ALAGAPPA UNIVERSITY

[Accredited with 'A+' Grade by NAAC (CGPA:3.64) in the Third Cycle and Graded as Category–I University by MHRD-UGC]

KARAIKUDI – 630 003

DIRECTORATE OF DISTANCE EDUCATION

M.Sc. [Botany]
346 24

PRACTICAL

LAB II: CELL BIOLOGY, GENETICS & PLANT BREEDING, PLANT ANATOMY & EMBRYOLOGY, PLANT PHYSIOLOGY AND BIOCHEMISTRY

II - Semester

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Author

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PRACTICAL - LAB II: CELL BIOLOGY, GENETICS & PLANT BREEDING, PLANT ANATOMY & EMBRYOLOGY, PLANT PHYSIOLOGY AND BIOCHEMISTRY

SYLLABI

Suggested Laboratory Exercises:

1. Study of different types of chromosomes.
2. Preparation of karyograms.
3. Study of different stages of mitotic cell division in suitable material.
4. Study of meiotic cell division.
5. Linear differentiation of chromosomes through banding techniques such as G-banding, C- banding and Q-banding.
6. Application of colchicines in genetics.
7. Studying pea plant as tool for investigating Laws of Inheritance.
8. Demonstration of Mendel's Law of segregation.
9. Demonstration of Mendel's Law of Independent Assortment.
10. Studying deviations from Mendel's laws and applying statistics.
11. Studying *Drosophila* as model organism.
12. Effect of chemicals on mutation.
13. Effect of radiations on mutation.
14. Isolation of DNA from different sources.
15. Study of cell structure from onion leaf peels; demonstration of staining and mounting methods.
16. Comparative study of cell structure in onion cells, *Hydrilla* and *spirogyra*.
17. Study of cyclosis in *Tradescantia* staminal hairs.
18. To study the permeability of plasma membrane using different concentrations of organic solvents.
19. To study the effect of temperature on permeability of plasma membrane.
20. To prepare the standard curve of protein and determine the protein content in unknown samples.
21. Separation of chloroplast pigments by solvent method.
22. Determining the osmotic potential of vacuolar sap by plasmolytic method.
23. Determining the water potential of any tuber.
24. Separation of amino acids in a mixture by paper chromatography and their identification by comparison with standards.
25. Comparison of the rate of respiration of various plant parts.
26. Protein, carbohydrate estimation.
27. Estimation of Nitrogenase activity.
28. Acid and alkaline acid phosphatase activity.
29. Isolation and identification of Rhizobium from leguminous plants.
30. Separation of amino acid through paper and column chromatography.

INTRODUCTION

NOTES

Cell biology is the study of cell structure and function, and it revolves around the concept that the cell is the fundamental unit of life. Focusing on the cell permits a detailed understanding of the tissues and organisms that cells compose. Some organisms have only one cell, while others are organized into cooperative groups with huge numbers of cells.

Genetics is a branch of biology concerned with the study of genes, genetic variation, and heredity in organisms. Gregor Mendel discovered genetics in the late 19th-century. Mendel studied 'trait inheritance', patterns in the way traits are handed down from parents to offspring. He observed that organisms (pea plants) inherit traits by way of discrete 'units of inheritance'. Trait inheritance and molecular inheritance mechanisms of genes are still primary principles of genetics in the 21st century, but modern genetics has expanded beyond inheritance to studying the function and behaviour of genes. Gene structure and function, variation, and distribution are studied within the context of the cell, the organism (dominance), and within the context of a population. Genetics has given rise to a number of subfields, including epigenetics and population genetics.

Plant breeding is the science of changing the traits of plants in order to produce desired characteristics. It has been used to improve the quality of nutrition in products for humans and animals.

Plant anatomy or phytotomy is the general term for the study of the internal structure of plants. Originally it included plant morphology, the description of the physical form and external structure of plants. Plant anatomy is now frequently investigated at the cellular level, and often involves the sectioning of tissues and microscopic examinations.

Embryology is the branch of biology that studies the prenatal development of gametes (sex cells), fertilization, and development of embryos and foetuses.

Plant physiology is a sub-discipline of botany concerned with the functioning, or physiology, of plants. Closely related fields include plant morphology (structure of plants), plant ecology (interactions with the environment), phytochemistry (biochemistry of plants), cell biology, genetics, biophysics and molecular biology. Fundamental processes, such as photosynthesis, respiration, plant nutrition, plant hormone functions, tropisms, nastic movements, photoperiodism, photomorphogenesis, circadian rhythms, environmental stress physiology, seed germination, dormancy and stomata function and transpiration, both parts of plant water relations, are studied by plant physiologists.

Biochemistry, sometimes called biological chemistry, is the study of chemical processes within and relating to living organisms. Biochemical processes give rise to the complexity of life. Biochemistry is closely related to molecular biology, the study of the molecular mechanisms by which genetic information encoded in DNA is able to result in the processes of life.

This book, *Cell Biology, Genetics & Plant Breeding, Plant Anatomy & Embryology, Plant Physiology and Biochemistry*, deals with the practical aspects of qualitative and quantitative analysis of the techniques used in the laboratory.

GENERAL INSTRUCTIONS AND LABORATORY ETHICS

NOTES

GENERAL INSTRUCTIONS

1. The students while coming to the laboratory for the practical class work, must check that they have the essential materials with them for the practical, namely practical notebook, pencil, pencil eraser, sharpener, scale, brush and a complete set of dissecting instruments.
2. The instruments should be sharp and according to the requirements.
3. To come prepared with the work you are supposed to do in the laboratory.
4. To keep your instruments, practical notebook and seat well-arranged and tidy.
5. Do not encourage the habit of lending either to or from your class fellows. Bring all the essential requirements of the day for the practical laboratory.
6. Listen carefully to the instructions given by your teacher before starting the work.
7. Discuss all your difficulties with your teacher and do not consult your class fellows for any help.
8. Never rub your pencil either on the floor or on the top of the working table. Use a sharpener for the work.
9. Clean the working table and arrange your seat before leaving the laboratory.
10. Maintain complete silence in the laboratory.

STUDENT'S BELONGINGS OR EQUIPMENT FOR LAB WORK

The students while coming to the laboratory for the practical work are required to bring certain compulsory equipment. However, it is not possible to list all that is required, but the following list is considered necessary:

1. The practical book or laboratory manual of the practical and practical record book.
2. The drawing pencil to draw or sketch the diagram of specimens, slides or equipment.
3. Pencil sharpener, pencil eraser and measuring scale.
4. Well maintained dissecting box with following instruments: scalpels, scissors, forceps, dissecting needles, blowpipe, and one edged safety razor or blade.
5. Brush, dropper (one), hand lens, a piece of clean cloth and dissecting pins or T pins.

Note: Do not mind bringing all these things regularly as you do not know when you may need them.

NOTES

INSTRUCTION ABOUT USING MICROSCOPE

Students while using microscope to observe permanent or temporary mount slides should keep in mind the following points:

1. The microscope should always be placed on a flat surface in a well-lit area.
2. Place the slide below the objective lens on the stage of the microscope. While doing so, make sure that the objective lens does not touch the slide.
3. Adjust the reflective mirror so that the slide is exposed to sufficient light.
4. Rotate the nosepiece to the lowest power (either 4X or 10X). It is very important to start with the lowest magnification in order to get proper focus. Higher magnification can be used once the focus is set.
5. Adjust the stage up and down as well as left and right to bring the specimen under the objective lens.
6. Focus the slides by rotating the coarse focus knob (the larger of the two knobs on the side of the microscope).
7. Once the slide is under focus, adjust the fine knob for fine focussing to obtain better clarity.
8. If required, increase the magnification of the objective lens by rotating the nosepiece to the next higher magnification and then adjust the focus.

INSTRUCTIONS TO STUDY AND DRAW THE MUSEUM SPECIMENS

1. Before leaving home for Zoology practical laboratory, check that you are equipped with a Zoology Practical Exercise Book, H.B. pencil, pencil sharpener, pencil eraser (good quality rubber) and a piece of soft cloth.
2. Try to obtain advance information about the slides or museum specimens to be drawn so that you come prepared for their study.
3. Special care should be taken to give a very correct proportion of the dimensions (length and breadth) of the slides or specimen.
4. Usually, draw only one diagram on a page and write their respective comments on the opposite page.
5. Only line diagrams should be drawn.
6. The shading should be avoided as far as possible.
7. Each diagram must be fully labelled with the help of Lab Manual.
8. The labelling should be horizontal and clear.

PRACTICAL NOTEBOOK (PRACTICAL RECORD BOOK)

1. The practical notebook should be neat, clean and up-to-date.
2. Write the date on the left-hand corner of the page of the notebook and details of the work on the top in the centre.
3. The diagrams should be correctly drawn and well-labelled.
4. The diagrams of all museum specimens, slides and dissections should be drawn and comments on all should be written.

STUDY OF SPECIMENS, SLIDES AND TIPS FOR SPOTTING

During the practical examination, the spotting is a very important exercise to obtain marks and merit. Students often face difficulty in spotting because of the lack of proper understanding of basic concepts and the method of commenting on a spot. Here are a few tips for good spotting.

1. First and foremost identify the spot along with the spot number.
2. Draw a well-labelled line diagram of the spot.
3. The important comments can be highlighted in the diagram.
4. The comments should be short and precise.
5. Always mention the special features of the spotting specimen.

MOUNTING AND PREPARATION OF PERMANENT SLIDES

While making the permanent slides (mountings) the following instructions should be strictly followed.

1. Never keep your mounting specimen material for less or more time than the desired time in an alcoholic grade or stain.
2. Keep the specimen material for slightly more time in 90% alcohol and absolute alcohol for complete dehydration.
3. Ensure that complete dehydration is done after putting the specimen material in absolute alcohol. To ensure that the specimen material is completely dehydrated put it in either Xylol or Benzene. If it gives turbidity with Xylol, then dehydrate it again.
4. Do the dehydration in closed specimen tubes or in covered cavity blocks.
5. Always use a brush and never the forceps for holding the mounting specimen material.
6. Put the required amount of Canada Balsam or D.P.X on the slides for mounting.

Note: Excess of Canada Balsam or D.P.X makes the slide dirty.

Experiment 1: Study of Different Types of Chromosomes

Principle

A chromosome is a DeoxyriboNucleic Acid (DNA) molecule with part or all of the genetic material (genome) of an organism, thus, DNA is packed into various units called chromosomes. In various organisms, DNA is powerfully associated with several types of proteins called histones that put the DNA tightly packed into the cell nucleus (in eukaryotes). In eukaryotes, the chromosomes are linear, whereas in most bacteria a single circular chromosome occur. All eukaryotic cells also contain a second genome inside them termed as the mitochondrial DNA (mtDNA). Genes present in mtDNA help to control the process of cellular respiration. In addition, several protists and plants contain a third genome in their chloroplasts which is known as chloroplast DNA (cpDNA), which is involved with photosynthesis.

In this lab, we will exclusively focus on nuclear chromosomes found in eukaryotes. Human somatic cells contain 46 chromosomes. As we know, humans are a diploid species, half of the set of chromosomes (23) originated from a sperm cell and other half set (23) originated from the egg cell. These are known as homologous chromosomes. For example, each person has two copies of chromosomes, one copy inherited from the father and one copy inherited from the mother. The portions of homologous chromosomes can exchange with each other during meiosis in a process known as recombination. During cell division, each homolog is also duplicated so that forming sister chromatids. The regions at which sister chromatids attach during cell division are known as the centromeres. Telomeres are regions found at the tips of chromosomes that consist of highly repetitive sequences that form a protective cap to the ends of chromosomes. Telomeres tend to shorten with each cell division, leading to cell aging and eventually cell death.

Human sex is determined genetically through the XY system of chromosomes—human females are XX and human males are XY. Researches have shown that human 'maleness' is determined by a gene that sits on the Y chromosome.

There is a large variation in the number of chromosomes among various species, but there tends to be a correlation between organismal complexity and genome size. A genome is an organism's complete set of deoxyribonucleic acid (DNA), a chemical compound that contains the genetic instructions needed to develop and direct the activities of every organism. DNA molecules are made of two twisting, paired strands. Each strand is made of four chemical units, called nucleotide bases. The bases are Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). Bases on opposite strands pair specifically, an A always pairs with a T, and a C always with a G. The human genome contains approximately 3 billion of these base pairs, which reside in the 23 pairs of chromosomes within the nucleus of all our cells. Each chromosome contains hundreds to thousands of genes, which carry the instructions for making proteins. Each of the estimated 30,000 genes in the human genome makes an average of three proteins. Some salamander genomes are much larger, but surprisingly only about 2% of their genome encodes a functional product, such as proteins. The remaining 98% is considered as 'junk DNA', although it is likely that other regions of the genome are involved in regulating gene

expression to some degree. To fit such a large genome inside the cell nucleus, it needs to be condensed substantially. The DNA is first wrapped around histone proteins, which then associate to form what are called nucleosomes. The nucleosomes further compress into what is called a 30 nm fiber. Additional coiling eventually gives chromosomes their highly compact, typical appearance during cell division.

Chromosome Classification

Multiple criteria is used to classify chromosomes. First, they are number from largest to smallest. For example, human chromosome 1 would be the largest chromosome, which contains 2,100 protein-coding genes and 249 million base pair (bp). The sex chromosomes are labelled properly as either XY in males or XX in females. It is noted that the X-chromosome is much larger than the Y-chromosome. So, the X- and Y- chromosomes are considered non-homologous, although they have a few homologous regions that are required for proper pairing during cell division.

The relative position of the centromere can also vary between chromosomes. Centromeres that are present exactly in the center of a chromosome are called **metacentric chromosomes**, resulting in equal length chromosomal arms. When the centromere is not found exactly in a central position, different chromosomal arm lengths result. These arms are referred to as q arms (long arms) and p arms (short arms). In addition to metacentric chromosomes, chromosomes can be **sub-metacentric, acrocentric**, and **telocentric**, according to their relative position of the centromere.

Aim/Objective

To study the Chromosomes structure and types.

Metacentric Chromosomes

Metacentric chromosomes have the centromere in the center, so that both the arms are of equal length. Human chromosome 1 and 3 are metacentric.

Sub-Metacentric Chromosomes

Sub-metacentric chromosomes have the centromere slightly offset from the center resulting to a slight asymmetry in the length of the two sections. Human chromosomes 4 through 12 are sub-metacentric.

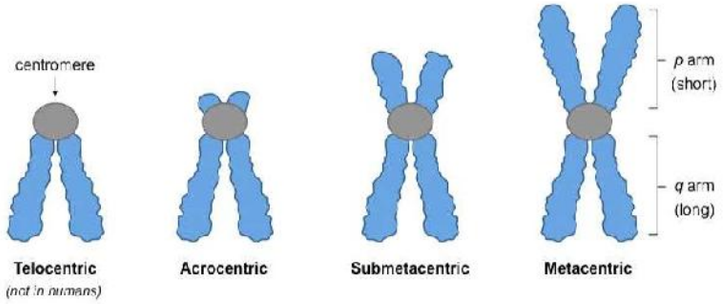
Acrocentric Chromosomes

In acrocentric chromosomes, centromere is severely offset from the center resulting to one very long and one very short section. Human chromosomes 13, 15, 21, and 22 are acrocentric.

Telocentric Chromosomes

Telocentric chromosomes have the centromere at the very end of the chromosome. Humans do not have any telocentric chromosome but they are found in other species such as mice.

Following figure illustrates the different types of chromosomes.



Results/Observations

After studying of the structure of Chromosome, the following significant information is recorded.

- In eukarytoic cells, chromosomes are composed of single molecule of DNA with many copies of five types of histones.
- Histones are proteins molecules and are rich in lysine and arginine residues, they are positively charged. Hence they bind tightly to the negatively-charged phosphates in the DNA sequence.
- A small number of non-histone proteins are also present, these are mostly transcription factors. Transcription factors regulate which parts of DNA to be transcribed into RNA.
- During most of the cell's life cycle, chromosomes are elongated and cannot be observed under the microscope.
- During the S phase of the mitotic cell cycle the chromosomes are duplicated.
- At the beginning of mitosis the chromosomes are duplicated and they begin to condense into short structures which can be stained and observed easily under the light microscope.
- These duplicated condensed chromosomes are known as dyads.
- The duplicated chromosomes are held together at the region of centromeres.
- The centromeres in humans are made of about 1-10 million base pairs of DNA.
- The DNA of the centromere are mostly repetitive short sequences of DNA, the sequences are repeated over and over in tandem arrays.
- The attached, duplicated chromosomes are commonly called sister chromatids.
- Kinetochores are the attachment point for spindle fibers which helps to pull apart the sister chromatids as the mitosis process proceeds to anaphase stage. The kinetochores are a complex of about 80 different proteins.
- The shorter arm of the two arms of the chromosome extending from the centromere is called the p arm and the longer arm is known the q arm.

Experiment 2: Preparation of Karyograms

Aim/Objective

Preparation of Karyograms.

Principle

A karyotype is the number and appearance of chromosomes in the nucleus of a eukaryotic cell. The term is also used for the complete set of chromosomes in a species or in an individual organism and for a test that detects this complement or measures the number. Karyotyping is known as a technique in which chromosomes are stained and visualized during the metaphase stage of cell division (mitosis or meiosis). In this lab, you will employ the role of a medical cytogeneticist who utilize human karyotypes to diagnose various abnormalities and diseases in humans. After this experiment, you should be familiar with what chromosomes are, what a karyotype is and how it is constructed, the various ways in which many chromosomal abnormalities might arise, and the basic terminology used to explain about chromosomes.

The bone marrow of long bones, like the limb bones, is an appropriate and convenient material for karyotype analysis of small animals. In animals like snakes, which do not have limbs, ribs can provide the marrow for analysis. Bone marrow also gives useful material in diagnosis of leukaemia. The air-dried chromosome preparation technique can also be used for other soft tissues, like, liver, spleen, kidney and gills. This is useful in those animals where marrow may be difficult to obtain, such as fish, new born mammals, etc. Most importantly, bone marrow cells can be incubated in vitro for at least one cell cycle without any exogenous mitogenic stimulation. Thus, they can be used for certain in vitro experiments, namely mutagenesis, cell metabolism and cell cycle, as very accurate representative of in vitro conditions. Such experiments can be done even in field, where power supply is not available. A hand centrifuge and a burner can be an easy substitute for incubator and power-driven centrifuge.

Materials Required

1. **Biological Materials:** Rat or Mouse.
2. **Chemicals/Reagents:** Colchicine, Hypotonic Solution: 0.56% KCl (should be kept pre-warmed to 37 °C before use), Fixative: (Aceto-Methanol: 1:3, Mix 25 mL Glacial Acetic Acid with 75 mL Methanol), Giemsa Stain.
3. **Glassware/Plastic Ware:** Centrifuge Tubes (15 mL Graduated), Brown Cellophane Tape, Sterile Disposable Syringe (5 mL) and Needles No. 22 and No. 18.
4. **Equipment:** Incubator (37-38 °C), Instruments for Dissection, Agitator (Cyclo-Mixer), Clinical Centrifuge, Pasteur Pipettes (with fire drawn long narrow tips), Camera lucida, Spirit lamp, Coplin jar.

Procedure

1. Harvesting Bone Marrow Cells

(i) Inject colchicine (0.2 mg/kg body weight) to the animal 2-3 hours prior to the dissection.

(ii) Using the cervical dislocation method dissect out femur bone (any long bone will do).

(iii) Cut both ends of the bone. Take about 0.5 mL of pre-warmed hypotonic solution in a 5 mL syringe (with No. 18 Needle for Rat and No. 22 for Mouse) and by inserting through one of the cut ends of the bone flush the marrow into a centrifuge tube.

(iv) Agitate the marrow with a rubber agitator (mix/cycle/rotate mixture at low speed) so that it breaks into a uniform cell suspension. Otherwise, we can use a long capillary tipped pasture pipette and aspirate up and down, the cell pellet, to make a suspension.

(v) Make up the volume to 10 mL with the pre-warmed hypotonic solution and keep in incubator (37 °C).

(vi) Centrifuge the cell suspension (1,000 rpm) for 5 minutes to get a cell pellet at the bottom of the tube.

(vii) Now, discard the supernatant.

(viii) Fix the cells by adding the fixative drop-by-drop (up to about 0.5 mL) and agitating the tube to make a suspension.

(ix) Make up the volume of the mixture up to 10 mL with the fixative.

(x) Keep aside for 15 minutes.

(xi) Agitate the cells and centrifuge at 1000 rpm for 5 minutes.

(xii) Obtain a cell pellet at the bottom of the tube and discard the supernatant.

(xiii) Add a small volume of fixative (0.3 - 0.5 mL) and re-suspend the pellet.

(xiv) Place a clean glass slide in a Petri dish, slating at an angle, with one end resting on the side of the Petri dish.

(xv) Use a Pasteur pipette and add 2-3 drops of the cell suspension one drop at a time, from about a foot height, at the centre of the slide such that each drop spreads after falling on the slide.

(xvi) Heat the slide over a gentle flame, by moving the slide in and out of the flame (spirit lamp/match stick flame) till the cell suspension almost dries. Do not overheat the slide.

(xvii) Keep the slide aside in a Petri dish to air dry.

(xviii) In the beginning, prepare only one slide and observe under the microscope to judge the density of cell suspension, an unstained slide can be examined for this purpose. If there are too many cells on the slide, dilute the suspension by adding more fixative. If the cells are too few, re-spin the tube and suspend the cells in a smaller volume of fixative.

2. Staining with Giemsa Stain

(i) After proper drying, stain the slide with Giemsa with following steps:

(a) Add three to four drops of Giemsa stain (undiluted) with a pipette so that the stain fully covers the smear area. Do not allow the stain to flow out of the slide.

(b) Immediately, cover it with the Petri dish and stain for 3 – 4 minutes.

(c) With a dropper or pipette, gently add few drops of deionized water on the stain such that the diluted stain spreads till all edges of the slide but does not flow out from the slide.

(d) Carefully observe a green shiny scum formed over the surface of the diluted stain.

(e) Gently cover the Petri dish and keep aside undisturbed for 15 minutes (differentiation).

(f) Now, open the Petri dish and lift the slide by one of its narrow ends and drain the diluted stain into the Petri dish.

(g) Put the slide in a Coplin jar for two minutes where a gentle stream of running water is directed. Do not allow the stream of water to fall directly on the smear.

(ii) Place the slide on strip of filter paper such that the smear surface is facing upwards.

(iii) When completely dried, mount with DPX mounting medium using a large cover glass.

(iv) Observe the slide first under the low power of the microscope (10X) and then under the high power (40X).

(v) Place a drop of immersion oil and focus the slide under the Oil Immersion Lens.

3. Preparation of Karyogram

(i) First set up a camera lucida and trace the chromosomes on to a graph paper.

(ii) Trace each chromosome separately on the graph paper. Note their magnifications.

(iii) Now, take a photocopy of the same (with enlargement) and cut out each individual chromosome.

(iv) Arrange the chromosomes in descending order of their sizes and stick them on a white paper.

Alternatively,

(i) Focus the area of the chromosome spread under 1000X of the microscope.

(ii) Using an image capture software obtain a microphotograph of the chromosome spread.

(iii) Print the microphotograph and obtain cut outs of each individual chromosome.

(iv) Arrange the chromosomes in descending order of their sizes and stick them on a white paper.

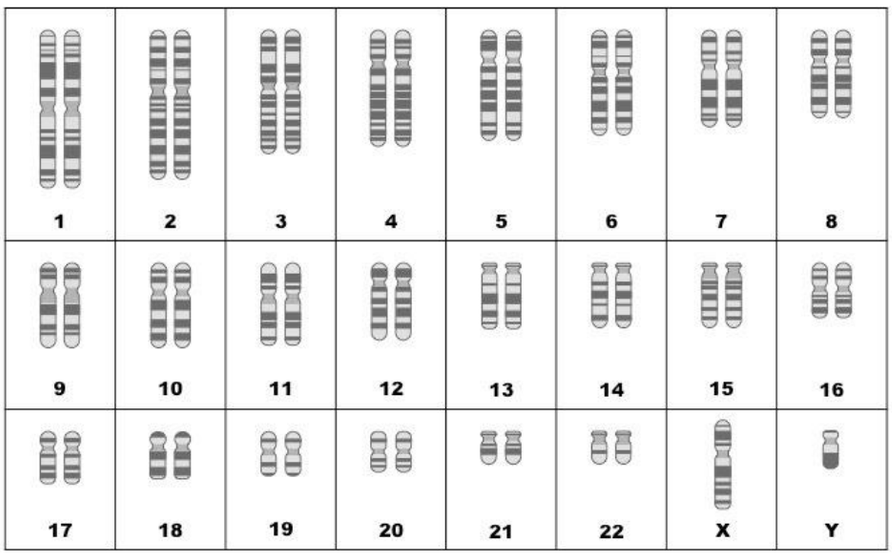
Observations

The rat species (*Rattus rattus*) has diploid number, 2n = 42. There are 14 metacentric and 26 acrocentric chromosomes and two sex chromosomes. The X chromosome is 11th of autosomal chromosome in length and Y chromosome is the shortest in the male genome.

Extension Activity

Use a good chromosome spread of human and carry out G-C banding of the chromosomes.

Figure given below illustrates the Karyogram of human chromosome.



Experiment 3: Study of Different Stages of Mitotic Cell Division in Suitable Material - Mitosis in Onion Root Cells

Aim/Objective

Our aim is to prepare a temporary mount of onion root tip to study mitosis.

Learning Outcomes:

- Students will understand the term mitosis.
- Students will understand the different events occurring during mitosis.

Principle

As we know, all organisms are made of cells. For an organism to grow, mature and maintain tissue, new cells must be formed. New cells are formed by division of pre-existing cells. The continuity of life also depends on cell division. There are two main methods of cell division known as mitosis and meiosis.

Mitosis

Mitosis is the process in which nucleus of eukaryotic cell splits in two followed by division of the parent cell into two daughter cells. The process of Mitosis is very important to life because it provides new cells for growth and replacement of dead cells.

Each cell division consists of two events, cytokinesis and karyokinesis, that occur simultaneously. Karyokinesis is the process of division of the nucleus while the cytokinesis is the process of division of cytoplasm.

Events during Mitosis

1. Prophase

(i) Mitosis begins at prophase with the thickening and coiling of the chromosomes.

(ii) The nuclear membrane and nucleolus shrinks and disappears.

(iii) The end of prophase is marked by the beginning of the organization of a group of fibres to form a spindle.

2. Metaphase

(i) The chromosome become thick and two chromatids of each chromosome become clear.

(ii) Each chromosome attaches to spindle fibres at its centromere.

(iii) The chromosomes are arranged at the midline of the cell.

3. Anaphase

(i) In anaphase each chromatid pair separates from the centromere and move towards the opposite ends of the cell by the spindle fibres.

(ii) The cell membrane begins to pinch at the centre.

4. Telophase

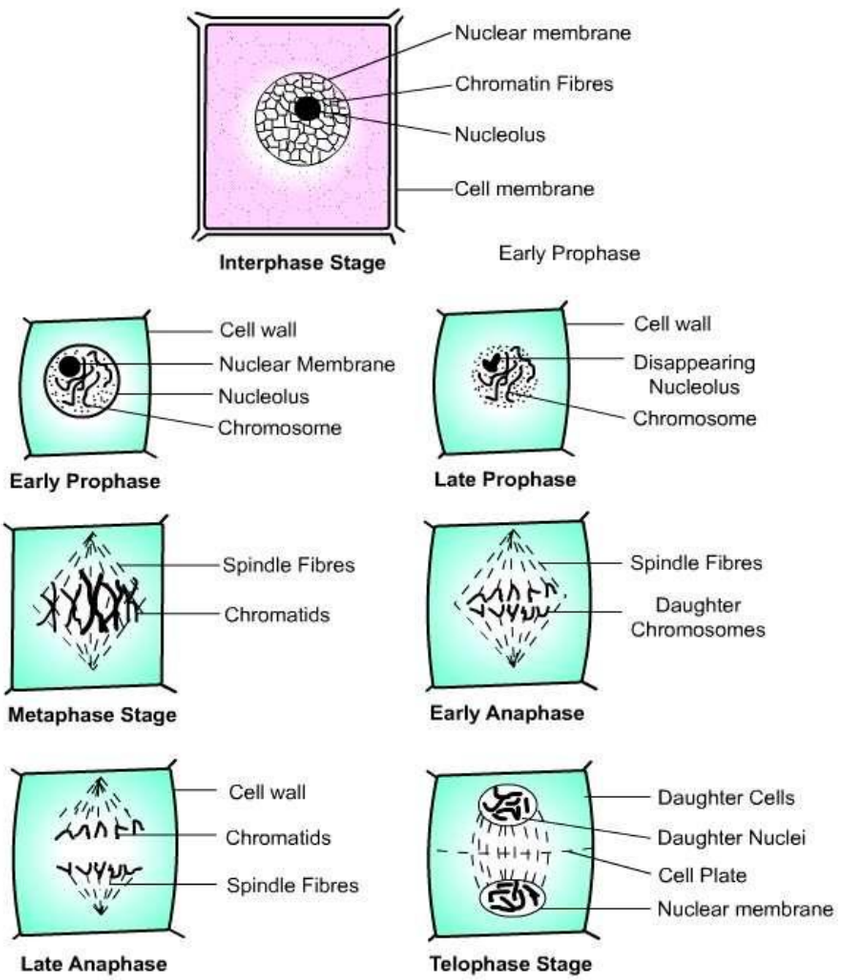
(i) Chromatids arrive at opposite poles of cell.

(ii) The spindle disappears and the daughter chromosome uncoils to form chromatin fibres.

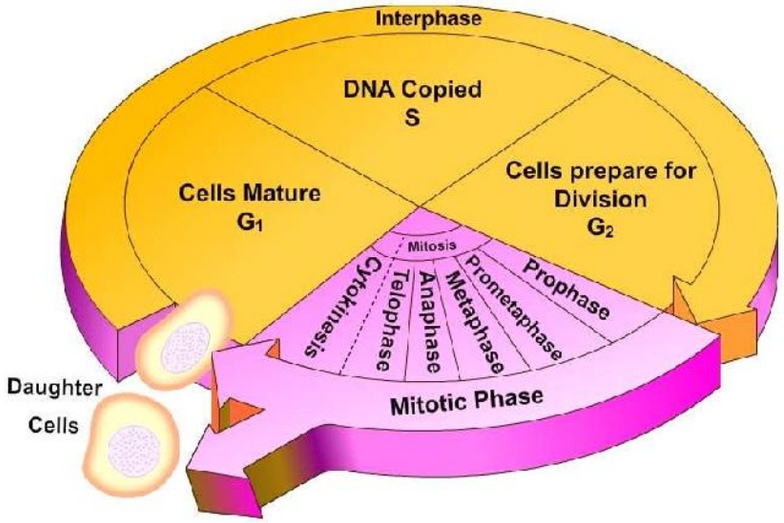
(iii) The nuclear membranes and nucleolus re-form and two daughter nuclei appear at opposite poles.

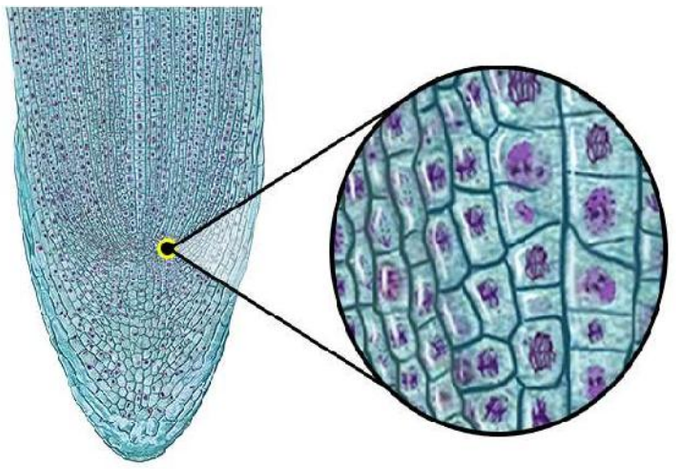
(iv) Cytokinesis or the partitioning of the cell may also begin during this stage.

The following figures illustrate the different stages/events of the Mitosis process in the cell.



After mitosis, the stage or phase that occur is known as **interphase**. It is the non-dividing phase of the cell cycle between two successive cell divisions. Mitosis is only one part of the cell cycle. Most of the life of a cell is spent in interphase. Interphase consist of three stages called G_1, S and G_2, as shown below in the given figure.





Mitosis in Onion Root Tip

The meristematic cells present in the root tips of onion provide the most suitable material for the study of mitosis. The chromosome of monocots is large and more visible, therefore, onion root tips are used to study the process of mitosis. On the basis of kind of cells and species of organism, the time taken for mitosis, may vary. The process of mitosis is affected by factors like temperature and time.

Cell divisions in plants localized in specialized regions are called meristems. Meristems are regions of active growth. Plants have two types of meristems: apical and lateral. Apical meristems are found at the tips of different plant organs (shoots and roots).

Materials Required

Acetocarmine stain, Burner, Filter paper, Watch glass, Cover slips, Aceto-alcohol, N/10 Hydrochloric acid, Glass slides, Onion, Forceps, Blade, Dropper, Needle, Compound microscope.

Laboratory Procedure

1. Take a bulb of onion and put it on the tile.
2. Remove carefully remove the dry roots by using a sharp blade.
3. Grow new root tips by placing the onion bulbs in a beaker filled with water.
4. Growing new roots may take 3–6 days to grow.
5. Cut off 2–3 cm of freshly grown roots and let them drop into a watch glass.
6. Using a forceps, transfer them to the vial containing freshly prepared fixative of aceto-alcohol (1:3 - Glacial acetic acid: Ethanol).
7. Before doing experiment, keep the root tips in the fixative for 24 hours.
8. By using a forceps, take one root and place it on a clean glass slide.
9. Place one drop of N/10 HCl on the root tip followed by 2–3 drops of acetocarmine stain by using a dropper.
10. Warm it a little on burner. It should be cared that the stain is not dried up.
11. Blot the excess stain carefully by using filter paper.
12. Through a blade, cut the comparatively more stained tip portion of the root, retain it on the slide and discard the remaining portion.
13. After that, put one drop of water on the root tip.
14. Mount a cover slip on the root tip by using a needle.
15. Now, tap the cover slip slowly by using the blunt end of a needle so that the meristematic tissues of the root tip below the cover slip is squashed properly and spread like a thin layer of cells.
16. This preparation of onion root tip cells is now ready for the study of mitosis.
17. Place the slide under the compound microscope and observe the different stages of mitosis.

Various stages of mitosis are prophase, metaphase, anaphase and telophase.

Experiment 4: Study of Meiotic Cell Division

Aim/Objective

To observe the different stages of Meiosis using permanent slides of pollen mother cell.

Materials Required

Permanent slides of different stages of meiosis.

Procedure

Observe permanent slides of different stages of meiotic division in pollen mother cell and report.

Principle

Meiosis is the special type of re-combinative and reductive cell division occurring only in the generation of the gametes or germ cells (oocyte and spermatozoa). For recombination, meiosis requires that homologous chromosomes are properly paired and aligned by the induction of DNA double-strand breaks during the prophase of the first meiotic division. Meiotic cell division also reduces (halves) the chromosomal content. The overall process of germ cell development is called 'gametogenesis' and includes not only meiosis but also the cellular morphological changes, that occur differently in male and female gametes.

Mitosis is used for almost all needs of cell division in our body. It adds new cells during growth, development and also replaces old and worn-out cells throughout our life. The main goal of mitosis is to produce daughter cells that are genetically identical to their parents, with not a single chromosome more or less.

On the other hand, Meiosis is used only for single purpose in the human body: the production of **gametes**—sex cells, or sperm and eggs. Its chief aim is to produce daughter cells with exactly half as many chromosomes as the starting cell.

To understand that in another way, **meiosis** in humans is a division process that takes part into a diploid cell—a cell with two sets of chromosomes—to haploid cells—cells with a single set of chromosomes. In humans, the haploid cells that made during meiosis, are sperm and eggs. When a sperm and an egg join in the process of fertilization, the two haploid sets of chromosomes form a complete diploid set: a new genome.

Phases of Meiosis

If we observe, in many ways, meiosis is similar to mitosis. The cell goes through similar stages and also uses same strategies to organize and separate chromosomes. However in meiosis, the cell has to do more complex task but it still needs to separate **sister chromatids** (the two halves of a duplicated chromosome), as in

mitosis. But it must also separate **homologous chromosomes**, the similar but non-identical chromosome pairs an organism receives from its two parents.

These goals are accomplished during meiosis which is a two-step division process. Homologous pairs of chromosomes separate during a first round of cell division, called **Meiosis I** while sister chromatids separate during a second round, called **Meiosis II**.

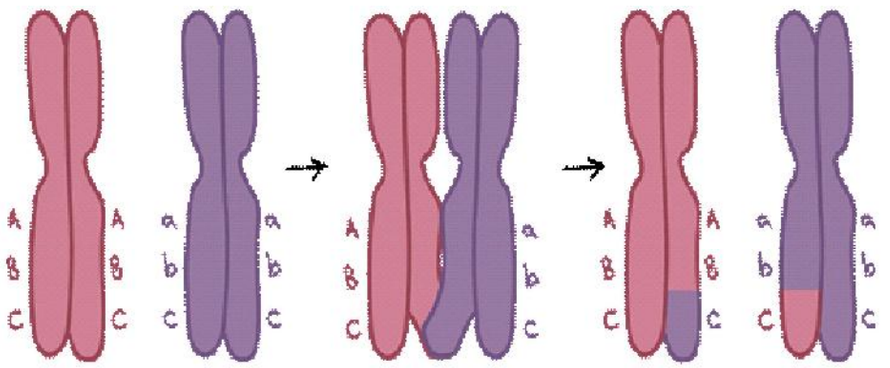
As, cell division occurs twice during meiosis, one starting cell can produce four gametes (eggs or sperm). In both phases of division, cells go through four stages: **Prophase, Metaphase, Anaphase,** and **Telophase.**

Meiosis I

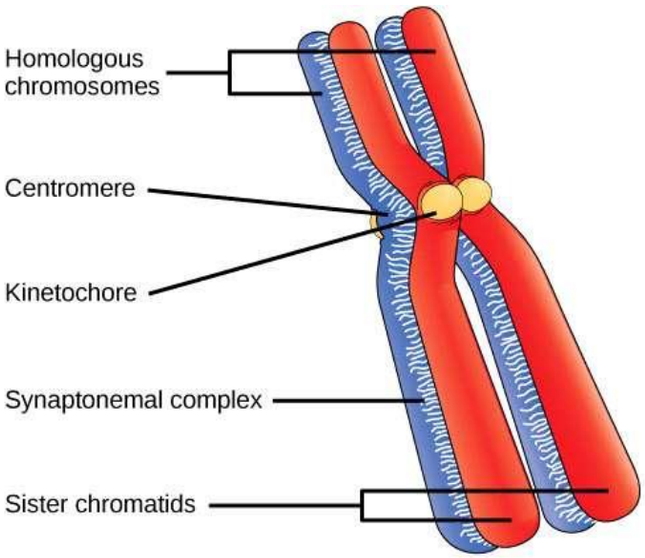
Before entering into meiosis I, a cell must first go through interphase. As in mitosis, the cell grows during G_1 phase, copies all of its chromosomes during S phase, and prepares for division during G_2 phase.

During **prophase I**, differences from mitosis usually start to appear. As in mitosis, the chromosomes begin to condense, but in meiosis I, they also pair up. Each chromosome carefully aligns with its homologue partner so that the two match up at corresponding positions along their full length.

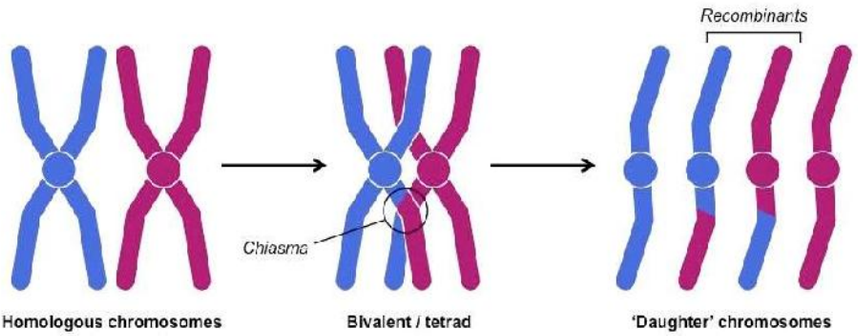
In the figure given below, the letters A, B, and C represent genes found at particular spots on the chromosome, with capital and lowercase letters for different forms, or alleles, of each gene. The DNA is split at the same spot on each homologue—here, between genes B and C—and reconnected in a criss-cross pattern so that the homologues exchange part of their DNA.



This process, in which homologous chromosomes exchange parts, is known as **crossing over**. It is helped along by a proteinaceous structure called the **synaptonemal complex** that holds the homologous together, as shown below in the figure.

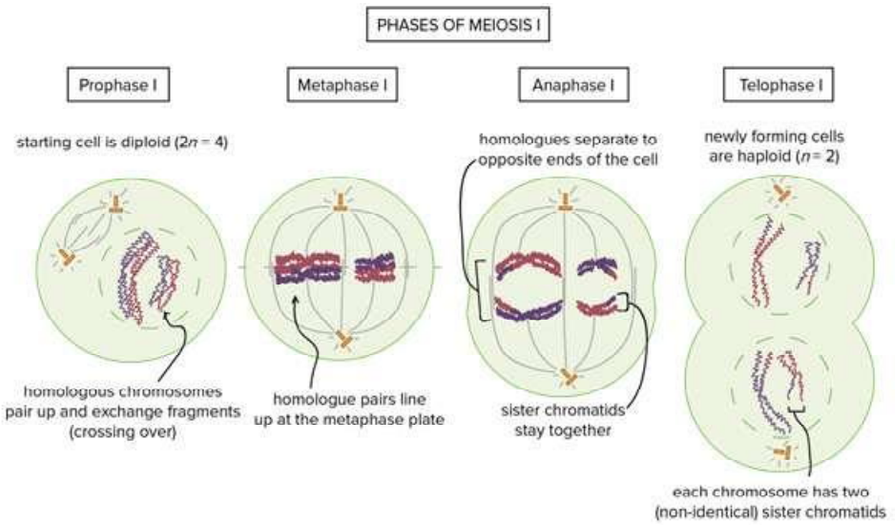


You can observe crossovers under a microscope as **chiasmata**, cross-shaped structures where homologues are linked together, as shown below in the given figure. Chiasmata keep the homologous connected to each other after the synaptonemal complex breaks down, so each homologous pair needs at least one.



The spots where crossing over happen are more or less random, leading to the formation of new, 'remixed' chromosomes with unique combinations of alleles.

After the process of crossing over, the spindle begins to arrest chromosomes and move them towards the center of the cell (metaphase plate). This may be like in mitosis, but there is a twist. Every chromosome attaches to microtubules from just one pole of the spindle, and the two homologues of a pair bind to microtubules from opposite poles. So, during **metaphase I**, homologue pairs—not individual chromosomes—line up at the metaphase plate for separation, as shown below in the given figure. When the homologous pairs line up at the metaphase plate, the direction of each pair is random.



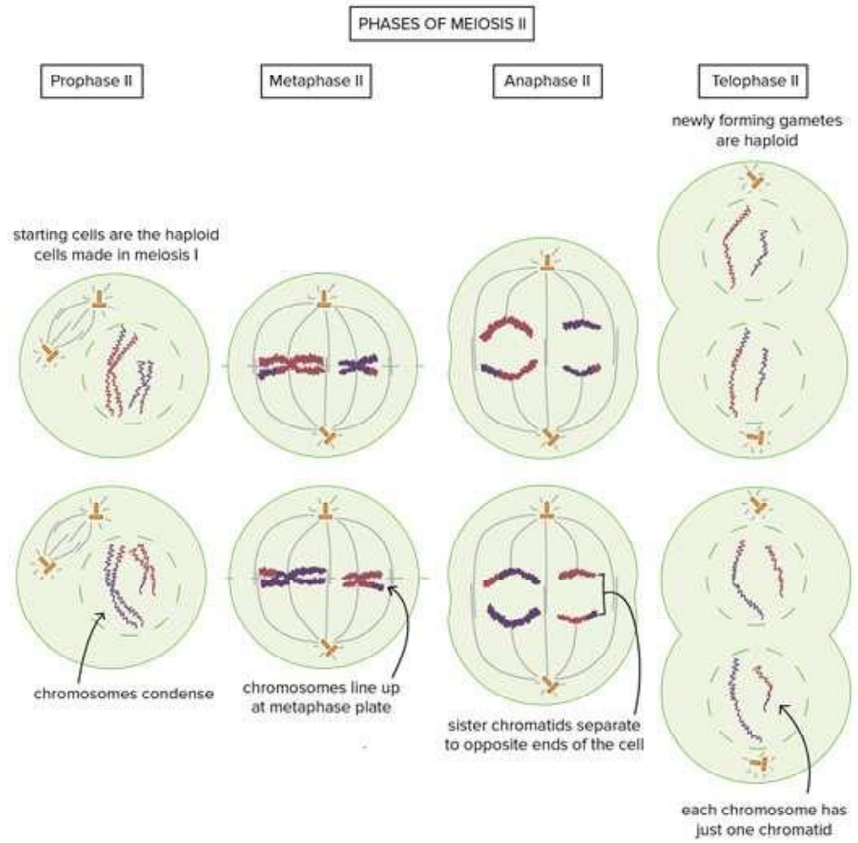
In **anaphase I**, the homologues are pulled apart and move apart to opposite ends of the cell. The sister chromatids of each chromosome, however, remain attached to one another and do not come apart.

Finally, in **telophase I**, the chromosomes reach at opposite poles of the cell. In some organisms, the nuclear membrane re-forms and the chromosomes decondense, although in others, this step is skipped—since cells will soon go through another round of division, meiosis II. Cytokinesis usually occurs at the same time as telophase I, forming two haploid daughter cells.

Meiosis II

Cells move from meiosis I to meiosis II without copying their DNA. Meiosis II is simpler and shorter process than meiosis I, and it is considered as mitosis for haploid cells formed during meiosis I.

The cells that enter meiosis II are the ones made in meiosis I. These cells are haploid—have just one chromosome from each homologue pair—but their chromosomes still consist of two sister chromatids. In meiosis II, the sister chromatids separate, producing haploid cells with non-duplicated chromosomes.



During **prophase II**, chromosomes condense and the nuclear envelope breaks down, if required. Now, the centrosomes move apart, the spindle forms between them, and the spindle microtubules begin to capture chromosomes.

The two sister chromatids of each chromosome are captured by microtubules from opposite spindle poles. In **metaphase II**, the chromosomes come up individually along the metaphase plate. In **anaphase II**, the sister chromatids separate and are moved towards opposite poles of the cell. In **telophase II**, nuclear membranes form around each set of chromosomes, and the chromosomes decondense.

Cytokinesis starts splitting the chromosome sets into new cells, forming the final products of meiosis: four haploid cells in which each chromosome has just one chromatid. In humans, the products of meiosis are sperm or egg cells.

Experiment 5: Linear Differentiation of Chromosomes through Banding Techniques - G-Banding, C-Banding and Q-Banding

Aim/Objective

Studying the linear differentiation of Chromosomes through banding techniques, such as the G-Banding, C-Banding and the Q-Banding.

Principle

Chromosomes as seen at metaphase stage appear uniformly stained all through their length. However, the chromosomes are made up of different structural compartments that manifest various structural and functional attributes of chromatin. The most obvious distinct domains are Euchromatin and Heterochromatin. At interphase satge, the 'Euchromatic' regions of chromatin decondense while the 'Heterochromatic' regions remain condensed. In genetic terms, Euchromatin comprises potentially active parts of the genome while heterochromatic regions are generally transcriptionally inert.

According to structural terms, heterochromatin is generally enriched with highly repeated base sequences. In contrast, Euchromatin, harbours unique sequences of DNA and is structurally not uniform. Through its length, different structurally as well as functionally distinct domains are encountered, and these domains are constant for any given species. However, these domains, invisible in routinely stained metaphase chromosomes, can be resolved if the chromosomes are treated with certain agents like trypsin. Trypsin treated chromosome preparations bring out transverse bands on chromosomes following staining with Giemsa. They are known as 'G–Bands'. Alternatively, the Heterochromatin region can be clearly visualized by treating chromosome preparations first with an alkali (a denaturing agent) and then with Saline Sodium Citrate (SSC) solution followed by Giemsa staining. The darkly stained Heterochromatin regions in such preparations are called 'C–Bands'. Besides unravelling certain aspects of chromosome structure and function, these techniques have been particularly useful in clinical cytogenetics and evolutionary studies. G–Banding helps in identifying homologous regions in chromosomes. It is also useful in evaluating chromosomal abnormalities and helps in understanding gene expression in cancerous cells. C–Banding is useful in paternity and in gene mapping studies. Additionally, the C–Banding is also useful in identifying the Y chromosomes in mammalian species.

The Q–Banding techniques differentiate the heterochromatic regions on chromosomes into two types, either exhibiting enhanced fluorescence or reduced fluorescence, when stained with fluorochrome dyes and observed under fluorescence microscope.

A. G – Banding of Metaphase Chromosomes

Materials Required

1. **Biological Materials:** Good Metaphase Chromosome Spread from Rat/ Mouse (Refer the protocol for preparing metaphase spread using mammalian bone marrow cells).
2. **Chemicals/Reagents:** Trypsin solution (30 mg/mL in 0.9% NaCl stored at –20 °C), 0.9% NaCl (stored at 4 °C), Giemsa stain, Phosphate buffer (stored at 4 °C).

   Solution 1: 9.073 g KH_2PO_4 in 1000 mL distilled water.

   Solution 2: 11.87 g $Na_2HPO_4.2H_2O$ in 1000 mL distilled water.

   Mix equal parts of Solution 1 and Solution 2.
3. **Equipment:** Microscope with camera attachment.
4. Distilled water (Deionized), Coplin jars.

Procedure for G–Banding

1. Take 1 mL of trypsin stock solution and dilute it up to 50 mL with 0.9% NaCl in a Coplin jar.
2. Adjust the pH of the solution with 1 M NaOH to 7.5-7.8 using pH paper (1-2 drops of 1 M NaOH may be required).
3. Pour 50 mL phosphate buffer to another Coplin jar.
4. Pour 7-10% Giemsa stain in phosphate buffer in another Coplin jar.
5. Pour distilled water (deionized) in another Coplin jar.
6. Take 3 – 4 slides with good spread of metaphase chromosomes.
7. Label the slides with a diamond tipped glass marker on the smear surface.
8. Dip one slide in trypsin for 5 seconds.
9. Rinse the slide (gently dip in and pull out the slide, 4 – 5 times) in phosphate buffer immediately after trypsin exposure.
10. Tap the narrow edge of the slide on filter paper to remove excess buffer.
11. Place the slide in Giemsa Stain for 3 – 5 minutes in Giemsa.
12. Rinse in water, and monitor under the microscope.
    (i) If stain is less, put again in Giemsa. If treatment is less, put the slide back again in trypsin for a few seconds and repeat the staining steps until satisfactory bands emerge.
    (ii) If the slide gets over-treated, i.e., chromosome will look hollow and crushed up, then take a new slide and repeat the process giving shorter trypsin treatment.
13. Air dry the slide, mount in DPX and observe under the microscope.

B. C–Banding of Metaphase Chromosomes

Materials Required

1. **Biological Materials:** Good Metaphase Chromosome Spread from Rat/ Mouse (Refer the protocol for preparing metaphase spread using mammalian bone marrow cells)
2. **Chemicals/Reagents:** 0.2 N HCl (1 mL Conc. HCl in 54 mL distilled water), Giemsa stain.
   (i) 5% Barium Hydroxide
      (a) Boil 100 mL of distilled water in a conical flask.
      (b) Add 5 g $Ba(OH)_2$ while the water is steaming.
      (c) Stir vigorously to get as much $Ba(OH)_2$ in solution as possible.
      (d) Filter into a Coplin jar and maintain the solution at 50 °C in a water bath.
   (ii) Saline Sodium Citrate (SSC) Solution (6X)
      (a) 52.6 g NaCl.
      (b) 26.5 g Sodium Citrate.
      (c) Dissolve in 800 mL deionized water in a 1 L standard flask.
      (d) Adjust pH to 7.0 with 10 M NaOH.
      (e) Dilute to 1000 mL mark with more deionized water.
   (iii) 2X SSC (pH 7.2)
      (a) 333 mL SSC Solution (6X).
      (b) 667 mL Deionized water.
      (c) Mix the two solutions well.
      (d) Giemsa stain.
3. **Glassware/Plastic Ware:** DPX mounting material, 500 mL Conical flask, Coplin jars.
4. **Equipment:** Microscope with camera attachment, Water bath set at 50 °C (for $Ba(OH)_2$ solution), Water bath set at 60 °C (for 2X SSC).

Procedure for C–Banding

1. Take 3 – 4 slides with good spread of metaphase chromosomes. Keep them aside for a week before further use.
2. Label the slides with a diamond tipped glass marker on the smear surface.
3. Pour 0.2 N HCl in a Coplin jar.
4. Pour distilled water in six different Coplin jars and mark them 1 to 6 serially.
5. Pour $Ba(OH)_2$ in a Coplin jar and keep the Coplin jar in water bath at 50°C for 30 minutes.
6. Pour 2X SSC in a Coplin jar and keep the Coplin jar in water bath at 60°C for 30 minutes.
7. Pour 7-10% Giemsa stain in phosphate buffer in another Coplin jar.

8. Dip the slides in 0.2 N HCl in a Coplin jar for 30 minutes.
9. Transfer slides into Coplin jar No. 1 of distilled water for 5 minutes.
10. Transfer slides into Coplin jar No. 2 of distilled water for 5 minutes.
11. Air dry the slides by keeping them in a dry Coplin jar.
12. Transfer the air-dried slides to $Ba(OH)_2$ in water bath at 50°C, treat different slides for varying time intervals ranging from 1 minute to 5 minute. Remove the precipitate formed in the solution, before placing slides in the $Ba(OH)_2$ solution.
13. Transfer slides into Coplin jar No. 3 of distilled water for 5 minutes.
14. Transfer slides into Coplin jar No. 4 of distilled water for 5 minutes.
15. Air dry the slides by keeping them in a dry Coplin jar.
16. Transfer the air-dried slides in 2X SSC in a water bath maintained at 60 °C for 120 minutes.
17. Transfer slides into Coplin jar No. 5 of distilled water for 5 minutes.
18. Transfer slides into Coplin jar No. 6 of distilled water for 5 minutes.
19. Air dry the slides by keeping them in a dry Coplin jar.
20. Transfer the slides to Giemsa stain and stain for 15-20 minutes.
21. After 10 minutes in Giemsa stain, intermittently take the slide out, tap the narrow end of the slide on a filter paper and check for proper staining under a microscope (Take care not to dry the slide).
22. After appropriate staining, air dry the slide and mount with DPX.

Recording Banding Pattern

1. Focus the area of the chromosome spread under low power and then under high power.
2. Locate chromosomes which are properly banded.
3. Focus them under the 1000X of the microscope.
4. Using an image capture software obtain a microphotograph of the banded chromosomes.
5. Print the microphotograph and obtain cut outs of each individual chromosome.
6. Arrange the chromosomes, in descending order of their sizes and stick them on a white paper.
7. Count the number of bands on each chromosome.
8. Record the number of bands seen on each chromosome in a table.

Observationa

After G–Banding, the long chromosomes show clearly distinct bands while in the short ones bands are not always visible. The homologous chromosomes can be paired easily based on banding patterns. In the male rat, G–Banding patterns have been recorded between 189 to 282 in 20 autosomes and two sex chromosomes. C–Bands are present in the centromeric regions of chromosomes and represent constitutive heterochromatin. This heterochromatin is highly condensed A–T or G–C rich, with multiple copies of DNA, with no known genes.

Experiment 6: Application Of Colchicines In Genetics

Aim/Objective

To study Colchicine effect on the DNA content and stomata size of *Glycyrrhiza glabra* var.glandulifera and *Carthamus tinctorius* L. cultured in vitro.

Principle

Licorice (*Glycyrrhiza glabra* L.) and safflower (*Carthamus tinctorius*) are medicinal plants containing various compounds and have long been used for medicinal and industrial purposes. The roots and stolons of G. *glabra* are used as natural sweetener and anti-inflammatory drug. Safflower pigments have been shown to have pharmaceutical properties and are also safely used in the food industry.

Developing a suitable method for increasing the secondary metabolites found in these plants and also enhancing their resistance against environmental stress can help the conservation of these precious species. The induction of artificial polyploidy has long been considered as a valuable tool that can improve genetic of many plants and as a result changes the morphological, anatomical, and physiological characteristics of plants. It also increases the production of important medicinal compounds and makes the plants more resistant to stresses. Colchicine as an anti-mitotic agent is used for chromosome doubling under in vitro conditions. The effect of colchicine for in vitro chromosome doubling is different regarding to its concentration, method and duration of treatment, and also genetic factors of the treated plants.

An important step in polyploidization is to determine ploidy levels in a quick and simple way. The measurement methods are classified into direct and indirect.

DNA content can also be a good indicator of ploidy level as it increases by chromosome doubling that happens in polyploidy. Therefore, optical density of extracted DNA, measured by spectrophotometry, will rise as well.

Another method for measuring DNA content is Flow Cytometry (FCM). Unlike spectrophotometry this method needs small amounts of tissue and can analyze a large number of cells especially when mixoploids or aneuploids exist.

In this study we used colchicine treatments in order to obtain polyploids of licorice and safflower. We used indirect methods such as spectrophotometer to compare optical density of DNA content in treated and control plants for estimating the DNA increase as the result of chromosome doubling. Also stomata size as an indirect method was used for recognition of putative polyploids in these species and then flow cytometry for confirming ploidy levels and comparing it with other methods of ploidy screening used in this experiment.

Materials Required Method

1. Seed Treatment

Seeds of Licorice (G. *glabra* var. glandulifera L.) were soaked in sulfuric acid 98% for 30 minutes in order to remove the hard seed coat that prevents the germination. Then seeds were sterilized in 70% for 1 minute and 1% sodium hypochlorite for 20 minutes and finally rinsed with distilled water for three times. Safflower's seeds also were sterilized using 0.1% $HgCl_2$ for 8–10 min followed by three rinses with distilled water. Seeds then were cultured on sterile liquid MS medium (Murashige and Skoog, 1962). Supplemented with colchicine (0%, 0.03%, 0.05%, 0.08%, 0.1%, pH = 6) for 24 or 48 H on an orbital shaker (100 rpm). Then they were transferred on solid MS media and allowed for germinating in the culture room under normal condition.

2. Ploidy Analysis

Putative polyploid plants were selected based on morphology, as they seemed to be thicker, darker and showed delayed growth. Size of the stomata in selected plants was measured and also cellular DNA content of them was analyzed by spectrophotometry, and finally flow cytometry as an accurate method was carried out to confirm the efficiency of above mentioned methods.

3. Size of Stomata

Epidermal cells of two leaves from 5 diploid and 5 putative tetraploid plants from each treatment were randomly examined. Imprint of epidermal cells of plantlets treated with different concentrations of colchicine for 24 H were obtained by applying thin layer of clear nail polish on the lower leaf surface and removing it after drying the polish (abaxial side). Imprints were stuck on a microscope slide and observed through the light microscope at 1000• magnification (for guard cells measurement). Stomata length can be measured as an indicator of stomata size which by itself is an indicator of ploidy using the Image tool software.

4. Spectrophotometry

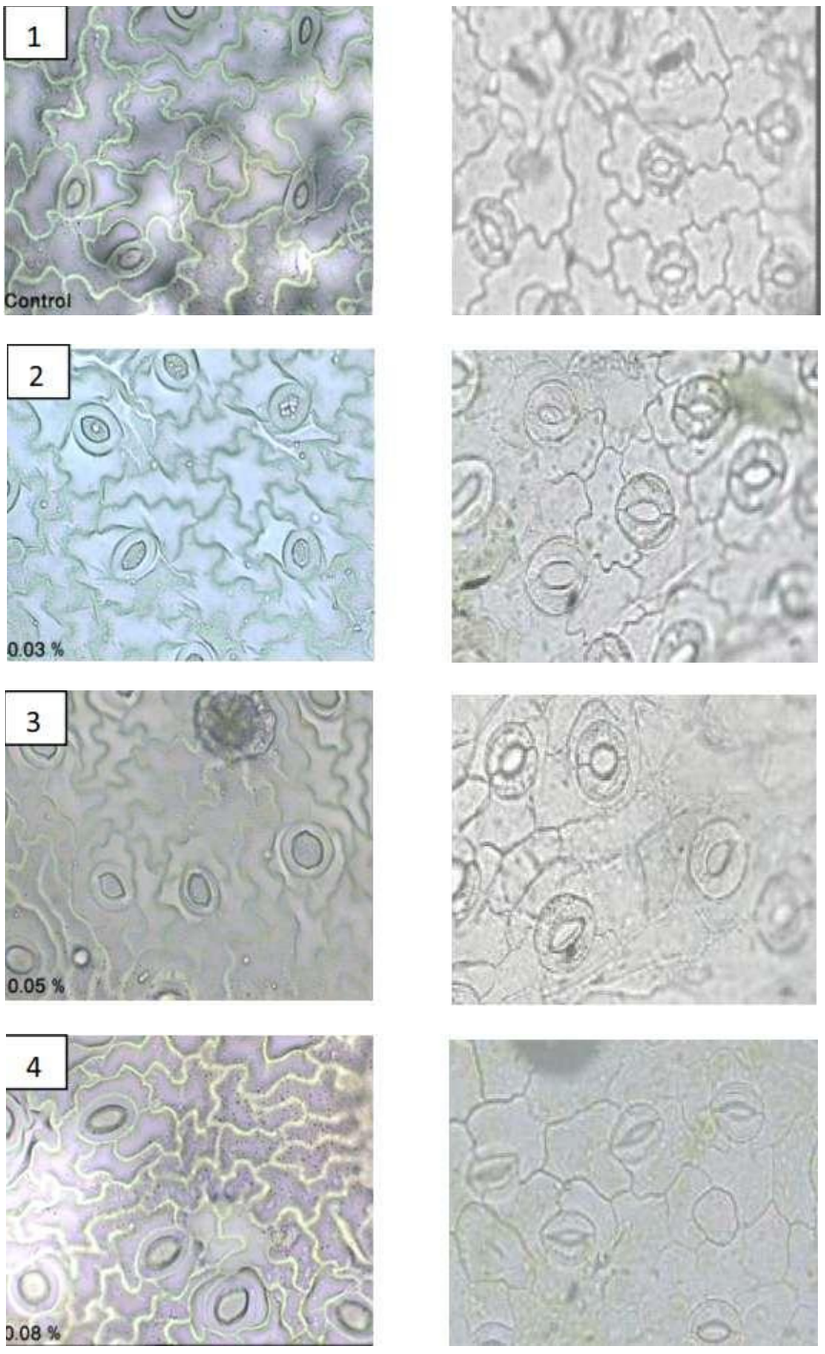
The DNA of 5 control and 5 treated plants from each treatment were randomly extracted by the standard CTAB procedure. Optical density of total DNA content was measured at 260 nm wavelength using spectrophotometry.

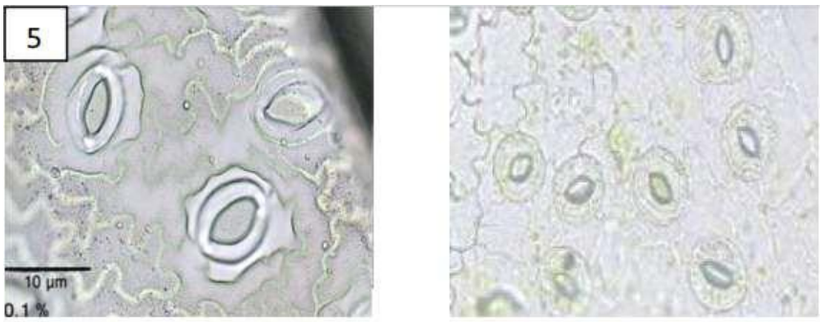
5. Flow Cytometry (FCM)

Flow cytometric analysis was carried out using a PA-1 flow cytometer. Leaf tissue (0.5 mg) from selected plants was chopped with a sharp blade in 0.5 ml CyStain DNA 1 step nucleus extraction buffer (HR, high resolution, A solution, Partec, CA) after being filtered through a 30-lm nylon mesh and then incubated in the same buffer for 5 min. The different flow cytometer parameters were adjusted

with untreated material to secure well defined and reproducible readings. The nuclear DNA of 150 colchicine treated plants was used in these determinations.

Figure below shows stomata of leaves obtained from treatments with (1) 0%, (2) 0.03%, (3) 0.05%, (4) 0.08%, (5) 0.1% of colchicine for 24 H in (A) licorice (B) safflower. Each stoma was obtained from the same part of leaves in all treatments.





(A) **(B)**

Results

1. In vitro plant polyploidization

The growth of seedlings under the effect of colchicine was assessed a month after treatment. All of the control seedlings and the seedlings resulted from treatment with different concentrations of colchicine for 24 H, survived. Not all of the seedlings treated for 48 H grew and just some in lower concentrations. It indicates that the 24 H colchicine treatments used were enough to induce cell polyploidization and did not have serious toxic effects, while 48 H treatments had some toxic effects and reduced the growth rate.

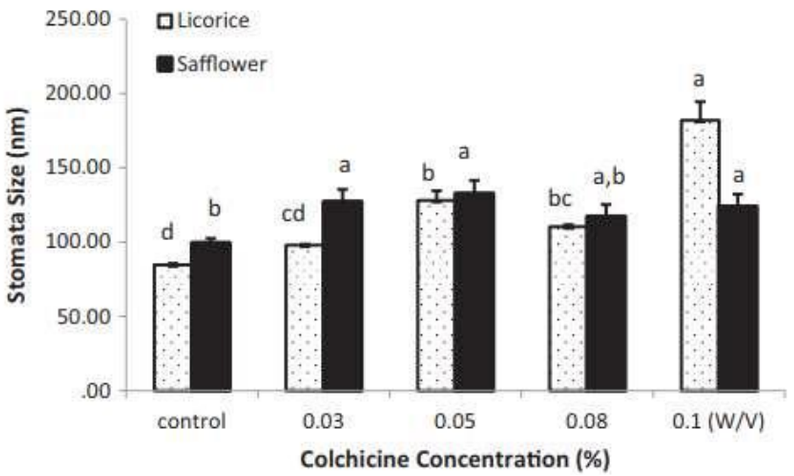
The morphological features of treated plantlets such as root shoot and leaf thickness were found to be increased, while their length was decreased. Also, treated plants' growth was slower than the control's especially in plantlets that resulted from 48 H of treatment in which only cotyledons and a few leaves emerged. So for the rest of the experiments for which we needed a large amount of tissues we used only plantlets that were treated for 24 H. These morphological changes were used for selecting putative polyploids. There was an inverse relationship between survival of plantlets and colchicine concentration and it is in agreement with similar reported result in ex vitro and in vitro polyploidization using other plants. These morphological changes were reported in similar studies. The slower rate in growth may be the result of physiological disturbance induced by colchicine, which causes a reduction in cell division rate. The growth of both control and treated plantlets was equally well in subculture, and it shows that colchicine only causes an initial reduction of growth.

2. Stomata Size

Length of the stomata in putative morphologically polyploidy plants was measured. We selected robust and darker green plants that were all from the group which was treated with different concentrations of colchicine for 24 H. The results suggested that treated plants both in licorice and safflower have a fair amount of larger stomata. In licorice length of the stomata in treated plants was larger significantly in plantlets treated with 0.05% and 0.1% of colchicine for 24 H with

average length of 128.01 and 181.86 nm respectively compared with 84.7 nm in control plantlets. In Carthamus average length of the stomata in plantlets treated with 0.03%, 0.05%, 0.1% of colchicine was 127.17, 132.67 and 123.83 nm respectively in which all were significantly larger in comparison with the control group with an average length of 99.44 nm. Stomata length as a measure of stomata size can be an indicator of ploidy levels and it has been used in different plant types for determining ploidy levels.

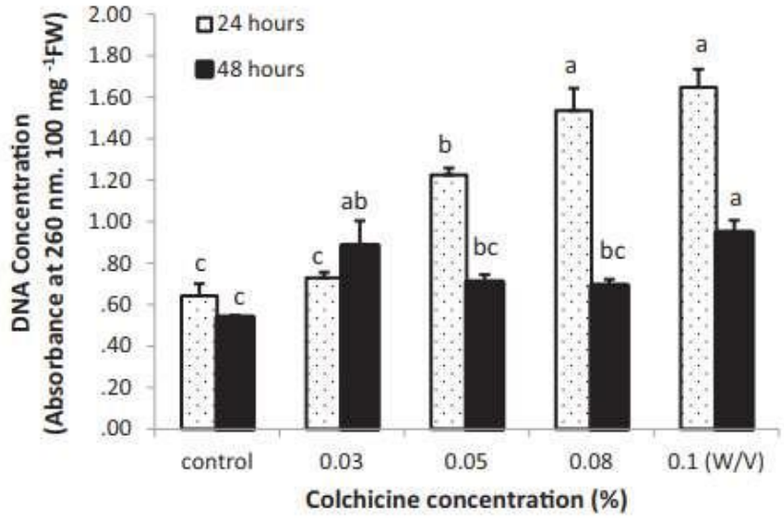
Figure below shows the effect of colchicine concentration on length of the stomata in licorice and safflower plants.



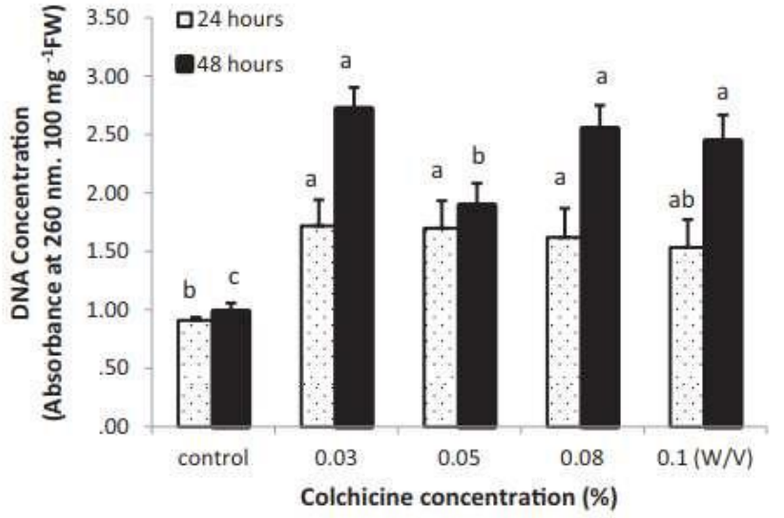
3. DNA Content

In this study, average concentrations of colchicine had the best effect on increasing the total DNA content of treated licorice plantlets. Optical density of total DNA content of licorice plantlets that were treated with 0.05% and 0.1% of colchicine for 24 h and 0.1% and 0.03% of colchicine for 48 H found to be almost doubled that confirms tetraploids. In safflower total DNA content of all treated plantlets for 24 H was almost twice more than that of control groups representing tetraploids and in 48 H treatments sometimes (in 0.03%, 0.08% and 0.1% of colchicine) three times more than that of control groups representing hexaploids. Total DNA content of plants increases as the result of chromosome doubling that happens in polyploidization. This can be used as an indicator for ploidy determination, so we extracted DNA and then measured its optical density in treated plants and compared it with the control group using a spectrophotometer. This method is easy and fast and reduces the time to determine ploidy of in vitro regenerated plants.

Figure below shows absorbance of extracted DNA content of regenerated seedlings of Licorice and safflower treated for (A) 24 and (B) 48 H with 0%, 0.03%, 0.05%, 0.08% and 0.1% of colchicine.



(A)



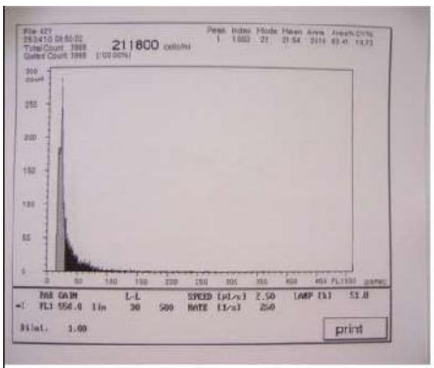
(B)

4. Flow Cytometric Analysis

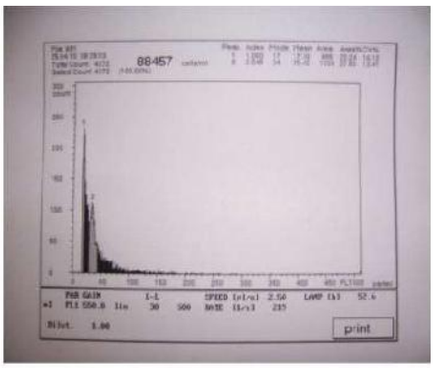
The putative polyploid plants were selected on the basis of morphology, stomata length and total DNA content for analyzing by flow cytometry. Results of flow cytometry in putative polyploid plants in licorice confirmed the previously mentioned results and suggested tetraploids in plantlets obtained from treatment with 0.08% of Colchicine and mixoploids in plantlets obtained from treatment with 0.1% of Colchicine. Figure below shows the histogram obtained by flow cytometry of a control plantlet treated with 0% colchicine and 2 treated plantlets treated with 0.08% and 0.1% of colchicine. In plantlets treated with 0.1% of colchicine mixture of diploid and tetraploid tissues was observed. Peak '1', with a mean relative

DNA content of 17.3, corresponds to the control cells, whereas peak '2', with mean relative DNA content of 35.4 corresponds to the tetraploid cells. In plantlets treated with 0.08% of colchicine a peak with mean relative DNA of 51.9 was observed which corresponds to tetraploids cells. In safflower the peak with mean relative DNA of 24.51 observed in control plantlets analysis corresponds to diploid cells. Figure below shows the histogram obtained by flow cytometry analysis of treated plantlets in which a peak with mean relative DNA of 49.26 was observed that corresponds to tetraploids cells. Flow cytometry is very accurate and powerful for estimating plant nuclear DNA content as it measures florescence intensity of stained nuclei sensitively within seconds.

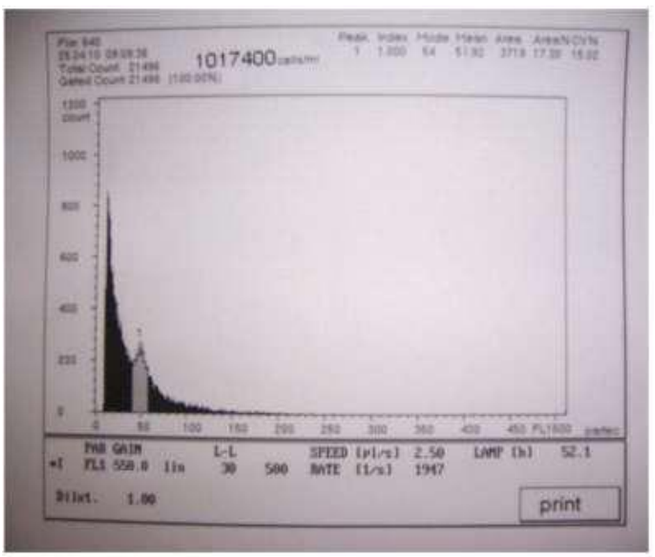
Figure below shows DNA-histograms of nuclei isolated from leaves of (1) Licorice (A) a diploid, (B) mixoploid and (C) tetraploid. (2) Safflower (A) a diploid, (B) tetraploid.



(A)

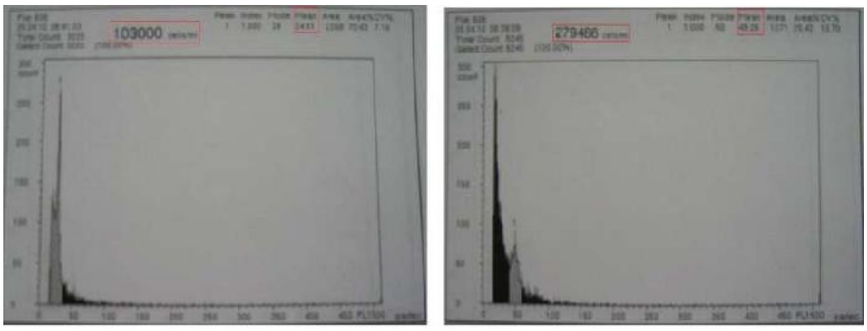


(B)



(C)

(1)



(A) (B)

(2)

Conclusions

In this study, efficient concentrations of colchicine for tetraploid licorice and safflower generation in in vitro were introduced. Measuring optical density of total DNA content makes it possible to easily recognize putative tetraploids. Also Tetraploids were morphologically thicker but shorter than controls and their growth was slower and they have larger stomata than the diploid plants. Nuclear DNA measurement via flow cytometry confirmed the above mentioned results. Using different methods we conclude that all methods can be efficient in determining ploidy levels, although flow cytometry is easier, faster and more accurate.

Experiment 7: Studying Pea Plant as Tool for Investigating Laws of Inheritance

Aim/Objective

To study pea plant for investigating the Gregor Mendel's Laws of Inheritance.

Principle

Gregor Mendel used around 29,000 pea plants to conduct hybridization experiments. Peas were an ideal choice for Mendel to do his experiments because they had easily observable traits and there were 7 of which he could manipulate. He started his experiments on peas with two initial conditions. The conditions were:

1. Possess constant differentiating characteristics.
2. Hybrids of such plants, during flowering period, be protected from the influence of all other foreign pollen.

The second condition was used to protect the plants from an accidental impregnation thus would cause misleading results.

Mendel planned to selectively cross pollinate the peas plants with one another to study their traits passed on and the results from each pollination. During his experiment he initially acquired about 34 varieties of peas and chose 22 different

types to conduct his experiments with which varied in colour and size. He took several years for breeding of constant family lines to perfect the original constant traits. Mendel used the following seven traits of pea plant in his experiments:

1. **Flower Colour** (Purple or White).
2. **Flower Position** (Axil or Terminal).
3. **Stem Length** (Long or Short).
4. **Seed Shape** (Round or Wrinkled).
5. **Seed Colour** (Yellow or Green).
6. **Pod Shape** (Inflated or Constricted).
7. **Pod Colour** (Yellow or Green).

The first generation of the hybrids were produced in a 3:1 ratio where there were 3 plants showing dominant traits and 1 showing recessive. The second generation produced a 2:1:1 ratio. This result showed there was one with the recessive trait, two with hybrid trait and one with dominant trait.

When crossing a green pod plant and a yellow pod plant, the first generation (F1) produced only green plants, given green was the dominant trait colour. But then the second generation (F2) produced a quarter yellow pea pods. These experiments allowed Mendel to conclude on two laws of 'Inheritance' - the 'Law of Segregation' and the 'Law of Independent Assortment'.

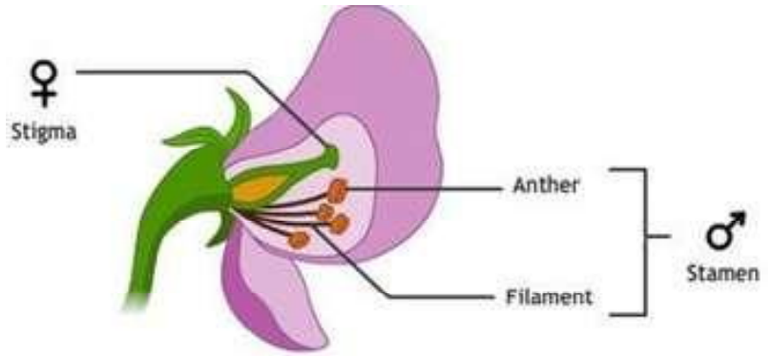
Materials Required

Mendel's chart on inheritance.

Procedure

Study the Mendel's theories and traits on inheritance and write the findings in your practical notebook.

Mendel studied inheritance in peas (*Pisum sativum*). Pea flowers contain both male and female parts, called stamen and stigma, and are usually self-pollinated, as shown below in the given figure. Self-pollination happens before the flowers open, so progeny are produced from a single plant.



Peas can also be cross-pollinated by hand, simply by opening the flower buds to remove their pollen-producing stamen (and prevent self-pollination) and dusting pollen from one plant onto the stigma of another.

Traits in Pea Plants

Mendel followed the inheritance of 7 traits in pea plants, and each trait had 2 forms. He identified pure-breeding pea plants that consistently showed 1 form of a trait after generations of self-pollination.

Pea Traits Studied By Mendel

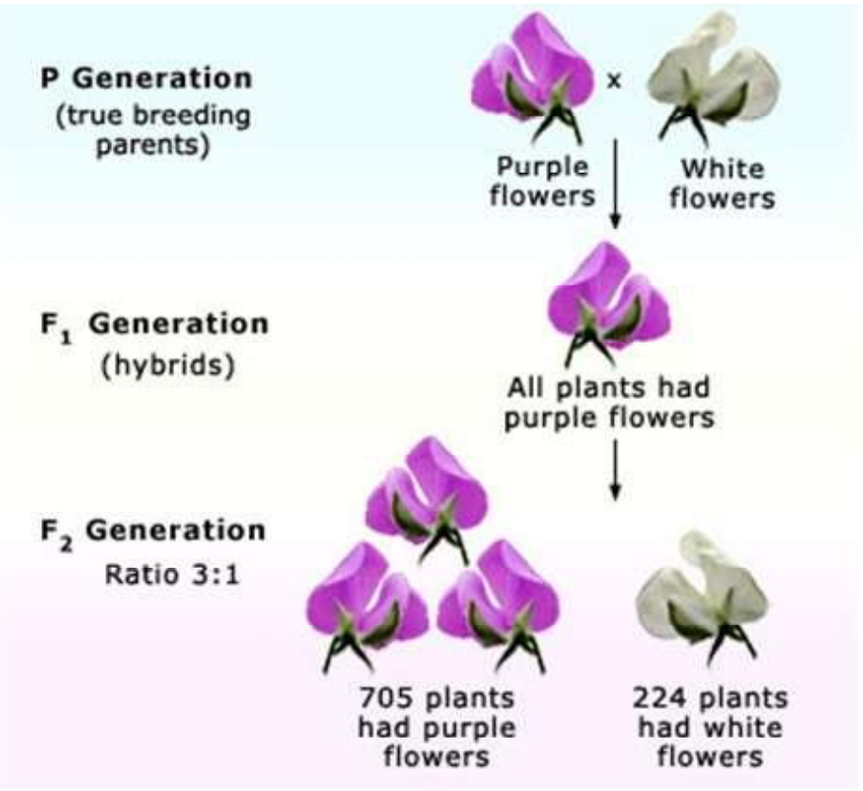
Mendel cross-bred peas with 7 pairs of pure-bred traits. First-generation (F1) progeny only showed the dominant traits, but recessive traits reappeared in the self-pollinated second-generation (F2) plants in a 3:1 ratio of dominant to recessive traits.

Mendel then crossed these pure-breeding lines of plants and recorded the traits of the hybrid progeny. He found that all of the first-generation (F1) hybrids looked like 1 of the parent plants. For example, all the progeny of a purple and white flower cross were purple (not pink, as blending would have predicted). However, when he allowed the hybrid plants to self-pollinate, the hidden traits would reappear in the second-generation (F2) hybrid plants.

Pea trait	Dominant trait		Recessive trait		Numbers in second generation (F2)	Ratio
Seeds						
Seed shape	Round		Wrinkled		5474:1850	2.96:1
Seed colour	Yellow		Green		6002:2001	2.99:1
Whole plants						
Flower colour	Purple		White		705:224	3.15:1
Flower position	Axial		Terminal		651:207	3.14:1
Plant height	Tall		Short		787:277	2.84:1
Pod shape	Inflated		Constricted		882:299	2.95:1
Pod colour	Green		Yellow		428:152	2.82:1

Dominant and Recessive Traits

Mendel described each of the trait variants as dominant or recessive, as shown below in the given figure. Dominant traits, like purple flower colour, appeared in the F1 hybrids, whereas recessive traits, like white flower colour, did not. Mendel did thousands of cross-breeding experiments. His key finding was that there were 3 times as many dominant as recessive traits in F2 pea plants (3:1 ratio), as shown in the figure given below.



Inheriting Traits in Peas

Mendel crossed pure lines of pea plants. Dominant traits, like purple flower colour, appeared in the first-generation hybrids (F1), whereas recessive traits, like white flower colour, were masked. However, recessive traits reappeared in second-generation (F2) pea plants in a ratio of 3:1 (dominant to recessive).

Traits are Inherited Independently

Mendel also experimented to see what would happen if plants with 2 or more pure-bred traits were cross-bred. He found that each trait was inherited independently of the other and produced its own 3:1 ratio. This is the principle of independent assortment.

Experiment 8: Demonstration of the Principle/Law of Segregation, Mendel's 'First Law'

Aim/Objective

To demonstrate the Principle/Law of Segregation - Mendel's 'First Law'.

Materials Required

Mendel's chart on inheritance.

Procedure

Study the Mendel's theories and traits on inheritance and write the findings in your practical notebook.

Principle

Mendel concluded his thoughts on this law after finding when breeding white and purple coloured flowered plants. The result of the experiment is not the mixture of these two colours, but really one colour was chosen over the other. It means one colour is dominant over the other. Following are the four different parts of the law that he included:

1. There are other forms of genes that can decide the heritable traits, are known as alleles.
2. Each offspring receives one allele from each parent.
3. Either a sperm or egg holds only one allele for each trait and those pair up during fertilization.
4. If the alleles are different, one is seen and the other is not, leading one trait is dominant and the other is recessive.

When he collected and planted the seeds produced in this cross, Mendel found that 100 percent of the plants in the next generation or the F1 generation had purple flowers.

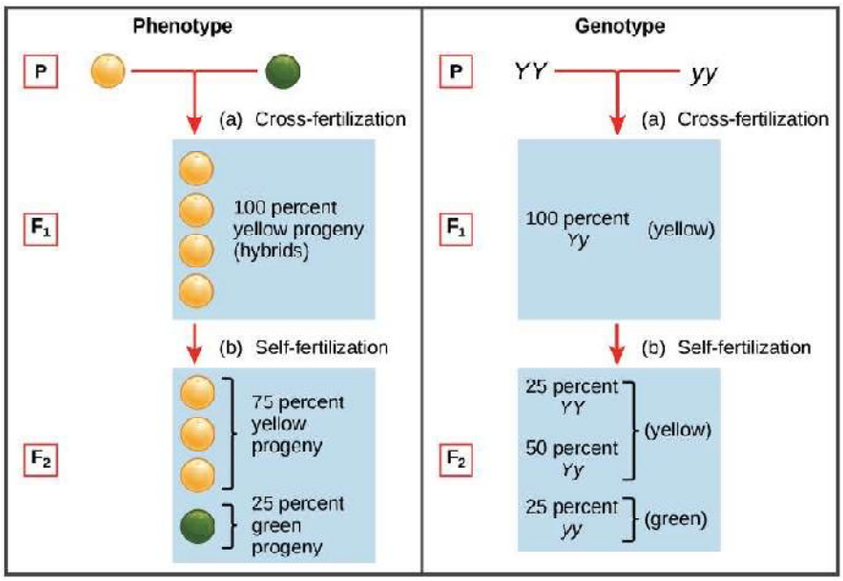
Conventionally that time, it would have predicted that the hybrid flowers should whitish purple that means the parents' traits should blend in the offspring. Instead, Mendel's experimental results showed that the white flower trait had completely disappeared. He termed the trait that was visible in the F1 generation (purple flowers) the **dominant trait**, and the trait that was hidden or lost (white flowers) the **recessive trait**.

Mendel did not stop his experimentation there. Instead, he let the F1 plants self-fertilize. Due to this cross, the produced offspring called F2 generation. He found that 705 plants had purple flowers and 224 had white flowers. Therefore the ratio was 3.15 purple flowers to one white flower, or approximately 3:1.

This 3:13:1 ratio was no fluke. Mendel also examined all the other characteristics in the same way and found same results in F1 and F2 generations as he found for flower colour.

Based on his results (including that magic 3:1 ratio), Mendel came up with a complete concept for the inheritance of individual characteristics, such as flower colour.

He concluded that parents pass along 'heritable factors', which we now call **genes** that determine the traits of the offspring. Each individual has two copies of a given gene, such as the gene for seed colour (*Y* gene) shown below. If these copies represent different versions of the gene, or **alleles**, one allele—the **dominant** one—may hide the other allele—the **recessive** one. For seed colour, the dominant yellow allele *Y* hides the recessive green allele *y*.

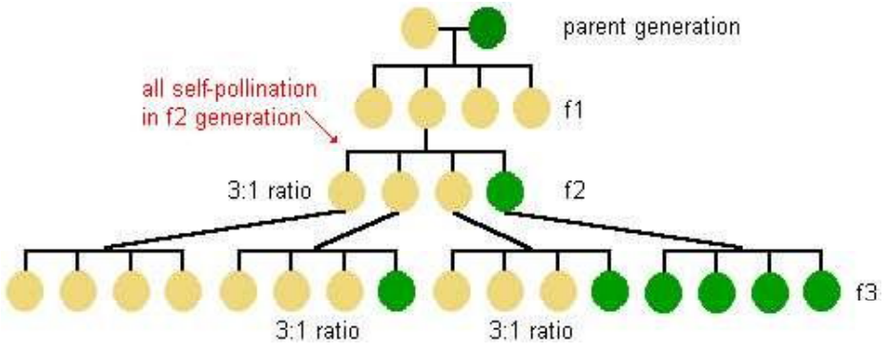


In the above figure the graphic representation illustrates the first column with the heading 'Phenotype' and the second one with the heading 'Genotype'. In the phenotype column, one yellow pea plant cross-fertilizes with one green pea plant. The first generation of offspring is 100 percent yellow pea plants. After self-fertilization of these yellow pea offspring, 75 percent of the second generation offspring have yellow peas and 25 percent have green peas. The genotype column shows the first generation offspring as 100 percent Yy, and the second generation as 25 percent YY, 50 percent Yy, and 25 percent yy.

The set of alleles carried by an organism is known as its **genotype**. Genotype determines **phenotype**, an organism's observable features. When an organism has two copies of the same allele (say, *YY* or *yy*), it is said to be **homozygous** for that gene. If, instead, it has two different copies (like *Yy*), we can say it is **heterozygous**. Phenotype can also be affected by the environment in many real life cases, though this did not have an impact on Mendel's work.

Observations/Results

The Principle/Law of Segregation, Mendel's 'First Law' was based on a direct result from watching the production of the F2 generation and the production of the 2:1:1 ratio. The second generation produces a 2:1:1 ratio. This showed there was one with the recessive trait, two with hybrid trait and one with dominant trait. In the second generation, after plants were allowed to self-fertilize (pollinate themselves), the hidden form of the trait reappeared in a minority of the plants. Specifically, there were always about 333 plants that showed the dominant trait (for example, tall) for every 111 plant that showed the recessive trait (for example, short), making a 3:1 ratio. The recessive traits only came when those were the only two being bred with each other.



On the basis of your observation, draw the Mendel's chart in your practical notebook showing both F1 generation and F2 generation.

Experiment 9: The Principle/Law of Independent Assortment - Mendel's 'Second Law'

Aim/Objective

To demonstrate the Principle/Law of Independent Assortment - Mendel's 'Second Law'.

Materials Required

Mendel's chart on inheritance.

Procedure

Study the Mendel's theories and traits on inheritance and write the findings in your practical notebook.

Principle

From the law of segregation, we can predict how a single feature associated with a single gene is inherited. In some cases, though, we might want to predict the inheritance of two characteristics associated with two different genes. To know about this, we need to understand whether the two genes are inherited independently

or not. We need to know that whether they 'ignore' one another when they are sorted into gametes, or whether they 'come together' and get inherited as a unit.

Gregor Mendel concluded that different genes were inherited independently of one another, following what's called the **law of independent assortment**. Mendel's **law of independent assortment** explains that the alleles of two (or more) different genes get sorted into gametes independently of one another. In other words, the allele a gamete receives for one gene does not influence the allele received for another gene.

Pea Colour and Pea Shape - Genes

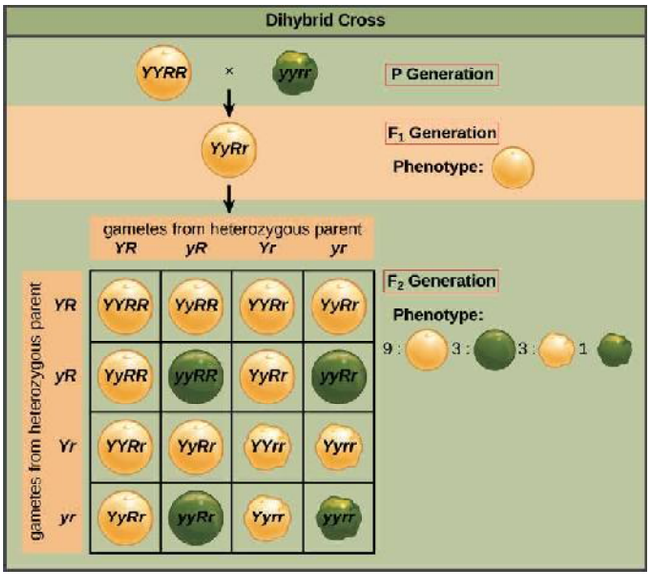
Consider an example of the law of independent assortment. If we cross two pure-breeding pea plants: one with yellow, round seeds (*YYRR*) and one with green, wrinkled seeds (*yyrr*). Because each parent is homozygous, so according to the law of segregation the gametes made by the wrinkled, green plant all are *ry*, and the gametes made by the round, yellow plant are all *RY*. That gives F1 offspring that are all *RrYy*.

The allele specifying yellow seed colour is dominant to the allele specifying green seed colour, and the allele specifying round shape is dominant to the allele specifying wrinkled shape, as shown by the capital and lower-case letters. This means that all the F1 plants are yellow and round. Because they are heterozygous for two genes, the F1 plants are called **dihybrids** (*di-* = two, *-hybrid* = heterozygous).

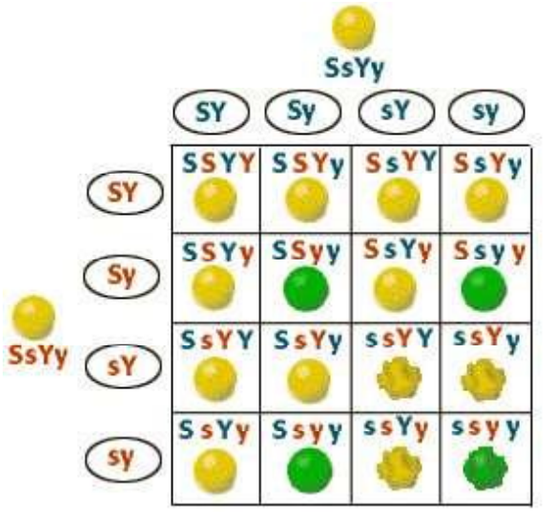
A cross between two dihybrids or equivalently self-fertilization of a dihybrid is known as a **dihybrid cross**. When Mendel did this cross and looked at the offspring, he found that there were four different categories of pea seeds: yellow and round, yellow and wrinkled, green and round, and green and wrinkled. These **phenotypic** categories appeared in a ratio of approximately 9:3:3:1.

This ratio was the key clue that led Mendel to the law of independent assortment. That is because a 9:3:3:1 ratio is exactly what is expected to see if the F1 plant made four types of gametes (sperm and eggs) with equal frequency: *YR*, *Yr*, *yR*, and *yr*. In other words, this is the result that predict if each gamete randomly got a *Y* or *y* allele and in a separate process, also randomly got an *R* or *r* allele (making four equally probable combinations).

Now, the link can be confirmed between the four types of gametes and the 9:3:3:1 ratio using the Punnett square as shown in the figure given below.



Mendel decided different pairs of alleles are passed on as individuals and not based upon each other. Mendel saw various combinations, which indicated all of the alleles are segregated from one another. When Mendel began mixing two traits and conducting dihybrid crosses he found a 9:3:3:1 ratio. Unless the traits are linked he concluded various traits are inherited independently and have no relation. The figure given below illustrates the Mendel's different pairs of allele's combination.



Observations/Results

On the basis of your observation, draw the following in your practical notebook.

1. Four types of gametes and the 9:3:3:1 ratio using the Punnett square.
2. Mendel's different pairs of allele's combination.

Experiment 10: Studying Deviations from Mendel's Law and Applying Statistics

Aim/Objective

To provide an introduction to basic concepts and techniques commonly used to study genetics.

Principle

The basic principles of Gregor Mendel's model of inheritance have held up for over a century. They can easily explain how many different characteristics are inherited in next generation, in a wide range of organisms including human beings.

Some of the key elements of Mendel's original model were:

1. Heritable traits are determined by various heritable factors, now called **genes**. Genes come in pairs, that is, are present in two copies in an organism.
2. Genes come in different versions, now called **alleles**. When an organism has two different alleles of a gene, one known as dominant allele will hide the presence of the other known as recessive allele and determine appearance.
3. During gamete production, each egg or sperm cell receives just one of the two gene copies present in the organism, and the copy allocated to each gamete is random (Law of Segregation).
4. Genes for different traits are inherited independently of one another (Law of Independent Assortment).

These rules still establish the base of our understanding of inheritance, that is, how traits are passed on and how an organism's **genotype** (set of alleles) determines its **phenotype** (observable features). Now you know of some exceptions, extensions, and variations, which must be added to the model in order to fully explain the inheritance patterns that you see around.

Probability, Statistics, and Measurements

The aim of this laboratory is to provide an introduction to basic concepts and techniques commonly used to study genetics. This is devoted to the concept of probability and how we can use basic theory of probability to understand genetic concepts and crosses. Genetic crosses are commonly used to study patterns of inheritance of traits. A trait, or a character, is generally any observable phenotypic characteristic of an organism, such as eye colour, skin colour, height, etc. Gregor Mendel, the father of genetics, performed many genetic crosses to quantify patterns of inheritance in pea plants. Based on the results of his experiments he came up with three laws:

Law 1: Law of Segregation

Alleles in diploid individuals separate during the process of gamete formation (meiosis).

It is the point to remember that a diploid cell contains two sets of chromosomes, one from the father and one from the mother. So, each gene will contain two alleles. The alleles can either be the same or homozygous and different or heterozygous. For example, if we predict that pea pod colour (green versus yellow) is controlled by a single gene with two alleles (R and r), RR and rr would represent homozygotes and Rr would indicate a heterozygote. During the formation of gamete, only one of the two alleles will be passed on to the sperm or egg. In other words, the two alleles segregate from one another.

Law 2: Law of Independent Assortment

Different genes randomly sort their alleles during the process of gamete formation (meiosis).

For example, going back to Mendel's experiments with pea plants, suppose that you are working with two genes, Gene 1 and Gene 2. Gene 1 controls pea pod colour and consists of two alleles (R = Green, r = Yellow). Assume that the R allele is dominant, meaning that RR and Rr genotypes produce green pods and rr genotypes produce yellow pods. Now assume that Gene 2 controls seed pod shape and also contains two alleles (Y = Constricted, y = Round). Assume that Y is dominant over y, such that YY and Yy genotypes produce constricted pods and yy genotypes produce round pods. Mendel's Law of Independent Assortment states that the alleles at these different genes will sort independently of one another during gamete formation. In other words, the R allele will not always be associated with the Y allele and the r allele will not always be associated with the y allele in each sperm or egg cell. All combinations of alleles are possible.

Law 3: Law of Dominance

According to this law, a heterozygous individual will express the phenotypic characteristics of the dominant allele.

For example, in the green versus yellow plant experiment, it is observed that the green allele (R) is dominant to the yellow allele (r) because both RR and Rr plants demonstrate the green phenotype.

According to Gregor Mendel and genetic crosses, it is must to understand the basic probability theory and how it can be used to predict the likelihood of particular outcomes.

Part 1: Probability and Statistics

Probability can be defined as the chance that any particular outcome will occur. For example, what is the probability of tossing a coin and obtaining heads? The answer would be ½ or 50%. Thus,

$$\text{Probability} = \frac{\text{Number of times a particular event occur}}{\text{Total number of possible outcomes}}$$

The probability would be observed of flipping a heads on one try. Thus, the Numerator = 1 (number of times a particular event will occur) and the Denominator = 2 (there are only two possible outcomes, heads or tails).

Try another example yourself and find out that what would be the probability of drawing a black card from a deck of cards on one try? What would be the probability of drawing the King of Hearts from a deck of cards on the first try?

Note down the observations and find out the possible probability.

A Random Sampling Error

While calculating probabilities, random sampling error can cause deviations from predicted probabilities. For example, if you tossed a coin six times you would predict that 50% of the tosses would be heads and 50% would be tails. However, it would also be possible that you tossed heads twice and tails four times, leading to a high random sampling error and a deviation from the expected value of 50%. Conversely, if you tossed the same coin 1000 times it is highly likely that the number of heads and tails would be closer to 50%.

Chi-Square Test

The topic of gene interaction includes a sometimes confusing array of different phenotypic ratios. A hypothesis is devised to explain the observed ratio. The next step is to determine whether the observed data are compatible with the expectations of the hypothesis. A statistical procedure called the χ^2 (Chi-square) test is used to help in making the decision to hold onto or reject the hypothesis.

The χ^2 test is simply a way of quantifying the various deviations expected by chance if a hypothesis is true. For example, consider a simple hypothesis that a certain plant is a heterozygote (monohybrid) of genotype A/a. To test this hypothesis, we would make a testcross to a/a and predict a 1:1 ratio of A/a and a/a in the progeny. Even if the hypothesis is true, we do not always expect an exact 1:1 ratio.

Experiment Example on the Gene Interaction

Consider the following example that specifies the gene interaction. In this, the two pure lines of plants are crossed, one with yellow petals and one with red petals.

The F1 generation are all orange. When the F1 generation is self-pollinated to give an F2 generation, the following result is obtained:

Orange	182
Yellow	61
Red	77
Total	320

What hypothesis can explain the results?

There are at least following two possibilities:

Hypothesis 1. Incomplete Dominance

F_1 (yellow) *G1/G1* × *G2/G2* (red)
G1/G2 (orange)

			Expected numbers
F_2	$\frac{1}{4}$ *G1/G1*	(yellow)	80
	$\frac{1}{2}$ *G1/G2*	(orange)	160
	$\frac{1}{4}$ *G2/G2*	(red)	80

Hypothesis 2. Recessive Epistasis of r (Red) on Y (orange) and y (Yellow)

F_1 (yellow) *y/y ; R/R* × *Y/Y ; r/r* (red)
Y/y ; R/r (orange)

			Expected numbers
F_2	$\frac{9}{16}$ *Y/– ; R/–*	(orange)	180
	$\frac{3}{16}$ *y/y ; R/–*	(yellow)	60
	$\frac{3}{16}$ *Y/– ; r/r*	(red)	80
	$\frac{1}{16}$ *y/y ; r/r*	(red)	

The statistic χ^2 is always calculated from actual numbers, not from percentages, proportions, or fractions. Sample size is therefore very important in the χ^2 test, as it is in most considerations of chance phenomena. Samples to be tested generally consist of several classes. The letter *O* is used to represent the observed number in a class, and *E* represents the expected number for the same class based on the predictions of the hypothesis. The general formula for calculating χ^2 is as follows:

Sum of $(O - E)^2/E$ for all classes

For hypothesis 1, the calculation is as follows:

	O	E	$(O - E)^2$	$(O - E)^2/E$
orange	182	160	484	3.0
yellow	61	80	361	4.5
red	77	80	9	0.1
				$\chi^2 = 7.6$

To convert the χ^2 value into a probability, we use Table on Critical Values of the $\chi 2$ Distribution for calculating Degrees of Freedom is given below, which shows χ^2 values for different degrees of freedom (df).

Table: Critical Values of the $\chi 2$ Distribution for Calculating Degrees of Freedom

					P					
df	**0.995**	**0.975**	**0.9**	**0.5**	**0.1**	**0.05**	**0.025**	**0.01**	**0.005**	**df**
1	.000	.000	0.016	0.455	2.706	3.841	5.024	6.635	7.879	1
2	0.010	0.051	0.211	1.386	4.605	5.991	7.378	9.210	10.597	2
3	0.072	0.216	0.584	2.366	6.251	7.815	9.348	11.345	12.838	3
4	0.207	0.484	1.064	3.357	7.779	9.488	11.143	13.277	14.860	4
5	0.412	0.831	1.610	4.351	9.236	11.070	12.832	15.086	16.750	5
6	0.676	1.237	2.204	5.348	10.645	12.592	14.449	16.812	18.548	6
7	0.989	1.690	2.833	6.346	12.017	14.067	16.013	18.475	20.278	7
8	1.344	2.180	3.490	7.344	13.362	15.507	17.535	20.090	21.955	8
9	1.735	2.700	4.168	8.343	14.684	16.919	19.023	21.666	23.589	9
10	2.156	3.247	4.865	9.342	15.987	18.307	20.483	23.209	25.188	10
11	2.603	3.816	5.578	10.341	17.275	19.675	21.920	24.725	26.757	11
12	3.074	4.404	6.304	11.340	18.549	21.026	23.337	26.217	28.300	12
13	3.565	5.009	7.042	12.340	19.812	22.362	24.736	27.688	29.819	13
14	4.075	5.629	7.790	13.339	21.064	23.685	26.119	29.141	31.319	14
15	4.601	6.262	8.547	14.339	22.307	24.996	27.488	30.578	32.801	15

For any total number of progeny, if the number of individuals in two of the three phenotypic classes is known, then the size of the third class is automatically determined. Hence, there are only *2 degrees of freedom* in the distribution of

individuals among the three classes. Generally, the number of degrees of freedom is the number of classes minus 1. In this case, it is 3 – 1 = 2. Looking along the 2-df line, we find that the χ^2 value places the probability at less than 0.025, or 2.5 percent. This means that, if the hypothesis is true, then deviations from expectations this large or larger are expected approximately 2.5 percent of the time. When values of less than 5 percent are obtained, the hypothesis is rejected as being too unlikely. Hence the incomplete dominance hypothesis must be rejected.

Experiment 11: Studying *Drosophila* as Model Organism

Aim/Objective

Studying *Drosophila* as a model organism.

Principle

Initially, a wide variety of animals have been used for animal testing including mice, flies, and monkeys. *Drosophila melanogaster (D. melanogaster),* a type of fruit fly is particularly known as a model organism. This fly belongs to the main invertebrate model used to study developmental genetics. It has now been used for over a century.

Reasons to Select *Drosophila melanogaster* as a Model Organism

Many different characteristics of *Drosophila melanogaster* make it an ideal model organism. The major benefit of using *Drosophila melanogaster* is that there are also no ethical issues surrounding their use, which is very common for using mammal models. The various reasons for selection are as follows:

Short Lifespan

One of the main advantage of selecting *Drosophila melanogaster,* is its short life cycle, which allows large quantity of flies to be produced within a short period.

An embryo emerges within 24 hours of egg fertilization. The embryo then goes through three different larval stages, eventually maturing into an adult *Drosophila melanogaster*. Development of an adult fly only takes 10 days after fertilization.

The female fly can produce up to 1500 eggs in its lifetime thereby providing a constant supply of new *Drosophila melanogaster* for further genetic studies.

Minimal Culturing Requirements

Another advantage of using *Drosophila melanogaster* is that they are very small and thus, very easy to maintain. Due to their small size and minimal requirements, many *Drosophila melanogaster* can be produced and tested within a small laboratory which does not have access time, space or funding.

Genetic Manipulation

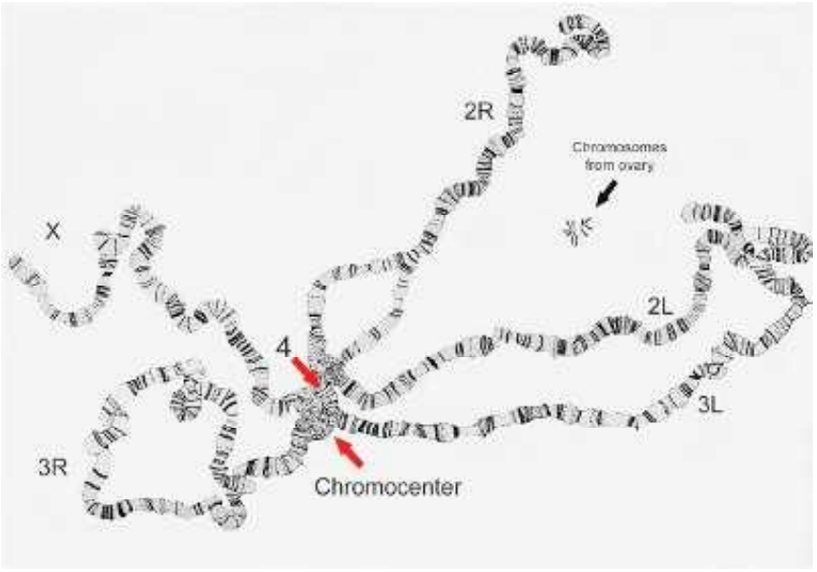
The genetic factors also make this fly an ideal model organism. *Drosophila melanogaster* has only four pairs of chromosomes as compared to 23 pairs of chromosomes in humans. This genetic simplicity was one of the reasons why they were first used in genetic studies; *Drosophila melanogaster* genes could be mapped easily to investigate genetic transmission.

The entire genome of *Drosophila melanogaster* has been sequenced and annotated as the human genome. Comparatively, the fly's genome is much smaller at 5% of its size.

However, if comparing the number of genes present in the genomes, 15,500 genes are present in the flies as compared to 2,200 genes in humans. About 60% of the *Drosophila melanogaster* genes are the same, as they originate from a common ancestor. Many of these shared genes are associated with cancer and other diseases, enabling investigation into qualities, such as inheritance.

Polytene Chromosomes

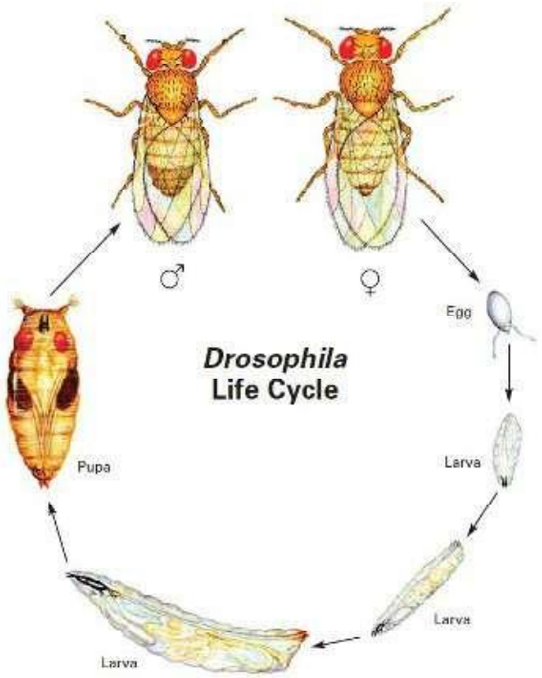
The giant chromosome found in the fly's salivary glands is known as polytene chromosomes (compared here with the chromosomes from the fly's ovary), as shown in the figure given below. This is another important characteristic that makes the *Drosophila melanogaster* fruit fly an important organism for laboratory studies. These easily visualized chromosomes provided a road map for early geneticists.



Anatomical Features

Drosophila melanogaster has some anatomical features (such as, wings and eyes) that allow for easy characterization. These genetic markers can be easily identified under a microscope.

Behaviours, such as eating, mating, and sleeping that are observed in humans are also seen in *Drosophila melanogaster*. Therefore, the possible effect of genetics upon human behaviour can also be assessed.



Structure and Organization of the *Drosophila melanogaster* Genome

The genome sequence of *Drosophila melanogaster* was published in the journal Science in March 2000.

In terms of base pairs, the fly genome is only around 5% of the size of the human genome, that is, 132 million base pairs for the fly, compared with 3.2 billion base pairs for the human.

The *Drosophila melanogaster* fly has approximately 15,500 genes on its four chromosomes, whereas humans have about 22,000 genes among their 23 chromosomes. Thus the density of genes per chromosome in *Drosophila melanogaster* is higher than for the human genome.

Humans and *Drosophila melanogaster* flies have retained the same genes from their common ancestor (known as homologs) over about 60% of their genome.

Based on an initial comparison, approximately 60% of genes associated with human cancers and other genetic diseases are found in the *Drosophila melanogaster* fly genome.

Observations/Results

After studying *Drosophila melanogaster* find the relationship between *Drosophila melanogaster* fruit fly and human gene. Write the details in your practical notebook.

Experiment 12: Effect of Chemicals on Mutation

Aims/Objectives

1. Know the Various Types of mutations: Base Substitution (Transition, Transversion), Deletion and Insertion.
2. Know the Causes of Mutation: Replication Errors, Environmental Factors:
   - Nitrous Acid and Alkylating Agents, such as Ethylene Oxide
   - UV and X-Ray Radiation
   - Intercalating Agents
   - Crosslinking Agents

Principle

Mutations are changes in the genetic sequence, and they are a main cause of diversity among organisms. These changes occur at many different levels, and they can have widely differing consequences. In biological systems that are capable of reproduction, we must first focus on whether they are heritable; specifically, some mutations affect only the individual that carries them, while others affect all of the carrier organism's offspring, and further descendants.

For mutations to affect an organism's descendants, they must:

- Occur in cells that produce the next generation.
- Affect the hereditary material. Ultimately, the interplay between inherited mutations and environmental pressures generates diversity among species.

Although various types of molecular changes exist, the word 'mutation' typically refers to a change that affects the nucleic acids. In cellular organisms, these nucleic acids are the building blocks of DNA, and in viruses they are the building blocks of either DNA or RNA. One way to think of DNA and RNA is that they are substances that carry the long-term memory of the information required for an organism's reproduction. This article focuses on mutations in DNA, although we should keep in mind that RNA is subject to essentially the same mutation forces.

Types of Mutation

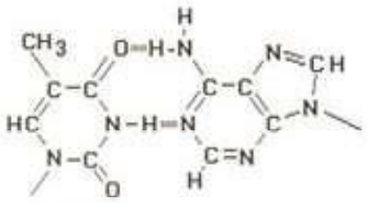
The following table displays schematics illustrating the different types of mutations. The mutated nucleotide in the double-stranded DNA is underlined.

Wild type gene	C G A C T G G C T G A C
Transition (AT pair replaced by GC pair)	C G **G** C T G G C **C** G A C
Transversion (AT pair replaced by TA pair)	C G **T** C T G G C **A** G A C
Insertion (GC pair inserted)	C G A **G** C T G G C T **C** G A C
Deletion (AT pair deleted)	C G C T G G C G A C

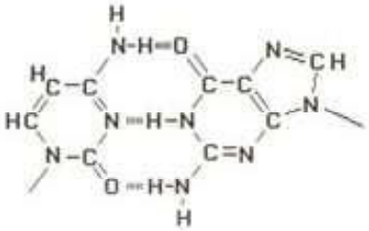
Causes of Mutation

Agents that cause mutations are *mutagens.* We will focus on mutagens which can alter the base-pairing properties.

Normal Base-Pairing



Thymine - Adenine basepair



Cytosine - Guanine basepair

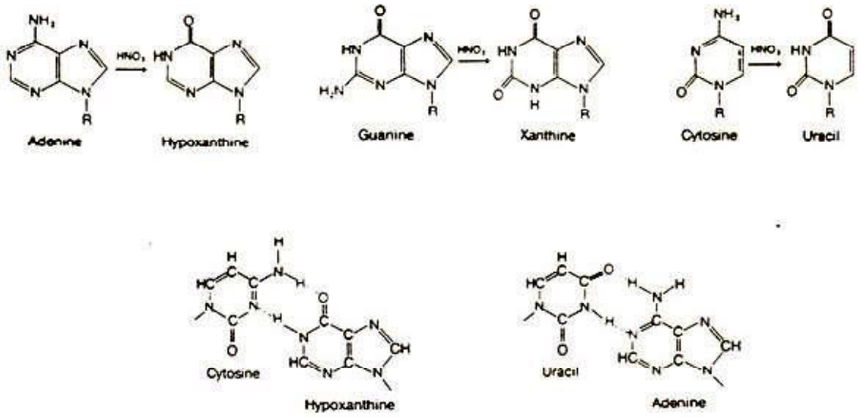
Chemical Agents

Chemical agents that change normal base-pairing can generate mutations. These chemicals can be further classified according to their mechanism of actions.

I. Chemicals that Deaminate Amino Groups

Chemicals that deaminate amino groups on the bases to keto groups, for example, A or Adenine to HypoXanthine (HX), G to Xanthine (X), C to U. The resultant products usually will have a different base-pairing property. HX will base pair with C and U will base pair with A. X has similar base pairing property as G. Deamination can be chemically induced, spontaneous, or Induced by reactive oxygen species that are produced as by-products of oxidative metabolism.

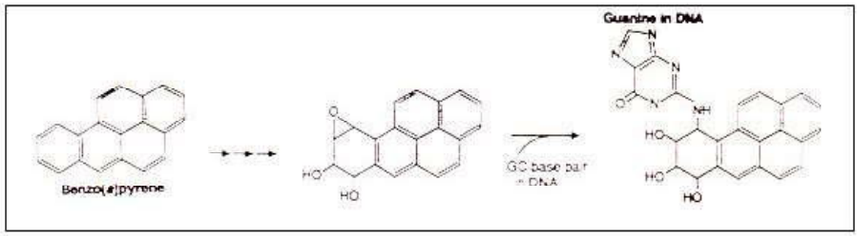
The figure below shows the examples of deamination on the subsequent DNA replication products.



II. Chemicals that Change a Base by Covalent Modifications

The following are some examples.

Alkylating agents, such as ethylene oxide and Benzo[a]pyrene in cigarette smoke or coal tar. Modification of guanine by Benzo[a]pyrene would result in a distortion of the DNA helical structure. Upon replication, the DNA product would contain an insertion.



Reactive oxygen species can modify guanine residues.

Ethylene oxide modifies Guanine (G) residues, leading to the formation of ethylGuanine (eG) which pairs with Thymine (T).

An example of the base sequence of the DNA product following a G -> eG mutation is as follows:

-A-T-T-**G**-A-	-A-T-T-**eG**-A-	-A-T-T-**eG**-A-		-A-T-T-**G**-A-
-T-A-A-**C**-T- →	-T-A-A—C- T- →	-T-A-A—**T**- T-	+	-T-A-A-**C**-T-
		mutant		wild-type

III. Chemicals that Cause Cross-Linking of DNA Strands

Cross-linking of DNA can lead to mutations during subsequent replication or transcription. There are several medical treatments that also take advantage of this phenomenon to stop replication of abnormal or cancer cells.

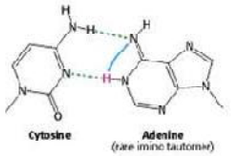
IV. Radiation Damage

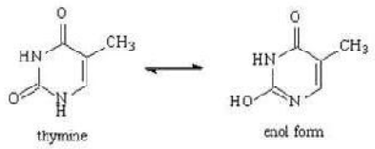
UV and X-ray radiation can result in:

- DNA fragmentation.
- Thymidine dimerization - UV light can cause two adjacent thymidine residues in a DNA molecule to form a covalently linked dimer, such as the structure shown below.
- Shift in the equilibrium of the tautomeric forms of bases. The minor tautomers differ from the major forms in their base-pairing properties.

Adenine (amino form, pairs with thymine)

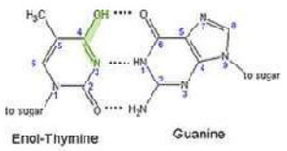
Adenine (imino form, pairs with cytosine)





Thymine (keto form, pairs with adenine)

Thymine (enol form, pairs with guanine)

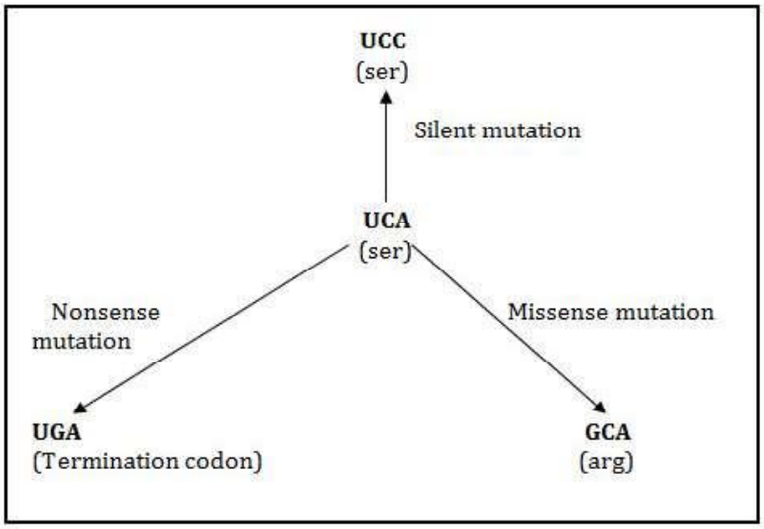


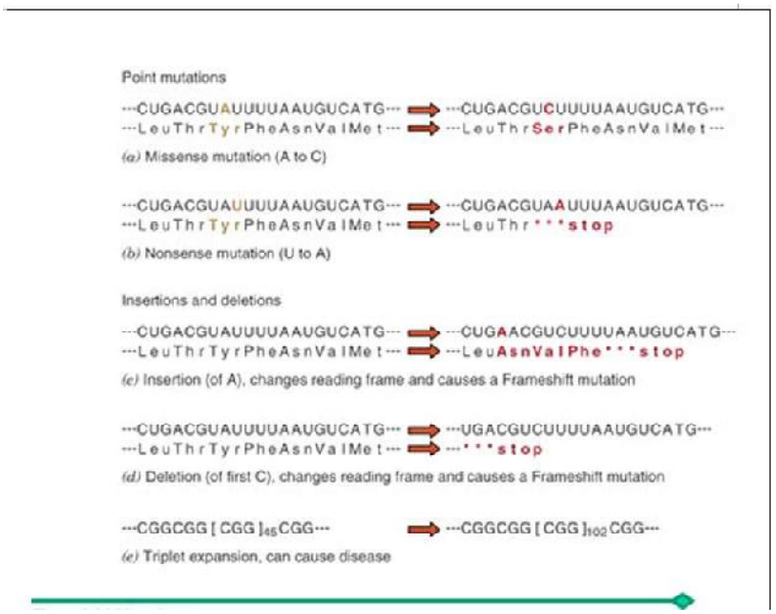
Consequences of Mutations

Mutations of a single nucleotide can result in:

- Silent Mutation
- Missense Mutation
- Nonsense Mutation

The following two diagrams present examples of the different mutations. The amino acid coded by the trinucleotide is shown in brackets below the nucleotide sequence.





Experiment 13: The Effects of Radiation on DNA Mutations

Aim/Objective

To study the effects of radiation on DNA mutations.

Principle

The information controlled by DNA is used by each cell to produce the proteins needed for life, which support the cells of the body and help govern cell behaviour. Mutations related to DNA lead to changes in the proteins the cell produces, which modify the way the cell behaves, and can ultimately lead to diseases such as cancer. Exposure to radiation is considered as a mutagen, it means that it causes mutations

in DNA. This is why exposure to radiation increases the risk of cancer. There are many different forms of radiation, which may differ in the ways by which they lead to mutations in cells.

Effect of UVA Radiation

UVA radiation is released from the sun, and we are exposed to UVA rays as sunlight. UVA rays are leading to indirect DNA mutation. Although the UV rays themselves do not have a direct effect on the DNA, they lead to subsequent changes in the cell that ultimately increase the rate of genetic mutation. UVA radiation usually works by generating molecules called free radicals within the cells. These free radicals are the molecules that are very reactive. They usually interact with the DNA molecules and affect the normal processes that prevent DNA damage, so ultimately, they increase DNA mutation.

Long term exposure to UVA increases an individual's risk for cancer, especially skin cancer. It is already reported that UVA rays penetrate deep into the skin, and prolonged exposure to this type of radiation leads to skin damage and cancer. The skin can be protected by harmful UV A rays by wearing a broad-spectrum sunscreen or sunblock daily. It decreases the rate of DNA mutation, and helps protect against the development of skin cancers.

Effect of UVB Radiation

UV B radiation is also emitted from the sun. UVB radiation is the main cause of sunburn and skin cancer because it directly damages DNA. It works by promoting the development of cross-links between certain chemicals within DNA, which leads to breaks in the strand of DNA.

When the cell repair these DNA breaks, there is an increased chance of mutation due to the possibility of a mistake in the repair. When the cell is exposed to prolonged UVB rays, the millions of resulting DNA breakages result in the development of genetic mutations that can result in cancer. Wearing sunscreen or sunblock protects the skin from UVB radiation.

Effect of Ionizing Radiation

Ionizing radiation is the type of radiation released by radioactive materials. It also contributes to DNA mutation. Like UVB radiation, ionizing radiation also causes direct DNA damage that lead to mutations. Exposure to ionizing radiation leads to break in double-stranded DNA, so both parts of the DNA molecule are broken at the same spot. This type of breakage is repaired by the cell by reattaching the DNA strands together.

If the cell is exposed prolonged to ionizing radiation, double-stranded breaks occur along the entire length of the DNA. Mutations occur if the repair mechanisms re-attach the wrong piece of DNA back together, so that a part of the DNA strand goes missing. This may lead to the deletion of important genes, or a change

in the location of a gene within the DNA. These types of mutations are linked to the development of a number of cancers, including leukemia.

Observation Table:

Name of the test performed	Observation	Interpretation

Experiment 14: Isolation of DNA from Different Sources

Aim/Objective

To study the isolation of DNA from different sources.

Principle

DeoxyriboNucleic Acid (DNA) is a molecule composed of two chains that coil around each other to form a double helix carrying the genetic instructions used in the growth, development, functioning, and reproduction of all known organisms and many viruses. DNA and RiboNucleic Acid (RNA) are nucleic acids; alongside proteins, lipids and complex carbohydrates (polysaccharides), nucleic acids are one of the four major types of macromolecules that are essential for all known forms of life.

DNA is made of chemical building blocks called nucleotides. These building blocks are made of three parts: a phosphate group, a sugar group and one of four types of nitrogen bases. To form a strand of DNA, nucleotides are linked into chains, with the phosphate and sugar groups alternating. The four types of nitrogen bases found in nucleotides are: Adenine (A), Thymine (T), Guanine (G) and Cytosine (C). The order, or sequence, of these bases determines what biological instructions are contained in a strand of DNA. For example, the sequence ATCGTT might instruct for blue eyes, while ATCGCT might instruct for brown.

DNA Extraction: Isolation of nucleic acids is the first step in most of the molecular biology studies. Extraction of nucleic acids from any biological material requires cell lysis, inactivation of cellular nucleases and separation of the desired nucleic acid from cellular debris. The common cell lysis procedures comprise; Mechanical disruption, for example, grinding, hypotonic lysis, Chemical treatment, for example, detergent lysis and Enzymatic digestion, for example, Proteinase K. The extraction medium typically contains an ionic detergent, which is needed to lyse the nuclei and release the DNA. The detergent also inhibits any nuclease activity present in the preparation. Combination of Phenol-chloroform and high concentrations of salt are often used to get rid of contaminants from nucleic acids. After cell lysis and

nuclease inactivation, cellular debris may easily be eliminated by precipitation. Nucleic acids are usually precipitated with isopropanol or ethanol.

Gel Electrophoresis: Electrophoresis is frequently used to determine size and purity of DNA. In the process, nucleic acids may be separated electrophoretically on gel systems according to their shape, size and overall charge density (charge per unit of mass). This separation process is commonly done on horizontal agarose gels. Due to the negatively charged phosphate backbone, nucleic acids usually move towards the anode in the electrical field. In the presence of Ethidium Bromide (EtBr), the separated nucleic acids are clearly visualized under UV light. EtBr intercalates between the two strands of DNA.

Spectrophotometry: The ratio of absorbance at $\lambda = 260$ to $\lambda = 280$ nm is one measure of the purity of a nucleic acid preparation. The 260/280 ratio of purified DNA is about 2. Higher ratio is often due to RNA contamination and lower values to protein contamination.

Most methods of DNA isolation involve the breakage or lysis of the cells to release nuclei and further breakage of nuclei to release the chromatin. DNA in cells exists as nucleoprotein complexes and therefore isolation of DNA involves removal of proteins and carbohydrates (if any) associated with it. Finally, the polymeric nature of DNA is utilised to precipitate it and make it free of small molecular contamination.

Materials Required

Tissue: Spleen/Heart/Testis/Kidney of any Vertebrate or Coconut Endosperm, Table-top, Spectrophotometer, Quartz cuvettes, Chilled blender , Saline Citrate Buffer, 2.6M NaCl, Absolute ethanol, Agarose, 0.5X TEB, 10X Loading buffer. Mortar and pestles or Glass homogeniser, Glass distilled water, Centrifuge (range 3000 to 10,000 rpm), pH meter (optional), 10 ml Centrifuge tubes, 30 ml Test tubes, Test tube rack, Bent glass rod, Sodium saline citrate solution (SSC-8 ml of 0.9% Sodium chloride solution + I5 ml of 0.5% Sodium citrate solution usually gives pH 7.4, if not adjust pH), 12% Sodium chloride solution (Dissolve 12 gm of Sodium chloride in 100 ml of Distilled water), Absolute alcohol (Double distilled alcohol).

Procedure

DNA Extraction

All the following steps to be carried out at 5°C:

1. Grind 200 mg of the Tissue (Coconut endosperm or Spleen/Heart/Testis/ Kidney of any Vertebrate) in about 5 ml of Saline Citrate Solution (85 ml of 0.9% Sodium Chloride Solution + 15 ml of 0.5% Sodium Citrate Solution – pH 7.4).
2. Now, transfer the homogenate into a centrifuge tube and make up the volume to 10 ml with Saline Sodium Citrate (SSC) Solution.

3. Centrifuge them at 3000 rpm for 8 minutes and discard the supernatant.
4. Re-homogenize the pellet with 5 ml of SSC.
5. Adjust the volume to 10 ml, centrifuge at 3000 rpm for 8 minutes and then discard the supernatant.
6. Now, suspend the pellet in 10 ml of 12% sodium chloride solution and centrifuge at 10,000 rpm for 15 minutes using a refrigerated centrifuge.
7. Thereafter, transfer the supernatant into a 30 ml test tube and add 2-3 volumes of absolute alcohol.
8. Mix it gently by inverting the tube. The white fibrous DNA precipitates.
9. Spool the fibrous white DNA by winding around a clean sterile bent glass rod.

Quantification of DNA

The presence of DNA in solution can be checked by the following methods:

1. Transfer the spooled fibrous DNA into a 1.5 ml Eppendorf tube, add 1 ml of 70% alcohol, centrifuge for 5 minutes at 10,000 rpm and discard the supernatant. Then the pellet containing the DNA is dried, dissolved in distilled water and optical density is read in a spectrophotometer at 260 nm wavelength.
2. The DNA in solution can be colorimetrically estimated by using diphenylamine colouring reagent. In brief, the deoxyribose purine in DNA in presence of acid forms hydroxy levulinic aldehyde which reacts with diphenylamine to give a blue colour. The formation of blue colour indicates the presence of DNA and intensity of the colour gives the concentration of the DNA in solution.
3. The DNA is sheared by violent agitation by passing through the small gauged needles as well as by boiling the DNA solution for 10 minutes and chilling it immediately on ice. This is needed to break the high molecular weight DNA, otherwise it cannot get into the gel. For agarose gel electrophoresis, the agarose gel can be prepared by dissolving 0.8% agarose with tris-acetate buffer, i.e., 4 mM Tris, 2 mM acetic acid, 0.2. mM EDT A, pH 8.1 (T AE) on boiling and pouring on to the casting tray after cooling the solution to 45°C and placing a slot creating comb before the polymerisation of the gel. Then place the polymerised gel into submarine electrophoretic chamber containing T AE buffer and load the DNA sample into the wells of the gel after mixing with tracking dye. After this connect the power supply and run the gel at 80 volts for 20-30 minutes. Remove the gel and stain with Ethidium Bromide (EtBr), a DNA intercalating dye. The DNA-et Br complex can be seen as an orange coloured fluorescent streaking band under ultraviolet light on a device called trans-illuminator.

DNA Analyses with Electrophoresis and Spectrophotometry

Agarose Gel Electrophoresis

1. Prepare 1% agarose (w/v) in 35 ml of 0.5X TEB. Boil it in microwave oven and let cool till about 55ºC. (Calculate: ..... g of agarose in 35 ml of 0.5X TEB).
2. Add 1.5 µl Ethidium Bromide (EtBr) to agarose solution, mix and pour into the gel tray. (Caution: Ethidium Bromide (EtBr) is a potential carcinogen so do not touch or inhale).
3. Let it polymerize for 10 min.
4. Prepare serial bovine DNA dilutions of 1:10, 1:100 and 1:1000.
5. Mix the samples (a control sample provided by the assistant and serial bovine DNA dilutions) with 6X loading buffer (Glycerol + Xylene Cyanol + Bromophenol Blue) in 5:1 ratio on a piece of Parafilm and load the samples into sample wells. Now, run the gel for about 10 min.
6. Visualize the gel under UV light and record to the computer. Notice the differences between the mobility patterns of control and your samples. Try to explain this difference.

Observation Table:

Name of the test performed	Observation	Interpretation

Spectrophotometry

1. Calculate the A260/A280 ratio will check the purity of the control and bovine DNA (Use 1:100 dilution of both control and bovine DNA).
2. Calculate the concentrations of both control and bovine DNA.

Experiment 15: Study of Cell Structure from Onion Leaf Peels

A. Study of Cell Structure from Onion Leaf Peels

Aim/Objective

To prepare a stained temporary mount of an onion peel.

Materials Required

Onion, Glass slide, Watch glass, Coverslip, Forceps, Needles, Brush, Blade, Filter paper, Safranin, Glycerine, Dropper, Water, Blotting paper, and Compound microscope.

Principle

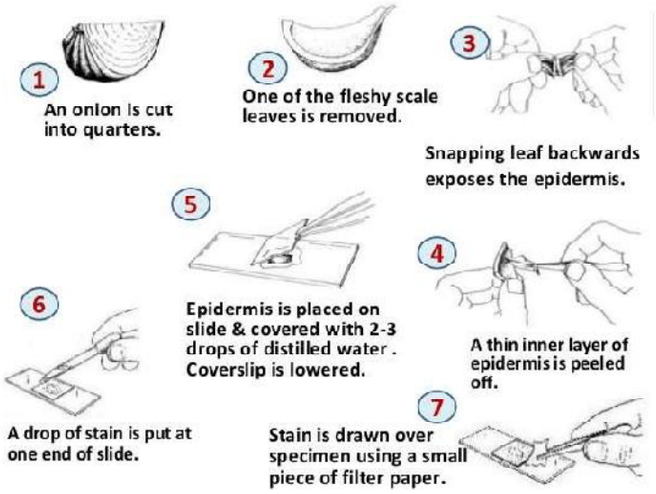
All living organisms are made up of cells. The size, shape and the number of cells vary in organisms. The three major components of a typical cell are the cell membrane, cytoplasm and nucleus. In a plant cell, a cell wall surrounds the cell membrane.

Onion is a multicellular plant. Like other plant cells, the cell of onion peel consists of a cell wall, cell membrane, cytoplasm, a large vacuole and a nucleus. The nucleus lies at the periphery of cytoplasm and vacuole is located in the centre. Presence of large vacuoles and cell wall confirms that cells of onion peel are plant cells.

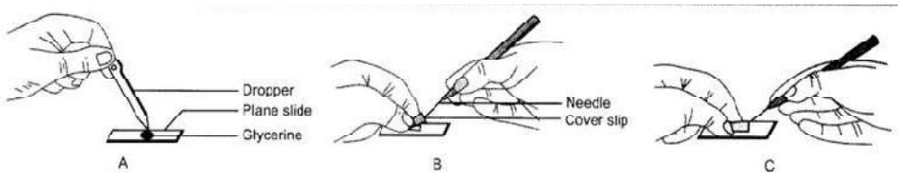
Procedure

1. Take a piece of onion and bend it to remove the transparent membranous structure called onion epidermal peel. With help of forceps remove the peel from its inner side.
2. Now, cut a small part from an inner scale leaf with the help of a blade.
3. Separate its thin, transparent peel from the convex surface of the scale leaf with the help of forceps.
4. Keep the peel in a watch glass containing water.
5. Now, add two drops of safranin stain in the watch glass to stain the peel.
6. Take a clean slide and put a drop of glycerine in the centre of the slide.
7. Transfer the peel on the slide with the help of a brush and needle. Glycerine prevents the peel from drying up.
8. Now, cover it with a coverslip carefully to avoid any air bubble from entering into the coverslip.
9. Blot any excessive glycerine with a filter paper.
10. Finally, observe the prepared mount of the peel under the low and high magnification of a compound microscope.

The following figure illustrates the steps for preparing a stained temporary mount of an onion peel.



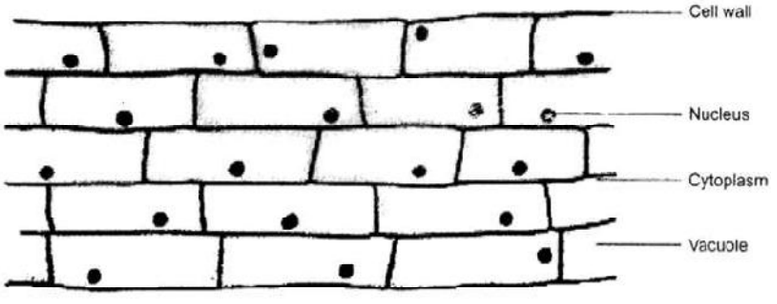
The following figure illustrates the stages to show the mounting of a slide.



Observations

1. A large number of rectangular cells with distinct cell wall can be observed.
2. Cytoplasm is seen as thin layer of deep coloured substance on inner surface of cell wall.
3. A big central vacuole is present in the cell.
4. A deeply stained round body called nucleus is seen in each cell.

The following figure illustrates the structure of an onion peel under a compound microscope.



Results

1. The epidermal peel of onion comprises of rectangular shaped cells. Each cell comprises of a nucleus, a central vacuole, thin layer of cytoplasm and cell wall.
2. As cell walls and large prominent vacuole are present in each cell, the cells placed under observation are plant cells.

Precautions

1. Peel should be properly stained. Avoid under-staining or excessive staining of the peel.
2. Always transfer the peel with the help of brush.
3. Mounting of the peel should be done in centre of slide.
4. Avoid folding of the leaf peel.
5. Remove extra glycerine with the help of blotting paper.
6. Avoid entry of air bubbles while placing the cover slip.
7. Clean and dry glass slide and coverslip should be used.
8. Coverslip should be put carefully avoiding any air bubbles.

B. Demonstration of Staining and Mounting Methods

Staining is a technique generally used in microscopy to improve contrast in a microscopic image. Stains and dyes are frequently used to highlight structures in microbes for viewing, often with the aid of different microscopes. Staining may be categorized as single or double.

Importance of Staining

- In microbiology the concept of staining is very important because it highlights the structures of microorganisms allowing them to be seen under a microscope (simple and electron microscope).
- It is also used to differentiate different microorganisms.
- Used for the identification of microorganisms like bacteria which may be either gram positive or gram negative.

I. Single Stain: Safranin or fast green is used to stain filaments of algae, fungi, sections of bryophytes, spores of pteridophytes, pollen grains of angiosperms, etc. Aniline blue or safranin is usually used for single staining methods.

Procedure

1. First keep material in watch glass.
2. Add few drops of stain to the water to another watch glass to dilute the stain.

3. The diluted stain is used to the material.
4. After taking up the stain by sample, the extra stain is washed with water.
5. Washing is repeated till the stain stops coming out.
6. Now, the stained material is ready for mounting.

II. Combined Stain: In this technique, two or more stains are used. Generally contrasting colours are used. The following combinations are commonly employed.

1. Haematoxylin and Safranin
2. Safranin and Fast Green
3. Safranin and Aniline Blue
4. Safranin and Crystal Violet
5. Crystal Violet and Erythrosine

Procedure

1. The section is initially stained with principle stain.
2. Now, the section is passed through a graded series of alcohol for degradation.
3. After that section is transferred to watch glass containing required amount of alcohol and water.
4. Watch glass should be covered by larger watch glass to avoid alcohol evaporation.
5. Now the counter stain is employed for few seconds.
6. The section is transferred to absolute alcohol for 5-7 minutes for complete dehydration.
7. Section is finally immersed in xylene for complete dehydration. Now the section is ready for mounting.

III. Gram Stain

Some of the bacteria like mycobacterium do not get stained with Gram stain due to their lipid capsule. Ziehl-Neelsen stain is applied to identify mycobacteria which are stained pink to red in colour.

Gram Staining

This reaction may depend on the difference in permeability of cytoplasmic membrane. During staining a dye iodine complex is formed within the cell, which is insoluble in water. The complex diffuses freely from gram negative organisms but diffuses less in Gram positive bacteria due to poor membrane permeability.

Preparation of Reagents

(i) Crystal Violet Solution

Crystal violet 1.0 gm

Absolute alcohol 20 ml

Ammonium oxalate (1%) 80 ml

(ii) Basic Fuchsin

Basic fuchsin 1.0 gm

Distilled water 100 ml

(iii) Gram's Iodine

Iodine crystal 1.0 gm

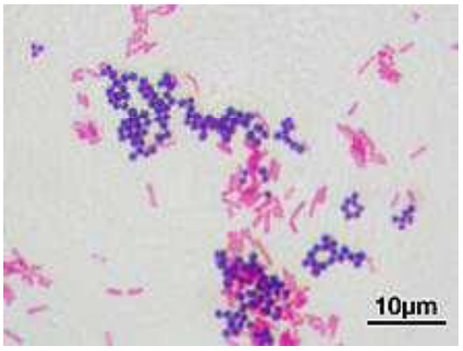
Potassium iodide 2.0 gm

Distilled water 300 ml

Procedure

1. Deparaffinize the section and bring it to water.
2. Put crystal violet for one minute.
3. Add Gram's iodine for 30 seconds.
4. Differentiate by dipping the section once or twice in alcohol.
5. Wash with water and counterstain with safranin for 45 seconds.
6. Wash with water.
7. Air dry and mount in DPX.RESULT Gram positive bacteria stain blue black.
8. Gram negative bacteria stain red or pink.
9. Some of the bacteria like mycobacterium do not get stained with Gram stain due to their lipid capsule. Ziehl-Neelsen stain is applied to identify mycobacteria which are stained pink to red in colour.

Figure below shows the bacteria stained with Gram stain.



The table below shows the Gram stain procedural steps.

Step	Procedure	Outcome
Primary stain(crystal violet)	Add several drops of crystal violet to the smear and allow it to sit for 1 minute. Rinse the slide with water.	Both Gram-positive and Gram-negative cells will be stained purple by the crystal violet dye.
Mordant (iodine)	Add several drops of iodine to the smear and allow it to sit for 1 minute. Rinse the slide with water.	Iodine "sets" the crystal violet, so both types of bacteria will remain purple.
Decolorization (ethanol)	Add drops of ethanol **one at a time** until the runoff is clear. Rinse the slide with water.	Gram-positive cells resist decolorization and remain purple. The dye is released from Gram-negative cells.
Counterstain(safranin)	Add several drops of safranin to the smear and allow it to sit for one minute. Rinse the slide with water and blot dry.	Gram-negative cells will be stained pink by the safranin. This dye has no effect on Gram-positive cells, which remain purple.

The table below shows some of the stains, their uses and specimen colour.

STAIN	USE	SPECIMEN COLOUR
Acid Fuchsin	Stains collagen fibers red and smooth muscle in contrast to collagen.	Red/magenta/violet
Congo Red	Used to stain amyloid. (A starchlike substance. A hard, waxy deposit consisting of protein and polysaccharides that results from the degeneration of tissue.)	Red
Crystal Violet	The (positive) dye of choice in Grams stain. Bacteria	Blue violet
Eosin B	Interchangeable with Eosin Y. Ionization – acid (Color is red)	Bluish cast
Eosin Y	Stains alkaline cell parts (like cytoplasm). Use on plants, animals and blood.	Pink
Eosin Y ws	Common counterstain to alum hematoxylin and Eosin method.	Red
Gram Stain	Positive – Staphylococcus, Streptococcus, Bacillus - subtilis (crystal violet) Negative – Neisseria, E. coli (Eosin Y ws)	Dark blue or purple Red
Hematoxylin	Stains nuclear chromatin using aluminum mordant. Acid resistant nuclear staining, muscle striations and some glial fibers (The delicate network of branched cells and fibers that supports the tissue of the central nervous system) with ferric salts mordant.	Yellow/brown
Iodine	Carbohydrates in plant and animal specimens Glycogen (Animal starch.. A polysaccharide, (C6H10O5)n, that is the main form of carbohydrate storage in animals.	Brown or Blue-Black Red
Mercurochrome	Marks tissue margins and faces for orientation. (Antiseptic) Similar to Eosin Y ws.	Red
Methyl blue	Stains collagen and connective tissue.	Blue
Methylene blue	Acidic cell parts (Use on animal, bacteria and blood specimens.	Blue

Saffron	Colors connective tissue yellow in contrast to the pink cytoplasm given by phyloxine.	Yellow orange
Safranin O	Commonly used for counterstaining nuclei red. Cartilage is stained yellowish.	Red
Toluidene blue	Stains acidic cell parts (like nucleus). Good to show mitosis in plant cells. MITOSIS = The process in cell division by which the nucleus divides, typically consisting of four stages, prophase, metaphase, anaphase, and telophase, and normally resulting in two new nuclei, each of which contains a complete copy of the parental chromosomes. Also called karyokinesis.	Dark Blue

IV. Mounting: Mounting is required for proper positioning of objects for clear view. Lactophenol, glycerin and glycerine jelly are used for temporary mounting while Canada balsam is used for permanent mounting.

Mounting Media

Following are some of the common mounting media:

1. **Canada Balsam:** It is resin obtained from conifer. It is most suited for permanent slide preparation.
2. **Lactophenol:** It is a mixture of equal parts of phenol crystals, lactic acids, glycerine and distilled water.
3. **Glycerine:** Pure glycerine diluted to 15-25% is widely used for semi-permanent and temporary preparations.
4. **Glycerine Jelly:** Jelly is used for mounting. It is made up of gelatin.
5. Many other mounting media like cedar oil, balsam, venetiane, turpentine, synthetic resin, etc., are also used.

V. Dry Mounts

1. Though rarely used for living objects, dry mounts can be useful for microscopic examination of non-living objects/materials.
2. Hair mounting can be done for a hair obtained from an individual and is visualized first under low power and then by using high power objective.
3. Similarly, examine a wool or cotton thread under the low power and then under high power to see its texture.

Mounting of Living Microorganisms

In this experiment we will examine, using wet mount techniques, different fluid environments to help become aware of the numbers and varieties of microbes found in nature. The microbes that are seen will be exhibiting either Brownian movement or true motility. Brownian movement is not true motility but rather the movement is caused by the molecules in the liquid striking an object and causing the object to shake or bounce. In Brownian movement the particles and microorganisms all vibrate at about the same rate and maintain their relative positions.

Motile microorganisms move from one position to another. Their movement appears more directed than Brownian movement and occasionally the cells may roll or spin.

Many kinds of microbes, such as protozoa, algae, and bacteria can be found in pond water and in infusions of any organic matter like urine, stool and broth culture. Direct examination by the hanging-drop method is very useful in determining size, shape, and movement.

Aim/Objective

1. To prepare and observe the mount slides and hanging drop slides.
2. Distinguish different types of microbes/cells in unstained preparations.
3. Distinguish true motility from Brownian movement.

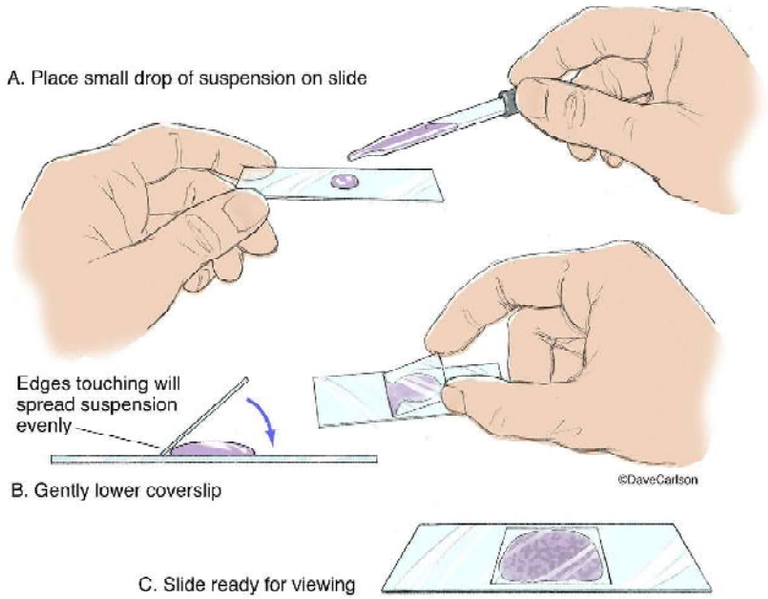
Materials Required

Slides, Cover slips, Hanging drop (depression) slide, Petroleum jelly/saline, Pasture pipettes, Bacteriological loop, Dirty pond water, Buccal mucosa scraping, Giardia cysts/parasitic ova in sterile saline.

Wet Mount Technique

1. Suspend the infusions by stirring or sharing carefully. Transfer a small drop of a given infusion/suspension using a Pasteur pipette, to a slide.
2. Handle the cover slip by its edges like a photograph and place it on the drop.
3. Gently press on the cover slip with the end of a pencil. Map excess infusion build with.
4. Place the slide on the microscope stage and observe with low power. Adjust the Iris diaphragm so that a small amount of light is admitted. Concentrate your observations on the larger, more rapidly moving organisms. At this magnification, bacteria are barely discernible as tiny dots.
5. Examine with the high-dry lens, then increase the light and focus carefully. Bacteria should now be magnified sufficiently to see them.
6. After recording your observations, examine the slide under oil immersion. Some microorganisms are motile while others exhibit Brownian movement.
7. If you want to observe the motile organisms further, place a drop of alcohol or Gram's iodine at the edge of the cover slip and allow it to run under and mix with the infusion. They can now be observed more carefully.
8. Record your observations, noting the relative size and shape of the organisms.
9. A wet mount should be made from the other suspensions/infusions and observed, using the low and high dry objectives. Record your observations.
10. Clean all the slides and return them to the slide box. Cover slips can be discarded in the disinfectant jar.

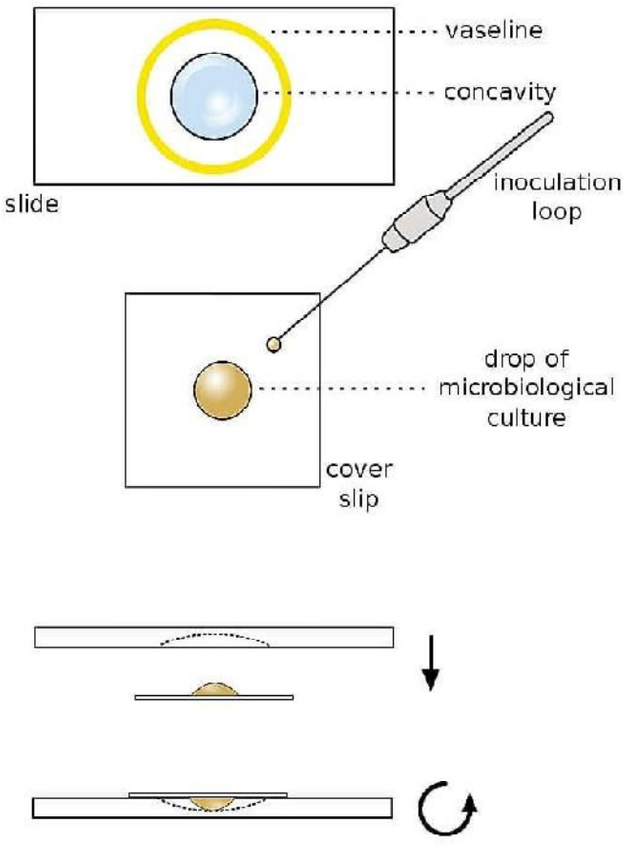
The figure below illustrates the wet mount technique.



VI. Hanging Drop Procedure

1. Obtain a hanging drop slide. If hanging drop slide is not available you can take a plain slide and make a small 'well' with the use of plastic.
2. Place a small amount of petroleum jelly on the palm of your hand and smear it into a circle about the size of a 1 rupee coin.
3. Pick up a cover slip (by its edges) and carefully scrape the petroleum jelly with an edge of the cover slip. Repeat with the other three edges, keeping the petroleum jelly on the same side of the cover slip.
4. Place the cover slip on a paper towel, with the petroleum jelly side up.
5. Transfer a drop of the prepared organic infusion or suspension on the cover slip.
6. Place a slide over the drop and quickly invert so that the drop is suspended.
7. Examine under low power by locating the edge of the drop and moving the slide so the edge of the cover slip crosses the center of the field.
8. Reduce the light with the Iris diaphragm and focus. Observe the different sizes, shapes, and types of movement.
9. Switch to high-dry and record your observations.
10. When finished, clean your slide, and, using a new cover slip, repeat the procedure with the culture of Bacillus. Record your observations.
11. Wipe the oil from the objective lens with lens paper and return your microscope to its proper location. Clean your slides well and return them.

The figure below shows the hanging drop preparation.



Experiment 16: Comparative Study of Cell Structure in Onion Cells, *Hydrilla* and *Spirogyra*

Aim/Objective

To do a comparative study of cell structure in Onion cells, *Hydrilla* and *Spirogyra*.

Materials Required

Onion cells, *Hydrilla* cell, *Spirogyra* cell, Watch glasses, Forceps, Glass slide, Cover slip, Dropper, Mounting needle, Brush, Blotting paper, Compound microscope, Knife/Scalpel, Glycerine, Safranin solution, Distilled water.

I. Onion Peel Cell

Principle

All living organisms are made up of cells. The size, shape and the number of cells vary in organisms. The three major components of a typical cell are the cell membrane, cytoplasm and nucleus. In a plant cell, a cell wall surrounds the cell membrane.

Onion is a multicellular plant. Like other plant cells, the cell of onion peel consists of a cell wall, cell membrane, cytoplasm, a large vacuole and a nucleus. The nucleus lies at the periphery of cytoplasm and vacuole is located in the centre. Presence of large vacuoles and cell wall confirms that cells of onion peel are plant cells.

Procedure

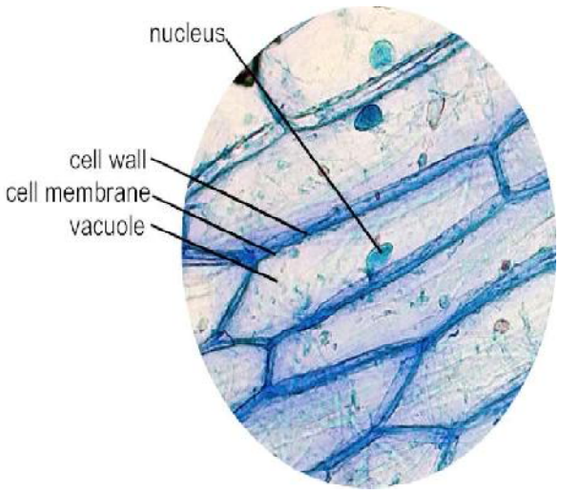
Onion Peel Cell

1. Pour some distilled water into a watch glass.
2. Take a piece of onion and with the help of a forceps gently pull a thin, transparent peel (epidermis) front it.
3. Take a thick onion scale and tear it from concave side to get transparent thin membrane like onion peel.
4. Using a brush, transfer the peel into the watch glass containing the methylene blue.
5. Place the peel in watch glass containing water and 2-3 drops of methylene blue.
6. Now, cut a small portion of peel and placed it on cleansed glass slide with a drop of glycerine.
7. Take a cover slip and place it gently on the peel with the aid of a needle.
8. Remove the extra glycerine using a piece of blotting paper.
9. Place this glass side on the stage of the compound microscope.
10. Make sure the low objective lens is over the specimen.
11. Carefully use the course focusing knob to lower the objective lens to just above the slide.
12. Look through the eye piece and carefully use the fine focusing knob to focus the image.

Observations

1. There are a large number of regularly shaped cells lying side by side and each cell has a distinct cell wall.
2. A distinct nucleus is present in the cell.
3. Lightly stained cytoplasm is observed in each cell.

The figure below shows the onion peel cell under a microscope.



Conclusion

As cell walls are clearly observed in all the cells, the cells placed for observation are plant cells.

Precautions

1. Use a brush to transfer the peel from one apparatus to another.
2. Staining of peel should neither be too dark, nor too light.
3. Extra glycerine stain should be removed using blotting paper.

II. *Hydrilla*

Principle

Hydrilla is a genus of aquatic plant, usually treated as containing just one species, *Hydrilla verticillata*, though some botanists divide it into several species. It is native to the cool and warm waters of the Old World in Asia, Africa and Australia, with a sparse, scattered distribution; in Australia from Northern Territory, Queensland, and New South Wales.

The stems grow up to 1–2m long. The leaves are arranged in whorls of two to eight around the stem, each leaf 5–20 mm long and 0.7–2 mm broad, with serrations or small spines along the leaf margins; the leaf midrib is often reddish when fresh. It is monoecious (sometimes dioecious), with male and female flowers produced separately on a single plant; the flowers are small, with three sepals and three petals, the petals 3–5 mm long, transparent with red streaks. It reproduces primarily vegetatively by fragmentation and by rhizomes and turions (overwintering), and flowers are rarely seen. They have air spaces to keep them upright. *Hydrilla* has a high resistance to salinity compared to many other freshwater associated aquatic plants.

Procedure

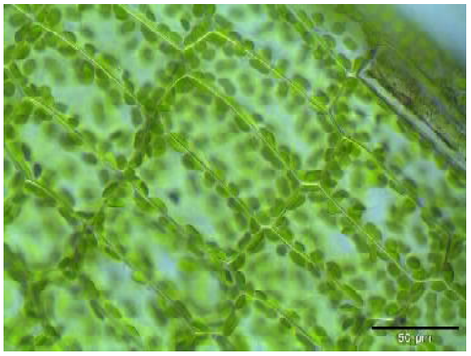
Hydrilla Cell

1. Take a cleansed glass slide.
2. Pour some distilled water into a watch glass.
3. Take a piece of *Hydrilla* cell and with the help of a forceps gently pull a thin, transparent peel.
4. Mount the *Hydrilla* cell on it with a drop of glycerine.
5. Using a brush, transfer the peel into the watch glass containing the methylene blue.
6. Place the peel in watch glass containing water and 2-3 drops of methylene blue.
7. Now, cut a small portion of peel and placed it on cleansed glass slide with a drop of glycerine.
8. Take a cover slip and place it gently on the peel with the aid of a needle.
9. Remove the extra glycerine using a piece of blotting paper.
10. Place this glass side on the stage of the compound microscope.
11. Make sure the low objective lens is over the specimen.
12. Carefully use the course focusing knob to lower the objective lens to just above the slide.
13. Look through the eye piece and carefully use the fine focusing knob to focus the image.

Observations

1. There are a large number of regularly shaped cells lying side-by-side and each cell has a distinct cell wall.
2. A distinct nucleus is present in the cell.
3. Lightly stained cytoplasm is observed in each cell.

The figure below shows the *Hydrilla* cell under a microscope.



Conclusion

As cell walls are clearly observed in all the cells, the cells placed for observation are plant cells.

Precautions

1. Use a brush to transfer the peel from one apparatus to another.
2. Staining of peel should neither be too dark, nor too light.
3. Extra glycerine stain should be removed using blotting paper.

III. *Spirogyra*

Principle

Spirogyra, common names include water silk, mermaid's tresses, and blanket weed, is a genus of filamentous charophyte green algae of the order Zygnematales, named for the helical or spiral arrangement of the chloroplasts that is characteristic of the genus. It is commonly found in freshwater habitats, and there are more than 400 species of *Spirogyra* in the world. *Spirogyra* measures approximately 10 to 100 ìm in width and may grow to several centimetres in length.

Spirogyra is very common in relatively clean eutrophic water, developing slimy filamentous green masses. In spring *Spirogyra* grows under water, but when there is enough sunlight and warmth they produce large amounts of oxygen, adhering as bubbles between the tangled filaments. The filamentous masses come to the surface and become visible as slimy green mats. *Spirogyra* has a cell wall, nucleus, pyrenoid and spiral chloroplasts. It is very rare among the plant-like protists.

Procedure

***Spirogyra* Cell**

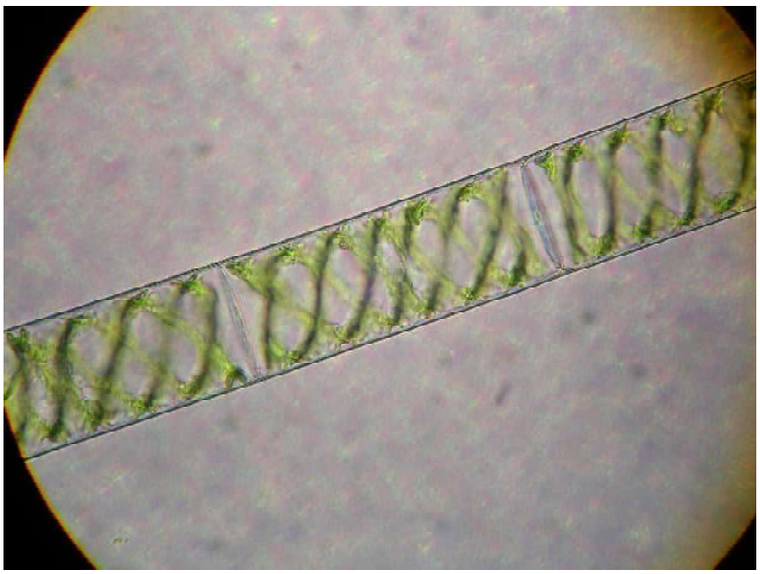
1. Take a cleansed glass slide.
2. Pour some distilled water into a watch glass.
3. Take a piece of *Spirogyra* cell and with the help of a forceps gently pull a thin, transparent peel.
4. Mount the *Spirogyra* cell on it with a drop of glycerine.
5. Using a brush, transfer the peel into the watch glass containing the methylene blue.
6. Place the peel in watch glass containing water and 2-3 drops of methylene blue.
7. Now, cut a small portion of peel and placed it on cleansed glass slide with a drop of glycerine.
8. Take a cover slip and place it gently on the peel with the aid of a needle.

9. Remove the extra glycerine using a piece of blotting paper.
10. Place this glass side on the stage of the compound microscope.
11. Make sure the low objective lens is over the specimen.
12. Carefully use the course focusing knob to lower the objective lens to just above the slide.
13. Look through the eye piece and carefully use the fine focusing knob to focus the image.

Observations

1. There are a large number of regularly shaped cells lying side by side and each cell has a distinct cell wall.
2. A distinct nucleus is present in the cell.
3. Lightly stained cytoplasm is observed in each cell.

The figure below shows the *Spirogyra* cell under a microscope.



Conclusion

As cell walls are clearly observed in all the cells, the cells placed for observation are plant cells.

Precautions

1. Use a brush to transfer the peel from one apparatus to another.
2. Staining of peel should neither be too dark, nor too light.
3. Extra glycerine stain should be removed using blotting paper.

Experiment 17: To Study Cyclosis from Tradescantia Stamen Hair

Aim/Objective

To study cyclosis from Tradescantia stamen hair.

Principle

Cyclosis is automatic movement of protoplasm of a cell. This movement may be usually due to the movement of various cell organelles like chloroplast in response to changing intensity of light. It is believed that cyclosis occurs due to Sol-gel interconversions or microfibrils.

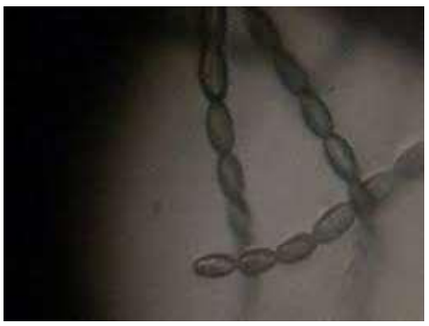
Materials Required

Tradescantia flowers, Watch glasses, Forceps, Glass slides, Cover slips, Dropper, Mounting needle, Brush, Blotting paper, Compound microscope, Knife/Scalpel, Glycerine, Safranin solution, Distilled water.

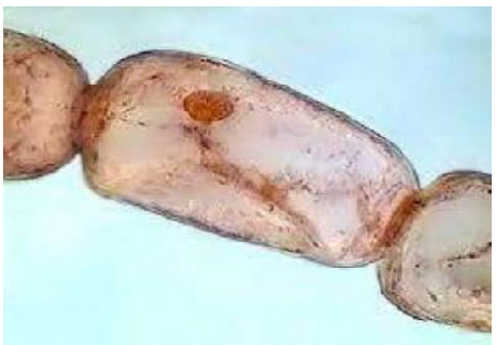
Procedure

1. Take a cleansed glass slide.
2. Pour some distilled water into a watch glass.
3. Take a piece of fresh Tradescantia flower and with the help of a forceps. Gently pull a thin, transparent terminal hair.
4. Mount the Tradescantia flower on it with a drop of glycerine.
5. Using a brush, transfer the peel into the watch glass containing the methylene blue.
6. Place the terminal hair in watch glass containing water and 2-3 drops of methylene blue.
7. Now, cut a small portion of terminal hair and place it on cleansed glass slide with a drop of glycerine.
8. Take a cover slip and place it gently on the peel with the aid of a needle.
9. Remove the extra glycerine using a piece of blotting paper.
10. Place this glass slide on the stage of the compound microscope.
11. Make sure the low objective lens is over the specimen.
12. Carefully use the course focusing knob to lower the objective lens to just above the slide.
13. Look through the eye piece and carefully use the fine focusing knob to focus the image.

The figure below shows the filament with staminal hairs under a microscope.



The figures below show the enlarged view of staminal hair under a microscope.



Conclusion

As cell walls are clearly observed in all the cells, the cells placed for observation are plant cells.

Precautions

1. Use a brush to transfer the peel from one apparatus to another.
2. Staining of peel should neither be too dark, nor too light.
3. Extra glycerine stain should be removed using blotting paper.
4. There should not be any air bubble while placing coverslip.

Experiment 18: To Study the Permeability of Plasma Membrane using Different Concentrations of Organic Solvents

Aim/Objective

To study the permeability of plasma membrane using different concentrations of organic solvents.

Principle

The plasma membrane serves as the interface between the machinery in the interior of the cell and the extracellular fluid that surrounds all cells. It comprises a phospholipid bilayer together with proteins that can span the bilayer (integral/ transmembrane) or are peripherally attached to one face or the other (peripheral). The components of the membrane bilayer frequently move laterally into other regions of the membrane, making it appear more fluid than static. This is known as the fluid mosaic model of the cell membrane. In addition, proteins present on the extracellular face can be heavily glycosylated, giving asymmetrical characteristics to the membrane.

The plasma membrane is selectively permeable, i.e., it allows only some substances to pass across it but not others. Molecules and ions move spontaneously down against their concentration gradient, i.e., from a region of higher to a region of lower concentration) by diffusion. Molecules and ions can be moved against their concentration gradient, but this process, called active transport, requires the expenditure of energy (usually from ATP). Most of the membranes are permeable to water. Osmosis is a special term used for the diffusion of water through cell membranes. When the concentration of the solute is the same on the inside and outside of the cell, the water moves equally in both directions. The solution outside the cell is called isotonic. If the solution contains a higher solute concentration than the cell, it is hypertonic, but if it is lower, the solution is hypotonic.

Materials Required

Onion bulbs, Glass slides, Cover slips, Watch glasses, Forceps, Dropper, Mounting needle, Brush, Blotting paper, Compound microscope, Knife/Scalpel, Glycerine, Distilled water, Stop watch, Glucose, Sucrose, Glycerol, Methyl alcohol, Tertiary Butyl Alcohol (TBA).

Procedure

1. Take a cleansed glass slide.
2. Pour some distilled water into a watch glass.
3. Take a piece of onion and with the help of a forceps gently pull a thin, transparent peel (epidermis) from it.
4. Now, cut the epidermal segment into small pieces, and place a piece of the epidermis in a few drops of each of the six 2M solutions of glycerol, glucose, methyl alcohol, ethylene glycol, sucrose and Tertiary Butyl Alcohol (TBA).

5. Using a brush, transfer the peel into the watch glass.
6. Place the peel in watch glass containing water and 2-3 drops of methylene blue.
7. Now, cut a small portion of peel and place it on cleansed glass slide with a drop of glycerine.
8. Take a cover slip and place it gently on each solvent immediately.
9. Remove the extra glycerine using a piece of blotting paper.
10. Place this glass side on the stage of the compound microscope.
11. Make sure the low objective lens is over the specimen.
12. Carefully use the course focusing knob to lower the objective lens to just above the slide.
13. Look through the eye piece and carefully use the fine focusing knob to focus the image.

Observations

Observe each slide very carefully for some time under microscope. We observe that plasmolysis start immediately. Record the time required for complete plasmolysis in each solution with the help of a stop watch.

Now, place a few drops of water on each epidermal segment and observe for about 15 minutes until the cells in the epidermal segments are completely deplasmolysed. Also, record the time required for plasmolysis with the help of stop watch.

Solvents (2 M strength)	Quantity/100 ml of water	Time required for plasmolysis *(in minutes)*	Time required for deplasmolysis *(in minutes)*
1. Glucose	18 gm		
2. Glycerol	146 ml		
3. Ethylene glycol	110 ml		
4. Methyl alcohol	81 ml		
5. Sucrose	29.2 gm		
6. Tertiary butyl alcohol (TBA)	188 ml		

Results

The total time of deplasmolysis in each epidermal segment depicts the rate of penetration or permeability of plasma membrane in different concentrations of organic solvents.

Precautions

1. Use a brush to transfer the peel from one apparatus to another.
2. Staining of peel should neither be too dark, nor too light.
3. Extra glycerine stain should be removed using blotting paper.
4. Avoid air bubbles while placing the cover slip.

Experiment 19: To Study the Effect of Temperature on Permeability of Plasma Membrane

Aim/Objective

The aim of the practical was to investigate the effect of temperature on membrane permeability in beetroot cells. This was tested by placing beetroot disks (attached to skewers) in 6 different temperatures for a minute and then in a water bath at room temperature for a minute. The water was then extracted and tested in a spectrophotometer at 530ppm. The results showed that once the temperature reached 60 degrees the absorbance rate increased greatly.

Principle

Cell membranes are composed of proteins and a phospholipid bi-layer, which serve to protect and organise cells and their composition. Phospholipid bi-layers are made up of Hydrophilic heads and hydrophobic tails. They function as a barrier to allowing for the division of not only extracellular and intracellular contents, but as well form the membrane of most intracellular organelles. The composition of a cell's membrane dictates its chemical and physical properties, and, therefore, the range of external environments in which the cell can exist.

Heat is a form of energy which when applied to cell membranes causes the phospholipid bi-layer to expand due to increased vibration within the molecules and as a consequence increases the permeability. In addition to this heat also causes proteins to denature which may enhance cell membrane permeability.

In the case of beetroot the cell membrane and the organelles are composed of phospholipid bi-layer so therefore when heat is applied their permeability increases and their intracellular contents are able to escape. Beetroot cells are a useful cell type to measure cell membrane permeability as they contain vacuoles which contain pigments called betacyanin and betaxanthin. These pigments absorb light at a wavelength of 530nm. When heat is applied to the beetroots cell membrane it becomes destabilised and these pigments are able to escape the cell.

***Hypothesis*:** As the temperature of the water increases the amount of absorbance increases.

***Independent variable*:** The temperature of the plant cell membrane.

***Dependent variable*:** The absorbance in 530ppm.

Controlled variables:

- The temperatures of the water.
- The calibration of the spectrophotometer.
- The amount of beetroot.

Materials Required

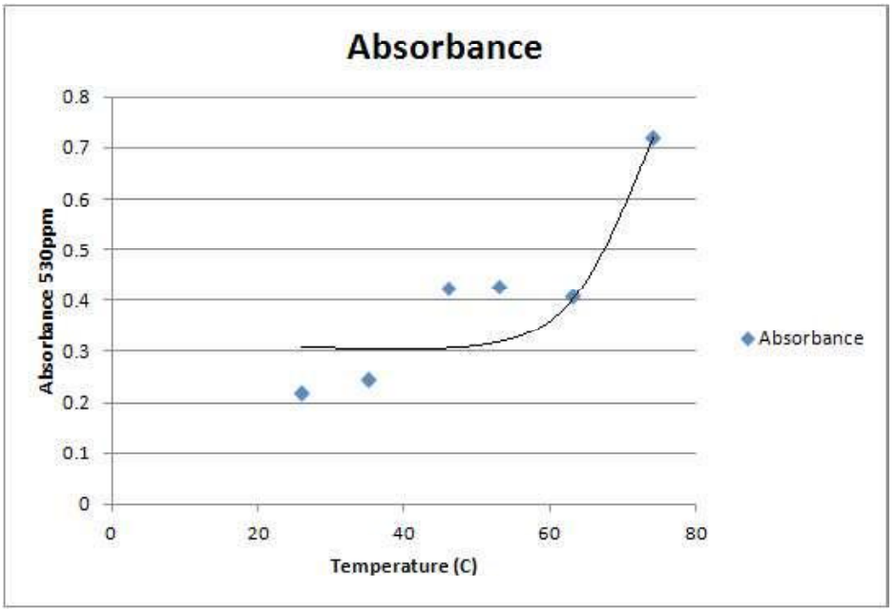
Beetroot	Measuring cylinder	Plastic pipette
6 Test tubes	Mounted needle	Sieve
Test tube racks	Large beakers	Marker pen
Ruler	2 Spectrophotometer cuvettes	Stopwatch
Cork borer (1cm diameter)	Thermometer (alcohol)	Spectrophotometer
White tile	Hot plate	Sharp Knife
Heat proof mat	Small beaker	Glass stirring rod

Procedure

1. A cork borer was used to cut beetroot cylindrically, which was then placed on a tile and cut into 35 disks at 3mm thickness. The disks were then placed in a small beaker containing distilled water, which was agitated for 3 minutes. This process was repeated 3 times until the washing solution remained clear. A mounting needle was then used to evenly space 5 beetroot disks, and this process was repeated to create a total of 6 beetroot stacks.
2. Six test tubes were labelled with different temperatures (30, 40,50,60,70 and 80) and 10mLs of distilled water was added to each. A water bath was then used to heat all six test tubes, where once the desired temperature was reached the test tube was removed and placed into a test tube rack where the temperature was recorded. Immediately after one of the beetroot stacks was placed into the test tube for exactly 1 minute and the test tube and beetroot stack were placed into a room temperature water bath for 30 minutes. This process was repeated for all six test tubes.
3. After 30 minutes, 3mLs of the solution from each of the test tube was placed into separate spectrophotometer cuvettes using a plastic pasture pipette. The machine was then calibrated using a spectrophotometer cuvette filled with distilled water that was set to have an absorbance of 0nm. Then each cuvette was placed into the machine and its respective absorbance was recorded.

Results

Temperatures	Absorbance
26	0.221
35	0.249
46	0.426
53	0.431
63	0.412
74	0.721



The relationship between water temperature and absorbance displays an exponential trend, where higher water temperatures generated an increase in absorbance. It increased greatly at 60 degrees.

Observations

As seen from the graph above as the temperature increases, so does the absorbance. This is due to the fact that as the heat increases the membrane vibrates, causing the proteins to denature and phospholipid bilayer to rupture, causing more of the pigment betacyanin and betaxanthin to escape from the vacuoles into the water. Therefore the higher the temperature the more of the pigment is released causing the absorbance to increase. The reason that the absorbance increases is because of the fact that pigments absorb light. The higher the amount of pigments, the higher the absorbance, hence the results.

The first error would be the misreading of the temperature (Random). This would cause the results to show different from what they actually are because the temperature was ready incorrectly. An improvement would be to take measurements multiple times until you are happy that the temperature reading is right. If needed multiple points of view.

Second some parts of the beetroot were redder than other parts of the beetroot (Random). This would cause some temperatures to have higher amounts of pigments causing some results to be invalid. An improvement could be when the beetroot is cut at the start making sure that all of the beetroot is the same colour.

Third, Size of the beetroot slices was not all the same (Random). This would cause some of the water samples to have higher amounts of pigments than others because of the fact that the slices were not all the same size. An improvement could be to allow enough time to precisely cut all of the beetroot sizes into exactly the same width to attempt to eliminate this error.

Lastly, the spectrophotometer cuvette not properly cleaned (Random). This would have caused the spectrophotometer to calibrate incorrect absorbance readings therefore making the results invalid. An improvement would be to make sure all cuvettes are thoroughly cleaned without any marks on them.

Class data was not made available to everybody. An improvement for next time would be able to make class data available for everybody to collate their data and provide comparisons.

Analysis

The results showed that when the temperature reached 60 degrees the absorbance increased greatly. The hypothesis 'As the temperature of the water increases the amount of absorbance increases' was supported. The evidence also states that when it reaches 60 degrees the membrane weakens which allows the pigments to be released. The temperature had low resolution because it didn't move in exact increments. The temperatures were increased in random increments. The results were adequately valid. The practical had lots of errors which caused outliers within the results, except we were able to create an adequate graph from the results. The sample size was also adequate; the sample had 6 replicates which provided enough sample size. The measuring equipment was accurate because the spectrophotometers were calibrated correctly. The first improvement which could be done to the procedure was to allow a larger time for the prac to be done. Because of time constraints the practical was rushed and mistakes were made. Second the beetroot disks could have been washed longer to make sure that the disks had all of the beetroot pigments washed out prior to putting them within the test tubes. Beetroot disks could have been placed within the tube instead of on skewers. The skewers could have potentially penetrated the membrane causing pigments to release.

Conclusion

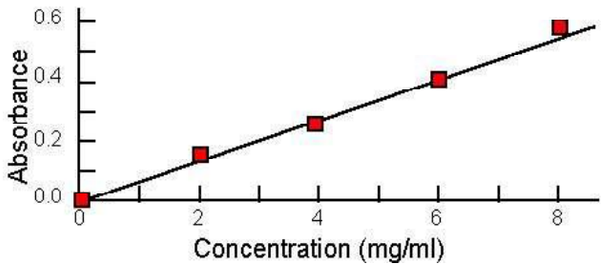
In conclusion the aim to test the effect of temperature on the permeability of membranes was successful. The hypothesis 'As the temperature of the water increases the amount of absorbance increases' was supported also. The results showed that once the temperature reached 60 degrees the absorbance increased.

Experiment 20: To Prepare the Standard Curve of Protein and Determine the Protein Content in Unknown Samples

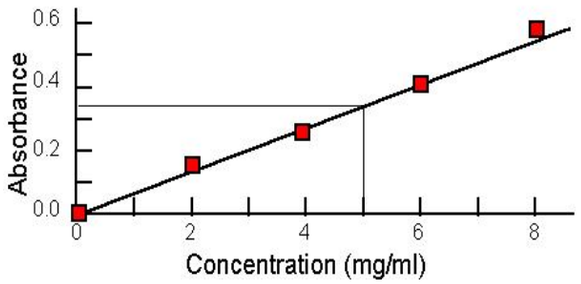
Principle

Standard curves are commonly used to determine the concentration of substances. They are obtained by relating a measured quantity to the concentration of the substance of interest in 'known' samples, i.e. Standards of known concentration. These standards supply a reference to determine unknown concentrations. Thus amounts chosen of standards need to span the range of concentrations expected to be found in the 'unknown' sample concentration.

The quantity (assay measurements as i.e. luminescence, radioactivity, fluorescence, and optical density of various known concentrations of a substance) graphed on y-axis and standard concentrations on x-axis. Data analyzed by fitting a line on curve as shown below in the figure.



To determine the unknown concentration of a substance in a sample (with same assay as for standards used), intersect across the assay measurement on y-axis with standard concentration, and down to x-axis. The concentration of substance in unknown sample is the value on x-axis.



Quantitative Estimation of the Amount of Proteins Present in an Unknown Solution

Aim/Objective

To determine protein content in an unknown solution requires:

1. An assay in which measurable quantity is related to concentration.
2. Standards for comparison.

Biuret Test

This is a method that forms a blue-violet colour on reaction of protein with copper ion in alkaline solution. It is a good protein assay.

Spectrophotometer

It is an optical instrument that usually measures the light energy transmitted throughout the continuous band of wavelength in spectromagnetic spectrum. A beam of light is focused by a lens onto an entrance slit, where it is collected by a second lens and refocused on the exit slit after being reflected and dispersed by a diffraction grating (used to select λ). After passing the slit, the light goes through the sample being measured and picked up by a phototube. The amount of light absorbed by the sample is read on the dial.

Colorimetry

White light passed through a solution containing coloured compounds.

Standard Solutions

These are known as concentrations of samples.

Procedure

1. Use the stock solution of concentrated bovine serum albumin. Check concentration.
2. Number 10 tubes, and place in each the following volumes of the bovine serum albumin: 0.0, 0.1, 0.2, and 0.3, up to 1.0 ml.
3. Bring total to 1 ml volume with distilled water.

These solutions of varying concentrations of protein will provide the data that will give the standard curve.

Experiment 21: Separation of Chloroplast Pigments by Solvent Method

Aim/Objective

To Carry out separation of chloroplast pigments by solvent method:

Principle

Chlorophyll is a green pigment found in the leaves of aerial part of plants such as young stem, fruits and sepals of flower. Chlorophyll is a green pigment consisting of a tetrapyrrole ring with a central magnesium ion. It has a long hydrophobic phytol chain in its structure. Six different types of chlorophyll are known viz, chl-a, b, c, d, e and bacteriochlorophyll. They are found to be distributed in the plant kingdom; of these chl-a and b are found in all higher green plants. The difference between these two chlorophyll molecules is that chl-a has a methyl group and chl-b has a formyl group. The ratio of chl-a to chl-b in higher plants is approximately 3:1. Chl-a is the primary pigment while chl-b is the accessory pigment that collects

energy and passes it on to chl-a. Chlorophyll absorbs light mainly in the red (650 – 700 nm) and the blue - violet (400 – 500 nm) regions of the visible spectrum. Green light (~550 nm) is not absorbed but reflected giving chlorophyll its characteristic color.

The chlorophyll content in an extract can be measured by using the following equation:

Chlorophyll a (mg/g tissue) = 12.7 (A663) - 2.69 × (A645) × V/ (1000 ×W)

Chlorophyll b (mg/g tissue) = 22.9 (A645) - 4.68 × (A663) × V/ (1000 × W)

Total Chlorophyll (mg/g tissue) = 20.2 (A645) + 8.02 × (A663) × V/ (1000 ×W)

Where, A = Absorbance at specific wave length, V = Volume of chlorophyll extract in 80% acetone, W = Fresh weight of the leaf tissue taken for the extraction.

Materials Required

Fresh leaves (Spinach or any plant species of that particular region), 80% Acetone, Spectrophotometer, Centrifuge, Mortar and pestle, Volumetric flask (100 ml), Water.

Procedure

1. Take 1g of finely cut and well mixed representative sample of leaf into a clean mortar.
2. The leaf tissue is now ground into a fine pulp with the addition of 20 ml 80% acetone.
3. Then, the homogenate is centrifuged at 5000 rpm for 5 minutes and the supernatant is transferred to a 100 ml volumetric flask.
4. The residue is ground with 20 ml of 80% acetone and centrifuged at 5000 rpm for 5 minutes. The supernatant is then transferred to the volumetric flask.
5. The above mentioned step is repeated till the residue is colourless.
6. The mortar and pestle are thoroughly washed with 80% acetone and the clear washings are collected in the volumetric flask and the volume is made up to 100 ml with 80% acetone.
7. The absorption at 663 nm, 645 nm and 652 nm are read against a blank of 80% acetone solution.

Calculations

Calculated the amount of chlorophyll present in the extract as mg chlorophyll per g tissue using the equation.

Chlorophyll a = 12.7 (A 663) - 2.69 (A 645) x V/ (1000 ×W)

Chlorophyll b = 22.9 (A 645) - 4.68(A 663) x V/ (1000 ×W)

Total Chlorophyll = 20.2 (A 645) + 8.02 (A 663) x V/ (1000 ×W)

Where 'V' final volume of chlorophyll extract in 80% acetone; 'W' is fresh weight of tissue collected.

Results

The total amount of chlorophyll present in the spinach leaf sample————

Amount of Chlorophyll a ———————— and Chlorophyll b is ————————

Precautions

1. Extractions are to be done carefully.
2. Calibrate the spectrophotometer with the blank before taking measurements of the sample.

Experiment 22: Determining the Osmotic Potential of Vacuolar Sap by Plasmolytic Method

Aim/Objective

To determining the osmotic potential of vacuolar sap by plasmolytic method

Principle

The phenomenon 'Osmosis' refers to the movement of water from a solution of higher water potential to one of lower water potential, across a differentially permeable membrane which separates the two solutions. The magnitude of osmotic forces in plant cells and tissues can be estimated in terms of solute potential (Ψ S), which was formerly termed as 'Osmotic Pressure'. The solute potential is expressed in bars with a negative sign.

There are several methods available at present for the measurement of solute potential in plant systems — using thermocouple psychomotor or by membrane osmometer, or by cryoscopic method, or by plasmolytic method.

In the plasmolytic method — which is based on the phenomenon of plasmolysis — a solution is identified which will cause only slight —just barely visible — separation of the protoplast from the cell wall.

This condition is known as 'incipient plasmolysis'. At incipient plasmolysis, the cell wall exerts no pressure on the cell contents (i.e. pressure potential is zero). Therefore, the water potential in the cell is equal to the solute potential.

Water potential (Ψ w) = Solute potential (Ψ s) + Pressure potential (Ψ p).

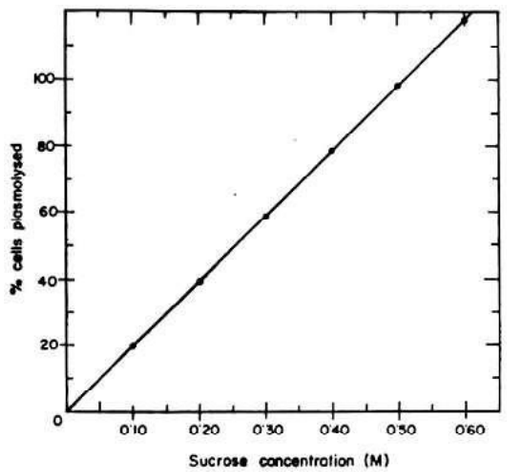
Materials and Equipment Required

1. 12 Beakers (50 ml) containing 20 ml of one of the following: Water, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50 and 0.60 Molar sucrose solutions.
2. Lower epidermal pealing's of Rhoeo leaves.
3. Microscope.
4. Slide, Cover glasses, Forceps, Scalpel, Graph papers, Blotting paper, etc.

Procedure

1. Take several thin pealing's of lower epidermal layer of Rhoeo leaves and then dip in water.
2. Examine the uniformness of the pealing's before placing them in different test solutions separately.
3. Dip the pealing's in test solutions for 20-25 minutes.
4. Examine the pealing's under microscope to determine the number of cells plasmolysed against each test solution. Finally calculate the % of plasmolysis in each test solution and graphically plot the data.
5. Record the molar concentration from the graph as shown below in the figure at which 50% plasmolysis is possible.

The figure given below illustrates the graphical representation of cell plasmolysis at different sucrose concentrations.



6. Solute potential in them determined by the following formula:

   $\Psi_s = miRT$

   Where, m = Molarity of solution

   i = Ionization constant, numerical value of 1 for sucrose

   R = Gas constant (0.083 litre bars/mol. degree)

   T = Absolute temperature (= °C + 273)

Results

Measurement of the solute potential of plant tissues by plasmolytic method.

Experimental sets	Sucrose Conc. (M)	No. of Observations	Total No. of cells observed per field	Total No. of Plasmolysed cells per field	Percentage cells plasmolysed (average)
I	0.00	1	—	—	
		2	—	—	—
		3	—	—	
II	11.05	1	—	—	
		2	—	—	—
		3	—	—	
III	0.10	1	—	—	
		2	—	—	—
		3	—	—	
IV	0.15	1	—	—	
		2	—	—	—
		3	—	—	
V	0.20	1	—	—	
		2	—	—	—
		3	—	—	
VI	0.25	1	—	—	
		2	—	—	—
		3	—	—	
VII	0.30	1	—	—	
		2	—	—	—
		3	—	—	
VIII	0.35	1	—	—	
		2	—	—	—
		3	—	—	
IX	0.40	1	—	—	
		2	—	—	—
		3	—	—	
X	0.45	1	—	—	
		2	—	—	—
		3	—	—	
XI	0.50	1	—	—	
		2	—	—	—
		3	—	—	
XII	0.60	1	—	—	
		2	—	—	—
		3	—	—	

Experiment 23: Determining the Water Potential of Any Tuber

Aim/Objective

To determining the water potential of any tuber:

Principle

The water potential of a plant tissue (tuber) can be determined by the following given principle. While experiment, if the tissue shows no net gain or loss of water when immersed in a solution of known molarity, its water potential is equal to that of the external solution. Samples of the tissue are allowed to come to equilibrium

in a range of solutions of different concentrations. When the tissue shows neither an increase nor a decrease in mass or length, the water potential of the potato tissue is the same as that of the external solution.

Materials Required

Potato, 1M Sucrose solution, Distilled water, Boiling tubes, Cork borer, Six discs, Filter paper, Blotting paper.

A. By Length

Procedure

1. Use 1M sucrose solution and distilled water to make a series of 10 cm^3 sucrose solutions in different boiling tubes of different concentrations: 1M, 0.8M, 0.6M, 0.4M 0.2M and 0.0M. Label these boiling tubes.
2. Use a cork borer to cut cylinders of potato tissue with the same diameter. Cut all of them to the same length of 5 cm. It is very important to work quickly to avoid loss of water through evaporation and this would lower the water potential of the tissue.
3. Immerse two potato cylinders in each tube and then cover the tubes with sealing film.
4. Leave the set up for one hour.
5. Remove the sealing and pick the cylinders from each tube. Measure the length of the cylinders, and calculate the percentage change in length using the formula given below:

$$\% \text{ change in length} = \frac{final\ length - initial\ length}{initial\ length} \times 100\%$$

6. Get the mean percentage change in length of the cylinders at each concentration.
7. Now, plot a graph of the mean percentage change in length (vertical axis) against the concentration of sucrose solution (horizontal axis).
8. From the graph, we can determine the concentration of sucrose solution that causes no change in length of the tissue.
9. The water potential of the potato tissue can be expressed in terms of the molarity of sucrose solution that causes no change in length of the tissue.

B. By Weight

Procedure

1. Repeat same Steps (1) and (2) given in method A.
2. Slice up each cylinder approximately equal thickness into six discs. Place each group of discs on a separate piece of filter paper to blot dry the water on the surfaces.

3. Weigh each group of discs and record the results.
4. Put the groups of discs in each of the labelled tubes. Cover the tubes with sealing film.
5. Leave the set up for one hour.
6. Remove the discs from each tube. Now, blot off any surplus fluid quickly and gently with filter paper and re-weigh them. Record the new weight of each group of discs.
7. Calculate the percentage change in weight using the formula given below:

   $$\% \text{ change in length} = \frac{\text{final weight} - \text{initial weight}}{\text{initial weight}} \times 100\%$$

8. Now, plot a graph of the percentage change in weight (vertical axis) against the concentration of sucrose solution (horizontal axis).
9. From the graph we can determine the concentration of sucrose solution which causes no change in weight of the tissue.
10. The water potential of the potato tissue can be expressed in terms of the molarity of the sucrose solution that causes no change in weight of the tissue.

Experiment 24: Separation of Amino Acids by Paper Chromatography

Aim/Objective

To separate amino acids by paper chromatography by ascending method.

Principle

Chromatography is a separation technique that relies on the different solubility of solutes in solvents. Therefore, the separation of components from a mixture can be achieved if the solutes are partitioned between two or more phases.

Paper chromatography is thus a type of partition chromatography in which the differences in the partition coefficients of the substances to be separated are used for separating them. When solvent system containing both hydrophilic and hydrophobic components (mobile phase) migrates on a paper, the hydrophilic solvent is absorbed on the cellulose, which acts as the stationary phase whereas the hydrophobic one does not.

Thus, a phase separation takes place at the micro level. When the solvent front reaches the spot where the compounds have been spotted, the compounds get partitioned as the solvent front migrates further by capillary action and separation is achieved. The separated amino acids are visualized by spraying a solution of ninhydrin (trihydrindene hydrate) and heating the paper. The amino acid spots appear purple. The two amino acids proline and hydroxyl proline appear yellow. Ninhydrin deaminates and decarboxylates the amino acids and at the same time gets reduced to hydrindantin. This hydrindantin then combines with another molecule of ninhydrin and one molecule of ammonia to form a purple coloured complex (Ruhemann's purple reaction).

Ratio of distance travelled by the solute to the distance travelled by the solvent from the origin of the spot is termed as Rf value (Retention value/factor). Different substances have different characteristic Rf values.

Materials Required

1. **Chemicals/Reagents:** Reference amino acids (leucine, lysine and proline), Mixture of unknown amino acids, Solvent system, Butanol: Acetic acid: Distilled water in a ratio of 4:1:5, 0.1 % Ninhydrin in Acetone.
2. **Equipment:** Chromatographic chamber.
3. **Glassware/Plastic ware:** Capillary tubes, Hot plate 3.
4. Pencil, Scale, Strip of Whatman No.1 chromatography paper (about 15 mm x 200 mm).

Procedure

1. Fill the chromatographic chamber to height of about 1 cm with solvent system.
2. Now, close the lid and allow equilibrating for at least 30 minutes.
3. On the end of the chromatographic paper, draw a light horizontal line with pencil about 1.5 cm from the bottom. This is called the base line.
4. On this line, mark 4 equidistant spots and label these spots as Le (for Leucine), Ly (for Lysine), P (for Proline), and M (for Mixture).
5. Transfer a very small drop of appropriate solution to the spots with the help of capillary tube. Allow to dry.
6. Insert the chromatographic paper into the chamber in such a way that the end near the spot is immersed into the solvent system and the spots are about 5-6 mm above the solvent level. Close the chamber and allow the solvent to run. Take care not to allow the paper to touch either the bottom or the sides of the chamber.
7. When the solvent has migrated to about 10-15 cm, remove it from the chamber and then mark the solvent level.
8. Dry the paper.
9. Spray ninhydrin and heat it on a hot plate or can be dry in a heated oven.
10. When the spots are visible, mark their outlines tightly with pencil.
11. Measure the distance from the origin (base line) to the solvent front (distance travelled by solvent).
12. Mark centre of spots, measure the distance travelled by solute and tabulate them.

Observations

It is found that:

1. Amino acid Leucine having the highest position coefficient migrates the longest distance.

2. Lysine with the lowest partition coefficient migrates the minimum distance.
3. Proline takes a position in between the two on spraying with ninhydrin.
4. Leucine and lysine develop into dark purple spots with strong heat.
5. Proline which is an amino acid develops yellow colour.
6. Measure the distance travelled by the solvent system and each amino acid and tabulate.

Name of the Amino Acid	Leucine	Lysine	Proline	Mixture
Distance Travelled by the Solvent				
Distance Travelled by the Amino Acid				

Calculations

$$\text{Rf values of each Amino Acids} = \frac{\text{Distance travelled by the solute}}{\text{Distance travelled by the solvent}}$$

Results

1. Rf value of Leucine = ——————————-
2. Rf value of Lysine = ——————————-
3. Rf value of Proline = ——————————

Conclusion

1. Rf value of given mixture matches with Rf value of reference amino acids, such as leucine, lysine and proline.
2. Leucine is a neutral, hydrophobic amino acid and migrates maximum distance because it has maximum solubility in butanol and a high partition coefficient.
3. Proline migrates a relatively closer distance because it has partial positive charge.
4. Lysine is a hydrophilic amino acid and migrate minimum distance as it is highly soluble in aqueous medium and least soluble in organic solvent.

Precautions

1. Use clean glassware for setting up the experiment.
2. Do not touch the chromatography paper with hands.
3. The chromatogram should remain in the stretched position on the rim of the petri dish.
4. The chromatogram should be dried properly before introducing it in the chromatography chamber.
5. The solvent mark should be marked immediately with a pencil after removing it from the chromatography chamber.
6. Be very careful while spraying Ninhydrin on the chromatogram (avoid skin contact).

Experiment 25: Comparison of the Rate of Respiration of Various Plant Parts

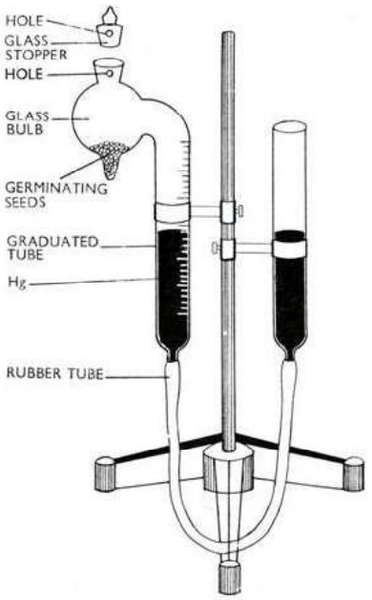
Aim/Objective

Determination of 'Rate of Respiration' in various plant parts.

Materials Required

Respiring materials (Germinating seeds, Floral buds, Roots, Leaves), Ganong's respirometer, 10% KOH solution.

The rate of respiration can be measured in various respiring materials with the help of Ganong's respirometer which is shown in the picture given below:



Description of the Apparatus

The apparatus consists of three levelling tube parts.

1. The bulb is used for the respiring material which ends in a 10% KOH win smaller bulb at the bottom. The bigger bulb is provided with a stopper having a lateral hole, through which atmospheric con-nection can be made by turning the stopper.
2. With the bulb a graduated manometer is fitted.
3. A levelling tube is connected with the manometer tube by rubber tubing. The whole apparatus is clamped on a stand.

Procedure

1. Two ml of each respiring material (measured by displacement of water) are placed into the bigger bulb of different respirometer.

2. A 10% solution of KOH is taken in the manometer tube. In the beginning of the experiment, the air around the material is brought to the atmospheric pressure by turning the stopper of the bulb until its hole coincides with that of the neck of the bulb.
3. The levelling of the reservoir tube on the right is so adjusted that the KOH solution in the tube is at the 100 ml mark at the bottom of the manometer. Two ml of respiring material is now surrounded by 100 ml of air.
4. The experiment is started by turning the glass stopper at the top and thus cutting off connection with the atmospheric air.

Results

As the respiration takes place in a closed space, the solution in the manometer tube rises up gradually. The reading should be taken up to 80 ml mark, i.e., up to 20 ml volume (since atmospheric oxygen is 20%) at an interval of 10 minutes, each time bringing the liquid in both the tubes at the same level, i.e., the liquid in the closed tube is brought under atmospheric pressure.

The volume of CO_2 evolved at an interval of 10 minutes is recorded in each case and rates of respiration are graphically plotted for each sample of plant material and compared.

Observation/Discussion

The released CO_2, on coming in contact with KOH solution, is absorbed by it, oxygen is consumed and as a result KOH solution rises up in the manometer tube. The rate of rise of KOH solution is taken as a measure of rate of aerobic respiration in terms of volume of O_2consumed per unit time per 2 ml of respiring material.

One-fifth volume of atmos-pheric air is O_2. Hence out of 100 ml of enclosed air within respirometer there is 20 ml of O_2. Hence reading should be taken up to 20 ml rise in volume of KOH solution. After that anaerobic respiration will start.

The rate of respiration is always higher in younger actively growing meristematic tissues than that of older and mature parts. There is a direct relationship between the amount of protoplasm and the rate of respiration the greater the protoplasm, the higher is the respiration rate.

The hydration of protoplasm and quantity of respiratory enzymes are always greater in young cells compared to mature and vacuolated cells. Hence the respiratory rate is always higher in young cells which are rich in protoplasm.

Experiment 26: Protein, Carbohydrate Estimation
A - Carbohydrate Estimation

Aim/Objective

To estimate the amount of carbohydrate present in the given sample by *Anthrone method*.

Principle

Carbohydrates are compounds produced during photosynthesis. In plants, they have two main purposes. First, they provide building blocks for plant structural components, such as cellulose (important in building cell walls). Secondly, carbohydrates are molecules that deliver energy for plant growth. The carbohydrates are stored in form of free sugars and polysaccharides. The basic units of carbohydrates are Monosaccharides. When carbohydrates hydrolyse, gives monosaccharides, but when monosaccharides hydrolyse it cannot be split into more simpler forms of sugars. The hydrolysed product of Polysaccharide are estimating by the resultant monosaccharides

Carbohydrates are dehydrated with concentrated H_2SO_4 to form "**Furfural**", which condenses with **Anthrone** to form a **green color complex** which can be measured by using colorimetrically at 620 nm (or) by using a red filter. Anthrone react with dextrins, monosaccharides, disaccharides, polysaccharides, starch, gums and glycosides. But they yields of colour where is to form carbohydrate to carbohydrate.

Materials/Reagents Required

1. 2.5 N HCl
2. ***Anthrone Reagent:*** Dissolve 200 mg Anthrone in 100 ml of ice cold 95% H_2SO_4. Prepare fresh before use.
3. ***Standard Glucose Solution:*** Stock – Dissolve 100 mg in 100 ml water. Working standard – 10 ml of stock diluted to 100 ml with distilled water. Store refrigerated after adding a few drops of toluene.
4. Weighing balance, Spectrophotometer, Test tubes, Beakers, Micropipette and Hot plate.

Procedure

1. Weigh 100 mg of the sample into a boiling tube.
2. Hydrolyse by keeping it in boiling water bath for 3 hours with 5 ml of 2.5 N HCl and cool to room temperature.
3. Neutralise it with solid sodium carbonate until the effervescence ceases.
4. Make up the volume to 100 ml and centrifuge.
5. Collect the supernatant and take 0.5 and 1 ml aliquots for analysis.
6. Prepare the standards by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard. '0' serves as blank.
7. Make up the volume to 1 ml in all the tubes including the sample tubes by adding distilled water.
8. Then add 4 ml of Anthrone reagent.
9. Heat for eight minutes in a boiling water bath.
10. Cool rapidly and read the green to dark green colour at 630 nm.
11. Draw a standard graph by plotting concentration of the standard on the *X*-axis versus absorbance on the *Y*-axis.
12. From the graph calculate the amount of carbohydrate present in the sample tube.

Calculation

Amount of Carbohydrate Present in 100 mg of the Sample = (mg of Glucose / Volume of Test Sample) X 100

Note

Cool the contents of all the tubes on ice before adding ice-cold Anthrone reagent.

B - Protein Estimation

Aim/Objective

To estimate the concentration of proteins by Lowry method from the given sample.

Principle

The Blue colour produced by the reduction of the Phosphomolybdic-Phosphotungastic components in the Folin-ciocalteau reagent by the amino acids Tyrosine and Tryptophan present in the protein plus the colour developed by the biuret reaction of the protein with the colour developed by the biuret reaction of the protein with the alkaline cupric tartarate are measured in the Lowry method.

Reagents for Lowry Method

1. **Lowry Reagent (or) Alkaline Copper Sulphate Solution:** Mix 50 mL of Solution A with 1 mL of Solution B, just prior to use.
   - **Solution A :** 2% sSodium carbonate in 0.1N NaOH.
   - **Solution B :** 0.5% Copper sulphate solution in 1% Sodium potassium tartarate solution (to be prepared fresh)
2. **Folin-Ciocalteau Reagent:** This is commercially available and has to be diluted with equal volume of water just before use.
3. **Standard Protein Solution:** Dissolve 200 mg of BSA in 100 mL of distilled water in a volumetric flask. (Concentration-2mg/mL)
4. **Working Standard:** Dilute 10ml of stock standard solution to 100ml of distilled water. (Concentration – 200mg/mL).

Procedure

1. Pipette out into a series of tubes 0.2, 0.4, 0.6, 0.8, and 1.0 of the protein solution and make up the total volume to 1ml with addition of water.
2. To each tube 5ml of the alkaline-copper sulphate solution is pipette out, mixed well and allowed to standard at room temperature for 10 to 15 minutes. 0.5ml of the reagent is pipettes out into each tube, mixing rapidly after each addition. The tubes are left as such for 30 minutes and the blue colour formed is measured at 700nm ((or) red filter).
3. Prepare a Blank with 1 mL of distilled water, instead of protein solution and with 1ml of unknown solution and proceeds as per standards.
4. Prepare a calibration curve with mg of protein on X-axis and O.D. on Y-axis and determine the amount of protein present in a given unknown sample.

Results

The concentration of protein in the given unknown sample is __mg/mL.

Experiment 27: Estimation of Nitrogenase Enzymes

Aim/Objective

To discuss about the principle, requirements and procedure for estimation of nitrogenase.

Nitrogenase is possessed by a group of prokaryotes which breaks the triple bond of elemental nitrogen (N≡N) present in the at-mosphere and convert it into NH_3, an as similable form for plants. This enzyme can also reduce acetylene to ethylene.

Principle

Nitrogenase reduces acetylene to ethylene and this ethylene is measured in a gas liquid chro-matograph (GLC). The activity is expressed as 'n' mole ethylene produced for unit time per gram of dry nodules.

Materials Required

1. Root nodules—Phaseolus.
2. Gas chromatograph with flame ioniza-tion detector.
3. Acetylene and ethylene gases.
4. Air tight syringes.
5. 100 mL conical flasks with small mouth to fit serum caps.
6. Conditions for operating GLC.
7. Carrier gas N_2/helium/argon having flow rate 30-45 mL.
8. Column-Poropak, NRT or Silica Gel

	Poropack	Silica gel
Temperature of oven/column	60°C	150°C
Temperature of injector	65°C	160°C
Temperature of detector	85°C	175°C
Minimum retention time for ethylene	1.3	1.5
Minimum retention time for acetylene	1.8	3.0

9. Gas for detector-H_2 and air.

Procedure

1. First inject pure acetylene and make the sample peak.
2. Without disturbing any nodule, remove Phaseolus from soil.
3. Now, excise root system as such. Do not sepa-rate nodules from roots.
4. Put the root system into a 100 mL coni-cal flask and seal the mouth of flask with serum cap.

5. By using an air tight syringe remove 10 mL of air from the flask and inject 10ml acetylene into the flask.
6. Incubate it at room temperature for 30- 60 minutes.
7. With an air tight syringe take 0.5 to 1.0 mL of gas mixture from the flask and inject it into a preconditioned GLC. Observe the acetylene and ethylene peaks and measure the height of the ethylene peak.
8. Remove nodules from the roots and get their dry weight.
9. A similar control assay should be con-ducted by injecting 10µg of pure ethylene into a 100 mL sealed conical flask.
10. Remove 0.5 to 1.0 mL of ethylene, inject into GLC and measure ethylene peak.

Calculations

1. Standard amount of Ethylene (E) in µ mol = 0.446 X2µL /Peak height in mm X attenuation.
2. Amount of ethylene produced in µ mol in the sample = E X Peak height of sam-ple ethylene in mm X attenuation.
3. Activity of nitrogenase = n mol or µ mol ethylene per unit sample per unit time (Unit sample = Dry weight of nodules).

Experiment 28: Acid and Alkaline Acid Phosphatase Activity

Aim/Objective

To study the acid and alkaline acid phosphatase.

Principle

Phosphatases liberate inorganic phosphate from organic phosphate esters. Acid phosphatase (3.1.3.2) hydrolyzes a number of phosphomonoesters and phosphoproteins. Alkaline phosphatase catalyses the hydrolysis of numerous phosphate esters, such as esters of primary and secondary alcohols, sugar alcohols, phenols and amines. Phosphodiesters are not hydrolyzed by either of them.

The enzyme phosphatase hydrolyzes p-nitro-phenol phosphate. This released p-nitro-phenol is yellow in colour in alkaline medium and is measured at 405 nm. The optimum pH for acid and alkaline phos-phatases are 5.3 and 10.5, respectively.

Materials Required

(i) Sodium Hydroxide 0.085 N: Dissolve 0.85 g sodium hydroxide in 250 mL water.

(ii) Substrate Solution: Dissolve 1.49 g EDTA, 0.84 g citric acid and 0.03 g p-nitro-phenyl phosphate in 100 mL water and adjust to pH 5.3.

(iii) Standard: Weigh 69.75 mg p-nitro-phenol and dissolve in 5.0 mL distilled water (100 mM).

(iv) Enzyme Extract: Homogenize 1 g fresh tissue in 10 mL of ice-cold 50 mM citrate buffer (pH 5.3) in a pre-chilled pestle and mortar. Filter through four layers of cheese cloth. Centrifuge the filtrate at 10,000 g for 10 min. Use the supernatant as enzyme source.

Procedure

1. 3 ml of substrate solution is incubated at 37°C for 5 minutes.
2. 0.5 mL enzyme extract is added and mixed well.
3. Immediately 0.05 mL is removed and mixed it with 9.5 mL of sodium hydroxide 0.085 N. This corresponds to zero time assay (blank).
4. The remaining solution (substrate + enzyme) is incubated for 15 min at 37°C.
5. 0.5 mL sample is drawn and mixed with 9.5 mL sodium hydroxide solution.
6. The absorbance of blank and incubated tubes is measured at 405 nm.
7. 0.2 to 1.0 mL (4 to 20 mM) of the standard is taken, and diluted to 10.0 ml with NaOH solution. The colour is read. Then draw the standard curve.

Calculation

Specific activity is expressed as m moles p-nitro-phenol released per min per mg protein.

Notes

1. For alkaline phosphatase extract the enzyme in 50 mM glycine NaOH buffer pH 10.4.
2. Alkaline phosphatase functions optimally at about pH 10.5. The assay procedure is similar to that for acid phosphatase, except for the substrate solution.

Prepare the Substrate Solution As Follows

Dissolve 375 mg glycine, 10 mg magnesium chloride, 165 mg p-nitro-phenyl phosphate in 42 ml of 0.1 N sodium hydroxide and dilute to 100 ml. Adjust to pH 10.5.

Experiment 29: Isolation And Identification Of Rhizobium From Leguminous Plants

Aim/Objective

Study about isolation and identification of rhizobium species from root nodules.

Principle

The green revolution has led to intensified agriculture to meet the ever increasing demands for food and fibre. In 2011, the administration of President Goodluck Ebele Jonathan launched an Agricultural Transformation Agenda to promote agriculture as a business, integrate the agricultural value chain and make agriculture the key driver of Nigeria's economic growth. However, the drive to intensify agriculture has constantly been challenged by poor soil fertility and to circumvent this, farmers have improved soil fertility using synthetic fertilizers.

Nitrogen is an essential plant nutrient being a component of amino acids, nucleic acids, nucleotides, chlorophyll, enzymes and hormones. However, almost all soils are deficient of nitrogen; hence nitrogen is considered a limiting element. Although nitrogen occupies about 78% of the atmospheric air, it is not readily accessible to plant unless in the form of soil nitrate. Synthetic fertilizers for improving soil fertility are rarely available to most farmers especially in the rural areas of Nigeria. In addition, these fertilizers are not environmental friendly and may induce soil acidification and reduce in efficiency after many cropping years, leading to a high dependence of soil on nitrogen fertilizers for optimum yield.

Interestingly, some plants (legumes) possess a unique ability to establish symbiotic association with nitrogen-fixing bacteria of the family Rhizobiaceae. Rhizobium inoculants significantly improves yield in many leguminous crops and can minimize the use of synthetic fertilizer which is rather expensive and deteriorates soil properties. Their ability to fix nitrogen in symbiosis makes them excellent colonizers of low nitrogen environment and economically friendly crop pasture. In addition, nitrogen from legume fixation is essentially 'free' for use by both the host plant and associated or subsequent crops. A well-established practice for maintaining soil fertility has been the cultivation of leguminous plants which replenish atmospheric nitrogen through symbiosis with Rhizobium species in rotation with non-leguminous plants. Groundnut and fluted pumpkin are two important indigenous food crops in Nigeria. Groundnut, also known as peanut (*Arachis hypogaea L.*) is crop of global importance. It is widely grown in the tropics and subtropics, and classified both as a grain legume and an oil crop. Like most other legumes, groundnuts harbour nitrogen-fixing bacteria in their root nodules, making then require less nitrogen-containing fertilizer and valuable in crop rotations. Fluted pumpkin (*Telfairia occidentalis*) is a tropical vine grown in West Africa as a leafy vegetable and for its edible seeds. It is indigenous to southern Nigeria, where it is used primarily in soup and herbal medicine. The fluted gourd is high in oil (30%), the shoot is high in potassium and iron, while the seeds are composed of 27% crude protein and 53% fat. The leaves contain a high amount of antioxidants and hepatoprotective and antimicrobial properties.

Although this vegetable is widely eaten in southern Nigeria for its nutritional and medicinal values, adequate information is lacking about its contribution to soil fertility and nutrient cycling. Therefore, this study was carried out to isolate and identify the nitrogen-fixing bacterium (Rhizobium spp.) from *Arachis hypogaea L.* and *Telfairia occidentalis* so as to explore their environmental friendly contributions to soil fertilization.

Methods

1. Collection of Nodulated Roots of *Arachis hypogaea L.* and *Telfairia occidentalis*

A total of ten nodulated plants (five each of pumpkin and groundnut) were collected from organic farms at Ezzamgbo and Ihiala (both in south-east, Nigeria) respectively. Healthy pumpkin plants were uprooted carefully and those plants possessing healthy nodules with pink colour were selected and

transported to the Department of Applied Microbiology Laboratory Complex of Ebonyi State University, Abakaliki in polythene bags for immediate processing.

2. Isolation of Rhizobium Species

Isolation of rhizobium was done using Yeast Extract Mannitol Agar (YEMA). In this, healthy, unbroken, firm and pink nodules were selected for the isolation. They were washed under tap water to remove adhering mud and soil particles, after which they were treated carefully with 5% hydrogen peroxide for surface sterilization. The nodules were repeatedly washed in sterile water for 3-4 minutes to get rid of the sterilant and then treated with 70% ethyl alcohol for about one minute and 0.1% $HgCl_2$ for two minutes. They were washed with sterile water (3 successive times) under aseptic conditions and crushed with sterile crucible. A suspension was made of the crushed nodules, plated on YEMA medium containing 1% Congo-red dye and incubated at 28±10C for 24 hours. Growth on YEMA plate was observed after the said incubation period.

3**. Identification of Rhizobium Species**

Pure cultures of the isolates were made and then subjected to Gram reaction. The Gram negative isolates were further subjected to biochemical tests including catalase, oxidase, voges-Proskauer and indole tests for confirmation. Flagellation test was carried out to test for motility using flagella mordant (Loffler's mordant).

4. Seed Inoculation of *Arachis hypogaea L.* and *Telfairia occidentalis* with Pure Culture of Rhizobium

Slants of modified Jensen's medium were made. Single seed producing radical in down position was aseptically transferred into slants. So that radical was in direct contact with the gelled medium. Then 2-5 drops of log phase culture of rhizobium isolated from pumpkin and groundnut were added and kept as uninoculated (control). The bases of the tubes were covered with black paper so as to create darkness required for growth of root system. Test tubes were plugged with non-absorbent cotton plug. The germinated seed were exposed to sunlight periodically. Tubes were incubated for 20-30 days and effect on growth in inoculated and uninoculated tubes was observed and recorded.

Results and Discussion

In this present study, strains of root nodulating bacteria were isolated from the root nodules of *Arachis hypogaea L*. and *Telfairia occidentalis* plants growing in South-East, Nigeria. During this study ten isolates of Rhizobium spp. were recovered from *Arachis hypogaea L*. and *Telfairia occidentalis* roots. The bacteria that nodulate these plants roots have been routinely considered as belonging to the miscellaneous group Bradyrhizobium spp., comprising a large number of slow-growing strains capable of nodulating several species of herbaceous legumes common in the tropical region. In this study, approximately all of isolates obtained

from the different samples showed rapid growth in culture medium indicating that the limit strain capable of *Arachis hypogaea L*. and *Telfairia occidentalis* nodular goes beyond the group's Bradyrhiobium spp. Some studies already showed the fast-growing Rhizobia are more common in arid regions. This feature is a survival strategy, since they are more drought tolerant than slow-growing and multiply rapidly in a short period of wet weather, which would explain its greater frequency in soils of semiarid regions.

The colonies of rhizobia species isolated from *Arachis hypogaea L*. and *Telfairia occidentalis* plants nodules isolated in this study were mucoid, rod-shaped, raised with smooth edges and musky odour of the colony was observed under low power microscope. Five samples each of root nodules from *Arachis hypogaea L*. and *Telfairia occidentalis* plants were found positive for Rhizobium spp. after screening through a series of various biochemical and sugar fermentative tests. The isolates showed hazy appearance on the motility media and also were positive for Catalase tests. The samples were found negative for Gram reaction tests. The results showed that all the isolates showed mucus production, although some have little mucus. The mucoid production would represent a mechanism involved in the process of adaptation and survival of Rhizobium in adverse conditions of soil and climate. The lack of description of strains that produce an excess of using exopolysaccharides in literature caused some omission on this group of bacteria for quite some time, believing that contaminants. The mucus production changes the permeability of the cells, making strains more resistant to biotic factors of competition on the soil in the presence of antibiotic producing microorganisms. There is a trend of increased mucus production by Bradyhizobium isolates, as a result of adaptation to conditions of acid soils of some areas. Isolated Rhizobium leguminosarum which were associated with groundnut. All the strains showed growth in three days and turned the yeast extract mannitol agar media containing bromothymol blue to yellow colour confirming that all were fast growers and acid producers.

The biochemical tests performed on the isolates showed that most were positive for catalase, Oxidase, Voges – Proskauer and indole tests. Only one Rhizobium isolate from pumpkin was negative to oxidase test.

Table below shows morphological and biochemical characteristics of Rhizobium species from Root Nodules of *Arachis hypogaea L*. (Groundnut) and *Telfairia occidentalis* (Fluted Pumpkin) in South-East, Nigeria.

Sample	*Colonial characteristics*	*GR*	*S*	*Catalase*	*Oxidase*	*Motility*	*VP*	*Indole*	*Suspected organism*
G1	Mg	-	Rd	+	+	+	+	+	*Rhizobium* spp.
G2	Mg	-	R	+	+	+	+	+	*Rhizobium* spp.
G3	Mg	-	Rd	+	+	+	+	+	*Rhizobium* spp.
G4	Mg	-	R	+	+	+	+	+	*Rhizobium* spp.
G5	Mg	-	Rd	+	+	+	+	+	*Rhizobium* spp.
P1	Mg	-	Rd	+	+	+	+	+	*Rhizobium* spp.
P2	Mg	-	R	+	+	+	+	+	*Rhizobium* spp.
P3	Mg	-	Rd	+	-	+	+	+	*Rhizobium* spp.
P4	Mg	-	R	+	+	+	+	+	*Rhizobium* spp.
P5	Mg	-	Rd	+	+	+	+	+	*Rhizobium* spp.

Key

Rd= Round Shape

R= Rod shape

Mg= Mucoid in Texture (Grey Colour)

GR= Gram Reaction

VP= Voges – Proskauer

S= Shape

Conclusion

Rhizobium is an important microorganism for the environment because of its nitrogen-fixing ability when in symbiotic relationship with plants (mainly legumes). This study confirmed that the root nodules of fluted pumpkin and groundnut plants harbor the nitrogen-fixing bacterium Rhizobium. It also showed that these plants when inoculated with Rhizobium isolates perform better. This organism will greatly enhance agricultural production, if they are often used to inoculate legume plants, thereby reducing the environmental threat of synthetic nitrogen fertilizers.

Experiment 30: Separation of Amino Acids by Paper Chromatography and Column Chromatography

Aim/Objective

To carry out separation of amino acids by paper chromatography:

Principle

Paper chromatography is a method through which a mixture of unknown amino acids can be separated and identified. The different positions of the amino acids in the chromatogram can be detected by spraying a chemical known as ninhydrin, which reacts with amino acids to yield highly coloured products.

Materials Required

Chemicals/Equipment

1. 2% Ammonia, (20 cm^3)
2. Propan-2-ol, (20 cm^3)
3. Aluminium foil
4. Ninhydrin spray (2% solution of ninhydrin in ethanol)
5. Four separate test tubes containing respectively: 0.05 M Glycine, 0.05 M Tyrosine, 0.05 M Leucine, and 0.05M Aspartic acid in 1.5% HCl, An unknown containing one to four of the above amino acids at a concentration of about 0.05 M each in 1.5% HCl.
6. Capillary tube, Chromatography paper (12 cm × 22 cm), 500 cm^3, Beaker, Oven.

Procedures

1. Mix 10 cm^3 of 2% ammonia solution with 20 cm^3 of propan-2-ol in a clean, 500 cm^3 beaker, and cover it tightly with a piece of aluminium foil. This mixture would be used as the solvent for the experiment.

Precaution/Warning: Propan-2-ol is flammable.

2. On a clean sheet of chromatography paper with size about 12 cm by 22 cm, mark a line with light pencil parallel to the bottom and about 1.5 cm away. On the line mark ten light crosses ("x") at intervals of about 2 cm.

   Label each cross as shown in Figure below.

   ('U' represents the unknown amino acid mixture.)

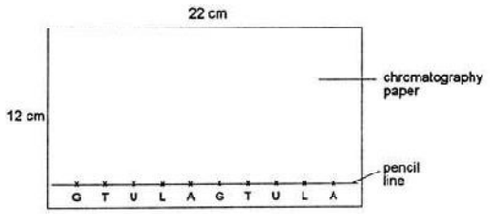


Figure 1

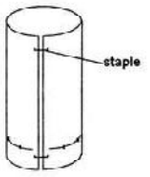


Figure 2

3. By using capillary tubes, place a small amounts of each appropriate solution on its two positions along the line on the chromatography paper. Avoid getting the spot on the paper larger than about 3 mm in diameter. Leave the paper for a few minutes in air to dry. Add a second portion of the unknown to one of its two positions, to make certain that sufficient quantities of each component of the unknown will be present for good visual observation when the paper is developed.
4. Roll the paper like a cylinder and staple the ends together in such a fashion that they do not touch each other. Otherwise the solvent will flow more rapidly at that point and form an uneven solvent front.
5. When the spots on the cylindrical paper become dry (it may be necessary to place the paper in an oven at about 100°C for a short time), place it in the beaker of solvent carefully, and then cover it tightly with the aluminium foil. It should be make sure that the paper does not touch any wall of the beaker.
6. Let the solvent rise up to the paper for at least 1.5 hours. If the time would be shorter, the components may not be sufficiently separated for easy identification. Remove the paper and place it upside down on the desk top to dry. When most of the solvent has evaporated, open the cylinder by tearing it apart where it was stapled and hang it in a fume cupboard. Now, spray ninhydrin solution on the paper lightly but completely, and leave the paper in the fume cupboard until the spray solution is dry.

   Precaution/Warning: The ninhydrin solution should be kept off the body because it reacts with proteins in the skin to form a rather long-lasting purple discoloration.

The teacher should ensure that student wear laboratory gowns, gloves and safety spectacles in carrying the experiment.

7. Dry the paper in an oven at 100-110°C for about 10 minutes, or until all the spots have developed.
8. Circle each spot with a pencil, and measure the distance each spot travelled (use the centre for the spot measurement). Measure the distance the solvent travelled at each position, and calculate the Rf values for each amino acid.

   Determine the composition of the unknown by visual comparison of spot colours and by comparing the Rf values.

   Rf1 =

   Rf2 =

   Rf3 =

   Rf4 =

To Separate Organic Compounds with the Help of Column Chromatographic Technique

Principle

Chromatography is a new method of separation of mixture of substances mainly when they are available in small amounts. This method is very useful when the components of a mixture have almost the same physical and chemical properties and hence they can't be separated by other usual methods of separations.

The separation methods in chromatography are based on the distribution of the components in a mixture between a fixed (stationary) and a moving (mobile) phase. The stationary phase may be a column of adsorbent, a paper, a thin layer of adsorbent on a glass plate, etc., through which the mobile phase moves on. The mobile phase may be a gas or a liquid. When a solid stationary phase is taken as a column, it is known as column chromatography.

Procedure

Here are the steps involved in Column Chromatography:

1. Preparation / Packing of an Adsorbent Column

Sucrose, starch, calcium carbonate, cellulose, calcium phosphate, calcium oxide, silica gel, charcoal, magnesium and alumina are the commonly used as adsorbents. Though, alumina is the strongest adsorbent giving best and accurate results.

Glass wool or cotton plug is utilised to give support to the column which is kept in a vertical position. The chromatography column is filled 1/3rd with the solvent and the adsorbent material is added slowly. It should be carefully poured down with a glass rod to prevent air bubble formation.

Wait till the suspension settles down and discard the excess solvent. Repeat the process until you get the column of 5 cm of height.

2. Solvent System / Mobile Phase

Column chromatography uses a single solvent. The commonly used solvents are petroleum ether, benzene, carbon tetrachloride, ethyl acetate, acetone, cyclohexane, ether, chloroform, etc.

The major role of the solvent in column chromatography is:

- Separate the mixture into different zones/bands.
- Introduce the mixture into the column
- Remove the pure components of each separated bands as eluents

3. Application of Samples

The study of sample can be applied to the top of the prepared column in various ways:

- Take away most of the mobile phase from above the column using a substrate and drain the remaining solvent into the column bed. Now, apply the sample carefully with a pipette and allow it to run in the column. To wash the resultant traces of the sample into the column bed, apply a small volume of the mobile phase. Carefully add the mobile phase to the column to the height of 2-5 cm and maintain it by connecting the column to a suitable reservoir of mobile phase.
- Add 1 % sucrose concentration to increase the density of the sample when the solution is layered into the liquid above the column bed; it will automatically sink to the surface of the column.
- Use capillary tubes or roller pumps to pass the sample directly to the column surface.

However, in all these cases, overloading with the sample should be avoided in the column, otherwise irregular separation will occur.

4. Sample Elution

After application of sample, the next step is to remove the material from the column using an appropriate solvent. This can be carried out in following ways:

(i) **Frontal development:** A large volume of the sample mixture is passed through a column of adsorbent. Pure solvent appears first followed by the least strongly held component of the mixture and so on. The components which are strongly held appear in pure form.

(ii) **Displacement development**: A solvent which has the higher affinity for the stationary phase than any of the components of the mixture is used. Consequently, all the components get displaced down of the column.

(iii) **Elution development:** Here, the components of the applied sample are separated by the continuous flow of the mobile phase through the column. The binding between the solvent and the column is weaker than the solute molecules. As a result, the bound molecule gets eluted from the column.

5. Collection and Analysis of Fraction

Collect the received fractions from the column into a series of test tubes, either with a fraction collector or manually. Now, analyse each fraction to know the presence of the compound. Coloured compounds can be detected by visual observation, but colourless compounds are detected by spectrophotometer or colorimetry or UV adsorption method.

M.Sc. [Botany]
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