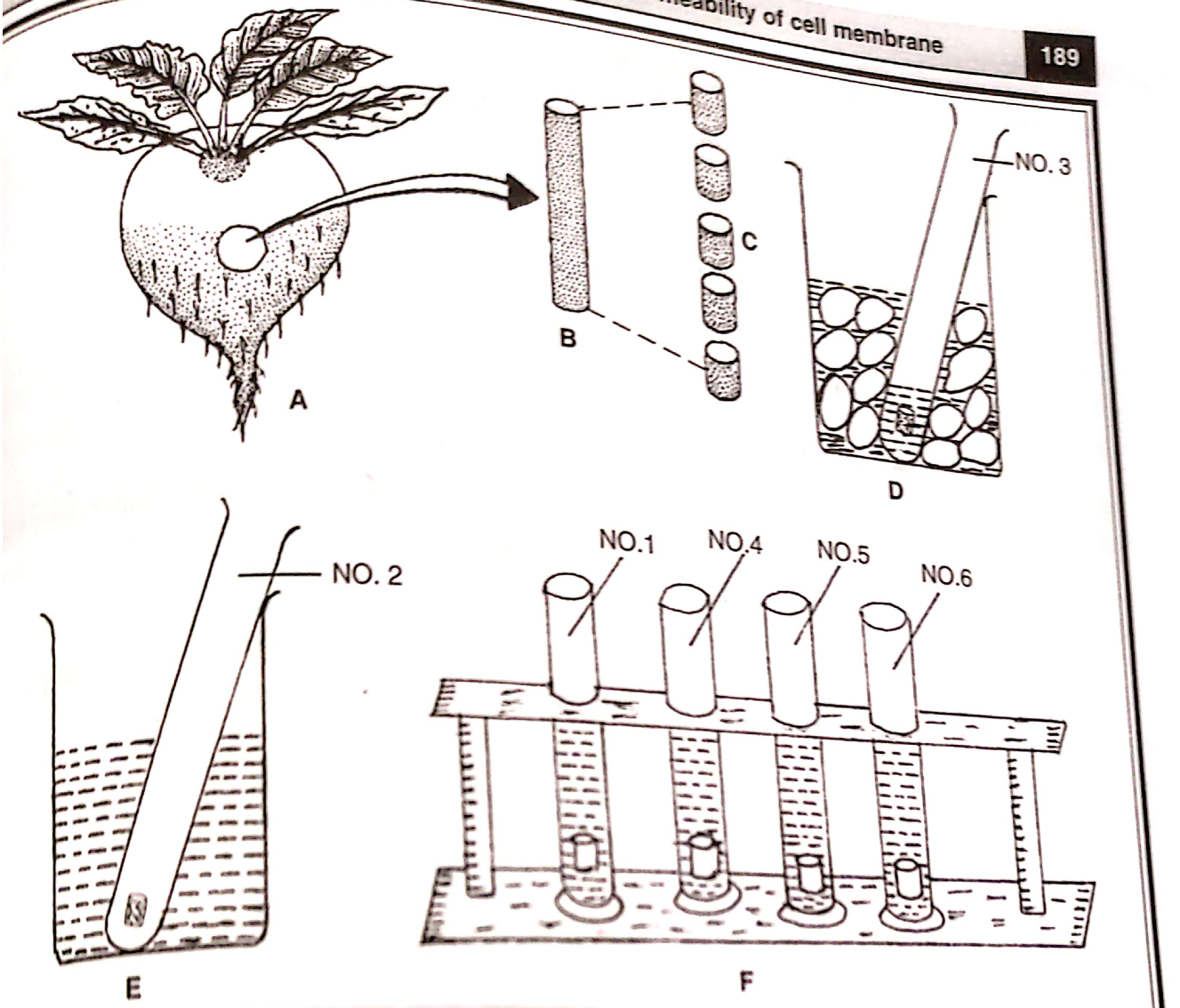

Fig. 5.1. Leaching of beet root experiment A. Beet Root B. A cylindrical piece of beet root C. Small pieces of beet root D. Incubation of beet slice in ice-cubes E. in boiled hot water, F. in distilled water (No. 1 test tube), methanol (No. 4 test tube), acetone (No. 5 test tube), and ethanol (No. 6 test tube).

Observation. The change in the intensity of the colour in different test tubes is observed as follows :

Sl. No./ Test tube No.	Temp./Chemical	Change in Colour	Degree of Leaching
1	Distilled water (control)	No change	No leaching
2	Boiled water (hot)	Light purple	Mild leaching
3	Ice-cold water	No change	No leaching
4	Methanol	Dark purple	Higher leaching
5	Acetone	Medium purple	Medium leaching
6	Ethanol	Dark purple	Higher leaching

Inference : The leaching of pigments in beet root when kept in high temperature or organic solvents like methanol, acetone or ethanol is due to increased cell permeability. Cell permeability is not altered when beet root slices are kept in distilled water or low temperature (ice-cold water). Boiling water or chemicals damage cell membrane enhancing cell permeability.

Precautions.

(1) The beet root slices should be of equal thickness.

(2) The slices should be thoroughly washed.

(3) Equal volume of the test solution should be taken.

(4) The test tubes should be shaken carefully after fifteen minutes of incubation.

(5) For maintaining high or low temperature, the test tubes should be kept in beakers containing boiled water or ice cubes respectively.

Conclusion. From the above experiment, it is concluded that high temperature and organic solvents enhance the cell permeability leading to leaching of pigments in coloured plant parts.

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CHAPTER 6

Numerical Problems Solving Mendel's Laws of Inheritance

EXPERIMENT NO. 6

Aim of the Experiments : To solve numerical problems of Mendel's laws of inheritance.

Requirements : Pea (*Pisum sativum*) or pigeon pea (*Cajanus cajan*) seeds of parental (P), F_1 and F_2 generations.

Theory : Gregor J. Mendel, the father of Genetics, had studied seven pairs of contrasting traits of pea plants and derived two important principles *i.e.* principle of segregation and principle of independent assessment. The former principle was derived from monohybrid experiment and the latter principle was derived exclusively from the dihybrid experiment.

Principle of segregation includes three laws : 1. law of unit character 2. law of dominance and 3. law of segregation. From principle of independent assortment, the law of independent assortment was subsequently derived. A character (gene of today) has two alternative traits (allelles of today) out of which one is dominant and the other is recessive.

Procedure : The characters and number of seeds of pea/pigeon pea are studied and counted respectively. Then the data are analysed and conclusions are derived.

Observations:

1. For law of unit character. The characters and traits of the supplied seeds of F_2 generation are studied and observed as follows :

Sl.No.	Characters	Alternative Trait-1	Alternative Trait-2
1.	Shape of the seed	Round	Wrinkled
2.	Colour of the seed coat	Grey	White
3.	Colour of the cotyledon	Yellow	Green

It is observed that the character shape of the seed has two alternative traits *i.e.* roundness and wrinkledness of the seeds. Likewise the character, colour of the seed coat has two alternative traits. *i.e.* grey and white. The seed coat of each seed is removed and the colour of the cotyledons is noted. It is observed that the character, colour of the cotyledon has also two alternative traits *i.e.* yellow and green. Thus Mendel's first law of inheritance (law of unit character) is verified.

2. For law of dominance: The traits of the seeds of F_1 generation are compared with that of the parental seeds and observed that a particular trait is expressed and another trait is suppressed in the F_1 generation. The suppressed trait is known as recessive trait and the expressed trait is known as dominant trait. From these seeds the following observations are noted.

Sl.No.	Character	Dominant Trait	Recessive Trait
1.	Shape of the seed	Round seed	Wrinkled seed
2.	Colour of the seed coat	Grey seed coat	White seed coat
3.	Colour of the cotyledon	Yellow cotyledon	Green cotyledon

Moreover, it was observed that the number of seeds having dominant trait is higher than that of the seeds with recessive trait.

Thus mendel's second law of inheritance is verified.

3. For law of Segregation: The character (seed shape) trait of the seeds of F_2 generations are observed and the number of seeds in each case is counted and presented below:

Sl.No.	Trait of the seed shape	No. of Seeds	% of Total	Ratio
1.	Round seed	62	76.54	3.06 ~ 3
2.	Wrinkled seed	19	23.46	0.94 ~ 1
Total		81	100	4

The percentage of round and wrinkledg seeds are found to be 76.54 and 23.46 respectively which is equal to 3 : 1 ratio. The F_1 seeds were found to be all round seeds. It indicates that the pair of traits of the character (shape of the seed) *i.e.* roundness and wrinkledness are segregated or separated from each other in the F_2 generation. Thus the third law of segregation is verified.

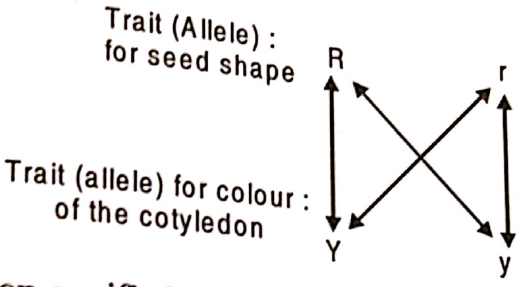
From the F_2 findings, the phenotypic monohybrid ratio of 3: 1 is also verified.

4. For law of independent assortment: The number of seeds of four types of samples *i.e.* (*i*) round seed with yellow cotyledon, (*ii*) round seed with green cotyledon (*iii*) wrinkled seed with yellow cotyledon and (*iv*) wrinkled seed with green cotyledon of F_2 pea plants are collected, counted and presented in the following table.

Sl.No.	Traits of the F_2 Seed	No. of Seeds	% of total	Ratio
1.	Round and Yellow	46	56.080	9.09 ~ 9
2.	Round and Green	16	19.75	3.16 ~ 3

3.	Wrinkled and Yellow	14	17.28	2.76 ~ 3
4.	Wrinkled and Green	5	6.17	0.98 ~ 1
Total		81	100	16

All the F_1 seeds are found to be with round shape and yellow cotyledon. From the above table it is observed that each trait of a pair *i.e.* either round or wrinkled seed is assorted or placed independently with each trait of the other pair of traits *i.e.* yellow and green cotyledon, so that 4 types of progenies are found in the F_2 generation in the ratio 9 : 3 : 3 : 1, known as the phenotypic dihybrid ratio.



Thus the above observation verified the fourth law of independent assortment that two pair of factors or traits (alleles) of an individual or F_1 hybrid are assorted (or placed) independently giving rise to different combinations with equal probability during gamete formation. Thus in the above case, each factor of a pair combines with each factor of the other pair giving rise to four types of gametes in equal frequency *i.e.* RY, R*y*, *r*Y and *ry* which produced four types of F_2 plants.

Conclusion. From the above observation and analysis, Mendel's four laws of inheritance are verified.

□□□□□

CHAPTER 7

Chromosome Mapping using Three Point test Cross Data

EXPERIMENT NO. 7

Aim of the experiment : To map a chromosome using three point test cross data.

Requirement : Graph paper, 3-point test cross data.

Theory : Genes (points) are present in a linear manner on the chromosome. All the genes present on a chromosome are known as **linked genes**. The distance between genes is known by recombination frequency represented by map unit, called **centimorgan** (cM).

1% recombination = 1 map unit (m.u.) = 1 cM

The linkage map using three point (or gene) test cross data can be plotted in a graph.

Procedure : The 3 point test cross data supplied is to be analyzed and the linkage map is plotted in a graph.

Three-point test cross data

Suppose the endosperm characters of maize are taken into consideration. The three characters and corresponding genes are :

1. Coloured aleurone : C versus colourless aleurone : C
2. Full endosperm : Sh. versus shrunken endosperm : sh
3. Non-waxy endosperm : Wx versus waxy endosperm : wx

The recombinant values can be known from the crossover values in a test cross between a coloured, shrunken and non-waxy homozygous parent with a colourless, full and waxy homozygous parent (3-point test cross), which are as follows:

1. % of single crossover between C and sh = 3.414%
2. % of single crossover between sh and Wx = 18.291%
3. % of double crossover between C and Wx = 0.0829%

Linkage map distance would be :

Between C and sh = 3.414 + 0.089 = 3.503 ~ 3.5 cM

Between sh and Wx = 18.291 + 0.089 = 18.38 ~ 18.4 cM

Between C and Wx = 3.414 + 18.291 + (2 × 0.89) = 21.883 ~ 21.9 cM

or

3.5 cm + 18.4 cm = 21.9 cM

The above data is plotted in a graph and the linkage map between the three genes *i.e.* C, sh and Wx is prepared.

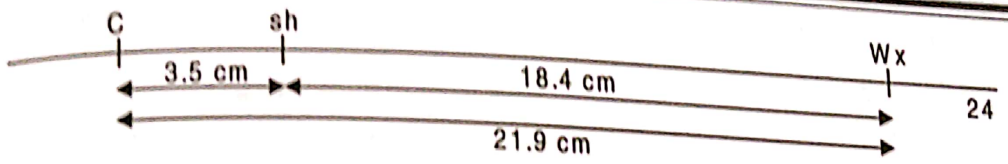


Fig. 7.1. Linkage map of 3 genes, C sh and Wx (scale : 1 cM = 0.5 cm)

Conclusion: From the above data, linkage map of three genes can be plotted in a graph which shows that the map distance between C and sh is 3.5 cM, between sh and Wx is 18.4 cM and between C and Wx is 21.9 cM. It shows that the gene sh is present in the middle.

□□□□□

CHAPTER 8

Hybridization Techniques : Emasculation and Bagging (Demonstration)

EXPERIMENT NO. 8

Aim of the Experiment : To demonstrate the hybridization techniques of emasculation and bagging.

Requirements : A plant having bisexual or hermaphrodite flower, forceps, polythene or plastic bag.

Theory and Procedure :

I. Emasculation : Removal of stamens or anthers or killing the pollen of a flower without the female reproductive organ is known as emasculation. In bisexual flowers, emasculation is essential to prevent of self-pollination. In monoecious plants, male flowers are removed, (castor, coconut) or male inflorescence is removed (maize). In species with large flowers e.g. (cotton, pulses) hand emasculation is accurate and it is adequate.

Methods of Emasculation : There are various methods of emasculation out of which some important ones are :

1. Hand Emasculation : In species with large flowers, removal of anthers is possible with the help of forceps. It is done before anther dehiscence. It is generally done between 4 and 6 PM one day before anthers dehisce. It is always desirable to remove other young flowers located close to the emasculated flower to avoid confusion. The corolla of the selected flower is opened with the help of forceps and the anthers are carefully removed with the help of forceps.

In cereals, one third of the empty glumes will be clipped off with scissors to expose anthers. In wheat and oats, the florets are retained after removing the anthers without damaging the spikelets. In all cases, gynoecium should not be injured. An efficient emasculation technique should prevent self pollination and produce high percentage of seed set on cross pollination.

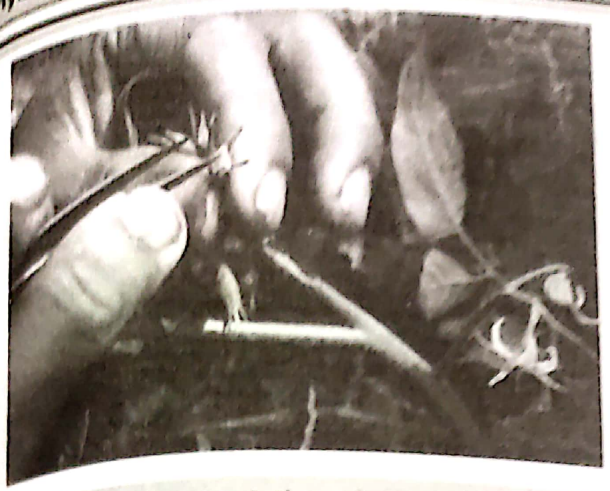


Fig. 8.1. Emasculation of a tomato flower with the help of a forcep

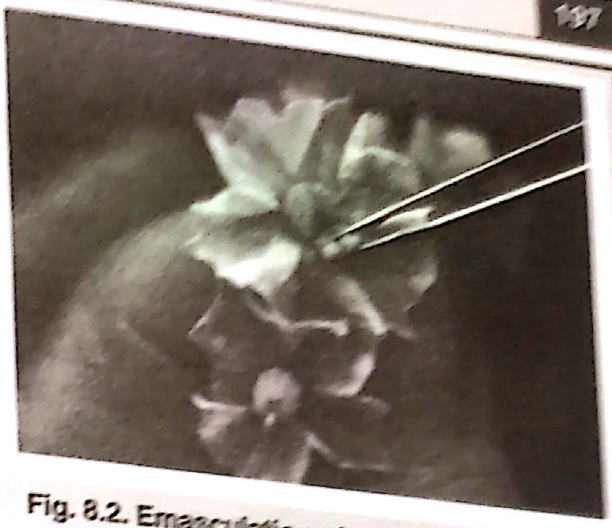


Fig. 8.2. Emasculation of a flower with the help of a forcep

2. Suction Method : It is useful in species with small flowers. Emasculation is done in the morning immediately after the flowers open. A thin rubber or a glass tube attached to a suction hose is used to suck the anthers from the flowers. The amount of suction used is very important which should be sufficient to suck the pollens and anthers but not gynoecium. In this method considerable self-pollination, upto 10% is like to occur. Washing the stigma with a jet of water may help in reducing self-pollination, However self pollination can not be eliminated in this method.

3. Hot Water Treatment : Pollen grains are more sensitive than female reproductive organs to both genetic and environmental factors. In case of hot water emasculation, the temperature of water and duration of treatment vary from crop to crop. It is determined for every species. For sorghum 42-48°C for 10 minutes is found to be suitable. In the case of rice, 10 minutes treatments with 40-44°C is adequate. Treatment is given before the anthers dehiscence and prior to the opening of the flower. Hot water is generally carried in thermos flask and whole inflorescence is immersed in hot water.

4. Alcohol Treatment : It is not commonly used. This method consists of immersing the inflorescence in alcohol of suitable concentration for a brief period followed by rinsing with water. In alfalfa the inflorescence immersed in 57% alcohol for10 second was highly effective. It is a better method of emasculation than suction method.

5. Cold Treatment : Cold treatment like hot water treatment kills the pollen grains without damaging gynoecium. In the case of rice, treatment with cold water 0.6°C kills the pollen grains without affecting the gynoecium. This is less effective than hot water treatment.

6. Use of Gametocide : It is also known as chemical hybridizing agent (CHA) chemicals which selectively kills the male gamete without affecting the female gamete. e.g. Ethrel, Sodium methyl sulphonate, Zinc methyl arsenate in rice, Maleic hydrazide for cotton and wheat.

II. Bagging : Immediately after emasculation the flower or inflorescence is enclosed with suitable bags of appropriate size to prevent random cross-pollination. Thus bagging is the process of covering the flowers of selected female parent plants by polythene or

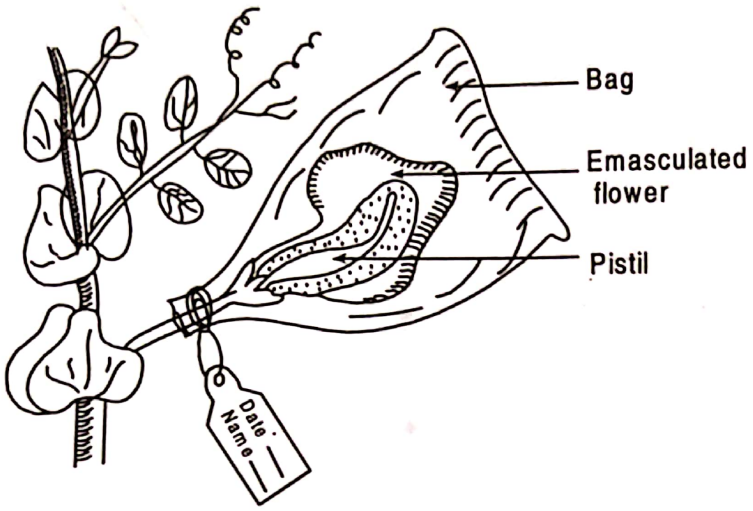


Fig. 8.3. Bagging of Emasculated Flower

plastic bags to avoid contamination by foreign undesirable pollen grains. The bagging will continue till the stigma is no more receptive or functionless. Bags are removed when fruits start to develop.

The flowers are tagged just after bagging. They are attached to the infloresence or to the flower with the help of a thread. The following may be recorded on the tag with pencil.

1. Date of emasculation
2. Date of pollination
3. Parentage
4. No. of flowers emasculated

Conclusion : From the above demonstrative experiment the techniques of emasculation and bagging during plant hybridization are studied.

□□□□

CHAPTER 9

Colorimetric Estimation of DNA by Diphenylamine Method

EXPERIMENT NO. 9

Aim of the Experiment : To estimate DNA colorimetrically by using diphenylamine reagent

Theory and Principle : Deoxyribonucleic acid (DNA) is the icon of modern bioscience. To estimate DNA, it is treated with diphenylamine under acidic condition so that a blue compound is formed with a sharp absorption maximum at 595 nm which can be measured by a colorimeter or spectrophotometer. This reaction is given by 2-deoxypentoses in general and not exactly for DNA. In acid solution, the straight chain form of the deoxypentose is converted to highly reactive hydroxy levuinaldehyde which reacts with **diphenylamine (D.P.A.)** to give a blue complex. In DNA only the **deoxyribose of the purine nuclcotides** reacts so that the value obtained represents half of the total deoxyribose present.

Material :

1. **Calf thymus** DNA (standard 1000 μg/ml) or salmon sperm (can be obtained from a standard company like Sigma Chemicals), spinach leaf.
2. D.P.A. reagent (1 g of pure diphenylamine + 100 ml glacial acetic acid + 2.5 ml conc., sulphuric acid).

   Note: D.P.A is a poisoning reagent.
3. All the reagents are made fresh.
4. Colorimeter/Spectrophotometer
5. Pipettes (1 ml and 5 ml).
6. 5 × 10 ml test tubes.
7. Water bath
8. Parafilm
9. Measuring cylinder 10. Digital balance.

Procedure :

1. Add the following amount in seven test tubes. (The weight of diffract chemicals can be taken with the help of a digital balance).

Reagents	1	2	3	4	5	6 (Un-known)	7 (Blank)
Standard DNA (1000 μg/ml)	0.1 ml	0.2 ml	0.3 ml	0.4 ml	0.5 ml	–	NIL
Unknown sample solution	–	–	–	–	–	0.5 ml	NIL
H_2O	1.4 ml	1.3 ml	1.2 ml	1.1 ml	1.0 ml	1.0 ml	1.5 ml
D.P.A.	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml	2.5 ml

The unknown DNA sample solution (should be aqueous) may be obtained from spinach leaf In the test tube No.6, 0.5 ml of this unknown DNA solution may be taken.

2. Place test tubes in 100°C water bath for 10 min.
3. Cool the tubes and measure the Optical density (O.D.) at 595 nm. ($^{A}595$) (A: Absorbancy).
4. A standard curve using the absorbance (A_{595}) readings against the conc. (μg/ml), is drawn in a graph. Then the conc. of DNA of unknown sample is determined.

Tabulation :

Sl.No.	Test tube No. and nature	A_{595}
1.	1 (known DNA, 0.1 ml)	say x_1
2.	2 (known DNA. 0.2 ml)	x_2
3.	3 (known DNA, 0.3 ml)	x_3
4.	4 (known DNA, 0.4 ml)	x_4
5.	5 (known DNA, 0.5 ml)	x_5
6.	6 (unknown sample)	y

Calculation : From the above tabulated value, a standard curve may be plotted with known concentration of DNA is X-axis and A_{595} in Y-axis. The standard curve will be a straight line passing through maximum possible points. From this standard curve value, the concentration of DNA present in the unknown sample can be calculated.

For example, if from the standard curve plotted in a graph (Fig. 9.1) the value of A_{595} with 0.5 ml of standard DNA soln gives 0.25 and the A_{595} of unknown sample is 0.15 (y) then the amount of DNA present in the unknown sample may be calculated as follows.

Standard DNA solution will contain 1000 μg DNA/ml

Hence, 0.5 ml standard solution will contain = $1000 \times 0.5 = 500$ μg

Thus, standard known solution with 500 μg DNA gives $A_{595} = 0.25$

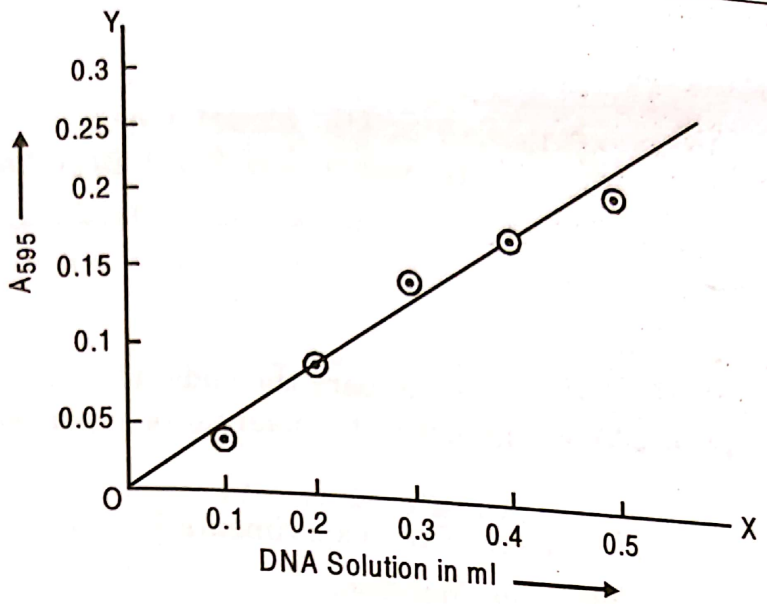


Fig. 9.1. A sample gaph showing standard curve for DNA by using diphenylamine reagent.

When A_{595} is 0.25, amount of DNA = 500 μg

So A_{595} with 0.15, amount of DNA $= \frac{500}{0.25} \times 0.15 = 300$ μg

Thus the unknown sample contains 300 μg DNA/0.5 ml or 600 μg/1 ml.

Precautions:

1. The weight or volume of different chemicals should be measured carefully and correctly.
2. The glasswares should be thoroughly cleaned, washed and dried before use.
3. Pipetting should be done carefully.
4. For plotting the standard curve in the graph for diphenylamine method, the straight line (standard curve) should satisfy all the five points and it may go directly through two points or more points.

Conclusion: From the above experiment, the amount of DNA of any sample can be estimately quantitatively through colorimetric analysis using diphenylamine reagent. From the standard curve, the value of A_{595} for a certain conc. of DNA can be measured which gives a more correct value. By the above experiment the amount of DNA present in the unknown sample was found to be 600 μg/lml (for example, but after actual calculation, put here the exact value).

□□□□

MODEL PAPER

B.SC. IIIrd - (Semester-V)
Botany Practical Model Paper
Cell Biology, Genetics and Plant Breeding

1. Perform the Experiment **A.**

   Perform squash on onion root tip, prepare the slide, identify at least one division stage. Write the procedure and draw the diagram of reported stage. $1 \times 15 = 15$ marks

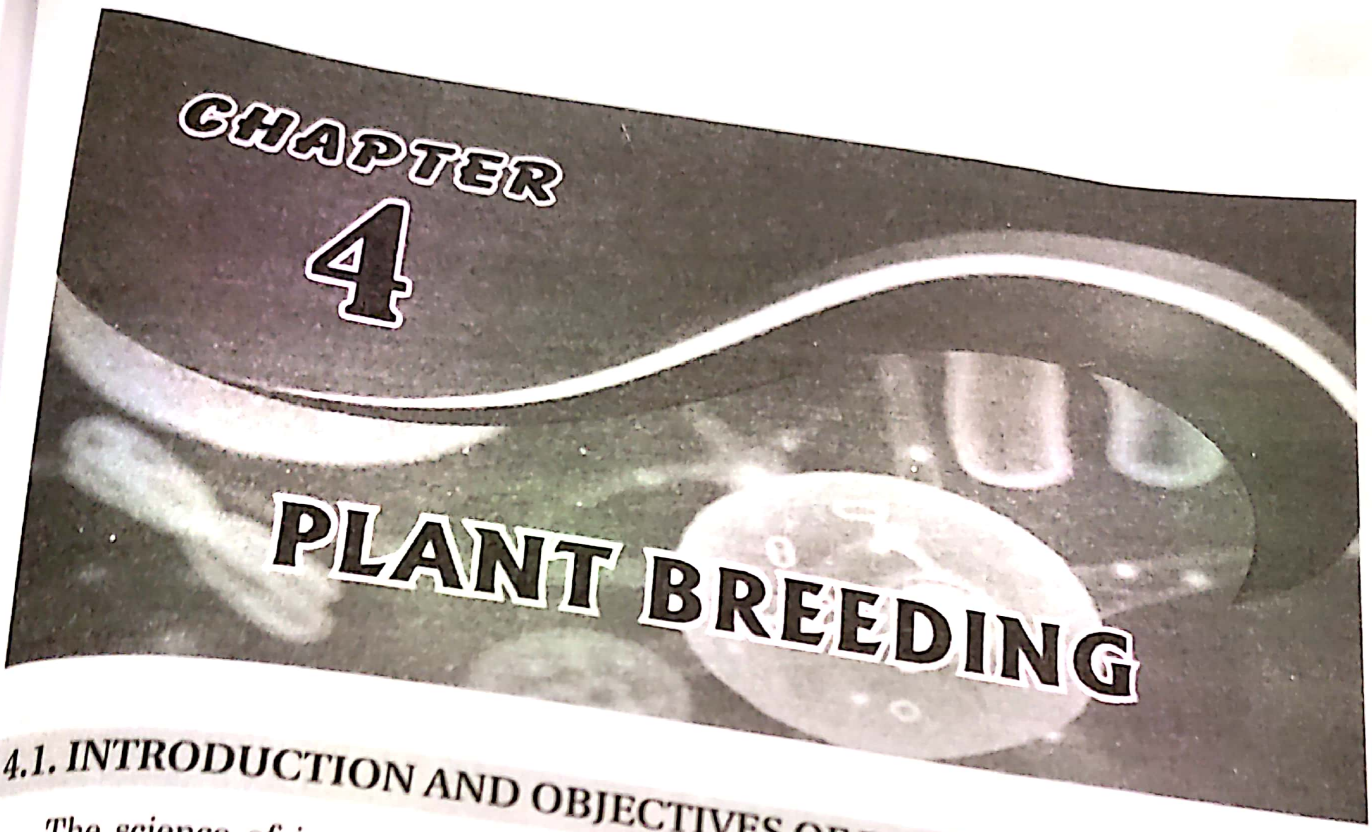
2. Give the experimental protocol of the experiments B $1 \times 10 = 10$ marks
3. Solving numerical problems on Mendelian in heritance C,D $2 \times 7\frac{1}{2} = 15$ marks
4. Record & Viva $= 10$ marks

Total = 50 marks

A-Onion root squash technique

B- Estimation of DNA by diphenylamine method

C&D Numerical problems on Mendelian Inheritance.



4.1. INTRODUCTION AND OBJECTIVES OF PLANT BREEDING

The science of improvement of various crop varieties or cultivars through different methods is called **plant breeding**. Plant breeding originated along with human civilization from time immemorial when human beings took interest in superior plants for their use and could recognize which plants were more beneficial and which were less. The early selectors were selecting the plants basing only on economic use and not on scientific basis. With the advancement in the field of science, plant scientists tried to select the plants on the basis of their cytology, genetics, including laws of inheritance. The scientists taking part in improvement of crop varieties are called **plant breeders**. The modern plant breeders are improving crops basing on the knowledge of **modern cytogenetics** and **genetic engineering** and **recombinant DNA technology**.

A **hybrid plant** is an offspring produced by crossing two plants having different characters which may be produced naturally or artificially. The first artificial hybrid was produced by **Thomas Fair Child** (1717) by crossing between sweet william and carnation flower i.e. *Dianthus barbatus* × *D. caryophyllus*. In the last century, several improved cultivars of crops have been produced in India and aboard leading to green revolution. The scientists who are famous in this aspect are **M.S. Swaminathan** (for rice and wheat), **K. Ramiah** (for rice), **B.P. Pal** (for wheat), **T.S. Venkatraman** (for sugarcane) and **J.K. Roy** (rice) etc. The famous breeding centres in rice are **Central Rice Research Institute** (CRRI), Bidyadharpur, Cuttack and **International Rice Research Institute** (IRRI), Philippines, Manila. According to report of IRRI, at present around 1,00,000

cultivars of rice are existing today in the whole world. The improved crop varieties have enhanced the food production of the country at a higher rate in recent times, so that India is now self-sufficient in food. Some of the well known achievements are the development of semidwarf rice and wheat cultivars, improved varieties of maize, soybean, bajra, jowar, sugarcane and many cereals, pulses, fruits and vegetables.

Crop improvement is defined as the changing the heredity of a crop for higher yield, better quality, resistance to diseases, environmental stresses, shorter duration and suitability to particular environment. Several methods are there for crop improvement *i.e.* selection, introduction, hybridization, polyploid breeding, induced mutations, tissue culture and genetic engineering.

4.2. METHODS OF CROP IMPROVEMENT

Plant breeding aims at improving the genetic makeup of the crop cultivars. Crop improvement involves changing heredity of crops for higher yield, better quality, resistance to disease and stresses, shorter duration, and suitability to particular environmental conditions.

The various methods used for crop improvement are as follows:

1. Selection
2. Introduction
3. Hybridization
4. Polyploid breeding
5. Mutation breeding

Selection, introduction and hybridization are discussed below. Mutation and polyploid breedings are discussed in the next chapter.

I. Selection

It is of two types :

(*a*) **Natural selection** : As proposed by **Charles Darwin**, origin of species is due to natural selection during course of organic evolution. Nature selects and preserves the desirable traits while discarding the undesirable variations. All the local or wild cultivars of crops are the result of natural selection.

(*b*) **Artificial selection** : It is the selection of the desired individual plants from a mixed population. It is of three types.

(*i*) **Mass selection**: It involves choosing seeds of a large number of phenotypically superior plants and sowing them in a mass in the next season without any progeny testing. It is a simple method which eliminates the undesirable traits and enhances the frequency of desirable traits. This selection is suitable to self-pollinated plants. However it may lead to inbreeding depression and loss of superior heredity.

(*ii*) **Pure line selection** : It is developed in plants obtained through self-pollination over a few generations from **single homozygous individual**. These developed plants are more pure and durable and exhibit homozygosity of desired traits. These plants may be released for mass cultivation as a new variety. **Example :** wheat variety PV-18 from V-18, Kalyan-sona from S-227, Ganga, Vijaya etc. Maximum and permanent improvement of the desired traits are observed in this type of selection. But this method takes a lot of time period and unsuitable for cross pollinated plants.

(*iii*) **Clonal selection**: It is the selection of plants with desirable traits which are vegetatively propagated. This selection is useful in maintaining superiority of **heterozygous, polyploid individuals**. **Example:** banana, sugarcane, potato, sweet-potato, mango, lemon, orange, apple. Specific cultivars are **navel orange, blood red sweet orange, Bombay green banana, Kufri safed potato** etc. A **clone** is a plant obtained vegetatively from a parent plant. **Superior clones** are obtained on the basis of their phenotypic characters. The selected clones are multiplied vegetatively before giving to the farmers for cultivation.

II. Introduction

Plants are introduced from one area to another area or from one country to another country where these plant cultivars are absent. The reasons for such transfer of plants are for

(*i*) use in agriculture and industry *e.g.* cereals, pulses, oil-, fibre-, sugar- or drug-yielding plants.

(*ii*) fruit and vegetable crops

(*iii*) ornamental purposes

(*iv*) increasing collection of plants in botanical gardens and parks

(*v*) improvement of local cultivars

After a few generations of the introduction of new plants, they are adapted to the new environment and this phenomenon is known as **acclimatization.** Example : The British people introduced cabbage, cauliflower, litchi, tea, cinchona etc. to India, Portuguese introduced tobacco, guava, maize, groundnut, pineapple, potato and sweet potato and the **Japonica variety of rice** from Japan etc. However, plants to be introduced are checked for any kind of disease, pest, weed etc. known as **plant quarantine** which is done under **Destructive Insects and Pests (DIP) Act, 1914.**

III. Hybridization

Definition : It is the crossing of two or more types or kinds of plants for bringing their traits together in the progeny.

Or

It is the mating or crossing of two plants or lines of different genotypes so as to obtain a new progeny.

The main objective of hybridization is to develop new plants with better characteristic features. Thus hybridization leads to creation of genetic variation. When two plants with different genotypes at parental generation are cross-pollinated, the F_1 plants, called F_1 **hybrids** contain genes from both the parents. In F_2 generation, segregation and recombination produce many new type of gene combinations which may be observed in later generations *i.e.* F_3, F_4, F_5 etc. called **segregating generation.** The degree of variation depends upon the number of heterozygous genes in F_1 hybrid and number of different traits in the parents. If the two parents are closely related, they differ in few genes and if distantly related, then differ in several genes, even in hundreds.

A. Types

Hybridization may involve.

(*i*) **Single cross** : A cross between two parents, example - A cross between *Oryza japonica* and *O. indica* producing the high yielding rice variety **ADT-37** which is a interspecific hybrid.

(*ii*) **Multiple cross** : A cross involving more than two parents. Example: **wheat variety C-306** was produced by multiple crosses between C-591 (Reagent 1974 × Ch2-3) and hybrid of P-19 × C-281.

Hybridization may further be categorized as:

1. Intervarietal or Intraspecific
2. Interspecific
3. Intergeneric

1. Intervarietal (Intraspecific) Hybridization : In this type of hybridization, the two parents belong to two different varieties or cultivars of the same species. It may involve simple cross or complex cross.

(*i*) **Simple cross** : In simple cross, two parents of the same species are crossed to produce the F_1 hybrid which in selfed to yield F_2 or is used in backcross programme. Example, the rice cultivar *Oryza sativa* var. Ratna is developed by a cross between two varieties *i.e.* TKM 6 and IR8 (developed at CRRI, Cuttack).

(*ii*) **Complex cross** : Here more than two parents are crossed to produce the hybrid, the F_1 hybrid is used to produce F_2 or is used for back cross programme. It is also called **convergent cross** as it brings together many genes from several parents into a single hybrid. An example is the **wheat variety C-306** as discussed above (multiple cross), the model may be as follows:

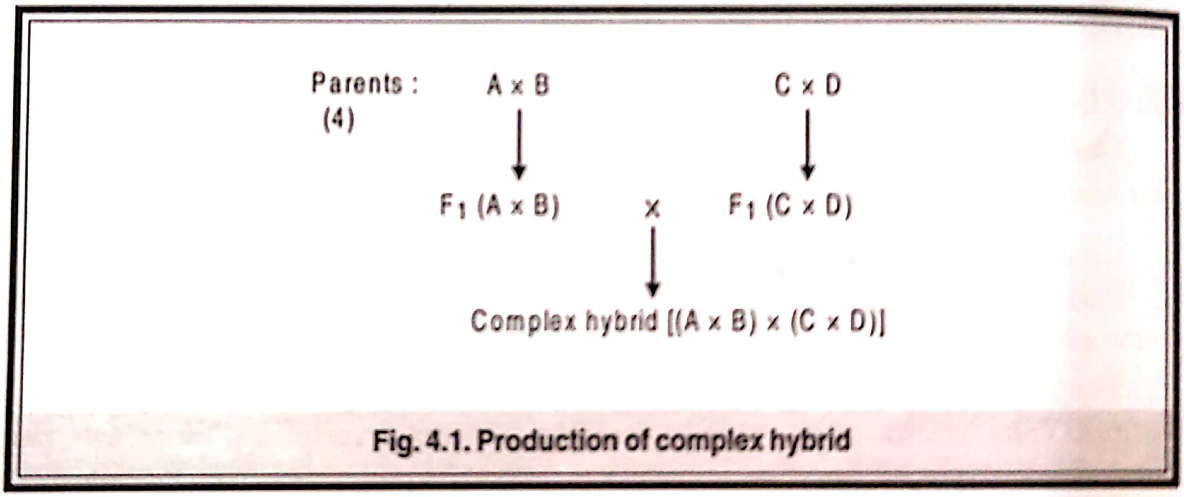


Fig. 4.1. Production of complex hybrid

2. Interspecific or Intrageneric hybridization: It involves crosses between two different species of the same genus. It may lead to the creation of a new species. All present day varieties of sugarcanes are produced by crosses between *Saccharum officinarum* and *S. barberi* or other *Saccharum* species. Other examples of interspecific crosses are as follows.

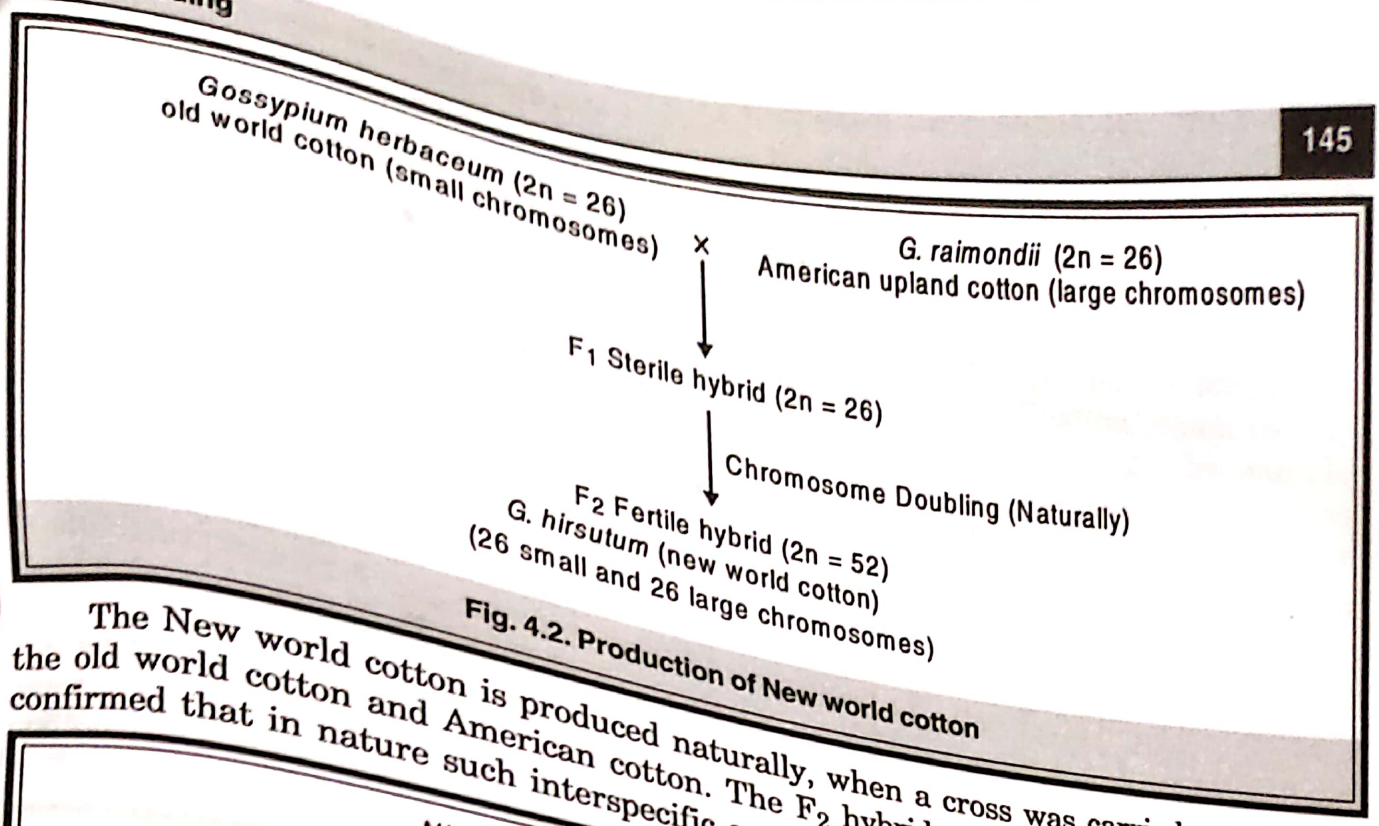


Fig. 4.2. Production of New world cotton

The New world cotton is produced naturally, when a cross was carried out between the old world cotton and American cotton. The F_2 hybrid resembled *G. hirsutum* which confirmed that in nature such interspecific cross had occurred.

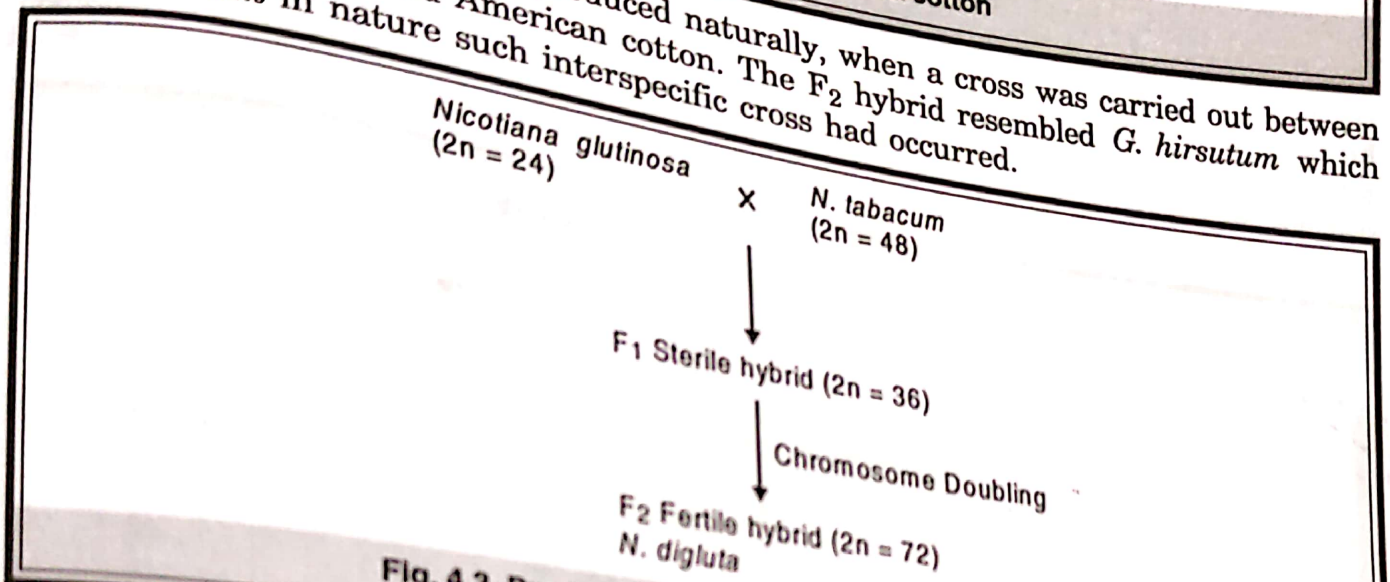


Fig. 4.3. Production of *N. digluta* by interspecific cross

3. Intergeneric hybridization : It involves crosses between two different species of two different genera producing a new species having a new genetic combination. *Raphanobrassica* is a classical example of intergeneric cross which is an **allopolyploid**. In 1927, **G.D. Karpechenko**, a Russian geneticist reported a cross between radish, *Raphanus sativus* (2n = 18) and cabbage, *Brassica oleracea* var. Capitata (2n = 18) producing F_2 fertile hybrid, *Raphanobrassica* (2n = 36), a **novel hybrid plant**. But this plant had roots like cabbage and shoots like radish which was not economically variable. However, it is a novel plant of genetic importance.

Another bright example of intergeneric cross is the production of **Triticale**, the first man-made crop, developed by a cross between wheat (*Triticum aestivum*) and rye (*Secale cereale*), reported by **A. Muntzing** (1939) from Sweden. However, triticale was first mentioned by **A. Stephen Wilson**, a Scottish botanist, who was successful in producing two Triticale plants, which were found to be sterile. Triticale, the **rye-wheat hybrid** is

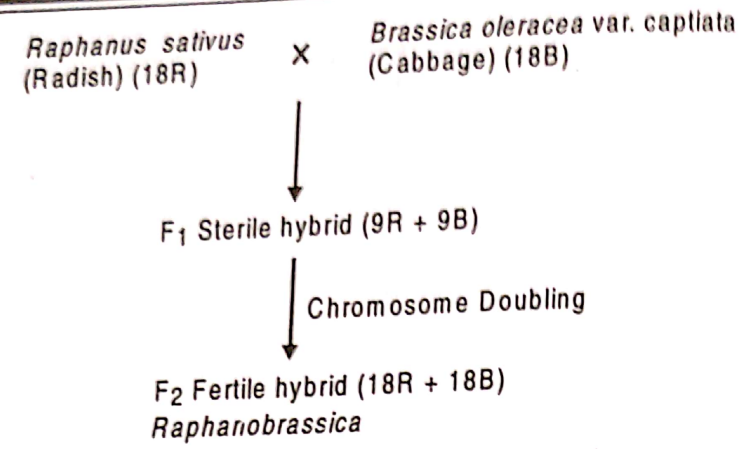


Fig. 4.4 Production of *Raphanobrassica*

resistant to drought. It is now grown on a commercial scale in more than one million hectares and research is in progress at several centres all over the world to improve this man made crop. Several hundred cultivars of triticale have been released in the last three decades. In 2014, according to the Food and Agriculture Organization (FAO), 17.1 million tonnes of triticale were harvested in 37 countries across the world.

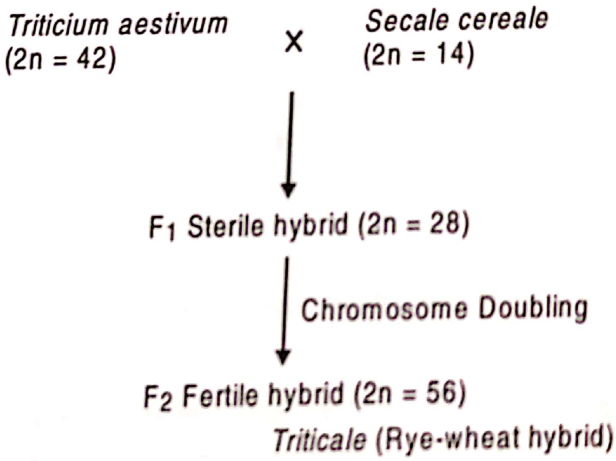


Fig. 4.5. Production of Triticale

B. Technique of Hybridization

The various steps involved in hybridization are as follows:

(*i*) **Selection :** The parent plants having desirable traits to be developed in the new crop are searched and listed. Two or more such types of plants are selected as the parental plants for different type of crosses.

(*ii*) **Selfing :** The selected parent plants undergo self breeding (self-pollination) for a few generations to get pure plants which contain homozygosity of the desired traits. In this process the defective plants are discarded. Healthy and vigorous pure plants are choosen for subsequent crossing.

(*iii*) **Emasculation :** The removal of stamens or anthers (male parts) from the bisexual flowers of female parent plant in the young stage is known as **emasculation**, which avoids chances of contamination from their pollens, thus self-pollination is avoided. For this process, the anthers are removed by the help of scalpels or scissors. **Male sterility** is an advantage for cross pollination which can be artificially induced in certain plants by (*a*) spraying inflorescence by **maleic hydrazide** (MH), **2,4-D** (2,4-dichlorophenoxy acetic acid) or **NAA** (Naphthelene acetic acid), (*b*) dipping the inflorescence of the female parent in **hot water** at 50°C for ten minutes *e.g.* **Sorghum** and (*c*) inverting a wide test tube having moist filter paper over the inflorescence when the anthers come out *e.g.* Ragi. Natural male sterility is found in plants like sunflower, cucumber, tomato, carrot, barley, maize etc. which can be taken as female parents.

(*iv*) **Bagging and cross pollination :** It is the process of covering the flowers of selected female parent plants by polythene or plastic bags to avoid contamination. Also after cross pollination, the flowers of the desired plants are also bagged till the stigma is no more receptive or functionless. The cross pollination is done by collecting the pollen grains from the flowers of desired male parent carefully by clean paper or test tubes or polythene bags. These pollens are then dusted over the receptive stigmas of the emasculated flowers of the selected female parent plant by clean brush. Bags are removed when fruits start to develop. Mature fruits and seeds are collected for testing.

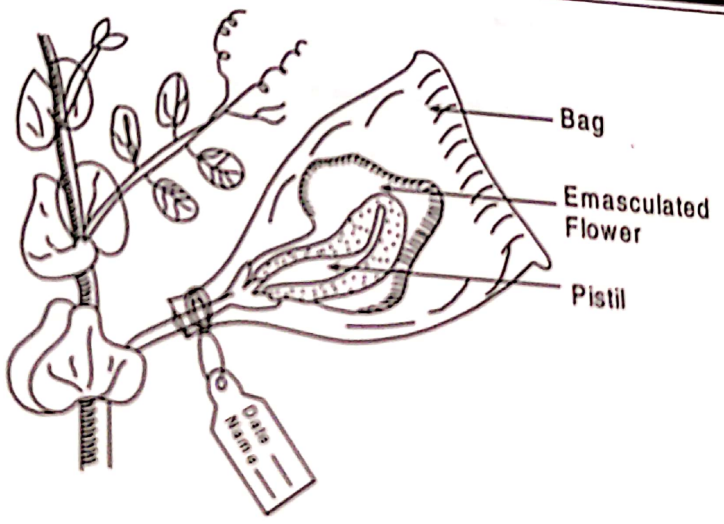


Fig. 4.6. Bagging of emasculated flower

(*v*) **Testing :** Seeds collected are now sown for the next generation in the next season which produce F_1 plants. The F_1 plants are evaluated for the desired traits and then the F_1 plants are selfed to produce the F_2 plants. The plants with desired traits are selected and allowed to inbreed for 2-3 generations.

(*vi*) **Back crossing :** If a desired trait is absent in the hybrid, the F_2 plants are backcrossed with the parent plants. The offsprings are then inbreed for

2-3 generations to obtain homozygosity of the desired traits, which ultimately released as a **new variety**.

C. Hybrid Vigour or Heterosis

Inbreeding leads to reduction in vigour and fertility and this phenomenon is known as **inbreeding depression**. However, hybridization which involves cross pollination between two different genotypes, results in increased vigour and fertility. Thus, the phenotypic superiority of the hybrid over either of its parents in one or more traits is known as **hybrid vigour** or **heterosis**. This term was coined by **A.F. Shull** (1912). However heterosis was first studied by **Kolreuter** (1763) followed by Charles Darwin (1876). Hybrid vigour leads to increased size, yield, vegetative growth, resistance to disease, insects, environmental stresses, agronomic superiority etc. This property of hybrid vigour has been exploited in commercial crops such as rice, maize, sorghum, tomato, bajra, sugarbeet, cabbage, cucumber, *Petunia* and *Zinnia* etc. Hybrid cotton produces large sized boll, cabbage produces large head, maize produces 30-50% higher yield in grain. Heterosis also leads to larger number and size of fruits in brinjal, tomato, mango, apple, larger tubers in potato, larger roots in radish, beet and carrot. Even in some cases the yield may be increased upto 300%.

By inbreeding, the hybrid vigour is lost gradually. Hence, seeds should be produced every year by crossing the pure parental lines so that optimum heterosis is maintained. However, heterosis is suitable for vegetatively propagated plants due to lack of genetic changes. For this reason vegetatively propogated hybrid varieties of mango, apple, guava, sugarcane, potato, rose, dahlia and *Chrysanthemum* are so popular and commercially profitable.

IMPORTANT QUESTIONS

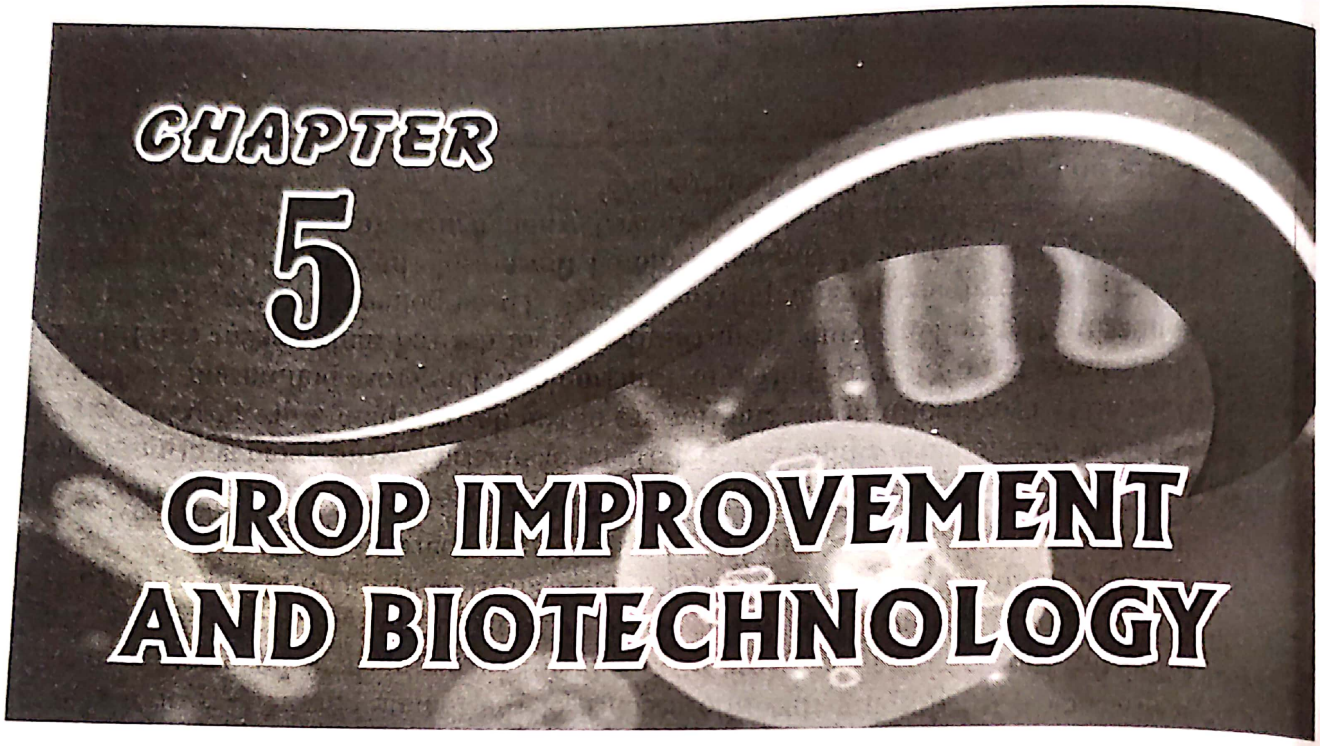
1. What in hybridization ? Describe the types and methods of hybridization.
2. Describe the different steps of hybridization.
3. Give a brief account of different types of breeding for crop improvement.
4. How the crops can be improved ?
5. Describe the role of heterosis in crop improvement.
6. How interspecific and intergeneric crosses help in crop improvement.
7. Enumerate plant selection and introduction.
8. How crop productivity can be enhanced by breeding programmes ?

Answer the following questions briefly :

1. Describe important techniques of plant hybridization.
2. Give a brief account of distant hybridization.
3. How artificial selection differs from natural selection ?
4. Discuss the hybridization programme in case of dioecious plants.
5. What is hybrid vigour ?

6. Discuss plant selection.
7. Enumerate plant introduction.
8. **Write notes on :**
   (a) Mass selection
   (b) Plant introduction
   (c) Heterosis
   (d) Pure line selection
   (e) Clonal selection
   (f) Emasculation
   (g) Inbreeding
   (h) Rye-wheat hybrid
   (i) *Raphano-brassica*
   (j) Amber colour wheat
   (k) Hybridization
   (l) Hybrid vigour
   (m) Interspecific crosses
   (n) Inbreeding depression
9. **Differentiate between the following :**
   (a) Mass and pure line selection.
   (b) Interspecific and intergeneric crosses.
   (c) Continuous variation and discontinuous variation.

□□□□□



CHAPTER 5

CROP IMPROVEMENT AND BIOTECHNOLOGY

5.1. ROLE OF MUTATIONS IN CROP IMPROVEMENT

I. Definition

The phenomenon of sudden heritable changes which alter the characters of organism is called **mutation**. Mutation is also a discontinuous variation in contrast to continuous variation as proposed by **Charles Darwin** in his principle of natural selection for creation of new species. Mutations are heritable due to abrupt changes in the genetic make up of the organisms. The individuals showing heritable changes are known as **mutants**. But an individual exhibiting only an altered phenotype is called a **variant** till it is established that the change is heritable. The allele of a gene producing the changed phenotype is called **mutant allele**. Thus, a mutation is a change in phenotype, which is sudden, **heritable** and is not produced due to segregation or recombination.

II. Types

In a broader sense mutations are of two types as follows :

1. **Chromosomal mutations.** Such mutations occur due to changes in chromosome number and structure leading to heritable changes in phenotype of the organisms. Changes in chromosome number is known as **polyploidy**, whereas changes in chromosome structure is known as **chromosomal aberrations** which may be due to **deletion, duplication, inversion** or **translocation** of chromosomal segments.

2. **Gene or point mutations.** Mutation occurring in the gene (also known as point) is known as gene mutation. At present, the term 'mutation' has been used as a synonym of gene mutation.

Mutation is the ultimate source of all the genetic variations existing in an organism. Without mutation all the genes would be present in only one form (the wild type alleles) and in the absence of their alternative forms or alleles, evolution and genetic studies would be impossible.

III. Brief development

The term 'mutation' was introduced by **Hugo de Vries** (1901) to describe sudden heritable changes in *Oenothera lamarckiana* (evening primrose), which later found to be due to chromosomal aberrations particularly due to translocation and de Vries proposed the **mutation theory**. **T.H. Morgan** (1910) discovered mutation in *Drosophila i.e.* the white eye mutant. Mutagenic action of X-rays was discovered by **H.J. Muller** in 1927 in *Drosophila* for which he was awarded Nobel prize in Medicine or Physiology in 1946. Mutagenic effect of X-ray in plants (barley) was discovered by **Stadler** in 1929. Mutagenic effect of mustard gas and other chemical mutagens were **discovered by Auerbach** and **Robson in 1946**. In 1929, the first plant breeding programme designed to exploit induced mutations now known as **mutation breeding** was initiated in Sweden, Germany, U.S.S.R. In India the first mutation breeding programme began is the early 1930's.

IV. Induction of Mutations

Both chromosomal and gene mutations may occur spontaneously (naturally) or artificially by induction. Induced mutations are caused by mutation causing agents known as **mutagens**. The mutagens are of two types *i.e.* **physical mutagens** and **chemical mutagens**. Some commonly used physical and chemical mutagens are listed in Table 5.1. However polyploidy can be induced by physical agents like heat/cold shock, dehydration, UV light, X-rays and centrifugation of seedlings, and by chemical agents like colchicine, acenaphthene, nitrous oxide, 8-hydroxyquinoline, chloral hydrate, veratrine, sulfanilamide, mercury chloride, ethylhexachloro cyclohexane etc. Colchicine is an alkaloid which is widely used to suppress mitotic spindle fibre production leading to chromosome doubling.

The rate of induced mutations varies considerable from one gene to another or from one chromosome to another and from species to species. Mutation can occur in any tissue or cell of an organism and at any developmental stage. However, treatment with mutagens during certain stages of cell division *e.g.* S phase may yield mutation at a rate higher than these obtained by treatments during other phases of cell cycle.

V. Role of Chromosome mutations in Crop Improvement

Out of the different chromosomal mutations, 'polyploid breeding' is of prime importance for crop improvement, which in discussed below.

Polyploidy is the phenomenon of increase in the number of genomes in addition to the normal diploid set. Depending upon the number of genomes (I genome = one haploid set), the polyploids may be as shown in Table 5.2.

Table 5.1. Some of the commonly used physical and chemical mutagens (Agents most commonly used in plants are marked with an asterik)

Class	Mutagen
Physical Mutagens	
Ionizing Radiations	alpha-rays (α-rays)
Particulate radiations	Beta-rays (β-rays)
	Fast neutrons*
	Thermal neutrons*
Nonparticulate radiations	X-rays*
	Gamma-rays (γ-rays)*
Nonionizing Radiations	Ultraviolet rays (UV rays)
Chemical Mutagens	
Alkylating Agents	Mustard gas or sulphur mustard, Nitrogen mustard, Ethylmethane sulphonate (EMS)*, Methylmethane sulphonate (MMS), Ethylethane sulphonate (EES), N-Methyl-N-nitro-N-nitrosogunanidine (NTG)
Base Analogues	5-Bromouracil (5-BU)
	2-Aminopurine (2-AP)
Acridine Dyes	Acriflavin, Proflavin, Acridine orange
Deamination Agents	Ethidium bromide
	Nitrous acid (HNO_2)
Other Chemical Mutagens	Hydroxylamine (HA), Sodium azide*, DNA sequences*

* Mutagens commonly used in plants.

Table 5.2. Types of Ploidy

No. of genomes	Type of ploidy
1n	Monoploid or haploid
2n	Diploid
3n	Triploid
4n	Tetraploid
5n	Pentaploid
6n	Hexaploid
7n	Heptaploid
8n	Octaploid
9n	Nanoploid
10n	Decaploid
12n	Dodecaploid

True polyploids or **euploids** are again devided into (*a*) **Autopolyploids** and (*b*) **Allopolyploids.** In case of autopolyploids, the genomes are similar, i.e. they are originated from parents having same genotypes. But in case of allopolyploids, the genomes are not similar, *i.e.* they originate from parents having different genotypes or from two different cultivars or species or even genera. In case of **aneuploids,** there is increase or decrease in the number of chromosome over or below the diploid number [$2n \pm \{1 \text{ to } (n\text{-}1)\}$] where n = haploid number.

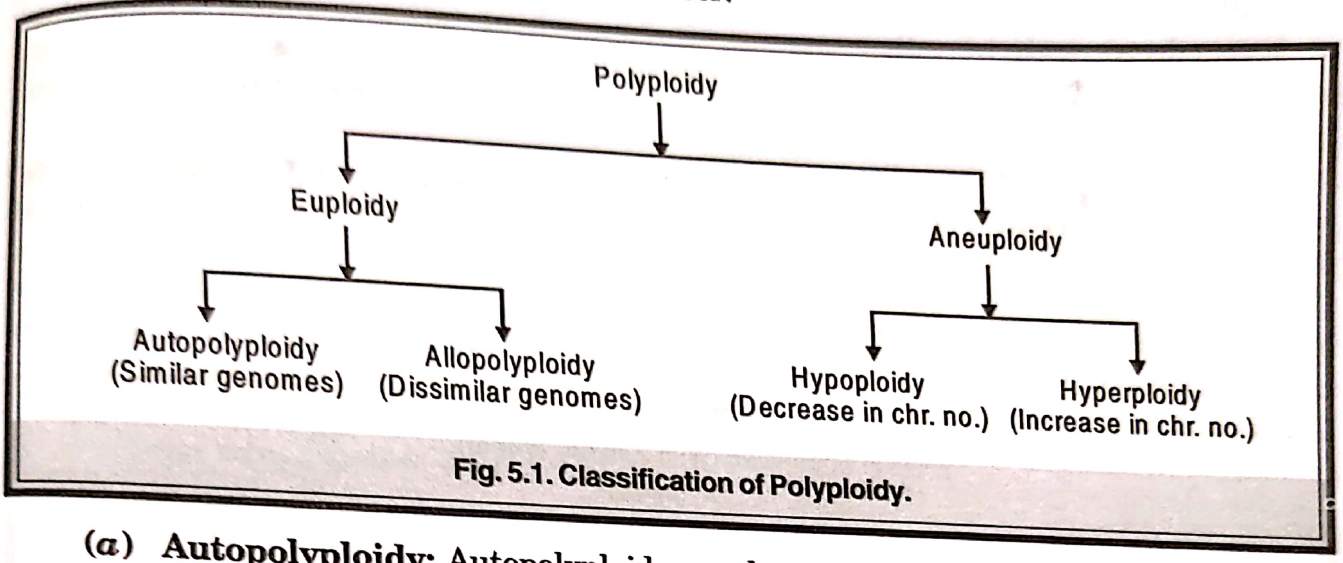


Fig. 5.1. Classification of Polyploidy.

(*a*) Autopolyploidy: Autopolyploids may be autotriploids is (AAA), autotetraploids (AAAA), autopentaploids (AAAAA) etc. However, autopolyploids having even set of genomes produce fertile seeds. Autopolyploids are produced due to abnormalities during mitotic or meiotic cell division. Failure in the anaphasic separation of chromosomes or cytokinesis leads to diploid gametes. If two diploid gametes fuse, a tetraploid is produced. If a diploid male gamete fuse with a normal egg, a triploid plant can be developed. If a tetraploid plant is crossed with a diploid plant also a triploid can be produced. All autopolyploids exhibit **gigantic effect** *i.e.* larger size of leaves, flowers, fruits and plants, more grain and more juice and sugar in the fruit etc. Examples of autotriploids : banana, sugarbeet, seedless watermelon, apple, pear etc, autotetraploids are tetraploid potato, groundnut, tomato, grape, strawberry, plum, coffee etc. The triploid sugarbeets have larger roots and more sugar content than the diploids. Likewise the triploid apple and pear varieties show larger size and more juicy fruits.

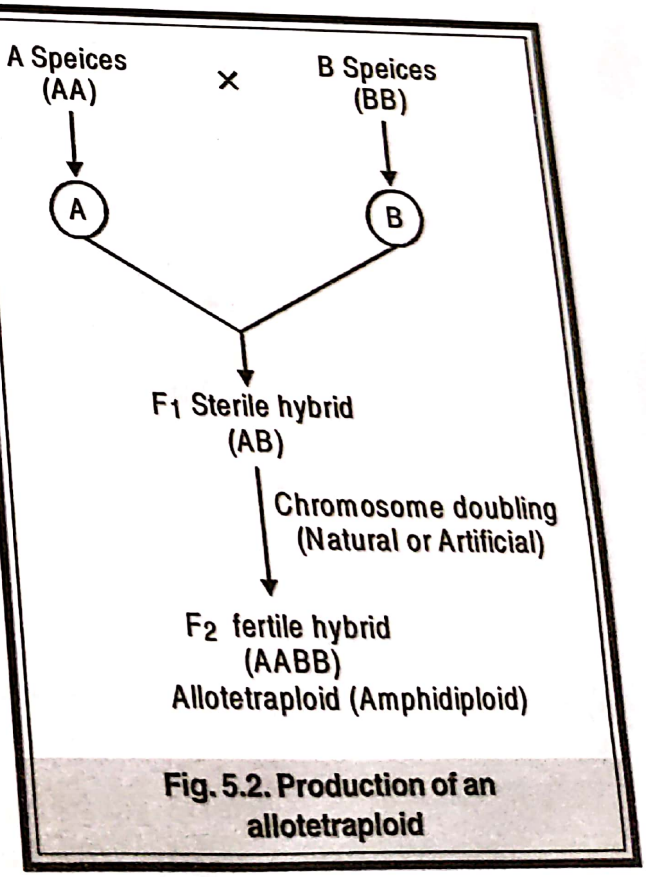


Fig. 5.2. Production of an allotetraploid

(b) Allopolyploidy: Allopolyploidy may be intervarietal, interspecific or intergeneric if the breeding involves between two different varieties or species or genera respectively. Allopolyploids may be allotriploids (AAB or ABB), allotetraploids (AABB), allopentaploids (AAABB or AABBB), allohexaploids (AAABBB or AABBBB or AAAABB) etc. Generally allopolyploid involves chromosome doubling of the F_1 sterile hybrid so as to produce the F_2 fertile hybrid.

Allopolyploid leads to creation of new species within a short period. Examples: *Triticale* or **Rye-wheat hybrid** (allooctaploid), Durum wheat (allotetraploid), bread wheat (allohexaploid), new world long stapled cotton (allotetraploid), *Brassica napus* variety (allotetraploid), tobacco (allotetraploid), *Raphano-brassica* (allotetraploid), *Helianthus tuberosus* (autoallopolyploid, AAAABB) etc.

Origin of Bread wheat (*Triticum aestivum*). Wheat is now called **'Staff of life'** which came under cultivation from 10 to 15 thousand years ago. It is an allohexaploid (AABBDD) having 3 sets of different genoemes coming from wild Einkorn wheat (*Triticum boeoticum*), and two wild grasses *i.e. Aegilops speltoides* and *A. squarrosa* during the course of organic evolution by mutation and polyploidy (Fig. 5.3). Currently *T. aestivum*

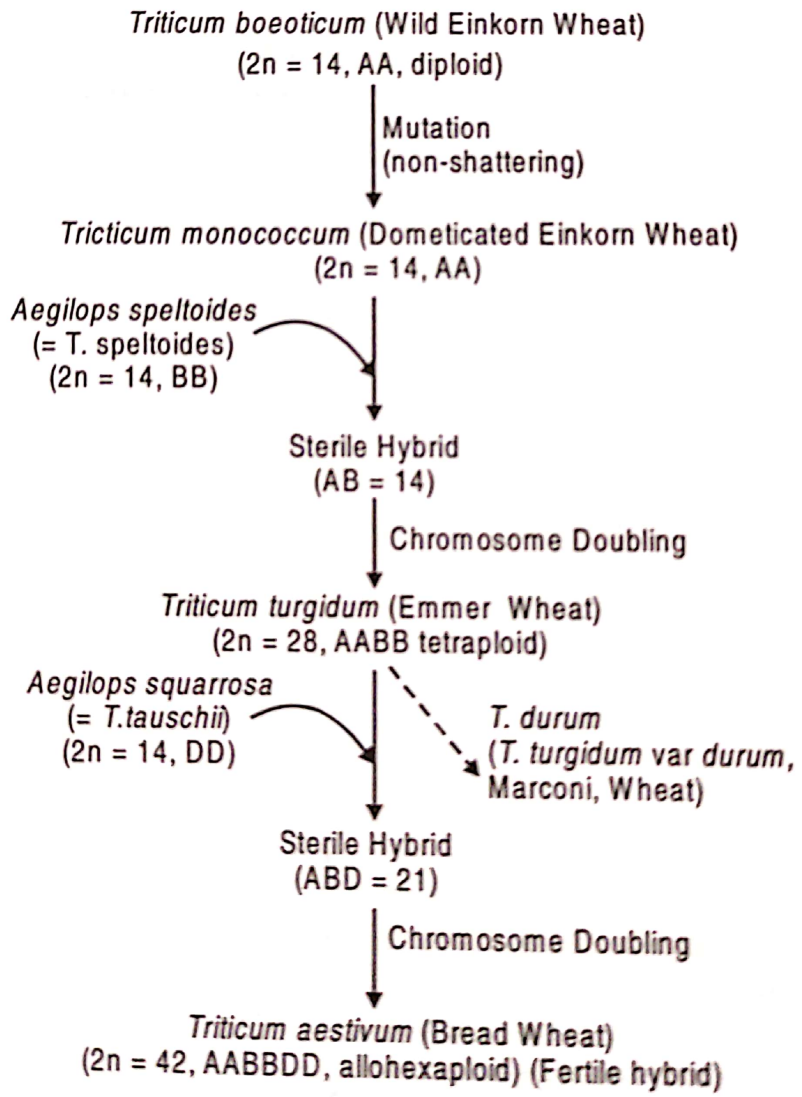


Fig. 5.3. Origin of Bread Wheat

has more than 20,000 cultivars. The first domesticated wheat is *T. monococcum* and it is now cultivated in Turkey and Yugoslavia. **Marconi wheat** is used for preparing pasta and noodles. It is a hard wheat cultivated in drier areas.

VI. Role of gene mutations in Crop improvement

1. Some **gene mutations** have beneficial effects and are useful in crop improvement. Mutations in both qualitative and quantitative traits have been exploited for the development of over 2,000 varieties of different crops, at present.
2. Mutations may be **germinal** occurring during reproductive stage or **somatic,** occurring in the vegetative stage. For crop improvement useful somatic mutations can be incorporated in vegetative propagated plants like delicious apple, navel orange, seedless grape, straw berries, Bhaskara banana, potato, superior shrub types of coffee plants. The somatic mutations are known as **'Sports'.** Somatic mutation breeding can also be applied to potato tuber, sugarcane, tapioca, beet, sugar-beet etc. Vegetative propagation is also beneficial in maintaining germinal variations obtained through sexual reproduction, *e.g.* mango, apple, sugarcane, potato etc.
3. The frequency or degree or rate of **spontaneous mutation** is very low. Hence mutations are artificially induced in crop varieties by different mutagens to increase the rate. **Stadler** first reported induced mutations by X-ray in plants, while in animals (fruit fly) it was first reported by H.J. Muller (1927) by X-ray. **UV radiation, ionising radiation** (X-ray, gamma ray, α ray, β ray, cosmic rays) are physical mutagens while **nitrous acid, nitrogen mustard, nitroethyl urea, ethyl methane sulphonate** (EMS) etc. are chemical mutagens. Semi-dwarfism in rice is brought about by X-ray while rice variety Pelita-1 was developed by gamma irradiation which increased the grain yield and resistane to brown hopper and had salt tolerance and good palatability. Another high yeilding variety of rie, **Reimei** is produced by gamma irradiation.
4. Irradiation with gamma rays also induced the production of amber coloured wheat **Sharbati Sonara** by M.S. Swaminathan *et al.* at IARI, N. Delhi. Another variety of wheat, **Pusa Lerma** was similarily produced from Lerma Rojo 64. Induced mutation in **Japonica variety** produced **Indica type** of grains in rice.
5. High yielding mutants of **barley** have been developed in Sweden. In wheat, several useful mutations *e.g.*, branched ears, lodging resistance, high protein and lysine content, amber seed colour and awned spikelets were obtained and utilized in plant breeding.
6. By induced mutation, **Todd's Mitcham variety of peppermint** and **Aruna variety** of **castor plant** have been developed. Examples of other crops are oat, pea, pigeon pea, tomato, cotton, mustard, groundnut, bajra, pearl millet, chili and jute etc. According to IAEA (International Aomic Energy Agency) upto 1991, 1542 varieties of plants have been released by induced mutation through out the world (Ref - Mutation Breeding News Letters, IAEA) which demonstrates enormous potential of mutation breeding in crop improvement.
7. In California, U.S.A. mutations were induced in groundnuts. Such thick shelled groundnuts do not crack easily and hence useful for transportation.

8. Some crop varieties developed in India by mutation breeding are wheat (NP 836), rice (Jagannath, IIT 60, K84 HM95), pea (Hans), cotton (Indore 2, Pusa Ageti), castor (Aruna), jute (JRO 514, JRO 412), french bean (Pusa Parvati), tomato (Pusa Lal, Meeruti, S 12), groundnut (TGI), ornamental plants like bougainvillea (Arjun), *Chrysanthemum* (Basant, Kanak, Anamika) etc.

9. Limitations of the mutation breeding:
   (*a*) Most induced mutations are lethal. Even some beneficial mutants pose problems due to lethality.
   (*b*) Large number of induced mutations are undesirable.
   (*c*) The breeder has to screen large populations to select the desirable mutations. This becomes even more difficult, the mutation rate being generally very low.
   (*d*) Most of the mutations are recessive. Their detection, therefore, is almost impossible. These can be expressed only if in homozygous condition.
   (*e*) Mutations must be produced in gametes, particularly the pollen grain so that it can be transferred to progeny through hybridization.
   (*f*) Most of the mutations are not stable and generally get reverted.

5.2. ROLE OF SOMACLONAL VARIATIONS IN CROP IMPROVEMENT

Micropropagation : Micropropagation is the production of a large number of plantlets from the callus tissues or suspension cell cultures through tissue culture technique within very short time period and space. The most advantage of micropropagation is the numerical one within time and space. Therefore, huge number of plantlets of a particular species or variety can be propagated all the year around. Example : banana, potato, tomato, grape, coffee, bamboo, cardamom, sal (*Shorea robusta*), *Begonia etc.*

Clonal propagation : *In vitro* clonal propagation is a type of micropropagation, where the plantlets are derived **asexually** through **tissue culture technique** so that these plantlets are genetically identical. Thus multiplication of **genetically identical copies of a species by asexual reproduction is called clonal propagation.** A plant population derived from a single donor in tissue culture technique is known as a **clone.** The plants with long seed dormancy can be generated faster by clonal propagation *in vitro,* than *in vivo* seed propagation, for example, sal seeds need long dormancy which can be avoided by this method of micropropagation. *In vitro* clonal propagation of orchids is commercially very much viable. It is also important in horticulture and sylviculture.

I. What is Somaclonal Variation

In plant tissue culture, a large number of isolated cells or tissues under sterile and controlled conditions are grown. During tissue or cell culture, unorganized mass of cells capable of cell division and growth are produced *in vitro* known as **'callus'.** From the callus root and/or shoot are produced by application of phytohormones like auxin and cytokinin. If both auxia and cytokinin concentration is equal then both roots and shoots are generated from the callus. Hence callus is cultured in specific ways as per the need.

In some callus cultures maintained for longer time priod, there is a tendency towards numerical variation of chromosomes in the cells mainly due to repeated division of the cells. This leads to **polyploidization** of cells and genetically variable plants can be produced in subsequent micropropagation. This is known as **somaclonal variation. Larkin** and **Scowcroft (1981)** have proposed a general term **'Somaclones'** for plant variants derived by tissue culture. Again it may be **'Calliclones'** if the plants are derived from callus culture or **'Protoclones'** if derived from protoplast culture. Such variant plants exhibit some useful characters like resistance to a particular disease or herbicide, stress tolerance, male sterility etc. which are valuable for crop improvement. Somaclonal variation may include, apart from polyploidization; point mutation, chromosomal aberrations, DNA amplification, changes in organelle DNA, epigenetic variation etc.

II. Somaclonal variations and crop improvement

Genetic variation is an essential component of any conventional crop breeding programme. The typical crop improvement cycle takes 10-15 years to complete and includes germplasm manipulations, genotype selection and stabilization, variety testing, variety increase, proprietary protection and crop production stages. Plant tissue culture is an enabling technology from which many novel tools have been developed to assist plant breeders. Tissue culture-induced somaclonal variation is akin to variations induced with chemical and physical mutagens and offers an opportunity to uncover natural variability for their potential exploitation in crop improvement. It reduces the time required for releasing the new varieties by at least two years as compared to mutation breeding by three years in comparison to back cross method of gene transfer. The *in vitro* induced somaclonal variation has the following advantages.

1. It is cheaper than other methods of genetic manipulation and does not require 'containment' procedures.
2. Tissue culture systems are available for more plant species than can be manipulated by somatic hybridization and transformation at the present time.
3. It is not necessary to have identified the genetic basis of the trait, or indeed, in the case of transformation, to have isolated and cloned it.
4. Novel variants have been reported among somaclones, and evidences indicate that both the frequency and distribution of genetic recombination events can be altered by passage though tissue culture. This implies that variation may be generated from different locations of the genome than those, which are accessible to conventional and mutation breeding.
5. There is no possibility of obtaining chimeric expression if somaclones are raised through cell culture.

Somaclonal variation has been most successful in crops with limited genetic systems (e.g., apomicts, vegetative reproducers) and/or narrow genetic bases. In ornamental plants, for instance, the exploitation of *in vitro* generated variability has become part of the routine breeding practice of many commercial enterprises.

Though techniques for selection of somaclones resistant to various biotic and abiotic stresses had been worked out in many horticultural crops, unfortunately, no *in vitro*

selection methods exist for complicated traits such as yield, soluble solids, sweetness, texture or shelf life. Somaclonal variation can become a part of plant breeding provided they are heritable and genetically stable. Only a limited number of promising varieties so far had been released using somaclonal variations. This is perhaps due to the lack of interaction between plant breeders and tissue culture scientists, and non-predictability of somaclones. Further, though the new varieties have been produced by somaclonal variation, in a large number of cases improved variants have not been selected due to certain constraints.

Some of the somaclonal variants so far is considered as boon to crop improvement are as follows :

1. **Sugarcane variants:** Resistant to eye spot disease caused by *Helminthosporium sacchari*, Fiji disease (by virus), and downy mildew (by *Sclerospora sacchari*). Most of the resistant lines exhibited a shift towards higher resistance.
2. **Potato.** Protoplast culture in potato cultivar, *Russet Burbank*, produced 1700 somaclones out of which only 15 stable somaclones were identified which show resistance to late blight of potato (by *Phytophthora infestans*) and early blight (*Alternaria solani*) of potato.
3. **Maize.** Somaclones of maize exhibited the characters of male sterility and toxin resistance and it is due to alterations in mt DNA (mitochondrial DNA) responsible for toxin tolerance.
4. **Wheat.** The somaclonal regenerants displayed phenotypic variations for the characters such as plant height, maturity, tiller number, presence of awns, glume colour, grain colour, appearance or disappearance of some specific bands of gladin protein.
5. **Tomato.** Tomato somaclones were isolated with variant phenotypes such as recessive mutations for male sterility, resistance to *Fusarium oxysporium*, joint less pedicel, tangerine pedicel, tangerine veriscent leaf, flower and fruit colour.

5.3. MOLECULAR BREEDING : USE OF DNA MARKERS IN PLANT BREEDING AND CROP IMPROVEMENT

Molecular Breeding or **Maker assisted breeding (MAB)** is the process of using the results of DNA tests to assist in the selection of individuals to become the parents in the next generation of genetic improvement programme. The choice among various methods of MAB depends on the complexity of the trait and prior knowledge on the genes or segments of chromosomes (known as **quantitative trait loci (QTL)**. Molecular markers facilitate coventional breeding, improve selection efficiency, reduce cost for developing new varieties, and/or quality control (ensuring line purity and genetic identity). Various schemes of MAB are being employed to accelerate variety development in cassava, maize and cowpea wheras genomic resource development or preparation is continuing for yam, banana, and soybean.

I. Molecular markers is plant breeding

The advent of molcuelar techniques played a significant role in increaseing our knowledge of cereal genetics and behaviour of cereal genomics. While **RFLP (Restriction fragment length polymorphism)** markers have been the basis for most work in crop

plants, valuable markers have been generated from **RAPD (Random amplification of polymorphic DNA)** and **AFLP (Amplified fragment length polymorphism)**. Recently, other improvised molecular markers such as **simple sequence repeats (SSR), microsatellite marker** have also been developed for major crop plants and initiate rapid advance in both marker development and implementation in breeding programme. Conventional plant breeding is time consuming and depends on environmental conditions, for example, breeding for new variety development takes between eight to twelve years without any guarantee of variety release. Hence, new molecular breeding technologies could make the entire breeding programme simple, speedy and efficient and also offers selection of desirable combination of traits. This approach can establish linkage between molecular marker and traits to be selected. In this approach breeding process can be conducted in the laboratory without waiting for expression of the gene for particular phenotype. As an example, resistance to plant pathogens can be evaluated in the absence of diseases. Every stress tolerance can be analysed in the seedling stage itself.

1. Mapping of plant genomes

Among serveral important crop plants, **rice** has been the target plant for intense mapping studies, apart from the other model plant, *Arabidopsis thaliana*. Currently, genetic mapping for rice and *Arabidopsis* has also been completed.

Mapping smaller size of genome is ideal for key strategy for the identification of gene in the complex genome. Molecular markers like RFLP marker from closely related species are good reliable markers for constructing gene map. **Microsatellite** is a useful tool for constructing gene map to each species whereas RFLP loci can be used to map at greater degree in related taxa.

2. Linkage of molecular marker to desired trait

Identification of genes responsible for useful trait may be established by a linkage analysis with markers on a genetic map of plant genome. Polymorphic marker is generally used to identity linked markers. Finding of linked markers can be accomplished by a certain useful technique like **bulked segregate analysis (BSA)**. This technique is used to detect polymorphism between two DNA samples made up of bulk of individuals from the segregating populations.

For example, one bulk sample DNA from one individual contains target gene while other DNA from individual is devoid of this gene. The segregating population derived sample contains both of the two bulks containing most genes.

Polymorphism between the bulks is likely to be linked to genes for the trait. The analysis of linkers for dominant (RAPD) and co-dominant marker (RFLP or microsatellite) requires separate unique analysis of mapping with F_2 population.

3. Accelerated back crossing

Marker assisted selection facilitates the acceleration of whole breeding process, allowing earlier release of commercial plants. This is achieved by two important methods like accelerated back crossing and selection for a desired trait. Introduction of desirable trait has been the favourite choice for plant breeders without altering other character.

This can be accomplished by repeated crossing of the plants with the genetic background. Each and every generation requires selection of introduced trait. This requires number

crossing and more number of generations since molecular marker facilitate selection of individuals with more of the recurrent genome at each generation. It can limit breeding programme to be completed with few generations.

4. Selection for a Desired Trait

Several desirable traits can be directly selected by molecular marker and can be screen at any stage in the breeding programme. In addition to the available marker for routine use, conversion of RFLP marker to PCR based marker helps significantly in the economy of molecular usage. In addition, usage of molecular marker for fruit selection in plant breeding requires availability of simple, inexpensive technique that provides rapid result in the assessment for next round of selection.

5. Molecular Breeding for Resistance

PCR-based marker has been used in the breeding of desirable resistance to viral and fungal pathogen in plants. **Barley yellow mosaic virus (BaYMV)** has been considered as important viral disease in Europe. Therefore, breeding for resistance to the disease is of special importance. Applications of closely linked PCR-based markers for the transmission of resistant gene(s) against barley yellow mosaic virus are now successful and efficient.

Similarly breeding for resistance to fungal pathogen has been advanced. **Fusarium head blight** is a serious disease of wheat. Molecular markers closely liked to the major QTL involved in Fusarium head blight (FHB) resistance have been identified and raised the possibility of **marker assisted selection (MAS)** for introducing resistant alleles into elite wheat variety. These are some of the safety strategy in breeding. The new varieties combined high yield performance and high level of resistance to *Fusarium* pathogen.

6. Identification of Breeding Lines

In the germplasm, labelling error can lead to breeding artifact because handling of large number of lines may create problem in identifying molecular marker used to confirm breeding lines.

7. Identification of Hybridity

Molecular marker can be used to identify hybrid nature of individuals especially in self-pollinating species. Production of hybrid through non-conventional hybridization method like somatic hybrid also can be identical using RAPD analysis.

8. Purity of Breeding Lines

Accidental mixing of seeds or cross-contamination in seeds harvested may lead to contamination of breeding lines. Molecular markers can be used to assist establishment of pure breeding lines and check contamination of breeding.

9. Prediction of Hybrid Performance (Heterosis)

Establishment of genetic distance between the parents used in the cross can able to ascertain the performance of hybridity. Genetic distance between possible parents can be estimated by employing molecular markers. RFLP microsatellite markers are selected as useful markers for these predictions.

10. Identifying Germplasm

Identification of several useful genetic resources of possible parents for use in breeding requires suitable molecular technique. RAPD marker is a useful tool for the survey of germplasm, for example, survey of rice germplasm using RAPD shows linkage between the presence of specific marker and quantitative trait loci **(QTL)** for novel character.

11. Marker Assisted selection (MAS)

As a shortcut, plant breeders now use marker-assisted selection (MAS). To help identify specific genes, scientists use what are called **molecular** or **genetic markers**. The markers are a string or sequence of nucleic acid which makes up a segment of DNA. The markers are located near the DNA sequence of the desired gene and are transmitted by the standard laws of inheritance from one generation to the next (Figure 5.4). Since the markers and the genes are close together on the same chromosome, they tend to stay together as each generation of plants is produced. This is called genetic linkage. This linkage helps scientists to predict whether a plant will have a desired gene. If researchers can find the marker for the gene, it means the desired gene itself is present.

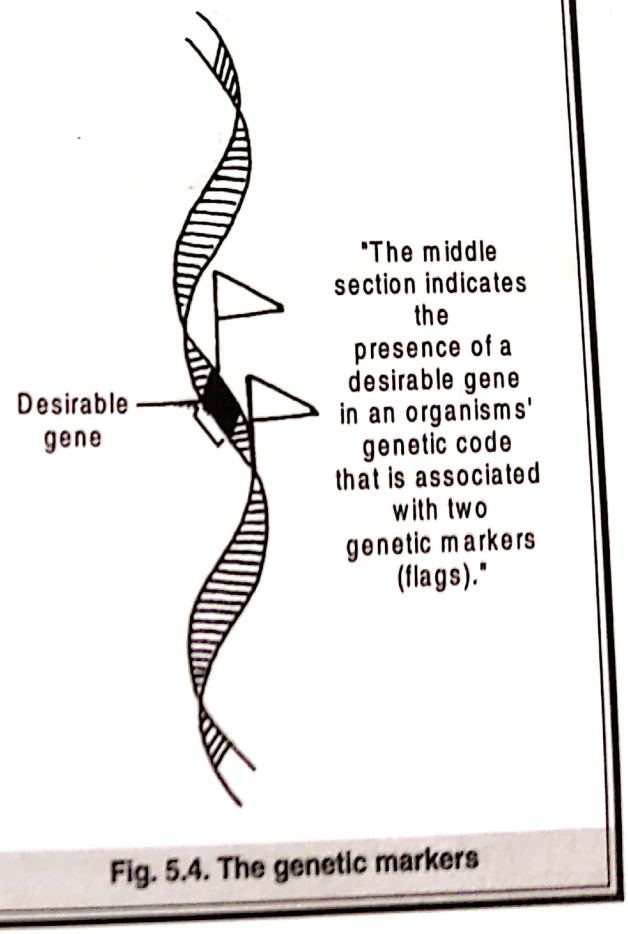


Fig. 5.4. The genetic markers

As scientists learn where markers occur on a chromosome, and how close they are to specific genes, they can create a genetic linkage map. Such a map would show the location of markers and genes, and their distance from other known genes. Scientists can produce detailed maps in only one generation of plant breeding.

Using very detailed genetic maps and better knowledge of the molecular structure of a plant's DNA, researchers can analyze only a tiny bit of plant tissue, even from a newly germinated seedling. Once the tissue is analyzed, scientists know whether that seedling contains the appropriate gene. If it doesn't, they can quickly move on and concentrate on analysis of another seedling, eventually working only with the plants which contain a specific trait.

Molecular breeding through MAS is somewhat limited in scope compared to genetic engineering or modification because:

1. It works only for traits already present in a crop;

2. It cannot be used effectively to breed crops which have long generation times (e.g. *Citrus*.); and
3. It cannot be used effectively with crops which are clonally propagated because they are sterile or do not breed true which includes many such as yams, bananas, plantain, sweet potato, and cassava).

1. DNA markers

Several marker systems have been developed and are applied to a range of crop species. These are the Restriction Fragment Length Polymorphisms (RFLPs), Random Amplification of Polymorphic DNAs (RAPDs), Sequence Tagged Sites (STS), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence Repeats (SSRs) or microsatellites, and Single Nucleotide Polymorphism (SNPs). The advantages and disadvantages of these marker systems are provided in Table 5.3.

Table 5.3. Comparison of most commonly used marker systems (adopted from Korzun, 2003)

Feature	RFLPs	RAPDs	AFLPs	SSRs	SNPs
DNA required (ng)	10	0.02	0.5-1.0	0.05	0.05
DNA quality	High	High	Moderate	Moderate	High
PCR-based	No	Yes	Yes	Yes	Yes
Number of polymorphic loci analyzed	1.0-3.0	1.5-50	20-100	1.0-3.0	1.0
Ease of use	Not easy	Easy	Easy	Easy	Easy
Amenable to automation	Low	Moderate	Moderate	High	High
Reproducibility	High	Unreliable	High	High	High
Development cost	Low	Low	Moderate	High	High
Cost per analysis	High	Low	Moderate	Low	Low

These molecular techniques have been widely used to monitor differences in DNA sequence in and among species. They also allow the creation of new sources of genetic variation by introducing new and desirable traits from wild varieties into elite lines. While RFLP markers have been the basis for most genetic work in crop plants, AFLPs and SSRs are currently the most popular techniques used due to ease in detection and automation. The adoption of the new marker system, SNPs, is now highly preferred, with the increasing amount of sequence information, and the determination of gene function due to genomic research.

2. Applications of molecular markers for crop genetic studies

The main uses of these molecular markers in crop genetic studies are as follows:

(*i*) Assessment of genetic variability and characterization of germplasm

(*ii*) Identification and fingerprinting of genotypes.

(*iii*) Estimation of genetic distances between population, inbreeds, and breeding materials.

(iv) Detection of monogenic and quantitative trait loci (QTL).

(v) Marker-assisted selection.

(vi) Identification of sequences of useful candidate genes.

3. MAS for pathogen Resistance in Tomato and other crop plants

One of the major constraints in tomato cultivation and production are severe harvest losses caused by a number of pathogens, including viruses, bacteria, fungi, and nematodes. Farmers have adopted control measures, such as applications of agrochemicals and use of resistant lines. Although conventional breeding has had a significant impact on improving resistance of tomato, the time-consuming process of making crosses and backcrosses, and the selection of the desired resistant progeny make it difficult to react adequately to the evolution of new virulent pathogens.

Molecular markers are now being widely used for breeding tomato. More than 40 genes that confer resistance to major classes of tomato pathogens have been mapped, cloned, and/or sequenced. These maps have allowed for "pyramiding" resistance genes in tomato through MAS, where several resistance genes can be engineered into one genotype. Currently, tomato breeding through MAS has resulted in varieties with resistance or tolerance to one or more specific pathogens.

Apart from tomato, marker-assisted selection for disease resistance has been performed in cassava, (*Manihot esculenta*) yam (*Dioscorea*), soybean (*Glycine max*) cowpea (*Vigna unguiculata*), banana (*Musa*) and wheat.

Some Imporant terms associcted with molecular breeding

AFLP: Amplified Fragment Length Polymorphism. A highly sensitive method for detecting DNA polymorphism. Following restriction enzyme digestion of DNA, a subset of the DNA fragments is selected for PCR amplification and visualization.

Genetic Map: A map of the relative positions of genetic loci on a chromosome, determined on the basis of how often the loci are inherited together.

Linkage Map: A map of relative positions of genes on a chromosome. Genes inherited together are close to each other on the chrormosome, and said to be linked.

Microsatellites: Very short DNA motifs (1-10 base pairs) which occur as tandem repeats at numerous loci throughout the genome. Also known as **simple sequence repeats (SSR), simple tandem repeats or simple repetitive sequences.**

Monogenic trait (Mendelian trait): a trait determined by the action of a single genetic locus

PCR: Polymerase Chain Reaction. A method for amplifying a DNA sequence in large amounts using a heat-stable polymerase and suitable primers to direct the amplification of the desired region of DNA.

Polymorphism: A detectable difference at a particular gene or marker occurring among individuals.

RAPD: Random Amplification of Polymorphic DNA. A widely-used technique for amplifying anonymous stretches of DNA using PCR with arbitrary primers.

RFLP: Restriction Fragment Length Polymorphism. Variations which occur in the length of DNA fragments produced when DNA is broken down by restriction enzymes

(enzymes which recognise specific sequences of DNA, usually 4-6 base pairs long, and cleave the DNA at these points, known as restriction sites).

SNP: Single Nucleotide Polymorphism. A common, but minute, variation that occurs in DNA sequences of a genome. These variations can be used to track inheritance in families or species.

QTL: Quantitative Trait Locus. Location of a specific gene that affects a measurable or quantifiable trait. These traits are typically affected by more than one gene, and also by the environment. Examples of quantitative traits are plant height (measured on a ruler) and body weight (measured on a balance).

Quantitative (continuous) traits: phenotypes that exhibit a range of measurable outcomes.

III. RAPD (Random amplification of polymorphic DNA)

RAPD (pronounced 'rapid') stands for 'Random Amplification of Polymorphic DNA'. It is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8-12 nucleotides), then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be obtained from a RAPD reaction.

No knowledge of the DNA sequence of the targeted genome is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared. However it is not suitable for forming a cDNA databank. Because it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species-specific DNA comparison methods, such as short tandem repeats. In recent years, RAPD has been used to characterize, and trace, the phylogeny of diverse plant and animal species.

RAPD markers are decamer (10 nucleotide length) DNA fragments from PCR amplification of random segments of genomic DNA with single primer of arbitrary nucleotide sequence and which are able to differentiate between genetically distinct individuals, although not necessarily in a reproducible way. It is used to analyse the genetic diversity of an individual by using random primers. Due to problems in experiment reproducibility, many scientific journals do not accept experiments merely based on RAPDs anymore. RAPD requires only one primer for amplification.

How it works

Unlike traditional PCR analysis, RAPD does not require any specific knowledge of the DNA sequence of the target organism: the identical decamer will or will not amplify a segment of DNA, depending on positions that are complementary to the primers' sequence. For example, no fragment is produced if primers annealed too far apart or 3' ends of the primers are not facing each other. Therefore, if a mutation has occurred in the template DNA at the site that was previously complementary to the primer, a PCR product will not be produced, resulting in a different pattern of amplified DNA segments on the gel.

IV. RFLP (Restriction fragement length polymorphism)

Restriction fragment length polymorphism, or **RFLP,** is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated. In **RFLP analysis,** the DNA sample is broken into pieces (and digested) by restriction enzymes and the resulting **restriction fragments** are separated according to their lengths by gel electrophoresis. Although now largely obsolete due to the rise of inexpensive DNA sequencing technologies, RFLP analysis was the first DNA profiling technique inexpensive enough to see widespread application. RFLP analysis was an important tool in genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing.

The basic technique for the detection of RFLPs involves fragmenting a sample of DNA by a restriction enzyme, which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as a **restriction digest**. The resulting DNA fragments are then separated by length through a process known as **agarose gel electrophoresis,** and transferred to a membrane via the **Southern blot** procedure. Hybridization of the membrane to a labeled DNA probe then determines the length of the fragments which are complementary to the probe. An RFLP occurs when the length of a detected fragment varies between individuals. Each fragment length is considered an allele, and can be used in genetic analysis.

RFLP analysis may be subdivided into **single-(SLP)** and **multi-locus probe (MLP) paradigms**. Usually, the SLP method is preferred over MLP because it is more sensitive, easier to interpret and capable of analyzing mixed-DNA samples. Moreover, data can be generated even when the DNA is degraded (e.g. when it is found in bone remains.)

Examples:

There are two common mechanisms by which the size of a particular restriction fragment can vary. In the first schematic (Fig. 5.5.) a small segment of the genome is being detected by a DNA probe (thicker line). In **allele "A"**, the genome is cleaved by a restriction enzyme at three nearby sites (triangles), but only the rightmost fragment will be detected by the probe. In **allele "a"**, restriction site 2 has been lost by a mutation, so the probe now detects the larger fused fragment running from sites 1 to 3. The second diagram(Fig. 5.6) shows how this fragment size variation would look on a Southern blot, and how each allele (two per individual) might be inherited in members of a family.

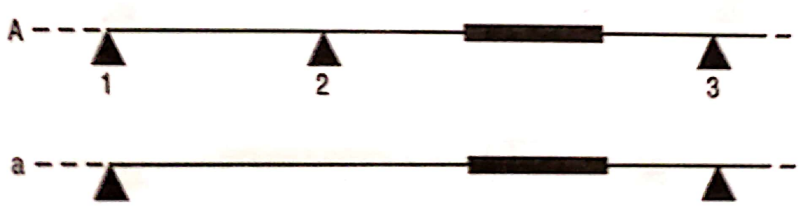


Fig. 5.5. Schematic for RELP by cleavage site loss

In the third schematic (Fig. 5.7) the probe and restriction enzyme are chosen to detect a region of the genome that includes a variable number tandem repeat segment (boxes in schematic diagram). In **allele "c"** there are five repeats in the **VNTR (variable number tandem repeat)**, and the probe detects a longer fragment between the two restriction sites. In **allele "d"** there are only two repeats in the VNTR, so the probe detects a shorter fragment between the same two restriction sites. Other genetic processes, such as insertions, deletions, translocations, and inversions, can also lead to RFLPs. RFLP tests require much bigger samples of DNA than do short tandem repeat (STR) tests.

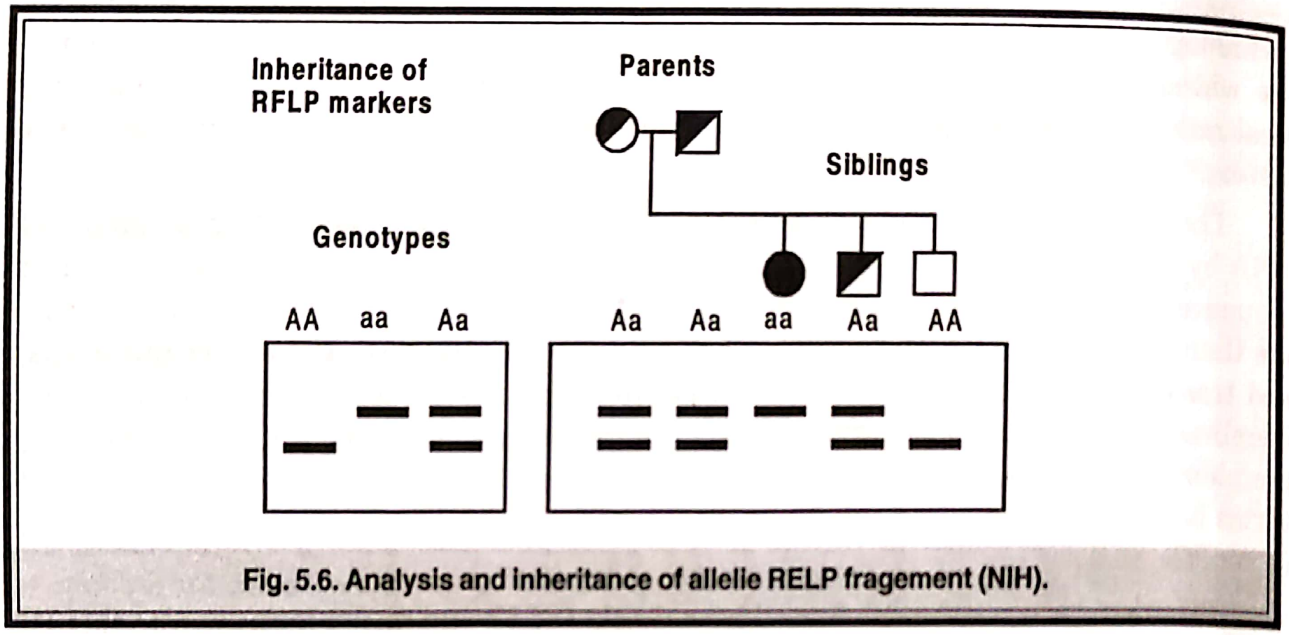


Fig. 5.6. Analysis and inheritance of allelle RELP fragement (NIH).

Applications

Analysis of RFLP variation in genomes was a vital tool in **genome mapping** and **genetic disease analysis**. If researchers were trying to initially determine the chromosomal location of a particular disease gene, they would analyze the DNA of members of a family afflicted by the disease, and look for RFLP alleles that show a similar pattern of inheritance as that of the disease. Once a disease gene was localized, RFLP analysis of other families could reveal who was at risk for the disease, or who was likely to be a carrier of the mutant genes.

RFLP analysis was also the basis for early methods of **genetic fingerprinting**, useful in the identification of samples retrieved from **crime scenes**, in the determination of **paternity**, and in the characterization of **genetic diversity** or **breeding patterns** in animal populations.

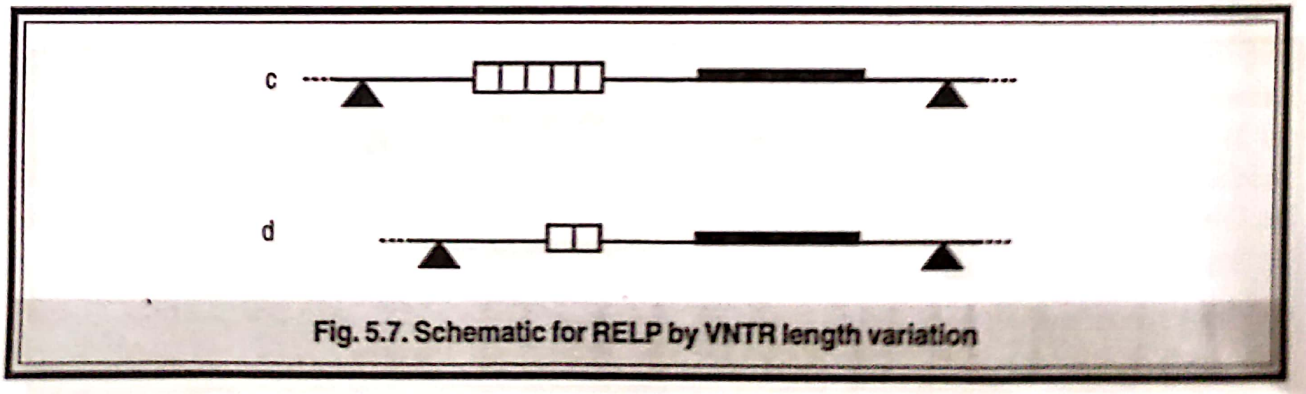


Fig. 5.7. Schematic for RELP by VNTR length variation

IMPORTANT QUESTIONS

1. Describe the role of chromosomal mutations in crop improvement.
2. Enumerate the role of gene mutations in crop improvement.
3. Give an account of role of somaclonal variations in crop improvement.
4. What is molecular breeding ? Describe the use of DNA markers in plant breeding.
5. Describe the role of molecular markers in crop improvement.
6. Write notes on :
   (*i*) Somaclonal variation
   (*ii*) Mutation breeding
   (*iii*) DNA markers in plant breeding
   (*iv*) Molecular breeding
   (*v*) MAS
   (*vi*) RAPD
   (*vii*) RFLP
   (*viii*) Molecular markers
   (*ix*) Molecular breeding for resistance
   (*x*) MAS for pathogen resistance in crop plants
   (*xi*) Application of RFLP

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