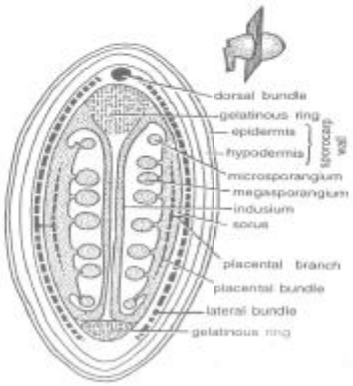
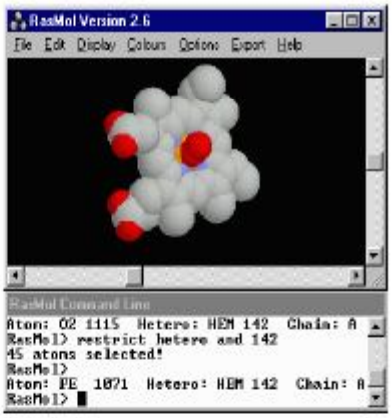
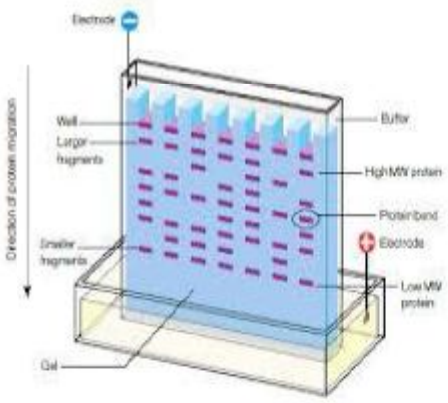
MBO-05

Vardhman Mahaveer Open University, Kota







Practical Botany-I

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School of Science & Technology
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Editing and Course Writing

Editors

Dr. Anuradha Dubey
Department of Botany
School of Science & Technology
Vardhman Mahaveer Open University,
Kota

Dr. Seema Bhaduriya
Department of Biology
JECRC University, Jaipur

Writers:

- **Dr. Seema Bhaduriya** 1,7,8,9
  Department of Biology
  JECRC University, Jaipur
- **Dr. Shalini Malhotra** 2,3,4,11, 12,13,14
  Department of Botany
  Tagore Biotech College, Jaipur
- **Dr. Anuradha Dubey** 10
  Department of Botany
  Vardhman Mahaveer Open
  University, Kota
- **Dr. Neerja Srivastava** 5,6,15,16
  Department of Botany
  Govt. College, Kota
- **Dr. Arvind Pareek** 17
  Director (Regional Centre)
  Vardhman Mahaveer Open
  University, Kota
- **Dr. Sujata Mathur** 18,19, 20
  Department of Botany
  Govt. College, Chimanpura

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Preface

The present book entitled **"Practical Botany-I"** has been designed so as to cover the unit-wise syllabus of MBO-05 course for M.Sc. Botany (Previous) students of Vardhman Mahaveer Open University, Kota. The basic principles and theory have been explained in simple, concise and lucid manner. Adequate examples, diagrammes, photographs and self-learning exercises have also been included to enable the students to grasp the subject easily. The unit writers have consulted various standard books and manuals on the subject and they are thankful to the authors of these reference books.

Unit - 1

Laboratory Introduction and Methodology

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Structure of the Unit

1.0 Objectives

After going through this unit you will be able to:

- Demonstrate the major parts and correct use of the compound light microscope
- Calibration of Microscope
- Cleaning of Glasswares
- The types of culture media needed to develop and maintain pure culture

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- The concept of sterility and the procedures necessary for successful subculturing of microorganisms
- Streak plate and spread plate inoculations for separation of microorganisms in a mixed microbial population for subsequent pure culture isolation

1.1 Introduction

Bacteria are found in every habitat on Earth: soil, rock, oceans and even arctic snow. Some live in or on other organisms including plants and animals including humans. There are approximately 10 times as many bacterial cells as human cells in the human body. A lot of these bacterial cells are found lining the digestive system. Some bacteria live in the soil or on dead plant matter where they play an important role in the cycling of nutrients. Some types cause food spoilage and crop damage but others are incredibly useful in the production of fermented foods such as yoghurt and soy sauce. Relatively few bacteria are parasites or pathogens that cause disease in animals and plants.

1.2 Laboratory Introduction and Lab rules

1. Wash hands before leaving lab.
2. Clean the lab table before and after lab with the 10% bleach solution provided.
3. If you are allergic to any antibiotics please inform the instructor immediately.
4. Because the microorganisms used in this class are potentially harmful (BL II), NO eating or drinking is allowed in the lab.
5. All materials and clothes other than those needed for the laboratory are to be kept away from the work area.
6. Any item contaminated with bacteria or body fluids must be disposed of properly. Disposable items are to be placed in the BIOHAZARD container. Reusable items are to be placed in the designated area for autoclaving prior to cleaning. Sharps are to be disposed of in the appropriate container.
7. Cuts and scratches must be covered with Band-Aids. Disposable gloves will be provided on request.
8. Long hair should be tied back while in lab.
9. All accidents, cuts, and any damaged glassware or equipment should be reported to the lab instructor immediately.

10. It is the responsibility of the student to know the location and use of all safety equipment in the lab (eyewash, fire extinguisher, etc.)

11. Reusable items should have all tape and marks removed by the student before being autoclaved.

NOTES

12. Read labs before coming to class and be on time. Lab instructions will not be repeated if you are late. Do not forget your lab manual. Wait for a laboratory introduction by the instructor before starting work.

13. You may want to wear old clothes to lab. We occasional work with stains that may permanently damage clothing. A limited number of lab coats are available upon request.

1.2.1 Microscopy

Microorganisms are too small to be seen with the naked eye so a microscope must be used to visualize these organisms. While a microscope is not difficult to use it does require some practice to develop the skills necessary to use the microscope to its maximum capabilities.

Bacteria and other cellular microorganisms are measured in micrometers (μm) or 1 x 10-6 meters. There microscopes used in an introductory microbiology laboratory is a compound light or bright-field microscope. All light microscopes have the same basic features shown in Fig. 1.1. A compound microscope consists of at least two magnifying lenses. One magnifying lens is in the ocular and one is in the objective. Each contributes to the magnification of the object on the stage. The total magnification of any set of lenses is determined by multiplying the magnification of the objective by the magnification of the ocular. The turret rotates allowing the objectives to change and thus change the magnification of the microscope. An iris diaphragm below the stage should be used to control the amount of light passing through a specimen. Less light is need at low magnification than at higher magnification.

Too much light at low magnification may mask the specimen, particularly something as small as a bacterial cell.

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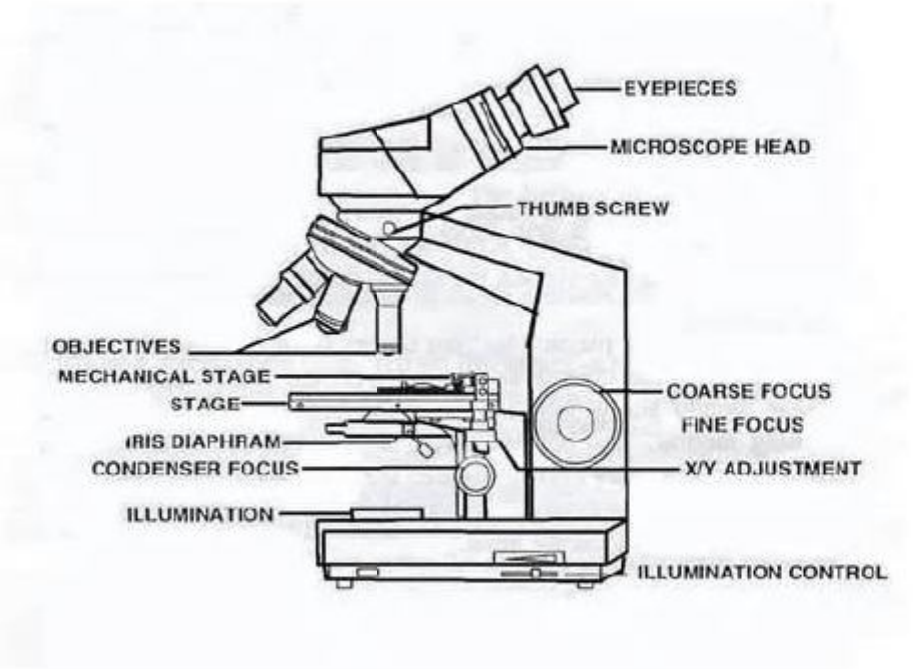

Fig. 1.1 : A Typical Compound Light Microscope

The distance between the specimen on the stage and the objective is known as the working distance. The coarse adjustme18nt knob will cause the working distance to visibly change while the fine adjustment knob is for final, fine focusing. The ability to see things using a microscope is limited by the resolving power of the microscope. The resolving power of a microscope is the distance two objects must be apart and still be seen as separate and distinct.

For the light microscope this is approximately 0.2 μm. Objects closer together than 0.2 μm will not be distinctly seen. Increasing the magnification will not make the objects more distinct, just bigger. Each objective has the magnification of the objective written on the objective. The magnification of the ocular is also inscribed on the ocular. Low magnifications are used for quickly examining the slide to find an appropriate area to examine. Higher magnifications allow the examination of a particular object on the slide. Examine your microscope and complete the table below. When you look through the ocular you will see a lighted circle. This is known as the field of view or the field. While looking through the microscope move the iris diaphragm lever and notice the changes in brightness of the light. As you move the objectives to provide increased magnification you will look at a smaller section of the slide. Be sure you move the object you want to view into the center of the field before moving to the next objective. These microscopes are parfocal. Once you have focused on an object using one objective the object will be approximately in focus on the next objective. Use of the fine focus knob will sharpen the focus.

Materials

i) Microscope
ii) Newsprint
iii) Stage micrometer
iv) Slides
v) Coverslips
vi) Transfer pipettes
vii) Prepared slides of bacteria
viii) Hay infusion
ix) Protoslow
x) Immersion oil
xi) Lens paper

NOTES

Procedure

1. Place a piece of newsprint on a microscope slide and cover with a coverslip.

Always Use A Coverslip!

2. Turn the microscope on and set the light source on its highest setting.
3. Use the coarse adjustment knob to obtain maximum working distance.
4. Place the slide on the stage. The slide should fit into the slide holder but is not placed under the slide holder. Use the stage adjustment knob to move the slide the edge of the coverslip bisects the hole in the stage.
5. Rotate the scanning objective (4X) into place.
6. Use the coarse adjustment knob to obtain the minimum working distance. Develop the habit of watching this process to be sure the objective does not crash into the slide.
7. Look through the oculars. Adjust the light with the iris diaphragm lever on the condenser if necessary. Slowly turn the coarse adjustment knob until the edge of the coverslip comes into focus. Use the fine adjustment knob to sharpen the focus.
8. Use the stage adjustment knob to locate the letter "e" in the newsprint. Note the orientation of the letter "e" in the newsprint.
9. Rotate a higher power objective (10X) into place. Use the fine adjustment knob to sharpen the focus. Do not use the coarse adjustment knob. Adjust the light using the iris diaphragm lever if necessary. The image is now magnified 100X (10X ocular x

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10X objective = 100X magnification). Draw the letter "e" as it appears in the microscope on the lab report sheet.

10. Place a stage micrometer on the stage and determine the diameter of the field of view for all four objectives. The micrometer is 2 mm in length. The ruler is divided into tenths. Record the distances on the lab report sheets.

11. **When using the high power objective (100X) use the following procedure.**

Rotate the turret halfway between the 40X and 100X objective. Place a drop of immersion oil on the slide and rotate the oil immersion objective (100X) into place.

The objective should be immersed in the oil on the slide. Use the fine adjustment knob to sharpen the focus. Adjust the light using the iris diaphragm lever if necessary.

Never use the coarse adjustment knob with high power.

12. Place a drop of water from the hay infusion on a microscope slide. Cover with a coverslip and view under all four objectives. Sketch two (2) of the organisms at 400X magnification.

13. Obtain a prepared slide for two bacterial species. View slides under the 1000X objective and sketch the bacteria. **Don't forget the immersion oil!**

14. When you are finished with the microscope clean the microscope, as described below, and return it to storage.

1.2.2 Procedure for Cleaning A Microscope

1. Turn off the light and unplug the cord. Store the cord appropriately.
2. Using the coarse adjustment knob to obtain maximum working distance and remove the slide from the stage.
3. Using **lens** paper clean all the lenses starting with the cleanest first—oculars, 4X through 100X objectives.
4. Clean any oil off of the stage using Kimwipes or paper towels.
5. Rotate the scanning objective into place. Use the coarse adjustment knob to obtain minimum working distance.
6. Return the microscope to the appropriate storage area.

1.3 Calibration of Microscope

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Determination or size of given microorganism using Micrometry

Principle

The diameter of a cell or length/diameter of subcellular components can be easily measured using an ocular micrometer which has graduation in arbitrary units. This arbitrary graduation of the ocular micrometer is calibrated using a stage micrometer by superimposing the two scales.

Materials Required

- Light microscope,
- ocular and stage micrometer,
- slide having cell preparations whose size is to be estimated.

Procedure

1. The ocular micrometer is placed on the circular shelf inside the eyepiece in such a way that the graduations sketched on the ocular, is visible when an observation is made using the microscope.
2. Place the stage micrometer on the stage of a microscope and focus the graduations using low power objectives. The graduations on stage micrometer are spaced 0.01mm (10μm) apart.
3. Superimpose the two scales and record the number of ocular division coinciding exactly with the number of divisions of the stage micrometer. The calibration factor or the least count of ocular micrometer is calculated as follows :

   If 13 ocular divisions coincide with 2 divisions (2X10μm=20μm) of stage micrometer

   Then 1 ocular division =

$$\frac{20\mu m}{13 \text{divisions}} = 1.54\mu m$$

4. Now remove the stage micrometer from the stage and place the slide having cell preparation under low power magnification. Position the cell being observed in such a

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way that the ocular micrometer is able to measure the diameter of a cell or the length/diameter of a cell component in arbitrary units. Calculate the size as shown below: If the diameter of a cell is occupying 5 divisions of ocular, the diameter of the cell will be:5 divisions X 1.54µm = 7.7µm

5. Similarly for high power objective the ocular micrometer calibration has to be done again following the same procedure and then cell diameter is can be measured focusing the cell in high magnification.

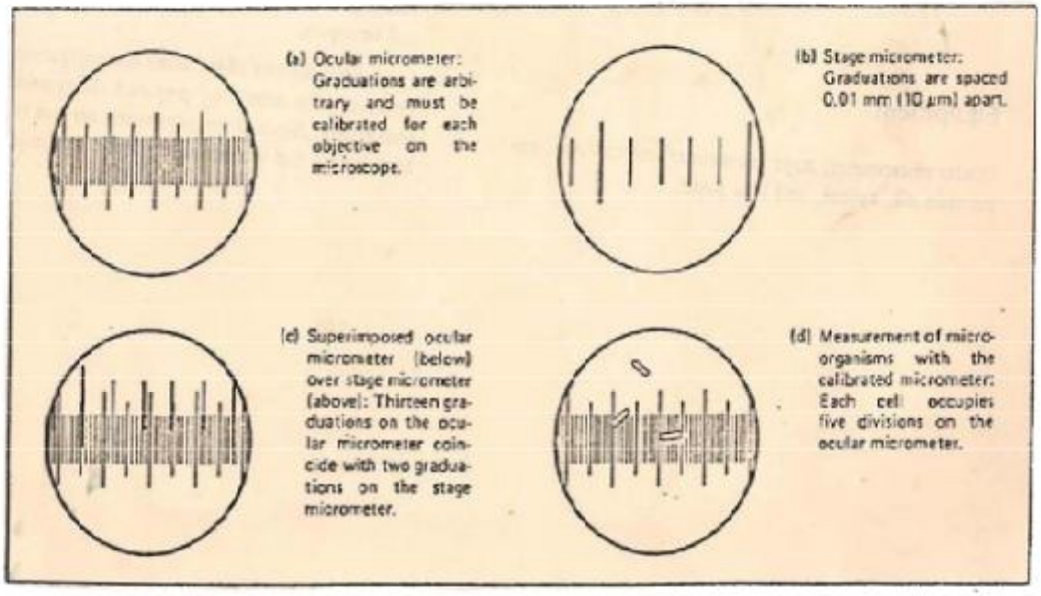

Fig. 1.2 : Different stages of Micrometry

1.4 Cleaning of Glasswares

Good laboratory technique demands clean glassware, because the most carefully executed piece of work may give an erroneous result if dirty glassware is used. In all instances, glassware must be physically clean; it must be chemically clean; and in manycases, it must be bacteriologically clean or sterile. All glassware must be absolutely grease-free. The safest criteria of cleanliness is uniform wetting of the surface by distilled water. This is especially important in glassware used for measuring the volume of liquids. Grease and other contaminating materials will prevent the glass from becoming uniformly wetted. This in turn will alter the volume of residue adhering to the walls of the glass container and thus affect the

volume of liquid delivered. Furthermore, in pipets and burets, the meniscus will be distorted and the correct adjustments cannot be made. The presence of small amounts of impurities may also alter the meniscus.

NOTES

Cleaning

Wash labware as quickly as possible after use. If a thorough cleaning is not possible immediately, put glassware to soak in water.

Most new glassware is slightly alkaline in reaction. For precision chemical tests, new glassware should be soaked several hours in acid water (a 1% solution of hydrochloric or nitric acid) before washing. Brushes with wooden or plastic handles are recommended as they will not scratch or abrade the glass surface.

1.4.1 Glassware Cleaners

When washing, soap, detergent, or cleaning powder (with or without an abrasive) may be used. The water should be hot. For glassware that is exceptionally dirty, a cleaning powder with a mild abrasive action will give more satisfactory results. The abrasive should not scratch the glass. During the washing, all parts of the glassware should be thoroughly scrubbed with a brush. This means that a full set of brushes must be at hand-brushes to fit large and small test tubes, burets, funnels, graduates and various sizes of flasks and bottles. Motor driven revolving brushes are valuable when a large number of tubes or bottles are processed. Do not use cleaning brushes that are so worn that the spine hits the glass. Serious scratches may result. Scratched glass is more prone to break during experiments. Any mark in the uniform surface of glassware is a potential breaking point, especially when the piece is heated. Do not allow acid to come into contact with a piece of glassware before the detergent (or soap) is thoroughly removed. If this happens, a film of grease may be formed.

1.4.2 Safe Use of Chromic Acid

If glassware becomes unduly clouded or dirty or contains coagulated organic matter, it must be cleansed with chromic acid cleaning solution1. The dichromate should be handled with extreme care because it is a powerful corrosive and carcinogen.

When chromic acid solution is used the item may be rinsed with the cleaning solution or it may be filled and allowed to stand.

The length of time it is allowed to stand depends on the amount of contamination on the glassware. Relatively clean glassware may require only a few minutes of exposure; if debris is present, such as blood clots, it may be necessary to let the glassware stand all night. Due to

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the intense corrosive action of the chromic acid solution, it is good practice to place the stock bottle, as well as the glassware being treated, in flat glass pans or pans made from lead or coated with lead, or plastic polymer pans determined compatible with the concentration of chromic acid you are using. Extra care must be taken to be sure chromic acid solution is disposed of properly.

Special types of precipitates may require removal with nitric acid, aqua regia or fuming sulfuric acid. These are very corrosive substances and should be used only when required.

Removing Grease

Grease is best removed by boiling in a weak solution of sodium carbonate. Acetone or any other fat solvent may be used. Strong alkalis should not be used. Silicone grease is most easily removed by soaking the stopcock plug or barrel for 2 hours in warm decahydronaphthalene.

Drain and rinse with acetone or use fuming sulfuric acid for 30 minutes. Be sure to rinse off all of the cleaning agents.

Rinsing

It is imperative that all soap, detergents and other cleaning fluids be removed from glassware before use. This is especially important with the detergents, slight traces of which will interfere with serologic and cultural reactions.

After cleaning, rinse the glassware with running tap water. When test tubes, graduates, flasks and similar containers are rinsed with tap water, allow the water to run into and over them for a short time, then partly fill each piece with water, thoroughly shake and empty at least six times. Pipets and burets are best rinsed by attaching a piece of rubber tubing to the faucet and then attaching the delivery end of the pipets or burets to a hose, allowing the water to run through them. If the tap water is very hard, it is best to run it through a deionizer before using.

Rinse the glassware in a large bath of distilled water. Rinse with distilled water. To conserve distilled water, use a five gallon bottle as a reservoir. Store it on a shelf near your clean-up area. Attach a siphon to it and use it for replenishing the reservoir with used distilled water. For sensitive microbiologic assays, meticulous cleaning must be followed by rinsing 12 times in distilled water.

1.4.3 Sterilizing Contaminated Glassware

Glassware which is contaminated with blood clots, such as serology tubes, culture media, petri dishes, etc., must be sterilized before cleaning. It can best be processed in the laboratory by placing it in a large bucket or boiler filled with water, to which 1-2% soft soap or detergent

has been added, and boiled for 30 minutes. The glassware can then be rinsed in tap water, scrubbed with detergent, rinsed again.

NOTES

You may autoclave glassware or sterilize it in large steam ovens or similar apparatus. If viruses or spore-bearing bacteria are present, autoclaving is absolutely necessary.

Handling and Storing

To prevent breakage when rinsing or washing pipets, cylinders or burets, be careful not to let tips hit the sink or the water tap.

Dry test tubes, culture tubes, flasks and other labware by hanging them on wooden pegs or placing them in baskets with their mouths downward and allowing them to dry in the air; or place them in baskets to dry in an oven2. Drying temperatures should not exceed 140°C. Line the drying basket with a clean cloth to keep the vessel mouths clean.

Dry burets, pipets and cylinders by standing them on a folded towel. Protect clean glassware from dust. This is done best by plugging with cotton, corking, taping a heavy piece of paper over the mouth or placing the glassware in a dust-free cabinet.

Store glassware in specially designed racks. Avoid breakage by keeping pieces separated.

Cleaning Specific Types of Glass Labware

Autoclaving

PyrexPlus labware can be successfully sterilized using liquids or dry cycle sterilization which involves no vacuum or low vacuum (<5 inches Hg).

Recommended cycles for automated autoclaves are:

Autoclave Type

Autoclave Cycle Gravity Prevacuum

Liquid Yes Yes

Dry Yes No

Prevac - No

CAUTION: Always autoclave vessels with loose caps or closures.

Steam sterilization time should not exceed 15 minutes at 121°C (250°F). Drying time should not exceed 15 minutes at 110°C (230°F). The actual cavity temperature of the autoclave should be checked to be sure the autoclave temperature does not exceed the recommended sterilization and drying temperature.

NOTES

Cleaning

As is common practice, clean all glassware before use. Any non-abrasive glassware detergent may be used for hand or automatic dishwasher cleaning. If using a dishwasher or glassware dryer, care should be taken to be sure the drying temperature does not exceed 110°C (230°F). Exposure to dry heat should be minimized.

Avoid brushes and cleaning pads which could abrade the glass or damage the coating. If using a chromic acid cleaning solution minimize contact of the solution with the coating.

Labeling and Marking

Use water-based markers for temporary marking or labeling of the PyrexPlus labware coating. Solvent-based markers, dyes and stains cannot be removed from the coating.

1.5 Aseptic Technique and Cultivation

Aseptic Technique

When working with microorganisms it is desirable to work with a pure culture. A pure culture is composed of only one kind of microorganism. Occasionally a mixed culture is used. In a mixed culture there are two or more organisms that have distinct characteristics and can be separated easily. In either situation the organisms can be identified. When unwanted organisms are introduced into the culture they are known as contaminants. Aseptic technique is a method that prevents the introduction of unwanted organisms into an environment. When changing wound dressings aseptic technique is used to prevent possible infection. When working with microbial cultures aseptic technique is used to prevent introducing additional organisms into the culture. Microorganisms are everywhere in the environment. When dealing with microbial cultures it is necessary to handle them in such a way that environmental organisms do not get introduced into the culture. Microorganisms may be found on surfaces and floating in air currents. They may fall from objects suspended over a culture or swim in fluids. Aseptic technique prevents environmental organisms from entering a culture.

Doors and windows are kept closed in the laboratory to prevent air currents which may cause microorganisms from surfaces to become airborne and more likely to get into cultures. Transfer loops and needles are sterilized before and after use in a Bunsen burner to prevent introduction of unwanted organisms. Agar plates are held in a manner that minimizes the exposure of the surface to the environment. When removing lids from tubes they are held in the hand and not placed on the countertop during the transfer of materials from one tube to another. All of these techniques comprise laboratory aseptic technique.

1.5.1 Cultivation

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Microorganisms must have a constant nutrient supply if they are to survive. Free-living organisms acquire nutrients from the environment and parasitic organisms acquire nutrients from their host. When trying to grow microbes in the lab adequate nutrition must be provided using artificial media. Media may be liquid (broth) or solid (agar). Any desired nutrients may be incorporated into the broth or agar to grow bacteria. Agar is the solidifying material used in solid media. It is an extract of seaweed that melts at 100°C and solidifies at about 42°C. Most pathogenic bacteria prefer to grow at 37°C so agar allows for a solid medium at incubator temperatures. Organisms grown in broth cultures cause turbidity, or cloudiness, in the broth. On agar, masses of cells, known as colonies, appear after a period of incubation. Certain techniques will allow bacterial cells to be widely separated on agar so that as the cell divides and produces a visible mass (colony), the colony will be isolated from other colonies. Since the colony came from a single bacterial cell, all cells in the colony should be the same species. Isolated colonies are assumed to be pure cultures. Colony morphology is described in terms of shape, margin or edge, elevation and color.

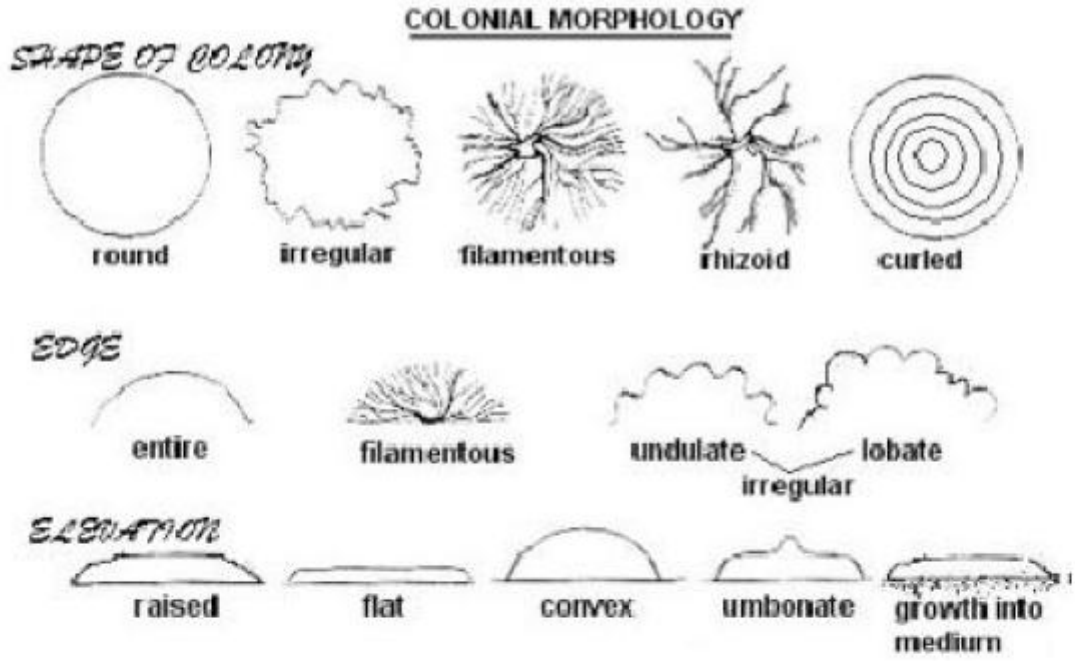

Fig. 1.3 : Morphology of Bacterial Colony

Materials

i. Mixed culture of: *Escherichia coli and Staphylococcus aureus*
ii. 2 Large nutrient agar plate
iii. 1 Small nutrient agar plate

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iv. Wire Inoculating loop
v. Bunsen burner
vi. Striker
vii. Sharpie marker

Procedure

1. Label the agar side of the large plates with your name, date and source material using the laboratory marker. This should become a habit and done every time you pick up a new plate.
2. Remove the lid and leave the plate exposed to the air for 2 minutes.
3. Obtain and label another plate.
4. Sterilize the inoculating loop in the inner flame of the Bunsen burner.
5. Obtain a loop of broth from the mixed culture tube using aseptic technique. **Do not set the test tube cap on the benchtop.**
6. Lift the agar plate from the lid and streak the first quadrant of the plate as shown in step one of Fig. 1.4 **Do not set the Petri dish lid on the bench top**. The loop should be parallel to the agar surface to prevent digging into or gouging the agar. Return the plate to the lid.
7. Re-sterilize the inoculating loop and return to the agar plate. Streak once through the first quadrant, which you have already inoculated, and continue on into the next quadrant in a zig-zag pattern, as shown in step 2 of Fig. 1.5.

   As a result of this process you will pick up fewer and fewer bacterial cells with each pass and distribute them farther and farther apart. In the end you should have several well isolated bacterial colonies (Fig. 1.5).
8. Place the plate in a 37°C incubator for 24-48 hours. Check your cultures the next day. If you do not have isolated colonies you will need to repeat the exercise immediately. **You must have isolated colonies for next weeks lab exercise.**
9. Use a lab marker to divide the back of the small nutrient agar plate in half. Mark it with your name, date and source on each half of the plate.
10. Lightly press your finger tips onto one half of the plate.

11. Use a sterile cotton swab to swab an area from the environment such as a door knob or the bottom of your shoe. Use this swab to lightly swab the other half of the small nutrient agar plate.

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12. Place the plate in a 37°C incubator for 24-48 hours. Examine you plates during the next lab session.

13. During the next lab period record your findings in terms of whether you obtained pure (axenic) cultures. Use the terms given above to describe the colony morphologies for both your isolation plate and your environmental sample plate.

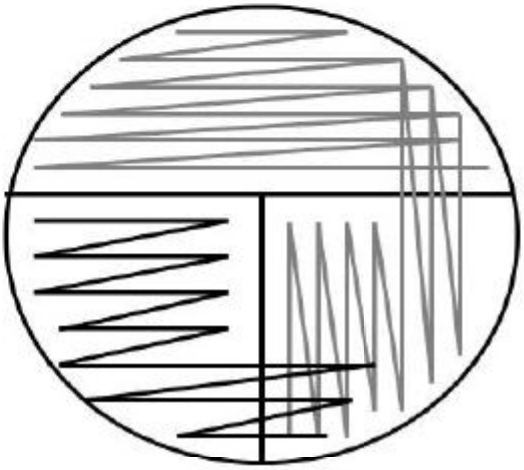

Fig. 1.4 : Streak isolation pattern of Bacteria

Fig. 1.5 : Streak isolation of Bacteria

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1.5.2 Preparation of Cultivation Media

Preparation of culture media-liquid and solid media

Introduction

Microorganisms are extraordinarily diverse in their requirements for growth. As you have learned in the lectures, microorganisms are greatly affected by environmental conditions and will grow in accordance to how these environmental niches support their individual needs. Factors that affect microbial growth include but are not limited to, **pH**, **osmolarity**, **water activity**, **temperature** and **oxygen levels**. There is a great deal of nutritional diversity among microorganisms; therefore, microbial growth is greatly affected by the nutrients that are available in their environment.

There are several nutritional classifications for bacteria. **Autotrophs** are organisms that are able to use inorganic carbon dioxide as their sole carbon source for the biosynthesis of macromolecules. **Heterotrophs**, require organic carbon for biosynthesis. Autotrophs can be further broken down into two categories: the **chemoautotrophs** derive energy from the oxidation of inorganic compounds such as iron, hydrogen sulfide and hydrogen gas. **Photoautotrophs**, such as the **cyanobacteria**, convert light energy into chemical energy. Heterotrophic organisms can also be divided into two major subgroups. **Photoheterotrophs**, use organic carbon sources for biosynthesis but use light energy to produce ATP (photosynthesis). **Chemoheterotrophs**, use organic compounds such as sugars, proteins and lipids as their source of energy. As chemoheterotrophs are more abundant and easier to work with, one usually works with these organisms in a typical teaching laboratory setting. Such bacteria include but are not limited to *Bacillus subtilis*, *Escherichia coli* and *Staphylococcus aureus*. Organisms of this classification that one might encounter in a hospital setting include, the *Clostridium* spp. that cause tetanus, botulism and gas gangrene, *Vibrio cholerae* and *Streptococcus pyogenes* (the causative agent of strept throat, impetigo, scarlet fever and cellulitis, i.e. the "flesh eating bacteria").

The specific nutritional requirements of heterotrophic bacteria can also be quite diverse. All microorganisms are made up of four biochemical molecules: proteins, lipids, carbohydrates and nucleic acids; however, these organisms may differ in their individual ability to synthesize these molecules. Some heterotrophs are metabolically flexible and require only a few organic compounds for energy production and biosynthesis of cellular components. Other heterotrophs require greater numbers of different organic compounds from their environment. Organisms that fall into the latter class are called **fastidious** organisms. The

successful cultivation of microbes in a laboratory setting requires that one understands the nutritional needs of an individual organism, as the absence of a single required nutrient would prevent growth. Fastidious organisms tend to require more ingredients in their growth media than less fastidious organisms.

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The media that one uses in a laboratory setting may be classified as **defined** or **complex (undefined)**. Complex media are composed of extracts from plants, animals or yeast and therefore are rich in nutrients. Such media is complex because the precise individual components of these media are unknown; however, as these media contain a wide range of nutrients that are well above the minimal nutritional requirements of the organism being cultured, these media support the growth of a wide range of organisms. A defined media is a media in which all of the constituents and the amounts of these constituents are known. Defined media typically supports a narrower range of heterotrophic microorganisms. Defined media typically consist of salts and a carbon source in the form of glucose. Depending on the fastidiousness of an organism, these media can be supplemented with vitamins, nucleic acids, cofactors and amino acids.

Using Selective, Differential and Selective-Differential Media in the isolation and identification of individual bacteria

Bacteria must be isolated from their natural environments before they can be characterized. As bacteria exist in mixed populations in the soil, water, food and within or on the human body, specialized media are often used to isolate individual bacteria from these populations and to characterize the isolated bacteria.

Selective media are used to select for the growth of some bacteria while inhibiting the growth of others. Such media take advantage of the differences in the nutritional needs of individual bacteria or exploit the ability of some organisms to grow in the presence of a noxious compound. For example, *Pseudomonas aeruginosa* is able to grow in the presence of a variety of antibiotics (compounds that inhibit bacterial growth or kill bacteria) while organisms like *Staphylococcus aureus* or *Escherichia coli* are **sensitive** to these antibiotics.

Differential media allow a variety of organism to grow but contain substances that allow the student to distinguish between the different types of bacteria growing on the media.

Selective-Differential Media have characteristics of both selective media and differential media. These media only allow a subset of bacteria to grow and allow the student to distinguish between the different types of bacteria that are able to grow on these media. For example, one can distinguish between bacteria that ferment lactose and those that do not in

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MacConkey Agar by adding a carbon source (lactose) and a pH indicator (methyl red)—bacteria that ferment lactose produce acids that imparts a color change to the media surrounding the individual bacterial colony.

In the following lab exercise, each student will examine the growth characteristics of different bacterial species on Starch Agar, MacConkey Agar, Mannitol Salt Agar and Hektoen Enteric Agar in terms of whether the media support the growth of these bacteria and in terms of the differences in the metabolic requirements of these bacteria.

Starch Agar

This is a differential medium used to determine whether a given bacterium is able to use starch as a carbon source and an energy source. Starch is a polymer (polysaccharide) of repeating glucose monomers and is too large to be transported into the cell. Organisms that use starch produce an exoenzyme called alpha-amylase that breaks the bonds between the glucose monomers such that the glucose monomers can be taken up into the cell and catabolized. Alpha-amylase is secreted outside of the cell but is still active at this location. To determine if the organism produces this exoenzyme, one adds Gram's Iodine to the plate that turns the intact starch in the medium purple. If the organism utilizes starch, the area around the colony will be clear because, the starch has been broken down into glucose monomers (starch positive). If the organism does not utilize starch then the media surrounding the organism will be dark purple (starch negative).

MacConkey Lactose Agar

This is a selective-differential media that selects for the growth of enteric bacteria. Enteric bacteria are gram-negative rods that are facultatively anaerobic. Enterics are most commonly found in the gastro-intestinal tract of humans and include *E. coli*, *Serratia* spp. and *Salmonella* spp. Enterics grow on MacConkey media because the bile salts that are present in the media inhibit the growth of non-enteric organisms while the crystal violet inhibits the growth of Gram positive organisms that might otherwise grow on this media. MacConkey media also differentiates between the noncoliforms and the coliforms that also grow on this medium. Enteric bacteria can be coliforms or noncoliforms. Enteric bacteria that ferment lactose to form acid and gas are coliforms, enteric bacteria that do not ferment lactose are noncoliforms. Coliforms will produce bright pink colonies on MacConkey lactose agar because a pH indicator (methyl red) is present in the media. The non-coliforms will grow on the media but the colonies will appear either light yellow or colorless.

Hektoen Enteric Agar

This is also a selective-differential media that contains bile salts to select for enteric organisms but distinguishes between these enterics by virtue of their abilities to ferment lactose, salicin or sucrose and to reduce sulfur to hydrogen sulfide gas. In addition to these fermentable sugars, H-E Agar contains ferric ammonium citrate that reacts with the hydrogen sulfide to form a black precipitate and the dyes acid fuchsin and bromothymol blue that are color indicators. This media is often used to isolate *Salmonella* and *Shigella* spp from other enterics. The enterics that ferment the sugars produce acids and form yellow to pink colonies on this medium. *Shigella* and *Salmonella* spp.do not ferment these sugars and produce blue colonies. *Salmonella* spp. (but not *Shigella* spp.) reduce sulfur to hydrogen sulfide forming colonies containing a black precipitate.

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Mannitol Salt Agar

It is another example of a selective differential media. The media contains a high percentage of NaCl (7.5% w/v) to select for organisms that prefer or can tolerate high salt conditions. Furthermore this media contains the sugar mannitol and the pH indicator phenol red to identify the organisms that can ferment this sugar. If the organism is able to ferment mannitol to produce acid then the pH of the media will drop around the colonies and cause the phenol red to turn yellow. This media is often used to isolate *Staphylococcus aureus* since this organism is halotolerant and able to use mannitol as a carbon source.

1.5.3 Preparation of Nutrient Agar Media for Bacterial Culture

Principle

Bacteriological media come an a wide range of types. Nutrient Agar is a complex medium because it contains ingredients with contain unknown amounts or types of nutrients. Nutrient Agar contains Beef Extract (0.3%), Peptone (0.5%) and Agar (1.5%) in water. Beef extract is the commercially prepared dehydrated form of autolysed beef and is supplied in the form of a paste. Peptone is casein (milk protein) that has been digested with the enzyme pepsin. Peptone is dehydrated and supplied as a powder. Peptone and Beef Extract contain a mixture of amino acids and peptides. Beef Extract also contains water soluble digest products of all other macromolecules (nucleic acids, fats, polysaccharides) as well as vitamins and trace minerals. Although we know and can define Beef Extract in these terms, each bach can not be chemically defined. There are many media ingredients which are complex: yeast extract, tryptone, and others. The advantage of complex media is that they support the growth of a wide range of microbes.

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Agar is purified from red algae in which it is an accessory polysaccharide (polygalacturonic acid) of their cell walls. Agar is added to microbiological media only as a solidification agent. Agar for most purposes has no nutrient value. Agar is an excellent solidification agent because it dissolves at near boiling but solidifies at 45 ^{0}C. Thus, one can prepare molten (liquid) agar at 45^{0}C, mix cells with it, then allow it to solidify thereby trapping living cells. Below 45^{0}C agar is a solid and remains so as the temperature is raised melting only when >95^{0}C is obtained.

Materials Required

1. Electronic or beam balances.
2. Weigh boats, tongue depressors.
3. Tripods, asbestos wire-gauze, asbestos gloves.
4. 10 ml nonsterile pipettes.
5. pH paper or pH meter with standard buffers.
6. 4 13x100 mm screw capped culture tubes.
7. Graduated Cylinder, 250 ml.
8. 2 500ml Erlenmeyer Flasks
9. Beef Extract, Peptone, Agar.
10. 3 N HCl, 3 N KOH.
11. 16 x 150 mm screw cap culture tubes.
12. Nonabsorbent cotton and gauze to make cotton stoppers.

Nutrient Agar

Beef Extract:	0.3%
Peptone:	0.5%
NaCl:	0.5%
Agar:	1.5%

Procedure

1. You will be making 200 ml of Nutrient Agar. To weigh out Beef Extract, first tare a tongue depressor, then dip it into the Beef Extract and weigh. Adjust the amount of Beef Extract until the correct amount is obtained. Be sure to be careful not to get Beef Extract

on to the balance! You need to weight out enough Beef Extract to get a 0.3% solution. Place the tongue depressor into the flask, beef extract side down.

2. Tare a weigh boat and weigh out enough Peptone and add that to the flask.
3. Add 200 ml of distilled water and swirl to dissolve the peptone and beef extract. Check the pH, it should be 7.0.
4. Tare a weigh boat and weigh out enough Agar and add that to the flask.
5. With a bunsen burner, tripod, asbestos wire-gauze, heat the medium to boiling to dissolve the agar
6. While the agar is still warm, but not hot, pipette 3 ml each into 4 13x100 mm screw cap culture tubes.
7. Label the flask and your tubes with your name.
8. After preparation of your medium, the instructor will take you to the autoclave.
9. Place your media in the autoclave with those of the rest of the class.
10. After discussion of the parts of the autoclave, autoclave the medium for 20 minutes.
11. The media will be saved and used in Experiments.

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1.5.4 Isolation of Pure Cultures

A pure culture theoretically contains a single bacterial species. There are a number of procedures available for the isolation of pure cultures from mixed populations. A pure culture may be isolated by the use of special media with specific chemical or physical agents that allow the enrichment or selection of one organism over another. The *differential* and *selective* procedures will be utilized later in this course. Simpler methods for isolation of a pure culture include: (i) spread plating on solid agar medium with a glass spreader and (ii) streak plating with a loop. The purpose of spread plating and streak plating is to **isolate** individual bacterial cells (colony-forming units) on a nutrient medium.

Both procedures (spread plating and streak plating) require understanding of the aseptic technique. Asepsis can be defined as the absence of infectious microorganisms. However, the term is usually applied to any technique designed to keep unwanted microorganisms from contaminating sterile materials.

Materials

- Seven 9-ml dilution tubes of sterile saline
- Seven nutrient agar plates
- ml and 0.1 ml pipets

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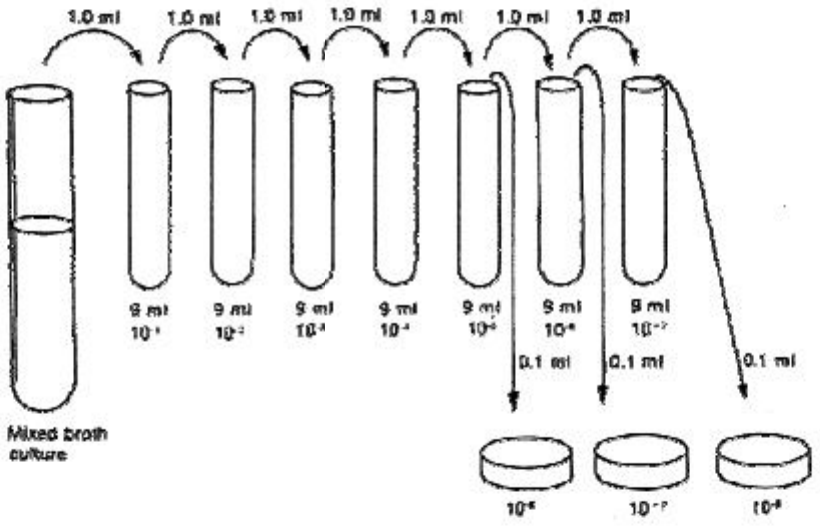
- Glass spreader aka "hockey stick"
- 95% ethyl alcohol in glass beaker
  (**WARNING**: Keep alcohol away from flame.)
- Mixed overnight broth culture of *Staphylococcus aureus* and *Serratia marce*

Procedure

A. Spread Plate Technique

In this technique, the number of bacteria per unit volume of sample is reduced by serial dilution *before* the sample is spread on the surface of an agar plate.

1. Prepare serial dilutions of the broth culture as shown below. Be sure to mix the nutrient broth tubes before each serial transfer. Transfer **0.1 ml** of the final three dilutions (10^{-5}, 10^{-6}, 10^{-7}) to each of three nutrient agar plates, and label the plates.



Preparation of 10^{-4} to 10^{-7} dilutions utilizing seven 9 ml sterile nutrient broth blanks.

Fig. 1.6 : Serial Dilution

2. Position the beaker of alcohol containing the glass spreader **away from the flame**. Remove the spreader and very carefully pass it over the flame just once (lab instructor will demonstrate). This will ignite the excess alcohol on the spreader and effectively sterilize it.

3. Spread the 0.1 ml inoculum evenly over the entire surface of one of the nutrient agar plates until the medium no longer appears moist. Return the spreader to the alcohol.
4. Repeat the flaming and spreading for each of the remaining two plates.
5. Invert the three plates and incubate at room temperature until the next lab period.

NOTES

B. Streak Plate Technique

The streak plating technique isolates individual bacterial cells (colony-forming units) on the surface of an agar plate using a wire loop. The streaking patterns shown in the Fig. below result in continuous dilution of the inoculum to give well separated surface colonies. Once again, the idea is to obtain isolated colonies after incubation of the plate.

1. Label two nutrient agar plates No. 1 and No. 2.
2. Prepare two streak plates by following two of the 3 streaking patterns shown in the Fig. below. Use the 10^{-1} dilution as inoculum.
3. Invert the plates and incubate at room temperature until the next lab period.

Various methods of preparing streak plates for the individual organisms.

Fig. 1.7 : Streak Plate Technique

NOTES

1.6 Viva-Voce

1. What is Bacteria?
2. Define Microbiology.
3. Who proposed the term bacteria?
4. Who discover microscope first?
5. Why cleaning is important for glasswares?
6. What is Sterilization?
7. Define media.
8. Define Cultivation.
9. Differentiate spread plate and streak plate technique.
10. Why screening is important for bacterial isolation?
11. How many types of media available for bacterial growth?
12. Define selective media.
13. What is serial dilution?
14. Name of the parts of microscope.

1.7 References

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Unit-2

Study of Micro-organism-I

NOTES

Structure of the Unit

2.0 Objectives

After going through this unit you will be able to understand:

- Structure of bacteria
- About isolation of microorganisms from different conditions
- visualization and differentiation between gram positive and gram negative

2.1 Study of Bacteria

2.1.1 Structure of Bacteria

Bacteria constitute a large domain of prokaryotic microorganisms. Typically a few micrometers in length, bacteria have a number of shapes, ranging from spheres to rods and spirals. Bacteria were among the first life forms to appear on Earth, and are present in most of its habitats. Bacteria inhabit soil, water, acidic hot springs, radioactive waste, and the deep portions of Earth's crust. Bacteria also live in symbiotic and parasitic relationships with plants and animals. They are also known to have flourished in manned spacecraft.

Bacteria are classified into coccus and bacillus

Coccus (Plural- Cocci): A coccus is a round or oval shaped bacterium. In most cocci, one axis of the cell is almost equal to any other axis. But a coccus may be slightly oval, pointed at the ends; lancet, bean or kidney shaped (Gonococcus).

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Typical cocci are about 0.5-1.0 pm in diameter. During multiplication, cocci may arrange themselves in irregular groups, chains, pairs or singles.

Bacillus (Plural- Bacilli) or Rod. Bacillus is rod shaped. Bacterium has one axis of the cell longer than the other one. Rods are mostly arranged singly. When the size of a rod is short and approximates to coccal form, it is called coccobacillus. Vibrio is comma-shaped single curved rod. Actinomyces and Nocardia are mycelial, filamentous or rods. Corynebacterium is club-shaped rod. Spirochaetes are long slender flexible spiral rods with several curves. Most are 5-20 pm x 0.1-0.2 pm. Fusobacterium is spindle shaped rod. Spirillum is spiral rod with several curves.

On Gram-staining, cocci and bacilli (except Mycobacterium) are classified into Gram-positive bacteria and Gram-negative bacteria. This difference in the staining depends on the composition of bacterial cell wall. It is used to study the shape, size and arrangement of bacteria beside the staining character. Pleomorphism indicates that there is considerable variation in the size and/or shape of a strain.

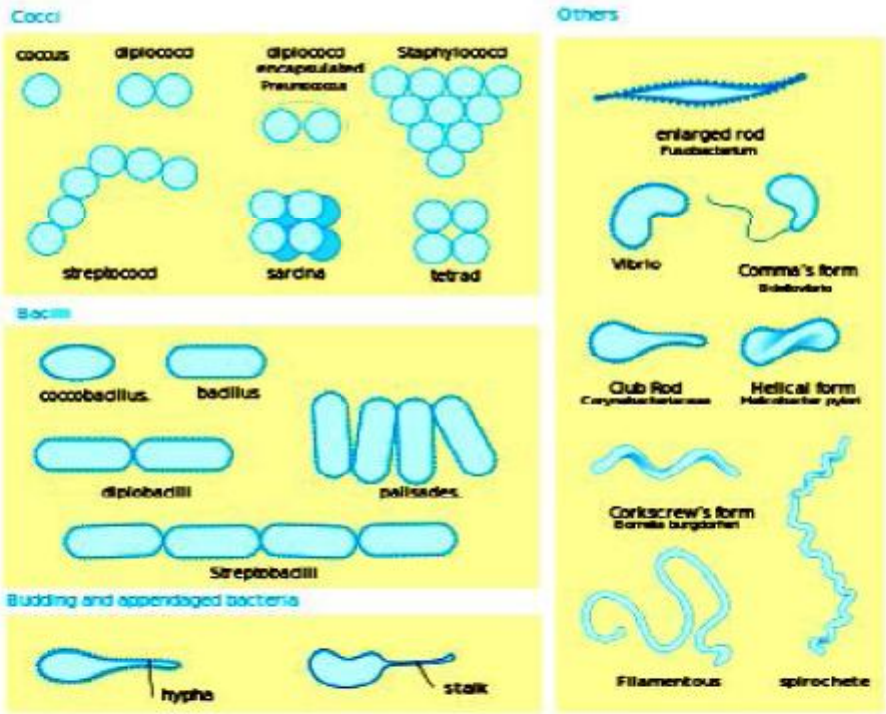

Fig. 2.1 : Bacterial Cell Morphology and Arrangements

Cell structure of Bacteria

Cell Envelope

The layers that surround the bacterial cells are referred collectively as the cell envelope. Cell envelope consists of cytoplasmic membrane, cell wall, and for some bacteria capsule and glycocalyx from inner to outer surface.

NOTES

Cell Wall

Layers of the cell envelope lying between the cytoplasmic membrane and capsule are the 'cell wall'. It is external to cytoplasmic membrane. A bacterial cell wall is prokaryotic. Mycoplasmas have no cell wall.

Functions: The cell wall is mostly rigid but also exhibit tensile strength. The strength depends on peptidoglycan, murein or mucopeptide which is the main component. The cell wall maintains the bacterial shape and protects the cell from lysis. The cell wall plays an important part in cell division. During cell division, a transverse partition is formed from the cell wall by its inward growth. Many antibacterial agents inhibit cell wall synthesis. Differential Gram-staining character leading to classification into Gram- positive and Gram-negative bacteria reside in the cell-wall. Peptidoglycan layer is common component, others are special components.

1. Cell-walls of Gram- positive bacteria

(a) Peptidoglycan Layer- Peptidoglycan comprises up to 50% of the cell wall material and may be 40 sheets. It is composed of N-acetyl muramic acid, N-acetyl glucosamine and peptides. Lysozyme acts on it, (b) Teichoic acids. Teichoic acids lie on the outside surface of the peptidoglycan layer in most Gram positive bacteria. Teichoic acids are polymers containing ribitol or glycerol. (c) Polysaccharides.

2. Cell walls of Gram-negative bacteria

(a) Peptidoglycan Layer. Peptidoglycan comprises 5 to 10% of the wall material. Only one or two sheets. (b) Lipoprotein- Lipoprotein is the most abundant protein of Gram negative cells. It probably stabilizes the outer membrane. (c) Outer membrane- Lipid in nature. Serves to protect the cell of enteric bacteria from bile salts. (d) Lipopolysaccharide (LPS). LPS molecule is toxic and is referred to as endotoxin of Gram negative bacteria. LPS is split into lipid A and polysaccharide. Lipid A is the toxin, and polysaccharide is the major surface antigen of the bacterial cell- called O antigen. Lipooligosaccharides (LOS) are important virulence factors and are antigenic. (e) Periplasmic space. The space between the inner and outer membrane. It contains gel-like solutions of proteins and murein layer.

Periplasmic proteins include (a) binding proteins for specific substrates like vitamins, sugar, amino acids, ions, and (b) enzymes like P-lactamase, alkaline phosphatase.

2.1.2 Isolation Techniques

NOTES

a) Media Preparation

Liquid Media

Bacteria are often cultured in liquid media (broth). The most common ingredients of basic media are beef extract and peptone. The Nutrient broth and Glucose broth have been considered as basic media for cultivation of bacteria.

Composition of Nutrient broth is –

1. Peptone- 5g
2. Beef extract-3g
3. Distilled water- 1 liter

Composition of Glucose broth is –

1. Peptone- 10g
2. Glucose- 5g
3. NaCl- 5g
4. Distilled water- 1 liter

Materials required

Glucose, peptone, beef extract, sodium chloride, sodium hydroxide pellets, hydrochloric acid, pH meter, heater, autoclave, beakers, conical flasks, measuring cylinders, cotton, glass rods, culture tubes.

Procedure

Nutrient Broth (pH= 7.0)

- Put the weighed amount of peptone and beef extract in 500 ml of distilled water.
- Heat with agitation to dissolve the constituents.
- Add more water to make the volume up to 1 liter.
- Adjust the pH of the medium to '7' with the help of a pH meter by adding either acid or alkali.
- Pour 10 ml of this medium per tube and apply cotton plugs. Autoclave at 121°C and 15 pound pressure for 15-20 minutes.
- Allow the autoclave to cool, remove the Nutrient broth tubes and store at room temperature.

- Cover with butter paper for further use.

Glucose Broth (pH=7.3)

- Glucose broth can be prepared in the same way as nutrient broth and the pH of the medium is to be adjusted to 7.3.

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Solid Media

Commonly used solid media for different bacterial cultures are – Glucose Peptone Agar (GPA), Yeast Extract Agar Medium (YEAM) and Nutrient Agar Medium (NAM).

Composition:-

1. Glucose Peptone Agar
   Glucose-40g, Peptone-10g, Agar-15g, distilled water-1 liter, pH-5.6
2. Yeast Extract Agar Medium
   Yeast extract-3g, Peptone-5g, Agar-15g, distilled water- 1 liter, pH-7.4
3. Nutrient Agar Medium
   Peptone-5g, Beef extract-1.5g, yeast extract-1.5g, Agar-15g, NaCl-5g, distilled water-1 liter, pH-7.4.

Materials required

Petri-plates, test tubes, Bunsen burner, glucose, peptone, agar, yeast extract, distilled water, beef extract, pH meter, measuring cylinders, heater, and autoclave.

Procedure

- Take 500ml of water in 1 liter beaker and add the chemicals required for a particular medium except agar.
- Boil it and add required agar bit by bit to the hot water to dissolve it.
- Bring the volume up to 1litre, dispense it in conical flask and plug with cotton.
- Sterilize it 121°C and 15 pound pressure for 15-20 minutes in an autoclave.

b) Streaking

In order to study the morphological and physiological characteristics of an organism or an individual species in a culture, it is essential to separate that species from other species. In other words we must have a pure culture of microorganism.

There are several methods of getting a pure culture from a mixed culture; one of them is 'steak plate method'.

Materials required

Bunsen burner, inoculating loop or needle, Petri plates, nutrient agar medium or any other suitable medium, incubator.

NOTES

Procedure

- Prepare the agar plate by pouring the medium in Petri plate under aseptic conditions.
- Take the master culture and with the help of sterilized inoculating loop full of mixed culture without touching the walls of test tube.
- Streak the plate with the loop containing inoculum.
- Incubate the plate in an incubator at 37°C for 24 hrs.

Streaking can be done in any of the five following ways:

(a) Quadrant streak- Steak one loop full of microorganisms from the culture near one edge of the plate (area 1), applying the loop lightly so that it does not gouge into the medium.

Flame the loop, cool it for 5 sec, and make 5-6 streaks from area 1 through area 2.

Flame the loop again, cool it, and make 6-7 streaks from area 2 through area 3.

Flame the loop again and make as many streaks as possible from area 3 into area 4, using up the remaining plate surface.

(b) Radiant streak- Spread a loop full of microorganisms in a small area near one edge of plate (area 1).

Flame the loop and allow it to cool for 5 sec.

From the edge of area 1 make 7-8 straight streaks to the opposite side of the plate.

Flame the loop again and cross streak the last streaks starting near area 1.

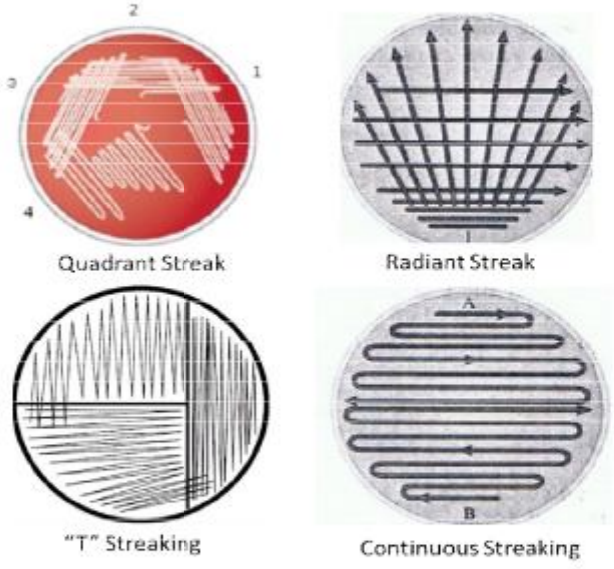

Fig. 2.2 : Isolation Techniques for Pure Culture

(c) "T" streak- Draw a "T" on the bottom of the plate (with a marking pencil), such that it divides the plate into one half and two quarters.

Inoculate the half portion with one loop full of microorganisms in a continuous line from the edge of the plate to the midline of the plate, taking care not to gauge the plate. Flame the loop, cool it and cross streak from area 1 into area 2 with one continuous streak filling area 2.

After flaming the loop again cross streak from the area 2 to area 3 with single streak.

(d) Continuous streak- Starting from the edge of the plate with a loop full of microorganisms, spread in a single continuous movement up to the center of the plate.

Rotate the plate by 180 degrees.

Without flaming the loop continue streaking the other half of the plate working towards center.

NOTES

2.1.3 Isolation of bacteria

a) From Air

Microorganisms are introduced in the air by various sources, the principal source being dust particles containing dry vegetative cells and spores of the microorganisms. The aerobic spore forming bacilli from the soil are also found frequently in the air. The number of organisms in the air is dependent on the activity of environment in that area. An untidy humid room has high bacterial contents; the air around rich fertile and cultivated soil contains more microorganisms. It is also seen that air around a bare surface shows high bacterial content than vegetative cells because it is more prone to air current and organisms can be blown easily. Humid air contains more microorganisms as compared to dry air.

Methods of isolation of microorganisms from air

1) Settled plate technique- It is used to determine the relative number of species of microorganisms present in air. In this method open plates of culture medium are exposed to air for given period of time, e.g. 30 min or 1 hr. These plates are incubated for 24 hrs. at 37^{o}C. The colonies grown on the plate give the relative estimates of members of organisms. The occurrence of pathogenic bacteria can be determined by using blood agar.
2) Slit sampler technique- It involves the use of special apparatus called slit sampler. A known volume of air directed towards a plate through 0.254 mm wide slit and for even distribution of microorganisms the plate is mechanically rotated. The slit allows one cubic foot of air per minute to pass through.

NOTES

Materials required

Petri plates, beakers, autoclave, medium like YEAM, GPA.

Procedure

- The autoclaved media are poured into sterilized Petri plates.
- The prepared plates are exposed to the different areas of choice for ½ an hour.
- These plates are then incubated at 37°C for 24 hrs. to 48 hrs.

b) From soil

Most of the soil microorganisms are found in soil surface layers and their number decreases in depth. A well aerated soil contains more microorganisms than that lacking abundance of oxygen.

Different methods are used to isolate the microbial populations from the soil. One of these methods is described below:

Agar Plate Method

This method gives the estimate of different types of microorganisms in soil capable of growing in NAM or any other selected media.

A weighed sample of soil is mixed with known volume of water and it is shaken thoroughly to separate as many microorganisms as possible from the colloidal material surrounding the soil.

Materials Required

YEAM/GPA/PDA/NAM, Petri plates, distilled water, incubator, compound microscope

Procedure

- Prepare the required medium and adjust its pH.
- Autoclave the medium and pour 10-15 ml of it into each Petri plate.
- Inoculate the plates by sprinkling soil or by pouring diluted soil on the plate.
- Incubate the inoculated Petri plates for 24 hours or 48 hours in incubator at 25°C-37°C depending upon the medium taken.

  Observe the colonies developed on the plate and prepare a slide and identify the organisms.

2.2 Gram Staining

NOTES

Gram staining, also called Gram's method, is a method of differentiating bacterial species into two large groups (gram-positive and gram-negative). The name comes from its inventor, Hans Christian Gram.

Gram staining differentiates bacteria by the chemical and physical properties of their cell walls by detecting peptidoglycan, which is present in a thick layer in gram-positive bacteria. In a Gram stain test, gram-positive bacteria retain the crystal violet dye, while a counterstain (commonly safranin or fuchsine) added after the crystal violet gives all gram-negative bacteria a red or pink coloring.

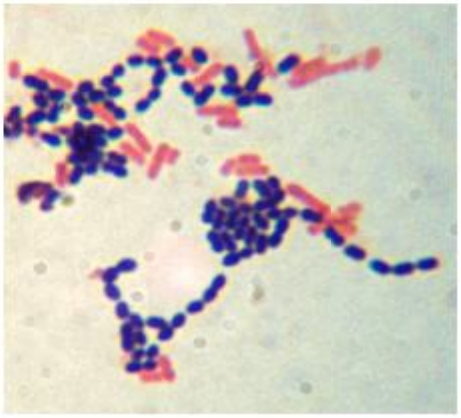

Fig. 2.2: Gram Staining of Bacteria

Materials Required

Clean glass slides, Inoculating loop, Bunsen burner, Bibulous paper, Microscope, Lens paper and lens cleaner, Immersion oil, Distilled water, 18 to 24 hour cultures of organisms

Reagents: Primary Stain - Crystal Violet, Mordant - Grams Iodine, Decolorizer - Ethyl Alcohol, Secondary Stain – Safranin.

Procedure

Part 1:- Preparation of the glass microscopic slide Grease or oil free slides are essential for the preparation of microbial smears. Grease or oil from the fingers on the slides is removed by washing the slides with soap and water. Wipe the slides with spirit or alcohol. After cleaning, dry the slides and place them on laboratory towels until ready for use.

Part 2:- Labeling of the slides- Drawing a circle on the underside of the slide using a glassware-marking pen may be helpful to clearly designate the area in which you will prepare the smear. You may also label the slide with the initials of the name of the

organism on the edge of the slide. Care should be taken that the label should not be in contact with the staining reagents.

Part 3:- Preparation of the smear

NOTES

- Bacterial suspensions in broth: With a sterile cooled loop, place a loop full of the broth culture on the slide. Spread by means of circular motion of the inoculating loop to about one centimeter in diameter. Excessive spreading may result in disruption of cellular arrangement. A satisfactory smear will allow examination of the typical cellular arrangement and isolated cells.
- Bacterial plate cultures: With a sterile cooled loop, place a drop of sterile water or saline solution on the slide. Sterilize and cool the loop again and pick up a very small sample of a bacterial colony and gently stir into the drop of water/saline on the slide to create an emulsion.
- Swab Samples: Roll the swab over the cleaned surface of a glass slide.

Note: It is very important to prevent preparing thick, dense smears which contain an excess of the bacterial sample. A very thick smear diminishes the amount of light that can pass through, thus making it difficult to visualize the morphology of single cells. Smears typically require only a small amount of bacterial culture. An effective smear appears as a thin whitish layer or film after heat-fixing.

Part 4:- Heat Fixing- Heat fixing kills the bacteria in the smear, firmly adheres the smear to the slide, and allows the sample to more readily take up stains. Allow the smear to air dry. After the smear has air-dried, hold the slide at one end and pass the entire slide through the flame of a Bunsen burner two to three times with the smear-side up.

Note: Take care to prevent overheating the slide because proteins in the specimen can coagulate causing cellular morphology to appear distorted.

Part 5:- Gram Stain Procedure

1. Place slide with heat fixed smear on staining tray.
2. Gently flood smear with crystal violet and let stand for 1 minute.
3. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
4. Gently flood the smear with Gram's iodine and let stand for 1 minute.
5. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle. The smear will appear as a purple circle on the slide.

6. Decolorize using 95% ethyl alcohol or acetone. Tilt the slide slightly and apply the alcohol drop by drop for 5 to 10 seconds until the alcohol runs almost clear. Be careful not to over-decolorize.
7. Immediately rinse with water.
8. Gently flood with safranin to counter-stain and let stand for 45 seconds.
9. Tilt the slide slightly and gently rinse with tap water or distilled water using a wash bottle.
10. Blot dry the slide with bibulous paper.
11. View the smear using a light-microscope under oil-immersion.

NOTES

Typical Gram-negative bacteria

1. *Bordetellapertusis*, the causative agent of whooping cough
2. *Salmonella typhi*, the causative agent of typhoid
3. *Vibrio cholera*, the causative agent of cholera
4. *Escherichia coli*, the normally benign, ubiquitous, gut-dwelling bacteria

Typical Gram-positive bacteria

1. Staphylococci such as *Staphylococcus epidermidis* and *Staphylococcus aureus* which is a common cause of boils.
2. Streptococci such as the many species of oral streptococci, Streptococcus pyogenes which causes many a sore throat and scarlet fever and *Streptococcus pneumoniae* which causes lobar pneumonia.
3. Clostridia such as *Clostridium tetani*, the causative agent of tetanus (lockjaw).
4. Actinomyces such as *Actinomycesodontolyticus* which is found in mouth.
5. Species of the genus Bacillus such as *Bacillus subtilis* which are common microbes living in soil.

2.3 Viva-Voce

1 What is difference between bacilli and cocci?

2 Which kinds of media are used to isolate bacteria?

3 What is the appearance of gram positive and gram negative after staining?

2.4 References

NOTES

- Practical in microbiology and biotechnology by Dr. Inderpalsoni, Dr.P.J.John
- www.amrita.edu/create Amrita University

Unit - 3

Study of Micro-organism- II

NOTES

Structure of the Unit

3.0 Objectives

After going through this unit you will be able to understand:

- Cyanobacteria and their types
- About mycoplasma and their pathogenic behavior
- Virus and Bacteriophage

3.1 Study of Cyanobacteria

Cyanobacteria are also known as Cyanophyta, is a phylum of bacteria that obtain their energy through photosynthesis. The name "cyanobacteria" comes from the color of the bacteria (kyanós = blue). They are often called blue-green algae, but some consider that name a misnomer as cyanobacteria are prokaryotic and algae should be eukaryotic, although other definitions of algae encompass prokaryotic organisms. By producing gaseous oxygen as a by-product of photosynthesis, cyanobacteria are thought to have converted the early reducing atmosphere into an oxidizing one, which dramatically changed the composition of life forms on Earth by stimulating biodiversity and leading to the near-extinction of oxygen-intolerant organisms. According to endosymbiotic theory, the chloroplasts found in plants and eukaryotic algae evolved from cyanobacterial ancestors via endosymbiosis.

NOTES

Cyanobacteria are photosynthetic nitrogen fixing group that survive in wide variety of habitats, soils and water. In this group photosynthetic pigments are cyanophycin, allo-phycocyanine and erythrophycocyanine. Their thallus varies from unicellular to filamentous and filamentous heterocystous. They fix atmospheric nitrogen in aerobic conditions by heterocyst, specialized cells, and in anaerobic conditions.

Nitrogen fixation

Cyanobacteria include unicellular and colonial species. Colonies may form filaments, sheets or even hollow balls. Some filamentous colonies show the ability to differentiate into several different cell types: vegetative cells, the normal, photosynthetic cells that are formed under favorable growing conditions; akinetes, the climate resistant spores that may form when environmental conditions become harsh; and thick-walled heterocysts, which contain the enzyme nitrogenase, vital for nitrogen fixation. Heterocysts may also form under the appropriate environmental conditions (anoxic) when fixed nitrogen is scarce. Heterocyst-forming species are specialized for nitrogen fixation and are able to fix nitrogen gas into ammonia (NH_3), nitrites (NO^{2-}) or nitrates (NO^{3-}), which can be absorbed by plants and converted to protein and nucleic acids (atmospheric nitrogen is not bioavailable to plants, except for those having endosymbiotic nitrogen-fixing bacteria, especially the Fabaceae family, among others).

a) Nostoc

Nostoc is a diverse genus of cyanobacteria. They are found in gelatinous colonies, composed of filaments called "trichomes" surrounded by a thin sheath. They are common in both aquatic and terrestrial habitats.

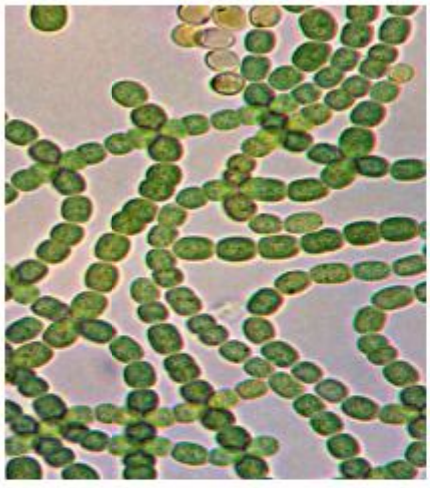

Fig. 3.1: ***Nostoc***

These organisms are known for their unusual ability to lie dormant for long periods of time and abruptly recover metabolic activity when rehydrated with liquid water. The

bacteria's ability to withstand freezing and thawing cycles make them well-adapted to living in extreme environments, such as the Arctic and Antarctica. They can fix atmospheric nitrogen, making them good candidates for environments with low nitrogen rates.

Nostoc, first discovered in the 19th century, is one of the most widespread phototrophic bacteria in the world. As a nitrogen fixer, these bacteria may provide plants with important nutrients and therefore can be used agriculturally. Nostoccommune, is found to harbor aUV-A/B absorbing pigment. This protective pigment has enabled them to survive not only while under hydration-related stress, but in areas of extreme UV radiation as well. Nostoc's genetics are worth studying because of the genus' unique adaptations which allow them to survive and even thrive in extreme environments. Also, a better understanding of soil-dwelling nitrogen fixers such as Nostoc may help advance fertilizer production and benefit agriculturalists.

Species

Nostoccalcicola, N. commune, N. cycadae, N. desertorum, N. edaphicum, N. ellipsosporum, N. entophytum, N. flagelliforme, N. indistinguenda, N. lichenoides, N. linckia, N. muscorum, N. paludosum, N. piscinale, N. punctiforme, N. sphaericum.

Cell Structure and Metabolism

Nostocs are photosynthesizers which use cytoplasmic photosynthetic pigments rather than chloroplasts in their metabolic process. They are single-celled, and lack a nucleus or other internal membrane systems; their cytoplasm is composed 70%-85% of water. The cells do not possess flagella, but are motile by a swaying motion. Division is by binary fission; some branching may occur. The cells form filamentous structures known as trichomes, which in turn make up colonies encased by a thin sheath; these colonies may be mat-like or spherical and are either micro- or macroscopic-spherical colonies.

Culinary use

Containing protein and vitamin C, Nostoc species are cultivated and consumed as a foodstuff, primarily in Asia. The N. flagelliforme and N. commune varieties are consumed in China, Japan and Java, N. commune is also consumed in the Andes. The preferred variety in Central Asia is N. ellipsosporum. Fat choy (Nostocflagelliforme), besides having no nutritional value, has also been found to contain Beta-methylamino L-alanine (BMAA), a toxic amino acid that could affect the normal functions of nerve cells.

b) *Oscillatoria*

NOTES

Oscillatoria is a cyanobacteria that is important because it can conduct photosynthetic activities. It has a long un-branching filamentous morphology and is color green due to the chlorophyll it contains. Some unique features that sets it apart from other cyanobacteria is that it is motile and can conduct anoxygenic photosynthesis. Within its long filamentous structure the cells within it are in the form of discoid and are surrounded by cell wall so it does not require heterocyst.

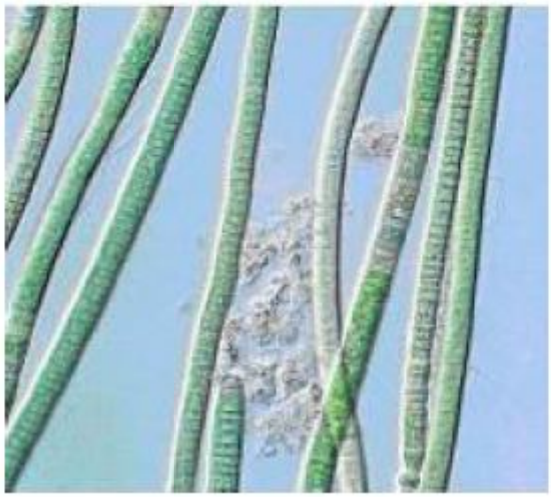

Fig. 3.2: *Oscillatoria*

Structure, Metabolism, and Life Cycle

Oscillatoria is motile and moves by the means of microfibrils. They move in a gliding motion and tend to use this mobility to move towards light. Its gliding motion has a unique feature though, it moves in oscillatory motion. *Oscillatoria* has a long un-branching filamentous structure that reproduces by binary fission. This cyanobacterium can conduct photosynthesis just like the other in its phylum but it can also conduct anoxygenic photosynthesis. Meaning that it can still obtain energy in the absence of oxygen, it can obtain it from the reduction of sulfate instead. This energy comes in the form of ATP and can be stored.

Oscillatoria species

Oscillatoriaamoena, Oscillatoriaanguiformis, Oscillatoriaanguina, Oscillatoriaannae, Oscillatoriabonnemaisonii, Oscillatoriachilkensis, Oscillatoriacrassa, Oscillatoriacroasdaleae.

c) Spirulina

The trichomes of *Spirulina* are cylindrical, loosdy or tightly coiled, varied; each cell is square or rectangular, spiral shaped, lack gas vasciles and has thylakoids. The opical cells are rounded. Hetrocysts and spoers are not present. Reproduce mainly by the formation of hormogones, occassinally propogate by fragmentation. This is most commonly found in lakes rich in sodium Carbonte. It grows naturally in warm and highly alkali lake in Tropical areas.

NOTES

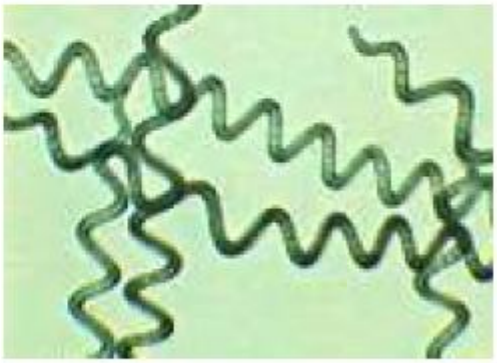

Fig. 3.3: *Spirulina*

The potential biotechnological application of *Spirulina* in the field of food, feed, medicines, cosmetics, biochemical and in the environmental depollution are well established and documented.

Spirulina is the only single, natural source providing the highest amount of protein (71%) ever known to man . It is commercially important due to its nutritional and therapeutic qualities. It have high contents of iron, pro-vitamin A, vitamin B-12, & essential fatty acids like (gamma) linolenic acid, so it is richest so of nutrients for malnourished population.

Spirulina have pharmaceutical role in arthritis, obesity and hypercholestrol related disease human. It perform as antiviral and anti inflamematory agents in cases of Herpes simple virus type and HIV-1.

The phytonutrients (pigments and polysaccharides) of *Spirulina* makes it useful in cosmetics, nutraceutical etc. A large number of formulations are available for the preparation of food using *Spirulina* powder like Soup , noodless, candies, low calorie bread.

It is also used as an agent for combating water pollution. Its polysaccharides have promising potentials in their utilization such as waste water treatment, clarification of water in reservoirs and sedimentation of colloids in chemical and food industries.

It is largly marketed in different form but still much more is required to be done for popularization of *Spirulina.*

NOTES

c) *Microcystis*

Microcystis aeruginosa is a species of freshwater cyanobacteria which can form harmful algal blooms of economic and ecological importance. They are the most common toxic cyanobacterial bloom in eutrophic fresh water. Cyanobacteria produce two groups of toxins, neurotoxins and peptide hepatotoxins, such as microcystin and cyanopeptolin. Microcystis is characterized by small cells (of only a few micrometers diameter), which lack individual sheaths. Cells usually are organized into colonies (large colonies of which may be viewed with the naked eye) that begin in a spherical shape, but lose their coherence to become perforated or irregularly shaped over time. The coloration of the protoplast is a light blue-green, appearing dark or brown due to optical effects of gas-filled vesicles; this can be useful as a distinguishing characteristic when using light microscopy. These vesicles provide the buoyancy necessary for M. aeruginosa to stay at a level within the water column at which they can obtain optimum light and carbon dioxide levels for rapid growth.

M. aeruginosa is favored by warm temperatures, but toxicity and maximal growth rates are not totally coupled, as the cyanobacterium has highest laboratory growth rates at 32°C, while toxicity is highest at 20°C, lowering in toxicity as a function of increasing temperatures in excess of 28°C. Growth has been found to be limited below 15°C.

The aquatic plant Myriophyllumspicatum produces ellagic, gallic, and pyrogallic acids and (+)-catechin, allelopathicpolyphenols inhibiting the growth of M. aeruginosa. M. aeruginosa can produce both neurotoxins (lipopolysaccharides-LPSs) and hepatotoxins (microcystins).

Economic importance

Because *M. aeruginosa*can form persistent microcystin toxins under the right environmental conditions, it has come to be a source of drinking water pollution (which can be rendered hepatotoxic). This can lead to increased economic costs such as water quality mitigation measures in the form of increased costs at water filtration facilities, as well as damage to local tourism caused by lake or other waterway closures due to toxicity concerns. *M. aeruginosa*is also the subject of research into the natural production of butylatedhydroxytoluene (BHT), an antioxidant, food additive, and industrial chemical.

Ecological importance

In addition, its effect on dissolved oxygen content in water can lead to fish kills and other marine life kills, also. In 2009, unprecedented mammal mortality in the southern part of the Kruger National Park led to an investigation which implicated *M. aeruginosa*. The

dead animals included grazers and browsers which preferred drinking from the leeward side of two man-made dams, a natural point of accumulation for drifting *Microcystis*blooms. Mammals such as elephants and buffalo which usually wade into water before drinking, were unaffected, as were the resident crocodiles. The source of nutrients which supported the *Microcystis*growth was narrowed down to the dung and urine voided in the water by a large resident hippo population, unaffected by the bloom. The immediate problem was solved by breaching of the dam walls and draining of the water. *M. aeruginosa*, the most abundant cyanobacterial genus in South Africa, may occur in toxic and harmless strains.

NOTES

3.2 Study of Mycoplasma

3.2.1 Structure of Mycoplasma

Mycoplasmas are the smallest free-living organisms that, unlike other bacteria, lack a cell wall. The outer layer is instead, a three layered membrane containing sterols. Diameters of these organisms may range from 0.2-0.3 μm and, due to their plasticity, are able to pass through the pores of a 0.2 micron filter with applied\ pressure. Because the morphology of Mycoplasma is pleomorphic, they occur as two different structural forms during a life cycle: coccoidal, a spherical or spheroidal shape, and filamentous, resembling rods. Because Mycoplasmas lack a cell wall, the organisms are poorly stained, if at all, by bacterial stains.

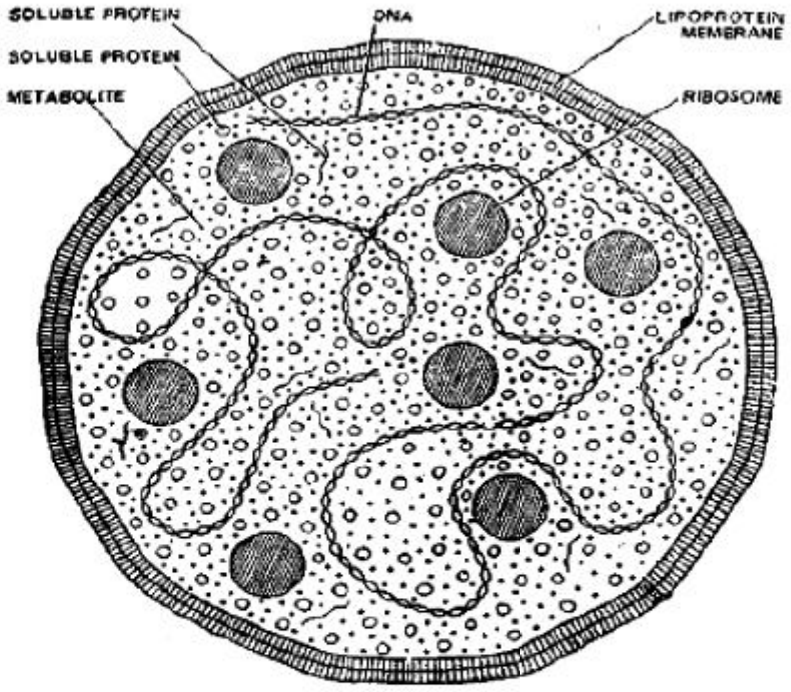

Fig. 3.4: Mycoplasma

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With the exception of *M.hyorhinis*, most Mycoplasma can be cultivated using standardized and Mycoplasma agar formulations, as well as in broth media, although growth is slow. When grown on agar, the colonies have a "fried-egg" appearance since the colony center grows into the agar and appears denser than the rest of the colony.

The mycoplasma cells are unicellular non-motile pleomorphic cells. They are characterised by very small coccoid bodies, swollen ring like forms and filamentous branched forms of variable length. The smallest mycoplasma, units, capable of independent growth, are small coccoids ranging 0.25 to 0.1μm in diameter. It is because of their small size that they easily pass through filters which otherwise do not permit bacteria to pass through them. Mycoplasmas, like animal cells are without cell wall and possess only a plasma membrane. The cell membrane is three layered or "Unit" type. It is rigid, externally thin, and resistant because of chemicals like sterol present in it. These sterols as a matter of fact, are not synthesized by these organisms but are taken up from the surrounding medium.

Internal Structure:- The internal structure of mycoplasms is similar to typical procaryotic cell and closely resemble with bacteria. The nuclear structure is in the form of DNA, which is in very less amount (4% in M. gallisepticum) than typical bacteria, but has sufficient genetic information necessary for independent existence of the cell. The DNA like higher organisms is a double standard helix. In addition to DNA approximately double amount of RNA (8% in M. gallisepticum) is found in mycoplasma cells. A good amount of ribosomes, soluble proteins and amino acids are also present with other metabolites in expected ratio. Mesosomes are conspicuously absent in mycoplasmas perhaps because of the elasticity of cell membrane. Enzyme systems and metabolic phenomenon of mycoplasma slightly differ from typical bacteria but the differences are not extreme.

3.2.2 Pathogenicity of Mycoplasma

Many species of Mycoplasmas and Mycoplasma like agents are causing serious diseases in human beings, animals and plants few examples of these diseases are given below.

In Human beings: The important human pathogen of mycoplasma group is the agent causing primary atypical pneumonia (PAP) and is now called as Mycoplasma pneumoniae showing symptoms of pneumonia and is confused with the pneumonia caused by Diplococcuspneumoniae causing misleading treatment. In addition to this M.mycoidesM.agalactiae, M.salivarium, M. pharyngis and M. ferments ns are some common pathogens causing severe diseases in human beings.

In Animals: Many species of Mycoplasmas have been reported recently causing various pathological conditions in animals like sheep, goat, dogs, rats and mice. In these animals the Mycoplasma causes rheumatic or arthritic diseases, infection in respiratory tracts and mammary glands.

NOTES

In Plants: Many plant diseases caused by Mycoplasma like organisms (MLOS). Now MLOS have been found causing about 75 types of plant diseases, which occur from tropical to temperate regions. But in tropical areas MLOS cause serious losses in crops like rice, maize, brinjal, cotton, citrus and potato etc. some common Mycoplasma diseases of crops are explained below.

Little leaf disease of Brinjal (eggplant)

Causal organism: - Mycoplasma is the causal agent of this disease. They are spherical to ovoid (40-3000 nm), MLB (Mycoplasma like Bodies) have been isolated from the phloem cells of diseased plant.

Symptoms: - The leaves of the eggplant become reduced in size, the newly developing leaves at the top of plants become progressively smaller and sterile (influence is not emerged). The lamina of leaves becomes soft glabrous and pale green. Internodes of top branches are shortened resulting in a bushy appearance of the affected plant.

Host Range: - Brinjal, Tomato, Potato, Datura and Asgandh etc. (Plants of Solanaceae family).

Transmission: - The disease is transmitted through Hishimonusphycitis.

Control: - Spraying with ledermycin at 500 ppm controls the disease and suppresses the symptoms.

Rice yellow dwarf

Causal organism: -This disease is caused by Mycoplasma like organism in U.P., Bihar, Orissa and Andhra Pradesh.

Symptoms: -The leaves become chlorotic, pale green to yellow causing stunted growth and profuse tillering. The disease is lethal to plants infected at early stage of seedlings. If plants are infected in late stage it reduces grain yield.

Host Range: -The Rice varieties most susceptible to disease are Taichimg Native 1, Taichung 65, Taiwan-3 and Kalim-pong-1.

Transmission: -The disease is transmitted through green leaf hopper called Nephotettiximpicticeps.

NOTES

Control: -Spray of insecticides like Furadan on plants checks the disease. In addition to this early sowing and destruction of diseased plants are the best prevention of disease. Since the vectors of this disease are grass hoppers, it can be easily controlled by aerial application of insecticides over large areas during rice crop season.

Sandal spike

It is a seriously destructive disease of Sandal wood, threatening whole of the Sandal industries throughout Sandal producing Karnataka state and neighboring areas.

Causal organism: -Mycoplasma like Bodies (MLB) have been investigated as causal organism of disease.

Symptoms: -The common symptom of the disease is the severe reduction of leaf size. The shortening of internode accompanied with reduced leaf size gives the appearance of rosette spike. There is no flowering and fruiting in the infected plant.

Host Range: -*Mi* the species of Sandal (Santalum) are infected by the mycoplasma.

Transmission: -The disease is transmitted by Jassid (Jassusindicus).

Control: -The disease can be easily controlled by Tetracycline. Latermycin and Terramycin are more useful, if a systemic fungicide 'benlate' is mixed and plants are treated with this mixture, it becomes most effective in controlling the disease.

3.3 Study of Viruses

3.3.1 Structure and general characters of virus and bacteriophages

Structure

A virus is a small infectious agent that replicates only inside the living cells of other organisms. Viruses can infect all types of life forms, from animals and plants to bacteria and archaea. Viruses are found in almost every ecosystem on

Earth and are the most abundant type of biological entity. The study of viruses is known as virology, a sub-specialty of microbiology. Virus particles (known as virions) consist of two or three parts: i) the genetic material made from either DNA or RNA, long molecules that carry genetic information; ii) a protein coat that protects these genes; and in some cases iii) an envelope of lipids that surrounds the protein coat when they are outside a cell. The shapes of viruses range from simple helical and icosahedral forms to more complex structures. The average virus is about one one-hundredth the size of the average bacterium. Most viruses are too small to be seen directly with an optical microscope.

The origins of viruses in the evolutionary history of life are unclear: some may have evolved from plasmids—pieces of DNA that can move between cells—while others may

have evolved from bacteria. In evolution, viruses are an important means of horizontal gene transfer, which increases genetic diversity. Viruses are considered by some to be a life form, because they carry genetic material, reproduce, and evolve through natural selection. However they lack key characteristics (such as cell structure) that are generally considered necessary to count as life. Because they possess some but not all such qualities, viruses have been described as "organisms at the edge of life".

All viruses contain the following two components: 1) a nucleic acid genome and 2) a protein capsid that covers the genome. Together this is called the nucleo-capsid. In addition, many animal viruses contain a 3) lipid envelope. The entire intact virus is called the virion. The structure and composition of these components can vary widely.

Viral Genomes: While the genomes of all known cells are comprised of double stranded DNA, the genomes of viruses can be comprised of single or double stranded DNA or RNA. They can vary greatly in size, from approximately 5-10 kb (Papovaviridae, Parvoviridae, etc.) to greater than 100-200 kb (Herpesviridae, Poxviridae). The known structures of viral genomes are summarized below.

DNA: Double Stranded - linear or circular

Single Stranded - linear or circular

Other Structures - gapped circles

RNA: Double Stranded - linear

Single Stranded - linear : These single stranded genomes can be either + sense, - sense, or ambisense. The sense strand is the one that can serve directly as mRNA and code for protein, so for these viruses, the viral RNA is infectious. The viral mRNA from - strand viruses is not infectious, since it needs to be copied into the + strand before it can be translated. In an ambisense virus, part of the genome is the sense strand, and part is the antisense.

The genome of some RNA viruses is segmented, meaning that a virus particle contains several different molecules of RNA, like different chromosomes.

Protein Capsid:- Viral genomes are surrounded by protein shells known as capsids. One interesting question is how capsid proteins recognize viral, but not cellular RNA or DNA. The answer is that there is often some type of "packaging" signal (sequence) on the viral genome that is recognized by the capsid proteins. A capsid is almost always made up of repeating structural subunits that are arranged in one of two symmetrical structures, a helix or an icosahedron. In the simplest case, these "subunits" consist of a single polypeptide. In many cases, however, these structural subunits (also called protomers) are

made up of several polypeptides. Both helical and icosahedral structures are described in more detail below.

NOTES

1) Helical Capsids:- The first and best studied example is the plant tobacco mosaic virus (TMV), which contains a SS RNA genome and a protein coat made up of a single, 17.5 kd protein. This protein is arranged in a helix around the viral RNA. Helical capsids can also be more complex, and involve more than one protein subunit. A helix can be defined by two parameters, its amplitude (diameter) and pitch, where pitch is defined as the distance covered by each turn of the helix.

This structure is very stable, and can be dissociated and re-associated readily by changing ionic strength, pH, temperature, etc. The interactions that hold these molecules together are non-covalent, and involve H-bonds, salt bridges, hydrophobic interactions, and Vander Waals forces. Several families of animal virus contain helical nucleocapsids, including the Orthomyxoviridae (influenza), the Paramyxoviridae (bovine respiratory syncytial virus), and the Rhabdoviridae (rabies).

2) Icosahedral Capsids:- In these structures, the subunits are arranged in the form of a hollow, quasi spherical structure, with the genome within. An icosahedron is defined as being made up of 20 equilateral triangular faces arranged around the surface of a sphere. Since proteins are not equilateral triangles, each face of an icosahedron contains more than one protein subunit. The simplest icosahedron is made by using 3 identical subunits. Each of these subunits could be a single protein or, more likely, a complex of several polypeptides. Many viruses have too large a genome to be packaged inside an icosahedron made up of only 60 polypeptides (or even 60 subunits), so many are more complicated. In these cases, each of the 20 triangular faces is divided into smaller triangles; and each of these smaller triangles is defined by 3 subunits. However, the total number of subunits is always a multiple of 60. When virus nucleo-capsids are observed in the electron microscope, one often sees apparent "lumps" or clusters on the surface of the particle. These are usually protein subunits clustered around an axis of symmetry, and have been called "morphological units" or capsomers.

Viral Envelope

In some animal viruses, the nucleocapsid is surrounded by a membrane, also called an envelope. This envelope is made up of a lipid bilayer, and is comprised of host-cell lipids. It also contains virally encoded proteins, often glycoproteins which are trans-membrane proteins. These viral proteins serve many purposes, such as binding to receptors on the host cell, playing a role in membrane fusion and cell entry, etc. They can also form channels in the viral membrane.

Bacteriophage

Bacteriophages are viruses that parasitize bacteria. Bacteriophages were jointly discovered by Frederick Twort and by Felix d'Herelle. Felix d'Herelle coined the term "Bacteriophage". Bacteriophage means to eat bacteria, and is called so because virulent bacteriophage can cause the compete lysis of a susceptible bacterial culture. They are commonly referred as "phage". Phages are obligate intracellular parasites that multiply inside bacteria by making use of some or all of the host biosynthetic machinery. They occur widely in nature and can readily be isolated from feces and sewage. There are at least 12 distinct groups of bacteriophages, which are very diverse structurally and genetically.

NOTES

Examples of phages:

- T-even phages such as T2, T4 and T6 that infect E.coli
- Temperate phages such as lambda and mu
- Spherical phages with single stranded DNA such as PhiX174
- Filamentous phages with single stranded DNA such as M13
- RNA phages such as Qbeta

Composition

Depending upon the phage, the nucleic acid can be either DNA or RNA but not both. The nucleic acids of phages often contain unusual or modified bases, which protect phage nucleic acid from nucleases that break down host nucleic acids during phage infection. Simple phages may have only 3-5 genes while complex phages may have over 100 genes. Certain phages are known have single stranded DNA as their nucleic acid.

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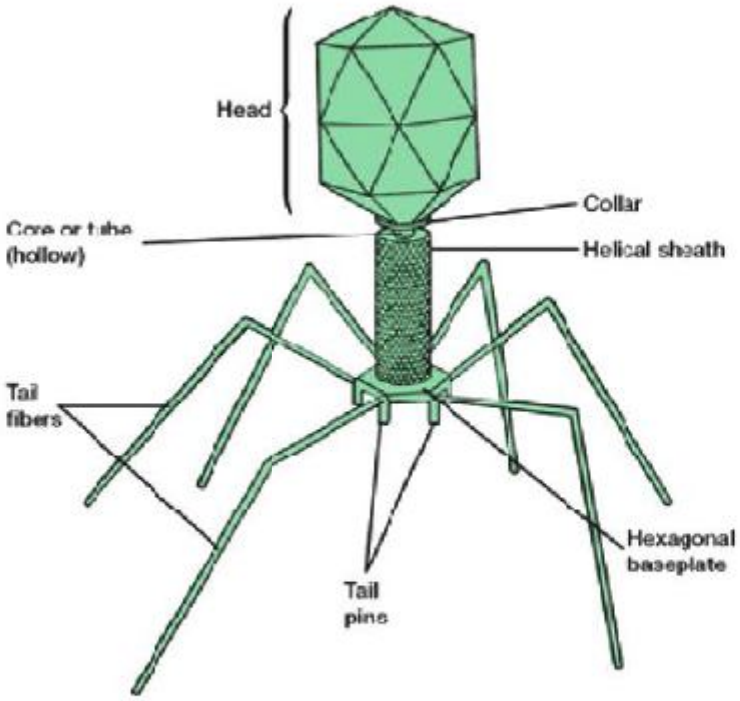

Fig. 3.4: Bacteriophage

Morphology

Most phages range in size from 24-200 nm in length. T4 is among the largest phages; it is approximately 200 nm long and 80-100 nm wide. All phages contain a head structure, which can vary in size and shape. Some are icosahedral (20 sides) others are filamentous. The head encloses nucleic acid and acts as the protective covering. Some phages have tails attached to the phage head. The tail is a hollow tube through which the nucleic acid passes during infection. T4 tail is surrounded by a contractile sheath, which contracts during infection of the bacterium. At the end of the tail, phages like T4 have a base plate and one or more tail fibers attached to it. The base plate and tail fibers are involved in the binding of the phage to the bacterial cell. Not all phages have base plates and tail fibers.

3.3.2 Animal and plant viruses

Animal viruses: -Viruses are important pathogens of livestock. Diseases such as foot-and-mouth disease and bluetongue arecaused by viruses. Companion animals such as cats, dogs, and horses, if not vaccinated, are susceptibleto serious viral infections. Canine parvovirus is caused by a small DNA virus and infections are often fatalin pups. Like all invertebrates, the honey bee is susceptible to many viral infections. However, mostviruses co-exist harmlessly in their host and cause no signs or symptoms of disease.

Plant viruses: - There are many types of plant virus, but often they cause only a lossof yield, and it is not economically viable to try to control them.Plant viruses are often spread from plant to plant by organisms,known as vectors. These are normally insects, but some fungi,nematode worms, and single-celled organisms have been shown tobe vectors. When control of plant virus infections is consideredeconomical, for perennial fruits, for example, efforts areconcentrated on killing the vectors and removing alternate hostssuch as weeds. Plant viruses cannot infect humans and otheranimals because they can reproduce only in living plant cells.

NOTES

Plants have elaborate and effective defense mechanisms against viruses. One of the most effective is the presence of so-called resistance (R) genes. Each R gene confers resistance to a particular virus by triggering localized areas of cell death around the infected cell, which can often be seen with the unaided eye as large spots. This stops the infection from spreading. RNA interference is also an effective defense in plants. When they are infected, plants often produce natural disinfectants that kill viruses, such as salicylic acid, nitric oxide, and reactive oxygen molecules. Plant virus particles or virus-like particles (VLPs) have applications in both biotechnology and nanotechnology. The capsids of most plant viruses are simple and robust structures and can be produced in large quantities either by the infection of plants or by expression in a variety of heterologous systems. Plant virus particles can be modified genetically and chemically to encapsulate foreign material and can be incorporated into supra-molecular structures for use in biotechnology.

3.4 Viva-Voce

1 Can any cyanobacteria be used as food supplement? If yes then give an example.

2 What are mycoplasmas?

3 Differentiate between bacteriophages and viruses.

3.5 References

- microbewiki.kenyon.edu/index.php/Nostoc
- microbewiki.kenyon.edu/index.php/Oscillatoria
- www.cellgro.com
- Biology@tutorvista NCS Pearson

Unit - 4

NOTES

Study of Micro-organism-III

Structure of the Unit

4.0 Objectives

After going through this unit you will be able to understand:

- Determination of the growth of microorganisms
- About Colorimetry and Spectrophotometry
- Determination of concentration of unknown solution

4.1 Determination of Growth of Microorganisms

Microbial growth rates and generation times can be measured by different methods. Since growth leads to increase both the number and the mass of the populations, either of the two may be followed. It is necessary to make it clear that no single technique is always best; the most appropriate approach depends upon the experimental situation.

Measurement of Cell Numbers

Breed Method

A known volume of microbial cell suspension (0.01 ml) is spread uniformly over a glass slide covering a specific area (1 sq. cm). The smear is then fixed by heating, stained and examined under oil immersion lens, and the cells are counted. Customarily, cells in a few microscopic fields are counted because it is not possible to scan the entire area of smear. The counting of total number of cells is determined by calculating the total number of microscopic fields per one square cm. area of the smear. The total number of cells can be counted with the help of following calculations:

(a) Area of microscopic field = πr^2. r (oil immersion lens) = 0.08 mm.

Area of the microscopic field under the oil immersion lens

$= \pi r^2 = 3.14 \times (0.08 \text{ mm})2 = 0.02$ sq. mm.

(b) Area of the smear one sq. cm. = 100 sq. mm. Then, the no. of microscopic fields = 100 / 0.02= 5000

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(c) No. of cells 1 sq. cm. (or per 0.01 ml microbial cell suspension) = Average no. of microbes per microscopic field x 5000

Counting Chamber Technique

The number of cells in a population can be measured by taking direct microscopic count using **Petroff-Hausser** counting chamber (for prokaryotic microorganisms) or **hemo-cytometers** (to larger eukaryotic microorganisms). Prokaryotic microorganisms are more easily counted if they are stained or if phase I contrast of florescence microscope is employed. These are specially designed slides that have chambers of I known depth with an etched grid on the chamber bottom. , Each square on the grid has definite depth and volume. Total number of microorganisms in a sample can be calculated taking the count of number of bacteria per unit area of grid and multiplying it by a conversion factor (depending on chamber volume and sample dilution used).

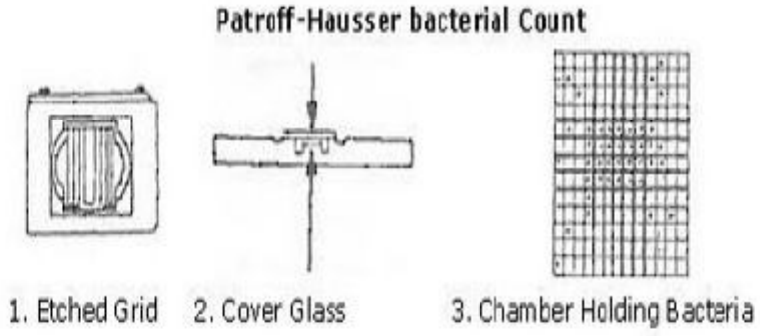

Fig. 4.1 : Counting Chamber Technique

Viable Count

The bacterial culture need not contain all living cells. There might be few dead as well. Only living cells will form colony when grown in proper solid medium and under standard set or growth conditions. This fact is used to estimate number of living or dead bacterial cells (viable count) in the given culture. Estimates thus obtained are expressed as a colony forming unit (CFU).

Viable count technique is very much useful in the dairy industry and the food industry for quantitative analysis of milk and spoilage of food products. For convenience, to obtain a colony count for bacteria in milk, 1 ml of well mixed milk is placed in 99 ml of sterile dilute solution (may be water or nutrient broth or saline solution).

NOTES

This results in s dilution of 1: 100 or 1 × 10-2. To the petri dish containing pre solidified medium 1 ml of 1: 100 dilution is transferred and incubated at desired is repeated for the preparation of further dilution as 1: 1000 or 1: 10, 0000 of bacteria per ml in original sample can be found by multiplying bacterial colony count by the reciprocal of the dilution and of the volume used.

For Example, CFU = 50 for 1: 10, 000 if volume used is 1 ml then

CFU = 50 × 10, 000 × 1

CFU = 5 × 105

Coulter Counter

Coulter counter is an electronic used to count number of microbes preferably protozoa microalgae and yeasts. In This method, the sample of microbes is forced through a small orifice (small hole). On the both sides of the orifice, electrodes are present measure the electric resistance or conductivity when electric current is passed through the orifice. Every time a microorganism passes through the orifice, electrical resistance increases or the conductivity drops and the cell is counted. The Coulter counter gives accurate results with larger cells. The precaution to be taken in this method is that the suspension of samples should be free of any cell debris or other extraneous matter.

Membrane-Filter Technique

Microbial cell numbers are frequently determined using special membrane filters possessing millipores small enough to trap bacteria. In this technique a water sample containing microbial cells passed through the filter. The filter is then placed on solid agar medium or on a pad soaked with nutrient broth (liquid medium) and incubated until each cell develops into a separate colony. Membranes with different pore sizes are used to trap different microorganisms. Incubation times for membranes also vary with medium and the microorganism. A colony count gives the number of microorganisms in the filtered sample, and specific media can be used to select for specific microorganisms. This technique is especially useful in analyzing aquatic samples

4.2 Principle of Colorimetry and Spectrophotometry

Spectrophotometry and colorimetry are based on the absorption of light by a matter. In colorimetry, polychromatic light and in spectrophotometry, monochromatic light is used. When measured in visible light, the compound tested must be coloured, in ultraviolet and infrared spectrum regions, the measured solutions may be colourless and it needs more complicated instruments. Monochromatic light passing into a coloured solution is partly absorbed (Io) and partly passes through. The intensity of arriving light (I) is decreased due to the absorption. The ratio I/Io is called transmittance T:

T=I/Io

the transmittance of a solution is in relation with absorbance (extinction, optical density) according to the equation

$$A=\log 1/T=\log Io/I$$

NOTES

Two empiric laws define the relations between the absorbance, the thickness of sample through which the light passes and the concentration.

Lambert's law - States that for a given solution the absorbance is proportional to the path length (l) of the light through the cell and is independent of Io.

Beer's law - States that the absorbance is proportional to the concentration (c) of the solution.

These laws were combined and are known as the Beer-Lambert Law.

$$A = \varepsilon.c.l$$

This law simply indicates a linear relationship between the sample concentration (c) and its absorbance (A). The thickness of the cell with a sample is constant for each instrument and ε is called the molar absorption coefficient. It is the absorbance of a molar solution of the compound, in a 1 cm cell, at the specified wavelength. Unfortunately, the linear dependence between the absorbance and the concentration is valid for diluted solutions (below 0.001 $mol.L^{-1}$) only, when the interaction among molecules does not affect the result. Another limitation lies in the construction of measuring instruments. To minimize these effects, absorption measurements are usually made at the wavelength corresponding to maximum absorption of measured substance. The spectrophotometry is widely used in biochemical and clinical laboratories for its rapidity and reproducible results. The spectrophotometry in visible light permits to determine not only the coloured compounds but also the compounds that can be converted quantitatively into coloured compounds.

Methods of determining the concentration of a compound in solution:-

1) Calibration curve

This method is based on the absorbance measurement of a set of standard solutions of known concentrations for which the Beer-Lambert law must be valid. Then a graph of light absorbance (axis y) against concentration (axis x) is plotted. The relationship of these values should be linear. A straight line may not actually pass through any of the measured values due to experimental errors. The line should be drawn so that there are approximately equal numbers of points on each side of it. Then it is possibleto find concentration of unknown sample from calibration curve if its absorbance is known.

2) Calibration factor

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The calibration factor is calculated from values that you measured for drawing the calibration curve. Divide the concentration with the appropriate absorbance to obtain a set of numbers, from which you can calculate a mean value the calibration factor. Then you can multiply the absorbance of unknown sample by calibration factor to get the concentration.This method is useful in routine practice.

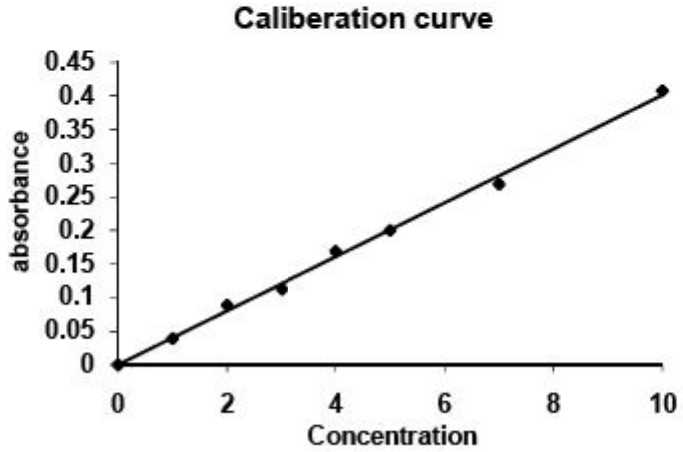

Fig. 4.2 : Caliberation Curve

3) Standard sample

This method of calculation of concentration is used predominantly in clinical biochemistry. The unknown sample (c_2) and the standard sample (c_1) are determined simultaneously. The concentration of a standard sample should be approximately in the middle of calibration curve.After measuring the absorbance of both samples we can calculate the concentration of unknown sample according to equation: $c_1 : A_1 = c_2 : A_2$ If we knowthe molar coefficientof absorbancewe can calculate the concentration directly from the Beer-Lambert law.

4.3 Viva Voce

1 What is generation time?

2 What is growth curve?

3 What is the difference between colorimetry and spectrophotometry?

4 What is the requirement of a standard plot?

4.4 References

- Clemens H.PostenBraunschweig, Federal Republic of Germany, Charles L. Cooney Cambridge, MA 02139 USA
- Chemistry 111 Lab: Introduction to spectrophotometry

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Unit – 5

Morphological Study and Microscopic Preparation of Algae

Structure of the Unit

5.0 Objectives

After going through this unit you will be able to :

- Describe the occurrence and distribution of *Prochloron, Dunaliella,Nitella, Botrydium, Navicula,Lamimaria,Gelidium*
- Discuss about the morphological characters of the a *Prochloron, Dunaliella,Nitella, Botrydium, Navicula,Lamimaria,Gelidium* genera.

5.1 Introduction

Description of each specimen was made elaborately using technical terms as seen under microscope. Identification of each specimen was made using manuals, monographs and identification keys etc.

5.2 *Prochloron*

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Distinguishing Features

Prochlorophyta was assigned as a new algal sub-class in 1976 by Ralph A. Lewin. *Prochlorophyta* were initially discovered in 1975 near the Great Barrier Reef and off the coast of Mexico. Prochlorophytes are very small microbes generally between 0.2 and 2 µm . They morphologically resemble Cyanobacteria (formally known as Blue Green Algae). *Prochlorophyta* are a photosynthetic prokaryote member of the phytoplankton group Picoplankton. They are abundant in nutrient poor tropical waters and use a unique photosynthetic pigment, divinyl-chlorophyll, to absorb light and acquire energy.Members of *Prochlorophyta* have been found as coccoid (spherical) shapes, like Prochlorococcus, and as filaments, like Prochlorothrix.Prochlorophytes can be classified as obligate photoautotrophic organisms.

Classification

Kingdom	:	Bacteria
Division	:	Cyanobacteria
Order	:	Prochlorales
Family	:	Prochloraceae
Genus	:	***Prochloron***

Cell Structure

1. The fine structure is described of *Prochloron*, a unicellular prokaryotic alga which contains both chlorophyll *a* and chlorophyll *b*.
2. The cell wall resembles that of a blue-green alga. The thylakoids and cytoplasm together occupy a wide peripheral band.

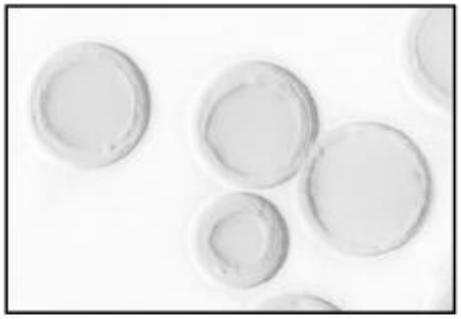

Fig. 5.1 *Prochloron*

3. However, the thylakoids are present not as single lamellae, as in blue-green algae, but in pairs or, sometimes, in thicker stacks.
4. Both thylakoids and cytoplasm are absent from a large central zone which is generally electron-transparent, but may contain electron-dense granules and fibrils.

The function of the central zone is not known. *Prochlorons are distributed within the tissues of Didemnid ascidians,* mainly in the cloacal cavities under the upper tunic of the

organism. Ascidians, or "sea squirts", are primarily known as a nusciance to humans. These sessile tunicates are found on most solid marine surfaces, including rocks, reefs, and algae.

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Reproduction

Reproduction in this divisions is strictly asexual, by simple cell division of fragmentation of the colony or filaments

Identification

Presence of differentiated plant axis containing nodes with lateral branches and internodes, reproductive organs complex with outer sterile covering.

Class : *Charophyceae*

Order : Prochlorales

Family : Prochloraceae

Genus : ***Prochloron***

The cell wall resembles that of a blue-green alga. The thylakoids and cytoplasm together occupy a wide peripheral band.

Prochloron is one of three known prochlorophytes, cyanobacteria that contain both chlorophyll *a* and *b* bound to a special light-harvesting protein (La Roche et al., 1996)

5.3 *Dunaliella*

The unicellular algae of the genus *Dunaliella* belong to the *Chlorophyta* (green algae). They fall into the class *Chlorophyceae*. Recently they were put into their own order of *Dunaliellales* and family of *Dunaliellaceae*. The genus *Dunaliella* consists of the two subgenera *Pascheria* (including five freshwater species) and *Dunaliella* (including 23 described saltwater species).

Classification

Phylum	:-	Chlorophyta
Class	:-	Chlorophyceae
Order	:-	Chlamydomonadales
Family	:-	Dunaliellaceae
Genus	:-	***Dunaliella***

Cell Structure

1. *Dunaliella tertiolecta* is a marine green flagellate with a cell size of 9−12 µm.

2. *Dunaliella* sp. are motile, unicellular, rod to ovoid shaped.
3. All species are unicellular and have two flagella in their motile vegetative phase
4. The organisms are relatively simple to cultivate and do not clump or form chains
5. This strain is reported to have oil yield of about 37% (organic basis).
6. *D. tertiolecta* is a fast-growing strain and that means it has a high CO_2 sequestration rate as well The best-known species is the extremely salt tolerant *Dunaliella salina.* all species possess a cup-shaped chloroplast.
7. For some species sexual reproduction has been reported with either homothallic or heterothallic behaviour.
8. It is known that the cell morphology changes throughout the life cycle. Moreover, cell morphology depends on environmental conditions.

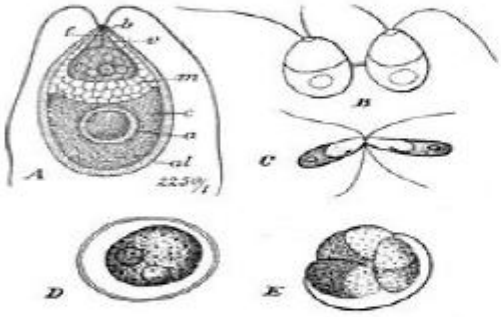

Fig.5.2 : *Dunaliella salina* Teodor. A: Vegetative cell, B: Zoospores in cell dvision, C: Mating gametes, D: Ripe zygospore, E: Zygospore germination

Identification

Presence of differentiated plant axis containing nodes with lateral branches and internodes, reproductive organs complex with outer sterile covering.

Class :Chlorophyceae

Order : Chlamydomonadales

Family : Dunaliellaceae

All species are unicellular and have two flagella in their motile vegetative phase

The organisms are relatively simple to cultivate and do not clump or form chains

Genus : ***Dunaliella***

Dunaliella sp. are motile, unicellular, rod to ovoid shaped

5.4 *Nitella*

Thallus Structure

The plant body has the appearance of a miniature horsetail. There is a main axis with alternate nodes and intermodes. The nodal zone bears a whole of lateral branches, often called 'leaves'; branches of limited growth with nodes and internodes just like the main axis may arise axillary to the leaves. The internode has a central cylindrical cell which is

NOTES

many times longer than broad and ensheathed by elongated narrow cells arising from basal nodes forming the cortex. The lowest node develops some multicellular rhizoids.

Reproductive Structure

NOTES

The lateral branches bear the reproductive organs, the globule and nucule. They are juxtaposed, the globule being directed upwards and a pair of nucules downwards. The globule is rounded, but nucule is somewhat short and oval with a roundish crown of ten cells in two tires and is relatively short. Other features of nucule and globule are similar to those of *Chara*.

Identification

Presence of differentiated plant axis containing nodes with lateral branches and internodes, reproductive organs complex with outer sterile covering.

Class : *Charophyceae*

Comparatively complex plant body being differentiate into root-like, shoot-like or leafy zones, sex organs stalked, multicellular with sterile jackets.

Order : *Charales*

Elaborately developed sex organs arising from nodes in pairs.

Family : *Characeae*

Presence of a pair of nucules and a globule in juxtaposed position on lateral branches, the globule being directed upwards and the nucules downwards, presence of ten-celled rounded two-tired crown on the tip of nucule.

Genus : *Nitella*

5.5 *Botrydium*

Thallus structure

The plant body is differentiated into a pear shaped or vesicular lobed aerial portion (assimilator) and a branched root-like underground rhizoidal portion. The plant body is coenocytic and cytoplasm contains many nuclei, lenticule to fusiform plastids, oil droplets and granules. The rhizoidal portion is without plastids.

Reproductive Structure

The mature thalli, the protoplast of aerial vesicle migrates into the rhizoidal portion and collects at the ends of the rhizoids which become inflated and swell to develop into rounded thick-walled tubers or cysts or coenocysts.

Identification

Thallus yellowish – green in colour, filamentous, coenocytic, branched, presence of oil as reserve food, sexual reproduction complex oogamous type.

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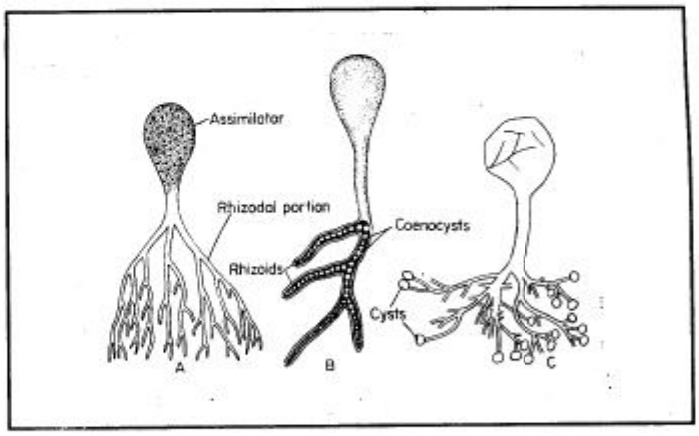

Fig. 5.3 : *Botrydium* Sp. A,B,C – Different stages of thallus developments

Class : *Xanthophyceae*

Plant coenocytic, balloon-shaped to filamentous, oil as reserve food present.

Order : *Heterosiphonales*

Plant body characterized by unicellular multinucleate vesicular lobed aerial portion and a colourless rhizoidal portion.

Family : *Botrydiaceae*

Plant body differentiated into vesicular lobed aerial portion and branched root like rhizoidal portion.

Genus : *Botrydium*

5.6 *Navicula*

Class : Bacillariophyceae

Order : Pennales

Family: Naviculodeaceae

Genus : *Navicula*

NOTES

Identification Characters

1. The thallus is diploid and unicellular.
2. It exhibits bilateral symmetry, therefore, it is included in pennales.
3. The cell wall is composed of two overlapping walls, the upper half is known as epitheca and the lower half is called hypotheca.
4. The cell is linear, lanceolate, ellipsoid, sigmoid or ovoid in shape.
5. The striations are arranged in a pinnate manner.
6. Central or axial strip called raphe is present.
7. The raphe extends from one end of the value to the other.
8. The raphe bears three round spots called nodules, one at the center and the other to one at each end.
9. It is commonly called pinnate diatom and constitutes the major constituent of fresh water phytoplankton.
10. Navicula halophila is boat shaped in valve view and rectangular on girdle view.
11. A single large nucleus lies embedded in the cytoplasmic band.
12. Usually one or two large parietal cgromotophores with 1 to many pyrenoids are present.
13. The pyrenoids are devoid of the starch sheath.
14. It exhibits slow or spontaneous gliding movement because of the presence of raphe.

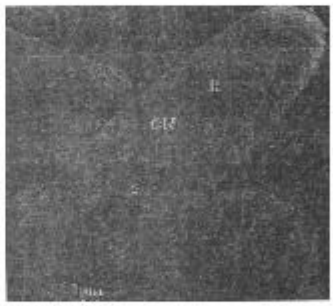

Fig. 5.4: Scanning electron micrograph of an epitheca of the fossil diatom *Navicula* subfossils

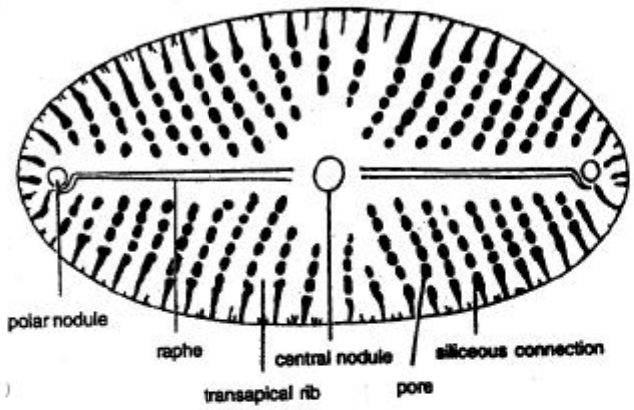

Fig. 5.5 : A *Navicula* Cell wall structure

Exercise

You have studied the identification characters of Navicula. You are provided with the materials and permanent slides of Navicula. Observe them carefully and draw well labeled diagrams and write the critical characters which you have observed.

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5.7 *Laminaria*

5.9.1 Classification

Class : *Phaeophyceae*

Order : *Laminariales*

Family : *Laminariaceae*

Genus : *Laminaria*

Laminaria is a ***brown alga*** *and* commonly known as ***Kelp***.

Laminaria is a ***marine*** alga and includes about 40 species.

It is widely distributed in temperature and Article zones. The common species are *Laminaria flavicans*.

Fig. 5.6: *Laminaria* Sporophyte

Structure

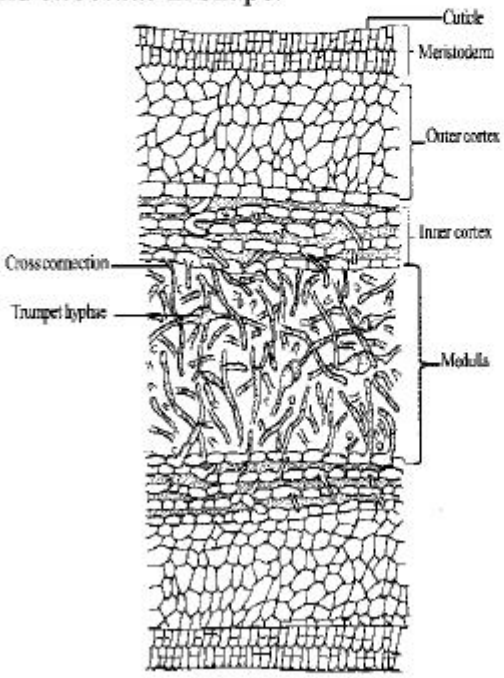
- A ***diploid sporophyte plant is*** consists of a ***holdfast, stipe*** and ***blade.***It is attached with substratum by a ***solid disc shaped holdfast.***
- It consists of ***hapteron*** which are root-like, branched or unbranched threads. From the holdfast ***smooth, cylindrical*** or ***flat*** stipeis are arises,which grows up to ***30-40 feet.***
- The stipe bears a ***single*** or ***many*** leaf like structures called ***blade*** or ***lamina.***
- The lamina is flat, long leaf-like and ribbon shaped. New lamina arises at the apex of the stipe.
- The lamina grows up to 2 meters.The margin of the blade contains fruit bodies called sori.
- The sori contain sporangia.*Laminaria* is ***heteromorphic.***
- The sporophytes are very large and the gametophytes are ***microscopic.***

Internal Structure of Thallus

- The transverse section of the thallus shows ***three regions*** namely an outer ***epidermis (meristoderm),*** a middle ***cortex*** and inner ***medulla.***

NOTES

- The epidermis consists of two layers of ***meristematic cells***. The outer layer is formed of thickened ***periclinal walls***.
- The thickening material forms a mucilaginous non-cellular layer called the ***cuticle***.
- The meristematic cells are ***small*** and ***cuboidal*** in shape.
- Between the ***meristoderm*** a ***medulla*** , cortex is present,which consists of ***large cells***.
- The cortex consist of **an outer cortex *(elongated broad cells)*** **a inner cortex(*elongated cells).***
- The elongated cells contain threa outgrowths or ***cross connection hyphae.***
- The inner cortex contains ***mucila canals*** filled with ***mucilage***.
- The inner layer is medulla and consists of loosely arranged cells, ***cross connections, hyphae*** and ***trumpet hyphae.***



like

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Fig. 5.7 A *: Laminaria* Internal Structure

- The cell consists of a ***cell wall, plasma membrane,*** **a *nucleus*** and ***chromatophores.***
- The ***fucosan*** is the stored ***reserve food.***

5.8 *Gelidium*

It is a widely distributed small seaweed growing attached to rocks in the littoral zone. *G. Corneum* grows on the rocks in pools in upper littoral belt in the Indian West Coast.

Vegetative Characters

1. It is in the form of an entangled mat of branched anastomosing polymorphic axes.
2. The dorsivental, creeping stolon, clusters of erect fornds tend to arise on the upper side. Peg-like holdfasts on the lower side.
3. Axes are pseudoparenchymatous and uniaxial.

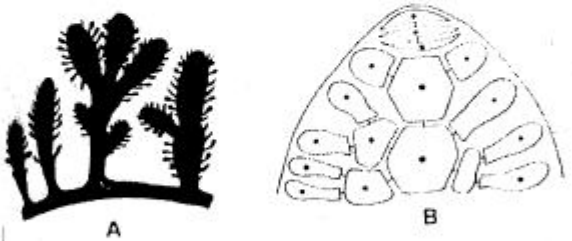

Fig. 5.7 : *Gelidium pulchellum*
A. Part of thallus B. Section of region

4. The apical cell is lenticular.
5. Internal hyphae serve as mechanical supporting function.
6. Fronds of *G. Corneum* are cartiliaginous, compressed, dichotomous, twice or triceprinnately branched.

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Reproduction

Vegetative Reproduction : Occurs from detached parts of fronds or stolons.

Sexual reproduction

i) The Spermatangia are carpogonia are formed from the cortical cells of upright fronds.

ii) The Cytoscarps are terminal in position or in specialized club-shaped lateral branches of limited growth.

iii) Cystocarp-bearing branches of *G. coreneum* are swollen.

iv) Cruciately dividing tetrasporangia are located terminally in unmodified vegetative blades.

v) In G. robustum, both tetrasporangial and carposporangial plants exhibit rhythmic activity in spore discharge.

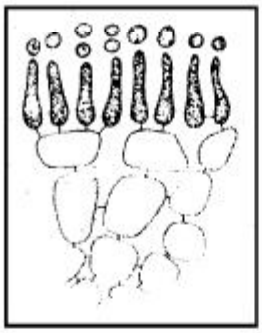

Fig. 5.8 A : *Gelidium sp.*, spermatangia

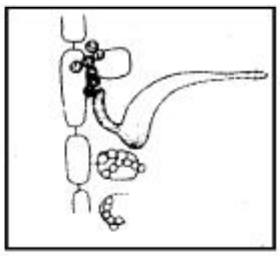

Fig. 5.8 B : *Gelidium sp.*, carpogonium and its fusion with nurse cells

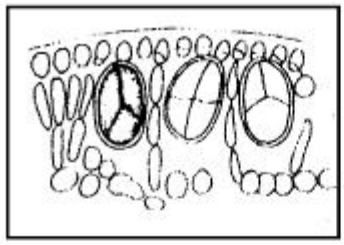

Fig. 5.8 C : *Gelidium sp.*, formation of tetrasprangia

Classification

Algae

(i) Simple thallus (ii) Presence of chlorophyll (iii) Cell wall cellulose

Rhodophyceae

i) Chromatophores pure red to dark purple.

ii) Photosynthetic reserve floridean starch or floridoside.

iii) Male gametes non-motile.

iv) Female reproductive organ with a receptive structure trychogyne.

v) Post-fertilization product a cystocarp.

NOTES

Floridae

(i) Thallus basically filamentous. (ii) Pit connection between sister cells.

(iii) Ells with more than one cromatophores. (iv) Carpogonium highly specialized.

Gelidiales

i) Thalli are macroscopic and cartilaginous.

ii) Internal Structure Uniaxial.

iii) Pit plugs have cap membranes and one layer pit caps.

iv) Carpogonia simple but aggregated.

Gelidiaceae

(i) Medulla parenchymatous (ii) Auxiliary cell absent

(iii) Three somatic phases (iv) Tetrasporangia cruciate

Gelidium

i) Branched anastomosing polymorphic axes.

ii) Axes are pseudoparenchymatous and umircial.

iii) Apicall cell is lenticular.

5.11 Viva-Voce

1 What is the 'algal bloom'?

2 Explain the haplodiplontic life cycle.

3 Name the type of reserve food in green algae.

4 Fill in the blanks :

(i) Fusion between gametes of unequal sizes is called ……..

(ii) A motile flagellated asexual cell is known as …….

(iii) Stonewort is the common name for ……

(iv) Botrydium belongs to family ……

(v) The zoospores in *Botrydium* is ……

(vi) In *Botrydium* sexual reproduction is ……

(v) *Dunalilla* belongs to family ……

5 Select the correct answer :

(i) Reserve food is starch in

(a) Chlorophyceae (b) Myxophyceae (c) Phaeophyceae

(d) Rhodophyceae

(ii) Asexual spores which are motile and have two flagella are called

(a) Zoospores (b) Synzoospores (c) Zygospores

(d) Chlamydospores

(iii) Which of the following has reserve food as starch ?

(a) Volvax (b) Vaucheria (c) Ectocarpus (d) Batrachospromum

(iv) *Botrydium* belongs to class :

(a) Chorophyceae (b) Charophyceae

(c) Xanthophyceae (d) Bacillariophyceae

(v) In *Botrydium* the sexual reproduction is

(a) Isogamous (b) Anisogamous (c) Heterogamous

(d) Oogamous

6 Name the type of reserve food in green algae.

NOTES

5.12 References

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Unit – 6

Morphological Study and Microscopic Preparation of Fungi

Structure of the Unit

6.0 Objectivess

After going through this unit you will be able to :

- Describe the occurrence and distribution of *Pilobolus, Chaetomium, Peziza, Morchella, Melampsora, Polyporus, Drechslera, Phoma.*
- Discuss about the morphological characters of the above genera.

6.1 Introduction

Description of each specimen was made elaborately using technical terms as seen under microscope. Identification of each specimen was made using manuals, monographs and identification keys etc.

6.2 *Pilobolus*

NOTES

Classification

Division	:	Eumycota
Sub-division	:	Zygomycotina
Class	:	Zygomycetes
Order	:	Mucorales
Family	:	Pilobolaceae

Pilobolus is atypical but fascinating coprophilous (dung-inhabiting) member of the order Mucorales. It is commonly called "hat-thrower". It grows very rapidly, and is one of the first fungi to fruit in the extended succession that occurs on dung.

The mycelium is aseptate, coenocytic and much branched. It produces rhizoids below and sporngiophores above, The rhizoids help to anchor the mycelium to the substratum and to absorb the nutrients.

Asexual Reproduction

- It reproduces by the formation of non motile spores formed on sporangiophores.
- Its unbranched sporangiophores are 2-4 cm tall, arise from the horizontal mycelium.
- They are erect and have a unique explosive dispersal mechanism.
- The sporangiophore consists of trophocyst, sporangiophore stalk, sub-sporangial vesicle and sporangium (also called mitosporangium.

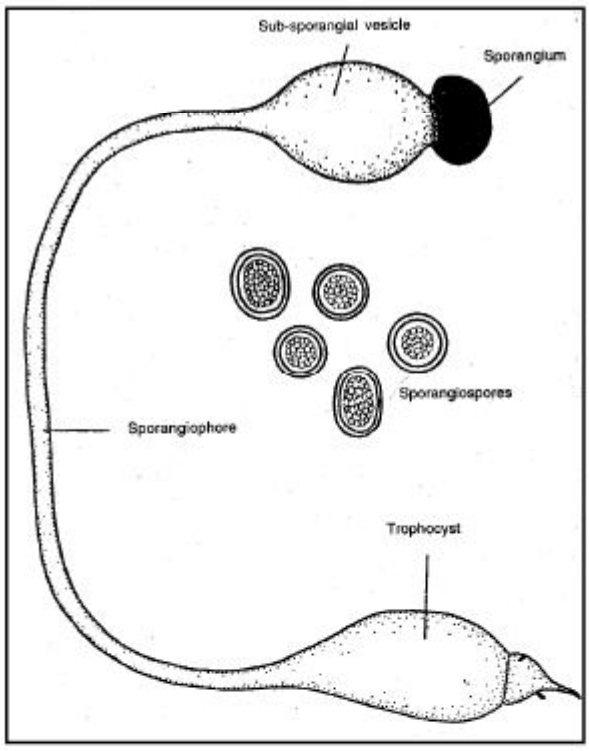

Fig. 6.1 : *Pilobolus longipes*, Sporangial apparatus

- The swollen base of the sporangiophore is called trophocyst (Gr.trophe=food+kysis=bladder) The stalk of the sporangiophore is narrow, tube like structure that holds a vesicle and sporangium at the apex.

- Beneath the black apical mitosporangium is a lens-like subsporangial vesicle, with a light-sensitive 'retina' at it base that controls the growth of the sporangiophore very precisely (above), aiming it accurately towards any light source.
- Bit longitudinally compressed, rigid, dark colored sporangium is found.
- Inside the sporangium columella is present.
- The vesicle is phototropic having osmotically active compounds causing pressure in the sporangiophore and the subsporangial vesicle to build up until it is more than 100 pounds per square inch (7 kilograms per square centimeter).
- This eventually causes the vesicle to explode, hurling the black sporangium away to a distance of up to 2 meters, directly towards the light.
- The mucilaginous contents of the subsporangial vesicle go with the sporangium, and glue it to wherever it lands on.

NOTES

Sexual Reproduction

- The members are heterothallic.
- Between tong-shaped suspensors zygospores are formed.
  - The wall of zygospores is thick, smooth and light brown to black in colour.

6.3 *Chaetomium*

Classification

Kingdom	:	Mycota
Division	:	Eumycota
Sub-Division	:	Ascomycotina
Class	:	*Pyrenomycetes*
Order	:	*Sphaeriales*
Family	:	Melanosporaceae
Genus	:	*Chaetomium*

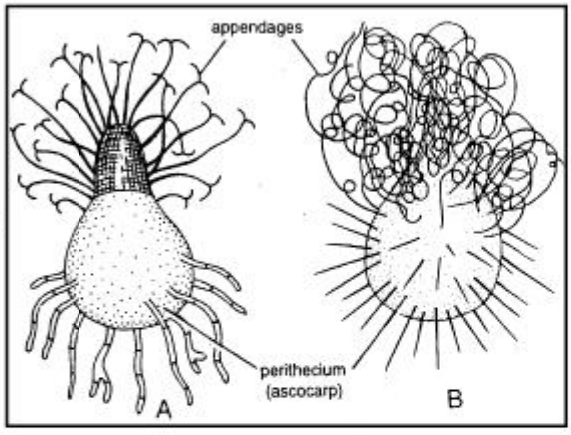

Fig. 6.2 : *Chaetomium*, Different types ascoarps

Practical work and study

[A] Study of perithecium

- The fruiting body is a perithecium.
- It is a flask-shaped structure.
- Perithecium consists of a multilayered

wall called peridium.

- The tubular asci project from the base of perithecium; towards the opening.
- The opening of the flask is called paraphyses.

NOTES

- It is lined with hairs called paraphyses.
- Thin, long and branched hairs occur along the outer surface of the ascocarp. The hairs in the apical part of the perithecium are either curly or forked.

[B] The L.S. of ascocarp

Study the Slide of L.s. of ascocarp.

- Flask-shaped is ascocarp found.
- A multilayered wall forms the outermost covering. It is called peridium.
- Numerous multicellular hairs project from the outer layer of the peridium.
- Asci are borne in groups at the base of the perithecium cavity.
- Tubular ascus has linearly arranged ascospores.
- Ascocarp opens outside by a hole called ostiole.
- Ostiole is lined with numerous hairs called paraphyses.

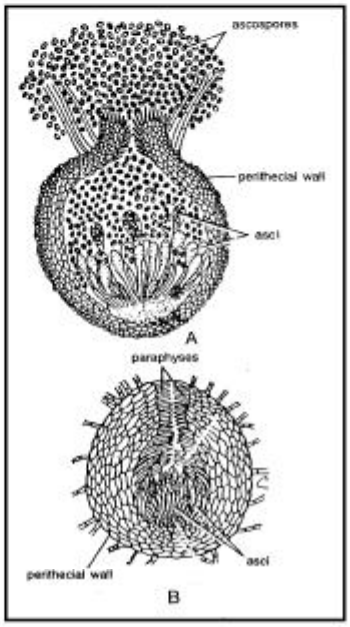

Fig. 6.3 A: A & B
Chaetomium
LS. Ascoarps

Identification

Kingdom – **Mycota.**

(1) Chlorophyll absent, (2) Reserve food glycogen, (3) Cell wall of fungal cellulose

Division – **Eumycota.**

Presence of definite cell wall throughout the vegetative phase.

Sub-Division - **Ascomycotina.**

(1) Mycelium septate, (2) Spores borne endogenously in ascus, (3) Spores in definite numbers, in multiples of two, usually eight.

Class – **Pyrenomycetes.**

Ascocarp a perithecium.

Order – **Sphaeriales.**

(1) Ascospores thread-like, (2) Perithecia within a well-developed stroma.

Family – **Melanosporaceae.**

(1) Perithecia beaked with globose or oval asci, (2) Each ascus with eight dark coloured ascospores.

NOTES

Genus – ***Chaetomium.***

(1) Long beaked perithecia covered with curly or forked hair, (2) The wall of the asci mucilaginous.

6.4 *Peziza*

Classification

Kingdom	:	Mycota
Division	:	Eumycota
Sub-Division	:	Ascomycotina
Class	:	*Discomycetes*
Order	:	*Pezizales*
Family	:	Pezizaceae
Genus	:	*Peziza*

Practical work and study

[A] Vegetative structure

Take apothecium, tear off a part to study mycelium.

- It is a common saprophyte growing on rich humus soils and decaying woods. Sometimes it becomes coprophilous (i.e. grows on dung.
- The mycelium is a complex system which penetrates the substratum.
- The mycelium is profusely branched, septate and the cells are multinucleate.

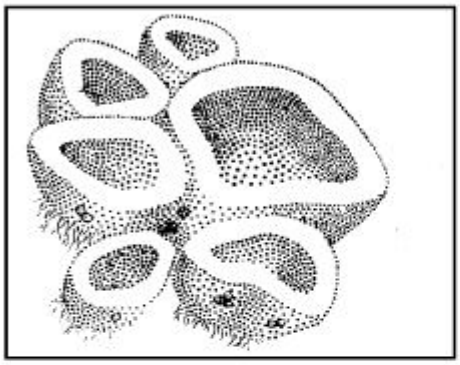

Fig. 6.4 : *Peziza* : Ascoarps

- The mycelium becomes visible only in the form of apothecial cups above the ground surface.

[B] Reproductive structures (Asexual)

Study the mycelium for the present of conidia/ chlamydospores.

- The 'condia' are the asexual reproductive bodies, produced rarely.
- Hyaline, to lightly coloured and elliptical conidia are present .
- In some species, thick walled and intercalary chlamydospores are produced singly or in series on mycelium.

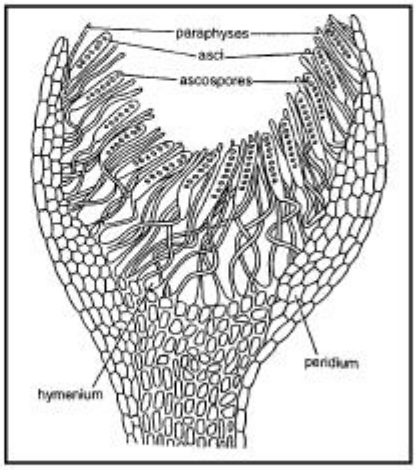

Fig. 6.5 : *A : Peziza :* V.S. Ascocarp

- The new mycelium is produced by germination chlamydospore.

[C] Ascocarp, asci and ascospores

Cut a V.S. of apothecial cup, stain with cotton blue, mount in lactophenol and study.

- An apothecium ascocarp is present.. It is fleshy, shortly stalked, about 5 cm in diameter with a bright red or bright grey lining.
- A vertical section of ascocarp shows a cup-shaped structure made up of mycelium. It shows 3 regions – hymenium, hypothecium and excipulum.

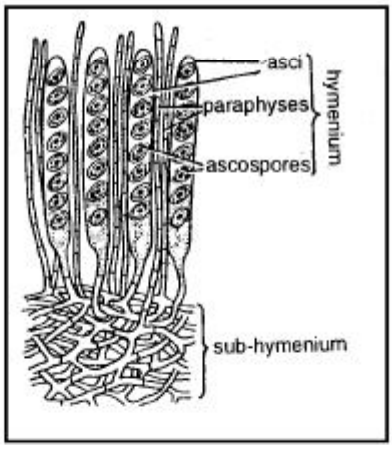

Fig. 6.5 B : *Peziza* : V.S. Assocarp

- Hymenium consists of asci and paraphyses arranged vertically in palisade-like layer, that is orange-red coloured.
- Thin and lightly coloured hyphae forms hypothecium that runs parallel to hymenium.
- Excipulum forms a basal large part of loosely interwoven hyphae of apothecium.
- The hymenium is encircled by densely interwoven hyphae forming the wall of the apothecium – the peridium.

- A single row of eight ascopores, arranged obliquely. Ascus is elongated in shap.
- Each ascospore is uninucleate, hyaline or faintly coloured, elliptical, surface smooth or coarsely reticulate and ellipsoidal. It germinates to form new mycelium.

NOTES

Identification

Kingdom – **Mycota.**

(1), Reserve food glycogen, Chlorophyll absent (3) Cell wall of fungal cellulose

Division – **Eumycota.**

Presence of a definite cell.

Sub-Division - **Ascomycotina.**

(1) Mycelium septate, (2) Spores borne endogenously in ascus, (3) Spores in definite numbers, in multiples of two, usually eight.

Class – **Discomycetes.**

Ascocarp an apothecium.

Order – **Pezizales.**

(1) Apothecia fleshy or leathery, and usually not in stroma.

Family – **Pezizaceae.**

(1) Apothecia cup-shaped or discoid, and not differentiated into stipe and pileus.

Genus – ***Peziza.***

(1) Apothecia 1 to 5 cm in diameter and conspicuous, (2) Vegetative mycelium penetrating the substratum, (3) Ascospores not apiculate

6.5 *Morchella*

Classification

Kingdom	:	Mycota
Division	:	Eumycota
Sub-Division	:	Ascomycotina
Class	:	*Discomycetes*
Order	:	*Pezizales*
Family	:	Helvellaceae
Genus	:	*Morchella*

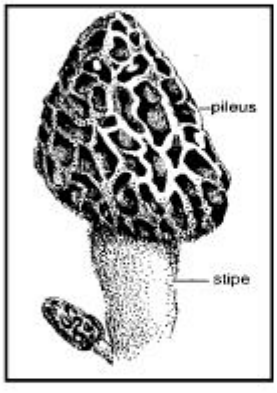

Fig. 6.6 : *Morchella*

NOTES

Practical work and study

[A] The vegetative structure

Pinch off a small piece of the fungus or cut a section. Stain in cotton blue, mount in lactophenol and study.

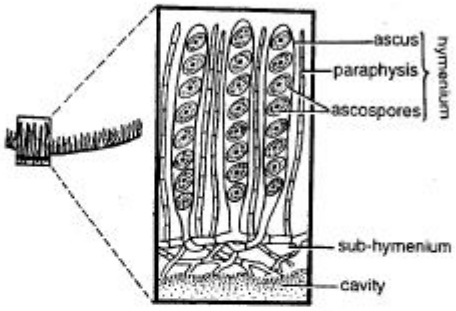

Fig.6.7 A *Morchella* : A portion of a section through depressing of pileus

- All the species are saprophytes and grow in deciduous forests on decaying wood or in humus soil. These are commonly known as morels.
- The mycelium is underground, growing a few inches deep in the soil.
- Mycelium is freely branched and the hyphae are septate. Each cell has many nuclei.
- The mycelium is woven to form a stalk, at the tip of which a conical pileus is present.
- The pileus is ridged and grooved. The grooves develop ascocarps.

[B] Ascocarp, asci and ascospores

Cut a L.S. through a groove that represents an apothecium, stain with cotton blue, mount in lactophenol and study.

- The ascocarp is an apothecium, varying from 1 to 5 inches in height.
- The colour of the apothecium varies from grayish white to dark brown depending on the species and age.
- The mature ascocarp consists of a stalk known as stipe, surmounted by a hollow conical cap the pileus, the fertile portion of the ascocarp. The stipe is cream coloured, thick, fleshly and hollow.
- The surface of the pileus is thrown into many strong longitudinal and transverse folds, so that it becomes coarsely pitted (i.e. with ridges and grooves).
- The ridges are the sterile areas whereas depressions are the fertile areas.
- A section of the pileus through the depression would reveal the hymenium with asci and paraphyses, the latter being sterile structures.
- Below the hymenium, the mycelium forms a close interwoven structure known as sub-hymenium.

- The asci and paraphyses in the hymenium are arranged perpendicular to the surface of depression and form a palisade-like layer,
- Each ascus is a long cylindrical structure and contains eight ascospores arranged obliquely and uniseriately.
- 10. The ascospores are large, hyaline, oval and arranged in single row.

NOTES

Identification

Kingdom – **Mycota.**

(1) Chlorophyll absent, (2) Reserve food glycogen, (3) Cell wall of fungal cellulose

Division – **Eumycota.**

A definite cell was present.

Sub-Division – **Ascomycotina.**

(1) Mycelium septate, (2) Spores borne endogenously in ascus, (3) Spores in definite numbers, in multiples of two, usually eight.

Class – **Discomycetes.**

Ascocarp an apothecium.

Order – **Pezizales.**

(1) Apothecia fleshy or leathery, (2) Apothecia usually not in stroma.

Family – **Helvellaceae.**

(1) Apothecia not cup shaped, (2) Apothecia differentiated into stipe and pileus.

Genus – ***Morchella.***

(1) Pileus costate (2) pileus thrown into many ridges and grooves, (3) Sterile stipe and fertile pileus distinct.

6.6 *Melampsora*

Work to be done

To study

[A] The symptoms of the disease.

[B] Diagnostic features of causal organism.

Practical work and study :

[A] Symptoms

NOTES

Study the diseased plant specimen of linseed-

- The rust of linseed (*Linum usitatissimum;* vern. Alsi; fam. Linaceae) is caused by a basidiomycetous fungus, *Melamspora lini.*
- The affected plants become bright orange in colour due to the presence of large number of uredosori.
- Uredosri occur on both the leaf surfaces and other aerial parts of the plant.
- The leaves die prematurely.
- Teleutosori appear later and are formed on the stem. These are brown to black crusts covered by epidermis.
- Control measures include sanitation, use of resistant varieties, seed treatment with chemicals to inactive teleutospores, etc.

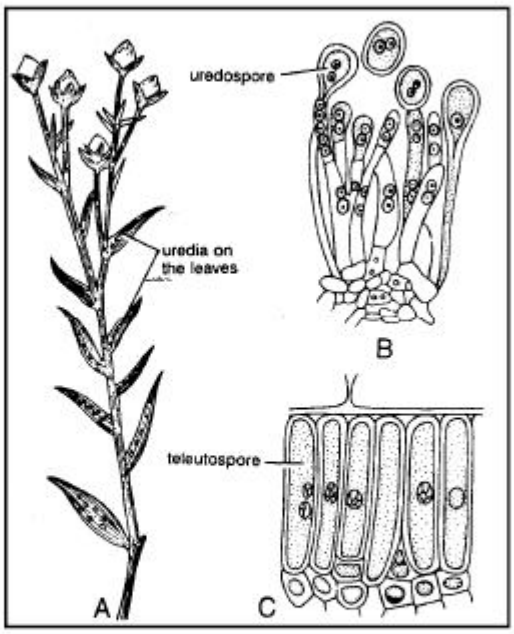

Fig. 6.8 Rust of linseed caused by *Melampsora lini.* A. Infected plant, B. Uredosorus, C. Teleutosorus

[B] Casual organism

Study the T.S. of leaf passing through uredosori and teleutosori.

- The pathogen causing rust of linseed is *Melamspora lini*, of basidiomycetes.
- It is an autoecious rust.
- About 5-10 amphigenous pycnia are grouped together.
- Aecia lack peridum, are epiphyllous and surround pycnia to form ring.
- Uredia are amphigenous and irregularly scattered. Paraphyses occur mixed with uredospores which are ellipsoidal, obovoidal. The wall is hyaline and finely echinualate.
- Telia are amphigenous, circular or elongated along the stems, often fused and covering large areas, subepidermal and black.

6.7 *Polyporus*

Vegetative Structure

- Thallus is mycelial type.
- Mycelium is composed of septate, branched hyphae,

- Dikaryotic somatic mycelium produces characteristic basidiocarp visible to naked eye and lies above the substratum.

Reproductive Structure :

NOTES

Basidiocarp – The basidiocarp is reflexed leathery and hard to brittle in texture. The upper surface is coloured, smooth with concentric zonations. The lower surface is with distinct pores of circular or angular outline. A T.S. through basidiocarp shows the presence of yellowish brown hymenial layer in the lower surface of basidocarp. Above the hymenial layer, there is context zone which is composed of thick-walled, yellowish brown hyphae. The hymenial layer is lined with basidia around the pores.

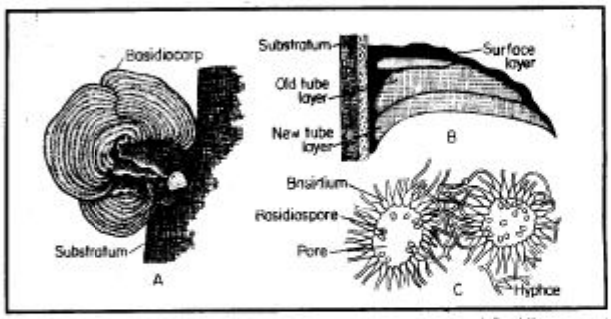

Fig. 6.9 *Polyporus Sp.* : A. Basidocarp, B & C – Sections through

Basidium – They are hyaline, clavate, and tetrasterigmatic. Each sterigma bears a single hyaline oval basidiospore.

Basidiospore – It is oval to ellipsoidal in shape and pale-coloured.

Classification

Mycelium septate, basidia and basidiospores present.

Class	:	*Basidiomycetes (Basidiomycotina)* Basidia of holobasidia type.
Sub class	:	Homobasidiomycetes
		Basidiocarp well-developed
Series	:	Hymenomycetes
		Hymenium unilateral or amphigenous, surface poroose.
Order	:	Aphyllophorales
		Hymenium lining shallow or deep pore tubes.
Family	:	Polyporaceae
		Pores circular to angular, pore tubes sunk to an even depth into context forming a distinct stratum.
Genus	:	Polyporus

Precaution : Stay away from beautifully coloured mushrooms for they are often poisonous.

NOTES

6.8 *Drechslera*

Division	:	Eumycota
Sub-division	:	Deuteromycotina
Class	:	Hyphomycetes
Order	:	Moniliales
Family	:	Dematiaceae
Genus	:	*Drechslera*

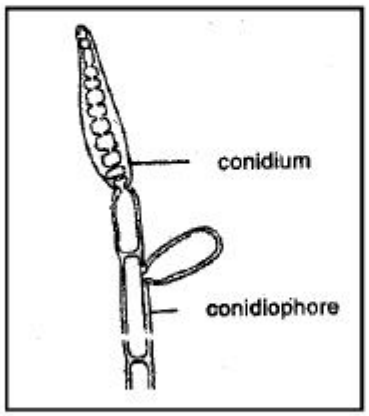

Fig. 6.10 : Conidiophore and condium of *Drechslera*

Mycelium branched, septate, subhyaline or brown. Conidiophores straight or flexuous, sometimes geniculate, mostly unbranched, brown, smooth, septate. Conidia solitary, sometimes catenate, straight or curved, clavate, ellipsoidal, fusoidal, cylindrical, rounded at the ends, straw coloured or pale, dark brown, many celled, sometimes end cells paler than intermediate cells mostly smooth, pseudoseptate.

6.9 *Phoma*

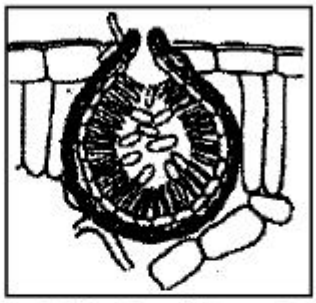

Fig. 6.11 : Pycnidium of *Phoma*

Mycelium branched, septate, subhyaline or brown, Pycnidia flask shaped or globose or slightly lens shaped with a small papilla at the apex, membranous to leathery made up or psuedoparenchymatous tissue. The wall is brown or almost carbonous, black. Conidiophores are present inside the pycnidium lining the pycnidial wall. Conidiophores thin, short or almost lacking, simple or sometimes forked. Conidia slimy, thin walled, ellipsoid, spindle-shaped, cylindric, pyriform or almost spherical, mostly one celled, hyaline, smooth, usually with two oil drops. Conidia are released along with slime in along thread called cirrhus.

6.10 Viva-Voce

1. Who proposed non-chlorophyllous unicellular origin of fungi?
2. Fungi are reported since ..... period.

3. Holocarpic fungus is : (a) Albugo (b) Agaricus (c) Synchytrium (d) Ustilago
4. In which form reserve food material is stored in fungi?
5. Name the subdivision of fungi having dolipore septum.
6. Name the subdivision of fungi having clamp connection.
7. The fungi whose perfect stage is not known are included under the group …….
8. Members of Mastigomycotina are characterized by the presence of ……
9. Members of Myxomycota are commonly known as ……
10. Who classified all fungi into Mycota and divided it into two divisions; Myxomycota and Eumycota?

NOTES

6.11 References

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NOTES

Unit - 7

Study of Morphology, Anatomy and Reproductive Structures and Microscopic Preparation of Bryophytes

Structure of the unit

7.0 Objectives

After going through this unit you will be able to:

- Describe the structure of *Pellia, Plagiochasma, Notothylas, Sphagnum,* and *Polytrichum.*
- Discuss about the reproduction of *Pellia, Plagiochasma, Notothylas, Sphagnum,* and *Polytrichum.*
- Discuss about the structure of sporophyte of *Pellia, Plagiochasma, Notothylas. Sphagnum,* and *Polytrichum.*

7 .1 Introduction

A liverwort is a flowerless, spore-producing plant with the spores produced in small capsules. Bryophytes have a **gametophyte** stage and a **sporophyte** stage. The spore capsule (possibly with a supporting stalk, or **seta**) is the sporophyte and this grows from the gametophyte stage.

Dorsiventrally differentiated gametophytes which are either simply thallose or with leaf like appendages and are always nerveless. Internal tissues are homogenous or differentiated and sex organs are borne terminally or developed from superficial dorsal cells. Sporophyte differentiated in to foot, seta and capsule- always of limited growth.

NOTES

A class of plants within the Bryophyta comprising the mosses, which are found in both damp (including freshwater) and drier situations. Mosses possess erect or prostrate leafless stems, which give rise to leafless stalks bearing capsules. Spores formed in the capsules are released and grow to produce new plants.

Bryopsida is the largest class of Bryophyta. It consists of about 660 genera and 14,500 species distributed all over the world. Plants are known as mosses. Bryopsida is devided into 3 subclasses, Sphagnidae, Andreadae and Bryidae. The subclass Sphagnidae is having only one order sphagnales. The gametophyte is differentiated into 2 stages, 1) a branched prostrate juvenile filamentous thalloid stage; peritonea a transitory stage and (2) an erect leafy shoot, the gametophores. The 85ntermodal85s consists of stem and spirally arranged leaves and bears the sex organs. The rhizoids are multicellular branched and provided with oblique cross walls. The sporogonium consists of foot, seta and capsule.

Description of each specimen was made elaborately using technical terms as seen under microscope. Identification of each specimen was made using manuals, monographs and identification keys etc.

- Study the external features of the gametophyte, both from dorsal and ventral surfaces.
- Place the thallus in the pith. Cut T.S. and stain either in safranin or fast green. Mount in glycerine and study.
- Cut L.S. of thallus through median portion. Stain in fast green , Mount in glycerine and study the antheridia.
- Cut L.S. of thallus through median portion. Stain in fast green , Mount in glycerine and study the archegonium.
- Cut L.S. of thallus through median portion. Stain in fast green , Mount in glycerine and study the sporophyte.

7.2 Plagiochasma

Systematic Position

Class - Hepaticopsida

Order - Marchantiales

Family - Marchantiaceae

NOTES

The genus *Plagiochasma* is represented by about 20 species, widely distributed in most parts of the world. Some 10 species of the genus occur in India. Most of the Indian species are xeromorphic and are more adapted to drier habitats. *P. appendiculatum* occurs in the plains of South India, Gujarat, Rajasthan and Madhya Pradesh. *P. simlensis* is found near water pools and streams in Simla hills and *P. articulatum* inhabits alpine region in the Himalayas at an altitude of about 4,500 meters.

Plagiochasma appendiculatum, P. articulatum, P. simlensis, P. intermedium, P. cordatum and P. quadricorrzutum are some common Indian species. Of these, *P. articulatum, P. simlensis, P. cordatum find P. quadricornutum* are endemic to India.

Practical work and study

Gametophyte

External Features

1. The plant body is large, prostrate, dorsiventral, thalloid and is of variable shape. It is oblong-obovate in *P. articulation*, obcordate in *P. appendiculatum* and (1 linear-elongate in *P. intermedium* and *P. simlensis*. The dichotomously branched thallus is dark green in colour, and its dorsal surface has an indistinct midrib.
2. The ventral surface bears scales and rhizoids along the median line. The scales are of appendiculate type. The scales are multicellular and one cell in thickness.
3. The unicellular rhizoids are of two types: (i) smooth walled, and (ii) tuberculate. The inner wall of the smooth walled rhizoids is smooth while those of the tuberculate rhizoids has peg-like ingrowths, which appear as small circular dots in surface view.
4. Sex organs are borne on special receptacles present on the dorsal surface of the mature thallus. The male receptacles (antheridiophores) are sessile and have a characteristic horse shoe shape. The female receptacles (archegoniophores) have a short stalk and are disc like.

Internal Structure

In a transverse section the thallus of *Plagiochasma* shows three distinct regions: (i) epidermal region, (ii) photosynthetic region, (iii) storage region.

[i] Epidermal region

This region includes both the dorsal and ventral epidermis. The dorsal (upper) epidermis forms a uniseriate covering over the photosynthetic region. It is interrupted by simple pores that are connected internally with air chambers. The air pores are slightly raised

above the surface of the thallus. Each pore is surrounded by 3-8 concentric rings, each with 6-8 cells. The ventral (lower) epidermis follows the storage region. It is made up of thin-walled cells. Many cells of the lower epidermis give rise to rhizoids and scales.

[ii] Photosynthetic region

NOTES

It consists of an irregular network of complex air chamber system. The walls of the air chambers are made up of a single layer of chlorophyllous cells. The air chambers are empty, i.e., without any photosynthetic filaments. They are interconnected through narrow passages. This region of the thallus is mainly concerned with the manufacture of food and is also called assimilatory region.

[iii] Storage region

The photosynthetic region is followed by a well-developed storage region made up of parenchymatous cells. These cells are rich in starch grains and reserve food material. Inter or intracellular mycorrhiza is occasionally present in the storage region.

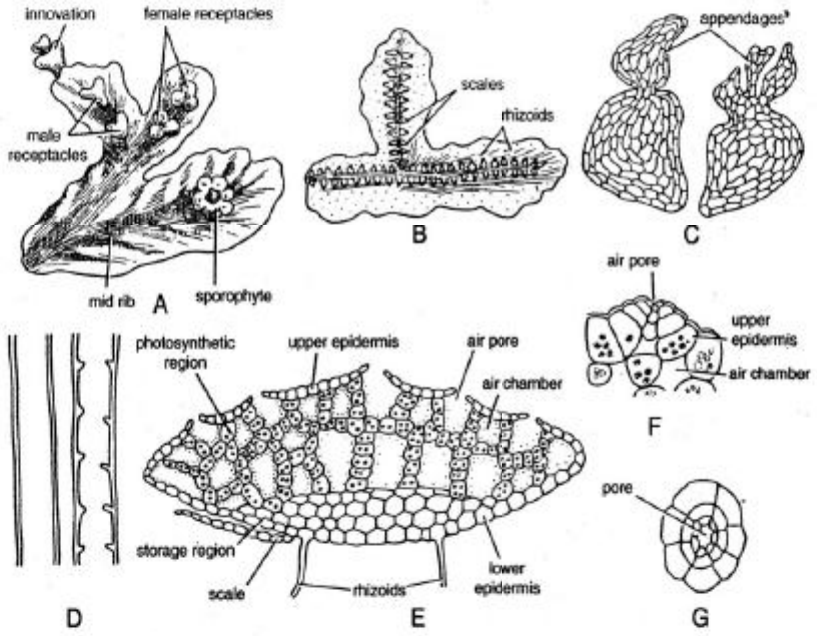

Fig. 7.1: *Plagiochasma:* Structure of Thallus with Rhizoids and Scales

Reproduction

Plagiochasma reproduces both vegetative and by sexual methods.

[I] Vegetative reproduction

Vegetative reproduction usually takes place by means of certain adventitious branches, known as innovations. These branches develop at the apex of the thallus. When detached from the parent thallus, they grow into new independent thalli.

NOTES

[II] Sexual reproduction Plants may be homothallic (e.g., *P. appendiculatum)* or heterothallic (e.g., *P. intermedium*, *P. articulatum, P. cordatum, P. quadricornutum*). Sex organs develop on specialized structures, known as receptacles.

1. Male receptacle. The internal structure of the receptacle is similar to that of the thallus. It contains several antheridial chambers, each with a single antheridium. Each antheridial chamber opens on the surface of the receptacle by a pore. A number of simple pores are also present on the receptacle and they open internally into air chambers. Antheridia develop acropetally on the surface of the male receptacle.

(a) Structure of antheridium. The mature antheridium is a club-shaped structure attached to the base of the antheridial cavity by a multicellular stalk. It has one-cell thick jacket layer of elongated or isodiametric cells which encloses a mass of androcyte cells. The androcyte cells metamorphose into slender filiform antherozoids. Each antherozoid has two flagella attached to its anterior end.

2. Female receptacle. The archegonia are present on special upright branches known as archegoniophores. They are sessile or stalked. The archegoniophore consists of a short stalk and a terminal disc. There is no rhizoidal furrow on the stalk as in *Marchantia.* The archegoniophore is encircled by minute, ovate to linear scales organised in a compound imbricate manner. The disc of the receptacle is 2-9 lobed and there is only one archegonium on each lobe. The anatomy of the archegoniophore is similar to that of the thallus, but it has barrel-shaped air pores.

(a) Position of archegonia on archegoniophore: On the young receptacle the archegonia are borne acropetally on the upper surface of the disc, and the necks of archegonia are directed upward. After fertilization, due to rapid growth of the central part of the disc, the archegonia come to lie on the flanks of the disc with their necks placed horizontally, i.e., parallel to the lobes of the receptacle.

(b) Structure of archegonium: Archegonia lie in grooves formed by the overgrowth of the involucre. A mature archegonium is a flask shaped structure attached to the lobe of the disc by a short stalk. It has a broad venter and a narrow neck. The venter contains a large egg and a venter canal cell. The neck of the archegonium is composed of six vertical rows of cells and it has 8-9 neck canal cells. There are four cover cells at the tip of the neck.

[II] Structure of mature sporophyte

The sporophyte of *Plagiochasma* is differentiated into a bulbous foot, a short seta and a spherical capsule.

1. Foot. It is the basal bulbous part of the sporophyte, composed of thin walled parenchymatous cells. It is an anchoring and absorbing organ of the sporophyte and

derives nutrients from the gametophyte.

2. Seta. It is present between the foot and the capsule. It is composed of several vertical rows of parenchymatous cells.

3. Capsule. The capsule is a nearly spherical structure. It has a single layered jacket of thin walled cells, enclosing spores and elaters. The spore wall is differentiated into three layers, the outermost perinium shows reticulate ornamentation. The elaters are long, narrow, spindle-shaped cells with 2-4 spiral thickening bands. in P. *intermedium* thickening bands are absent and instead the elaters are uniformly thickened. The elaters are hygroscopic and they coil and uncoil with the changes in atmospheric humidity. Thus they help in separation and scattering of spores.

NOTES

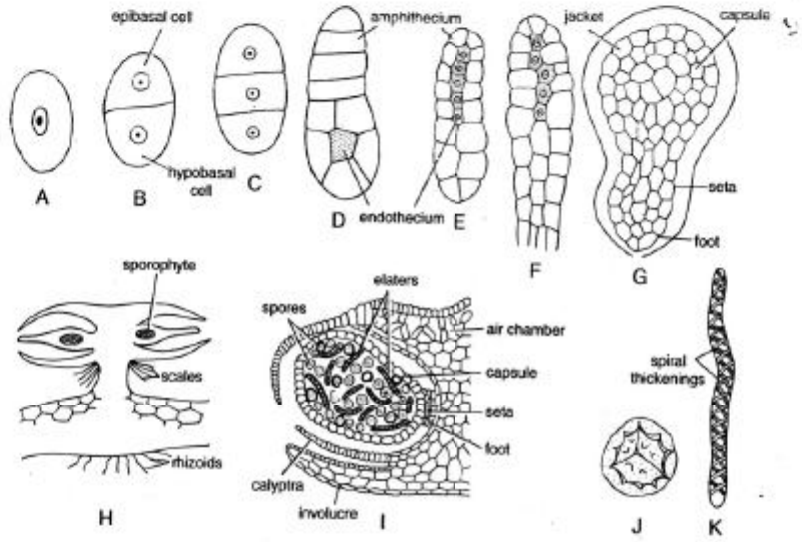

Fig. 7.2: *Plagiochasma:* Sporophyte

7.3 *Pellia*

Pellia is an anacrogynous genus of thalloid form of the family Pelliaceae of Jungermanniales. There are about 4 species of *Pellia* and of these 3 are available in India, *P. epiphilla, P. fabroniana (P calycina) and P. neesii.*

Usually occur on moist soils or on rocks by the side of ditches and springs, growing as green patches among mosses or grasses

Practical work and study

External Structure:

1. The plant body is thin dorsiventral, prostrate, dichotomously branched with somewhat wavy margin. Dorsal surface is smooth with thick at the mid rib.

2. On the ventral surface smooth unicellular rhizoids are present below the mid rib. Scales and pegged rhizoids are absent.

Internal Structure:

NOTES

1. Internal structure of the gametophyte is simple, mainly consists of parenchymatous cells with a mid rib made up of many cells in thickness; the marginal region is single cell in thickness.
2. The cells of marginal region and the upper cells of mid rib with chloroplasts. Starch grains are present in all the cells.
3. In the lower surface unicellular, smooth walled rhizoids are present in the mid rib region (some internal thickenings or bands are present in some species of *Pellia*).

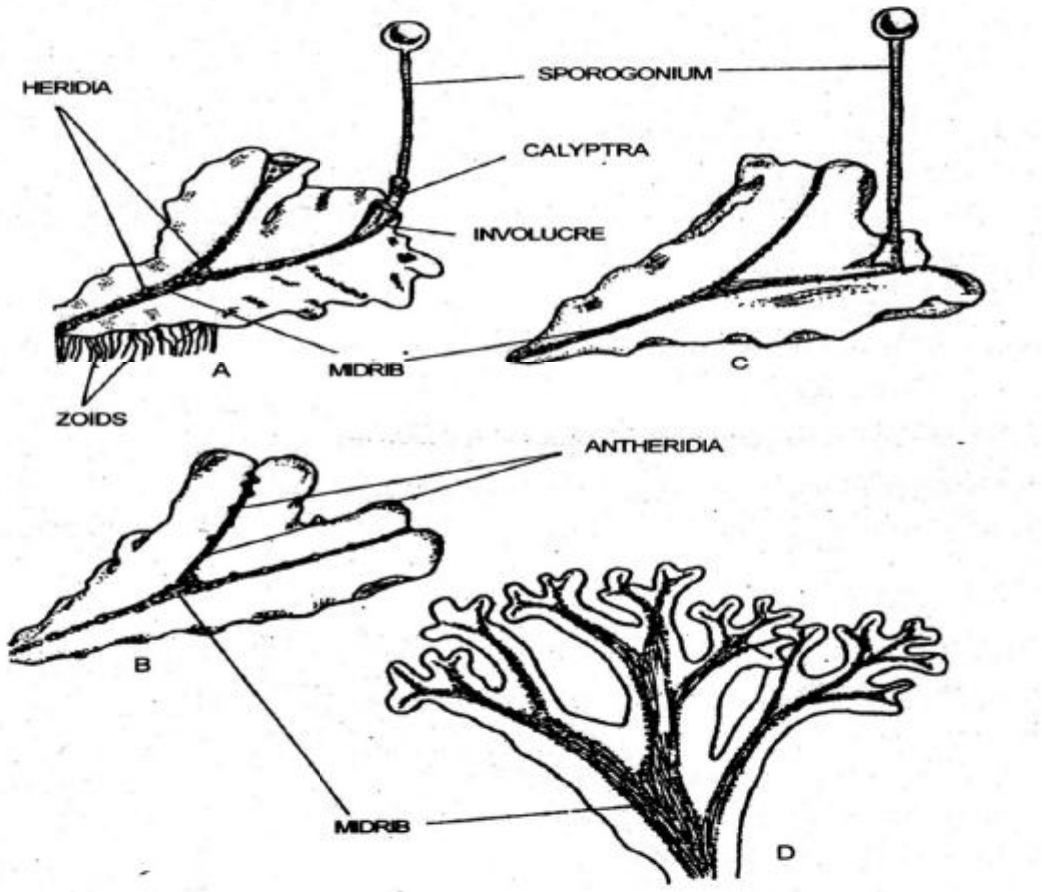

Fig. 7.3: *Pellia* External Features of Gametophyte

Reproduction

Vegetative Reproduction: *Pellia* produces adventitious branches which arise on the dorsal surface near the margin. These branches separate from the plant and develop in to individual plants. By death and decay of the thallus the young branches will develop in to new plants.

Sexual Reproduction: Pellia may be monoecious or dioecious. The sex organs are formed in two or more rows on the dorsal side of mid rib.

Antheridia: Antheridia are born on the dorsal surface of the thallus along the mid rib. Their position is indicated by projections and each projection marks the antheridial cavity in which single antheridium is present.

NOTES

Archegonia: The archegonia are formed in groups of four to twelve at the anterior end of the branch. The group of archegonia is protected by an involucre.

At maturation the archegonium has a multicellular stalk, a dilated venter and a long neck (The venter becomes two cells in thickness).

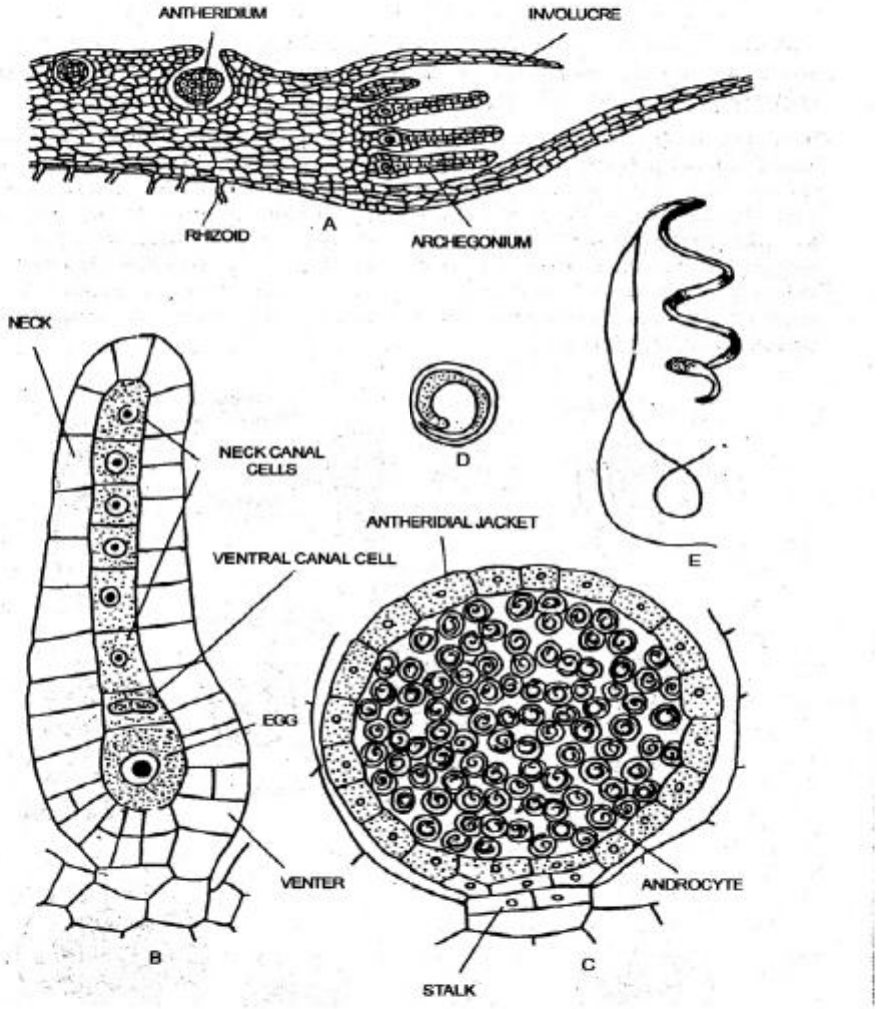

Fig. 7.4: *Pellia* with sex organs: Antheridium and Archegonium

Sporophyte

The mature sporangium consists of foot, seta and capsule. The foot is conical in form and it is produced like a collar around the seta. The seta consists of regular longitudinal rows of cells which are short in young condition but during the maturity the cells are very much

elongated. The capsule is a spherical body and the outer jacket is composed of two or more layers of cells. The jacket provide with four lines of thin walled cells which denotes the line of dehiscence.

NOTES

7.4 *Notothylas*

Systematic Position

Class - Hepaticopsida

Order - Anthocerotales

Family - Anthocerotaceae

Notothylas includes about 11 species and is distributed in tropical and temperate regions four species *(N. indica, N. levieri, N. chaudhurii* and *N. javanicus)* have been reported from India. It usually grows in damp, shady places, either on moist rocks or earth, or on the walls and floors of old buildings.

Practical work and study

The Gametophyte

External Structure

1. The gametophyte plant body is a thallus which is prostrate, and delicate. It is usually light green or yellowish in colour.
2. The outline of the thallus is orbicular or suborbicular or somewhat circular. The thallus is lobed and the lobe margins may be entire, serrate or fimbriate.
3. On the ventral surface there are found only the smooth-walled rhizoids, the tuberculate rhizoids and scales being absent.

Internal Structure

1. The thickness of the thallus in the middle varies from 6 to 8 cells, gradually thinning out to I to 3 cells towards the margin.
2. The internal cells are twice as big as the cells of the upper and lower limiting layers.
3. The mucilage cavities containing the *Nostoc* colonies are commonly found but in some species, such as *N. javanicus,* the thallus is solid, and there is no trace of mucilage cavities.

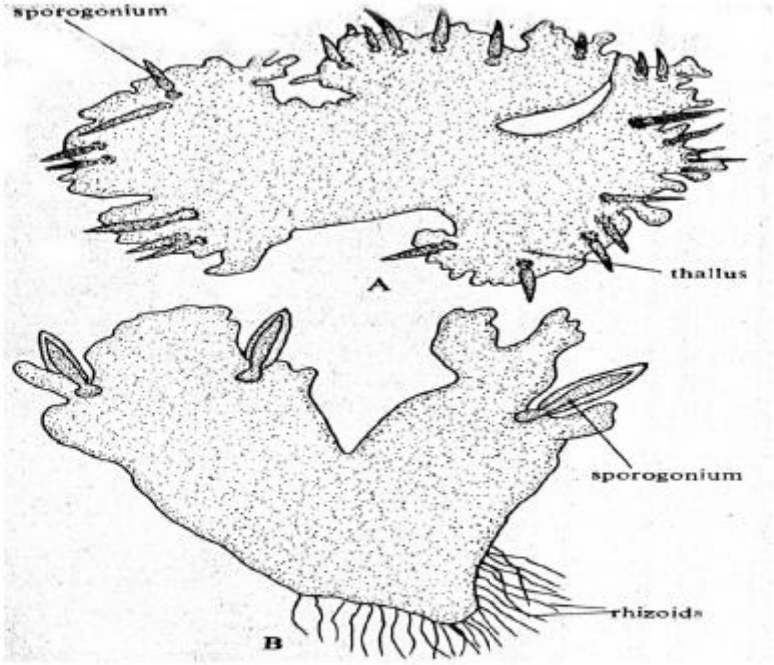

Fig. 7.5: *Notothylas* external features of Gametophyte

Sexual Reproduction

Notothylas may be monoecious or dioecious. All the 3 Indian species, viz., *N. indica, N. levieri and N. chaudhurii* are monoecious and protandrous.

The Antheridium:

1. The antheridia are developed endogenously, and are usually formed near the growing point. The usual number of antheridia inside an antheridial chamber is 3 to 4, but sometimes as many as six may be met with.
2. Antheridia occur singly or in group within closed cavities, known as antheridial chamber. These chambers develop on the dorsal surface of the thallus, but they do not open on the thallus.
3. The jacket cells contain chloroplast and as such the mature antheridium is green in colour. The primary androgonial cells undergo many regular divisions to form a mass of androcyte mother cells. The androcyte mother cell divides to form two androcytes, each of these metamorphoses into a biflagellate antherozoid.

The Archegonium

Archegonium arises in acropetal secquence on the dorsal surface of the thallus. In monoecious species, they are formed on the same thallus which had borne antheridia.

At maturation the archegonium has a multicellular stalk, a dilated venter and a long neck, four cover cells, 6 neck cells, upper small venter canal cell and lower large egg cell.

7.5 *Sphagnum*

NOTES

Class	:	Bryopsida
Sub Class	:	Sphagnidae
Order	:	Sphagnales
Family	:	Sphagnaceae
Genus	:	*Sphagnum*(Dill.)

Practical work and study

The common names of *Sphagnum* are 'bog moss', turf moss' and ' peat moss'. Species of *Sphagnum* are aquatic or semi-aquatic which grows in dense masses or in cushions in ponds, swamps, lake margins, and wet hill sides. *Sphagnum* is perennial and the upper part continues to grow while the lower part dies away without disintegration. Thus constantly increasing the mass of dead gametophores which accumulates and it is known as peat, hence the name peat moss.

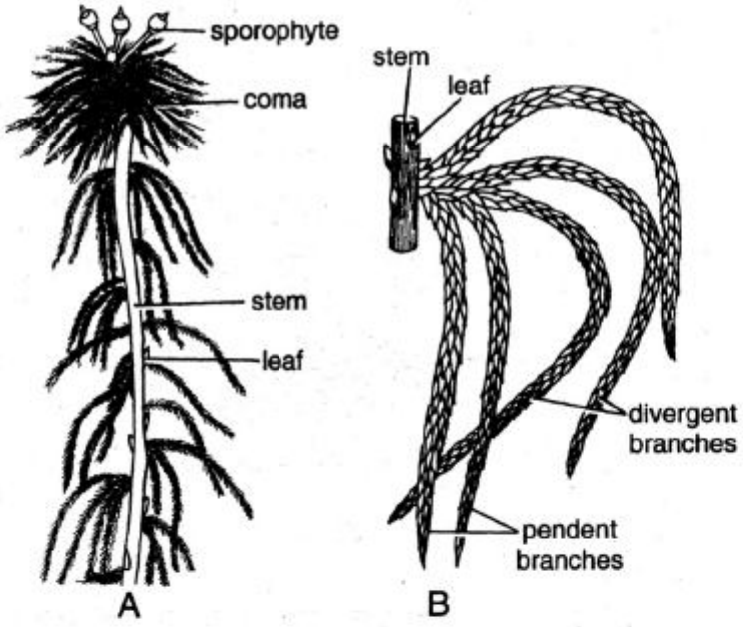

Fig. 7.6 : *Sphagnum* External Features of Gametophyte

The Mature Gametophyte

External Structure:

1. The gametophytic phase starts with the spore and is made up of two stages the juvenile stage and the leafy gametophyte stage.

2. The stems are individually slender and fragile but they gain support by their growth in masses and are thus able to remain erect when growing in cushions above the surface of water.
3. Near the apex of the main stem, the branches are short, of limited growth and densely crowded to form a compact head or coma.
4. Elongated branches, usually in tufts of 3-8 in the axil of every fourth leaf. In aquatic species growing in bogs and pools, all the branches are similar in form and structure and the pendent branches are scarce or absent. But in species which do not grow submerged in water, the branches forming a tuft are of two sorts those which grow upwards towards the apex of the stem from their point of insertion and are known as upright or divergent branches; the others which are elongated, flagelliform and hang downwards are termed as pendent branches.

Reproduction

Sexual Reproduction. The sex organs are borne on slightly modified short lateral branches present near the apex of the gametophores. Some species of *Sphagnum* are monoecious, others are dioeciously the antheridia and archegonia in either case are formed on different branches. In a monoecious species, the antheridial branches appear first near the apex of the male shoot but are displaced downwards by the eventual growth of the apex. The leaves on the antheridial branch are closely imbricate and are often more brightly coloured (yellow, red, brown, purple or even dark green). They are much shorter than the foliage leaves.

The Antheridium. Antheridia are axillary in position on a male branch and are cut off in acropetalous succession.

A mature antheridium has a globular body borne on a long stalk of two to four rows of cells. The jacket of the antheridium is single layered and encloses a large number of androcytes each of which gives rise to an antherozoid. The apical cell of the antheridium is also utilized for one of the jacket cells.

NOTES

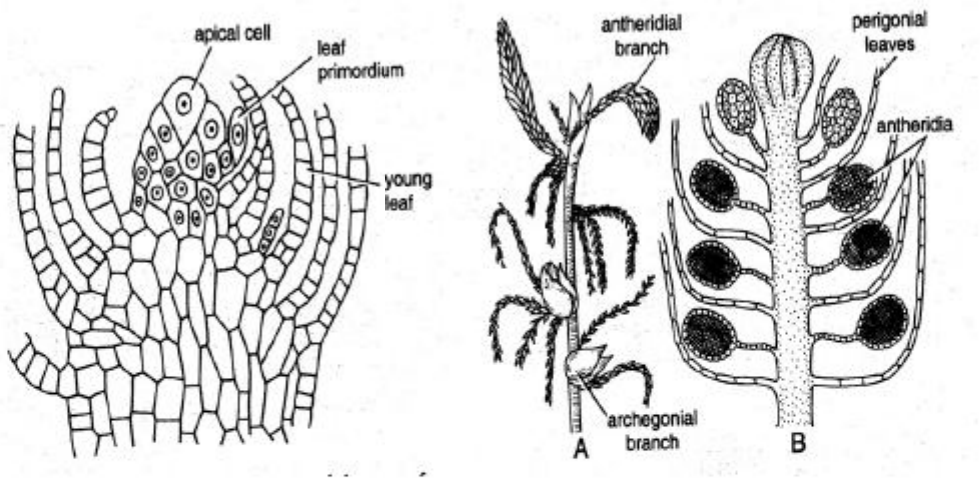

Fig. 7.7: *Sphagnum* Apical Growth of Thallus and (A) lateral branch with sex organs (B) Antheridial branch

The Archegonium: The archegonia are terminal on short, green bud-like archegonial branches. The leaves on these branches are usually much larger than any other leaves on the plant. In anatomical structure, they differ from the branch leaves in having few fibres in the hyaline cells, and from stem-leaves in having abundant chlorophyll in the green cells. These leaves manufacture food material for the developing sporogonium in the early stages of its growth. They also serve as protective organs to the young archegonium and constitute the perichaetium during post-fertilization stages. The number of archegonia borne on an archegonial branch may range from one to five.

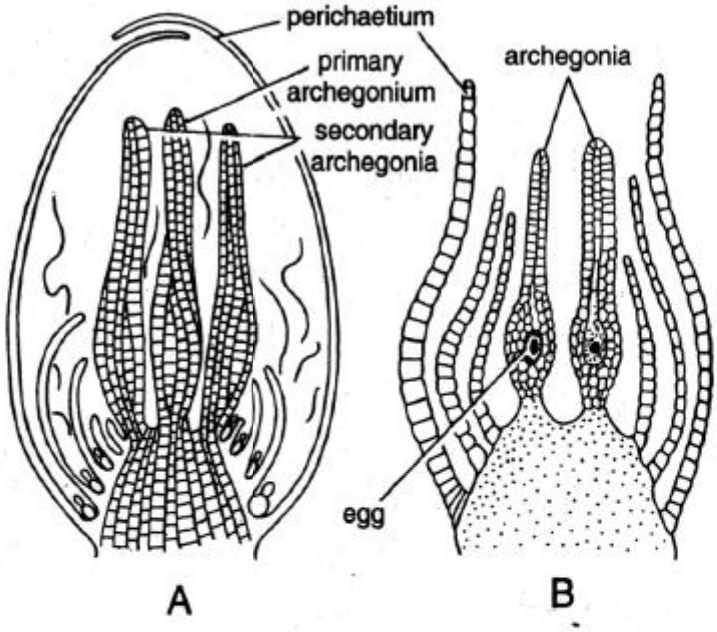

Fig. 7.8: *Sphagnum* Archegonial branches showing Archegonia

Structure of the Arehegonium: A fully developed archegonium has a long stalk, a massive venter and usually a long twisted neck. The venter and lower portion of neck are two or three celled thick whereas the upper part of neck always remains one cell in thickness. The primary cover cell forms the terminal portion of the neck but this is not well demarcated as in archegonia of Hepaticopsida. The axial row of cells consists of an egg, a venter canal cell and 8 to 9 neck canal cells.

NOTES

Structure of mature sporophyte

1. The mature sporophyte is differentiated into foot, seta and capsule. The foot is bulbous or cylindrical and haustorial in function. It is composed of parenchymatous cells.
2. The seta is an inconspicuous narrow region between the foot and the capsule.
3. The capsule is an almost spherical structure of dark brown or black color. The wall of the capsule is 3-7 layered. The outermost layer, which has thick walls, firms epidermis. It has many non-function and rudimentary stomata. The cells of the capsule wall are compactly arranged and they contain chloroplasts.
4. The presence of rudimentary stomata and chloroplast in the capsule wall show photosynthetic ability of the sporophyte of the ancestors of *Sphagnum.*
5. A circular biconvex disc-shaped lid is present at the apex of the capsule. It is called operculum. The operculum is seperated from rest of the capsule by a circular groove of thin walled cells, known as annulus. At maturity, the operculum breaks off from the annulus and allows dispersal of spores.
6. The central part of the capsule is occupied by a cylinder of sterile cells, called columella. The sporogenous tissue forms a dome shaped arch over the columella.
7. The young sporophyte is enclosed within calyptra and perichaetium, but at maturity the axis of the archegonial branch elongates, pushing out the capsule from the protective coverings. This elongated and leafless axis of archegonial branch, present at the base of the sporophyte, is known as pseudopodium. It develops after fertilization.
8. The distal end of the pseudopodium together with the basal part of the calyptra forms a sac like structure, known as vaginula. The foot of the sporophyte is embedded in vaginula.

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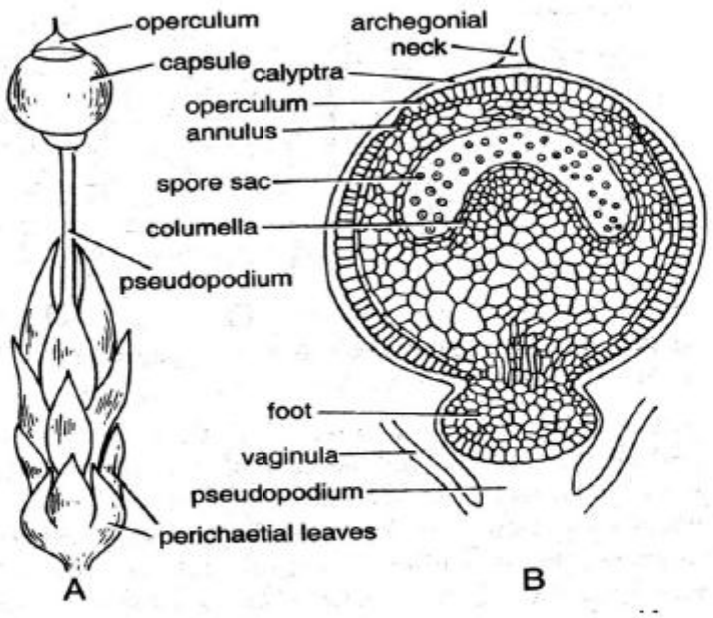

Fig. 7.9: *Sphagnum* : Vertical section of Sporophyte

7.6 *Polytrichum*

Class	:	Bryopsida
Subclass	:	Bryidae
Order	—	Polytrichales
Family	—	Polytrichaceae
Genus	—	*Polytrichum Hedw.*

Polytrichum, a large genus of about 100 species is widely distributed throughout the world, chiefly in temperate and trophical countries. Only four species have been reported from India (Bruhl, 1931) namely *P. densifolium, P. xanthopilum, P. juniperinum,* and *P. alpinum*—these are known from very high Himalayan ranges. *Polytrichum commu*ne is cosmopolitan in distribution.

The genus grows on a wide variety of habitats ranging from dry woody peaty, moors, damp soil, and sandy bank to margin of lakes and ponds.

Practical work and study

The Mature Gametophyte

External Structure.

1. The plant body of *Polytrichum* is a gametophore differentiated into two regions: a horizontal, underground so-called rhizome from which arises the slightly branched,

aerial leafy shoot which may attain a height of 20 centimeters or more in some species as *P. commune*.

2. In *P. commune*, there is a transitional middle region between the rhizome and the upper leafy shoot which is brown in colour and three sided.

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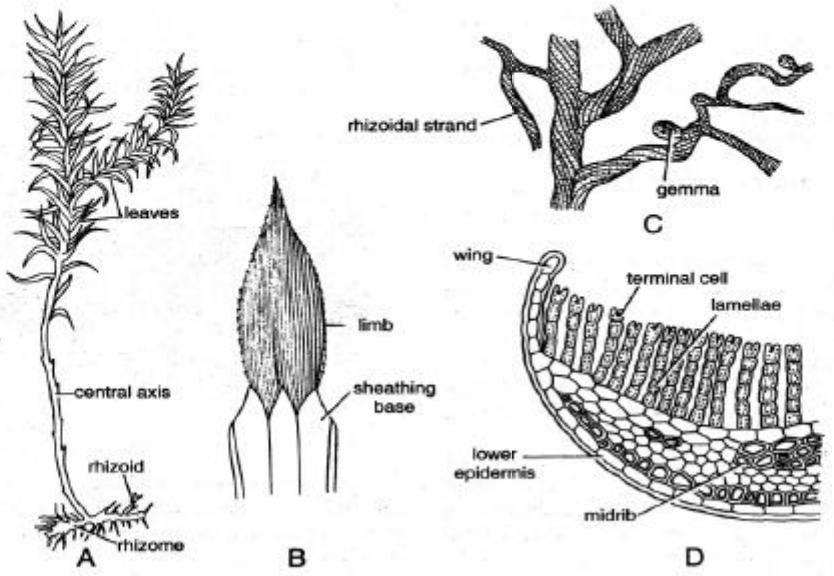

Fig. 7.10 : *Polytrichum* External features of Gametophyte

Reproduction

Polytrichum is usually dioecious; the antheridia and archegonia arise in clusters at the top of separate gametophores.

The Antheridium: Antheridia are borne in clusters at the apex of the male shoot within an involucre of perigonial leaves (The leaves bearing the antheridia are termed the perigonial leaves). The position of the antheridia in the centre surrounded by the involucre of perigonial leaves looks almost a conspicuous flower-like open cup. The perigonial leaves differ in form and colour from the vegetative leaves occurring lower down on the gametophore. The perigonial leaves are dull red or reddish-brown in colour and each leaf possesses a very broad sheathing base terminating in a short bristle point.

The perigonial or involucral leaves are arranged spirally from the vegetative axis outwards. The antheridia arise in groups at the base of each perigonial leaf in the position of lateral buds.

A mature antheridium consists of a club-shaped body, borne on a short stalk. In the centre of the antheridium are androcytes surrounded by the peripheral one-layered jacket. Interspersed with the antheridia are the paraphyses. Some of these are simply filamentous;

other has the terminal cell of the Filament broadened into a spathulate single celled thick mass of cells.

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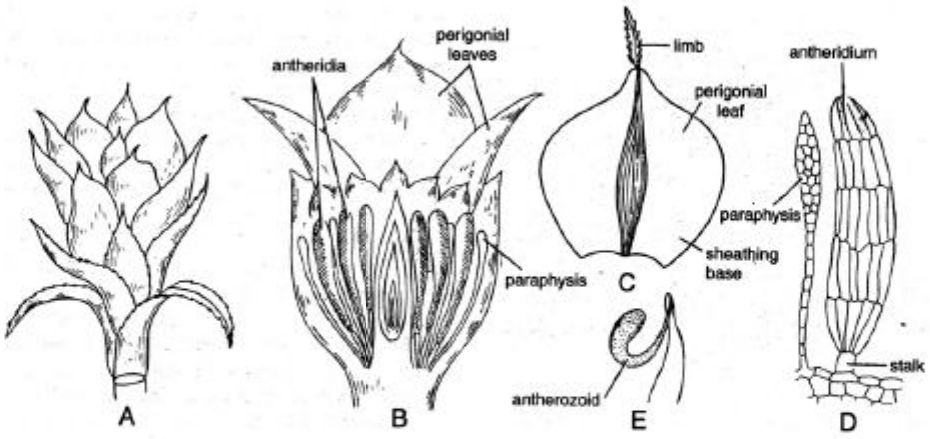

Fig. 7.11 : *Polytrichum:* Male Sex Organs with Antheridia

The Archegonia. The archegonia are borne in clusters at the apex of the leafy stems within an involucre of leaves. The number of archegonia in a cluster may be three (as in *P. commune*) or more. As the apical cell of the shoot itself gets converted into archegonial initial, the further growth of the shoot is checked with the formation of the sporogonium. The structure and development of the archegonium is essentially similar to that found in *Funaria* but the number of neck canal cells is rather large and often variable.

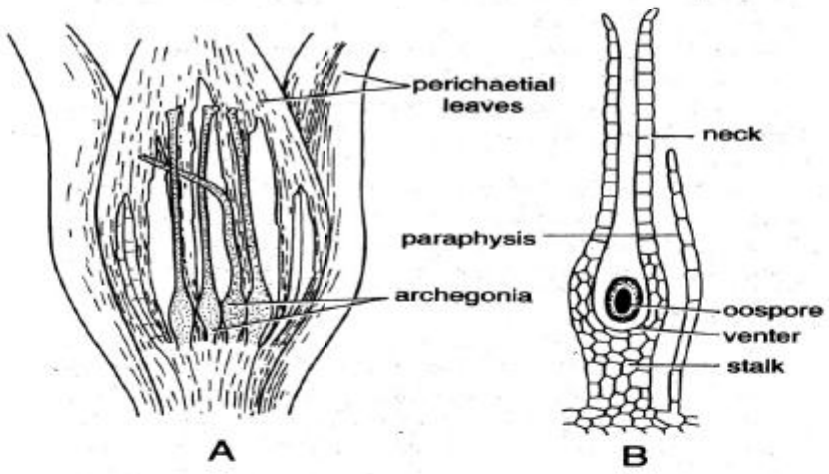

Fig. 7.12: *Polytrichum:* Female sex organ with Archegonia

Sporophyte

1. The mature sporogonium consists of a foot, a long seta and a capsule.
2. The foot is made up of thin-walled parenchymatous cells. Just above the foot is a long slender seta which carries the capsule at its apex. A transverse section of the seta shows a thick-walled superficial layer.

3. At the base of the capsule, the seta enlarges to form the apophysis which is considerably larger than that of *Funaria* and is separated from the sporogenous region.
4. The capsule of *Polytrichum* is usually angular and shows a square outline in cross section. In the region of theca proper (middle portion of capsule) there is a several-layered wall formed of cells containing chloroplast. The outermost layer is the epidermis consisting of cells with thick outer walls. Lying internal to the epidermis is the air space or outer lacuna which is traversed by radially situated assimilatory filaments which connect the epidermis with the outer wall of the spore sac.
5. There is a similar inner lacuna consisting of photosynthetic filaments and connecting the spore sac to the central columella.
6. The spore sac is surrounded on each side by two layers of thin walled cells both on the outer and inner surface. The archesporium consists of one layer in the young and 4-6 layers of sporogenous cells in the adult sporogonium. All cells of the sporogenous tissue are potential spore mother cells capable of giving rise to spores. The centre is occupied by the columella.
7. The apex of the capsule consists of an operculum which appears a conical lid with a long beak or rostrum. There is no well marked annulus seen in *Polytrichum* although the usual thickened diaphragm or rim is present in the region of the theca.
8. The peristome is formed of 32-64 short and stout teeth, composed of bundles of curved crescent-shaped sclerotic cells.
9. In the mature capsule, the peristome consists of 32 to 64 Pyramidal teeth united below with the capsule wall and above with margin of the epiphragm by their tips. The top of the columella is expanded into a flat disc, the epiphragm which is a transverse band of thin membranous tissue formed of pale compressed cells without intercellular spaces. The diaphragm expands like a drum-head and closes the opening of capsule; it fills the space inside the ring of peristome teeth and attached to their tips.

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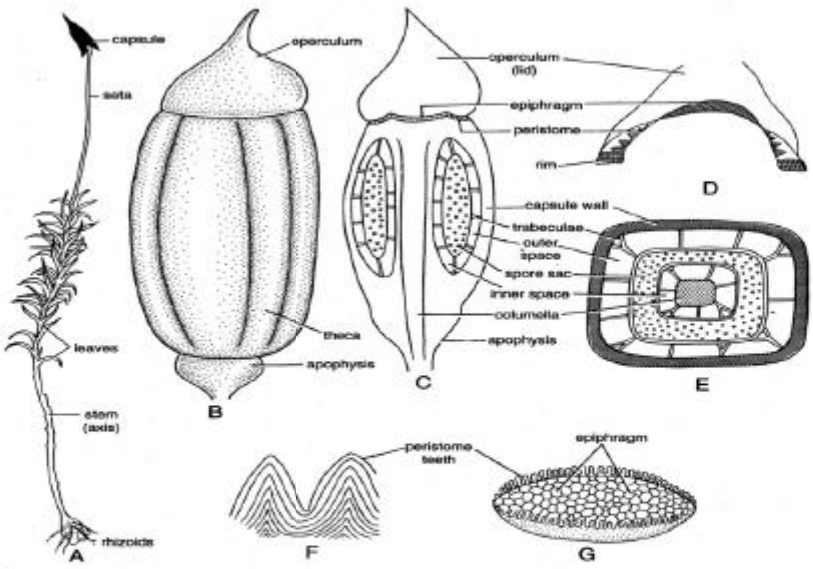

Fig. 7.13: *Polytrichum* Structure of Sporophyte

7.7 Viva - Voce

1. Define Bryophytes.
2. Define Amphibians.
3. What are mosses?
4. Liver worts are also known as……
5. Horn wort is Commanly known as……..
6. Differentiate hornwort and liver worts.
7. Most primitive sporophyte of Bryophyte is ……
8. What is the shape of sphagnum capsule?
9. Sporophyte is divided in to how many parts in Plagiochasma?
10. Explain vegetative thallus of Notothylus.
11. What is operculum?
12. What is peristomial teeth?
13. Define elaters.
14. What is the role of foot and seta in sporophytes.
15. In which genera antheridiophore is found?
16. What is the role of columella?
17. What is gametophyte?

18. What is sporophyte?

7.8 References

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Unit - 8

Study of Morphology, Anatomy and Reproductive Structures and Microscopic Preparation of Pteridophytes

Structure of the Unit

8.0 Objectives

After going through this unit you will be able to:

- Describe the external morphology and internal anatomy of the prominent Pteridophytes like *Lycopodium, Isoetes, Ophioglossum, Pteris, Marselia and Azolla*.
- Differentiate the vegetative and reproductive stages of gametophyte and sporophyte of *Lycopodium, Isoetes, Ophioglossum, Pteris, Marselia and Azolla* .

8.1 Introduction

Pteridophytes possess an independent well developed sporophyte stage Alternation of independent heteromorphic generation is showen by *Lycopodium, Isoetes, Ophioglossum, Pteris, Marselia and Azolla*. The order Ophioglossales is represented by herbaceous sporophytic plants. The order contains a single family Ophioglossaceae. Genus Ophioglossum has been discussed here in detail. The living Lycopodiales are the

representative of a group which, during the Carboniferous period formed the chief vegetation. The order Marsileales includes a single family, Marsileaceae, the family include *Marsilea,* which is heterosporous fern. *Isoetes and Stylites* are two living genera of Isoetales, here we discussed only *Isoetes Marselia* is the best known cosmopolitan genous of family Marsileaceae. *Azolla* is the one of living genera which is placed in family Salviniaceae.

NOTES

Description of each specimen was made elaborately using technical terms as seen under microscope. Identification of each specimen was made using manuals, monographs and identification keys etc.

8.2 *Lycopodium*

Division : Lycophyta

Class : Eligulopsida

Order : Lycopodiales

Family : Lycopodiaceae

Genus : *Lycopodium*

Lycopodium is a cosmopolitan genus with about 400 species and chiefly in subtropical and tropical forests. They are commonly known as *"club mosses"* or *"ground pines"*, because they possess moss like leaves, club shaped cones at the tips of stem and branches with cones appear like pine trees.

Practical work and study

External Morphology

Generally the species are creeping, erect or scrambling, erect or scrambling, except *L. phlegmaria*, which is an epiphyte. Genus *Lycopodium* is divided into two sections which can be distinguished as follows.

S.No.	1. Section : Urostachya	2. Section : Rhopalostachya
1.	Plants erect in terrestrial forms and pendent in epiphytes.	Plants creepers with trailing stems.
2.	They have roots only at base of the stem	The roots are adventitious on the lower portions
3.	Bulbils are formed, used for	Bulbils are not formed, reproduction

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	vegetative reproduction	takes place by sporophylls only
4.	Branching is dichotomous	Branching is dichotomous but in majority of creepers, it appears like monopodial due to unequal development of the branches.

Internal Structure

Root:

1. The transverse section of root is well distinguished into epidermis, cortex and stele. Epidermis is consists of a single layer of thin walled cells and is covered by numerous root hairs.
2. The epidermal cells produce root hairs in pairs. Epidermis is persistent in the aerial roots.
3. The outer layers of cortex in old roots are developed into sclerenchymatous zone.The inner cortex is composed of parenchymatous cells. The stele may be monarch, diarch or triarch according to the number of protozylem masses.
4. In *L. selago* the stele is diarch with"U" shaped xylem terminated with protoxylem at tips. The phloem is present at centre. Rarely, the same root at one region is diarch and tetrarch in other region. In *L. calvatum,* the stele is polyarch.

Leaf:

1. The internal structure of leaf shows the outermost epidermis with stomata on both surfaces.
2. The epidermis is a single layer of compactly arranged cells. In *L. voubile*, the leaves are dimorphic in which the stomata are restricted to lower surface only.
3. Mesophyll is undifferentiated and consists of loosely arranged chlorophyllose cells. Prenchymatous cells are angular without intercellular spaces or circular with intercellular spaces.
4. The midrib shows a central mass of endarch xylem. The young leaves do notpossess and endodermis while both of them are distinct in matured leaves.

Stem:

1. The T.S. of stem shows distinction into epidermis, cortex and stele. Epidermis is single layer of parenchymatous cells with cutinized outer walls and stomata.
2. Cortex is divided into three layers, the outer zone which is thick walled cells and inner zones are scelerified but the middle layer is large and thin walled parenchymatous.

3. Entire cortex is sclerenchymatous. The endodermis is distinct with thickened radial walls, which is followed by pericycle. Pericycle in composed of parenchymatous cells.
4. The vascular tissue is arranged in different types. It may vary in different parts of stem even with in the same species. Xylem is protostelic without central pith. The protostele shows distinct variations in different species.

NOTES

(a) Actinostele: In which the xylem appears star shaped with protoxylem situated at tips of the star shaped projections and is exarch This is the simplest type of stele, seen in *L. serratum*.

(b) Polyarch Actinostele: The lobes of the xylem are irregular and not separated into isolated strands e.g. *L. phlegamria.* Phloem is present, but parenchyma separates it from xylem. The above two types of arrangements are generally found in erect and terrestrial forms.

(c) Plectostele: In which the xylem in a transverse section appears to be in form of separate plates of variable sizes with phloem in between them. This type of stele is commonly seen in creeping and scrambling stems (*L.clavatum*). In *L. squarrosum,* the isolated stands of phloem lined with parenchyma occur as islands within the radiating arms of xylem.

(d) Mixed Haplostele: The Xylem occurs as strands, embedded in the phloem and the whole thing looks like a mesh, e.g. *L. cernuum.*

5. There is no secondary growth and so the stems remain slender even after several years. The leaf traces are inserted on protoxylem and passes through cortex into leaf base as midrib.

The sporophytes:

1. The sporophytes of *Lycopodium* are commonly known as club mosses or ground pines.
2. They produce fruiting bodies, called sporangia, borne singly on the upper surface of the leaf. Based on the presence of sporangium, the leaf is called sporophyll.
3. The sporangium consists of outer jacket cells and inner sporogenous or archesporial cells. The archesporial cells produce the sporogenous tissue by repeated divisions in anticlinal and periclinal planes.
4. The sporocytes are round, separated and float on a viscous fluid inside the sporangium. The later sporocytes undergo meiosis and produce four haploid spores in the form of tetrad of spores.

NOTES

5. As the sporangium matures, a stomium is developed in the outer most wall layer at the apex, marking the future line of dehiscence. The wall breaks transversely along the stomium and liberates the spores.
6. Spores are round, very small and covered with thin wall. All the spores produced in *Lycopodium* are similar, hence, it is homosporous.

Sex Organs: The gametophytes are monoecious, consists of both sex organs in the same organism.

Antheridium:

A mature antheridium is either completely embedded in the tissue of the prothallus or slightly projecting. It has a single layered antheridial wall with a distinct triangular opercular cell. The antheridium is well differentiated into other jacket layer and inner sperm mother cells or androcytes. The sperm mother cells develop into the antherozoids which possess two flagella at the narrow anterior end. The sperms are liberated by breaking down of opercullar cell .

Archegonium:

A mature archegonium is distinguishable into a slightly broader and embedded lower portion called the venter and a projecting part called the neck. The neck of the neck varies considerably in the genus. It consists of neck canal cells in neck, and ventral canal cell and egg in the basal venter . The venter has no wall of its own but is protected and surrounded by the tissue of the prothallus.

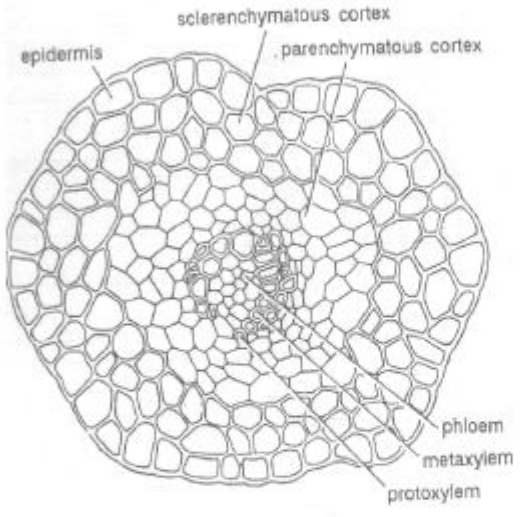

Fig.: 8.1 : *Lycopodium*: Complete plant **Fig. 8.2 *Lycopodium* T. S.of Root**

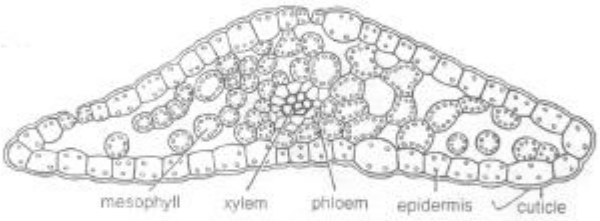

Fig. 8.3 : *Lycopodium* T.S.of Leaf

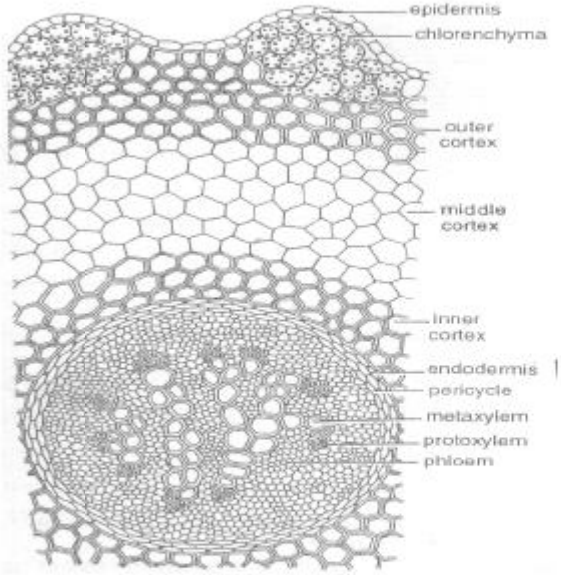

Fig. 8.4 :*Lycopodium* T.S. of Stem

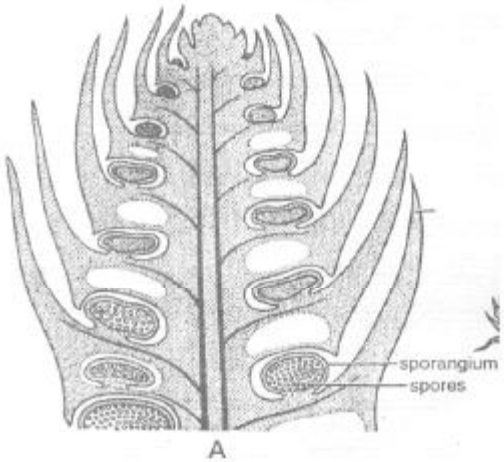

Fig. 8.5 : *Lycopodium* L.S. of Strobilus

8.3 Isoetes

Systematic Position

Division : *Lycophyta*

Class : *Lycopsida*

Order : *Isoetales*

Family : *Isoetaceae*

Genus : *Isoetes*

Isoetes and stylites are two living genra of isoetales, along with a fossil genus *Nathorstiana*. Species of *Isoetes* are commonly called Quilwort.

Practical work and study

NOTES

Isoetes is represented by about 70 species and shows a world-wide distribution extending from tropics to near arctic regions. Nine species have been reported from India (Panigrahi, 1981), of which *I. coromandelina* is the most common. Other Indian species include *I. disitii, I. panchananii, I. sahayadrii, I. sampatkumarinii, I. unilocularis* and *I. pantii. I. coromandelina* grows wild at different places in India including the Coromandel Coast, Bombay, Baroda, Varanasi and Merrut.

A majority of the species of *Isoetes* grow in swampy areas or remain submerged in water. However, a few species are terrestrial. A majority of the Indian species are amphibious as well as terrestrial.

External Morphology

1. The plant body is sporophytic and appears like that of a Liliaceous or Graminaceous monocotyledon because of their characteristic linear leaves arising from an underground bulbous axis.
2. Superficially it resembles the 'leek', a vegetable similar to the onion. Plants are 5-50 cm. tall, and remain differentiated into roots, stem and leaves .

A. *Axis :* It is the underground, bulbous portion of the sporophyte. **Axis**, divide into an upper leaf-bearing portion called **stem** and a lower root-bearing portion called **rhizomorph**. The exact external demarcation between stem and rhizomorph become difficult because the axis is greatly reduced.

B. *Leaf* : Many, densely crowded, long, broad based leaves cover the upper part of the stem. They are very stiff, awl-shaped and form a crowded rosette. In *I. japonica* the leaves may reach up to 50 cm. in length. The lower basal part of each leaf remains expanded in the form of a broad membranous sheath. Leaves taper abruptly from a broad base to an elongate acicular portion. On the adaxial surface of each leaf is present a single ligule at the juncture of basal and acicular portions. Each leaf remains traversed by a single median, unbranched vein. Some lower or megasporangium, and thus called microsporophylls or megasporophylls.

C. *Roots :* Roots develop on the lower part of the axis. They are well-developed and dichotomously branched. The position of the roots is related to the place of the grooves.

Internal Structure

Anatomy of Root

Roots remain surrounded by an irregular epidermis made up of cells of no definite size. Cortex is multilayered and composed of thin-walled prenchymatous cells. Roots are

characterized by a central lacuna and a single vascular bundle. The vascular cylinder is a monarch protostele on the side of lacuna facing the xylem is located towards the cortex. Casparian strips are clearly seen in the radial walls of the endodermis. The central lacuna or air cavity is formed by the breakdown of the cells.

NOTES

Anatomy of Leaf

An epidermal layer with a thin cuticle covers the leaf all over. Stomata are present only in the leaves present outside the water. Mesophyll is not differentiated into palisade and spongy parenchyma. One to four chloroplasts remain filled in the cells of mesophyll. In the awl-shaped portion of the leaf, the mesophyll is traversed by four longitudinally running cavities or air spaces. Each air space remains separated from one another by a several-layered thick partition wall.

Anatomy of Stem

Very young stems remain surrounded by a single layered epidermis followed by a well-developed cortical region containing many leaf traces. Endodermis is not clearly defined, and the centrally located vascular cylinder is made up of a protostele in which xylem remains surrounded by the phloem.

Even slightly **mature stems** show secondary growth. The stele is anchor shaped. From the vertical part of the stele develop many leaf traces, and from the bilobed portion develop the traces for roots. Stele consists of four radiating arms in plants that contain a trilobed axis. Cortex is made up of starch filled parenchymatous cells. Many intercellular spaces are present in the cortex. Endodermis is absent. Few layered cambium is present. Towards the outer side cambium produces secondary cortex which pushes the primary parenchymatous cortex towards the outer side which ultimately gets sloughed off in the form of cork. Towards the inner side cambium produces a mixture of xylem, phloem and parenchyma in the form of a special tissue called *prismatic tissue.* Xylem consists of tracheids mixed with parenchyma. Distinct sieve plates are present in the sieve tubes.

Sporophylls: ***Isoetes*** is heterosporous because it produces both microspores as well as megaspores. Microspores are produced in microsporangium and megaspores in megasporangium. Each leaf of the sporophytic plant body is potentially a sporophyll because each bears a sporangium, normal or abortive. The sporophylls are not grouped into a definite strobilus. Three sets of the sporophylls are typically recognized in a mature plant at the end of the growing season : (a) Outermost, megasporangia-bearing megasporophylls produced first in the growing season; (b) middle, microsporangia-bearing **microsporophylls** formed later in the growing season; and (c) innermost, abortive

sporangia-bearing leaves produced at the end of the growing season. The sporophylls produced in the previous season fall off by the end of the growing season.

NOTES

1. Sporangia : *Isoetes* is peculiar among the present-day spore-producing or living non-seed-bearing plants in producing the largest sporangia with the highest spore output. They are also peculiar in possessing trabeculae. In each megasporangium, 50 to 100 megaspores, each with a diameter of 250 to 900 μ are present. According to Pant and Srivastava (1962) each megasporangium bears 703 to 2345 megaspores in *I. indica*.

2. Each sporangium is situated on the adaxial surface of the sporophyll near its expanded base in between the base and the ligule . Mature sporangium remains covered in a protective membranous covering called **velum**. The velum develops below the liglue. On the adaxial side of the sporophyll the velum leaves an oval opening called **foramen.** The sporangial chamber remains incompletely and irregularly divided by transverse to oblique sterile bars called **trabeculae**. The innermost layer of the multilayered sporangial wall represents the tapetum

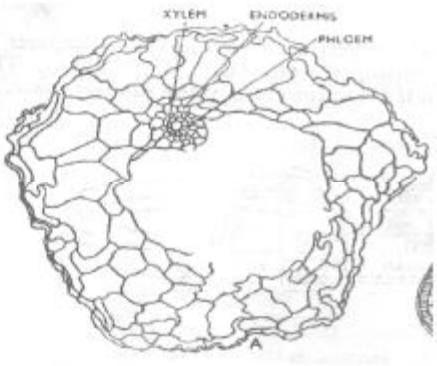

Fig. 8.6 Isoetes: T.S.of Root

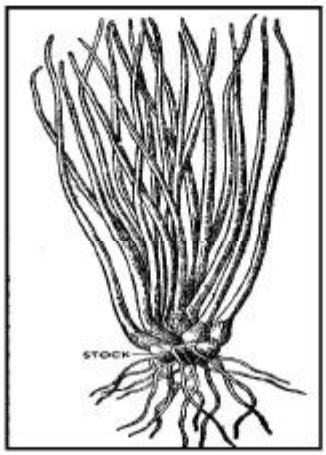

Fig. 8.7 Isoetes: Complete Plant

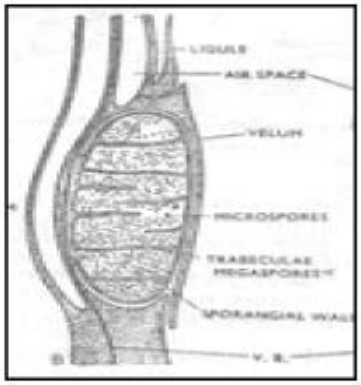

Fig. 8.8 Isoetes: L.S.of Sporangium

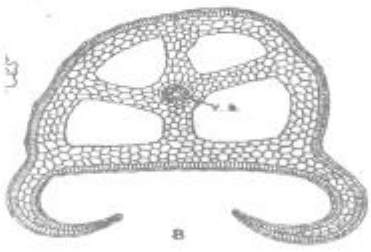

Fig. 8.9Isoetes: T.S.of Leaf

8.4 *Ophioglossum*

NOTES

Systematic Position

Class	:	Pteropsida
Sub – class	:	Eusporangiatae
Order	:	Ophioglossales
Family	:	Ophioglossaceae
Genus	:	*Ophioglossum*

In India, *Ophioglossum* is represented by more than twelve species, majority of the species are among them are *Ophioglossum aitchinsoni, O. costatum, O. lucitanicum, O. pendulum* and *O. vulgatum* (commonly called **adder's tongue fern**). Recently, Khandelwal and Goswami (1984) reported a new *Ophioglossum* species (*O. eliminatum*) from India, and Bir and Bhusri (1985) reported one species of *Ophioglossum* and four species of *Botrychium* from Simla hills.

Nearly all the species are perennial herbs of small or moderate size. Some species are evergreen plants found in damp grassland. *O. lucitanicum* is found in grassy cliff tops.

Practical work and study

External Morpholgy

1. *Roots* : Many thick adventitious roots are present on the rhizome. Root hairs are absent and are heavily infected with an entophytic fungus . A root develops singly below each leaf scar of the previous season and below each leaf of the current season. The roots may be branched (*O. pendulum*) or unbranched.
2. *Leaves*: The sporophyte may be monophyllous or polyphyllous The leaves are borne in an irregular spiral on the stem but sometimes their arrangement is two-ranked. Most of the species of *Ophioglossum* produce only one leaf in one growing season. But in some species 4-5 leaves are produced in every growing season.
3. The leaves are erect A well-developed leaf is differentiated into a **blade** or lamina and **petiotle**. The lamina or blade is entire and narrowly linear to broadly ovate, but in *O. palmatum* the blade is palmately lobed In the epiphytic *O. pendulum* the leaves are very long and reach up to 1.5 meters in length.

Internal Structure

Anatomy of Rhizome

NOTES

It is somewhat round in shape. Many leaf bases and adventitious roots are attached to it. Internally the rhizome is composed of epidermis, cortex and stele .

1. *Epidermis :* The outermost layer is which is epidermis and lack of any stomata.
2. *Cortex :* The cortex is made up of oval or ellipsoidal shaped and thin-walled cells. Many intercellular spaces are also present. In older parts of rhizome .Periderm layers are present superficially in some cases but according to Maheshwari and Singh (1934) functional cork cambium is absent. The innermost cortical layer functions as a layer of endodermis in very young rhizomes, but endodermis is not distinguishable in mature rhizomes . In the cortial cells abundant starch is present .
3. *Stele :* The stele is variable in different species of *Ophioglossum*. In *O. reticulatum* the young basal portions of the rhizome shows a protostelic condition, but in the mature portions, it becomes medullated and changes into an **ectophloic siphonostele** by the development of pith. Sometimes the siphonstele is being perforated by one or more leafs gaps may overlap one another and the ectophloic solenostelic condition changes into a **dictyostelic** condition. The individual bundle of a dictyostele is called a **meristele**. Thus different conditions of the stele can be observed in different regions of the rhizome.
4. *Meristele :* In each meristele the xylem is endarch in arrangement. The phloem is extermally placed and collateral. The complete inner face of the xylem is lined by protoxylem .
5. *Pith :* In the centre of the rhizome pith is present. It is made up of large, thin-walked, parenchymatous cells.

Anatomy of Root : Anatomically, the root consists of epidermis, cortex and stele.

1. *Epidermis :* A distinct epidermal layer is present ,which is single-layered, with the outer wall of its cells being specially thickened and suberized. Root hairs are absent.
2. *Cortex :* The cortex is wide and divisible into two regions , i.e. outer cortex and inner cortex. The outer cortex composed of angular, parenchymatous cells with no intercellular spaces. It is 3 to 6 cells thick and its cells are occupied by endophytic **mycorrhizal fungus.** The inner cortex is composed of rounded or ovel , starch-filled cells with small intercellular spaces. Pericycle is absent.

3. *Stele :* The stee3. Stele: xylem is monarch to tetrarch but in majority of the species it is either monarch O. reticulatum diarch. In the diarch roots the xylem is in the form of a plate having a protoxylem group at both its ends. On either side of the xylem is present a mass of phloem. With the help of 2-3 cells thick parenchymatous tissue the phloem groups are separated from the xylem. In some monarch roots the xylem and phloem are present radial to each other.

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Anatomy of Lamina

It consists of a layer of epidermis which is present on both the sides of the lamina. Epidermal cells are somewhat elongated or more or less globular in shape, and their continuity is broken by stomata. Large stomata are present .The stomatal frequency of both the epidermal layers is different. In between the epidermal layers *mesophyll* region is present which is not differentiated into palisade and spongy parenchyma. It is composed of loosely arranged, thin-walled, chlorophyll-containing cells. Many intercellular spaces are present in this region. The mesophyll is traversed by a large number of delicate veins.

Each vascular bundle is encircled by a bundle sheath. The vascular bundles are collateral and endarch as in petiole.

Life History

Spore-bearing Organs

(a) *Fertile Spike :* The spores are present in sporangia which are borne on a fertile spike. The mature spike has a lower cylindrical sterile portion called stalk or peduncle. There is a stalk at the base of the lamina of the sterile leaf on its ventral side. Two rows of sporangia are embedded on either side of the spike. The conical tip of the spike is free from sporangia. Depending upon the size of the plant, the number of sporangia in a fertile spike varies between 6 and 20. In the axis of the plant, the number of sporangia in a fertile spike varies between 6 and 20. In the axis of the spike many vascular strands run longitudinally. From this longitudinally running vascular duct arise many lateral vascular strands which traverse between sporangia .

(b) *Sporangium :* Each mature sporangium is large, sub spherical, fused with the leaf and ranges between 0.5 and 3 mm in diameter. It remains surrounded by a wall composed of several layers of cells. In the outermost layer of the sporangial wall are present stomata. The innermost wall layer functions as **tapetum**. The sporangial cavity is filled with spore mother cells or sporogenous cells. Generally, all the spore mother cells are functional and develop into spore tetrads. The number of spores per

sporangium in *O. pendulum* may reach up to 15,000 (Bower, 1935). *Ophioglossum* is homosporous.

NOTES

(c) *Morphological nature of Fertile Spike :* Regarding the morphological nature of the fertile spike, many views have been expressed by different workers from time to time. Some of them are as follows :

1. Bower (1896 and 1908) has mentioned that the fertile spike is a single septate sporangium.
2. According to Chrysler (1910) the fertile spike is the result of the fusion of two basal pinnae of a leaf.
3. According to Goebel (1915) the fertile spike of *Ophioglossum* is equivalent to a single pinna.
4. The view of Zimmermann (1930), suggests that the fertile spike and the sterile lamina are the two dichotomics of a shoot.

Gametophyte

Ophioglossum is homosporous and the spores germinate into bisexual prothalli. The prothalli in all the three genera of Ophioglossaceae are mycorrhizal.

(a) *Spore :* Largr number of spores are produced They are tetrahedral, dust-like, yellow, small, and remain surrounded by two-layered thick wall.The outer layer in exosporium which is thick, sculptured and colourless and inner endosporium, which is delicate. In each spore many oil droplets and a centrally located nucleus are present. The young spores are green but chlorophyll is absent in the mature spore.

(b) *Spore Germination:* About the spore germination and early development of gametophyte in *Ophioglossum detail* information in not avialable. According to Eames,(1936) the spore germination in different species of *Ophioglossum* may either take place a within a few days or may require even many years.

At the time of the germination, the spore absorbs water and swell. The ultimate result is the exine splits along the triradiate ridge. Two equal cells are formed by a transverse division . it is followed by a vertical division in the lower cell, and thus a 3-celled stage is attained . Beyond this stage the further development stages of the spore have not been properly studied. After the three celled stage, further development takes place only after the infection of mycorrhizal fungus. The infection of the fungus usually takes place through the lower cells.The upper cell divided only occasionally. Campbell (1911) has observed 13-celled stage in his culture of *Ophioglossum* prothalli. Smith (1955) collected the prothalli of *O. pendulum*.

(c) *Mature Prothallus :* Prothalli are Brownish, yellowish or light-greyish coloured or hyaline ,fleshy, subterranean,and mycorrhizal structures. They usually lack rhizoids They are ovoid, linear, stellate or cylindrical and branched or unbranched structures. The size of prothallus varies from 5 to 10 mm (*O. moluccanum* and *O. vulgatum*). has stated The prothalli in some species of *Ophioglossum* may survive even upto 20 years (Eames ,1936). Adventitious buds are commonly seen on the prothalli of this genus. They help in the vegetative propagation.

NOTES

An apical cell, with three or four cutting faces, is present in the growing apex of gametophyte. The Rhizoids are generally, absent on the gametophyte (Bruchmann, 1904). .According to (Campbell, 1907)in some species many rhizoids are present in the older portions. Starch grains are present in Interior cells and heavily infected by mycorrhizal fungus.

The prothalli are *monoecious*. On the entire surface of prothallus the two sex organs remain irregularly distributed. In *O. pendulum* the sex organs develop in acropetal succession.

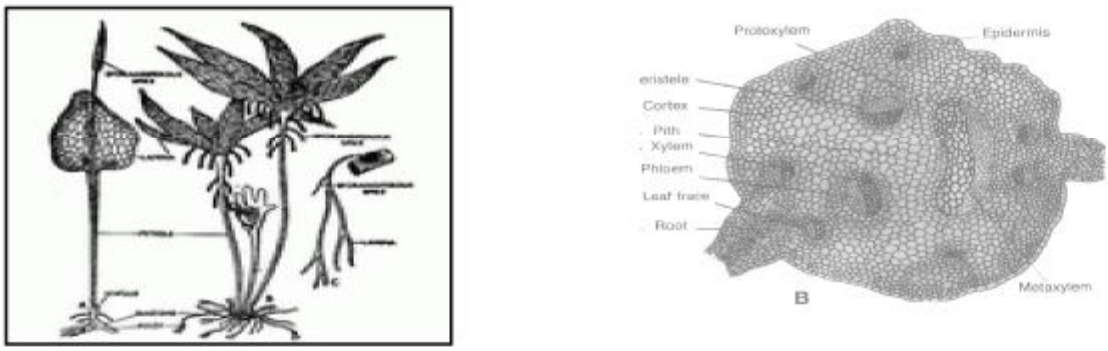

Fig. 8.10 : *Ophioglossum:* Complete Plant and T.S.of Rhizome

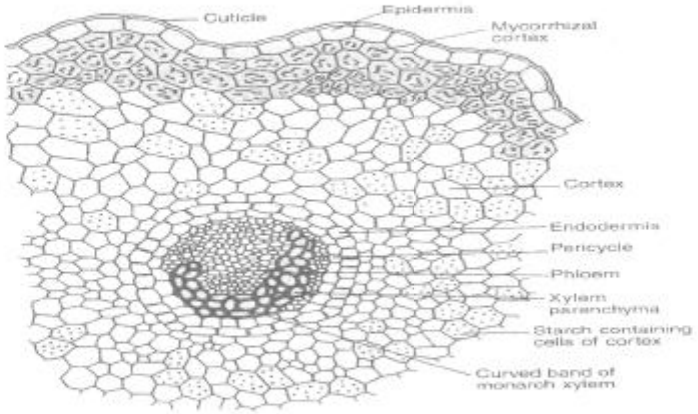

Fig. 8.11 *: Ophioglossum:* T.S.of Root

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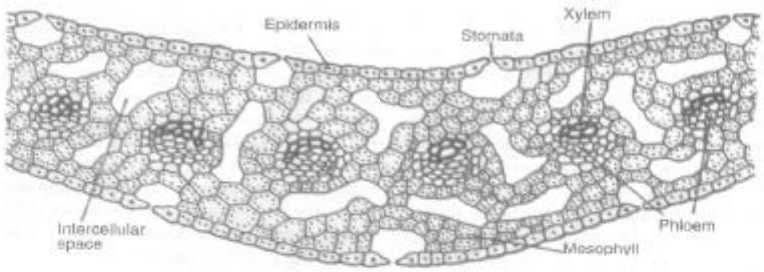
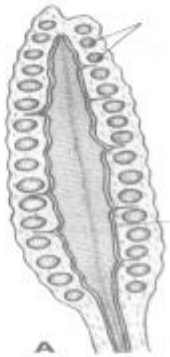


Fig. 8.12 *: Ophioglossum: T.S.of Lemina*



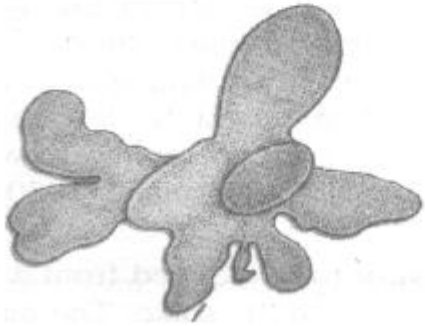


Fig. 8.13 *:Ophioglossum*V.S. of young spike Fig. 8.14 *: Ophioglossum:* Mature prothallus

8.5 *Pteris*

Systematic Position

Sub-Division	-	Pteropsida
Class	-	Leptosporangiatae
Order	-	Filicales
Family	-	Polypodiaceae

Pteris is a widely distributed genus with more than 250 species. It grows abundantly in the sub tropical and tropical regions of the world. *P.vittata,P.cretica L.,P.biaurita L.,P.stenophylla Wall* .,are common species which are found in India. The genus is characterized by great variations in the habitat and morphology of its members. It is mostly found in rock-crevices along roadsides. *Pteris vittata*, a fairly common species occurs in open sunny places. In India, the genus is widely distributed both in north and south. *Pteris cretica, P. quadriaurita, P. stenophylla* and *P. wallichiana* are common

species of the north and *P. crenata, P. aquiline, P. otaria, P. hookeriana* and *P. pellucid* are found in South India.

Practical work and study

External Morphology

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The main plant body is sporophytic in nature. It is differentiated into **root, rhizome** and **leaves.**

As in most of the ferns, the **primary root** is ephemer. Numerous slender fibrous adventitious roots are present in rhizome. They arise in acropetal succession throughout the length of the rhizome. The adventitious roots are branched and arise endogenously .

The **rhizome** is usually short, slender and creeping. Usually branched **rhizome** is always covered with scales. The scales are short and narrow *(P. grevilleana),* long *(P. merlensioides)* or with broad margins (e.g., *P. longipinnula).*Simple hairs are also present besides scales.

The young leaves are circinately coiled and. vary in length from 20 to 100 cm The basal part of the petiole is covered with scales or hairs. The leaves are **simply pinnate** (e.g., *P. vittata).* **Bipinnate** (e.g., *P. crenata)* or **bi-or tripinnate** (e.g., *P. boivini).*

In sterile fronds vein endings are within the margins and often have large terminal hydathodes. In fertile fronds, however, all vein endings are connected laterally by a vascular commissure below the receptacle of the sorus .

Internal structure

Root. In a transverse section, the root is almost circular in outline. **Epiblema** is the outermost layer which is made of compactly arranged thin walled cells. Some of the cells of epiblema form thin walled unicellular root hairs . **The cortex is consist of two zones** – outer cortex **and inner cortex.** The outer cortex is many layered and consists of thin walled parenchymatous cells. These cells store the food material. The inner cortex is made of 3-5 layered deep and cosists of thick walled sclerenchymatous cells, provides mechanical support to the root.**Endodermis lies inner to the cortex** . The radial and inner tangential walls of the endodermal cells possess casparian thickenings. Inner to the endodermis are one or two layers of thin-walled cells.Preicycle is situated inside the endodermis. It consists of one or two layers of thin walled cells.**Diarch** and **exarch stele is present.** The **xylem** consists of two central metaxylem tracheids with small groups of protoxylem elements on either side. Groups of phloem are present on their alternate radii.. Lateral roots originate from the cells of the endodermis present opposite to the protoxylem groups.

NOTES

Rhizome. In a transverse section rhizome is irregular in outline. Internally, it is differentiated into epidermis, hypodermis, ground tissue and stele.The **epidermis** is the outermost protective layer .It is made of compactly arranged cells which are is covered with a thick layer of cuticle.Almost continuous layer of **hypodermis is present**,which is made up of thick walled sclerenchymatous cells. It provides mechanical support to the rhizome and is relatively thick in sub-erect or upright rhizomes than in horizontal rhizomes.Inner to the hypodermis is parenchymatous **ground tissue**. The cells of the ground tissue store food material in the form of starch grains.

The complexity of the steler organization also varies with the increase in the size of the rhizome. For example, in *P. podophylla* the rhizome is protostelic at the base which gradually becomes medullated protostele (i.e., siphonostele) and solenostele upward. A little higher up a second ring of vascular strand appears in the medullary region.

Leaf. The anatomy of leaf can be studied under two headings.

(a) Petiole. The petiole is more or less oval in outline with a slight depression on the dorsal(upper) side. **Epidermis** is the outermost layer and is made of compactly arranged cells. It is surrounded by a thick layer of cuticle. In the basal region of the petiole, some of the epidermal cells form multicellular hairs.

Inner to the epidermis is **hypodermis.** It is made of 3-4 layers deep and made up of sclerenchymatous cells which provide mechanical support to the long petiole. The remaining part of the hypodermis is composed of parenchymatous cells. The region is called ground tissue. Some of the cells of the ground tissue contain tannin. The central part of the ground tissue is occupied by a single C-shaped, U-shaped or V-shaped **vascular strand**. The outermost layer surrounding the vascular strand is endodermis .One or more layered pericycle in present inner to the endodermis. The central part of the vascular strand is occupied by xylem. These show mesarch condition. Phloem occupies the region between pericycle and the xylem.

The vascular strand of the petiole gives vascular supply to the pinnae. These traces may divide further if the leaves are bi-or tripinnate.

Pinna. The **epidermal cells** of the pinnae are wavy in outline (sinuous). Stomata are present only on the lower epidermis .The **mesophyll** is consists of large chlorenchymatous cells with intercellular space and usually not differentiated into palisade and spongy tissue. It consists of large chlorenchymatous cells with intercellular spaces. Occasionally, the mesophyll cells lying just below the upper epidermis become elongated and cylindrical and look like palisade tissue.

The **vascular bundle** in the midrib region has a central core of xylem which is surrounded by phloem. It is surrounded by distinct endodermis and pericycle. The vascular bundle also has sclerenchymatous bundle steath. The lateral veins, present in the region of wings, have small collateral bundles.

NOTES

Life History

Pteris reproduces mainly by spores. *Pteris is* a **homosporous** fern. The spores are produced in sporangia which develop on the abaxial side of the pinnules in very large numbers. In most of the species of *Pteris* all the fronds bear sporangia and the fertile fronds are known as **sporophylls.** A few species are, however, distinctly **dimorphic**. For example, in *P. ensiformis* the sterile fronds have the pinnae in lower region whereas in fertile fronds the pinnae are forked once near the base only and the lobes are much narrower than in sterile pinnae.

Development of Sorus. *Pteris* is characterised by the presence of marginal continuous sori developing on a slender filiform receptacle. The sori are covered by a continuous upper marginal indusium only; the lower indusium is completely absent.

Mature Sporangium. Linear continuous sori of *Pteris* (also called coenosorus) (also called coenosorus) are fully covered by the marginal indusium (the lower indusial lip is absent). As sori are of mixed type, sporangia of different ages are irregularly distributed. Mature sporangium is differentiated into **stalk** and a **capsule.** The stalk is long, slender and composed of three vertical rows of cells. The capsule is oval or biconvex. The sporangial jacket is single layered. An **annuals** consisting of 13 thick walled cells extends from the base to almost three fourth of the capsule. At its distal end the annuals joins a strip of thin walled

cells, the **stotium.** 32-64 spores are present in the capsule. (Fig. 4.6 E)

Gametophyte

Spore

In *Pteris* all spores are bluntly or roughly triangular and have a distinct triradiate mark , they are nearly spherical (0.03 mm in diameter), haploid and uninucleate. The spore wall is dirrerentiated into an outer dark and relatively thick **exine** and in inner thin **intine.**

Development of Prothallus

In the gametophyte generation haploid spore is the mother cell. If moisture and suitable temperature is available spores are germinates immediately after dispersal. If germination is delayed than viability of spores gradually decreases. The spore absorbs water and swells. Consequently, the exine ruptures at the triradiate mark and the intine protrudes out

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in the form of a **germ tube**. The germ tube usually grows into a short multicellular chlorophyllous filament. The basal cell of the filament gives rise to colourless rhizoids which attach the developing prothallus to the soil. The cells of the filamentous prothallus divide repeatedly and eventually form a flat green cordate (heart shaped) prothallus. Young prothallus is essentially a single-layered sheet. The peripheral part remains single layered even at maturity but the central region, below the emarginated apex, becomes massive cushion-like due to cell divisions. The mature prothallus is 3-8 mm in diameter. It is capable of independent physiological existence, nourishing itself by absorption from the soil and by photosynthesis.

Sex organs

Both, male (antheridia) and female (archegonia) sex organs are borne on the same prothallus. Antheridia which usually appear first, are confined to the basal central or lateral regions and archegonia develop near the apical notch.

(i) **Antheridium. The anthridia are small and globular structures.** An antheridium develops from a superficial cell of the prothallus. The **antheridial initial** grows as a papillate outgrowth which is cut off from the vegetative cell by a transverse wall. The initial cell now divides transversely into a lower **first ring cell** and an upper **daughter cell.** Due to higher turgor pressure in the upper daughter cell, the wall between these two cells is pressed downward and as a result the upper cell becomes dome-shape. The upper cell divides by an arched periclinal wall into an outer **dome cell** and an inner **primary androgonial cell** . Dome cell now divides transversely into a **cover cell** and a **second ring cell** . The cover cell and the first and second ring cells eventually form a single-layered jacket of the antheridium by anticlinal divisions. The primary androgonial cell divides repeatedly and gives rise to 30-40 androcytes. Each androcyte metamorphoses into a multiflagellate antherozoid.

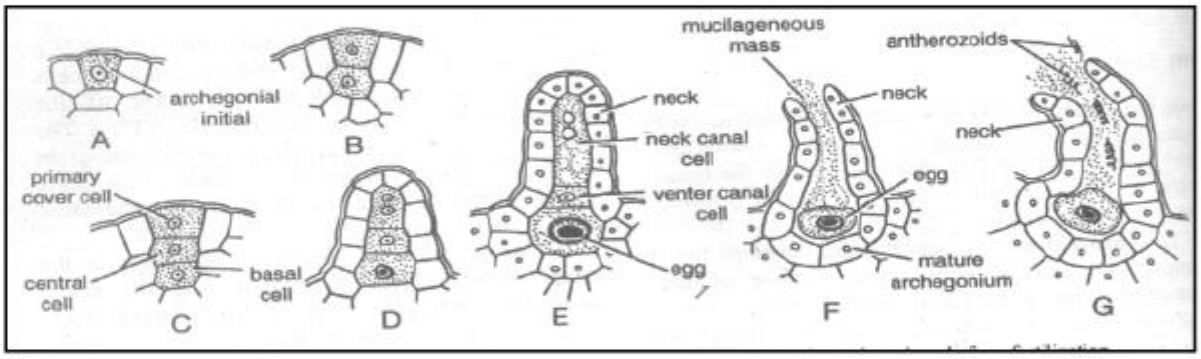

Fig. 8.15 : *Pteris*- Mature Gametophyte

(ii) **Archegonium.** The archegonium develop from a single superficial cell the archegonial initial . The **archegonial initial** divides transversely into an upper **primary cover cell** and a lower mother cell or the **central cell**. The primary cover cell divides by two vertical walls at angles to each other, as a result four **neck initials** are formed. The neck initials, by repeated transverse divisions, give rise to 5-7 celled high neck of the archegonium. The central cell divides transversely and eventually forms three cells – a **neck canal cell,** a **venter canal cell** and an **egg**. The nucleus of the neck canal cell may divide further but it is not accompanied by wall formation.

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Thus a mature archegonium is differentiated into a 5-7 cells high neck composed of four vertical rows of cells and a venter. The neck canal is occupied by a neck canal cell and a venter canal cell. A single large egg is found in the venter. The neck of the archegonium is curved towards the posterior side of the prothallus as the two anterior neck canals cells grow faster than the posterior cells. The neck projects above the surface of the prothallus, whereas the venter remains embedded in it. Due to unequal growth of its cells the neck become curved.

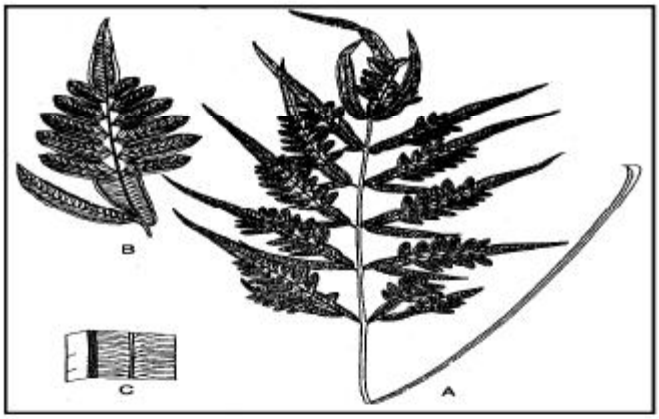

Fig. 8.16: A-*Pteris*- Complete Plant

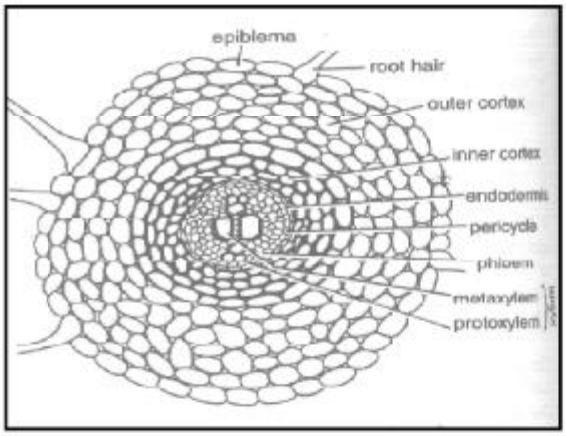

Fig. 8.17 : B-*Pteris*- T.S.of Root

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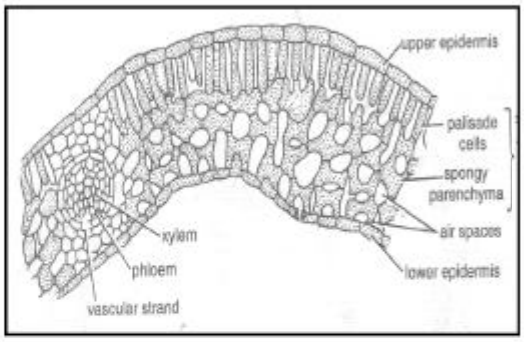

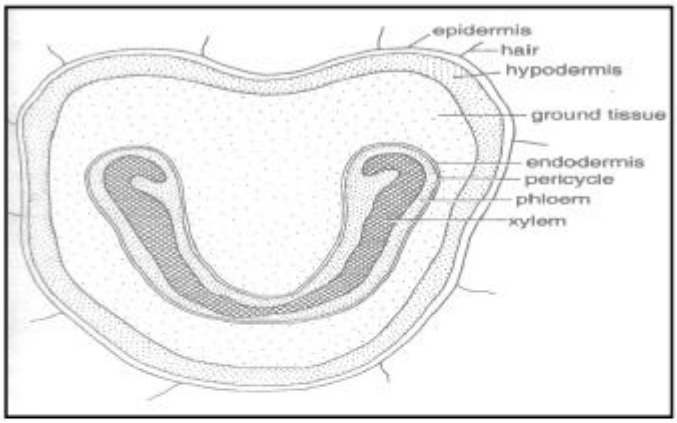

Fig. 8.18: *Pteris* - T.S. of young Rachis

Fig. 8.19: *Pteris* - T.S. of Pinnule

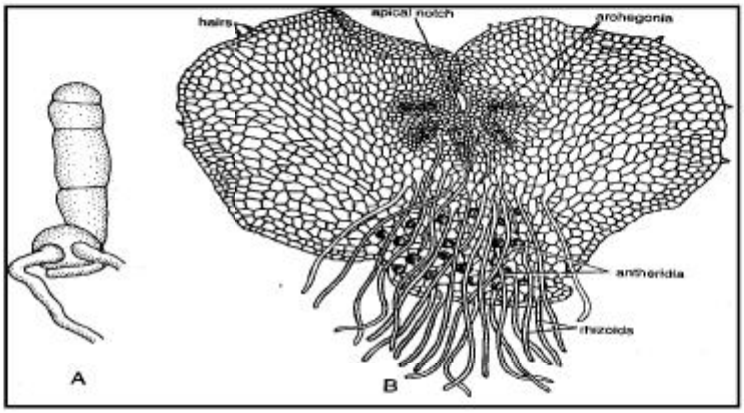

Fig. 8.20: *Pteris*- Prothallus

8.6 *Marsilea*

Division	:	Pteriophyta
Class	:	Leptosporangiopsida
Order	:	Marsileales
Family	:	Marsileaceae
Genus	:	*Marsilea*

There are about 60-65 species of *Marsilea* are known which are found in almost all the parts of the world. They are commonly found in tropical regions such as Africa and Australia. Out of 60-65 species, about 10-12 species are found in India (Gupta and Bhardwaja,1957) *M. minuta* is most common species from India which is aquatic or semi-aquatic hydrophyte. *M. aegyptiaca, M. condensate* and *M. rajasthanensis* are xerophytic and seen growing on dry soil.

Practical work and study

External Morphology

The plant of *Marsilea* is herbaceous sporiphyte and is differentiated into rhizome, leaves and roots.

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Rhizome

Rhizome is differentiated into nodes and internodes. Each node gives arise leaves on the upper side and roots on the lower side. Internode are long when the plants grow in water but become short under dry habitats. The rhizome of *M. minuta* is creeper with dichotomous branching.

Root

From the lower surface of rhizome arise numerous adventitious roots, which fix plant firmly into mud or soil. Roots are unbranched or branched monopodial. Primary roots are short lived soon replaced by adventitious roots. Adventitious roots develop in acropetal order that is oldest at the base and youngest at the apex.

Leaves

Leaves arise singly from each node of acropetal order that is youngest at the apex of the rhizome. They are compound, alternately arranged with circinate vernation (found in young leaves). Leaves with long petiole, pinnately compound which arise from the upper surface of rhizome on nodes. The petiole of the plant growing in water is long, weak and flexible. According to Puri and garg (1933), four leaflet of leaf are seen floating on the surface of water. Lamina is divided into four equal leaf-lets (pinnae). They are ovate to nearly elliptical in shape. The venation is multicostate, reticulate and veins are divided dichotomously. The leaf-lets show sleeping movement at night. Sporocarps are situated on the lower side of petiole of leaf. In leaves, dichotomously branched veins are present i.e. it lacks a mid-rib. These veins combine together on the margins of leaflet.

Internal Structure

Rhizome or Stem

The transverse section of stem (rhizome) shows following structures. It is differentiated in epidermis, cortex and stele.

Epidermis is the outermost, single layered thick, continuous and without cuticle and stomata.Below epidermis, broad cortex is present and is differentiated into three regions. **Outer Cortex is** consists of many layer of compact pranchymatous cells with inter cellular spaces and contains tannin containing cells. It maintains the cylindrical form of

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stem. **Middle Cortex** is made up of pranchymatoes cells in one layer. The cells are large radiating with air chambers (or lacunae). Air spaces are separated from each other by means of septum. Air chambers in few xerophytic species are absent. **Inner Cortex** is the compact zone of one layer made of parachenymatous cells. A few tannin cells are scattered in the inner cortex. It functions as storage tissue.

Stele

Amphiphloic siphonostele type (solenostele) is found. In the center of pith vascular cylinder is situated. In aquatic species, the pith is parenchymatous however; it is sclerenchymatous in those species which grow in dry and terrestrial habitat. It has siphon-like vascular cylinder in which the xylem is surrounded on both outer and inner sides by phloem, pericycle and endodermis, that is xylem is present as central ring and outer phloem, outer pericycle and outer endodermis are present towards its outer side. The section passing through or near the node shows arc-shaped or C-shaped stele with small curved leaf traces at the opening of C, therefore, in *Marsilea*, when endodermis, two pericycle, two phloem one xylem and one pith are present in such position, it is called amphiphloic siphonstele.

Petiole

Transverse section of *Marsilea* petiole shows differentiation of epidermis, hypodermis, endodermis, pericycle and stele.**Epidermis** is the outermost layer shows more or less circular outline and consists of circular or rectangular cells. Very thin cuticle is present on it.**Hypodermis** is made up of one or more layers of thin-walled parenchymatous thin walled cells. It is also called outer cortex.**Cortex** is differentiate outer and inner cortex. **Outer cortex** is made up of aerenchyma having many air chambers. Air chambers are absent in xerophytic species. Air spaces are separated from each other by septum. Sometimes the cells may be sclerenchymatous. **Inner cortex** is the compact zone made up of parenchymatous cells whose cells contain tennin and starch grains. The tannin cells are scattered.

Endodermis is one layered and present inside the cortex.

Pericycle is one layered and present inside the cortex.

More or less triangular and protostele type of ***Stele*** is present.. It is present in the centre with V-shape xylem. Both the arms of xylem are free towards downward. Metaxylems are present in between the arm of 'V' shaped xylem and protoxylem on both the ends. Phloem surrounds the xylem.

Leaf Let

The T.S. of leaf shows single layer of upper and lower epidermis. In floating species stomata are present only on the upper surface, while stomata are present on both upper and lower epidermis in plants growing under dry conditions. Stomata are slightly sunken. Multilayered mesophyll is present in between upper and lower epidermis. It is differentiated into upper palisade tissues and lower spongy parenchyma Air chambers are large in sized and present in spongy parenchyma which are separated by means of transverse septum. Vascular bundles are concentric and each vascular bundle encloses within a single layered endodermis and pericycle. The central solid core of xylem is surrounded by phloem from all side.

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Root

Root is differentiated into epidermis, cortex, endodermis, pericycle and stele.***Epidermis*** is the outermost, thick layer of circular outline. The cells are compact, tangentially elongated and thin walled. It is also called piliferous layer.Below the epidermis broad zone of cortex is present. It is differentiated into outer and inner cortex. **Outer cortex** has big air chambers (aerenchymatous) which are separated by radial septa.

Inner cortex is compact and parenchymatous. The cells are filled with starch grains. In xerophytic species, the cells towards inner side may become thick walled.

Endodermis and Pericycle are one layered. They surround stele (endodermis is followed by single layer pericycle).Stele is protostele which is plate like, found in vascular bundle. Xylem is diarch and exarch, surrounded by phloem from both the sides. The protoxylem lies towards the smaller metaxylem, almost touching the pericycle.

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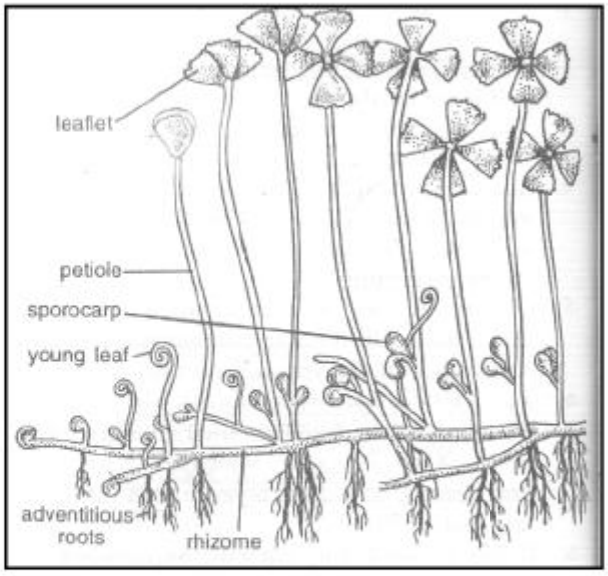

Fig. 8.21 : *Marsilea*: Complete Plant

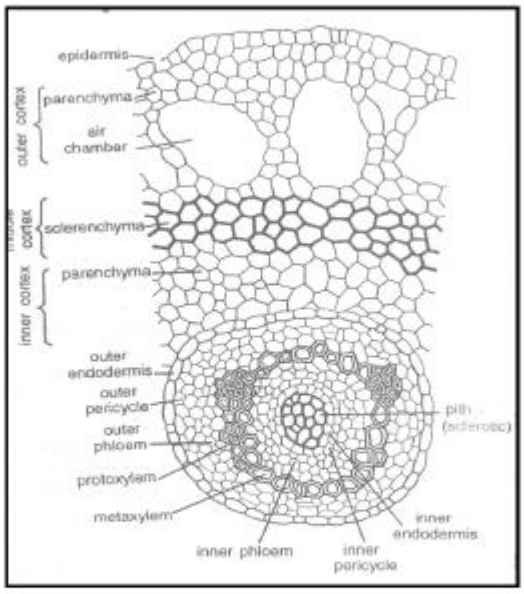

Fig. 8.22 : *Marsilea*: T.S.of Stem

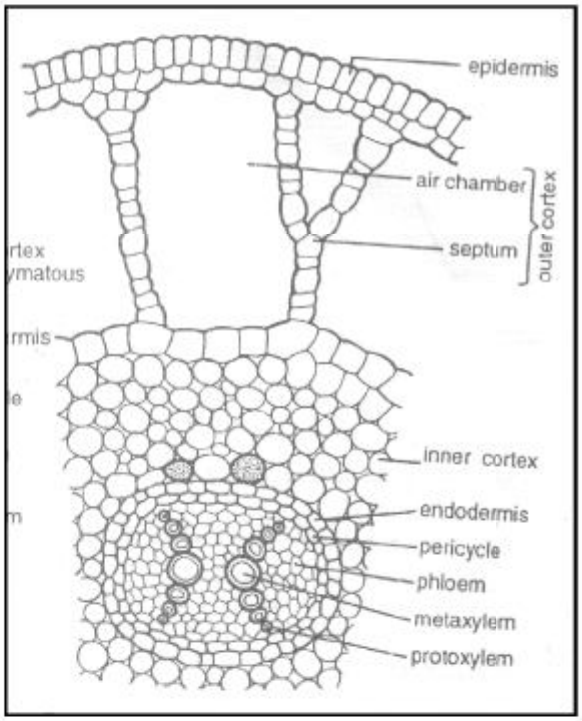

Fig. 8.23 : *Marsilea*: T.S. of Petiole

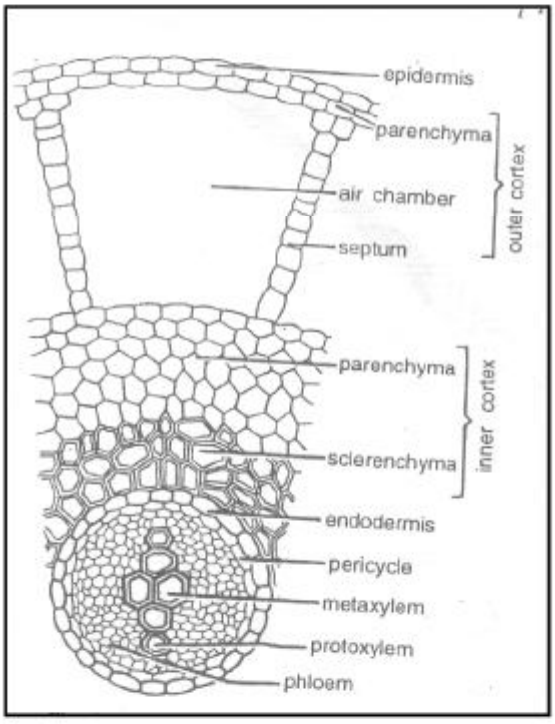

Fig. 8.24 : *Marsilea*: T.S. of Root

Life History

Reproduction by Spore

Marsilea is heterosporus aquatic fern. The spores are produced in **sporocarp**. The spores are of two kinds and present in two types of sporangia microsporangia (contains microspores) and megasporangia (contains megaspores).

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Sporocarp

External morphology : It is the spore producing organ or fruiting body. They are bisporangiate (produce two kinds of spore). Both kinds of sporangia are produced in separate sporangium(micro- and megasporangium). They are small oblong to oval resembling bean seed. In *M. aegyptiaca* it is squarrish of rectangular shaped. It is green soft with small hairs at young stage, while brown to black and hard, nut-like at mature stage.

Sporocarps are borne laterally on the adaxial side of the petiole of leaf with the help of small stalks, which are called peduncles. The part of peduncle, which is fused with the body of sporocarp is called **raphe**. The raphe is seen as distinct ride on one side of sporocarp. The sporocarp beyond the raphe bears one or two horn-like tubercles or teeth on its dorsal surface.

Internal Structure of Sporocarp : It is more or less bilaterally symmetrical. The body can be divided into two halves. The wall is thick and hard. The wall consists of three layers. The outermost layer is called epidermis, just below it lies two layer of hydromel palisade cells an after this the inner most layer composed of three celled thick parenchymatous zone.

- **Epidermis:** It is made up of cuboidal or columnar cells it is interrupted with sunken stomata and covered with a thick layer of cuticle.
- **Hypodermal layer:** Below epidermis two layers of palisade, radially elongated cells with chloroplast are present, called hypodermis. The upper hypodermis contains thick-walled cells. The first palisade layer consists of long cells which surrounds the sporcarp form all sides. The lower hypodermis is made up of thin walled cells.
- **Zone (ring) of gelatinous tissue:** It is also called parenchymatous portion. It encircles the central cavity of the sporocarp in dorsiventral plane. It consists of parenchymatous cells which become gelatinous on maturity. It is more prominent on dorsal side as compared to ventral.
- **Vascular supply of Sporocarp:** The single vascular supply in each sporocarp transverse through the stalk and begins from the penduncle of the sporocarp and runs

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directly into the raphe. It further bends near the lower tooth and passes up to dorsal portion of the sporocarp. This is called dorsal bundle which gives out many dichotomously branched veins in the lateral branches in each valve and alternate each other. It runs left and right parts of the sporocarp. A small branch develops dichotomy from each lateral branch and runs into the receptacle. It is called receptacular or placental branch, which further divided in two branches and run dorsally and ventrally into receptacular ridge. Their number corresponds to number of the sori. Many veins on the lower surface of the sporocarp divide to form a net of veins.

Anatomy of Sporocarp

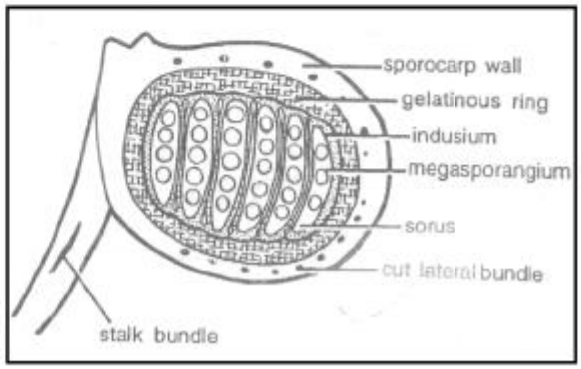


Fig. 8.25: *Marsilea* - V.T.S. Sporocarp

V.T.S. Sporocarp: The vertical tyransverse section shows that the wall of sporocarp consists of three layers and surrounds the sporocarps from all sides. Gelatinous ring is observed on both other sdides of sporocarp. In V.T.S. only two sori of sporocarp can be observed. If the section of sporocarp is cut passing through megasporangia, only then megasporangia can be observed in both the sori. Internally, each sorus is covered with an indusium. Dorsal bundle, lateral bundle and placental bundle can be observed in V.T.

H.L.S. of Sporocarps: The horizontal longitudinal section shows the wall of sporocarp is three-layered which surrounds the sporocarp. A gelatinous ring appears on both the sides of sporocarp. Numerous sori are arranged alternately in the sporocarp. Each sorus is covered with an indusium and has a receptacle which contains one apical megasporangium and two microsporangia on the lateral sides. In this section dorsal bundle, lateral bundle and placental bundle are distinctly observed.

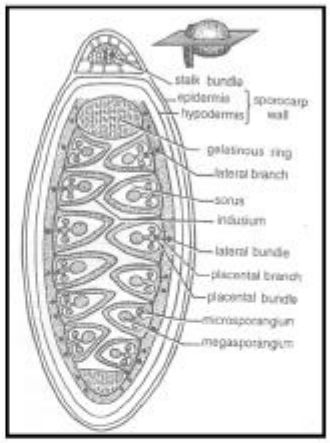
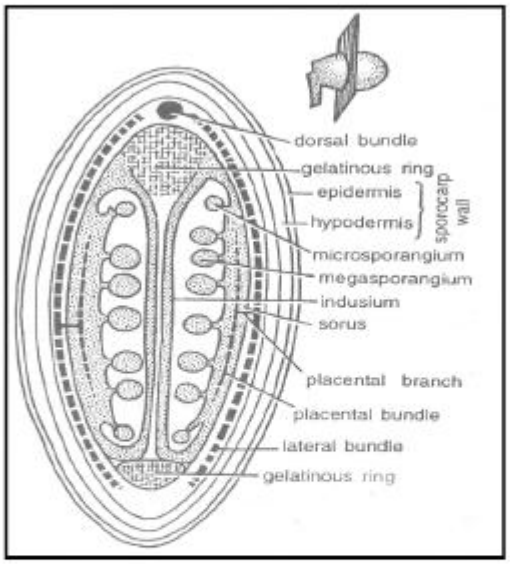


Fig. 8.26: *Marsilea* - H.L.S. Sporocarps



V.L.S. of Sporocarp: The vertical longitudinal section shows that the wall of sporocarp is surrounded by three layers. The sori are present in the middle of the sporocarp. It is surrounded by gelatinous ring form all the sides. The sorus can be observed either with only microsporangia. If the section is form the middle portion of the sporocarp megasporangia can be observed, however, if the section is little away from the middle, microsporangia can be observed. Each sorus is cover with an indusium.

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F.g. 8.27 : *Marsilea* – V.L.S. of Sporocarp

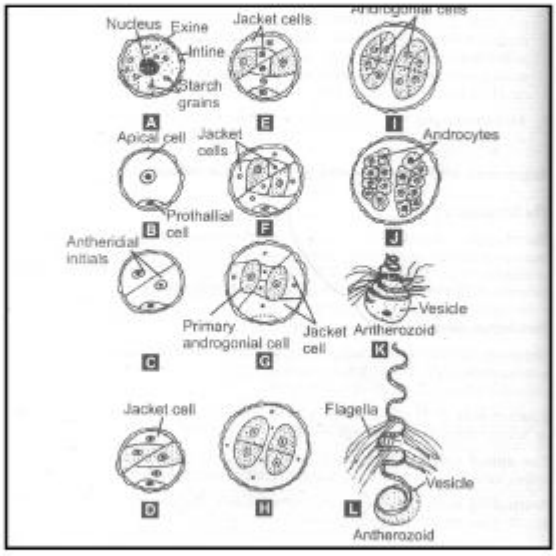
Sporangia

Morphology

Marsilea is a heterosporous and sporangia are of two types: Micro and megasporangium. Each microsporangium has numerous microspores but megasporangium contains only one megaspore. Each sorus has a receptacle which bears one terminal megasporangium and two microsporangia on the lateral sides.

Development

Development of sporangia of *Marsilea* is leptosporangiate type. During development one layered jacket and two layered tapetaum are fomed in micro and megasporangia and are filled with 32 or 64 spores. In microsporangium all 32 or 64 spores remain alive. In megasporangium only one spore remains alive and rest die.



Microspore

Microspore of *Marsilea* is haploid and globose in shape. It bears yellow colour with a diameter of 40 to 60µ. The cytoplasm consists of one nucleus and numerous starch grains.

Development of Male Gametophyte

Each microspores germinates under favourable environmental conditions and develops into male gametophytes. During

germination the nucleus of microspore increases in size and shifts to the lower side. Microspore divides into two cells (one smaller and another larger cell) by a wall. Small cell is called prothallial cell and large cell is called apical cell.

NOTES

Prothallial cell: It does not divided further.

Large apical cell: It divides by an oblique wall and form two antheridial initials.

- **Antheridial cell :** It divides by periclinal wall to form cell wall or fist jacket cell. Second cell again divide to form one big outer cell and three other small second jacked cell. Big outer cell again divides three times to form third jacket cell and a primary androgonial cell towards the nucleus. Each primary androgonial cell divides many times to form 16 androcytes. Each androcyte develops into single uninucleate, multiflagellate antherozoid.
- **Antherozoids :** It is also known as spermatozoid. It is spirally coiled, funnel shaped with multiflagella. Each antherozoids has about 12 flagella.

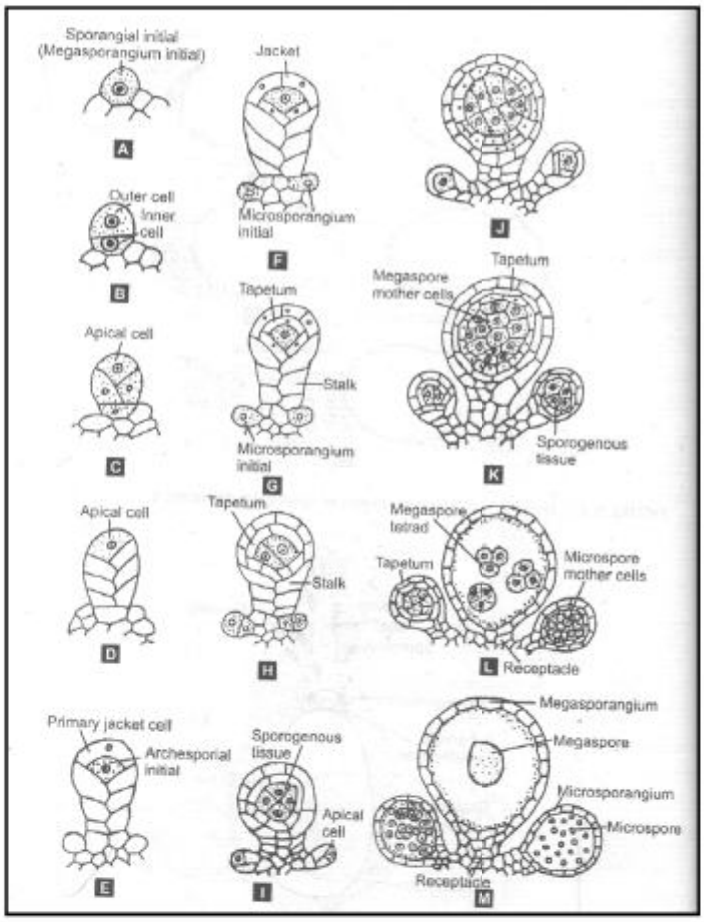


Fig. 8.29: *Marsilea Sp-*: Various stages of development of micro and megasporangia

Megaspores and Development of Female Gametophyte

The Megaspore

The megaspore is oval with an apical protuberance. The apical protuberance of megaspore has three radiating ridges with a nucleus in s present in this apical protuberance and the rest part is filled with starch grains. Gupta (1962) observed presence of oil and albumin in the cytoplasm of megaspore

Development of Female Gametophyte

Megaspore of *Marsilea* is haploid n) and on germination develops into female gametophyte. It divides by transverse wall and results into one upper smaller cell and one big prothallial cell or nutritive cell.

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Smaller cell: This cell develops into female gametophyte. This cell may divide for several times to form an apical cell.

The apical cell functions as an archegonial initial cell. The development of archegonium occurs form an archegonial initial just like other fern.

Prothallial cell : It is also called nutritive cell. It does not divide further and directly functions as food storage cell.

8.7 *Azollla*

Division	:	Pteriophyta
Class	:	Leptosporangiopsida
Order	:	Salviniales
Family	:	Azollaceae
Genus	:	*Azolla*

Thirty one species of *Azolla* are reported from tropical and temperature regions all over the world out of which six are extant species. The common Indian species is *Azolla pinnata.* It is widely distributed.

Practical work and study

External Morphology

The sporophyte of *Azolla* is small in size , free floating in ditches and ponds with a thin, delicate, pinnately branched rhizome. Because of vegetative multiplication by decay of older parts and setting free of growing branches often the water surface is completely covered. The floating mass looks reddish when growing vigorously. The roots are adventitious.The roots have root caps The rhizomatous stem bears two rows of alternating leaves densely packed to overlap one another on the upper surface and long adventitious roots on the under surface here and there. Each leaf has two lobes of almost equal size. The upper lobe has photosynthetic tissue (palisade parenchyma) and stomata above and a large hollow below containing the alga *Anabaena azolla* which fixes free nitrogen and the association seems to be symbiotic. There is a single vein in the leaf. The leaf surface is papillate to prevent wetting. The lower submerged leaf is colourless and only one cell in

thickness except for a few chloroplasts in the thicker base. This lobe seems to be an absorbing organ.

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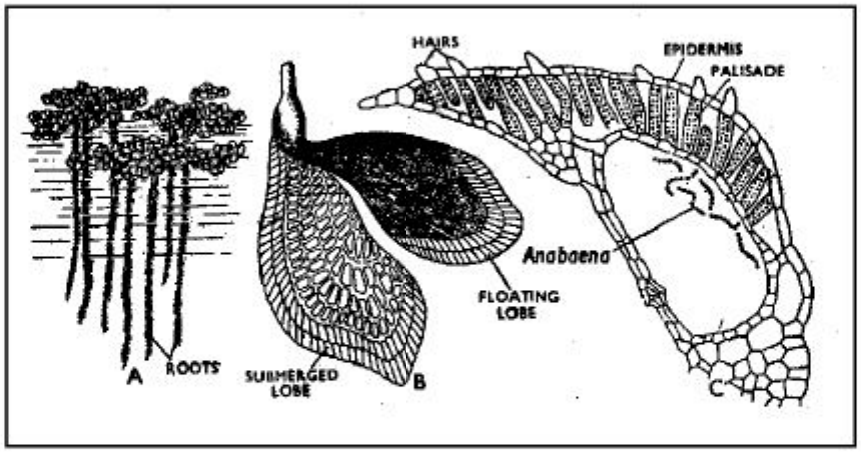


Fig. 8.30: ***Azolla*** **- A Complete Plant**

Internal Structure

The stem grows by an apical cell of three cutting faces. The *vascular cylinder* is slender and siphonostelic with *endodermis* and *pericycle* of one layer each. There are a few *xylem* elements in a ring broken up by leaf *traces*. The *phloem* is better developed on the lower surface of the stem. The *pith* is almost non-existent .

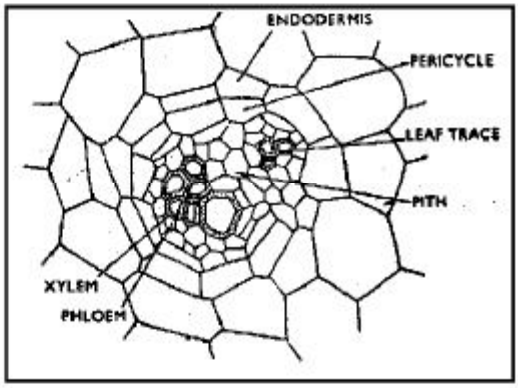
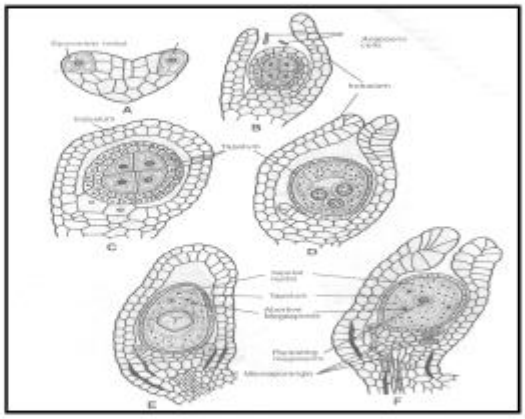


Fig. 68.31 : ***Azolla*** **- T.S. of axial**

It has a cortex of large thin-walled cells and a narrow, exarch, tetrarch xylem with a patch of phloem inside.

Life History



Sporocarps are borne only on fertile leaves, the lower-most leaf of each branch at the end of the annual season. The oldest leaf of a lateral branch is always fertile .The submerged lobe of this leaf is split, into two division (rarely four) and one sporocarp is borne on the tip of each division. Of the two sporocarps one is larger and male *(microsporocarp)* and the other smaller and

Fig. 8.32 : Azolla _ Sporocarp

female *(megasporocarp)* . Each sporocarp is a sorus covered by its *indusium.* The development of the sporocarp fallows avery simpal course. The indusium develops from the base and is two-layered when mature. There is a central raised cushion on which The sporangia develop basipetally (*gradate*). Both the sporocarps seem to be potentially bisexual, the tip one developing as a *megasporangium* and the side ones as *microsporangia*. In the *megasporcap* only the tip *megasporcap* only the tip megasporangium develops and the lower microsporangia abort. In the megasporangium only one *megasphore mother cell* gives rise to four *megaspores* of which, again, only one megaspores enlarges and becomes functional while the other three degenerate . In the *microsporocarp* . In the microsporocarp the reverse takes place. The apical megasporangium aborts and all the lower microsporangia mature. Each microsporangium has a *topetum* below the one-layered jacket and 16 *microspore mother cells* inside, which latter give rise to 64 *microspores*. Microsporangia of certain species have been reported to show vestigial oblique *annuls* suggesting a Hymenophyllaceous origin. But, usually there is no annulus.

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In the *microsporangium* a multinucleate *periplasmodium* is formed out of the tapetal cells. This increases greatly with the increases greatly with the increase in the size of the sporangium and completely surrounds the microspores which soon take up a pheripheral position .

Subsequent to this, the periplasmodium organises into four or more quadartely arranged *massulae* of alveolar, solidified structures each of which embeds some spores on the periphery. In some species the massulae develop on the surface tubular anchor-like structures called *glochidia*.

The single *megasporangium* within the megasporocarp also develops similarly. In it, the *periplasmodium* develops four *massulae* of which a large one at the base contains the functional megaspores while the three smaller ones hanging from top many each contain an aborted megaspores.

The sporocarp walls rot away and the sporangial walls burst at this stage liberating the massulae. The male massulae float away separately while four female massuale stick together causing better buoyancy. One or more male massulae stick to the female mass of massulac which latter usually retains the top of the sporocarp wall above it like a cap.

The Gametophytes

The germination of the megaspore is endosporic and is completed within 7-8 days. The microspores germinate into *male gametophytes* while still within a massula. Only a single

antheridium with 8 *sperm mother cells* develops and this peeps out of the massula. each sperm mother cell gives rise to a coiled multiciliate *sperm*.

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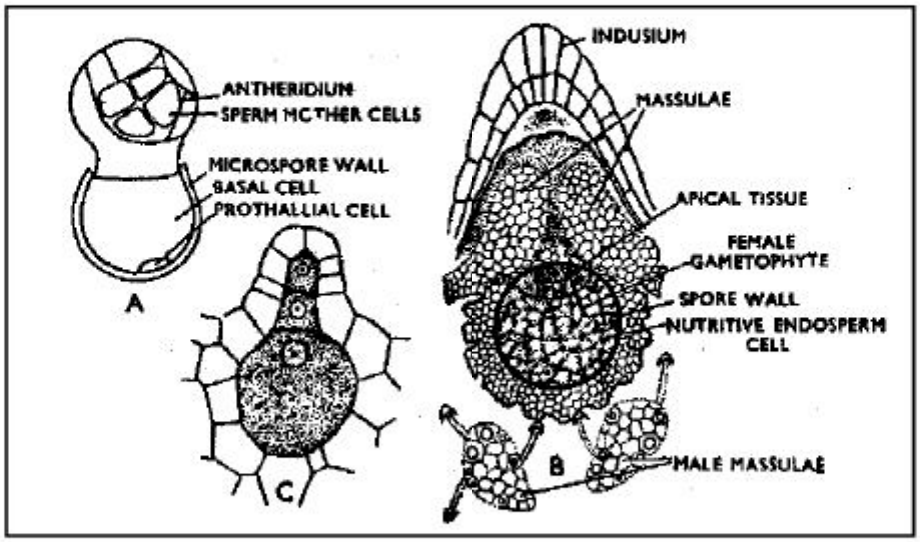

Fig. 8.33 : Azolla - Gametophyte

The megaspore, still within the lower massula, develops a *female gametophyte*. An *apical tissue* and a large, basal, free nucleate *nutritive endosperm* is formed . The apical tissue develops a number of archegonia and gets exposed bursting open the top of the megaspore wall but never turns green. A mature *archegonium* shows a short, protruding *neck canal cell* whose nucleus may or may not divide and a *ventral canal cell* which sometimes may be absent and an *egg*.

Fertilization –The neck canal cell and the ventral cell disorganize and the neck cells spread apart to create a passage for the spermatozoids to enter and effect fertilization.Large number of sperms gather around the open necks,but only one is able to penetrate the egg and effect fertilizatioetrate the egg and effect fertilizatioetrate the egg and effect fertilizatioetrate the egg and effect fertilizatioetrate the egg and effect fertilization.

The New Sporophyte

The zygote or the oospore increases in size .The first division of the *zygote* is transverse and not vertical. Subsequently, the four quadrants – *cotyledon, stem root* and *foot* are organized as in other leptosporongiates. The growing embryo comes out by pushing off the upper massulae, sporocarp wall top, etc. The cotyledon grows first and then the root and stem when the new sporophyte gets established.

8.8 Viva- Voce

1. What are the specific characters of Pteridophytes?
2. Define protostele.
3. What is stelar system?
4. What is the commom name of *Pteris?*
5. What is Sporocarp?
6. Differentiate VLS and HLS.
7. What is homospory?
8. What is heterospory?
9. Classification of *Pteris?*
10. Classification of *Marsilea?*
11. Classification *fo Azolla?*
12. What is exarch and endarch condition?
13. What is rhizome?
14. How many types of spores available in *Lycopodium?*
15. What is symbiosis?
16. Differentiate Root and Rhizome.
17. What is vascular system?
18. What is vascular bundle?
19. Define monoecious?
20. Define dioceous?

NOTES

8.9 References

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NOTES

Unit – 9

Comparative Study of Morphology, Anatomy, Reproductive Structures and Microscopic Preparation of Gymnosperms

NOTES

Structure of the Unit

9.0 Objectives

Scholars will able to understand following concepts about Gymnosperms:-

- Introducton of Gymnosperms.
- Structure and reproduction in *Zamia, Araucaria, Taxus* and *Ginkgo.*
- Structure and reproduction in *Welwitschia* and *Gnetum.*

9.1 Introduction

Description of each specimen was made elaborately using technical terms as seen under microscope. Identification of each specimen was made using manuals, monographs and identification keys etc.

Gymnosperms are mostly cone-bearing seed plants. In gymnosperms, the ovules are not completely enclosed by sporophyte tissue at pollination. There are four groups of living gymnosperms, Conifers, Cycads, Gnetophytes, and *Ginkgo*. In all of them the ovule,

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which becomes a seed, rests exposed on a scale (modified leaf) and is not completely enclosed by sporophyte tissues at the time of pollination. The name gymnosperm combines the Greek root 'Gymnos' or "naked," with 'sperma' or "seed." In other words, gymnosperms are naked-seeded plants. Details of reproduction vary somewhat in gymnosperms, and their forms vary greatly.

The most familiar gymnosperms are conifers (phylum Coniferophyta), which include pines, spruces, firs, cedars, hemlocks, yews, larches, cypresses, and others. The coastal redwood (*Sequoia sempervirens*), a conifer native to northwestern California and southwestern Oregon, is the tallest living vascular plant; it may attain nearly 100 meters (300 feet) in height. Another conifer, the bristlecone pine (*Pinus longaeva*) of the White Mountains of California is the oldest living tree; one is 4900 years of age. Conifers are found in the colder temperate and sometimes drier regions of the world.

Cycads are slow-growing gymnosperms of tropical and subtropical regions. The sporophytes of most of the 100 known species resemble palm trees with trunks that can attain heights of 15 meters or more. Unlike palm trees (which are flowering plants) cycads produce cones and have a life cycle similar to that of pines.

In Gnetophytes, there are three genera and about 70 living species. These are the only gymnosperms with vessels (a particularly efficient conducting cell type) in their Xylem (a common feature in angiosperms). The members of the three genera differ greatly from one another in form. One of the most specific of all plants is *Welwitschia,* which occurs in the Namib and Mossamedes deserts of southwestern Africa. The stem is shaped like a large, shallow cup that tapers into a taproot below the surface. It has two strap-shaped, leathery leaves that grow continuously from their base, splitting as they flap in the wind. The reproductive structures of *Welwitschia* are conelike, appear toward the bases of the leaves around the rims of the stems, and are produced on separate male and female plants.

In Ginkgoales, the fossil record indicates that members of the Ginkgo family were once widely distributed, particularly in the northern hemisphere; today only one living species, the maidenhair tree (*Ginkgo biloba*), remains. The tree, which sheds its leaves in the fall, was first encountered by Europeans in cultivation in Japan and China; it apparently no longer exists in the wild. Like the sperm of cycads, those of *Ginkgo* have flagella. The Ginkgo is diecious, that is, the male and female reproductive structures of Ginkgo are produced on separate trees. The fleshy outer coverings of the seeds of female Ginkgo plants exude the foul smell of rancid butter caused by butyric and isobutyric acids. Due to this, male plants vegetatively propagated from shoots are preferred for cultivation. Because it is resistant to air pollution, Ginkgo is commonly planted along city streets.

9.2 *Zamia*

NOTES

Systematic Position

Kingdom:	Plantae
Division:	Cycadophyta
Class:	Cycadopsida
Order:	Cycadales
Family:	Zamiaceae
Genus:	*Zamia*

The western genus, *Zamia,* with more than a third of all the species in the family Zamiaceae has a wide distribution, its various species ranging from Florida through the West Indies, through Mexico, Central America, the northernpart of South America, and down the Andes into Chili. Some of the species are local, well marked, and easily recognizable, while others range widely and are so variable that identifications are uncertain. The name *Zamia* comes from the Greek azaniae, meaning "a pine cone". The smallest cycad known, Zamia pygmaea, grows in the Microcycas region. The leaves of adult coning specimens are some-times only 4 or 5 cm. long. Associated with *Z. pygmaea*, but in soil not quite so bad, is *Z. kickxii*. It is quite possible that *Z. pygmaea* might become *Z. kickxii* under equally favorable conditions. The largest species of Zamia are arborescent, and have leaves over a meter in length.

Sporophyte: Vegetative

The genus comprises deciduous shrubs with aerial or subterranean circular stems, often superficially resembling palms. They produce spirally arranged, pinnate leaves which are pubescent, at least when young, having branched and simple, transparent and coloured hairs. The articulated leaflets lack a midrib, and are broad with subparallel dichotomous venation. Lower leaflets are not reduced to spines, though the petioles often have prickles. The emerging leaves of many *Zamia* species are striking, some emerging with a reddish or bronze cast (*Z. roesli* is an example). *Zamia picta* is even more distinctive, being the only truly variegated cycad (having whitish/yellow speckles on the leaves).

Sporophyte : Reproductive

Zamia sporophylls are born in vertical rows in cones, and the megasporophyll apices are faceted or flattened, not spinose. The fleshy seeds are subglobular to oblong or ellipsoidal, and are red, orange, yellow or rarely white. The endosperm is haploid, derived from the female gametophyte. The embryo is straight, with two cotyledons that are usually united at the tips and a very long, spirally twisted suspensor.

Sperms:

The sperm of the genus are large, as is typical of cycads, and *Zamia roezlii* is an example; its sperm are approximately 0.4 mm long and can be seen by the unaided eye.

NOTES

Fig. . 9.1 *Zamia:* Complete Plant

Fig. 9.2 *Zamia*: Strobili and Seeds

9.3. *Araucaria*

Systematic Position

Kingdom:	Plantae
Division:	Pinophyta
Class:	Pinopsida
Order:	Pinales
Family:	Araucariaceae
Genus:	*Araucaria*

Araucaria is a genus of evergreen coniferous trees in the family Araucariaceae. There are 19 extant species in the genus, with a Gondwanan natural distribution in New Caledonia (where 13 species are endemic), Norfolk Island, eastern Australia, New Guinea, Argentina, Chile, and southern Brazil. *Araucaria* is mainly large trees with a massive erect stem, reaching a height of 30–80 metres (98–262 ft). The horizontal, spreading branches grow in whorls and are covered with leathery or needle-like leaves. In some species, the leaves are narrow and lanceolate, barely overlapping each other, in others they are broad and flat, and overlap broadly.

NOTES

Sporophyte: Vegetative

The trees are mostly dioecious, with male and female cones found on separate trees, though occasional individuals are monoecious or change sex with time. The female cones, usually high on the top of the tree, are globose, and vary in size between species from 7 to 25 centimetres (2.8 to 9.8 in) diameter. They contain 80–200 large, edible seeds, similar to pine nuts though larger. The male cones are smaller, 4–10 cm (1.6–3.9 in) long, and narrow to broad cylindrical, 1.5–5.0 cm (0.6–2.0 in) broad. The genus is familiar to many people as the genus of the distinctive Chilean pine or monkey-puzzle tree (Araucaria araucana). The genus is named after the Spanish exonym Araucano ("from Arauco") applied to the Mapuches of central Chile and south-west Argentina whose territory incorporates natural stands of this genus. The Mapuche people call it pehuén, and consider it sacred. Some Mapuches living in the Andes name themselves Pehuenches ("people of the pehuén") as they traditionally harvested the seeds extensively for food.

Sporophyte: Reproductive

The two Argentinean species of *Araucaria* are dioecious. The pollen cones of both species are single and occur laterally on the tips of the branches. The mature cones in *A. araucana* are sub-cylindrical, slightly curved and reddish and up to 13 cm long and up to 6 cm wide. In *A. angustifolia*, the mature cones are cylindrical, brown, straight, longer (up to 20 cm long) and narrower (up to 3 cm wide). The microsporphllys are disposed in a dense and imbricate spiral on the cone axis. They are peltate, and numerous cylindrical microsporangia, which are parallel to and free from the stalk, hang on their abaxial face. The sporophylls in A. araucana have a distal portion that end in a long flattened and obtuse apex. They are strongly curved and overlap the adjacent sporophyll. *A. angustifolia* have a rhomboidal expanded distal portion. The number of sporangia is up to 20 per sporophyll in *A. araucana* and up to 22 in *A. angustifolia*. The dehiscence in both species is longitudinal.

Sperms:

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Pollens of *Araucaria angustifolia A. angustifolia* is asaccate, inaperturate, and 84 µm in diameter. The pollen is spheroidal in outline in polar view, while it is ellipsoidal in lateral view. The pollen also has a subequatorial annular area. Broad exine depressions are seen in dehydrated specimens. The exine is 2-4 µm thick and atectate with a nexine that is equal to or thinner than the sexine. In the annular area, the exine shows a thickening of the sexine (3 µm thick), in which it is observed sexine detachments. The exine has a conspicuously granulate sculpture of Pilate elements, as seen in the optical. The granulate elements (less than 1 µm in diameter) are irregularly dispersed, showing different degrees of coalescence and forming granules of different sizes. Each granule has microgranule-like and isolated microspinule-like elements on its surface. The ectexine is defined by granules, which decrease gradually in diameter towards the endexine: the most external are large, dark, electron dense, and spherical- to irregular-shaped because of the microspinule-like and the microgranule-like elements. The inner granules are smaller, less electron dense, spherical, and loosely disposed with numerous interstices. The endexine is formed of two layers: endexine I and endexine II. Endexine I have from 10 to 12 parallel lamellae, where the innermost are compactly arranged and the outermost are sinuous, loosely disposed, and closely related to the innermost granules of the ectexine. Each lamella shows a central, less electron-dense area surrounded by darker sporopollenin Endexine II is a thin and amorphous layer contiguous to the intine. This layer is 2.0 µm thick and is composed of irregularly disposed microfibrillae. At the contact with the endexine, the intine appears as a regular, darker layer.

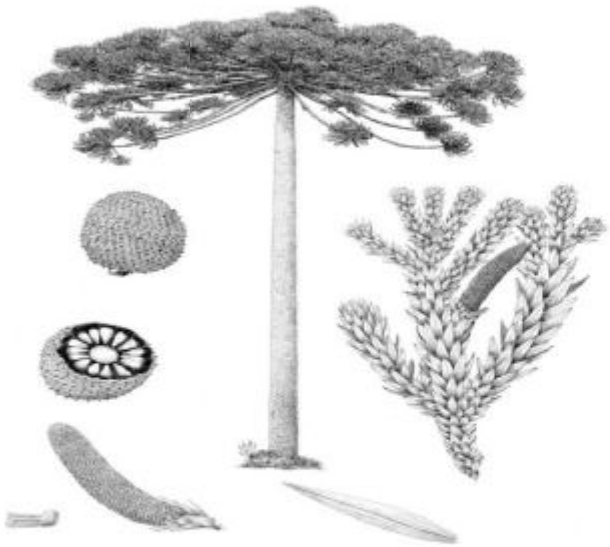

Fig. 9.3: *Araucaria angustifolia:* Complete Plant, Leaves and Strobili

9.4 *Taxus*

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Systematic Position

Kingdom:	Plantae
Division:	Pinophyta
Class:	Pinopsida
Order:	Pinales
Family:	Taxaceae
Genus:	*Taxus*

Taxus is a genus of yews, small coniferous trees or shrubs in the yew family Taxaceae. They are relatively slow-growing and can be very long-lived, and reach heights of 1–40 m, with trunk diameters of up to 5 m.[1] They have reddish bark, lanceolate, flat, dark-green leaves 1–4 cm long and 2–3 mm broad, arranged spirally on the stem, but with the leaf bases twisted to align the leaves in two flat rows either side of the stem. The most distinct is the Sumatran yew (*T. sumatrana*, native to Sumatra and Celebes north to southernmost China), distinguished by its sparse, sickle-shaped yellow-green leaves. The Mexican yew (*T. globosa*, native to eastern Mexico south to Honduras) is also relatively distinct with foliage intermediate between Sumatran yew and the other species. The Florida yew, Mexican yew and Pacific yew are all rare species listed as threatened or endangered.

Sporophyte: Vegetative

It is a small- to medium-sized evergreen tree, growing 10–20 metres (33–66 ft) (exceptionally up to 28 metres (92 ft)) tall, with a trunk up to 2 metres (6 ft 7 in) (exceptionally 4 metres (13 ft)) diameter. The bark is thin, scaly brown, coming off in small flakes aligned with the stem. The leaves are flat, dark green, 1–4 centimetres (0.39–1.57 in) long and 2–3 millimetres (0.079–0.118 in) broad, arranged spirally on the stem, but with the leaf bases twisted to align the leaves in two flat rows either side of the stem, except on erect leading shoots where the spiral arrangement is more obvious. The leaves are highly poisonous.

Sporophyte: Reproductive

The seed cones are highly modified, each cone containing a single seed 4–7 mm long partly surrounded by a modified scale which develops into a soft, bright red berry-like structure called an aril, 8–15 mm long and wide and open at the end. The arils are mature 6–9 months after pollination, and with the seed contained are eaten by thrushes, waxwings and other birds, which disperse the hard seeds undamaged in their droppings; maturation

NOTES

of the arils is spread over 2–3 months, increasing the chances of successful seed dispersal. The male cones are globose, 3–6 mm diameter, and shed their pollen in early spring. Yews are mostly dioecious, but occasional individuals can be variably monoecious, or change sex with time.[2]

Sperms:

The pollen is spheroidal in outline in polar view, while it is ellipsoidal in lateral view. The pollen also has a subequatorial annular area. Broad exine depressions are seen in dehydrated specimens. The exine has a conspicuously granulate sculpture of Pilate elements, as seen in the optical. The granulate elements are irregularly dispersed, showing different degrees of coalescence and forming granules of different sizes. Each granule has microgranule-like and isolated microspinule-like elements on its surface. The ectexine is defined by granules, which decrease gradually in diameter towards the endexine: the most external are large, dark, electron dense, and spherical- to irregular-shaped because of the microspinule-like and the microgranule-like elements. The endexine is formed of two layers: endexine I and endexine II. Endexine I have from 10 to 12 parallel lamellae, where the innermost are compactly arranged and the outermost are sinuous, loosely disposed, and closely related to the innermost granules of the ectexine. Each lamella shows a central, less electron-dense area surrounded by darker sporopollenin Endexine II is a thin and amorphous layer contiguous to the intine. This layer is thick and is composed of irregularly disposed microfibrillae.

Fig. 9.4 *Taxus baccata:*Various plant parts

9.5 Ginkgo

Systematic Position

NOTES

Kingdom:	Plantae
Division:	Ginkgophyta
Class:	Ginkgoopsida
Order:	Ginkgoales
Family:	Ginkgoaceae
Genus:	*Ginkgo*
Species:	*Ginkgo biloba*

Ginkgos are large trees, normally reaching a height of 20–35 m (66–115 feet), with some specimens in China being over 50 m (164 feet). The tree has an angular crown and long, somewhat erratic branches, and is usually deep rooted and resistant to wind and snow damage. Young trees are often tall and slender, and sparsely branched; the crown becomes broader as the tree ages. During autumn, the leaves turn a bright yellow, then fall, sometimes within a short space of time (one to 15 days). A combination of resistance to disease, insect-resistant wood and the ability to form aerial roots and sprouts makes ginkgos long-lived, with some specimens claimed to be more than 2,500 years old. Although Ginkgo biloba and other species of the genus were once widespread throughout the world, its range shrank until by two million years ago, it was restricted to a small area of China. For centuries, it was thought to be extinct in the wild, but is now known to grow in at least two small areas in Zhejiang province in eastern China, in the Tianmushan Reserve. However, recent studies indicate high genetic uniformity among ginkgo trees from these areas, arguing against a natural origin of these populations and suggesting the ginkgo trees in these areas may have been planted and preserved by Chinese monks over a period of about 1,000 years. This study demonstrates a greater genetic diversity in Southwestern China populations, supporting glacial refugia in mountains surrounding eastern Qinghai-Tibet Plateau, where several old-grow, candidates to wild populations have been reported.Whether native ginkgo populations still exist has not been demonstrated unequivocally, but evidence grows favouring these Southwestern populations as wild, from genetic data but also from history of those territories, with bigger Ginkgo biloba trees being older than surrounding human settlements. Where it occurs in the wild, it is found infrequently in deciduous forests and valleys on acidic loess (i.e. fine, silty soil) with good drainage. The soil it inhabits is typically in the pH range of 5.0 to 5.5.

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In many areas of China, it has been long cultivated, and it is common in the southern third of the country. It has also been commonly cultivated in North America for over 200 years and in Europe for close to 300, but during that time, it has never become significantly naturalized

Ginkgo is a relatively shade-intolerant species that (at least in cultivation) grows best in environments that are well-watered and well-drained. The species shows a preference for disturbed sites; in the "semiwild" stands at Tian Mu Shan, many specimens are found along stream banks, rocky slopes, and cliff edges. Accordingly, ginkgo retains a prodigious capacity for vegetative growth. It is capable of sprouting from embedded buds near the base of the trunk (lignotubers, or basal chi chi) in response to disturbances, such as soil erosion. Old individuals are also capable of producing aerial roots on the undersides of large branches in response to disturbances such as crown damage; these roots can lead to successful clonal reproduction upon contacting the soil. These strategies are evidently important in the persistence of ginkgo; in a survey of the "semiwild" stands remaining in Tianmushan, 40% of the specimens surveyed were multistemmed, and few saplings were present.

Sporophyte: Vegetative

Ginkgo branches grow in length by growth of shoots with regularly spaced leaves, as seen on most trees. From the axils of these leaves, "spur shoots" (also known as short shoots) develop on second-year growth. Short shoots have very short internodes (so they may grow only one or two centimeters in several years) and their leaves are usually unlobed. They are short and knobby, and are arranged regularly on the branches except on first-year growth. Because of the short internodes, leaves appear to be clustered at the tips of short shoots, and reproductive structures are formed only on them (see pictures below - seeds and leaves are visible on short shoots). In ginkgos, as in other plants that possess them, short shoots allow the formation of new leaves in the older parts of the crown. After a number of years, a short shoot may change into a long (ordinary) shoot, or vice versa.[The leaves are unique among seed plants, being fan-shaped with veins radiating out into the leaf blade, sometimes bifurcating (splitting), but never anastomosing to form a network. Two veins enter the leaf blade at the base and fork repeatedly in two; this is known as dichotomous venation. The leaves are usually 5–10 cm (2–4 in), but sometimes up to 15 cm (6 in) long. The old popular name "maidenhair tree" is because the leaves resemble some of the pinnae of the maidenhair fern, Adiantum capillus-veneris. Ginkgos are prized for their autumn foliage, which is a deep saffron yellow.

Leaf:

Leaves of long shoots are usually notched or lobed, but only from the outer surface, between the veins. They are borne both on the more rapidly growing branch tips, where they are alternate and spaced out, and also on the short, stubby spur shoots, where they are clustered at the tips.

NOTES

There is great variation in the size and in the lobing of the leaves of *Ginkgo*. The blades are often deeply cut and with more than two lobes, and the same tree may show every gradation between deeplylobed leaves and those with nearly entire margins.

In the surviving genus *Ginkgo*, the lobed condition is always found in the leaves of seedlings, and usually on the long shoots, leaves from the top of a tree being particularly deeply cleft. The leaves on the dwarf shoots are usually nearly entire, but when the bud of a dwarf shoot develops into a long shoot, the lobed character ofthe leaf is suddenly resumed, the contrast between the entire leaves of the dwarf shoot and the lobed leaves of the new long shoot being quite marked. The young leaves develop abundant hairs, especially on the petiole; and the hairs are numerous on the scale leaves, forming a distinct woolly fringe to the petiole. There is an evident but not very thick cuticle; the stomata are mostly restricted to the abaxial surface, the guard cells being somewhat below the level of the epidermis; there is a distinct pallisade tissue in the larger leaves, but not in the leaves of the dwarf branches; and between the veins the very loose inner mesophyll cells are elongated parallel with the plane of the leaf surface.

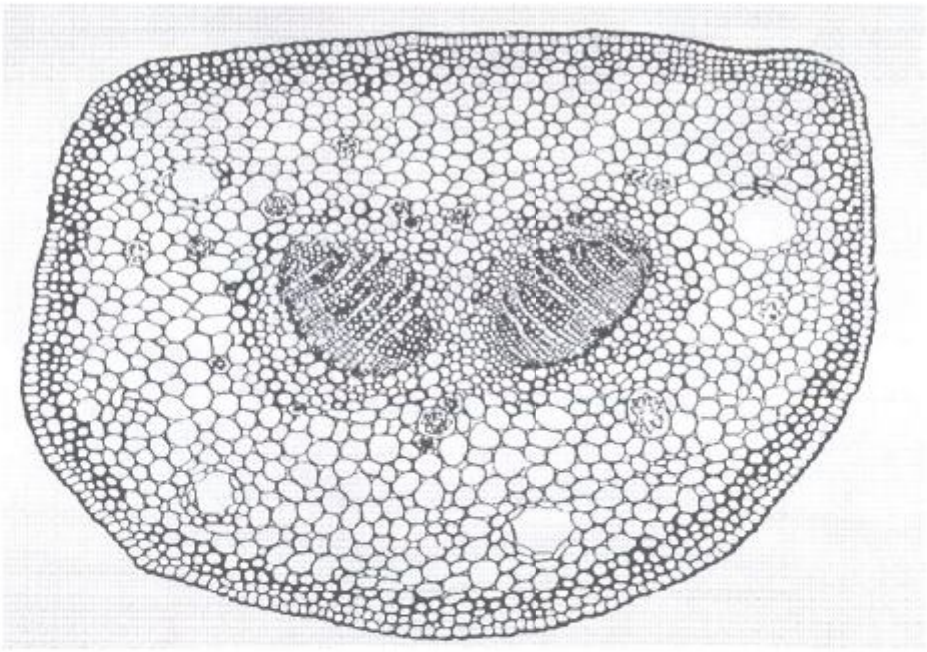

Fig. 9.5 : *Ginkgo biloba* T. S. of leaf

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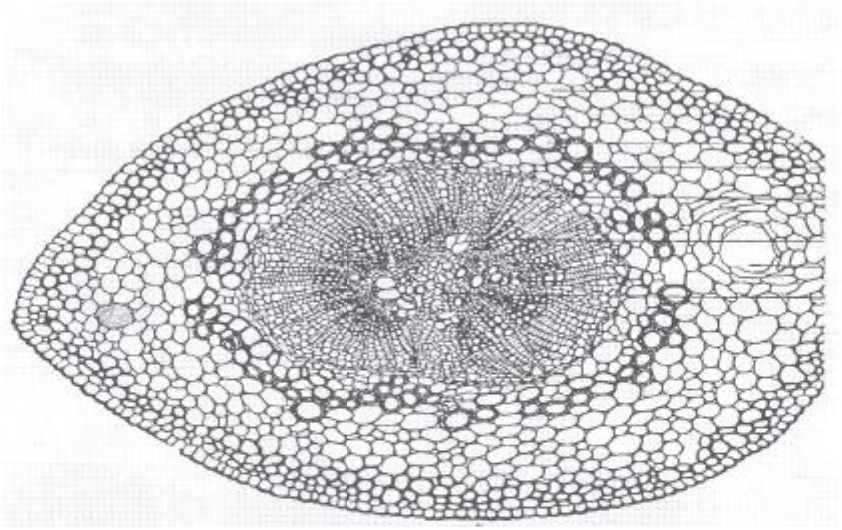

Fig. 9.6 : *Ginkgo biloba* T. S. of rachis

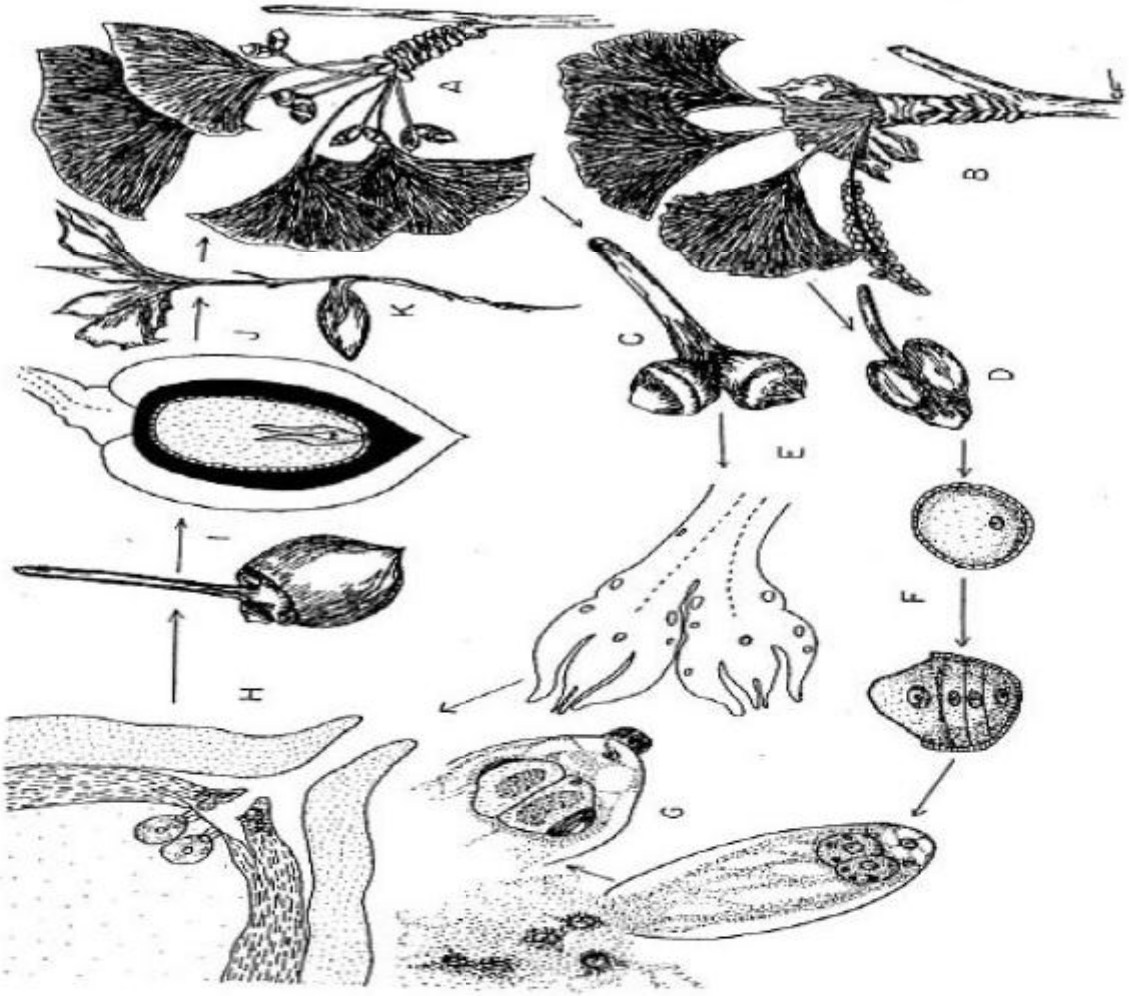

Fig. 9.7 : Life cycle of *Ginkgo biloba*

This species is dioecious: ovulate (A) and staminate (B). two ovules on a common peduncle (C), two mature microsporangia from a staminate strobilus (D). Pollen develops from a microspore (F), in which the microgametophytes germinates (F) and develops (G) in the pollen chamber of the ovule (H). Sperm nuclei are delivered to the archegonia by pollen tubes (G). The embryo (I) develops within a seed that has a fleshy berry-like outer integument (I & J).

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Root:

The root has a diarch cylinder, except when there are three cotyledons, in which case it is triarch. A short distance back from the root tip, the outer layers of cells are suberized and just within the suberized layers, tannin is very abundant. Starch, as in most roots, is very abundant. Within a millimeter of the tip, the endodermis can be distinguished, and the next layers of cells outside it have characteristic ring thickenings. The mature root looks much like the mature stem, with annual rings and with similar bordered pits.

Sporophyte : Reproductive

Ginkgos are dioecious, with separate sexes, some trees being female and others being male. Male plants produce small pollen cones with sporophylls, each bearing two microsporangia spirally arranged around a central axis. Female plants do not produce cones. Two ovules are formed at the end of a stalk, and after pollination, one or both develop into seeds. The seed is 1.5–2 cm long. Its fleshy outer layer (the sarcotesta) is light yellow-brown, soft, and fruit-like. It is attractive in appearance, but contains butyric acid (also known as butanoic acid) and smells like rancid butter or vomit when fallen. Beneath the sarcotesta is the hard sclerotesta (the "shell" of the seed) and a papery endotesta, with the nucellus surrounding the female gametophyte at the center.

The fertilization of ginkgo seeds occurs via motile sperm, as in cycads, ferns, mosses and algae. The sperm are large (about 70–90 micrometres) and are similar to the sperm of cycads, which are slightly larger. Ginkgo sperm were first discovered by the Japanese botanist Sakugoro Hirase in 1896. The sperm have a complex multi-layered structure, which is a continuous belt of basal bodies that form the base of several thousand flagella which actually have a cilia-like motion. The flagella/cilia apparatus pulls the body of the sperm forwards. The sperm have only a tiny distance to travel to the archegonia, of which there are usually two or three. Two sperm are produced, one of which successfully fertilizes the ovule. Although it is widely held that fertilization of ginkgo seeds occurs just before or after they fall in early autumn, embryos ordinarily occur in seeds just before and after they drop from the tree.

Ginkgos are dioecious, with separate sexes, some trees being female and others being male. Male plants produce small pollen cones with sporophylls, each bearing two microsporangia spirally arranged around a central axis. Female plants do not produce

NOTES

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Sperms:

The sperms in *Ginkgo* are more elongated than in the cycads, and the spiral, ciliated band, with fewer turns, is more confined to the apical region. *Ginkgo* and the cycads are the only known living seed plants which have retained the swimming sperm of their very remote ancestry.

9.6 *Welwitschia*

Systemic	**Position**
Kingdom:	Plantae
Division:	Gnetophyta
Class:	Gnetopsida
Order:	Welwitschiales
Family:	Welwitschiaceae
Genus:	*Welwitschia*

Welwitschia is amonotypic gymnosperm genus comprising solely the very distinct *Welwitschia mirabilis*. The plant is commonly known simply as Welwitschia, and has various common names in local languages. It is the only genus of the family Welwitschiaceae and order Welwitschiales, in the division Gnetophyta. Informal sources commonly refer to the plant as a "living fossil". *Welwitschia mirabilis* is endemic to the Namib desert within Namibia and Angola.

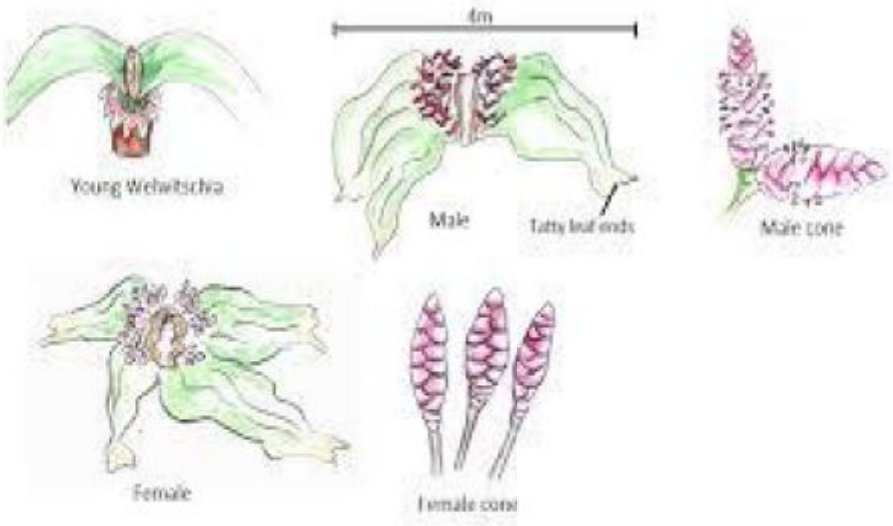

Fig.9.8 Welwitschia mirabilis : Various plant parts

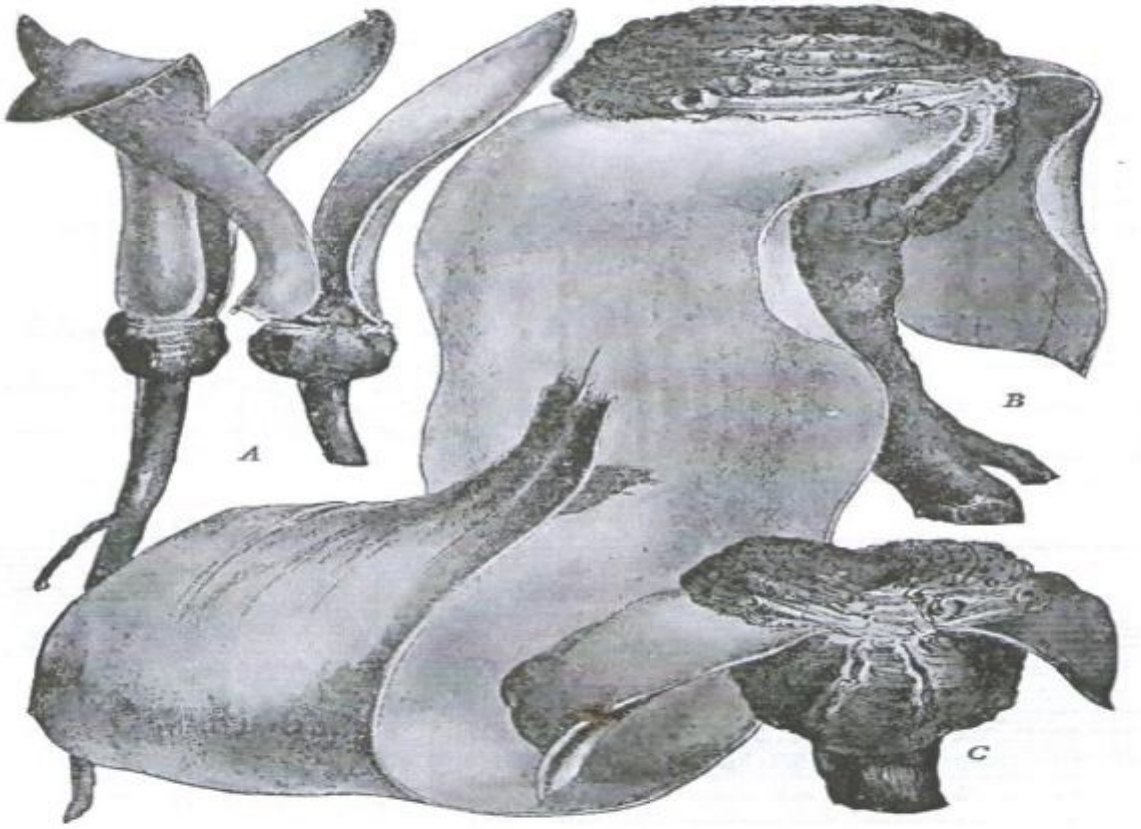

Fig. 9.9. : *Welwitschiales mirabilis* A. Young plant B.Older plant C. Top view of plant

Sporophyte: Vegetative

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Welwitschia has an elongated, but relatively shallow root system and a woody, fibrous unbranched stem. Bornman appears to have investigated the matter more intimately at first hand than most authors, and describes the root system as "rather shallow and simple, consisting of a tapering taproot with one or more non-tapering extensions, some pronounced lateral roots, and a network of delicate spongy roots." He offers the guideline that the roots extend to a depth roughly equal to the span of the living leaves from tip to tip.

Sporophyte : Reproductive

The species is dioceous, with separate male and female plants. Fertilization that is, the transfer of the pollen from the male to the female strobili, is carried out by insects. The insects responsible include flies and true bugs. The commonest of the true bugs attending *Welwitschia* is a member of the family Pyrrocoridae. *Odontopus sexpunctatus*, though much associated with *Welwitschia mirabilis* however, *Odontopus sexpunctatus* may not be a *Welwitschia* specialist; there are claims that it also occurs on other plants, such as *Adansonia*, though it is not clear how well-supported these claims might be. Infrequently, wasps and bees also play a role as pollinators of *Welwitschia*. At least some of the pollinators are attracted by "nectar" produced on both male and female strobili.

9.7 Gnetum

Systematic	**Position**
Kingdom:	Plantae
Division:	Gnetophyta
Class:	Gnetopsida
Order:	Gnetales
Family:	Gnetaceaea
Genus:	*Gnetum*

Gnetum is a genus of gymnosperms, the sole genus in the family Gnetaceae and order Gnetales. They are tropical evergreen trees, shrubs and lianas. Unlike other gymnosperms they possess vessel element in the xylem. Some species have been proposed to have been the first plants to be insect pollinated as their fossils occur in association with the extinct pollinating scorpionflies. Molecular phylogenies based on nuclear and plastid sequences from most of the species indicate hybridization among some of the Southeast

Asian species. Fossil-calibrated molecular-clocks suggest that the *Gnetum* lineages now found in Africa, South America, and Southeast Asia are the result of ancient long-distance dispersal across seawater.

NOTES

Sporophyte: Vegetative

It is a small to medium-size tree, growing to 15–20 m tall. The leaves are evergreen, opposite, 8–20 cm long and 3–10 cm broad, entire, emerging bronze-coloured, maturing glossy dark green. The fruit-like strobilus consist of little but skin and a large nut like seed 2–4 cm long inside. Fleshy strobili weigh about 5.5g, the seed alone 3.8 g. Strobili mature mainly from June to September in Philippines. The red strobili are eaten by birds, mammals and reptiles.

Sporophyte : Reproductive

Gnetum sporophylls are born in vertical rows in strobili, and the megasporophyll apices are faceted or flattened, not spinose. The fleshy seeds are subglobular to oblong or ellipsoidal, and are red, orange, yellow or rarely white. The endosperm is haploid, derived from the female gametophyte. The embryo is straight, with two cotyledons that are usually united at the tips and a very long, spirally twisted suspensor.

Sperms:

The gametes of the genus are small, as is typical of Gnetophytes, and *Gnetum* is an example.

Fig. 9.10 *Gnetum*: Plant parts and Reproductive structure (strobili)

9.8 Viva - Voce

1. What is the meaning of naked seed plant.
2. What is the difference between angiosperm and gymnosperm?
3. Name of living fossil?
4. Identify specific characters of gymnosperms.

NOTES

5. Give examples of gymnosperm.
6. Give examples of conifers
7. Identify the term coralloid root.
8. Define Megasporophyll.
9. What is cone.
10. Economic importance of *Taxus* plant.
11. Name of the plant, which is important in timber industry.
12. *Zamia* belongs to which order?
13. What is the classification of *Araucaria?*
14. *Ginkgo* belongs to which family?
15. What is the structure of *Welwitschia* plant?
16. What are the specific characters of *Gnetum*?
17. What is gametophyte?
18. What is sporophyte?
19. Cone bearing plant is monoecious or dioceous?
20. Give the name of largest gymnosperm.

9.9 References

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Unit -10

Study of Important Fossil Plants

NOTES

Structure of the Unit

10.0 Objectives

After going through this unit you will be able to understand

- General Characters, Vegetative and Reproductive structures of fossil plants : *Lyginopteris, Medullosa, Caytonia,* Glossopteris belongs to pteridospermales.
- General Characters, vegetative and Reproductive structures of fossil plants : Cycadeoidea & Williamsonia belongs to cycadeoidales.
- General Characters, Vegetative and Reproductive structures of fossil plants: Cordites belongs to cordaitales.

10.1 Introduction

Pteridospermales or Cycadofilicales came into existence during upper Devonian and reached their climax in Mesozoic era. Their leaves were fern like. Secondary wood was manoxylic. Seeds were directly borne on modified megasporophyll. Ovule was present in cupulate structure and microsporangia were grouped to form synangia. Male gametes were motile. *Lyginopteris, Medullosa, Caytonia,* Glossopteris belongs to pteridospermales.

Cycadeoidales is a group of fossil gymnosperms, existed during Mesozoic era from Triassic to Cretaceous period. Cycadeoidea & Williamsonia belongs to cycadeoidales.

Cordaitales is also a group of fossil gymnosperms appeared in the Devonian and reached its zenith in the carboniferous period of Paleozoic era. Cordites belongs to cordaitales.

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10.2 *Lyginopteris*

The genus *Lyginopteris oldhamia* was studied in detail. This was earlier known as *Calymatotheca hoeninghausii* found in coal ball horizon of Lancashire and Yorkshire.

This was described by Williamson Scott, Brongniart, Binney, Potonie, Oliver and Scott. Members of this family have been recorded from upper & lower Carboniferous periods of Palaeozoic era.

(i) External features

1. Stem is aerial, weak, radially symmetrical, branched, 2 mm – 4 cm in diameter, supported by prop roots.
2. Leaves are *Sphenopteris hoeninghausii*. Leaves are bipinnate or tripinnate, 0.5 meter long, spirally arranged on stem and pinnae are arranged opposite to each other on rachis. Pinnules are alternately arranged on pinnae. Leaflets or pinnules are cuneate. Whole plant except the root is covered by capitate glands. Long (1963) described the petiole under the form genus *Lyginorachis*.
3. *Kaloxylon hookeri* is a form genus of root. Roots are adventitious, 7 mm in diameter, emerge from stem among the leaves.

Fig.10.1 : The Frond of *Lyginopteris oldhami*

(ii) Anatomy

(a) Root

1. Transverse section of root shows epidermis, a well developed cortex, endodermis, pericycle and vascular bundles.

2. Outer cortex is 2-3 layered , made up of thin walled parenchyma cells. Inner cortex is broader comprising cells with dense cytoplasmic contents.
3. Root is polyarch with radial vascular bundles. The exarch protoxylem elements show spiral thickening.

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(b) Stem

1. The transverse section of stem shows outer epidermis, many layered cortex, pericycle and vascular bundles.
2. Outer cortex shows fibrous strands in form of vertical network. They provide mechanical strength to the weak stem. Inner cortex is parenchymatous. The pericycle consists of smaller cells and a number of thick walled scerotic cells. Each strand of 5 vascular bundles is mesarch, collateral and open. Xylem is present towards centre and phloem towards outer side. The xylem consists of tracheids with spiral thickening. The protoxylem is surrounded by metaxylem on all sides. The tracheids of centripetal metaxylem have multiseriate bordered pits arranged on radial walls and the centrifugal metaxylem has scalariform tracheids.
3. Vascular bundles of stem traverse through cortex and divide into two. These two strands again unite at the base of petiole to form a V-shaped leaf trace. It travels through the petiole as a single trace and branches only to enter rachis. Next to the cortex is the periderm and then pericycle, crushed primary phloem and secondary vascular tissue are present. The outer pericycle becomes meristematic to give rise phellogen which produces periderm on its outer side. The vascular bundle in the petiole has trough shaped xylem, many groups of protoxylem at the base and phloem surrounds the xylem.
4. Except the roots plant is covered by capitate glands. Capitate gland is a flask shaped, 3 mm in length with stalk and a head of 0.1-0.4 mm diameter. They are parenchymatous and some cells of head may secrete oil or wax for protection of young portions from fungi and bacteria.

(c) Leaf

1. Anatomy of Pinnules shows distinct upper and lower epidermis. Upper epidermis is covered by thick cuticle.
2. Mesophyll is distinguished into palisade and spongy tissue.
3. Lower epidermis shows stomata.
4. Flask shaped glandular outer growth or capitates glands are also present.

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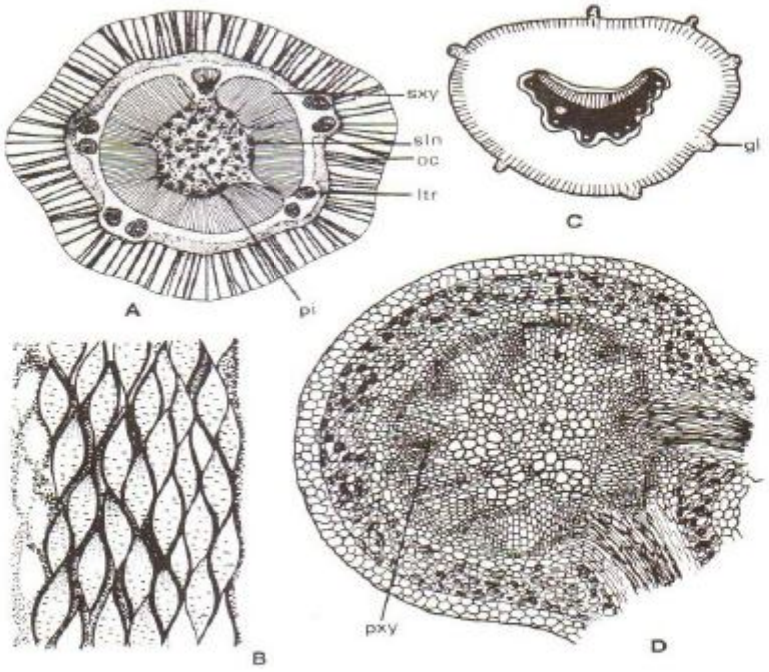

Fig. 10.2 : (A) *L. oldhamia*; T.S. Stem, (B) T.L.S. Outer Cortex, (C) T.S. Petiole (D) T.S. Root (gl=Gland, Sxy=Secondary xylem, Pxy=Primary xylem, Sln=Sclerenchyma, OC = Outer cortex, Pi = Pith, Ltr = Leaf trace)

(iii) Reproductive Structures

(a) Female Fructification

1. *L. oldhamia* is heterosporous. The seeds and ovules are enclosed within a lobed cupules. Seeds and ovules were described under form genus *Lygenostoma lomaxi* by Oliver and Scott.

2. Ovule is radially symmetrical, barrel-shaped orthotropus with single integument.

3. The integument is vascularised by 9 vascular strands. The integument is fused with nucellus except near the micropyle. The integument forms a canopy around pollen chamber and divided into 9 loculi with a vascular strand. This structure is believed to contain water and supply it to the seed. The integument is differentiated into outer fleshy layer sarcotesta, middle stony sclerotesta and inner fleshy endotesta.

4. The nucellus is elongated with a pollen chamber (Lagenostome) at apical end. Nucellus is not vascularised.

5. Pollen chamber is narrow and conical. A central column arises from the base of pollen chamber, leaving a narrow space for the reception of pollen grains. It may be possible that this pollen chamber is formed by the disintegration of nucellar tissue around the central column.

6. *Lagenostoma ovoides* having many archegonia in female gametophyte.

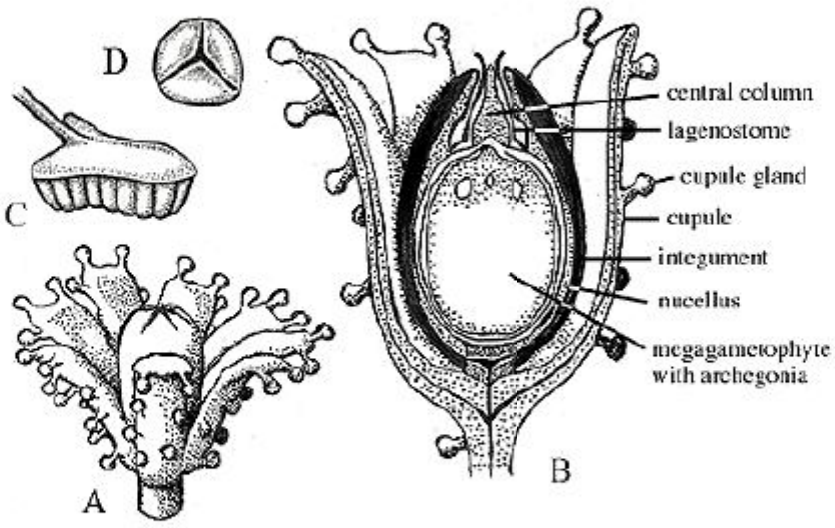

Fig. 10.3 : A. The Seed of *L. oldhamia* covered by Cupule, B. V.S. Ovule*(Lygenostoma lomaxi)* , C. Microsporangia (*Crossotheca)*, D. Microspore

(b) Male fructification

1. Microsporangia have been described under the form genus *Crossotheca,* discovered by Kidston (1905). They have been found in form of impressions with the frond genus Sphenopteris of *Lyginopteris oldhamia*. The microsporogia borne on the ultimate branches of frond.

2. The fertile fronds look like a minute brushes. The pinnules became flattened and microsporangia borne as elongate, pendent appandages.

3. Each microsporangium is bilocular, lack annulus and about 3 mm long. Microspores were 50-70 μ in diameter.

4. Pollen grains spherical with trilete structure on proximal end. Its exine is homogeneous with 2 distinct layers. *Crossotheca* pollen shows poor ornamentation on sexine.

10.3 *Medullosa*

According to Stewart (1983) this group was prevalent during lower carboniferous and continued up to the Permian. Petrified fossils were found in coal balls.

(i) External features :

1. It was 3.5-4.5 m tall plant look like tree fern. Stem was covered with leaf bases. Stewart & Delevoryas (1956) reconstructed *M. noei*.

2. Adventitious roots develop from the lower portion of stem.
3. Leaves are pinnately compound, arranged spirally in form of crown. The fronds are large and brached .

NOTES

Fig. 10.4 : *Medullosa noei*

(ii) Anatomy

(a) Root

1. It is triarch in *M. anglica* and tetrarch in *M. noei.*
2. The root showed secondary wood.
3. Rays were broad which develop wedge shaped segments against each protoxylem strand.

(b) Stem

1. Stem has more than one vascular bundle. Stem and its vascular bundles grew in size with secondary growth. Simultaneously, parenchymatous tissue in primary bundles, rays in secondary xylem and cortex also increased in size by division.
2. Basinger *et. al. (*1974) believed that stem in monostelic and from the dissection of one protostele many steles have evolved. The individual vascular bundle is a vascular segment and not a stele. All these segment form a eustele.
3. In *Medullosa noei* 5-6 sympodia (axial bundles with 5-6 protoxylem points) for 2 or 3 vascular segments may present and in *M. primaeva* 4-5 vascular segments and 13 sympodia may be present.
4. Transverse section of stem is triangular with 3- many vascular segments and many wide rays. *M. Noei* and *m. anglica* show 2-3 vascular segments. Each one is mixed protostele, surrounded by manoxylic secondary xylem and parenchymatous ground tissue with secretory cells.

5. Cortex is divided into outerand inner zone by layer of internal periderm. Many leaf trace strands, sclerenchymatous strands and accessory strands are present in outer cortex.
6. Leaf traces are concentric with one or more protoxylem points. These protoxylem were surrounded by secondary xylem. As it traversed through cortex, divided dichotomously and lost secondary xylem.
7. Its petiole was earlier mistaken for monocot stem. Number of vascular strands could vary from 2-20 or more. 2 or 3 vascular segments tangentially expand or proliferate to give rise many smaller segments. In *M. stellata* vascular tissue are seen in form of a complete ring, which surrounds ground tissue in which are embedded many protostelic vascular segments with their own secondary xylem.

(c) Leaf

1. Leaf petiole *Myeloxylon* is round or oval with a diameter of few millimeters in upper region and few centimeters at the base.
2. Transverse section of petiole shows outer epidermis, ground tissue, scattered vascular segments, strands of radially elongated sclerenchymatous cells and gum canals.
3. The petiole (*Myeloxylon lanatum*) of *Medullosa noei* has irregular sclerenchymatous strands. Vascular bundles are arranged in ring followed by inner ring of gum canals.
4. Each vascular bundle shows compact xylem with scalariform or spirally thickened tracheids, phloem, parenchyma between xylem and phloem and a sclerenchymatous bundle sheath.

(iii) Reproductive Structures

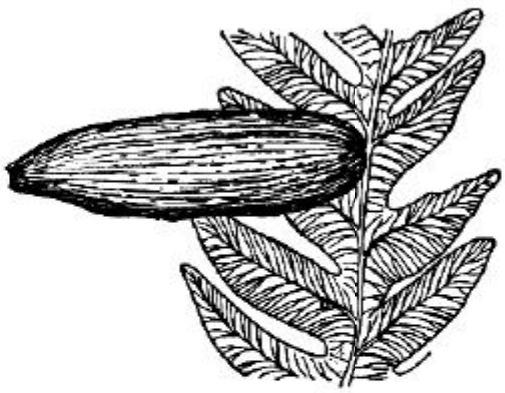

Fig. 10.5 : *Alethopteris* bearing *Pachytesta* type Ovule

NOTES

(a) Female fructification

1. The connection between seed forms and stem forms was not found.

2. Fronds have bifurcated rachis and petiole is large with many traces.

3. The seeds of Medullosa are named as form genus *Trigonocarpales*.

(b) Male fructifications

1. Pollen bearing organs have been rarely found with stem. The pollen organs of *Medullosa* show fusion of the adjacent microsporangia to give rise a synangium. They are found in form of impressions. It is represented by many form genera- *Whittleseya, Goldenbergia, Dolerotheca, Codonotheca, Aulacotheca, Parasporotheca, Boulaya fertilis.*

2. Many sporangia fused side by side with a hollow cavity in centre to form a cup shaped structure. Due to compression it looks like a leaf with parallel venation. Each sporangia gives the appearance of a vein.

*3. Dolerotheca i*s largest pollen organ of Medullosaceae. There are 4 independent radial synangia, which are fused to form a compound structure. Each radial synangia is homologous to a single *Whittleseya* type of structure. It is hemispherical, 4 cm in diameter with eccentrical pedicel in *D. formosa*.

4. A vascular trace is supplied to each microsporangium.

5. Microsporangia dehisce longitudinally and dehiscing surfaces of opposite microsporangia face one another.

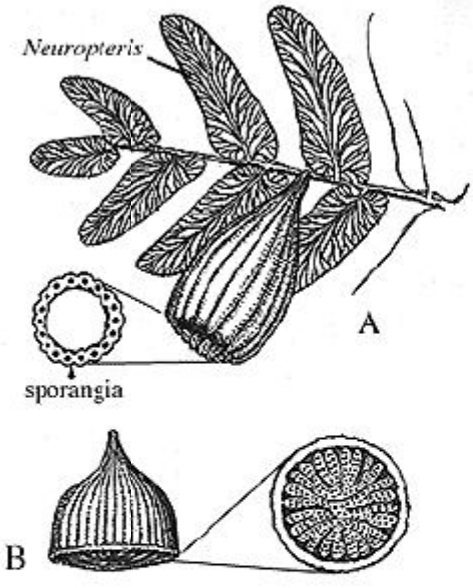

Fig. 10.6 : A. *Whittleseya media* Synangium attached to *Neuropteris,*

B. *Dolerotheca* showing hundreds of tubular Sporangia.

10.4 *Caytonia*

This group was discovered by Thomas in 1925 from Middle Jurassic rocks of Cayton Bay in Yorkshire coast of England.

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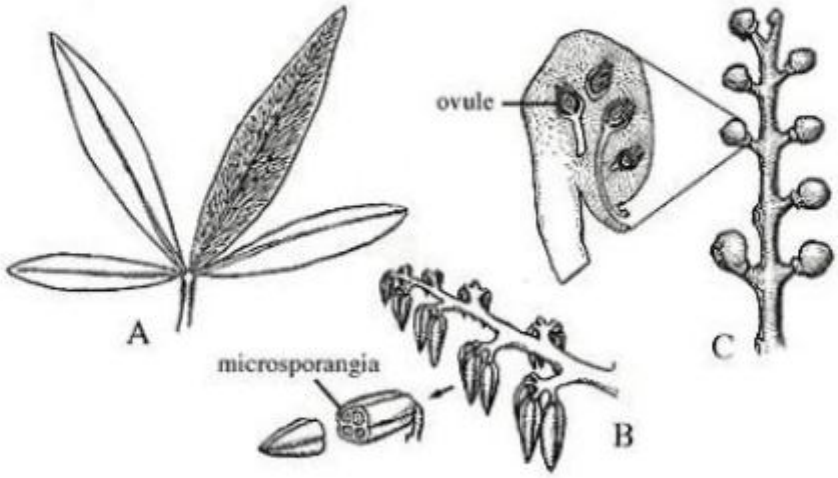

Fig. 10.7 : A. *Sagenopteris phillipsii*, B. Microsporophyll with anther like Synangia, C. Megasporophyll and L.S. Cupule to show arrangement of ovules

(i) External features

1. The leaf *Sagenopteris* is palmately compound.
2. Leaflets are 3-6, lanceolate, 2-6 cm in length with prominent midrib and reticulate venation. The veins end freely at the margin.

(ii) Anatomy

1. Leaf anatomy shows palisade tissue and elongated transfusion tissue.
2. Stomata are present only on lower side and they are haplocheilic.
3. Leaf shed after the formation of abscission layer like dicotyledons.

(iii) Reproductive Structures

(a) Female fructification

1. Female fructification was named as *Caytonia* first described by Thomas in 1925.
2. Megasporophyll is about 5 cm in length.
3. The seeds are enclosed within fruit like structures or cupule. It is small sac like structure with an outgrowth near the stalk called lip. There is a minute opening or mouth between lip and stalk. The mouth is always found to be closed. The mouth and lip of a mature fruit show transversely arranged bars equal to the number of seeds arranged in a single or double arched row. The number of seeds varies in different species.

(b) Male Fructification

NOTES

1. Pollen bearing structures were described by Phillips (1829) from Yorkshire. These were described as leaves.
2. The form genus of microsporangiate fructification is *Caytonanthus*. It was a modified pinnate microsporophyll (Harris, 1964).
3. The central axis is dorsiventral. The pinnae are opposite and branched irregularly. Each branchlet bears a single terminal, tubular pendent and tetralocular synangium.
4. The 4 sporangia or pollen sacs separate out except the tip after dehiscence.

10.5 *Glossopteris*

Glossopteris flora was flourished in Gondwana Island.

(i) External features

1. The Glossopteris is form genus of leaf. The *Glossopteris* plant was reconstructed by Pant (1977) and Gould & Delevoryas (1977). Evidences suggest that it was a large tree, may attain a height of 6 meter.
2. The trunk *Dadoxylon* was supported on root system called *Vertebraria*. Schopf (1965) and Gould (1975) confirmed it to be a root system.
3. The leaves found in Upper Carboniferous- Lower Permian era have also been reported in Jurassic deposits of Mexico (Delevoryas, 1969).
4. Leaves were arranged spirally or in whorls on the stem (Pant, 1977). They were sessile, rarely petiolate (e.g. *G. petiolata*), lanceolate, small or large up to 30 cm, acute or obtuse with prominent midrib and reticulate venation.
5. Scale leaves have been found associated with *Glossopteris*. They help in protection of vegetative buds. They are shed during the growing season. They are rounded or lanceolate with broad truncate or cordate base, indistinct midrib, reticulate venation and distinct cuticle.

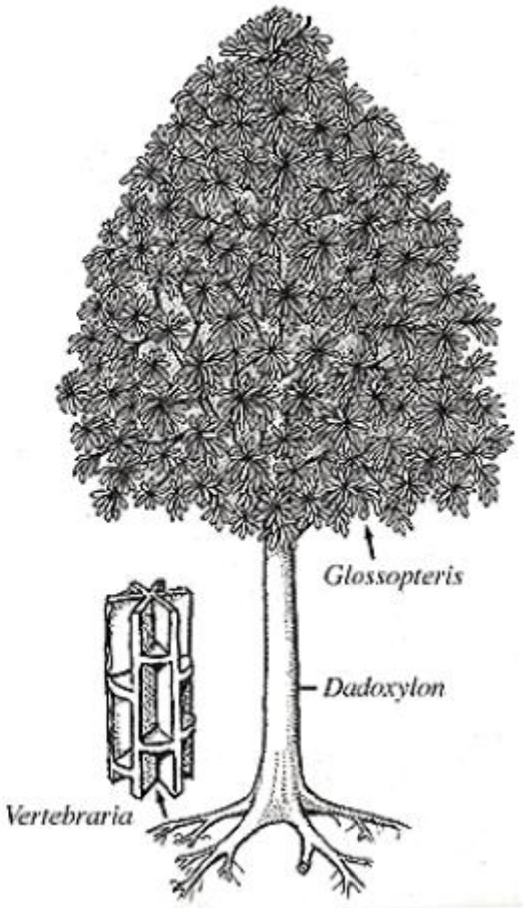

Fig. 10.8 : *Glossopteris* plant

(ii) Anatomy

(a) Root

1. Transverse section of root shows solid polyarch protostele.
2. It was surrounded by wedge like sectors of secondary xylem.
3. The Secondary xylem alternate with protoxylem.

(b) Leaf

1. The leaves are dorsiventral and hypostomatic.
2. The stomata are irregularly dispersed between the veins, haplocheilic and sunken. Their cells have sinuous or straight walls.
3. In some species hypodermis is present .Mesophyll shows palisade and spongy parenchyma.
4. The midrib shows many longitudinally running veins. The tracheids of midrib and the subsidiary veins show scalariform thickening, a few show bordered pits.

(iii) Reproductive Structures

Accodring to surange and Chandra (1976) the plants were dioecious. Pant and Singh (1974) have described an axillary fructification.

(b) Male Fructifications

1. They are named as *Eretmonia, Glossotheca* and *Nesowelesia*.

2. Their sporangia are *Arberiella.* It is foliate structure.

NOTES

3. *Eretmonia* consists of a stalked and triangular lamina. Two branches are borne on lamina and each bears whorls of microsporangia. Each microsporangium ruptures longitudinally. Pollen grains are striated and bisaccate.

4. Glossotheca has more sporangia bearing pedicels. In *Arberiella* the stalk of fertile leaf may bear 3 or more pedicels. Each bifurcates into two branches. Each branch divides by repeated dichotomy and ultimate branches bear micro sporangia.

(a) Female Fructification

1. Ovule bearing structures of *Glossopteris* is foliar.

2. There are evidences to show that *Glossopteris* belong to Gymnosperm. These are- the presence of gymnospermous wood in *Vertebraria*, the axes to which leaves have been found attached, the cuticle, stomata and xylem in the leaves and seeds found attached to leaves.

3. The seeds contained bisaccate pollen similar to those seen in pollen sacs of glossopterid microsporangiate organs.

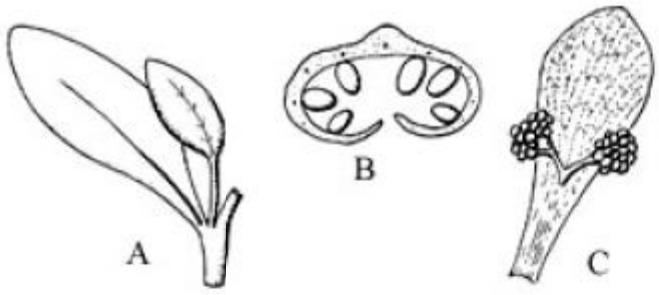

Fig. 10.9 : A. Ovuliferous Capitulum and subtending leaf in *Glosspteris* , B. T.S. ovuliferous Capitulum showing position of ovules, C. Fertile leaf of Eretmonia bearing microsporangia in clusters

10.6 *Cycadeoidea*

The only genus of the family is *Cycadeoidea* distributed from upper Jurassic to upper Cretaceous of Mesozoic era. The family was constituted by Sporne, represented by about thirty species of *Cycadeoidea.*

(i) External features

1. It has a short stout branched or unbranched, conical to irregular trunk. The trunks are massive about 1 meter in length and 50 cm in diameter except *C. jenneyana* which attained a height of several meters.
2. The trunk bears a crown of pinnately compound leaves. Its surface is covered with rhomboidal leaf bases with multicellular hairs in between.

NOTES

Fig. 10.10 : *Cycadeoidea* sp. showing External Features

(ii) Anatomy

(a) Stem

1. A cross section of stem shows a rough circular outline with leaf bases and hair like ramenta.
2. Large parenchymatous cortex is traversed by leaf traces and mucilage canals.
3. Vascular bundles are conjoint, collateral, endarch, open arranged in a ring. Secondary xylem encircles primary xylem. Pith is wide, parenchymatous, encircled by vascular tissue.
4. Secondary xylem consists of tracheids with scalariform and circular bordered pits. Secondary medullary rays traverse the secondary xylem and secondary phloem. They are uni- to biseriate. It shows that wood is manoxylic.
5. The leaf trace is c-shaped at the time of its origin and enter the leaf circling around the cortex, i.e. like cycads there is no girdling traces.

(b) Leaf

1. Transverse section of pinnule shows upper and lower epidermis. Epidermal cells are thick walled.

2. The mesophyll is differentiated into palisade and spongy parenchyma.
3. Each vascular bundle is surrounded by bundle sheath.

NOTES

(iii) Reproduction

1. Fructifications (flowers) are bisexual in most of the species and plant flowered only once in its life time (monocarpic).
2. A bisexual flower of *Cycadeoidea* is short, pedicellate, 5-10 cm in diameter and 6 cm long at maturity.
3. About 150 hairy bracts protect the microsporophyll and megasporophyll. These bracts are spirally arranged. The receptacle is conical as in *C. decotensis* and slightly convex in some other species.
4. Androecium: It consists of 19-20 unipinnate appendages or microsporophylls, arranged in whorls . They are united at base to form a disc.
5. According to Wieland (1906, 1916) each microsporophyll consists of a central rachis and many pinnae. Each pinna bears two rows of kidney shaped, stalked synangia, arranged in two rows on the inner surface of each pinnule.

10.7 *Williamsoniaceae*

The family is constituted by Sporne (1965) includes the genus *Williamsonia* (the only completely reconstructed genus). Williamson (1870) discovered it from Jurassic of Yorkshire and named as *Zamia gigas*. In India five species – *W. sewardiania, W. indica, W. sahni, W. microps and W. santalensis* have been discovered from the Rajmahal hills of Bihar and other from different regions of World.

(i) External features

1. In India Late Prof. Birbal Sahni (1932) reconstructed *W. sewardiana* of Jurassic period, collected from Rajmahal Hills of Bihar. It was the first reconstruction of a fossil plant from India.
2. Stem consists of slender, branched and columnar stem *(Bucklandia indica*) about 2 m tall with spirally arranged rhomboidal leaf scars and a crown of unipinnately compound leaves (*Ptilophyllum cutchense*).
3. Two types of lateral shoots can be recognized (i) vegetative shoots and (ii) the fertile shoots which terminate in a flower. A prominent constriction is present at the base of lateral shoot. It is believed that they served as a means of vegetative propagation after detachment.
4. The leaves are of two types :

(i) The foliage leaves which are unipinnately compound and ;

(ii) Small scale like pointed leaves.

The leaves belong to the type genera *Pterophyllum, Ptilophyllum*, *Dictyozamites* and *Otozamites*. Long pinnae have parallel venation rarely reticulate and attached to rachis by means of broad base.

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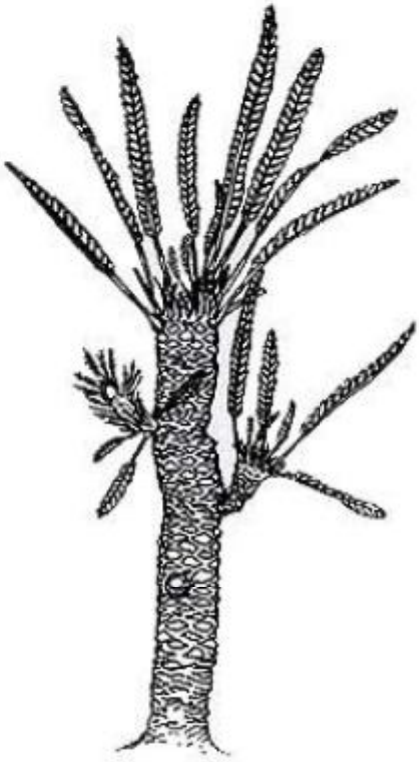

Fig. 10.11 : *W. sewardiania*

(ii) Anatomy

1. A cross section of stem shows a rough circular outline, wide parenchymatous cortex and narrow parenchymatous pith.
2. Both cortex and pith possesse secretory sacs.
3. Vascular bundles are endarch, conjoint, collateral open and arranged in ring.
4. Secondary wood is pycnoxylic, i.e. compact and consisted of scalariform bordered pitted tracheids.
5. Radial walls also have multiseriated bordered pits. Secondary medullary rays are uni-,bi- or triseriate.

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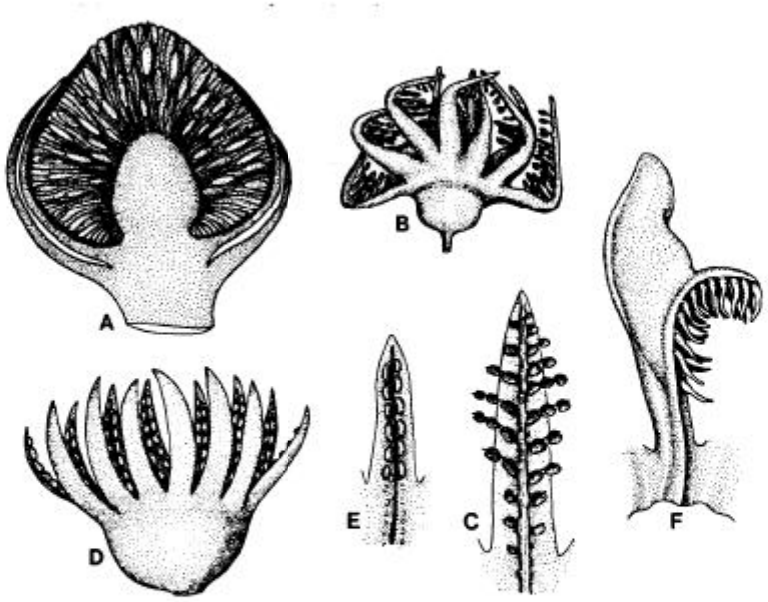

Fig. 10.12 : A. *Williamsonia harrisiana* L.S. of cone, B. *Weltrichia spectabilis* fructification C. *Weltrichia spectabilis* microsporophyll showing attached synangia D. *W. whitibiensis* fructification, E. *W. whitibiensis* microsporophyll with synangia, F. *W. santalensis* microsporophyll bearing two rows of synangia

(iii) Reproductive Structures

(a) Male fructification

1. All species of *Williamsonia* consist of a whorl of microsporophylls, united at the base to form a cup like structure. They are branched. The male fructification is referred to the genus *Weltrichia.*
2. In *W. campanulatiformis* the bell shaped receptacle have broken microsporophylls.

(b) Female Fructification:

1. The lateral fertile branch of *Williamsonia* has been shown to terminate in a female flower. These branches arise from the axial of a leaf and bear scale and foliage leaves both.
2. The female flower of *W. sewardiana* and *W. gigas* have a conical receptacle surrounded by perianth like bracts with scales at their base and hairy ramenta above. The interseminal scales and stalked ovules were arranged spirally around the receptacle. The tip of receptacle is naked.

3. Ovule is orthotropus, unitegmic and stalked. The integument is fused with nucellus and was vascularised except at the tip, where it is prolonged beyond the surface layer, fromed by the enlarged tips of interseminal scales. The nucellus extands to form a nucellar beak and pollen chamber. The interseminal scales are fleshy with expanded tips. They form a protective covering with small openings through which micropyles project. As the ovule mature, the micropylar canal becomes wide. Female gametophyte consists of thin walled cells. Cellular endosperm and dicotyledonous embryo are reported from some nucellar cavities.

NOTES

10.8 *Cordaites*

The name *Cordaites* is applied to the whole plant.

(i) External features

1. The roots are named *Ameylon*. They were branched arose from the base of stem.
2. The stem is long slender about 65 cm in diameter. The branching was lateral, confined to the top of the tree.
3. The leaf has been described under the generic name *'Cordaites'*. Leaves are spirally arranged, petiolate, simple, spathulate, subutate, entire, obtuse or acute with parellal venation. They were found in form of compressions.

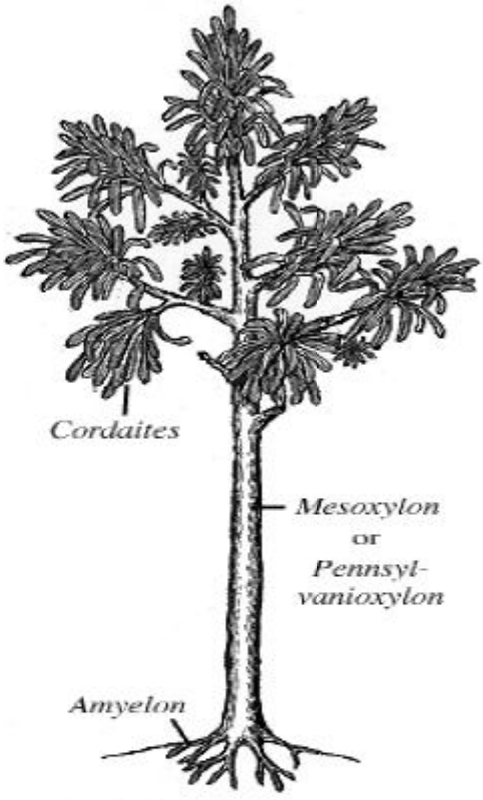

Fig. 10.13 : *Cordaites* Plant

NOTES

(ii) Anatomy

(a) Root

1. The roots *Ameylo*n are triarch, exarch and protostelic. It showed periderm, cortex, secondary wood and pith.

2. Secondary xylem formed a distinct ring from that of primary xylem. The inner cortex had some cells infected with mycorrhiza.

(b) Stem

1. It had a large parenchymatous cortex with patches of sclerenchyma and secretory sacs. Large air chamber is present in pith. They are separated with diaphragms.

2. The transverse section of stem shows 2-3 large and many small cavities as the petrified material reveals the diaphragms to be incomplete. A narrow zone of primary xylem is present around the pith. Large number of endarch protoxylem is present.

3. Vascular bundles were open and sclerenchymatous patches in pith region were present.

4. Secondary wood is compact or pycnoxylic.

5. Tracheids were narrow and long. The borderd pits were hexagonal,restricted to radial walls.

6. They occurred usually in uniseriate rows and some scaleriform pitting in metaxylem.

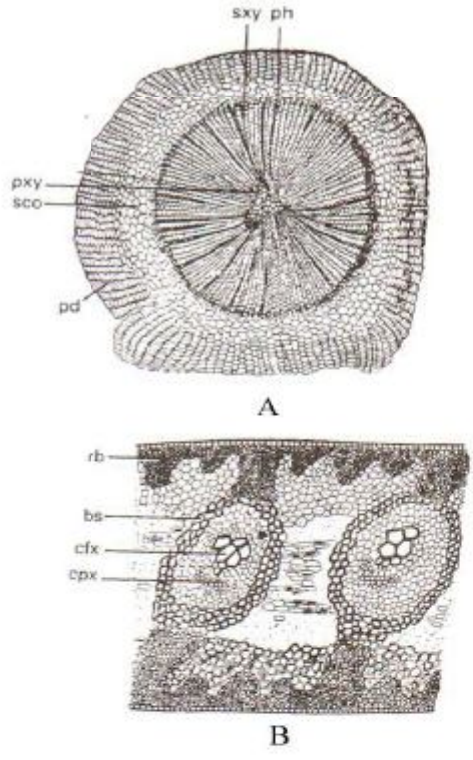

Fig. 10.14 : (A) T.S. Root *Amyelon radicans* (pd, periderm; sco, secondary cortex, pxy protoxylem; sxy; secondary xylem; ph phloem), (B) T.S. Leaf of *Cordaites angulostriatus* (rb, rib; bs bundle sheath; cfx, centrifugal xylem; cpx, centripetal xylem)

(b) Leaf

1. Leaves show xerophytic internal structure. Vertical section of leaf shows distinct upper and lower epidermis. The cells are thick walled covered with thick cuticle.
2. The stomata in most species have been seen on lower epidermis only. They are arranged in longitudinal bands in the furrows by the parallel viens, stomata were haplocheilic. The hypodermis is sclerenchymatous.
3. In *C. lingulatus* the mesophyll tissue shows clear distinction into palisade and spongy parenchyma. Palisade layer is interrupted by sclerenchymatous bundle sheath extensions.
4. The Vascular bundles are mesarch and surrounded by sclerenchymatous bundle sheath. The phloem is located below the xylem.

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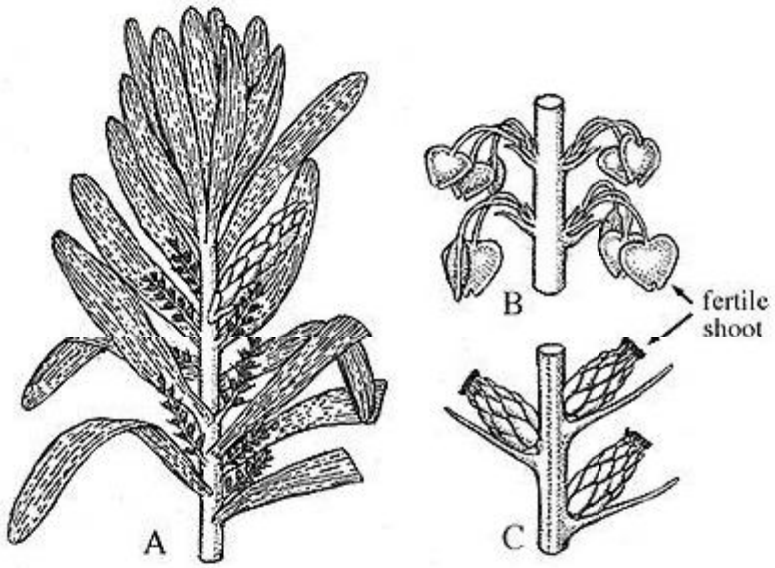

Fig. 10.15 : A. *Cordaites* branch showing leaves and fertile shoots, B. primary shoot of *Cardiocarpus* bearing ovulate secondary shoot with pendent ovules, C. *Cordaianthus concinnus* microsporangiate short shoot

(iii) Reproductive Structures

1. Both male and female fructifications of *Cordaites* have been described under form genus *'Cordaianthus'*.
2. They are compound structure. It consists of a primary axis with spirally arranged bracts. Each bract had an axillary fertile dwarf shoot of about 6 mm. long which consists of a central axis with spirally arranged sporophylls. The compound strobili were unisexual.

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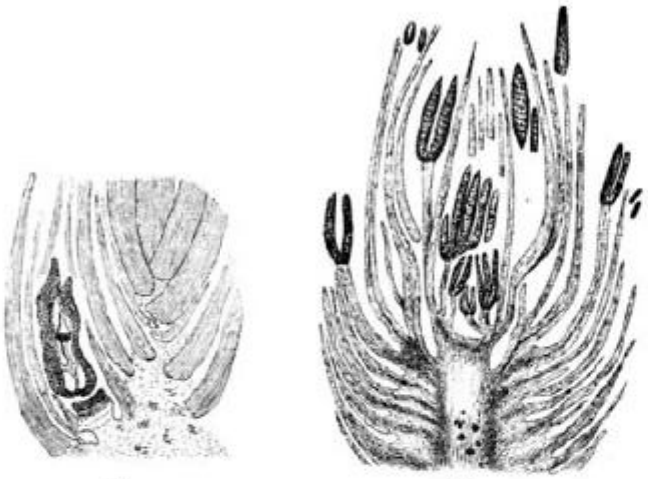

Fig. 10.16 : (A) *Cordaianthus* L.S. Female Strobilus;

(B) L.S. Male Strobilus

(a) Male Fractification

1. Microsporangiate dwarf shoot had a central axis with spirally arranged microsporophylls. 5-10 fertile microsporophylls were present at the distal end of dwarf shoot.
2. They possessed 4-6 microsporangia or pollen sacs. Microsporangia are elongated finger like structure. Each microsporangium is vascularized. Vascular bundles are mesarch. Only one trace which enters the secondary shoot divides into many branches. Each branch enters one microsporophyll.
3. The vascular strands divide dichotomously. Wall of microsporangia is single layered.
4. Microspores were 65 μ to 150 μ in diameter. The microspore had an air cavity enclosed by exine. The intine enclosed a central cavity. The nexine is thick and lamellate and sexine is thin, irregular. Some of the pollen grains were observed in pollen chamber of the ovule. Presence of 6 prothallial cells and 4 spermatogenous cells have been reported.
5. Pollen grains were monolete to trilete, monosaccate with saccus attached on both proximal and distal poles.

(b) Female Fructification

1. Dwarf shoots arise on the main axis in the axil of the bract. Each dwarf shoot consists of a central axis with spirally arranged sporophylls.

2. Out of 16-20 bracts, 4-6 distal bracts are fertile.

3. The fertile bract bear one-three terminal pendulous, bilaterally symmetrical, flattened heart shaped ovules. In *C. zeilleri* and *C. williamsonii* there is only one ovule.

4. Mitrospermum and Cardiocarpus are the 2 form genera of ovules The distal fertile sporophylls are greatly elongated in *C. pseudofluitans.*

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5. The outer fleshy sarcotesta froms a wing like extension. It composed of large thin walled cells in outer region and small cells in the inner region. The sclerotesta has spine like projection into sarcostesta. Sclerotesta is hard stony layer.

6. The nucellus is free from integument except at the base. The distal portion of nucellus forms a beak and small pollen chamber.

10.9 Viva-Voce

(1) What do you mean by Cupule?

(2) What is *'Cordaianthus'*?

(3) Name any 2 fossil gymnosperms.

(4) Describe male reproductive structures of *Medullosa*.

(5) Briefly explain about *Glossopteris*.

(6) Highlight the contribution of Prof. Birbal Sahani.

(7) Explain characteristic features of *Wlliamsonia.*

(8) Draw a labelled drawing of Cupule of Lyginopteris.

(9) Write 10 comments on fossil plants.

(10) Describe external morphology of Cycadeoidea.

10.10 References

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Unit-11

Cell Biology

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Structure of the Unit

11.0 Objectives

After going through this unit you will be able to understand:

- Structure of a plant cell, some specialized plant cells and cell organelles
- Isolation of mitochondria and other organelles
- Isolation and estimation of DNA and running it on agarose gel electrophoresis
- Isolation of various proteins
- Cell Division

11.1 Study of Plant Cell and Cell Organelles

Each cell is a community of subcellular components. Each type of component has its own particular set of functions. The individual parts could not survive for long outside the cell, but within the cellular environment they support each other so effectively that the cell as a whole is a viable entity. This subcellular cooperation not only ensures survival, but also provides for growth and multiplication of the cell (if given the necessary nutrients) and ultimately differentiation for a particular function. Looking at a higher level of organization, multicellular organisms are cooperative communities of cells, tissues and

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organs, all analogous to the subcellular components in that each contributes in a specialized way to the life of the system of which it is a part. No matter how complex the system, however, it is the cell that is the simplest, indivisible, unit which is viable hence the common statement that the cell is the unit of life.

All living systems have the structural and functional similarities of plant and animal cells. There are, in addition, cellular features in which the two kingdoms differ, mostly deriving from two major events in the evolution of living organisms - the development of a cell wall and the acquisition of photosynthetic capabilities.

Structurally, plant and animal cells are very similar because they are both eukaryotic cells. They both contain membrane-bound organelles such as the nucleus, mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, and peroxisomes. Both also contain similar membranes, cytosol, and cytoskeletal elements. The functions of these organelles are extremely similar between the two classes of cells (peroxisomes perform additional complex functions in plant cells having to do with cellular respiration). However, the few differences that exist between plant and animals are very significant and reflect a difference in the functions of each cell. Plant cells can be larger than animal cells. The normal range for an animal cell varies from 10 to 30 micrometers while that for a plant cell stretches from 10 to 100 micrometers. Beyond size, the main structural differences between plant and animal cells lie in a few additional structures found in plant cells. These structures include: chloroplasts, the cell wall, and vacuoles.

Plant cell

Plant cells vary in the extent to which different functions are developed, for, as with most multi-cellular organisms, plants exhibit division of labour. As a result of the varied requirements of maintaining life and supporting growth and development, specialized cells develop for protection, mechanical support, synthesis or storage of food reserves, transport, absorption and secretion, meristematic activity, reproduction, and the vital role of interconnecting the more specialized tissues.

Various plant cell organelles

Plant tissues are composed of the non-living extracellular region and the living protoplasm of the cells proper. The former consists of intercellular spaces and cell walls. Each protoplast consists of a nucleus (or sometimes several nuclei) and the cytoplasm. Within these are the various membranous and non-membranous components which are:

1. Plasma membrane

The bounding membrane of the protoplast normally is in close contact with the inner face of the cell wall.

2. Cytoplasm

A collective term for everything outside the nucleus, out to and including the plasma membrane. Includes membranous and other inclusions, and also the general matrix, or cytosol, in which the cytoplasmic components reside.

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3. Nucleus

This is bounded by the nuclear envelope and contains genetic material in the form of chromatin, and the nucleolus (or, if more than one, nucleoli in a matrix of nucleoplasm. The cell nucleus is one of the largest organelles found in cells and also plays an importantbiological role. It composes about 10% of the total volume of the cell and is found near thecenter of eukaryotic cells. Its importance lies in its function as a storage site for DNA, ourgenetic material. The cell nucleus is composed of two membranes that form a porousnuclear envelope, which allows only select molecules in and out of the cell.The DNA that is found in the cell nucleus is packaged into structures called chromosomes.Chromosomes contain DNA and proteins and carry all the genetic information of anorganism. The nucleus gains support from intermediate filaments that both form thesurrounding nuclear lamina and makes direct contact with the endoplasmic reticulum.The nucleus is also the site of DNA and RNA synthesis.

4. Nucleoplasm

Everything enclosed by the nuclear envelope falls in the category of nucleoplasm, just as objects outside it are constituents of the cytoplasm. The word is often, however, used to denote the ground substance in which the chromatin and nucleolus lie.

5. Chromatin

Chromatin contains the genetic material of the cell, i.e. information in the form of DNA that is passed from parent cell to daughter cell during the multiplication of cells and reproduction of the organism. It can exist in less dense (euchromatin) and more dense (heterochromatin) forms. During division of nuclei it is condensed into discrete units, chromosomes.

6. Nucleolus

A mass of filaments and particles, largely a sequence of identical repeating units of specialized genetic material together with precursors of ribosomes produced from that genetic information.

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7. Nuclear envelope

A cisterna (a general term meaning a membrane-bound sac) wrapped around the contents of the nucleus. The space between the two membranous faces of the cisterna is the peri-nuclear space.

8. Nuclear envelope pores

Elaborate perforations in the nuclear envelope, involved in transport between nucleus and cytoplasm and in the processing of messenger and ribosomal RNA molecules that are being exported from the nucleus.

9. Endoplasmic reticulum

Membranous cisternae that ramify through the cytoplasm, occasionally connected to the outer membrane of the nuclear envelope. The bounding membrane segregates the contents of the cisterna from the cytoplasm. The outer face frequently bears attached ribosomes and polyribosomes. Endoplasmic reticulum is described as rough, or granular, and forms that lack ribosomes as smooth, or agranular. A special form that lies just inside the plasma membrane is called cortical ER. ER cisternae may or may not have visible contents, which distend the cisternae when present in bulk.

The endoplasmic reticulum, or ER, is a very important cellular structure because of its function in protein synthesis and lipid synthesis. For example, the ER is the site of production of all transmembrane proteins. Since nearly all proteins that are secreted from a cell pass through it, the ER is also important in cellular trafficking. In addition to these major roles, the ER plays a role in a number of other biological processes. There are two different types of ER: smooth ER and rough ER. The rough ER has its name because it is coated with ribosomes, the structures most directly responsible for carrying out protein synthesis. Smooth ER lacks these ribosomes and is more abundant in cells that are specific for lipid synthesis and metabolism.

In addition to protein and lipid synthesis, the ER also conducts post-synthesis modifications. One such modification involves the addition of carbohydrate chains to the proteins, though the function of this addition is unknown. Another major modification is called protein folding, whose name is rather self- explanatory. Another role of the ER is to capture calcium for the cell from the cytosol. Finally, the ER can secrete proteins into the cell that are usually destined for the Golgi apparatus.

10. Ribosomes

Small particles of RNA and protein lying free in the cytoplasm or else attached to the endoplasmic reticulum. They aggregate in clusters, chains, spirals, or other polyribosome configurations when they are engaged in protein synthesis.

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11. Golgi bodies

Each Golgi body (= Golgi stack) consists of layered cisternae together with many small vesicles that are involved in traffic to and from the Golgi apparatus and between its constituent cisternae. The Golgi apparatus is usually located near the cell nucleus. It is composed of a series of layers called Golgi stacks. Proteins from the ER always enter and exit the Golgi apparatus from the same location. The cis face of the Golgi is where proteins enter. A protein will make its way through the Golgi stacks to the other end called the trans face where it is secreted to other parts of the cell.

In the Golgi apparatus, more carbohydrate chains are added to the protein while other chains are removed. The Golgi stacks also sort proteins for secretion. After sorting, the membrane of the Golgi buds off, forming secretory vesicles that transport proteins to their specific destination in the cell. A protein's destination is often signaled with a specific amino acid sequence at its end. A protein secretion most often travels back to the ER or to the plasma membrane or to the lysosomes.

12. Vacuole

Compared with the surrounding cytoplasm, these are usually empty looking spaces, spherical when small. They are often very large, and can occupy 90% or more of the volume of the cell in mature tissues. Vacuoles are large, liquid-filled organelles found only in plant cells. Vacuoles can occupy up to 90% of a cell's volume and have a single membrane. Their main function is as a space-filler in the cell, but they can also fill digestive functions similar to lysosomes (which are also present in plant cells). Vacuoles contain a number of enzymes that perform diverse functions, and their interiors can be used as storage for nutrients or provide a place to degrade unwanted substances.

13. Mitochondria

These pleiomorphic bodies consist of a compartment, the matrix, surrounded by two membrane barriers, a double envelope. The outer membrane of the double envelope is more or less smooth, but the inner is thrown into many folds - mitochondrial cristae - that project into the matrix.

14. Tonoplast

Is the membrane which bounds a vacuole. Except for its position in the cell it looks very like the plasma membrane.

15. Plastids

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This is a group name for a whole family of cell components. In the young root-tip cells of the first three plates this group is represented by the structurally simplest member, which is called the proplastid. Proplastids are usually larger than mitochondria, but, like them, have a double membrane envelope surrounding (in these examples) a fairly dense ground substance, the stroma. Starch grains may be present in them. Other members of the plastid family are: chloroplasts, etioplasts, amyloplasts, and chromoplasts.

16. Chloroplast

In animal cells, the mitochondria produce the majority of the cells energy from food. It does not have the same function in plant cells. Plant cells use sunlight as their energy source; the sunlight must be converted into energy inside the cell in a process called photosynthesis. Chloroplasts are the structures that perform this function. They are rather large, double membrane bound structures (about 5 micrometers across) that contain the substance chlorophyll, which absorbs sunlight. Additional membranes within the chloroplast contain the structures that actually carry out photosynthesis.

Chloroplasts carry out energy conversion through a complex set of reactions similar to those performed by mitochondria in animals. The double membrane structure of chloroplasts is also reminiscent of mitochondria. The inner membrane encloses an area called the stoma, which is analogous to the matrix in mitochondria and houses DNA, RNA, ribosomes, and different enzymes. Chloroplasts, however, contain a third membrane and are generally larger than mitochondria.

17. Microbodies

These are bounded by a single membrane, and are distinguished from vesicles by their size and dense contents (sometimes including a crystal).

18. The Cytoskeleton

The cytoskeleton is similar to the lipid bilayer in that it helps provide the interior structure of the cell the way the lipid bilayer provides the structure of the cell membrane. The cytoskeleton also allows the cell to adapt. Often, a cell will reorganize its intracellular components, leading to a change in its shape. The cytoskeleton is responsible for mediating these changes. By providing "tracks" with its protein filaments, the cytoskeleton allows organelles to move around within the cell. In addition to facilitating intracellular organelle movement, by moving itself the cytoskeleton can move the entire

cells in multi-cellular organisms. In this way, the cytoskeleton is involved in intercellular communication.

The cytoskeleton is composed of three different types of protein filaments: actin, microtubules, and intermediate filaments.

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19. Peroxisomes

Peroxisomes are single-membrane structures found in all eukaryotic cells. They are small, membrane-bound structures that use molecular oxygen to oxidize organic molecules. The structure is one of the major oxygen utilizing organelles, the other being the mitochondria. Peroxisomes contain oxidative enzymes and other enzymes that help produce and degrade hydrogen peroxide. Because of their varying enzymatic compositions, peroxisomes are diverse structures. Their main function is to help breakdown fatty acids. They perform specific functions in plant cells.

20. Cell wall

This is a thin structure in meristematic cells, but it can be very massive and elaborate in mature cells. It is external to the living protoplast, but nevertheless contributes very significantly to the life of the plant cell; indeed, along with plastids, it is the major determinant of the lifestyle of plants. One of its main constituents is microfibrillar cellulose - the most abundant macromolecule on Earth.

11.2 Specialized Plant Cell Types

The major classes of cells differentiate from undifferentiated meristematic cells (analogous to the stem cells of animals) to form the tissue structures of roots, stems, leaves, flowers, and reproductive structures. All plant cells have several common features, such as chloroplasts, a cell wall, and a large vacuole. In addition, a number of specialized cells are found only in vascular plants. They include:

Meristematic cells

They give rise to all three fundamental mature cell types. Their major function is cell division, and so their cell cycle indeed cycles. The walls are thin, the vacuole is largely missing, the plastids are immature, etc.

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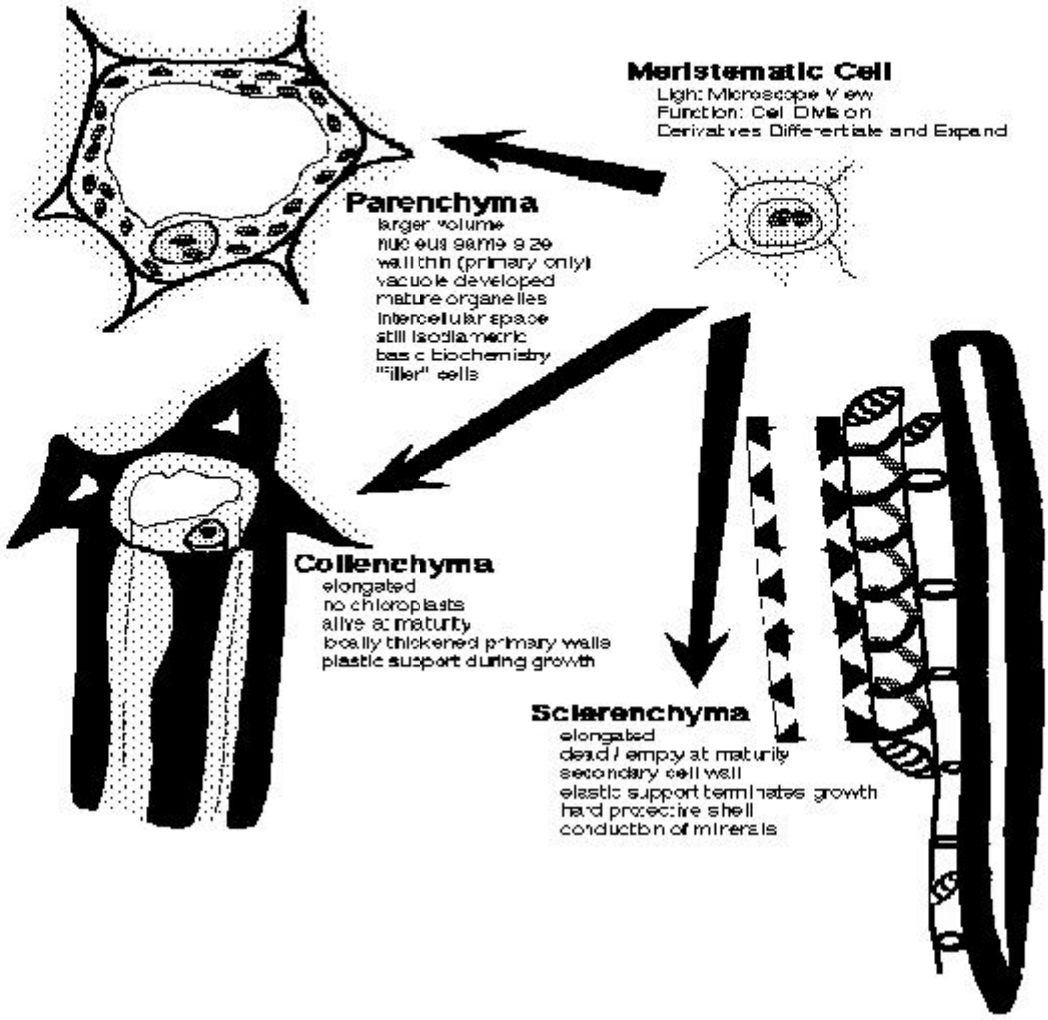

Fig. 11.1 : Specialized Plant Cell Types

Parenchyma cells

Parenchyma (from the Greek para, meaning "beside," and en + chein, meaning "to pour in") cells are the most common cells found in leaves, stems, and roots. They are often spherical in shape with only primary cell walls. Parenchyma cells play a role in food storage, photosynthesis, and aerobic respiration. They are living cells that have functions ranging from storage and support to photosynthesis and phloem loading (transfer cells). Apart from the xylem and phloem in their vascular bundles, leaves are composed mainly of parenchyma cells. Some parenchyma cells, as in the epidermis, are specialized for light penetration and focusing or regulation of gas exchange, but others are among the least specialized cells in plant tissue, and may remain totipotent, capable of dividing to produce new populations of undifferentiated cells, throughout their lives. Parenchyma cells have thin, permeable primary walls enabling the transport of small molecules between them, and their cytoplasm is responsible for a wide range of biochemical functions such as nectar secretion, or the manufacture of secondary products that discourage herbivores.

Parenchyma cells that contain many chloroplasts and are concerned primarily with photosynthesis are called chlorenchyma cells. Others, such as the majority of the parenchyma cells in potato tubers and the seed cotyledons of legumes, have a storage function. Other than support functions, this cell type is the basis for all plant function.

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Collenchyma cells

Collenchyma (from the Greek term kola, meaning "glue") cells have thickened primary cell walls and lack secondary cell walls. They form strands or continuous cylinders just below the surfaces of stems or leaf stalks. The most common function of collenchyma cells is to provide support for parts of the plant that are still growing, such as the stem. Similar to parenchyma cells, collenchyma cells are living cells at maturity and have only a primary wall. These cells mature from meristem derivatives that initially resemble parenchyma, but differences quickly become apparent. Plastids do not develop, and the secretory apparatus (ER and Golgi) proliferates to secrete additional primary wall. The wall is most commonly thickest at the corners, where three or more cells come in contact, and thinnest where only two cells come in contact, though other arrangements of the wall thickening are possible.

Pectin and hemicellulose are the dominant constituents of collenchyma cell walls of dicot angiosperms. Collenchyma cells are typically quite elongated, and may divide transversely to give a septate appearance. The role of this cell type is to support the plant in axes still growing in length, and to confer flexibility and tensile strength on tissues. The primary wall lacks lignin that would make it tough and rigid, so this cell type provides what could be called plastic support – support that can hold a young stem or petiole into the air, but in cells that can be stretched as the cells around them elongate. Stretchable support (without elastic snap-back) is a good way to describe what collenchyma does. Parts of the strings in celery are collenchyma.

Collenchyma cells are also alive at maturity and have only a primary wall. These cells mature from meristem derivatives. They pass briefly through a stage resembling parenchyma, however they are determined to differentiate into collenchyma, and this fact is quite obvious from the very earliest stages. Plastids do not develop and secretory apparatus (ER and Golgi) proliferates to assist in the accumulation of additional primary wall. This is laid down where three or more cells come in contact. Areas of wall where only two cells come in contact remain as thin as those of parenchyma cells.

Sclerenchyma cells

Sclerenchyma cells (from the Greek skleros, hard) are hard and tough cells with a function in mechanical support. They are of two broad types – sclereids or stone cells and fibers.

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The cells develop an extensive secondary cell wall that is laid down on the inside of the primary cell wall. The secondary wall is impregnated with lignin, making it hard and impermeable to water. Thus, these cells cannot survive for long' as they cannot exchange sufficient material to maintain active metabolism. Sclerenchyma cells are typically dead at functional maturity, and the cytoplasm is missing, leaving an empty central cavity.

Functions for sclereid cells (hard cells that give leaves or fruits a gritty texture) include discouraging herbivory, by damaging digestive passages in small insect larval stages, and physical protection (a solid tissue of hard sclereid cells form the pit wall in a peach and many other fruits). Functions of fibers include provision of load-bearing support and tensile strength to the leaves and stems of herbaceous plants. Sclerenchyma fibers are not involved in conduction, either of water and nutrients (as in the xylem) or of carbon compounds (as in the phloem), but it is likely that they may have evolved as modifications of xylem and phloem initials in early land plants.

These cells are hard and brittle. The cells develop an extensive secondary cell wall (laid down on the inside of the primary wall). This wall is invested with lignin, making it extremely hard. Lignin, plus suberin and/or cutin make the wall waterproof as well. Thus, these cells cannot survive for long as they cannot exchange materials well enough for active (or even maintaining) metabolism. They are typically dead at functional maturity. The cytoplasm is missing by the time the cell can begin to carry out its function.

Functions for sclerenchyma cells include discouraging herbivory (hard cells that rip open digestive passages in small insect larval stages, hard cells forming a pit wall in a peach fruit), support (the wood in a tree trunk, fibers in large herbs), and conduction (hollow cells lined end-to-end in xylem with cytoplasm and end walls missing).

Sclerenchyma includes the fibers used for making thread and fabric. Particularly the fibers from flax those are spun and woven into linen. Sclerenchyma cells provide rigid support for the plant. There are two types of sclerenchyma cells-fiber and sclereid. Fiber cells are long, slender cells that usually form strands or bundles. Sclereid cells, sometimes called stone cells, occur singly or in groups and have various forms. They have a thick, very hard secondary cell wall. Most sclerenchyma cells are dead cells at maturity.

Xylem

Xylem (from the Greek term xylos, meaning "wood") is the main water-conducting tissue of plants and consists of dead, hollow, tubular cells arranged end to end. The water transported in xylem replaces that lost via evaporation through stomata. The two types of water-conducting cells are tracheids and vessel elements. Water flows from the roots of a

plant up through the shoot via pits in the secondary walls of the tracheids. Vessel elements have perforations in their end walls to allow the water to flow between cells.

Phloem

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The two kinds of cells in the food-conducting tissue of plants, the phloem (from the Greek term phloios, meaning "bark"), are sieve cells and sieve-tube members. Sieve cells are found in seedless vascular plants and gymnosperms, while sieve-tube members are found in angiosperms. Both types of cells are elongated, slender, tube-like cells arranged end to end with clusters of pores at each cell junction. Sugars (especially sucrose), other compounds, and some mineral ions move between adjacent food-conducting cells. Sieve-tube members have thin primary cell walls but lack secondary cell walls. They are living cells at maturity.

Epidermis

Several types of specialized cells occur in the epidermis including guard cells, trichomes, and root hairs. Flattened epidermal cells, one layer thick and coated by a thick layer of cuticle, cover all parts of the primary plant body.

11.3 Isolation of some Cell Organelles

Isolation of mitochondria

Mitochondria are rod-shaped structures ranging from 2 to 8 μm in length. They are found throughout the cytoplasm and may account for up to 20% of the cell's volume. The number of mitochondria in a cell depends upon the metabolic requirements of that cell, and may range from a single large mitochondrion to thousands of the organelles. Mitochondria are considered as the "power house of the cell" because It produces Adenosine Tri Phosphate (ATP), the energy currency by extracting energy from nutrient molecules. Number of enzymes and proteins are present in the mitochondria, which helps in processing fats and carbohydrates obtained from food. ATP powers the cell's metabolic activities. This process is called aerobic respiration, which is the reason for animals breathing oxygen. The cells in the higher animals obtain energy from anaerobic respiration (in the absence of oxygen), so it does exist without mitochondria.

Isolation of mitochondria involves cell disruption and centrifugation. The process of cell disruption involves breaking open of cell so as to spill out the contents within the cell. Centrifugation is the process by which mixtures of cell components are separated by centrifugal force. The more dense particles migrate away from the axis, while less dense components of the mixture migrates towards the axis of centrifuge. The centrifugal

technique which is used to separate the cell components from whole cell is called differential centrifugation. Differential centrifugation gives only a crude extract.

Disruption of the Tissue:-

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The aim is to rupture as many of the cells in the tissue as possible in order to release the mitochondria, but without disrupting them. Whereas many mammalian tissues are easy to homogenize, plant tissues are much tougher mainly because of the cell walls. There are a number of standard homogenizers, including mixers with rotating knives or razor blades, juice extractors, ball mills, and mortar and pestle.

Homogenization Medium:-

The medium for homogenizing rat liver only contains an osmoticum and a buffer. That will not work with plant tissues because of the disruption of the large central vacuole (absent in mammalian cells), which releases its fairly acidic content often containing phenols and other potentially harmful substances.

Osmoticum is required to prevent the mitochondria from losing their matrix content because of swelling and bursting. For the isolation of plant mitochondria, there is a tradition of using sucrose or mannitol at 0.2–0.5 M; whereas 0.15 M KCl is standard for the isolation of mammalian mitochondria.

Buffer- The pH in the cytosol of plant cells is around 7.5, and the homogenate should be buffered at pH 7–8. We choose an inexpensive one with a pKa of 7–8 (e.g., 4-morpholinopropane sulfonic acid = MOPS) and be sure to use enough to counter balance the acidic vacuolar content. The tissue type, the tissue/medium ratio, and the buffer concentration determine whether the acidic vacuolar content is properly neutralized. Differential Centrifugation:-

Differential centrifugation is the most common method of fractionating cell. Fractionation is separation of different organelles within a cell. It is a classical procedure used to isolate different particles by stepwise successive centrifugations at increasing RCF's (Relative Centrifugal Forces). Centrifugation separates particles in a suspension based on differences in size, shape and density that together define their sedimentation coefficient. The tube containing the suspension of particles is rotated at a high speed, which exerts a centrifugal force directed from the center of the rotor towards the bottom of the tube. Centrifugal Force 'G' is more commonly expressed as the Relative Centrifugal Force (RCF) or g value in multiples of the earth's gravitational field 'g'.

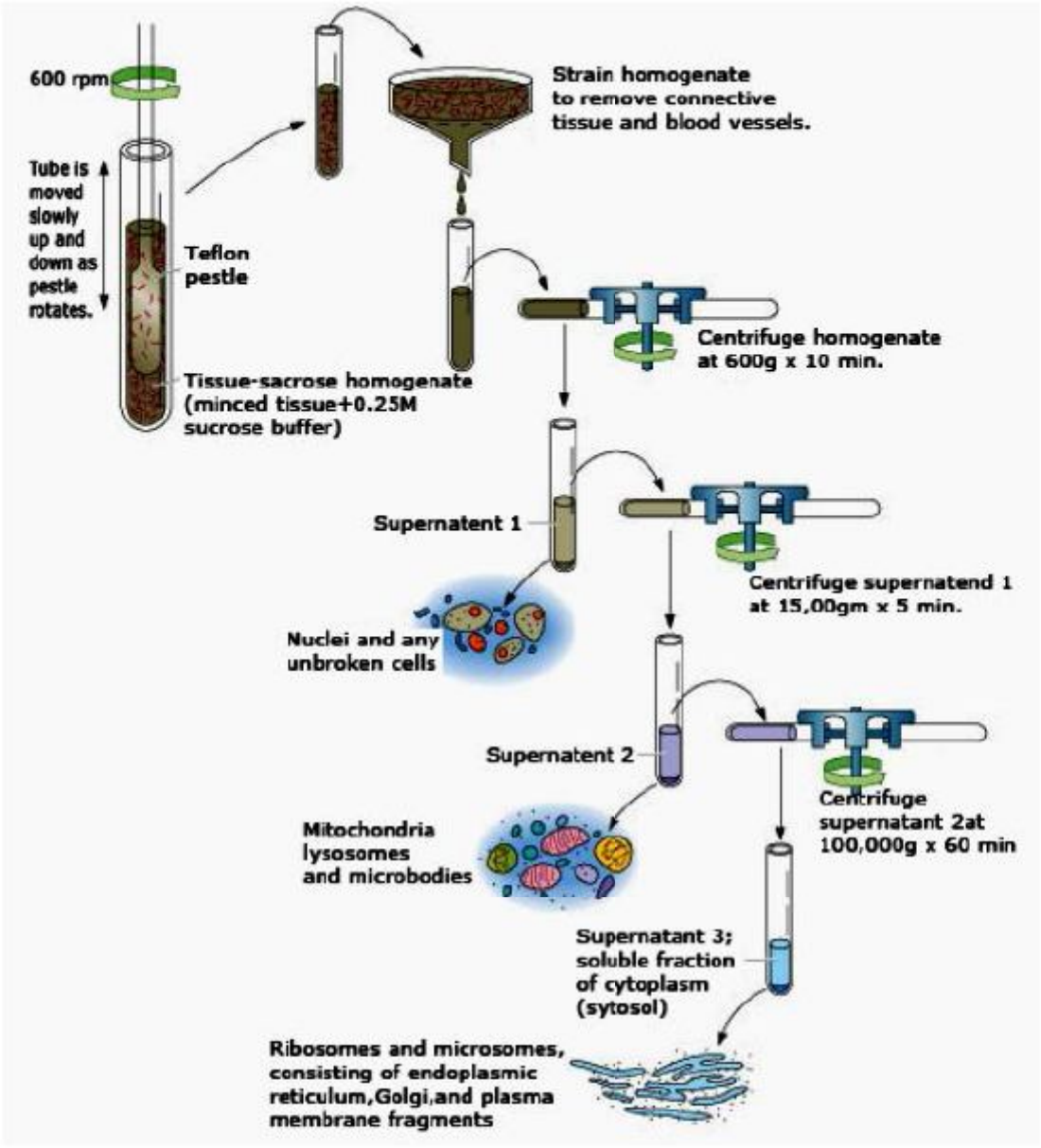

Fig. 11.2 : Isolation of Cell Organelle

11.4 Isolation of Plant DNA and Estimation by Spectrophotometer

Introduction

The search for a more efficient means of extracting DNA of both higher quality and yield has led to the development of a variety of protocols, however the fundamentals of DNA extraction remains the same. DNA must be purified from cellular material in a manner that prevents degradation. Because of this, even crude extraction procedures can still be adopted to prepare a sufficient amount of DNA to allow for multiple end uses.

DNA extraction from plant tissue can vary depending on the material used. Essentially any mechanical means of breaking down the cell wall and membranes to allow access to

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nuclear material, without its degradation is required. For this, usually an initial grinding stage with liquid nitrogen is employed to break down cell wall material and allow access to DNA while harmful cellular enzymes and chemicals remain inactivated. Once the tissue has been sufficiently ground, it can then be resuspended in a suitable buffer, such as CTAB. In order to purify DNA, insoluble particulates are removed through centrifugation while soluble proteins and other material are separated through mixing with chloroform and centrifugation. DNA must then be precipitated from the aqueous phase and washed thoroughly to remove contaminating salts. The purified DNA is then re-suspended and stored in TE buffer or sterile distilled water. This method has been shown to give intact genomic DNA from plant tissue. To check the quality of the extracted DNA, a sample is run on an agarose gel, stained with ethidium bromide, and visualised under UV light.

Materials- CTAB buffer, Microfuge tubes, Mortar and Pestle, Liquid Nitrogen, Microfuge, Absolute Ethanol (ice cold), 70 % Ethanol (ice cold),7.5 M Ammonium Acetate, 55° C water bath, Chloroform : Iso Amyl Alcohol (24:1), Water (sterile), Agarose, 6x Loading Buffer, 1x TBE solution, Agarose gel electrophoresis system, Ethidium Bromide solution.

CTAB buffer 100ml

2.0g CTAB (Hexadecyltrimethyl-ammonium bromide), 10.0 ml 1 M Tris pH 8.0 4.0 ml 0.5 M EDTA pH 8.0 (EthylenediaminetetraAcetic acid Di-sodium salt), 28.0 ml 5 M NaCl, 40.0 ml H2O, 1g PVP 40 (vinylpyrrolidinehomopolymer) Mw 40,000) Adjust all to pH 5.0 with HCL and make up to 100 ml with H2O.

1 M Tris pH 8.0

Dissolve 121.1 g of Tris base in 800 ml of H2O. Adjust pH to 8.0 by adding 42 ml of concentrated HCL. Allow the solution to cool to room temperature before making the final adjustments to the pH. Adjust the volume to 1 L with H2O. Sterilize using an autoclave.

5x TBE buffer

54 g Tris base, 27.5 g boric acid, 20 ml of 0.5M EDTA (pH 8.0) Make up to 1L with water. To make a 0.5x working solution, do a 1:10 dilution of the concentrated stock.

1% Agarose gel

1 g Agarose dissolved in 100 ml TBE

Procedure

- Grind 200 mg of plant tissue to a fine paste in approximately 500 µl of CTAB buffer.

- Transfer CTAB/plant extract mixture to a microfuge tube.

- Incubate the CTAB/plant extract mixture for about 15 min at 55°C in a recirculating water bath.

- After incubation, spin the CTAB/plant extract mixture at 12000 g for 5 min to spin down cell debris. Transfer the supernatant to clean microfuge tubes.

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- To each tube add 250 µl of Chloroform :Iso Amyl Alcohol (24:1) and mix the solution by inversion. After mixing, spin the tubes at 13000 rpm for 1 min.

- Transfer the upper aqueous phase only (contains the DNA) to a clean microfuge tube.

- To each tube add 50 µl of 7.5 M Ammonium Acetate followed by 500 µl of ice cold absolute ethanol.

- Invert the tubes slowly several times to precipitate the DNA. Generally the DNA can be seen to precipitate out of solution. Alternatively the tubes can be placed for 1 hr. at -20°C after the addition of ethanol to precipitate the DNA.

- Following precipitation, the DNA can be pipetted off by slowly rotating/spinning a tip in the cold solution. The precipitated DNA sticks to the pipette and is visible as a clear thick precipitate. To wash the DNA, transfer the precipitate into a microfuge tube containing 500 µl of ice cold 70 % ethanol and slowly invert the tube. Repeat. (Alternatively the precipitate can be isolated by spinning the tube at 13000 rpm for a minute to form a pellet. Remove the supernatant and wash the DNA pellet by adding two changes of ice cold 70 % ethanol).

- After the wash, spin the DNA into a pellet by centrifuging at 13000 rpm for 1 min. Remove all the supernatant and allow the DNA pellet to dry (approximately 15 min). Do not allow the DNA to over dry or it will be hard to re-dissolve.

- Resuspend the DNA in sterile DNase free water (approximately 50-400 µl H2O; the amount of water needed to dissolve the DNA can vary, depending on how much is isolated). RNaseA (10 µg/ml) can be added to the water prior to dissolving the DNA to remove any RNA in the preparation (10 µlRNaseA in 10ml H2O).

- After resuspension, the DNA is incubated at 65°C for 20 min to destroy any DNases that may be present and store at 4°C.

- Agarose gel electrophoresis of the DNA will show the integrity of the DNA, while spectrophotometry will give an indication of the concentration and cleanliness.

DNA quality confirmation:-

– Prepare a 1 % solution of agarose by melting 1 g of agarose in 100 mL of 0.5x TBE buffer in a microwave for approximately 2 min. Allow to cool for a couple of minutes then add 2.5 µl of ethidium bromide, stir to mix.

NOTES

– Cast a gel using a supplied tray and comb. Allow the gel to set for a minimum of 20 min at room temperature on a flat surface.

– Load the following into separate wells

• 10 µL 1kb ladder

• 5 µL sample + 5 µL water + 2 µL 6x Loading Buffer

– Run the gel for 30 min at 100 V

– Expose the gel to UV light.

– Confirm DNA quality, presence of a highly resolved high molecular weight band indicates good quality DNA, presence of a smeared band indicates DNA degredation.

Estimation of DNA

Principle:-

When DNA is treated with diphenylamine under the acidic condition a bluish green colored complex is formed which has an absorption peak at 595nm.This reaction is given by 2 deoxypentose in general. In acidic solution deoxypentose are converted into a highly reactive β hydroxyl leavulinic aldehyde which reacts with diphenylamine gives bluish green colored complex. The colour intensity was measured using a red filter at 595nm.

Reagent required:-

1. Stock Standard Solution: 50mg of DNA was dissolved in 50ml of Saline Sodium citrate buffer. Concentration 1mg/ml
2. Working Standard Solution: 5ml of stock solution was diluted to 50ml with distilled water. Concentration 100µg\ml
3. Diphenylamine Reagent: 10g of pure diphenylamine was dissolved with 25ml of concentrated sulphuric acid which was made up to 1ml with glacial acidic acid the solution must be prepared freshly.
4. Buffered Saline pH 7.4: 0.14N Sodium chloride and 0.02M sodium citrate.
5. Unknown Solution: The given unknown solution is mad up to 100ml with distilled water.

Procedure:-

1. 0.5-2.5ml of working standard solution is pipetted out into 5 test tubes labeled as s1- s5 where concentration ranging from 50-250µg.
2. 1ml and 2ml of unknown solution is pipetted out into two test tube u1 and u2.
3. The volume in all test tubes is made up to 3ml with distilled water and 3ml of distilled water alone serve as a blank.
4. 4ml of diphenylamine reagent was added to all the tubes. The tubes were kept in a boiling water bath for 20min. The tubes were than cooled and the bluish color developed is read at 595nm.
5. A standard graph is drawn taking concentration of DNA on x-axis and absorption on y-axis. From the standard graph the amount of DNA present in the unknown solution is calculated.

NOTES

11.5 Isolation of DNA and RNA by Agarose Gel Electrophoresis

Gel electrophoresis is a widely used technique for the analysis of nucleic acids and proteins. Almost every molecular biology research laboratory routinely uses agarose gel electrophoresis for the preparation and analysis of DNA.

Principle

Electrophoresis is a method of separating substances based on the rate of movement while under the influence of an electric field.Agarose is a polysaccharide purified from seaweed.An agarose gel is created by suspending dry agarose in a buffer solution, boiling until the solution becomes clear, and then pouring it into a casting tray and allowing it to cool.The result is a flexible gelatin-like slab.During electrophoresis, the gel is submersed in a chamber containing a buffer solution and a positive and negative electrode.The DNA to be analyzed is forced through the pores of the gel by the electrical current.Under an electrical field, DNA will move to the positive electrode (red) and away from the negative electrode (black).Several factors influence how fast the DNA moves, including; the strength of the electrical field, the concentration of agarose in the gel and most importantly, the size of the DNA molecules.Smaller DNA molecules move through the agarose faster than larger molecules.DNA itself is not visible within an agarose gel.The DNA will be visualized by the use of a dye that binds to DNA.

Materials Required: Agarose, TAE Buffer, 6X Sample Loading Buffer, DNA ladder standard, Electrophoresis chamber, Power supply, Gel casting tray and combs, DNA stain, Staining tray, Gloves, Pipette and tips.

Preparations

TAE Buffer: - 4.84 g Tris Base, 1.14 ml Glacial Acetic Acid, 2 ml 0.5M EDTA (pH 8.0), bring the total volume up to 1L with water

NOTES

Add Tris base to ~900 ml H2O. Add acetic acid and EDTA to solution and mix. Pour mixture into 1 L graduated cylinder and add H2O to a total volume of 1 L.

Note – for convenience a concentrated stock of TAE buffer (either 10X or 50X) is often made ahead of time and diluted with water to 1X concentration prior to use.

6X Sample Loading Buffer: -1 ml sterile H2O, 1 ml Glycerol enough bromophenol blue to make the buffer deep blue (~ 0.05 mg)

Protocol

Preparing the agarose gel

- Measure 1.25 g Agarose powder and add it to a 500 ml flask
- Add 125 ml TAE Buffer to the flask.(The total gel volume well vary depending on the size of the casting tray)
- Melt the agarose in a microwave or hot water bath until the solution becomes clear.(If using a microwave, heat the solution for several short intervals - do not let the solution boil for long periods as it may boil out of the flask).
- Let the solution cool to about 50-55°C, swirling the flask occasionally to cool evenly.
- Seal the ends of the casting tray with two layers of tape.
- Place the combs in the gel casting tray.
- Pour the melted agarose solution into the casting tray and let cool until it is solid (it should appear milky white).
- Carefully pull out the combs and remove the tape.
- Place the gel in the electrophoresis chamber.
- Add enough TAE Buffer so that there is about 2-3 mm of buffer over the gel.

Note – gels can be made several days prior to use and sealed in plastic wrap (without combs).If the gel becomes excessively dry, allow it to rehydrate in the buffer within the gel box for a few minutes prior to loading samples.

Loading the gel

- Add 6 ml of 6X Sample Loading Buffer to each aliquot.

- Record the order each sample will be loaded on the gel, including who prepared the sample, the DNA template - what organism the DNA came from, controls and ladder.
- Carefully pipette 20 ml of each sample/Sample Loading Buffer mixture into separate wells in the gel.
- Pipette 10 ml of the DNA ladder standard into at least one well of each row on the gel.

NOTES

Running the gel

- Place the lid on the gel box, connecting the electrodes.
- Connect the electrode wires to the power supply, making sure the positive (red) and negative (black) are correctly connected.
- Turn on the power supply to about 100 volts.Maximum allowed voltage will vary depending on the size of the electrophoresis chamber – it should not exceed 5 volts/cm between electrodes.
- Check to make sure the current is running through the buffer by looking for bubbles forming on each electrode.
    - Check to make sure that the current is running in the correct direction by observing the movement of the blue loading dye – this will take a couple of minutes (it will run in the same direction as the DNA).
- Let the power run until the blue dye approaches the end of the gel.
- Turn off the power.
- Disconnect the wires from the power supply.
- Remove the lid of the electrophoresis chamber.
- Using gloves, carefully remove the tray and gel.

Gel Staining

- Using gloves remove the gel from the casting tray and place into the staining dish.
- Add warmed (50-55°) staining mix.
- Allow gel to stain for at least 25-30 minutes (the entire gel will become dark blue).
- Pour off the stain (the stain can be saved for future use).
- Rinse the gel and staining tray with water to remove residual stain.

NOTES

- Fill the tray with warm tap water (50-55°).Change the water several times as it turns blue.Gradually the gel will become lighter, leaving only dark blue DNA bands.De-stain completely overnight for best results.
- View the gel against a white light box or bright surface.
- Record the data while the gel is fresh, very light bands may be difficult to see with time.

11.6 Isolation of Proteins by using SDS PAGE

The term electrophoresis refers to the movement of charged molecules in response to an electric field, resulting in their separation. In an electric field, proteins move toward the electrode of opposite charge. The rate at which they move (migration rate, in units of cm2/V Sec) is governed by a complex relationship between the physical characteristics of both the electrophoresis system and the proteins. Factors affecting protein electrophoresis include the strength of the electric field, the temperature of the system, the pH, ion type, and concentration of the buffer as well as the size, shape, and charge of the proteins. Proteins come in a wide range of sizes and shapes and have charges imparted to them by the dissociation constants of their constituent amino acids. As a result, proteins have characteristic migration rates that can be exploited for the purpose of separation. Protein electrophoresis can be performed in either liquid or gel-based media and can also be used to move proteins from one medium to another (for example, in blotting applications).

Over the last 50 years, electrophoresis techniques have evolved as refinements have been made to the buffer systems, instrumentation, and visualization techniques used. Protein electrophoresis can be used for a variety of applications such as purifying proteins, assessing protein purity (for example, at various stages during a chromatographic separation), gathering data on the regulation of protein expression, or determining protein size, isoelectric point (pI), and enzymatic activity. In fact, a significant number of techniques including gel electrophoresis, isoelectric focusing (IEF), electrophoretic transfer (blotting), and two-dimensional (2-D) electrophoresis can be grouped under the term protein electrophoresis.

When electrophoresis is performed in acrylamide or agarose gels, the gel serves as a size-selective sieve during separation. As proteins move through a gel in response to an electric field, the gel's pore structure allows smaller proteins to travel more rapidly than larger proteins. For protein separation, virtually all methods use polyacrylamide as an anticonvective, sieving matrix covering a protein size range of 5-250 kD. Some less common applications such as immune-electrophoresis and the separation of large proteins or protein complexes >300 kD rely on the larger pore sizes of agarose gels. In most PAGE

applications, the gel is mounted between two buffer chambers, and the only electrical path between the two buffers is through the gel. Usually, the gel has a vertical orientation, and the gel is cast with a comb that generates wells in which the samples are applied. Applying an electrical field across the buffer chambers forces the migration of protein into and through the gel.

When proteins are separated in the presence of SDS and denaturing agents, they become fully denatured and dissociate from each other. In addition, SDS binds non-covalently to proteins in a manner that imparts:

- An overall negative charge on the proteins. Since SDS is negatively charged, it masks the intrinsic charge of the protein it binds.
- A similar charge-to-mass ratio for all proteins in a mixture, since SDS binds at a consistent rate of 1.4 g of SDS per 1 g protein (a stoichiometry of about one SDS molecule per two amino acids).
- A long, rod-like shape on the proteins instead of a complex tertiary conformation.

As a result, the rate at which SDS-bound protein migrates in a gel depends primarily on its size, enabling molecular weight estimation.

Materials Required: -

To Pour Gels: -30% acrylamide, 10% SDS, 10% APS (make fresh each time), TEMED, 1.5 M Tris, pH 8.8 (resolving gel), 1.0 M Tris, pH 6.8 (stacking gel)

5x SDS Running Buffer (1 L):- Tris 15 g, Glycine 72 g, SDS 5 g

Coomassie Blue Stain: -10% (v/v) acetic acid, 0.006% (w/v) Coomassie Blue dye, 90% ddH2O

Isopropanol Fixing Solution: - 10% (v/v) acetic acid, 25% (v/v) isopropanol, 65% ddH2O

SDS sample loading buffer (40 ml):- ddH2O 16 ml, 0.5 M Tris, pH 6.8 5 ml, 50% Glycerol 8 ml, 10% SDS 8 ml, 2-βmercaptoethanol 2 ml (add immediately before use), bromophenol blue

10% (v/v) acetic acid

Protocol:

1. Make the separating gel:-

Set the casting frames (clamp two glass plates in the casting frames) on the casting stands.

Prepare the gel solution (as described above) in a separate small beaker.

Swirl the solution gently but thoroughly.

NOTES

Pipet out appropriate amount of separating gel solution and pour it into the gap between the glass plates.

To make the top of the separating gel horizontal, fill in water (either isopropanol) into the gap until an overflow.

Wait for 20-30min to let it solidify.

Make the stacking gel:-

Discard the water and you can see separating gel left.

Pipet in stacking gel until an overflow.

Insert the well-forming comb without trapping air under the teeth. Wait for 20-30min to let it solidify.

2. Make sure a complete gelation of the stacking gel and take out the comb. Take the glass plates out of the casting frame and set them in the cell buffer dam. Pour the running buffer (electrophoresis buffer) into the inner chamber and keep pouring after overflow until the buffer surface reaches the required level in the outer chamber.

3. Prepare the samples:

Mix your samples with sample buffer (loading buffer).

Heat them in boiling water for 5-10 min.

4. Load prepared samples into wells and make sure not to overflow. Don't forget loading protein marker into the first lane.

Then cover the top and connect the anodes.

5. Set an appropriate volt and run the electrophoresis when everything's done.

6. As for the total running time, stop SDS-PAGE running when the down most sign of the protein marker almost reaches the foot line of the glass plate. Generally, about 1 hour for a 120V voltage and a 12% separating gel. For a separating gel possessing higher percentage of acylamide, the time will be longer.

Note: Various factors affect the properties of the resulting gel.

• Higher concentration of ammonium persulfate and TEMED will lead to a faster gelation, on the other hand, a lower stability and elasticity.

• The optical temperature for gel gelation is 23°C-25°C. Low temperature will lead to turbid, porous and inelastic gels.

• The pH is better to be neutral and the gelation time should be limited in 20-30 min.

11.7 Study of Cell division: Mitosis and Meiosis

NOTES

1. Cell division

a) Mitosis

Mitosis is the process, in the cell cycle, by which the chromosomes in the cell nucleus are separated into two identical sets of chromosomes, each in its own nucleus. In general, mitosis is followed immediately by cytokinesis, which divides the cytoplasm, organelles, and cell membrane, and later karyokinesis, which divides the nucleus, dividing the cell into two new cells containing roughly equal shares of these cellular components. Mitosis and cytokinesis together define the mitotic (M) phase of the cell cycle—the division of the mother cell into two daughter cells, genetically identical to each other and to their parent cell. This accounts for approximately 20% of the cell cycle.

Phases of mitosis: -

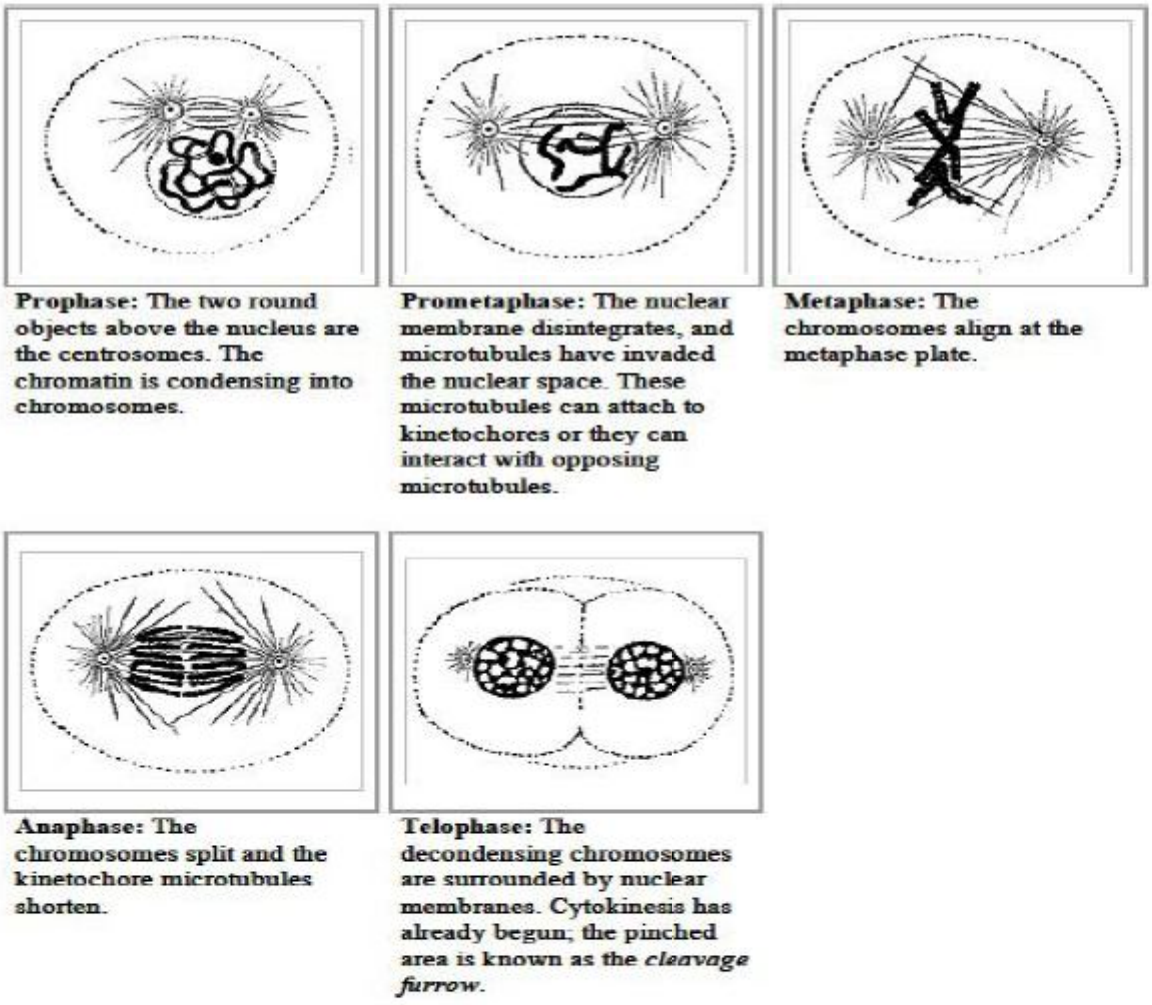


Fig. 11.3 : Mitosis

Study of mitosis by preparing squash of onion root tip: -

NOTES

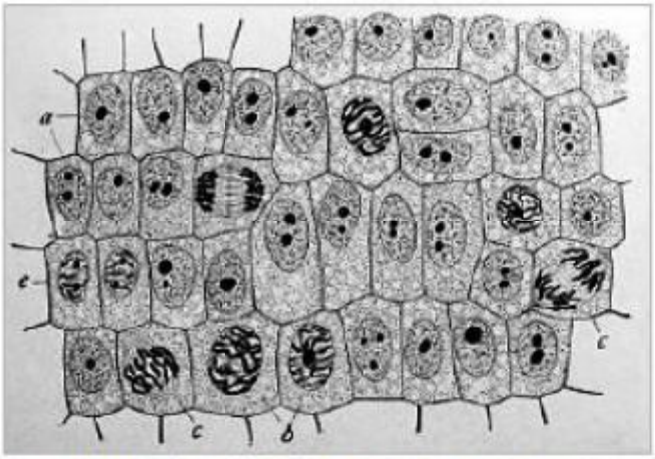
Materials required:-

Onion root tips, acetic acid, aceto-carmine, glass slides, cover slips, etc.

Allow the onion bulbs to grow in bottles filled with water. If the lower root portion of the bulb dips in water, it quickly sends forth large number of roots. Cut the root tips between 9 a.m. to 12 noon and fix them in Carnoy's fluid.

Procedure

- Place the fixed root tips in a drop of 45% acetic acid.
- Place the cover glass over the tip and diffuse aceto-carmine.
- Tap and apply uniform pressure over the cover glass.
- The squash is ready.
- Different phases of mitosis are analyzed under light microscope.



Onion (Allium) cells in different phases of the cell cycle enlarged 800 diameters.
a. non-dividing cells
b. nuclei preparing for division (spireme-stage)
c. dividing cells showing mitotic figures
e. pair of daughter-cells shortly after division

Fig. 11.4 : Cell Division in Onion Cells

b) Meiosis

Meiosis is a special type of cell division necessary for sexual reproduction which occurs or has occurred in all eukaryotes, including animals, plants and fungi, including both multi-celled and single-celled organisms. The number of sets of chromosomes in the cell undergoing meiosis is reduced to half the original number, typically from two sets (diploid) to one set (haploid). The cells produced by meiosis are either gametes (the usual

case in animals) or otherwise usually spores from which gametes are ultimately produced (the case in land plants). In many organisms, including all animals and land plants (but not some other groups such as fungi), gametes are called sperm in males and egg cells or ova in females. Since meiosis has halved the number of sets of chromosomes, when two gametes fuse during fertilisation, the number of sets of chromosomes in the resulting zygote is restored to the original number.

NOTES

Meiotic division occurs in two stages, meiosis I and meiosis II, dividing the cells once at each stage. Before meiosis begins, during S phase of the cell cycle, the DNA of each chromosome is replicated, so that each chromosome has two sister chromatids; a diploid organism now has a tetraploid DNA amount in the cell. The first stage of meiosis begins with a cell that has (if it is from a diploid organism) two copies of each type of chromosome, one from each of the mother and father, called homologous chromosomes, each of which has two sister chromatids. The homologous chromosomes pair up and may exchange genetic material with each other in a process called crossing-over. Each pair then separates as two cells are formed, each with one chromosome (two chromatids) from every homologous pair. The chromatids composing a chromosome may differ from one another if crossing-over occurred. The chromosomes present in each of the two cells will be complementary subsets from the original set, some originally from the mother and some originally from the father. In the second stage, each chromosome splits into two; each half, each sister chromatid, is separated into two new cells, which are haploid. (Note: The instant the "sister chromatids" are separated, they are called daughter chromosomes, not chromatids.) This occurs in both of the cells formed in meiosis I. Therefore from each original cell, four genetically distinct haploid cells are produced. These cells can mature into gametes.

Phases of Meiosis:

Meiosis is divided into meiosis I and meiosis II which are further divided into Karyokinesis I and Cytokinesis I &Karyokinesis II and Cytokinesis II respectively.

Meiosis I:

Meiosis I separates homologous chromosomes, producing two haploid cells (N chromosomes, 23 in humans), and thus meiosis I is referred to as a reduction division. A regular diploid human cell contains 46 chromosomes and is considered 2N because it contains 23 pairs of homologous chromosomes. However, after meiosis I, although the cell contains 46 chromatids, it is only considered as being N, with 23 chromosomes. This is because later, in Anaphase I, the sister chromatids will remain together as the spindle fibers pull the pair toward the pole of the new cell. In meiosis II, an equational division

similar to mitosis will occur whereby the sister chromatids are finally split, creating a total of 4 haploid cells (23 chromosomes, N) - two from each daughter cell from the first division.

NOTES

Prophase I:

It is the longest phase of meiosis. During prophase I, DNA is exchanged between homologous chromosomes in a process called homologous recombination. This often results in chromosomal crossover. This process is critical for pairing between homologous chromosomes and hence for accurate segregation of the chromosomes at the first meiosis division. The new combinations of DNA created during crossover are a significant source of genetic variation, and may result in beneficial new combinations of alleles. The paired and replicated chromosomes are called bivalents or tetrads, which have two chromosomes and four chromatids, with one chromosome coming from each parent. The process of pairing the homologous chromosomes is called synapsis. At this stage, non-sister chromatids may cross-over at points called chiasmata (plural; singular chiasma).

Leptotene:

The first stage of prophase I is the leptotenestage, also known as leptonema, from Greek words meaning "thin threads". In this stage of prophase I, individual chromosomes; each consisting of two sister chromatids; change from the diffuse state they exist in during the cell's period of growth and gene expression, and condense into visible strands within the nucleus. However the two sister chromatids are still so tightly bound that they are indistinguishable from one another. During leptotene, lateral elements of the synaptonemal complex assemble. Leptotene is of very short duration and progressive condensation and coiling of chromosome fibers takes place.

Zygotene:

The *zygotene*stage, also known as *zygonema*, from Greek words meaning "paired threads", occurs as the chromosomes approximately line up with each other into homologous chromosome pairs. This is called the bouquet stage because of the way the telomeres cluster at one end of the nucleus. At this stage, the synapsis (pairing/coming together) of homologous chromosomes takes place, which is facilitated by assembly of central element of the synaptonemal complex. Pairing is brought about in a zipper-like fashion and may start at the centromere (procentric), at the chromosome ends (proterminal), or at any other portion (intermediate). Individuals of a pair are equal in length and in position of the centromere. Thus pairing is highly specific and exact. The paired chromosomes are called bivalent or tetrad chromosomes.

Pachytene:

The pachytene stage, also known as pachynema, from Greek words meaning "thick threads", is the stage when chromosomal crossover (crossing over) occurs. Nonsister chromatids of homologous chromosomes may exchange segments over regions of homology. Sex chromosomes, however, are not wholly identical, and only exchange information over a small region of homology. At the sites where exchange happens, chiasmata form. The exchange of information between the non-sister chromatids results in a recombination of information; each chromosome has the complete set of information it had before, and there are no gaps formed as a result of the process. Because the chromosomes cannot be distinguished in the synaptonemal complex, the actual act of crossing over is not perceivable through the microscope, and chiasmata are not visible until the next stage.

NOTES

Diplotene:-

During the diplotenestage, also known as diplonema, from Greek words meaning "two threads", the synaptonemal complex degrades and homologous chromosomes separate from one another a little. The chromosomes themselves uncoil a bit, allowing some transcription of DNA. However, the homologous chromosomes of each bivalent remain tightly bound at chiasmata, the regions where crossing-over occurred. The chiasmata remain on the chromosomes until they are severed in anaphase I. In human fetal oogenesis all developing oocytes develop to this stage and stop before birth. This suspended state is referred to as the dictyotene stage and remains so until puberty.

Diakinesis:

Chromosomes condense further during the diakinesisstage, from Greek words meaning "moving through". This is the first point in meiosis where the four parts of the tetrads are actually visible. Sites of crossing over entangle together, effectively overlapping, making chiasmata clearly visible. Other than this observation, the rest of the stage closely resembles prometaphase of mitosis; the nucleoli disappear, the nuclear membrane disintegrates into vesicles, and the meiotic spindle begins to form.

Metaphase I:

Homologous pairs move together along the metaphase plate: As kinetochore microtubules from both centrioles attach to their respective kinetochores, the homologous chromosomes align along an equatorial plane that bisects the spindle, due to continuous counterbalancing forces exerted on the bivalents by the microtubules emanating from the two kinetochores of homologous chromosomes. The physical basis of the independent assortment of chromosomes is the random orientation of each bivalent along the

metaphase plate, with respect to the orientation of the other bivalents along the same equatorial line.

Anaphase I:

NOTES

Kinetochore (bipolar spindles) microtubules shorten, severing the recombination nodules and pulling homologous chromosomes apart. Since each chromosome has only one functional unit of a pair of kinetochores, whole chromosomes are pulled toward opposing poles, forming two haploid sets. Each chromosome still contains a pair of sister chromatids. During this time disjunction occurs, which is one of the processes leading to genetic diversity as each chromosome can end up in either of the daughter cells.

Telophase I:

The first meiotic division effectively ends when the chromosomes arrive at the poles. Each daughter cell now has half the number of chromosomes but each chromosome consists of a pair of chromatids. The microtubules that make up the spindle network disappear, and a new nuclear membrane surrounds each haploid set. The chromosomes uncoil back into chromatin. Cytokinesis, the pinching of the cell membrane in animal cells or the formation of the cell wall in plant cells, occurs, completing the creation of two daughter cells. Sister chromatids remain attached during telophase I.

Meiosis II:

Meiosis II is the second part of the meiotic process, also known as equational division. Mechanically, the process is similar to mitosis, though its genetic results are fundamentally different. The end result is production of four haploid cells (23 chromosomes, N in humans) from the two haploid cells (23 chromosomes) produced in **meiosis I.**

The four main steps of Meiosis II are: Prophase II, Metaphase II, Anaphase II, and **Telophase II.**

In prophase II we see the disappearance of the nucleoli and the nuclear envelope again as well as the shortening and thickening of the chromatids. Centrioles move to the Polar Regions and arrange spindle fibers for the second meiotic division.

In metaphase II, the centromeres contain two kinetochores that attach to spindle fibers from the centrosomes (centrioles) at each pole. The new equatorial metaphase plate is rotated by 90 degrees when compared to meiosis I, perpendicular to the previous plate.

This is followed by anaphase II, where the centromeres are cleaved, allowing microtubules attached to the kinetochores to pull the sister chromatids apart. The sister

chromatids by convention are now called sister chromosomes as they move toward opposing poles.

The process ends with telophase II, which is similar to telophase I, and is marked by uncoiling and lengthening of the chromosomes and the disappearance of the spindle. Nuclear envelopes reform and cleavage or cell wall formation eventually produces a total of four daughter cells, each with a haploid set of chromosomes. Meiosis is now complete and ends up with four new daughter cells.

NOTES

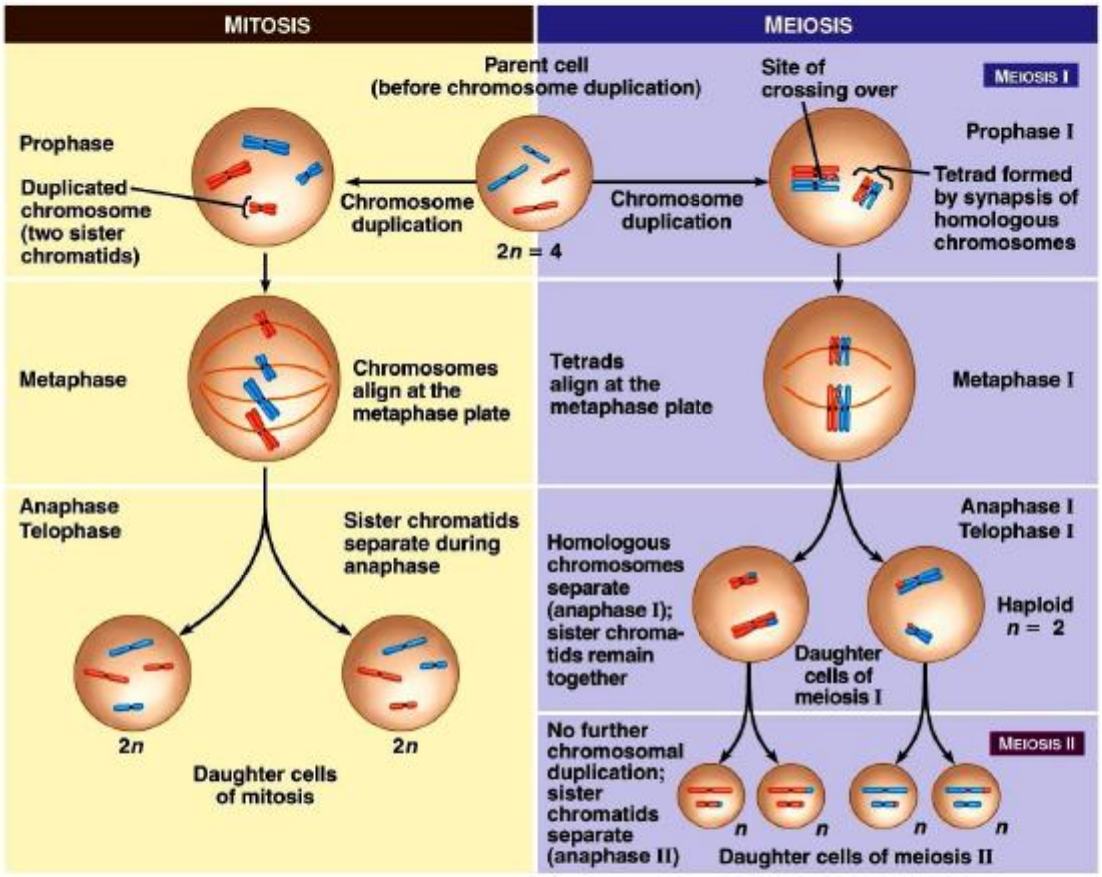


Fig. 11.6 : Mitosis & Meiosis

11.8 Viva-Voce

1 What is the difference between plant cell and animal cell?

2 In which organelle photosynthesis occurs in plant cell?

3 What are parenchyma cells?

4 What is differential centrifugation?

5 What is the full form of SDS PAGE?

6 What is the basic difference between mitosis and meiosis?

11.9 References

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Unit-12

Immunological Techniques

NOTES

Structure of the Unit

12.0 Objectives

After going through this unit you will be able to understand:

- Qualitative and quantitative assays to determine protein antigen
- Separation of Proteins from gels
- Blood agglutination reactions

12.1 ELISA (sandwich & dot)

Principle

Antibody-sandwich ELISAs may be the most useful of the immunosorbent assays for detecting antigen because they are frequently between 2 and 5 times more sensitive than those in which antigen is directly bound to the solid phase. To detect antigen, the wells of microtiter plates are coated with specific (capture) antibody followed by incubation with test solutions containing antigen. Unbound antigen is washed out and an antigen-specific antibody conjugated to enzyme (i.e., developing reagent) is added, followed by another incubation. Enzyme labeled antibody can be produced in the same animal that produced passively adsorbed antibody, or from a different species immunized with the same antigen that is captured. Unbound conjugate is washed out and substrate is added. After another

incubation, the degree of substrate hydrolysis is measured. The amount of substrate hydrolyzed is proportional to the amount of antigen in the test solution.

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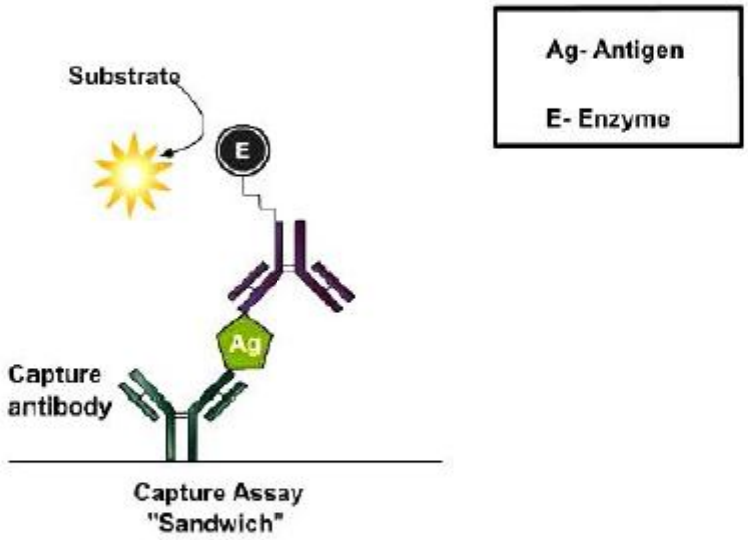

Fig. 12.1 : ELISA

Stages of Sandwich ELISA

Coating ELISA Plates:

Coating is achieved through passive adsorption of the antibody to the assay microplate. This process occurs though hydrophobic interactions between the microtiter plate and non-polar protein residues. Although individual proteins may require specific conditions or pretreatment for optimal binding, the most common method for coating plates involves adding a 2-10 μg/ml solution of protein dissolved in an alkaline buffer such as phosphate-buffered saline (pH 7.4) or carbonate-bicarbonate buffer (pH 9.4).The buffer contains no other proteins that might compete with the target antigen for attachment to the microtiter plate. Antigens, which are protein in nature will attach passively to the microtiter well plate during incubation in incubator at 37°C.

Washing step:

After incubation any excess antibody is removed by washing steps by flooding and emptying the wells with neutral phosphate buffered saline (PBS) or deionized water. Washing steps are necessary to remove unbound reagents and decrease background, thereby increasing the signal to noise ratio. Insufficient washing will allow high background, while excessive washing might result in decreased sensitivity caused by elution of the antigen from the well.

Add blocking buffer

The binding capacity of microplate wells is typically higher than the amount of protein coated in each well and the residual binding capacity of the plate is blocked in this step. The ideal blocking buffer will bind to all potential sites of nonspecific interaction, eliminating background altogether, without altering or obscuring the epitope for antibody binding. The blocking buffer is effective if it improves the sensitivity of an assay by reducing background signal and improving the signal-to-noise ratio. Tween 20 (0.05%) by itself is more effective at blocking than any protein tested, but because the combination of protein and Tween 20 may be more effective than Tween 20 alone in some cases, bovine serum albumin (BSA; 0.25%) is included in the blocking buffer. Coated plates can be used immediately or dried and stored at 4°C for later use, depending on the stability of the coated protein.

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Add antigen solution:

This step involves the addition of detecting soluble antigens (in test sample) being directed against the coated antibody. The antigen is usually diluted in blocking buffer to prevent nonspecific attachment of protein in the antiserum on the solid phase. The antibody present in the serum which are specific to the antigen, binds the coated antigen on incubation.

Washing step:

Excess antigen or unbound antigen are removed by washing step and is followed by addition of blocking solution.

Add antibody enzyme conjugate:

The next step is the addition of antibody enzyme conjugate, diluted in blocking buffer directed against the antigen. The choice of antibody enzyme conjugate is determined by the goals of the assay. If it is necessary to detect all antibodies that bind to antigen, conjugates prepared with antibodies specific for Igκ and λ light chains should be used. Alternatively, protein A or protein G–enzyme conjugates may be preferable when screening monoclonal antibodies. Such antibodies are produced against immunoglobulins (Igs) of species in which the detecting antibodies are produced and are termed antispecies conjugates. Thus, if detecting antibodies are produced in rabbits, the enzyme-labeled antibodies would have to be anti-rabbit Igs in nature. This allow greater flexibility in use of anti-species conjugates in that different specificities of conjugate can be used to detect particular Igs binding in the assay. For example, the antispecies conjugate could be anti-IgM, IgG1, IgG2 and so on. The enzyme can be linked to a protein such as streptavidin if the primary antibody is biotin labeled. The most commonly used enzyme labels

horseradish peroxidase (HRP) and alkaline phosphatase (AP). Other enzymes have been used as well, but they have not gained widespread acceptance because of limited substrate options. These include β-galactosidase, acetylcholinesterase and catalase.

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Washing step: Unbound antibody enzyme conjugate is washed away after incubation phase.

Adding substrate:

Substrates are critical for the detection and visualization steps of an ELISA. The step involves the addition of suitable substrate solution for the particular enzyme conjugated to the antibodies. The objective is to allow development of color reaction through enzyme catalysis. A large selection of substrates is available for performing the ELISA with an HRP or AP conjugate. TMB (3, 3', 5, 5'-tetramethyl benzidine) is the most commonly used substrate for the enzyme horseradish peroxidase (HRP). The substrates of alkaline phosphatase (AP) ,4-methylumbelliferyl phosphate (MUP) and pNPP (p-Nitrophenyl-phosphate) are nontoxic and relatively stable. Solutions of p-nitro-phenyl phosphate (NPP) are stable for months at 4°C, while solutions of 4-methylumbelliferyl phosphate (MUP) can be kept for months at room temperature without any significant spontaneous hydrolysis. The biggest disadvantage if NPP is used as a substrate is that, the yellow color of the nitrophenyl product is relatively difficult to detect visually. Using the substrate MUP instead of NPP can greatly enhance the sensitivity of the assay. The fluorogenic system using MUP is 10 to 100 times faster than the chromogenic system using NPP, and appears to be as sensitive as an enhanced chromogenic assay in which alkaline phosphatase generates NAD+ from NADP. The disadvantage of using fluorogenic substrates is that they require a microplatefluorometer costing twice as much as a high quality microtiter plate spectrophotometer.

The choice of substrate depends upon the required assay sensitivity and the instrumentation available for signal-detection (spectrophotometer, fluorometer or luminometer)

Stop solution:

The reaction is allowed to progress for a defined period after which the reaction is stopped by altering the pH of the system. Stop Solution is a used to terminate the enzyme substrate reaction for ELISA applications after attaining the desired color intensity which is an indication of analyte level. The TMB substrate reacts with immobilized horseradish peroxidase (HRP) conjugated antibodies to produce a blue solution. Reaction may be stopped by 0.2 M sulphuric acid which offers a yellow end product read at 450 nm. AP

stop solution (0.5M NaOH) does not change the yellow color or the absorbance of the chromogen, and so the absorbance is read at 405 nm to 420 nm.

Quantification:

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Specially designed spectrophotometers are available which reads through the microtiter wells either singly or in rows. Several ELISA plate readers are available, with increasing levels of sophistication. Some of these provide a measurement of optical density while some tabulate data and apply statistical analysis. Compatibility with a small computer and availability of a suitable program to process the results and transform the optical density readings into concentrations of protein are important additional things to look for when selecting an instrument. Most ELISA readers can be set to measure the absorbance of the colors produced by the action of antibody conjugated enzymes on their respective substrates. The microplate reader works by shining a particular type of light at each of the samples in the microwell plate. Common detection modes for microplate assays are absorbance, fluorescence intensity, luminescence, time-resolved fluorescence and fluorescence polarization. A light source illuminates the sample using a specific wavelength (selected by an optical filter, or a monochromator), and a light detector located on the other side of the well measures how much of the initial (100%) light is transmitted through the sample, the amount of transmitted light will typically be related to the concentration of the (100%) light is transmitted through the sample, the amount of transmitted light will typically be related to the concentration of the molecule of interest. This is called absorbtion detection. The range of application of fluorescence intensity detection is much broader than when using absorbance detection, but instrumentation is usually more expensive. Microplate readers feed the absorbance or fluorescence measures into a computer program that analyses the particular information being collected.

Dot - ELISA

Principle:

Dot-ELISA (Enzyme Linked Immuno Sorbent Assay) is an extensively used immunological tool in research as well as analytical/diagnostic laboratories. In sandwich Dot- ELISA, the antigen is sandwiched directly between two antibodies which react with two different epitopes on the same antigen. Antigen in the test sample first reacts with the immobilized antibody and then with the enzyme-linked secondary antibody. The amount of enzyme linked antibody bound is assayed by incubating the strip with an appropriate chromogenic substrate, which is converted to a coloured, insoluble product. The latter precipitates onto the strip in the area of enzyme activity, hence the name Dot-ELISA. The

enzyme activity is indicated by intensity of the spot, which is directly proportional to the antigen concentration. In this method, ELISA strips are having three well defined zones:

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- **Negative control zone** that is blocked with an inert protein.
- **Test zone** having an antibody immobilized on it and then blocked with an inert protein.
- **Positive control zone** having the antibody immobilized on it, blocked with inert protein and has a specific antigen bound to the immobilized antibody.

These strips will be used to detect the antigen in the test serum samples by using a secondary antibody conjugated to Horse radish peroxidase (HRP). HRP is then detected using hydrogen peroxide as a substrate and Tetramethylbenzidine (TMB) as a chromogen. HRP acts on hydrogen peroxide to release oxygen, which oxidizes the TMB to TMB oxide. The TMB oxide is deposited wherever enzyme is present and appears as a blue spot.

$$H_2O_2 \xrightarrow{HRP} H_2O + [O]$$
$$TMB + [B] \rightarrow TMBO(Blue)$$

If the test sample does not contain the antigen specific to the antibody, there will be no enzyme reaction to occur and no spot develops.

Negative Control Zone: In this zone, immobilize antibody is not present and hence, there is no reaction when the reagents are added.

Positive control zone: In this zone, antigen is bound to immobilize antibody. The antigen binds to antibody enzyme conjugate and develops spot.

Test Zone: The result depends on the presence or absence of antigen and will occur according to the test performed.

Procedure

1. In a vial, take 1ml of 1X assay buffer and 50µl of serum sample. Mix thoroughly and insert a Dot-ELISA strip.
2. Allow the reaction to occur at room temperature for 20 minutes.
3. Wash the strip three times by dipping it in 1 ml of 1X assay buffer for about 5 minutes each. Replace the buffer each time.
4. Take 1 ml of 1X assay buffer in a fresh vial; add 10µl antibody-HRP conjugate to it. Mix thoroughly. Dip the strip; allow the reaction to take place for 20 minutes.
5. Wash the strip as in step # 3, three times.

6. In a fresh vial, take 100ul of 10X TMB/H2O2 and 900ul of distilled water, mix thoroughly. Dip the strip in this substrate solution.

7. Observe the strip after 10-20 minutes for appearance of a blue/grey spot.

8. Rinse the strip with distilled water.

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Interpretation:

- Spot in positive control zone and no spot in the negative control zone indicates proper performance of test.
- Spot in test zone indicates presence of specific antigen in the sample.

Note: Intensity of the spot will vary depending upon the test sample used.

- No spot in the test zone indicates the absence of specific antigen in the sample.

12.2 Western Blotting

Western blotting is a widely used technique for the detection and analysis of proteins based on their ability to bind to specific antibodies.. It was first described by Towbin, et.al in 1979 and has since become one of the most commonly used methods in life science research. Western blotting is an accomplished rapidly, using simple equipment and inexpensive reagents, it is commonly used laboratory technique. The specificity of the antibody- antigen interaction enables to a target protein to be identified in the midst of a complex protein mixture.

It is an analytical method where in a protein sample is electrophoresis on an SDS- PAGE and electro transferred on to PVDF membrane or nitrocellulose membrane. The transferred protein is detected using specific primary and secondary enzyme labeled antibody.

Antibodies bind to specific sequences of amino acids, known as the epitope. Because amino acid sequences are different from protein to protein, antibodies can recognize specific proteins among a group of many. Therefore, a single protein can be identified in a cell lysate that contains thousands of different proteins and its abundance quantified through western blot analysis. First, proteins are separated from each other based on their size. Second, antibodies are used to detect the protein of interest. Finally, a substrate that reacts with an enzyme is used to view the antibody/protein complex.

Principle

Western blotting is the transfer of proteins from the SDS- PAGE gel to a solid supporting membrane. There are two types of blotting apparatus used to transfer proteins to solid supports; these facilitate either wet transfer (tank blotting) or semidry transfer. Both of

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them give good result. Electrophoresis is used to separate complex mixtures of proteins denaturing discontinuous one dimensional gel electrophoresis separates proteins only based on molecular size as they move through a SDS- polyacrylamide gel(SDS PAGE) toward the anode with the smaller protein migrating faster and bigger proteins running slower. The SDS-PAGE is a separating gel topped by stacking gel and secured in an electrophoresis apparatus. Sample proteins are solubilized by boiling in the presence of SDS and equal amount of the protein in solution are loaded into a gel lane, and the individual proteins separated electrophoretically. 2-mercaptoethanol and dithiothreitol are added to reduce disulfide bonds.

A protein sample is subjected to polyacrylamide gel electrophoresis. After this the gel is placed over a sheet of nitrocellulose and the protein in the gel is electrophoretically transferred to the nitrocellulose. The nitrocellulose is then soaked in blocking buffer (3% skimmed milk solution) to "block" the non-specific binding of proteins. The nitrocellulose is then incubated with the specific antibody for the protein of interest. The nitrocellulose is then incubated with a second antibody, which is specific for the first antibody. For example, if the first antibody was raised in mouse, the second antibody might be termed "goat anti-mouse immunoglobulin". What this means is that mouse immunoglobulin were used to elicit an antibody response in goats. The second antibody will typically have a covalently attached enzyme which, when provided with a chromogenic substrate, will cause a color reaction. Thus the molecular weight and amount of the desired protein can be characterized from a complex mixture (e.g. crude cell extract) of other proteins by western blotting.

12.3 Blood Agglutination

Principle

It was in 1901, that Austrian-American immunologist and pathologist Karl Landsteiner discovered human blood groups. Karl Landsteiner's work helps to determine blood groups and thus opened a way for blood transfusions which can be carried out safely. He was awarded the Nobel Prize in Physiology or Medicine in 1930 for this discovery. Death of the patient was the result in most cases before 1900, when blood transfusion was attempted. Blood transfusion was made much safer by the discovery of blood groups, as blood of the same ABO group could be chosen for each patient. However, there were still many cases of unexplained blood transfusion reactions. Biologists still went in search of these unexplained questions.

In 1902, the fourth main type, AB was found by Decastrello and Sturli. It was the observations of Levine and Stetson in 1939, and Landsteiner and Weiner in 1940 that laid

the foundations of our knowledge about the remaining major blood group the Rhesus system. Once reliable tests for Rhesus grouping had been established, transfusion reactions became rare.

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At times, it was observed that mixing blood from two individuals led to blood clumping or agglutination. Later it was understood that the agglutinated red cells can clog blood vessels and stop the circulation of the blood to various parts of the body. The agglutinated red blood cells also crack and their contents leak out in the body. The RBCs contain hemoglobin which becomes toxic when outside the cell. This must have been the phenomena that occurred in the blood transfusion cases that ended up with fatality of the patient at the receiving end. Karl Landsteiner discovered that blood clumping was an immunological reaction which occurs when the receiver of a blood transfusion has antibodies against the donor blood cells! People learned that, compatibility of blood groups needed to be checked before anything else was done. If they are not, the red blood cells from the donated blood will agglutinate. This can have fatal consequences for the patient.

ABO blood grouping system

In this system two different types of antigens are present on cell surface as a marker. They help in recognizing of self-cells.

If somebody possesses antigen-a on its cells then he has 'a' blood group.

If somebody possesses antigen-b on its cells then he has 'b' blood group.

If somebody possesses both antigen-a and antigen-b on its cells then he has 'ab' blood group.

If somebody possesses no antigen on its cells then he has 'o' blood group.

Rh (Rhesus) factor is found on the RBC's surface in most people. Like A and B, this is also an antigen and those who have it are called Rh+. Those who lack the antigen on the surface of RBCs are called Rh-. A person with Rh- blood does not have Rh antibodies naturally in the blood plasma. But a person with Rh- blood can develop Rh antibodies in the blood plasma if he or she receives blood from a person with Rh+ blood, whose Rh antigens can trigger the production of Rh antibodies (as the immune system is triggered by the presence of an unknown antigen in the system). A person with Rh+ blood can receive blood from a person with Rh- blood without any problems.

Blood clumping or Agglutination observation

Compatibility between the blood groups of donor and recipient determines the success of a blood transfusion.

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The ABO and Rh blood groups are looked at while conducting the test. In a diagnostic lab, Monoclonal antibodies are available for A, B and Rh antigen. Monoclonal antibody against Antigen A (also called Anti-A), comes in a small bottles with droppers; the monoclonal suspension being BLUE in color. Anti-B comes in YELLOW color. Anti-D (monoclonal antibody against Rh) is colorless. All the color codes are universal standards. When the monoclonal antibodies are added one by one to wells that contain the test sample (blood from patient), if the RBCs in that particular sample carry the corresponding Antigen, clumps can be observed in the corresponding wells. A drop of blood is left without adding any of the antibodies; it is used as a control in the experiment. The monoclonal antibody bottles should be stored in a refrigerator. It is recommended to tilt the bottle a couple of times before use in order to re-suspend the antibodies that have settled at the bottom of the bottle.

Table -1 : Blood Group System

Blood Group	Antigens	Antibodies	Can Donate To	Can Receive from
A Rh+	A and Rh	B	A Rh+ AB Rh+	A Rh+ A Rh- O Rh+ O Rh-
A Rh-	A	B (Can develop Rh antibodies)	A Rh+ A Rh- AB Rh+ AB Rh-	A Rh- O Rh-
B Rh+	B and Rh	A	B Rh+ AB Rh+	B Rh+ B Rh- O Rh+ O Rh-
B Rh-	B	A (Can develop Rh antibodies)	B Rh+ B Rh- AB Rh+ AB Rh-	B Rh- O Rh-
O Rh+	Rh	A and B	O Rh+ A Rh+ B Rh+ AB Rh+	O Rh+ O Rh-
O Rh-	None	A and B (Can develop Rh antibodies)	AB Rh+ AB Rh- A Rh+ A Rh- B Rh+ B Rh- O Rh+ O Rh-	O Rh-
AB Rh+	A, B and Rh	None	AB Rh+	AB Rh+ AB Rh- A Rh+ A Rh- B Rh+ B Rh- O Rh+ O Rh-
AB Rh-	A and B	None (Can develop Rh antibodies)	AB Rh+ AB Rh-	AB Rh- A Rh- B Rh- O Rh-

12.4 FISH

NOTES

FISH (fluorescent *in situ* hybridization) is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence complementarity. Fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosomes. FISH is often used for finding specific features in DNA for use in genetic counseling, medicine, and species identification. FISH can also be used to detect and localize specific RNA targets (mRNA, lncRNA and miRNA) in cells, circulating tumor cells, and tissue samples. In this context, it can help define the spatial-temporal patterns of gene expression within cells and tissues.

Often parents of children with a developmental disability want to know more about their child's conditions before choosing to have another child. These concerns can be addressed by analysis of the parents' and child's DNA. In cases where the child's developmental disability is not understood, the cause of it can potentially be determined using FISH and cytogenetic techniques. Examples of diseases that are diagnosed using FISH include Prader-Willisyndrome, Angelman syndrome, 22q13 deletion syndrome, chronic myelogenous leukemia, acute lymphoblasticleukemia, Cri-du-chat, Velocardiofacial syndrome, and Down syndrome.In medicine, FISH can be used to form a diagnosis, to evaluate prognosis, or to evaluate remission of a disease,such as cancer.

12.5 RID & ODD

RID (radial immuno diffusion)

Principle

Single immunodiffusion (RID) is used extensively for the quantitative estimation of antigen. The antigen-antibody precipitation is made more sensitive by the incorporation of antiserum in the agarose. Antigen is then allowed to diffuse from wells cut in the gel, in which the antiserum is uniformly distributed. Initially, as the antigen diffuses out of the well, its concentration is relatively high and soluble antigen-antibody complex reacts with more amount of antibody resulting in a lattice that precipitate to form a precipitation ring. By running a range of known antigen concentrations on the gel and by measuring the diameters of their precipitin rings, a calibration graph is plotted. Antigen concentrations of unknown samples, run on the same gel can be found by measuring the diameter of precipitin rings and extrapolating this value on the calibration graph.

Materials: Conical flask, Measuring cylinder, Alcohol, distilled water, Micropipette, Tips, Moist chamber (box with wet cotton)

Procedure:

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1. Prepare 6ml of 1.0% agarose solution in 1x assay buffer by heating slowly till agarose dissolves completely and allow the molten agarose to cool to 55° C.
2. Add 120ul of antiserum to 6ml of agarose solution. Mix thoroughly for uniform distribution of antibody.
3. Pour agarose solution containing the antiserum on to a glass plate set on a horizontal surface. Leave it undistributed to form a gel.
4. Cut wells using a gel puncher, using the template provided.
5. Add 20ul of the given standard antigens and test antigens to each wells.
6. Keep the gel plate in moist chamber and incubate overnight at room temperature.
7. Mark the edges of the circle and measure the diameter of the ring.
8. Plot a graph for diameter of the ring on y-axis vs. conc. of antigen on x-axis and determine the unknown concentration.

ODD: - Ouchterlony double diffusion

The key reaction of immunology and immune defense is the interaction of antibodies and antigens. This interaction is responsible for the body's defense against viral and bacterial infections and other toxins. The body's defense mechanism recognizes foreign substances, or antigens, and raises specific antibodies against them. The antibodies bind to the antigens and form large macromolecular complexes. These complexes are formed due to the fact that each antibody can associate and bind with more than one antigen and each antigen can be bound by more than one antibody molecule. The non-covalent interactions that form the basis of antigen-antibody (Ag-Ab) binding include hydrogen bonds, ionic bonds, hydrophobic interactions, and Vander Waals interactions. The formation of the large macromolecules results in their precipitation and the resulting precipitate is cleared by the body by various mechanisms. The interaction of antigen and antibody, resulting in precipitation, is also useful in research and diagnostics.

The specificity of antigen-antibody interactions has led to the development of a variety of immunologic assays, which can be used to detect the presence of either antibody or antigen. Immunological assays play an important role in diagnosing diseases, monitoring the level of the humoral immune response, and identifying molecules of biological or medical interest. These assays differ in their speed and sensitivity; some are strictly qualitative, others are quantitative.

Immune precipitates can form in an agar matrix. When antigen and antibody diffuse toward one another in agar, or when antibody is incorporated into the agar and antigen diffuses into the antibody containing matrix, a visible line of precipitation will form. As in a precipitation reaction in fluid, visible precipitation occurs in the region of equivalence, whereas no visible precipitate forms in regions of antibody or antigen excess. Two types of immunodiffusion reactions can be used to determine relative concentrations of antibodies or antigens, to compare antigens, or to determine the relative purity of an antigen preparation. They are radial immune-diffusion (the Mancini method) and double immune-diffusion (the Ouchterlony method); both are carried out in a semisolid medium such as agar.

The Ouchterlony double diffusion (ODD) technique is one of the simplest techniques extensively used to check antisera for the presence of antibodies for a particular Ag and to determine its titre. This method has been widely used for detection and qualitative diagnostic procedures. The method is called "double" referring to the fact that in this procedure, antigen and antibody are allowed to migrate towards each other in a gel and a line of precipitation is formed where the two reactants meet. This precipitation reaction is highly specific. The method is even today widespread and used by people working with diagnosis or protein detection or comparing antigens or antisera. The method is not very sensitive. The technique involves cutting wells into an Agarose solidified in a glass plate. The wells are filled with antibody or antigen and the plate is incubated. When homologous antigen and antibody diffuse toward each other from the individual wells, a precipitin line will form somewhere between the two wells. Precipitation occurs because the antigen is multivalent i.e. has several antigenic determinants per molecule to which antibodies can bind. Antibodies have at least two antigen binding sites, thus large aggregates or lattices of antigen and antibody are formed. Precipitation will not occur if excess antigen is present or if excess antibody is present. Cross-linking and lattice formation will only occur when antigen and antibody concentrations are optimal. An increasing amount of antigen is added to a constant amount of antibody in solution. This is called the antibody-excess zone (Prozone phenomenon). The Ag and Ab concentrations are relatively higher near their respective wells. As they diffuse farther from the wells, their concentration decreases. An antigen will react with its specific antibody to form an Ag-Ab complex. As more antigens are added, the amount of protein precipitated increases until the antigen/antibody molecules are at an optimal ratio. This is known as the equivalence zone or equivalence point. When the amount of antigen in solution exceeds the amount of antibody, the amount of precipitation will decrease. This is known as the antigen excess zone.

Different patterns of lines obtained onOuchterlony double diffusion:-

Pattern of Identity: A

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The antibodies in the antiserum react with both the antigens resulting in a smooth line of precipitate. The antibodies cannot distinguish between the two antigens. i.e., the two antigens are immunologically identical.

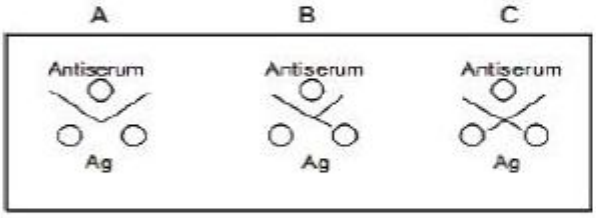

Fig. 12.2 : Oucheterlony Double Diffusion

Pattern of Partial Identity: B

In the 'pattern of partial identity', the antibodies in the antiserum react more with one of the antigens than the other. The 'spur' is thought to result from the determinants present in one antigen but lacking in the other antigen.

Pattern of Non-Identity: C

In the 'pattern of non-identity', none of the antibodies in the antiserum react with antigenic determinants that may be present in both the antigens, i.e., the two antigens are immunologically unrelated as far as that antiserum is concerned.

12.6 RIA (Radio Immuno Assay)

Principle

Radioimmunoassay was developed by the Berson and Yalow for the measurement of insulin in human plasma. RIA principles have found by an application in the field of drug analysis, pharmacokinetic studies, drug therapy monitoring.Specifically RIA measures the actual effect change in concentrations of a particular substance present in a biological fluid based on in vitro system consisting of radioactive standards of the same substance and a specific antibody.Before the emergence of RIA as an acceptable analytical technique, a number of other methods were employed for the analysis of drugs in the plasma or TLC, GLC, Spectroflourimetry.

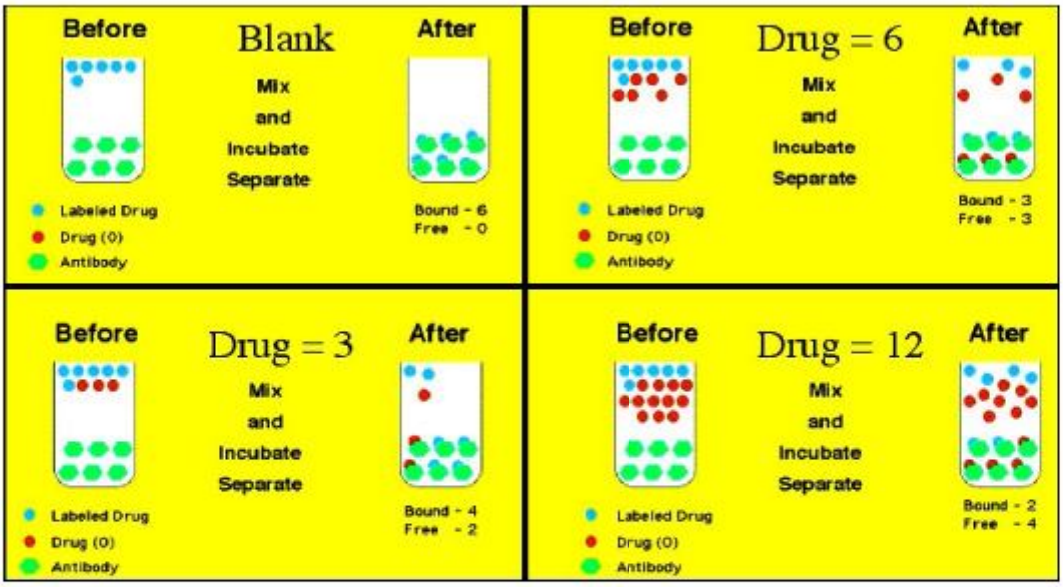

Fig. 12.3 : Radioimmunoassay (RIA)

Radioimmunoassay (RIA) is a very sensitive in vitro assay technique used to measure concentrations of antigens (for example, hormone levels in the blood) by use of antibodies. As such it can be seen as the inverse of a radio binding assay, which quantifies an antibody by use of corresponding antigens.Although the RIA technique is extremely sensitive and extremely specific, requiring specialized equipment, it remains the least expensive method to perform such tests. It requires special precautions and licensing, since radioactive substances are used. Today it has been supplanted by the ELISA method, where the antigen-antibody reaction is measured using colorimetric signals instead of a radioactive signal. However, because of its robustness, consistent results and low price per test, RIA methods are again becoming popular. It is generally simpler to perform than a bioassay.

The RAST test (radio allergo sorbent test) is an example of radioimmunoassay. It is used to detect the causative allergen for an allergy.

Method

To perform a radioimmunoassay, a known quantity of an antigen is made radioactive, frequently by labeling it with gamma-radioactive isotopes of iodine attached to tyrosine. This radio-labeled antigen is then mixed with a known amount of antibody for that antigen, and as a result, the two specifically bind to one another. Then, a sample of serum from a patient containing an unknown quantity of that same antigen is added. This causes the unlabeled (or "cold") antigen from the serum to compete with the radio-labeled antigen ("hot") for antibody binding sites. As the concentration of "cold" antigen is increased, more of it binds to the antibody, displacing the radio-labeled variant, and reducing the ratio of antibody-bound radio-labeled antigen to free radio-labeled antigen.

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The bound antigens are then separated from the unbound ones, and the radioactivity of the free antigen remaining in the supernatant is measured using a gamma counter. Using known standards, a binding curve can then be generated which allows the amount of antigen in the patient's serum to be derived.

Requirements:

1. Preparation and characterization of an antigen (Ligand to be analyzed)
2. Radiolabelling of the antigen
3. Preparation of the specific antibody
4. Development of assay system.

Procedure:

- Mix sample containing drug with fixed quantity of labeled drug and antibody
- Allow to incubate
- Separate drug bound to antibody from unbound drug
- Measure radioactivity associated with bound labeled drug
- low drug concentration means more bound radioactivity and higher measurement
- High drug concentration means less bound radioactivity and lower measurement
- Determine standard curve
- Non-linear plot of radioactivity versus concentration

12.7 Viva Voce

1 What is the full form of ELISA?

2 What is the objective to use western blotting?

3 What will be the outcome if Rh^+ blood is transfused into Rh^- bearing person?

4 Differentiate between RID and ODD?

5 Why do we use RIA?

12.8 References

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Unit-13

Genetics - I

NOTES

Structure of the Unit

13.0 Objectives

After going through this unit you will be able to understand:

- Differentiate various chromosomal parts
- Giant chromosomes
- Various problems arising due to changes in chromosomes

13.1 Linear differentiation of Chromosomes through Banding Techniques

G-banding

G banding, or Giemsa banding is a technique used in cytogenetics to produce a visible karyotype by staining condensed chromosomes. It is useful for identifying genetic diseases through the photographic representation of the entire chromosome complement. The metaphase chromosomes are treated with trypsin (to partially digest the chromosome) and stained with Giemsa stain. Heterochromatic regions, which tend to be rich with adenine and thymine (AT-rich) DNA and relatively gene poor, stain more darkly in G-banding. In contrast, less condensed chromatin which tends to be rich with guanine and cytosine (GC-rich) and more transcriptionally active incorporates less Giemsa stain, and these regions appear as light bands in G-banding. The reverse of G-bands is obtained in R-banding. Banding can be used to identify chromosomal abnormalities, such as

translocations, because there is a unique pattern of light and dark bands for each chromosome. It is difficult to identify and group chromosomes based on simple staining because the uniform color of the structures makes it difficult to differentiate between the different chromosomes. Therefore, techniques like G-banding were developed that made "bands" appear on the chromosomes. These bands were the same in appearance on the homologous chromosomes, thus, identification became easier and more accurate. The acid-saline-Giemsa protocol reveals G-bands.

NOTES

Solutions Needed

2 X SSC = 8 .8 g NaCl

4 .4g tri sodium citrate

Make up to 500 ml with distilled water

Trypsin -Giemsa solution

1.0 ml KaryomaxGiemsa

45 ml Gurr pH 6.8 phosphate buffer

4 drops 0.0125% trypsin

0.9 % NaCl = 0 .9g NaCl in 100 ml distilled water

Protocol

Make air dried preparations by dropping small droplets of cell suspension on the slides and blow dry. Bands are sharper if preparations are aged 7-10 days at room temperature, but this is not essential.

2. Incubate slides in Coplin jars (5-6 per jar) in 2 X SSC at 60 -65°C for 11/2 hrs.

3. Transfer all slides to 0.9 % NaCl at room temperature. Then rinse each slide in fresh NaCl and drain . Thorough rinsing is critical.

4. Stain 4-6 minutes in trypsin -Giemsasolution (below). Remove the metallic film which forms on the stain surface with a cotton ball before placing slides inCoplin jar or float the film off with running water before removing slides.

5. Transfer all slides in jar to fresh buffer (1:1 buffer:dist water).

6. Rinse slides individually in 2 changes of buffer (1:1 buffer:dist water). Thorough rinsing is critical. Shake off excess liquid and blow dry with an air jet.

C Banding

Protocol

NOTES

1. Germinate seeds in petri dishes on moist filter paper for 3–4 days.
2. Collect root tips when 1.5–2.5-cm long and pretreat with 0.05% colchicine for 3 h at room temperature. The colchicine pretreatment results in a lower mitotic index compared with ice water pretreatment but give better chromosome morphology and band contrast.
3. Fix in Carnoy's I for at least 1 h, but complete metaphase spreads are more easily obtained when the material is stored in fixative solution for a longer time (2 weeks to several months) at 4°C. For meiotic chromosomes, fix anthers that are at the appropriate stage of division without pretreatment in Carnoy's I.
4. Make squash preparations in 45% acetic acid; a 2–3-min pretreatment with 45% acetic acid before squashing softens the tissue and makes squashing easier. If possible, remove the very tip of the root with a razor blade, squeeze the meristematic tissue out with a scalpel, and squash using only gentle heat. Check quality of preparations under phase contrast.
5. Remove cover slips by placing on dry ice until frozen.
6. Immediately place slides in 99% ethanol. Slides should be kept in ethanol overnight, and the staining procedure continued the next day.
7. Air dry the slides for several minutes.
8. Incubate slides for 1 min in 0.2 N HCl in a water bath at 60°C. The time and temperature of the treatment is critical for good contrast. Prepare a 2 N HCl stock solution, which is 86 mL concentrated HCl per 500 mL distilled water. This solution can be stored for several months and should be diluted 1:9 before use to obtain a 0.2 N treatment solution.
9. Wash briefly in distilled water.
10. Incubate the slides for 7 min in a saturated $Ba(OH)_2$ at room temperature. Prepare a saturated $Ba(OH)_2$ solution in distilled water, stir for 30 min at room temperature, and filter before use. This solution needs to be prepared fresh each time.
11. Wash briefly in distilled water.
12. Incubate slides for 30 min in 2 X SSC in a water bath at 60°C.

20 X SSC stock solution

tri-sodium-citrate-2-hydrate- 88.2 g

NaCl -175.3 g

Distilled water dH_2O to 1 L

NOTES

13. Move slides directly from 2 X SSC into the staining solution, which is a 10% solution of Giemsa stain in Soerensen phosphate buffer, pH 7.2. Begin with a low Giemsaconcentration and control the staining under the microscope. If necessary, add more Giemsa stain. Staining time will vary (10–45 min) between different cells on the same slide and also between different slides. Slides should be individually checked every few minutes for best contrast. A staining time of about 30 min is optimal.

Soerensen's buffer

Stock Stain

Part A- sodium phosphate dibasic (Na_2HOP_4) 9.47g / L H_2O 58 mL

Part B- potassium dihydrogenphosphate (KH_2PO_4) 9.07g / L H_2O 42 mL

14. Dip the slides in distilled water to rinse and air dry.

15. For permanent slides, soak in xylene and mount in Permount.

Q Banding

Reagents/materials:
QuinicineDihydrochloride: dissolve 0.25g in 50mldistilled water store away from light.

Store desiccated in light free container at -20°.

MacILvaine Buffer

Solution A: 0.1 mol/L citric acid (19.2g to 1 L distilled water)

Solution B: 0.2 mol/L Na2HPO4 (28.4g to 1 L distilled water)

Q-Banding Method:

1. soak slide in methanol for 5 minutes. Pour methanol off and add thestain.
2. Stain for 5 minutes with QM
3. Rinse 3 times in distilled water
4. Expose in MacILvaine buffer for 30 seconds
5. Mount with a coverslip using MacILvainebuffer diluted one part to four parts distilled water.
6. Examine by fluorescent microscope (390-490 excitation; suppression: 515nm)
7. Scan cells & photograph.

13.2 Study of Polytene Chromosomes

Polytene chromosomes are over-sized chromosomes which havedeveloped from standard chromosomes and are commonly found inthe salivary glands of Drosophila melanogaster. Specialized cellsundergo repeated rounds of DNA replication without cell division(endo-mitosis), to increase cell volume, forming a giant polytenechromosome. Polytene chromosomes form when multiple rounds ofreplication produce many sister chromatids that remain synapsedtogether.

NOTES

Polytene chromosomes have characteristic light and dark banding patterns that can be used to identify chromosomal rearrangements and deletions. Dark banding frequently corresponds to inactive chromatin, whereas light banding is usually found at areas with higher transcriptional activity. The banding patterns of the polytene chromosomes of *Drosophila melanogaster* were sketched in 1935 by Calvin B. Bridges, in such detail that his maps are still widely used today. The banding patterns of the chromosomes are especially helpful in research, as they provide an excellent visualization of transcriptionally active chromatin and general chromatin structure. For example, the polytene chromosomes in *Drosophila* have been used to support the theory of genomic equivalence, which states that all of the cells in the body maintain the same genome. Chromosome puffs are diffused uncoiled regions of the polytene chromosome that are sites of RNA transcription. A Balbiani ring is a large chromosome puff. Polytene chromosomes are about 200 μm in length. The chromonemata of these chromosomes divide but do not separate. Therefore, they remain together to become large in size. Another form of chromosomal enlargement that provides for increased transcription is the lampbrush chromosome.

13.3 Study of Lampbrush Chromosomes

Lampbrush chromosomes are a special form of chromosomes that are found in the growing oocytes (immature eggs) of most animals, except mammals. Lampbrush chromosomes of tailed and tailless amphibians, birds and insects are described best of all. Chromosomes transform into the lampbrush form during the diplotene stage of meiotic prophase I due to an active transcription of many genes. They are highly extended meiotic half-bivalents, each consisting of 2 sister chromatids.

Lampbrush chromosomes are clearly visible even in the light microscope, where they are seen to be organized into a series of chromomeres with large chromatin loops extended laterally. Amphibian and avian lampbrush chromosomes can be microsurgically isolated from oocyte nucleus (germinal vesicle) with either forceps or needles. A given loop

NOTES

always contains the same DNA sequence, and it remains extended in the same manner as the oocytes grow. These chromosomes are producing large amounts of RNA for the oocyte, and most of the genes present in the DNA loops are being actively expressed. Each lateral loop contains one or several transcription units with polarized RNP-matrix coating the DNA axis of the loop. The majority of the DNA, however, is not in loops but remains highly condensed in the chromomeres on the axis, where genes are generally not expressed. It is thought that the interphase chromosomes of all eukaryotes are similarly arranged in loops. Although these loops are normally too small and fragile to be easily observed in a light microscope, other methods can be used to infer their presence. For example, it has become possible to assess the frequency with which two loci along an interphase chromosome are paired with each other, thus revealing candidates for the sites on chromatin that form the closely apposed bases of loop structures. These experiments and others suggest that the DNA in human chromosomes is organized into loops of different lengths. A typical loop might contain between 50,000 and 200,000 nucleotide pairs of DNA.

Giant chromosomes in the lampbrush form are useful model for studying chromosome organization, genome function and gene expression during meiotic prophase, since they allow the individual transcription units to be visualized. Moreover lampbrush chromosomes are widely used for high-resolution mapping of DNA sequences and construction of detail cytological maps of individual chromosomes.

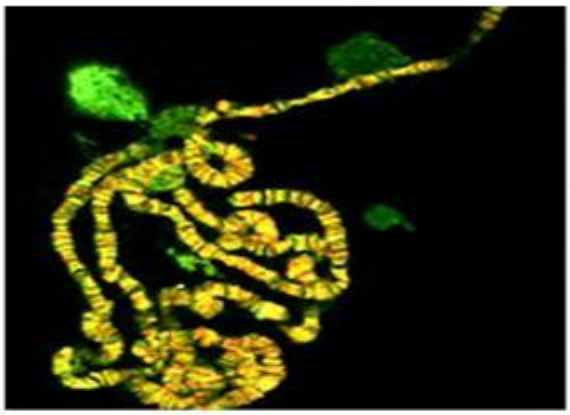

Fig. 13.1 : Polytene Chromosomes

Fig. 13.2 : Lampbrush chrompsome from the cell nucleus of an ovarial egg from Triton sp., a salamander.

13.4 Study of B Chromosomes

In addition to the normal karyotype, wild populations of many animal, plant, and fungi species contain B chromosomes (also known as supernumerary or accessory

chromosomes). These chromosomes are not essential for the life of a species, and are lacking in some (usually most) of the individuals. Thus a population would consist of individuals with 0, 1, 2, 3 (etc.) supernumeraries.

NOTES

Most B chromosomes are mainly or entirely heterochromatic (and so would be largely non-coding), but some, such as the B chromosomes of maize, contain size-able euchromatic segments. In general it seems unlikely that super numeraries would persist in a species unless there was some positive adaptive advantage, which in a few cases has been identified. For instance, the British grasshopper *Myrmeleotettixmaculatus* has two structural types of B chromosomes: metacentrics and submetacentrics. The supernumeraries, which have a satellite DNA, occur in warm, dry environments, and are scarce or absent in humid, cooler localities. In plants there is a tendency for B chromosomes to be present in the germ-line, but to be lost from other tissues such as root tips and leaves.

General properties of B chromosomes

Form and size-B chromosomes are smaller than A chromosomes except in afew cases, in which they are of equal size. They often have distinctcentromere positions and can be readily identified at mitosis. Variants include b chromosomes as isochromosomes or telocentrics, and, in a few species, they appear as microchromosomes.

Structural polymorphism- the normal situation is for only one form of a B chromosome, withvariants arising at mutation frequency.

Chromatin- B chromosomes are described as heterochromatic in about half ofplants that carry them (Zea mays). In general, theirheterochromatic content is similar to that of their A chromosomes.

Inheritance-

The inheritance of B chromosomes is non-mendelian and irregularowing to vagaries in the levels of paring, to degrees of meioticelimination and to various drive processes. Drive is mostly caused bydirected nondisjunction of sister chromatids at the first pollen mitosis,such that the generative nucleus carries the unreduced number, whichthen forms the sperm (e.g. many species of Gramineae). In rye,unusually, this drive happens on both the male and the femaleside, and, in maize, the nondisjunction happens at the second pollenmitosis, followed by preferential fertilization by the B-chromosomecontainingsperm. Meiotic drive and accumulation at earlier developmentalstages in the germ line operate in a few cases. Theseirregularities in transmission generate a numerical polymorphism inpopulations, with a spectrum of B chromosome numbers includingindividuals with none. There is usually a modal number

and anequilibrium frequency based on a balance between drive and theharmful effects caused by high numbers. Drive is by no means auniversal process: it is known in,60% of species for whichtransmission data are available. In the others, there is no known driveand no real understanding of how the population equilibriumfrequencies are maintained.

NOTES

13.5 Chromosomal Aberrations

The chromosome set of a species remains relatively stable over long periods of time. However, within populations abnormalities can be found involving the structure or number of chromosomes. These alterations arise spontaneously from errors in the normal processes of the cell. Their consequences are usually deleterious, giving rise to individuals who are unhealthy or sterile, though in rare cases alterations provide new adaptive opportunities that allow evolutionary change to occur. In fact, the discovery of visible chromosomal differences between species has given rise to the belief that radical restructuring of chromosome architecture has been an important force in evolution.

Structural Aberrations: -These occur due to a loss or genetic material, or a rearrangement in the location of the genetic material. Theyinclude: deletions, duplications, inversions, ring formations, and translocations.

Deletions: A portion of the chromosome is missing or deleted. Known disorders include Wolf-Hirschhorn syndrome, which is caused by partial deletion of the short arm of chromosome 4; and Jacobsen syndrome, also called the terminal 11q deletion disorder.

Duplications: A portion of the chromosome is duplicated, resulting in extra genetic material. Known disorders include Charcot-Marie-Tooth disease type 1A which may be caused by duplication of the gene encoding peripheral myelin protein 22 (PMP22) on chromosome 17.

Translocations: When a portion of one chromosome is transferred to another chromosome. There are two main types of translocations. In a reciprocal translocation, segments from two different chromosomes have been exchanged. In a Robertsonian translocation, an entire chromosome has attached to another at the centromere; these only occur with chromosomes 13, 14, 15, 21 and 22.

Inversions: A portion of the chromosome has broken off, turned upside down and reattached, therefore the genetic material is inverted.

Rings: A portion of a chromosome has broken off and formed a circle or ring. This can happen with or without loss of genetic material.

Iso-chromosome: Formed by the mirror image copy of a chromosome segment including the centromere.

Structural aberrations also include some disorders which are characterized by chromosomal instability and breakage. One example is the creation of a fragile site on the X chromosome - Fragile X syndrome. Boys are worse affected by this because they only have one X-Chromosome but even in girls, Fragile X syndrome can cause learning difficulties.

Most chromosome anomalies occur as an accident in the egg or sperm, and are therefore not inherited. The anomaly is present in every cell of the body. Some anomalies, however, can happen after conception, resulting in mosaicism (where some cells have the anomaly and some do not). Chromosome anomalies can be inherited from a parent or be "de novo". This is why chromosome studies are often performed on parents when a child is found to have an anomaly.

Numerical aberrations: - In genetics as a whole there are few topics that impinge on human affairs quite so directly as this one. Foremost is the fact that a large proportion of genetically determined ill health in humans is caused by abnormal chromosome numbers. Additionally, manipulation of chromosome number is routinely used by breeders to improve agriculturally important species. Changes in chromosome number are of two basic types: changes in whole chromosome sets (resulting in a condition of aberrant euploidy) and changes in parts of chromosome sets (resulting in aneuploidy).

Aberrant Euploidy

Organisms with multiples of the basic chromosome set are called euploid. Eukaryotes such as plants, animals, and fungi carry in their cells either one chromosome set (haploid) or two sets (diploid). In these species, the haploid and diploid states are both cases of normal euploidy. Organisms that have more or less than the normal number of sets are aberrant euploids. Polyploids are individual organisms in which there are more than two chromosome sets. They can be represented by $3n$ (triploid), $4n$ (tetraploid), $5n$ (pentaploid), $6n$ (hexaploid), and so forth. In essentially diploid taxa, an individual organism with only one chromosome set (n) is called a monoploid to distinguish it from species in which all individuals are normally haploid (also n).

Monoploidy:- Male bees, wasps, and ants are monoploid. In the normal life cycles of these insects, males develop partheno-genetically from unfertilized eggs. However, in most species, monoploid individuals are abnormal, arising in natural populations as rare aberrations. The germ cells of a monoploid cannot proceed through meiosis normally, because the chromosomes have no pairing partners. Thus, monoploids are characteristically sterile. (Male bees, wasps, and ants bypass meiosis; in these types, gametes are produced by *mitosis*.)

NOTES

Polyploidy: - In aberrant euploids, there is often a correlation between the number of copies of the chromosome set and the size of the organism and its component parts. For example, typically a tetraploid organism looks very similar to its diploid counterpart in its proportions, except that the tetraploid is bigger as a whole and in its component parts. The higher the ploidy level, the larger is the size. In the realm of polyploids, we must distinguish between autopolyploids, which are composed of multiple sets originating from within one species, and allo-polyploids, which are composed of sets from two or more different species. Allopolyploids form only between closely related species; however, the different chromosome sets are only partly homologous (homologous), not fully homologous, as they are in autopolyploids.

Aneuploidy

Aneuploidy is the second major category of chromosome aberrations in which chromosome number is abnormal. An aneuploid is an individual organism whose chromosome number differs from the wild type by part of a chromosome set. Generally, the aneuploid chromosome set differs from wild type by only one chromosome or by a small number of chromosomes. An aneuploid can have a chromosome number either greater or smaller than that of the wild type. Aneuploid nomenclature is based on the number of copies of the specific chromosome in the aneuploid state. For example, the aneuploid condition 2n−1 is called monosomic (meaning "one chromosome") because there is only one copy of some specific chromosome present instead of the usual two found in its diploid progenitor. For autosomes in diploid organisms, the aneuploid 2n+1 is called trisomic, 2n−1 is monosomic, and 2n−2 (where the −2 represents homologs) is nullisomic. In haploids, n+1 is di-somic. Special symbolism has to be used to describe sex-chromosome aneuploids, because we are dealing with two different chromosomes (X and Y) and the homogametic and heterogametic sexes have different sex-chromosome compositions even in euploid individuals. The symbolism merely lists the copies of each sex chromosome, such as XXY, XYY, XXX, or XO (the "O" stands for absence of a chromosome and is included to show that the symbol is not a typographical error).

Nondisjunction:-

The cause of most aneuploid conditions is nondisjunction in the course of meiosis or mitosis. Disjunction is another word for the normal segregation of homologous chromosomes or chromatids to opposite poles at meiotic or mitotic divisions. Nondisjunction is a failure of this process, and two chromosomes or chromatids go to one pole and none to the other. In meiotic nondisjunction, the chromosomes may fail to disjoin at either the first or the second division. Either way, n+1 and n−1 gametes are produced. If

an n−1 gamete is fertilized by an n gamete, a monosomic 2n−1 zygote is produced. The fusion of an n+1 and an n gamete yields a trisomic 2n+1.

13.6 Viva-Voce

1 What are giant chromosomes?

2 What is the difference between B chromosome and A chromosome?

3 What is euploidy?

4 What are monosomics?

13.7 References

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Unit-14

Genetics - II

NOTES

Structure of the Unit

14.0. Objectives

After going through this unit you will be able to understand:

- Problems based on Mendel's laws

14.1 Problems based on Mendel's laws

Gregor Mendel was a monk who lived in an Austrian monastery. He spent his time crossing the pea plants. As he did this over & over, he noticed some patterns to the inheritance of traits from one set of pea plants to the next. By carefully analyzing his pea plant numbers, he discovered three laws of inheritance.

Mendel's Laws are as follows:

1. The Law of Dominance

2. The Law of Segregation

3. The Law of Independent Assortment

Problems

Que.1 Which cross would best illustrate Mendel's Law of Segregation?

A. TT x tt

B. Hh x hh

C. Bb x Bb

D. rr x rr

Que.2 In the cross Yy x Yy, what percent of offspring would have the same phenotype as the parents?

A. 25%

B. 50%

C. 75%

D. 100%

NOTES

Que.3 In a certain plant, purple flowers are dominant to red flowers. If the cross of two Purple-flowered plants produces some purple-flowered and some red-flowered plants, what is the genotype of the parent plants?

A. PP x Pp

B. Pp x Pp

C. pp x PP

D. pp x pp

Que. 4-8 Use this information to solve these questions.

A white-flowered plant is crossed with a pink-flowered plant. All of the F1 offspring from the cross are white.

Que.4 Which phenotype is dominant?

Que.5. What are the genotypes of the original parent plants?

Que.6. What is the genotype of all the F1 offspring?

Que.7. What would be the percentages of genotypes & phenotypes if one of the white F1 plants is crossed with a pink-flowered plant?

Que.8. Which of Mendel's Laws is/are illustrated in this question?

Que.9 The phenotype of a pea plant can best be determined by:

A. analyzing its genes

B. looking at it

C. crossing it with a recessive plant

D. eating it

Que.10. Mendel formulated his Law of Segregation after he had:

A. studied F1 offspring

B. studied F2 offspring

C. produced mutations

D. produced hybrids

NOTES

Que.11. Which cross would produce phenotypic ratios that would illustrate the Law of Dominance?

A. TT x tt

B. TT x Tt

C. Tt x Tt

D. tt x tt

Que.12. The mating of two curly-haired brown guinea pigs results in some offspring withbrown curly hair, some with brown straight hair, some with white curly hair, and evensome with white straight hair. This mating illustrates which of Mendel's Laws?

A. Dominance

B. Segregation

C. Independent Assortment

D. Sex-Linkage

Que.13. Considering hybridization in a trait like the color of the flowers of a given plant species (red/yellow) conditioned by a pair of different alleles in relation to complete dominance (red dominant/ yellow recessive), why in the F1 generation is one of the colors missing?

Que.14. Considering hybridization in a given trait like the color of the hair of a mammalian species (white/black) conditioned by a pair of different alleles under complete dominance (black dominant, B/ white recessive, w), how can the phonotypical proportion obtained in the F2 generation be explained? What is this proportion?

Que.15. Why can the crossing of an individual that manifests dominant phenotype with another that manifests recessive phenotype (for the same trait) determine whether the dominant individual is homozygous or heterozygous?

Answers

Ans.1 C, both parent show dominant trait, but some recessive offspring will be produced (each parent carries a "b").

Ans.2 C, in the completed p-square, 3 of 4 boxes will have at least 1 "Y", producing the dominant phenotype (same as parents).

Ans.3 B, for any offspring to be recessive, each parent must have at least one "p".

Ans.4 white

Ans.5 WW(pure white) X ww(pink)

Ans.6 Ww (white)

Ans.7 50% heterozygous white & 50% homozygous recessive pink.

Ans.8 Dominance is illustrated by the original cross (WW x ww).

NOTES

Ans.9 B

Ans.10 B, he crossed two hybrids (F1's) and got a second generation (F2).

Ans.11 A, one parent tall, the other short, all offspring would be tall.

Ans.12 C, the question involves two different traits(hair color & hair texture), this is the only law that deals with two different traits.

Ans.13 In this monohybrid cross one of the colors does not appear in the F1 generation because their parental generators are pure, i.e., homozygous, and in F1 all descendants areheterozygous (each parental individual forms only one type of gamete). Since onlyheterozygous genotypes appear and red is dominant over yellow the individuals of the F1generation will present only red flowers.

Ans.14 Monohybrid crossis conditioned by two different alleles,the F1 generation presents only heterozygous individuals (Bw). In F2 there is one individual BB, two individuals Bw and oneindividual ww. In relation to the phenotype there are in F2 two black individuals and onewhite individual, since black is the dominant color. So the proportion is 3:1, three blackhairedto one white-haired.

Ans.15 From the crossing of an individual having recessive phenotype with another having dominant phenotype (for the same trait) it is possible to determine whether the dominantindividual is homozygous or heterozygous. This is true because the genotype of therecessive individual is obligatorily homozygous, for example, aa. If the other individual isalso homozygous, AA, the F1 offspring will be only heterozygous (aa x AA = only Aa). If theother individual is heterozygous there will be two different genotypes, Aa and aa inthe 1:1 proportion. So if a recessive phenotype appears in the direct offspring the parentalindividual that manifests dominant phenotype is certainly heterozygous.

14.3 Viva-Voce

1 What is the meaning of heterozygous?

2 What do you understand by term homozygous?

3 How will you differentiate between allele and genes?

4 What is phenotype?

5 What is genotype?

14.3 References

NOTES

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Unit-15

Biostatistics-I

NOTES

Structure of the Unit

15.0 Objective

After going through this unit you will be able to-

- Understand and compute Mean, Mode& Median
- Understand and explain the various methods of data analysis.
- Compute standard deviation

15.1 Introduction

Biostatistics is the application of statistical methods to the problems of biology, including human biology, medicine and public health. Statistical methods include the collection, organization, summary-classification and analyzing and measured evaluation of facts to reach some inference.

A population can be expressed by means of a location parameter, around which most of the values of the population should be clustered. Since this central value locates the population, the location parameters are also called *measures of central tendency.* There are at least three measures of central tendency which are commonly used (i) arithmetic mean, (ii) median and (iii) mode.These measures will not tell the researcher how the scores tend to be distributed. For this, we use a different set of measures which are called measures of *'variability'* or measures of *'spread'* or *'dispersion'*. The most commonly used measures of variability include the variance and standard deviation.Standard deviation of means, also called standard error.

15.2 Calculation of Central Tendencies Median and Mode Method

NOTES

Central tendency is a number or a quantity which is typical or representative of a set of data. Measures of this type are known as averages.

[I] The Median

Median or (middle item) is the middle value (or the mean of the two middle values) of a set of numbers arranged in order of magnitude.

e.g. in the set of numbers : 1,2,4,7,8,9,10.

The median is 7

Similarly, in the set of numbers : 2,3,4,5,6.7

The median is $\frac{3+4}{2} = 3.5$

The median number is calculated by

$\left(\frac{N+1}{2}\right)$, where N is the total number of observations.

Example

Calculation of median when the number of items is odd.

Find the median of the following observations.

5,6,7,9,6,9,11,12,13,12,13,14,14

Step 1

Median number = $\frac{13+1}{2} = 7$

Step 2

Now arrange the Fig.s in ascending order. 5, 6, 6,7, 9, 9, 11, 12, 12,13, 13, 14, 14.

Step 3

Here 7^{th} Fig. is 11, therefore, median is 11.

[II] The Mode

The mode is the most commonly occurring value or the value of that variable which has the maximum frequency.

e.g. if the set of numbers is 2,2,5,7,7, 7, 9, 10, 11, 12, then the mode is 7.

Example

Computation of mode from continuous series with all items having different frequencies.

NOTES

Find out the mode from the following data.

Numbers of stomata Per sq. mm	Frequency
51-52	10
52-53	19
53-54	17
54-55	16
55-56	22
56-57	18
57-58	19
58-59	12
59-60	14
60-61	13
61-62	15

Here the modal class would be 55-56 with maximum frequency.

Now use the following formula.

Formula

$$\text{Mode} = l_1 + \frac{\Delta_1}{\Delta_1 + \Delta_2} \times i$$

Where l_1 = lower limit of modal class

Δ_1 = difference between the frequencies of the modal class and the next lower class

Δ_2 = difference between the frequencies of the modal class and the next higher class

i = width of the modal class

NOTES

Now calculate the values and substitute

$l_1 = 55, \Delta_1 = 22 - 16 = 6, \Delta_2 = 22 - 18 = 4, i = 1$

$$\text{Mode} = 55 + \frac{6}{6+4} \times 1, \quad = \frac{55+6}{10} \times 1$$

Mode = 55 + 0.6 x 1 = 55.6

15.3 Calculation of Standard Deviation

Method

It is the most important measure of dispersion that gives the measure of the amount of deviation of individuals from the mean. It is calculated by one of the following formula :

$$\text{Formula} = S = \sqrt{\frac{\sum(\bar{x} - x)^2}{n}}$$

Where S or is the standard deviation

$\sum$ = sign of summation

$\bar{x}$ = arithmetic mean

x = various values

n = number of values (items)

Example

Find the standard diviation of the set of values 50, 60, 70, 88, 32

Formula

Step 1

Calculate the arithmetic mean ($\bar{x}$) of the set of values.

x/n = $\bar{x}$, x = 50+60+70+88+32 = 300; n = 5, 300/5 = 60, $\bar{x}$ = 60

Step 2

Calculate the difference of the items from this average (sign may be ignored).

$\bar{x}$ - x, 10,0,10,28,28

NOTES

Step 3

Calculate the squares of these differences.

$(\bar{x}-x)^2$ 10 x 10 = 100, 0 x 0 = 0, 10 x 10 = 100, 28 x 28 = 784, 28 x 28 = 784

Step 4

Calculate the sum of squares of differences to get the quantity known as the sample sum of squares.

$(\bar{x}-x)^2$ 100 + 0 + 100 + 784 + 784 = 1768

Step 5

Divide the sample of sum squares by the number of items (n=5). This the quantity known as 'sample variance'.

$$\sqrt{\frac{(\bar{x}-x)^2}{n}} = \sqrt{\frac{1768}{5}} = \sqrt{353.6}$$

Step 6

Take square root of variance to obtain the standard deviation.

$$S = \sqrt{353} = 18.80$$

15.4 Calculation of Standard Error

Method

This method can be used when ratio between two classes is to be tested –

$$S.E.r = \sqrt{\left(\frac{P.q}{n}\right)}$$

Where P = one of the obtained percentages (denoted by decimals)

q = another of the obtained percentages (1-P)

n = total number (P+q)

Deviation = the difference between expected and obtained values.

If the ratio $\frac{Deviation}{S.E.r}$ is less than 1.96, the obtained results are said to be a good fit.

NOTES

Example

In F_2 generation Mendel obtained as follows –

Round seeds	Wrinkled seeds	Total
5,574 % = 0.74	1,850 % = 0.26	7,324

Expected ratio : 3:1 i.e. 0.75 : 0.25

P = 0.74 q = 0.26 n = 7,324

Deviation = 0.75 – 0.74 = 0.1

Substituting the values

$$S.E.r = \sqrt{\left(\frac{P \times q}{n}\right)} = \sqrt{\frac{(0.74 \times 0.26)}{7,324}} = \sqrt{\frac{0.1924}{7324}}$$

$$\therefore \frac{Deviation}{S.E.r} = \frac{.01}{.005118} = 1.95$$

i.e. less than 1.96 and hence good fit.

Thus, the result obtained are close to the expected ratio and the hypothesis is acceptable.

15.5 Viva-Voce

1 Define biostatistics.

2 What is inferential biostatistics?

3 Who is called father of Biostatistics

4 When are two variables positively related?

Fill in the Blanks :

(1) When values of two variables deviate in the same direction, it is called _____ correlation. (negative/positive)

(2) Two variables are said to be _____ if the change in one variable results in corresponding change in other variable. (correlated/partially correlated)

15.6 References

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NOTES

Unit-16

Biostatistics - II

NOTES

Structure of the Unit

16.0 Objectives

After going through this unit you will be able to-

- Understand 't-test' for significance
- Understand and explain the Calculation of x^2 (chi-square)
- Compute Correlation and Analysis of variance (ANOVA)

16.1 Introduction

If two samples are available and their means calculated, one is always interested in finding whether these samples came from same population of iron two populations having different means. In such a case, the standard error needs to be calculated by t -test for sinificance. Chi-square test is most frequently used for testing whether the observed results are in agreement with the hypothetical results expected on the basis of sound principles The statistical technique used to compare means of variations of more than two populations is called **'analysis of variance (ANOVA).** The concept of **ANOVA** or analysis of variance was introduced by **R.A. Fisher.**

16.2 't-test' for Significance

Method

This is a method used to find out whether the differences between the two different samples are significant or mere fluctuations or errors.

$$t = \frac{\text{mean difference}}{\text{standard error of differences}}$$

$$\text{mean difference} = \frac{\text{sum of difference}}{\text{number of trials (n)}}$$

standard error of difference

$$\frac{\sqrt{(\text{sum of square of difference}) - \frac{(\text{sum of differences})^2}{\text{n}}}}{(n-1).n}$$

NOTES

Example

The table below shows some hypothetical data on amino acid content of soyabean grown in ten different localities.

Locality or trials	Amino acid content data of Soyabean (in %) Amount of amino acid in %			
	Variety A	Variety B	Difference	Square of difference
1.	3	8	5	25
2.	6	4	2	4
3.	9	6	3	9
4.	15	11	4	16
5.	10	13	3	9
6.	12	17	5	25
7.	7	9	2	4
8.	13	12	1	1
9.	8	6	2	4
10.	12	8	4	16
Sum			31	113

NOTES

Step 1

Calculate the mean difference.

$$\text{mean difference} = \frac{\text{sum of difference}}{\text{number of trials (n)}}$$

$$= \frac{31}{10} = 3.1$$

Step 2

Calculate the standard error of differences.

$$\frac{\sqrt{(\text{sum of square of difference}) - \frac{(\text{sum of differences})^2}{\text{n}}}}{(n-1)\times n}$$

Sum of square of difference = 113

$(\text{sum of differences})^2 = (31)^2 = 961$

n = 10,

$(\text{sum of differences})^2/\text{n} = 961/10 = 96.1$

$$\sqrt{\frac{113-96.1}{(n-1).n}} = \sqrt{\frac{16.9}{(10-1).10}}$$

$$= \sqrt{\frac{16.9}{90}} = \sqrt{0.187}$$

$\sqrt{0.187} = 0.433$(standard error of differences)

Step 3 Substitute the value

$$t = \frac{\text{mean difference}}{\text{standard error of differences}} = \frac{3.1}{0.433} = 7.15$$

Step 4

Find out the value of p for 9 degrees of freedom (d.f. is one less than the number of trials or comparisons)

Degree of freedom (d.f.)	**Probability of larger value of t (P)**	
	0.05	0.01
1.	12.71	63.66
2.	4.30	9.92

3.	3.18	5.84
4.	2.78	4.60
5.	2.57	4.03
6.	2.45	3.71
7.	2.36	3.50
8.	2.31	3.36
9.	2.26	3.25
10.	2.23	3.17
11.	2.20	3.11
12.	2.18	3.06
13.	2.16	3.01
14.	2.14	2.98
15.	2.13	2.95
16.	2.12	2.92
17.	2.11	2.90
18.	2.10	2.88
19.	2.09	2.86
20.	2.09	2.84

NOTES

For 9 d.f.t = 2.26 for P of 0.05 and 3.25 for P of 0.01. Hence P is much < 0.01. This means that chances are much less than 0.01 (or 1% or 1.99). This difference is a sample chance or error. As such the amino acid content difference observed is highly significant. In t tests P <0.05 only is considered significant.

16.3 Calculation of X^2 (Chi-Square)

NOTES

Method

This is one of the most versatile methods in the statistical theory. It permits the test-whether observed frequencies in a distribution differ significantly from the frequencies which can be expected according to some hypothesis.

x^2 is calculated by the following formula –

$$x^2 = \Sigma\left[\frac{(X_0 - X_e)^2}{X_e}\right] \qquad \ldots\ldots (1)$$

Where $\sum$ = sign of summation;

x_0 = Observed numbers;

x_e = expected numbers

Since $x_0 - x_e = d$ i.e. deviation

$$x^2 = \frac{d^2}{X_e} \qquad \ldots\ldots (2)$$

Example

In F_2 generation, Mendel obtained 787 tall plants and 277 dwarf, out of the total of 1,064. As might be expected for 3:1 ratio, plants should have been 798 tall and 266 dwarf.

		Tall	**Dwarf**
1.	Observed no (X_0)	787	277
2.	Expected no. (X_e)	798	266
	$(X_0 - X_e)$	11	11
	$(X_0 - X_e)^2$	121	121
	$(X_0 - X_e)^2$	121 +	121
	X_e	798	266

$$x^2 = \frac{121}{798} + \frac{121}{266} = 0.15 + 0.45 = 0.60$$

Now value of x^2 is to be tested

Degree of freedom (d.f.)	Probability of larger value of Chi-square (P)		
	0.99	0.50	0.05
1	0.00016	0.455	3.841
2	0.0201	1.386	5.991
3	0.115	2.366	7.815
4	0.297	3.337	9.488
5	0.254	4.351	11.070

d.f. = degree of freedom

= number of actual classes – 1

In this case d.f. = 2-1 = 1

Find out 0.60 value against the d.f. 1.

It falls between P 0.05 and 0.50. The probability, therefore, is 0.05 or 5% or 5 times in hundred that a higher value may be obtained. Begin within the limits of higher values mentioned in the table the values are considered to

16.4 Correlation

Defination

According to **Connor** 'if two or more quantities vary in sympathy and the movements in one tend to be accompanied by corresponding movements in the other, then these two quantities are said to be correlated

Correlation analysis- we are concerned whether two variables are independent or they vary together in positive or negative direction. In correlation the two variables are not related as independent and dependent variables. It means in correlation both the variables are affected by a common cause and the degree to which these variables vary together is estimated. The concept of correlation analysis and term correlation originated with **Galton** in 1888

Regression Analysis,-- the dependence of one variable on another variable is determined. Therefore, the two variables are related as independent and dependent variables.

Regression analysis is employed to predict or estimate the value of one variable corresponding to a given value of another variable. Regression equations are applied to determine changes in Y due to changes in X variable.

NOTES

Significance of Correlation

The study of correlation is of great significance in practical life, because of the following reasons :

1. The study of correlation enables us to know the nature, direction and degree of relationship between two or more variables.
2. Correlation studies help us to estimate the changes in the value of one variable as a result of change in the value of related variables. This is called **regression analysis.**
3. Correlation analysis helps us in understanding the behaviourof certain events under specific circumstances. For example, we can identify the factors for rainfall in a given area and how these factors influence paddy production.
4. Correlation facilities the decision making in the business world. It reduces element of uncertainty in decision-making.
5. It helps in making predictions.

Types of Correlation

Depending on its extent and direction the correlation between two variables may be of following types :

1. Positive and Negative Correlations

The positive and negative correlations are based on the direction of change in the value of two variables :

1. **Perfect Positive Correlation :** When two variables move proportionately in the same direction, i.e., the increase in the value of one variable leads to corresponding increase in the values of other variable, the correlation between them is called **perfect positive**. For example, increase in body weight with the increase in height presents positive correlation. It is also called **direct correlation**.
2. **Moderately Positive Correlation :** When two variables are partially positively correlated the correlation is termed **moderately positive correlation**, e.g., tallness of plants and the quantity of manure used etc.
3. **Perfect Negative Correlation :** The two variables show negative correlation when one variable increases with a constant interval and another decreases with constant interval. Thus, variables deviate in opposite directions. This is also called **inverse**

correlation. Examples of perfect negative correlation are very rare in nature but some approaching to that extent are temperature and lipid content of the body , etc.

4. **Moderately Negative Correlation :** When two variables are partially negatively correlated, the correlation is termed as **moderately negative correlation.** e.g., economic condition of state and cases of tuberculosis, income and infant mortality rate, etc.

5. **Absolutely no Correlation :** When two variables are completely independent of each other, the correlation is termed as **absolutely no correlation,** e.g., body, weight and I.Q. In this case no imaginary mean line is formed which could indicate the trend of correlation.

NOTES

2. Linear and Non-linear Correlations

The correlation can also be classified as linear or nonlinear on the basis of ratio of variations in the related variables :

1. **Linear Correlation :** Correlation between two variables is said to be linear if there is some constant relationship between the two variables. When the values of two variables are plotted as points in the XY plane, a straight line is formed
2. **Non-Linear Correlation :** The relationship between two variables is said to be **nonlinear** or **curvilinear** if corresponding to a unit change in one variable, the other variable does not change at the same constant rate but fluctuates.

3. Simple, Partial and Multiple Correlation

Based on the number of variables involved, the correlation may be of following three types :

1. **Simple Correlation :** In simple correlation only two variables are involved. Therefore, in simple correlation the relationship is between two variables such as intelligence of students and their performance (marks) in the examination.
2. **Multiple Correlation :** In multiple correlation relationship between three or more variables is studied. Simultaneous study of relationship between yield of wheat per acre, the amount of rainfall and the amount of fertilizer applied are the examples of multiple correlation.
3. **Partial Correlation :** In partial correlation, relation between more than two variables is considered but correlation is studied only between two variables. Other variables are assumed to be constant. For example, the correlation between the amount of fertilizers and the yield of wheat per acre is partial correlation in case rainfall is assumed to be normal.

NOTES

Correlation analysis measures the degree of association of two variables. Following methods are used to measure the correlation between two variables :

1. Scatter diagram method
2. Karl Person's coefficient of correlation
3. Spearman's rank correlation coefficient

1. Scatter Diagram Method Or Scatter Plot Method

Scatter diagram is the simplest method of studying relationship between two variables. It is in the form of graphic representation of degree and direction of correlation between two variables.

Say we take two variables X and Y for n number of samples (X_1, X_2, X_3, ...X_n) and plot X_1 against Y_1 as a dot (.) in the XY-plane, the diagram of dots so obtained is known as **scatter diagram** or **dot diagram**. It is customary to take dependent variable along Y-axis, i.e., along vertical axis and independent variable along X-axis or horizontal axis. Placement of dots on the graph reveals whether the changes in the variable are in the same direction or in opposite direction.

2. Karl Pearson's Correlation Coefficient

A scattered diagram, like a histogram, is a convenient way of displaying existence of correlation and direction of correlation, but is does not give any correlation value. To measure correlation, the coefficient of correlation is worked out by Karl Pearson's Coefficient of Correlation.

Coefficient of correlation is the degree to which two variables are inter-related. T is mathematical method of measuring the tendency of linear relationship between two variables. This was introduced by **Karl Pearson** (1867-1936). This measure of correlation is also known as **Pearsonian Correlation Coefficient**. If two variables are denoted by X and Y, the coefficient of correlation between them is represented by rxy or r. Pearson's coefficient correlation method can be used to measure correlation for individual series as well as for grouped data be very good fit.

16.5 Analysis of Variance (Anova)

The statistical technique used to compare means of variations of more than two populations is called **'analysis of variance (ANOVA).** The concept of **ANOVA** or analysis of variance was introduced by **R.A. Fisher.** ANOVA is based on two types of variations :

1. Variations existing within the sample

2. Variations existing between the sample

The ratio of these two variations is an indication of sample differences. Therefore, ANOVA helps in estimating whether more variations exist among the group means or within the groups. The ratio of these two variations is denoted by **'F'**. The **F- value** is the indication of differences between the samples.

NOTES

Test of Anova

For performing the test of analysis of variance following statistical steps are carried out :

1. **Sum of Squares (S.S.)**

   Sum of squares is computed as the sum of squares deviation of the values for mean of the sample. It means :

$$S.S. = \sum \left(X - \overline{X}\right)^2$$

$$S.S. = \sum x^2 - \frac{\sum x^2}{n}$$

   Here, X represents sum of squares

   $\overline{X}$ represents mean of sample

   x represents deviation

   n represents total number of observations.

2. **Mean Square (M.S.)**

   Mean square is the mean of all the sum of squares. It is obtained by dividing S.S. by appropriate degree of freedom (df) :

$$M.S. = \frac{\text{Sum of squares}}{\text{Degree of freedom}} = \frac{S.S.}{df}$$

   Therefore, following three values or quantities are required for computing ANOVA :

   1. Total sum of squares
   2. Between sample sum of squares
   3. Residual sum of square

16.6 Viva-Voce

1 What is correlation ?

2 Who proposed coefficient of correlation ?

3 Under what conditions P.E. can be used ?

Fill in the Blank

NOTES

(1) The ratio of unexplained variation to the total variation is called coefficient of _____. (non-determination/determination)

(2) Covariance frequency method was suggested by _____ (Karl Pearson/Craxton)

(3) _____ Diagram enables to obtain approximate estimating line or line of best fit. (Chi square/Scattered)

Multiple choice questions

1 Scatter diagram tells about

(a) The relationship of two variables

(b) The nature of relationship between two variables whether their relationship is positive or negative

(c) Both of them

(d) None of them

2 Concurrent deviation method is associated with

(a) Quantitative measurement

(b) Qualitative measurement

(c) Variables of qualitative characters

(d) Correlation coefficient of variables of two qualitative characters

3 The ratio of unexplained variation to the total variation is called

(a) Coefficient of non-determination

(b) Coefficient of determination

(c) Partial correlation (d) Coefficient of alienation

4 Probable error can be used only when

(a) Data is drawn from a normal population

(b) Conditions of random sampling are prevailing during selection of samples

(c) None of the two

(d) Both of them

16.7 References

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NOTES

Unit - 17

Bioinformatics

NOTES

Structure of the Unit

17.0 Objectives

After going through this unit you will able to –

- Search local alignment for any nucleic acid or protein sequence using BLAST tool
- Visualize three dimension structure of protein molecule Using RASMOL/Cn3D

17.1 Introduction

17.1.1 Sequence Alignment

A sequence alignment is a way of arranging the sequences of DNA, RNA, or protein to identify regions of similarity that may be a consequence of functional, structural, or evolutionary relationships between the sequences.

Computational approaches to sequence alignment generally fall into two categories: global alignments and local alignments

Global Alignment: Calculating a global alignment is a form of global optimization that "forces" the alignment to span the entire length of all query sequences.

Local alignment: Local alignment identifies regions of similarity within long sequences that are often widely divergent overall. Local alignments are often preferable, but can be more difficult to calculate because of the additional challenge of identifying the regions of similarity.

Local alignment aims at identifying the best pair of regions, one from each sequence, such that the optimal (global) alignment of these two regions is the best possible. This relies on a scoring scheme that maximizes a similarity score because otherwise an empty alignment would always yield the smallest distance. Naively, the algorithm to compute a local alignment would need to inspect every pair of regions and apply a global alignment algorithm to it. The decisive idea of Smith and Waterman was to offer the maximization in each cell of the matrix a fourth alternative: a zero to signify the beginning of a new alignment. After filling the dynamic programming matrix according to this scheme, backtracking starts from the cell in the matrix that contains the largest value.

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17.1.2 Three Dimension Structure

Protein structure is the biomolecular structure of a protein molecule. Proteins are polymers – specifically polypeptides -sequences formed from various L-α-amino acids. Tertiary structure refers to the three-dimensional structure of a single, double, or triple bonded protein molecule. The alpha-helixes and beta pleated-sheets are folded into a compact globular structure. The folding is driven by the *non-specific* hydrophobic interactions, the burial of hydrophobic residues from water, but the structure is stable only when the parts of a protein domain are locked into place by *specific* tertiary interactions, such as salt bridges, hydrogen bonds, and the tight packing of side chains and disulfide bonds. The disulfide bonds are extremely rare in cytosolic proteins, since the cytosol (intracellular fluid) is generally a reducing environment.

The generation of a protein sequence is much easier than the determination of a protein structure. However, the structure of a protein gives much more insight in the function of the protein than its sequence. Therefore, a number of methods for the computational prediction of protein structure from its sequence have been developed.[9] *Ab initio* prediction methods use just the sequence of the protein. Threading and homology modeling methods can build a 3-D model for a protein of unknown structure from experimental structures of evolutionarily-related proteins, called a protein family.

17.2 Searching local alignment for given sequence using BLAST tool

Go with URL: http://blast.ncbi.nlm.nih.gov/Blast.cgi

Choose BLAST Programme :

Choose a BLAST program to run.

nucleotide	Search a **nucleotide** database using a **nucleotide** query

NOTES

blast	*Algorithms:* blastn, megablast, discontiguousmegablast
protein blast	Search **protein** database using a **protein** query *Algorithms:* blastp, psi-blast, phi-blast, delta-blast
blastx	Search **protein** database using a **translated nucleotide** query
tblastn	Search **translated nucleotide** database using a **protein** query
tblastx	Search **translated nucleotide** database using a **translated nucleotide** query

3. Enter QUERY SEQUENCE (Given sequence)
4. Choose search set & programme
5. Do BLAST
6. The result shows the alignment between two sequences on the basis of Expect value, Identities, Gaps.

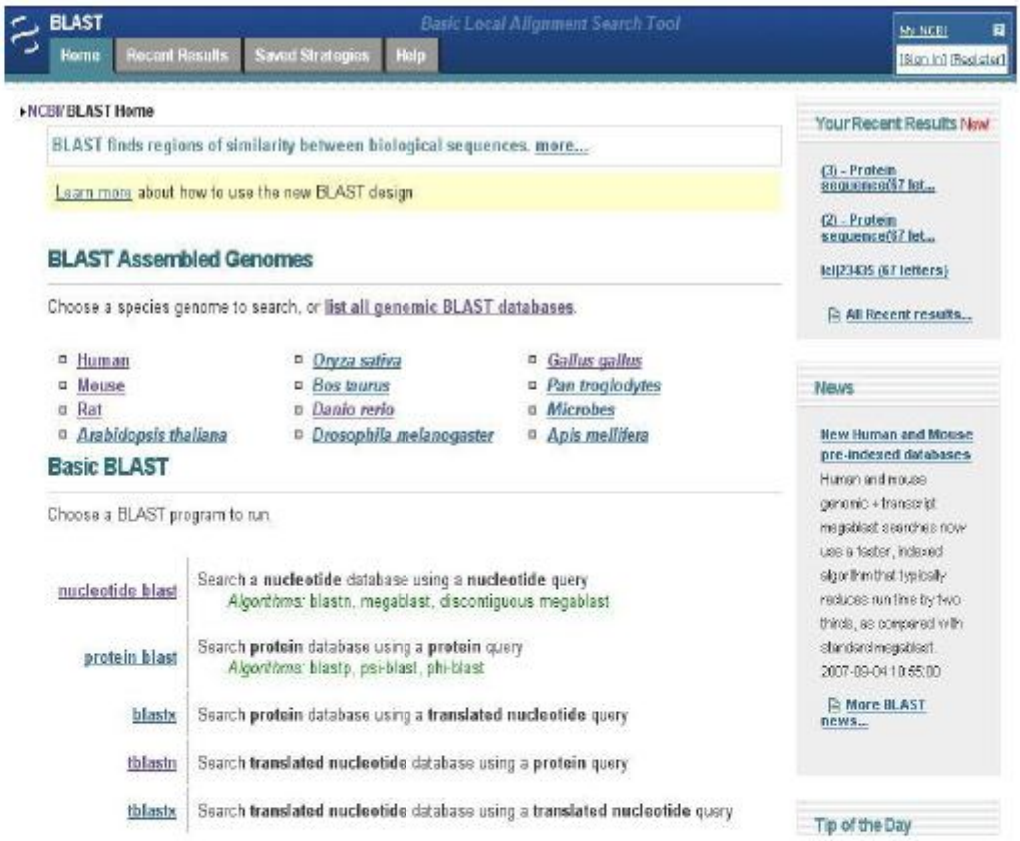

Fig. 17.1: Window showing BLAST tool

17.3 Visualization of 3D structure of protein molecule using RASMOL /Cn3D

NOTES

RasMol sand Cn3D are the programs for molecular graphic visualisation in 3 dimensions. One can Directly download both the programs from internet (RASMOL from PDB and Cn3D from NCBI site)to use on window or linux program.

17.3.1 Instruction to use RASMOL:

To open a file: **File/Open** from within RasMol. From within Netscape, click on a RasMol viewable molecule (a .pdb file). (RasMol should automatically start and load in the molecule.)

To open multiple files (i.e., can view up to 5 molecules simultaneously): **File/Open** (for each file, without closing the previous files) from within RasMol. From within Netscape, just click on another RasMol viewable molecule.

To select a molecule: **Click on molecule name in the Molecules window** (the secondary window)

To close a file (a molecule): **File/Close** when the molecule is selected.

Moving the molecule(s):

Action	PC	MAC
Rotation	Left-mouse button (Click & Hold)	Mouse button (Click & Hold)
Translation	Right-mouse button (Click & Hold)	<OpenApple> and mouse button
Zoom	<alt><SHIFT> and left-mouse button	<SHIFT> and mouse button
Z-Rotation	<alt><SHIFT>and right-mouse button	<option><SHIFT> and mouse button

To change to different representations (i.e., CPK, stick, ribbon, etc.): **Display/Stick**

To determine distances, angles, dihedral angles:

Click on appropriate icon in the **Molecules window** (the secondary window)

Click once on the appropriate number of individual atoms. (With angles, clicking on atoms must be in the appropriate order.)

To rotate bond (Note: This changes the angle.):

Click on **rotate angle** icon in the **Molecules window** (the secondary window)

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ii. Click on 2 atoms

iii. Click on **rotate angle** icon in the **Molecules window** (the secondary window) again

iv. Click on 3rd atom

v. Use mouse button to rotate

I. To color molecule in different ways:

i. To color by the protein-secondary structure: **Colours/Structure**

- a -helices: magenta
- b -sheets: yellow
- turns: pale blue
- all other residues: white

ii. To color by residue type (i.e., each type of residue is colored a specific color): **Colours/Shapely**

iii. To color by atom type (below are the default colors): **Colours/CPK**

- Carbon: gray
- Hydrogen: white
- Oxygen: red
- Nitrogen: blue
- Sulfur: yellow
- Iron: yellow

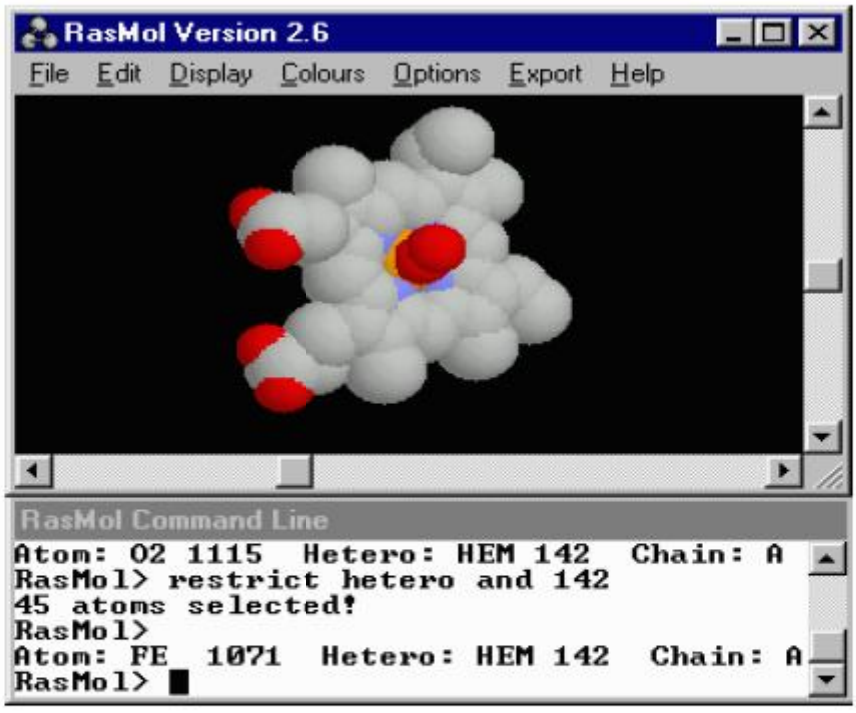

Fig.17.2: A RASMOL window

17.3.2 Instruction to use Cn3D

The File menu controls the input of data into Cn3D, and output to a variety of data and image file types; these are fairly self-explanatory. In particular, the File:Save action saves the structure along with the current view, drawing settings, and any user annotations, so that work can be saved and retrieved.

NOTES

The View menu contains items that control how the structure as a whole is displayed. The whole structure can be made larger or smaller with View:Zoom In and View:Zoom Out, and the view returned to its original size and orientation as stored in the data file with View:Restore. View:Reset willfit the entire structure into the window. If there is more than one structure being viewed (e.g. a VAST alignment), or if the structure contains multiple models (e.g. an NMR structure, downloaded with all models), then each structure will be assigned its own "frame." The various View:Frame items control which frame is currently displayed. The Show/Hide menu contains operations that allow certain substructures to be shown or hidden. The Show/Hide:Pick Structures dialog lets you choose which individual structures, chains, and domains are turned on or off. The other items in the Show/Hide menu are more relevant to alignment views. The choices in the Style menu affect the shape and color of the different parts of the structure. For example, the default display for single structures is acombination of Style:Rendering Shortcuts: Worms and Style: Coloring Shortcuts: Secondary Structure which shows a worm backbone, no side chains, and solid objects - arrows and cylinders - to represent strands and helices. The colors are green for helices, strands orange, and coil blue. (The location and extent of these secondary structure elements is determined by NCBI, and does not represent the helix and strand notations in the original PDB file.) Note that the arrows on the strands (and optionally on helix cylinders) always point in the N-to-C direction. The best way to learn what the other different styles are is simply to try them out!

Cn3D intentionally keeps color separate from drawing style. The Style:Coloring Shortcuts menu choices determine what properties are used to map different parts of the structure to different colors. Using these options, one can easily visualize a structure's NCBI-determined domain composition (Domain), or crystallographic temperatures (Temperature) from the PDB data, etc. The Style:Rendering Shortcuts choices determine the shape of the various parts of the structures, like Worms or Ball and Stick.

The style panel The style panel (Style:Edit Global Style) contains detailed controls for all drawing styles, colors, and labels, and is where much of Cn3D's flexibility is contained. In fact, all of the options in the Style:Rendering Shortcuts and Style:Coloring Shortcuts menus are simply convenient shortcuts to different combinations of options in the style panel. The style panel's Settings tab has four columns of settings for the various structure

elements identified on the left; these settings are applied globally to all structures. "On/Off" controls whether the elements are displayed (as well as what type of backbones are used), "Rendering" controls the geometry used to render the elements, and "Color Scheme" controls the elements' colors.

NOTES

Fig.17.3: Cn3D window

17.4 Viva-Voce

1. What do you understand by sequence alignment?
2. How will you differentiate between local and global alignment?
3. Define 3D satructure.

17.5 References

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UNIT - 18

Plant Physiology- I

Structure of the Unit

18.0 Objectives

After going through this unit you will be able to understand the following physiological experiments;

- Effect of time & enzyme concentration on the rate of reaction of enzyme,
- Effect of substrate concentration on activity of any enzyme and determination of its Km value;
- Separation of isozymes by PAGE, Determination of Water Potential,
- Effect of temperature on membrane permeability, Determination of total and titrable acidity and
- Determination of stomatal frequency and index.

18.1 Effect of Time & Enzyme Concentration on the Rate of Reaction of Enzyme

Introduction

Enzymes are organic catalysts that speed up reactions by decreasing the activation energy needed to start the chemical reaction. Therefore, my hypothesis is that as the enzyme

concentration increases, the speed of the chemical reaction will also increase. Using the 0% enzyme concentration should lead to the disk not rising because of the lack of reaction between the distilled water and hydrogen peroxide.

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Principle

Enzymes are proteins that speed up the rate of a chemical reaction without being used up. Enzymes are usually specific to particular substrates. The substrates in the reaction bind to active sites on the surface of the enzyme. The enzyme-substrate complex then undergoes a reaction to form a product along with the original enzyme. The rate of a chemical reaction is affected by the total number of enzymes as well as the concentration of substrates. We can describe the reaction rate with a simple equation to understand how enzymes affect chemical reactions.

Procedure

The manipulated variable is the enzyme concentration, while the responding variable is the amount of time it takes for the paper disk to rise to the top of the hydrogen peroxide solution. First, extract the catalase enzyme from the potato. Then dip a construction paper disk into different solutions that consist of 2.5 ml, 5 ml, 7.5 ml, or 10 ml enzyme and enough distilled water to have a final volume of 10 ml. afterwards, place the paper disk into a solution of hydrogen peroxide. Using a stopwatch, measure the time it takes for the paper disk to rise to the top of the hydrogen peroxide solution. This measurement is a way of recording the speed and efficiency of the rate of reaction. For each enzyme solution, take results from ten trials.

Observation and Results

The results show that as the enzyme concentration increases, the time for the disk to rise decreases. The distilled water, or 0% enzyme concentration, leads to no chemical reaction at all. According to the statistical results, or standard deviations, the results of this experiment are reliable. The highest catalase enzyme concentration was able to speed up the reactions by quickening the process of the disk rising. When absorbed in an enzyme concentration, the disk would rise, but when dipped into the 0 ml enzyme concentration, or distilled water, there was no reaction between the water and hydrogen peroxide, causing the disk to sink. This experiment can prove that increasing the amount of the enzyme concentration results in a faster reaction.

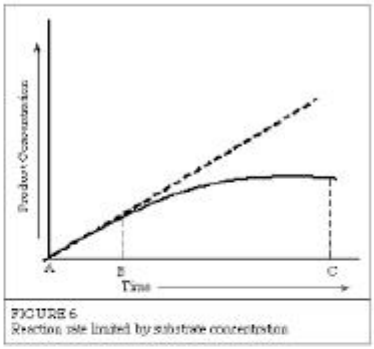

Fig. 18.1 : Reaction Rate Inhibited by Substrate Concentration

Enzyme Concentration

In order to study the effect of increasing the enzyme concentration upon the reaction rate, the substrate must be present in an excess amount; i.e., the reaction must be independent of the substrate concentration. Any change in the amount of product formed over a specified period of time will be dependent upon the level of enzyme present. Graphically this can be represented as:

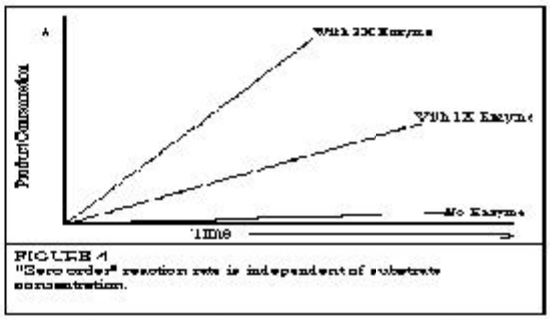

Fig. 18.1 : Zero Order Reaction Rate is Independent of Substrate Concentration

These reactions are said to be "zero order" because the rates are independent of substrate concentration, and are equal to some constant k. The formation of product proceeds at a rate which is linear with time. The addition of more substrate does not serve to increase the rate. In zero order kinetics, allowing the assay to run for double time results in double the amount of product.

18.2 Effect of Substrate Concentration on Activity of any Enzyme and Determination of its Km Value

Introduction

Enzymes are protein molecules that act as biological catalysts by increasing the rate of reactions without changing the overall process. They are long chain amino acids bound together by peptide bonds. Enzymes are seen in all living cells and controlling the metabolic processes in which they converted nutrients into energy and new cells. Enzymes also help in the breakdown of food materials into its simplest form. The reactants of

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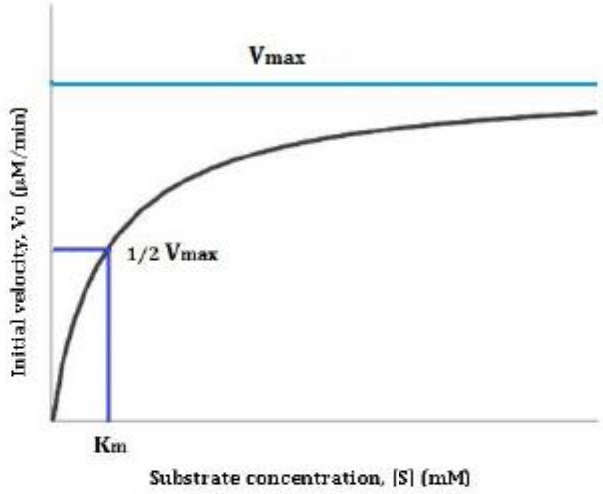
enzyme catalyzed reactions are termed as substrates. Each enzyme is quite specific in character, acting on particular substrates to produce particular products. The central approach for studying the mechanism of an enzyme-catalyzed reaction is to determine the rate of the reaction and its changes in response with the changes in parameters such as substrate concentration, enzyme concentration, pH, temperature etc .This is known as enzyme kinetics.

Principle

The enzyme Amylase can catalyze the hydrolysis of internal -1, 4-glycosidic bond present in starch with the production of reducing sugars. In the study of substrate concentration on enzyme kinetics, the enzyme is kept constant where as the concentration of Starch is taken in increasing order. As the substrate concentration increases, the amount of products produced in every successive tube also increases. This was explained by Michealis and others that an enzyme catalyzed reaction at varying substrate concentrations is diphasic i.e. at low substrate concentration the active sites on molecules (enzyme) are not occupied by substrate and the enzyme rate varies with substrate molecules concentration (phase1). As the number of substrate molecules increases, the enzyme attains the saturation level, since there are no more reaction sites remaining for binding. So the enzyme can work with full capacity and its reaction rate is independent of substrate concentration.

One of the important parameters affecting the rate of a reaction catalyzed by an enzyme is the substrate concentration, [S]. During enzyme substrate reaction, the initial velocity V0 gradually increases with increasing concentration of the substrate. Finally a point is reached, beyond which the increase in V0 will not depend on the [S]. When we plot a graph with substrate concentration on the X axis and corresponding velocity on Y axis. It can be observed from the graph that as the concentration of the substrate increases, there is a corresponding increase in the V0. However beyond a particular substrate concentration, the velocity remains constant without any further increase. This maximum velocity of an enzyme catalysed reaction under substrate saturation is called the Vmax, Maximum velocity.



Michaelis – Menten Equation

Leonor Michaelis and Maud Menten postulated that the enzyme first combines reversibly with its substrate to form an enzyme-substrate complex in a relatively fast reversible step:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \longrightarrow \text{Eqn.1}$$

In the next step, this ES complex is breaks down in to the free enzyme and the reaction product P:

$$ES \xrightarrow{k_2} E + P \longrightarrow \text{Eqn.2}$$

Since the second step is the rate limiting step, the rate of overall reaction must be proportional to the concentration of the ES that reacts in the second step. The relationship between substrate concentration, [S] and Initial velocity of enzyme, V0 has the same general shape for most enzymes. This can be expressed algebraically by the Michaelis-Menten equation. Based on their basic hypothesis that the rate limiting step in enzymatic reactions is the breakdown of the ES complex to free enzyme and product, Michaelis and Menten derived an equation which is;

$$V_0 = \frac{V_{max}[S]}{K_m + [S]} \longrightarrow \text{Eqn.3}$$

The necessary terms in this reaction are [S], V0, Vmax, and Km (Michaelis constant),. All these terms can be measured experimentally.

Observation and Results

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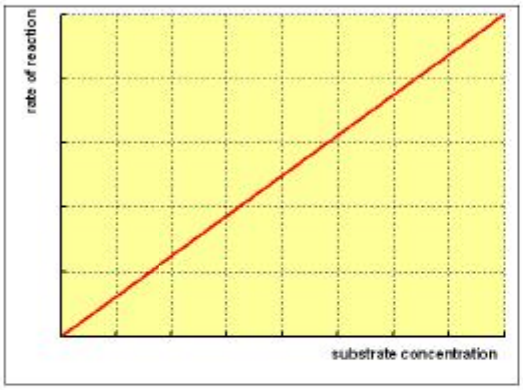
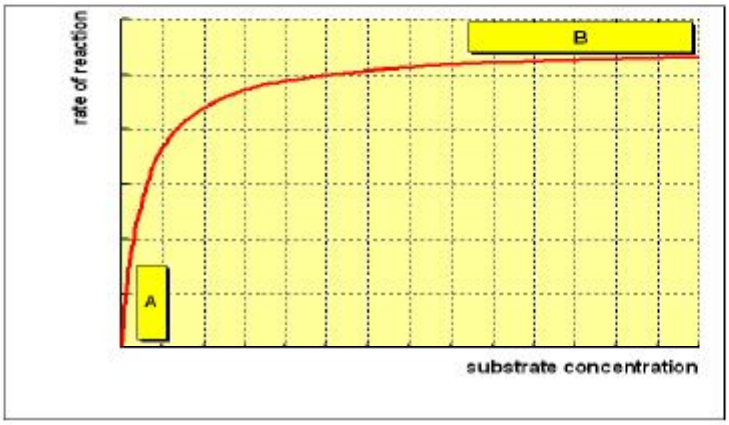
A simple chemical reaction with a single substrate shows a linear relationship between the rate of formation of product and the concentration of substrate, as shown below:



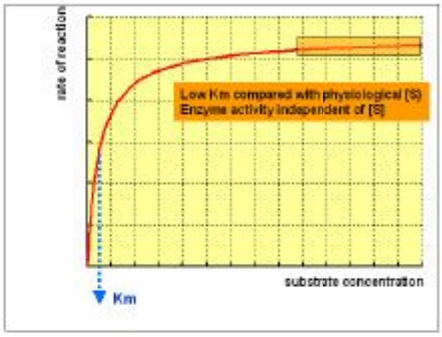
For an enzyme-catalysed reaction, there is usually a hyperbolic relationship between the rate of reaction and the concentration of substrate, as shown below:



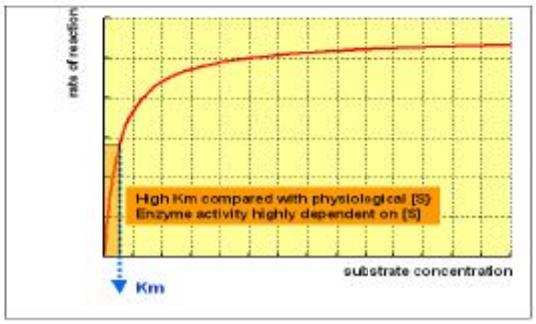
At low concentration of substrate, there is a steep increase in the rate of reaction with increasing substrate concentration. The catalytic site of the enzyme is empty, waiting for substrate to bind, for much of the time, and the rate at which product can be formed is limited by the concentration of substrate which is available. As the concentration of substrate increases, the enzyme becomes saturated with substrate. As soon as the catalytic site is empty, more substrate is available to bind and undergo reaction. The rate of formation of product now depends on the activity of the enzyme itself, and adding more substrate will not affect the rate of the reaction to any significant effect.

The importance of determining Km and Vmax

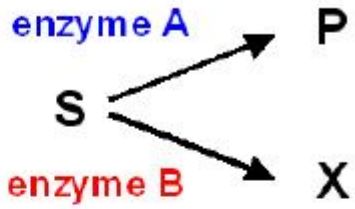
The Km of an enzyme, relative to the concentration of its substrate under normal conditions permits prediction of whether or not the rate of formation of product will be affected by the availability of substrate.



An enzyme with a low Km relative to the physiological concentration of substrate, as shown above, is normally saturated with substrate, and will act at a more or less constant rate, regardless of variations in the concentration of substrate within the physiological range.



An enzyme with a high Km relative to the physiological concentration of substrate, as shown above, is not normally saturated with substrate, and its activity will vary as the concentration of substrate varies, so that the rate of formation of product will depend on the availability of substrate.



If two enzymes, in different pathways, compete for the same substrate, then knowing the values of Km and Vmax for both enzymes permits prediction of the metabolic fate of the substrate and the relative amount that will flow through each pathway under various conditions.

18.3 Separation of Isozymes by PAGE

Introduction

Proteins - as the primary products of structural genes - are very alluring for the direct genetic studies. Variation in the DNA coding sequences frequently causes variation in the

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primary conformation of the proteins. In un-natural environments the detection of this variation is very difficult, because in such conditions the base of the separation is only the size of the protein (molecular weight). In natural environments the change of a single amino acid can detectably modify the migration. The extraction from a single tissue can contain a lot of proteins, which - in the case of non-specific (e.g. Comassie blue) staining - can result in a complex pattern, that makes it difficult to identify the homolog (allelic) and non-homolog enzymes. This problem can be solved by the application of enzyme-specific staining after the electrophoresis. The analysis of isozymes and their functions is the subject of functional genomics.

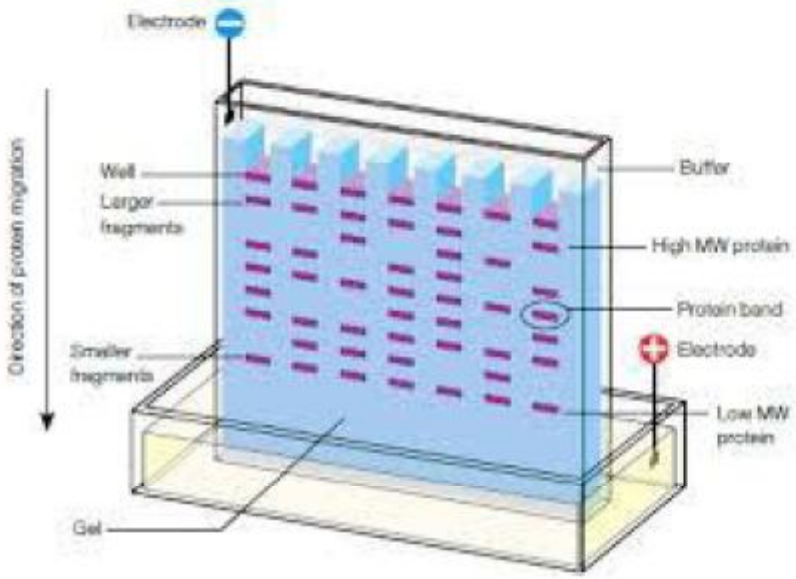

Fig. 18.3 : Electrophoresis

Principle

Polyacrylamide gel electrophoresis (PAGE), describes a technique widely used in biochemistry, forensics, genetics, molecular biology and biotechnology to separate biological macromolecules, usually proteins or nucleic acids, according to their electrophoretic mobility. Mobility is a function of the length, conformation and charge of the molecule. Isoenzymes can be separated by electrophoresis or isoelectric focusing. The isozymes under given proper circumstances show peculiar patterns in the gel, which are called zymogramm.

Electrophoresis is a type of chromatography. The power for the separation of proteins is the difference in voltage between the two ends of the gel. The movement of proteins in the Electric field is effected by their weight, shape and charge. The gel for the separation can be made from starch, agarose or acrilamide (Fig 2.). A standardized method of starch gel electrophoresis is used by UPOV (1996) for the analysis of identity of plant cultivars by isozyme analysis .Advantages of the starch gel are that it is non-toxic, and more isozymes

can be analysed by the slicing of a thick gel. More recently polyacrylamide gels are used because of their larger resolution. The porous structure of polyacrylamide gel is formed through a process of polymerization of acrylamide (CH2=CH-CO-NH2) and bis-acrylamide (CH2=CH-CO-NHCH2- NH-CO-CH=CH2). As a result of polymerisation a colourless, diaphanous, flexible, and consistent gel arises, which is resistant to scalding or chilling. The density, viscosity and size of poles are determined by the concentration of acrylamide and bis-acrylamide.

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Requirements

Electrophoresis unit, Power supply, Chemical buffer, Counterion balance, Acrylamide (C3H5NO; mW: 71.08)., Sodium Dodecyl Sulphate (SDS) (C12H25NaO4S; mW: 288.38)., Urea (CO(NH2)2; mW: 60.06) Ammonium persulfate (APS) (N2H8S2O8; mW: 228.2).. TEMED (N, N, N', And N'-tetramethylethylenediamine) (C6H16N2; mW: 116.21).

Procedure

In gel electrophoresis, molecules may be run in their native state, preserving the molecules' higher-order structure or a chemical denaturant may be added to remove this structure and turn the molecule into an unstructured linear chain whose mobility depends only on its length and mass-to-charge ratio. For nucleic acids, urea is the most commonly used denaturant. For proteins, sodium dodecyl sulphate (SDS) is an anionic detergent applied to protein sample to linearize proteins and to impart a negative charge to linearized proteins. This procedure is called SDS-PAGE. In most proteins, the binding of SDS to the polypeptide chain imparts an even distribution of charge per unit mass, thereby resulting in a fractionation by approximate size during electrophoresis. Proteins that have a greater hydrophobic content, for instance many membrane proteins, and those that interact with surfactants in their native environment, are intrinsically harder to treat accurately using this method, due to the greater variability in the ratio of bound SDS.

Observations and Results

To determine the molecular weight of an unknown protein, you should separate the sample on the same gel with a set of molecular weight standards. After running the standards and the unknown protein sample, the gel is processed with the desired stain and then de-stained for about 12 to 14 hours to visualize the protein bands.

After running the gel, you should then determine the relative migration distance (*Rf*) of the protein standards and the unknown protein. The migration distance can be determined using the following equation:

$$Rf = \frac{\text{migration distance of the protein}}{\text{Migration distance of the dye front}}$$

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Based on the values obtained for the bands in the standard, the logarithm of the molecular weight of an SDS-denatured polypeptide and its relative migration distance (*Rf*) is plotted into a graph. Please take note that you will generate a linear plot for most proteins if your samples are fully denatured and the gel percentage is appropriate for the molecular weight range of the sample. If you get a sigmoidal curve, it means that the sieving effect of your matrix is either too large that it restricts the penetration of the molecules into the gel or is nearly negligible that it allows protein molecules to migrate almost at their free mobility.

Interpolating the value from this graph will then give you the molecular weight of the unknown protein band. The accuracy of this method in determining the molecular weight of an unknown protein typically ranges from 5% to 10%. The presence of polypeptides such as glycol- and lipoproteins usually leads to erroneous results since they are not fully coated with SDS and thus, would not behave as expected.

So for the example pictured, the unknown protein has an Rf of 0.7084. Using the equation for the linear plot we can calculate the Log (MW). (-2.0742 x 0.7084) +2.8 = 1.3305. So the inverse log is 10^1.3305= 21.4kDa for the molecular weight of the unknown protein.

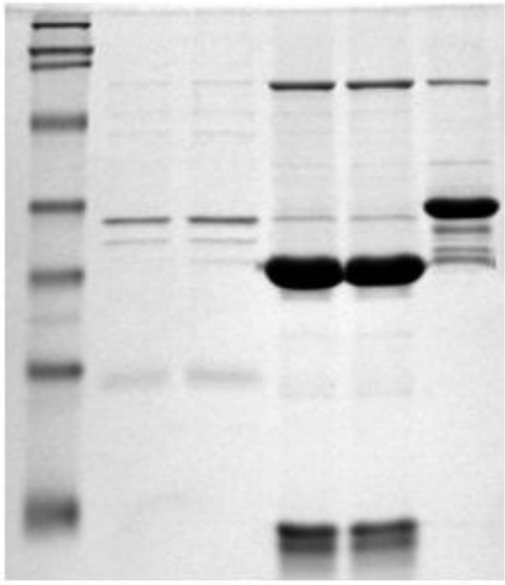

Fig. 18.4 : Protein Bands on Gel

18.4 Determination of Water Potential

Introduction

Water potential is the measure of the tendency of water to move from one place to another. Water always moves from an area of high water potential to a region of lower

water potential. The addition of solutes decreases water potential making it more negative. Therefore it is less likely to move from one place to another. Pure water has a water potential of zero, by adding solutes you make the water potential more negative.

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Water potential = Solute Potential + Pressure Potential

Water Potential is a Physical property of water that determines the direction that water will flow. In the world of water potential 0 is high! Usually it is a negative number, occasionally a positive number. Determined by the solute concentration and pressure. Water potential is equals the solute potential (solute potential is always negative), as the pressure potential is negligible. However pressure potential is pressure exerted when water enters a cell and makes it turgid. Increasing pressure increases the tendency of water to move, therefore increases water potential. Pressure potential is always positive therefore making water potential less negative. In my experiment

Water potential = Osmotic Potential + Pressure Potential

$$p\psi + s\psi = \psi$$

Measured in bars a metric measure of pressure

$$\psi = s\psi + p\psi$$

$s\psi$ = the solute (or osmotic) potential

$$s\psi = -I\,CRT$$

i = ionization constant (number of particles formed)

Glucose (1)

NaCl (2)

CaCl 2 (3)

5. $\psi = s\psi + p\psi$

$s\psi$ = the solute (or osmotic) potential

$s\psi = -iCRT$

i = ionization constant (number of particles formed)

C = osmotic molar concentration of the solute

R = pressure constant (handbook value R=0.0831 litre bars/mole o K)

T = temperature in o K (273 + o C) of solution Water Potential

$\psi = s\psi + p\psi$

$s\Psi$ = the solute (or osmotic) potential

$s\Psi = -iCRT$

NOTES

$p\Psi$ = the pressure potential

A solution open to the atmosphere has a pressure potential of 0 bars

Principle

Water always moves from high water potential to low water potential .Water potential is a tendency of water to move from high free energy to lower free energy.

Distilled water in an open beaker has a water potential of 0 (zero).

Addition of solute decreases water potential

Addition of pressure increases water potential

In cells, water moves by osmosis to areas where water potential is lower.

A hypertonic solution has lower water potential

A hypotonic solution has higher water potential

Requirements

Beakers, Test tubes, pipettes, Aluminium foils, Balance, fresh potato tubers.

Procedure

Dispense 10 mL of water or sucrose (0.1 - 0.8 molal) into each of nine appropriately-labelled containers. (Sorbitol, mannitol or polyethylene glycol can be used in place of sucrose). Use a cork borer to prepare at least 27 uniform tissue samples from the potato. Cut them to the same length with a razor blade. Be sure not to include any fragments of the skin. Work quickly to minimize evaporation and keep the tissue wrapped in a moist towel. Weigh two or preferably three cores, record data and then place the cores in one of the test solutions. Repeat for all solutions. Weigh the cores to the nearest 0.01 g. If necessary, add more of the appropriate sucrose solution to completely submerge the cores – but, the final volume in each tube must be the same. Incubate the cores for 1.5- 2.0 hours. After 1.5 - 2.0 hours, record the temperature of the solutions. Then remove the tissues, gently blot on paper towels and reweigh. Record data and examine the cores and weigh them. Also describe their relative turgor.

Observations and Results

Equation

To calculate the percent change in weight for each tissue by the following equation:

$$\% \text{ change} = (\text{final} - \text{initial})/\text{initial} \times 100$$

Plot % change in weight vs. sucrose concentration (molality). Draw the best fit line for data.

From the graph, determine the concentration of the sucrose solution in which there was no net weight gain (i.e., % change = 0). At this point, the water potential of the solution equals the water potential of the potato cores. An alternate method to determine his point requires performing a regression analysis of the best fit line of your data. The equation for this line is in the form, Y = mx + b. Substitute in this equation, Y = O, and then solve for X (the point at which the line crosses the X axis and equals the sucrose concentration in which there is no net change in weight of the cores = water potential of the cores). Which method do you think will be more accurate?

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Calculate the osmotic (= water) potential of this solution.

Table 1: Change in weight of potato cores incubated in sucrose solutions

[Sucrose] (molality)	Initial Weight (g)	Final Weight (g)	Change in Weight (final - initial) (g)	% Change in Weight
0				
0.1				
0.2				
0.3				
0.4				
0.5				
0.6				
0.7				
0.8				

18.5 Effect of Temperature on Membrane Permeability

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Introduction

The cell membrane is a biological membrane that separates the interior of all cells from the outside environment. The cell membrane is selectively permeable to ions and organic molecules and controls the movement of substances in and out of cells. The basic function of the cell membrane is to protect the cell from its surroundings. It consists of the phospholipid bilayer with embedded proteins. Cell membranes are involved in a variety of cellular processes such as cell adhesion, ion conductivity and cell signalling and serve as the attachment surface for several extracellular structures, including the cell wall, glycocalyx, and intracellular cytoskeleton.

Principle

All living cells are protected by their cellular membrane which is made up of phospholipids with embedded proteins. The structure of the cell membrane is very specific and not only protects the cell and the organelles within the cell but also supports the functions of the cell. Changes in the cell's environment, such as changes in temperature, can affect the cell's structure and functions.

Requirements

Apparatus

Beaker, Corer size 4, White tile, A Beetroot, Automatic Water Bath, Segregated knife, thermometer, Stopwatch.

Procedure

First take the white tile and the corer. Then collect a cylinder of beetroot by pushing the order into the beetroot and withdrawing it. The cylinder remains inside the corer- so push it out with the end of a pencil. Collect 3 cylinders, and then cut them into 6 pieces of 3 cm with a segregated knife. The beetroot was cut to 1cm. Because the beetroot has been cut some of the cell membranes had been broken, which means some anthocyanin will leak out. This must be completely washed off in order to maintain the reliability of the results. The water bath must then be heated to 20oC (the first temperature for the experiment) .Once the water bath is at the correct temperature (measured using the thermometer), one piece of beetroot is placed into the hot water directly and left for exactly1 minute (using a stopwatch).The beetroot piece is then placed into a tube of 5 cm of distilled water. This procedure will be repeated with the other four pieces of beetroot and the temperature should be changed accordingly. The temperatures will be using are 20oC, 40 C , 60oC and 80oC .Each time a piece of beetroot is removed from the heated water, it will be left in the distilled water for exactly 30 minutes, before being discarded. The fluid in each of the test

tubes will be analysed using a colorimeter and compared against the control, which is distilled water to check for any variations in the colour of the water. The variables kept constant ·the same diameter corer is used so to keep the surface area of each beetroot piece the same size. When the beetroot has been cut some of the cell membranes is broken, which means some anthocyanin will leak out. This must be completely washed off in order to maintain the reliability of the results. Distilled Water can also be used to test with a colorimeter.

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Observations and Results

Observe the colour density of water with increased temperatures and measure its OD at 620 nm using Colorimeter.

S.No	Temperature (C)	OD at 620 nm
1	30	
2	40	
3	50	
4	60	
5	70	
6	80	
7	90	
8	100	

18.6 Determination of Total and Titrable Acidity

Introduction

Plant cell contains several acids like- amino acids, organic acids, fatty acids, proteins etc. Titrable Acidity (TA) is an index that represents the total amount of organic acid in a sample (primarily tartaric, malic and citric acid). The total acidity includes both the undissociated and the dissociated hydrogen ions, while the titrable acidity contributed by weak or strong organic acids.

Principle

The total acidity includes both the Titrable Acidity and the dissociated hydrogen ions, while the actual acidity depends only on the latter and is conveniently designated by the P, + numbers of Sorensen, which are the negative common logarithms of the numbers

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expressing hydrogen ion concentration. Thus if the hydrogen ion concentration is 1 X 10-7.5 N, the P, + number is 7.5. The titrable acidity is expressed as % lactic acid and is determined by titration of a known amount of reconstituted milk with 0.1 N NaOH using phenolphthalein as indicator.

Requirements

Apparatus

1. Analytical balance 0.1 mg
2. Methrom autoburette
3. Solubility index mixer, Snijders, The Netherlands.Speed 3800-4000 rpm
4. 100 ml Erlenmeyer flask
5. Centrifuge
6. 20 ml pipette, other sizes may be used
7. Beaker

Chemicals and Reagents

1. Titrisol, 0.1 N NaOH - R 35, and S 26, 27, 37/39
2. Phenolphthalein
3. 96% Ethanol
4. 0.1 N NaOH. Dilute the Titrisol solution to 1 litre. Standard Method no. R-7.1
5. 1 % Phenolphthalein solution; Dissolve 1g of phenolphthalein in 50 ml 96% ethanol and dilute to 100 ml with deionized water.
6. 0.1 N HCl
7. Bromothiol Blue

Procedure

1. Disperse and dissolve the following amount of powder in 100 ml of deionized water using the mixer.

Powder:	
Skim or buttermilk:	10 g
Whole milk:	13 g
Whey:	6 g

2. Allow the mixture to stand for approx. 1 hour, stir gently.
3. Pipette 20 ml into a 100 ml Erlenmeyer flask.
4. Add 0.5 ml of phenolphthalein and titrate with 0.1 N NaOH until a faint pink colour persists for 30 sec.

Observations and Results

$$\% \text{ titratable acidity} = \frac{ml \times N \times 90 \times 100}{V \times 1000}$$

where

ml = ml 0.1 NaOH used

N = Normality of 0.1 N NaOH

V = ml milk solution used

Titratable acidity is expressed as % lactic acid, (CH_3-CHOH-COOH, MW = 90)

Reproducibility ± 0.01% lactic acid

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18.7 Determination of Stomatal Frequency and Index

Introduction

Stomata are minute aperture structures on plants found typically on the outer leaf skin layer, also known as the epidermis. They consist of two specialized cells, called guard cells that surround a tiny pore called a stoma. Stomata are present in the sporophyte generation of all land plant groups except liverworts. Dicotyledons usually have more stomata on the lower epidermis than the upper epidermis. Monocotyledons, on the other hand, usually have the same number of stomata on the two epidermises. In plants with floating leaves, stomata may be found only on the upper epidermis and submerged leaves may lack stomata entirely.

Principle

Stomata control the movement of gases in and out of a leaf, making carbon dioxide available for photosynthesis, and controlling the loss of water from the leaf through transpiration. Stomatal frequency is a function of both the number of stomata plus the size of the epidermal cells. It is affected both by the initiation of stomata and the expansion of epidermal cells. This expansion is a function of many variables (e.g. light, temperature, water status, position of leaf on crown, and intra-leaf position), and can overprint the signal reactive of stomatal initiation. As it turns out, CO2 plays a stronger role in stomatal initiation than in epidermal cell expansion (this is discussed in detail below). Salisbury (1927) introduced the concept of stomatal index (SI), which normalizes for the effects of this expansion (i.e. density of epidermal cells). It is defined as the percentage number of stomata as compared to all the epidermal cells (including stomata) in a unit area of leaf.

SI= {S / (E+S)}*100

where,

I=Stomatal Index

S=number of stomata for unit area

E= number of epidermal cells per unit area.

Requirements

Microscope, Occulometer, Slides, Petri dishes, cover slips, brushes, plant material.

NOTES

Procedure

1. Take a leaf with leaflet as sample which is mature.
2. Remove a leaflet and determine its surface area using the Tracing Technique. Record your data in Table 1.
3. Prepare an epidermal strip from the abaxial surface of the leaf and mount in a drop of water.
4. Count the number of stomata in 10 randomly selected fields at 400 x. Record your data in Table 2. Record the magnification used.
5. Repeat with the adaxial surface. Record your data in Table 2.
6. Measure the diameter of the microscope field at 400 x with a stage micrometer. Record your data in Table 3.

Observations and Results

This is expressed as: (number of stomata per mm2 × 100) /(number of stomata per mm2 + number of epidermal cells per mm2). This value has been found useful in comparing leaves of different sizes. Relative humidity and light intensity during leaf development affect the value of stomata index. Complete Tables 1, 2, and 3. Calculate % of the leaf that is open space for each side of the leaf .To perform this calculation assumes that an open stoma of Broad bean forms a rectangular pore (9 μm x 40 μm). Write the equation that you used.

Table-1

Leaf area data for a Broad bean leaflet	
dimensions of paper standard (mm x mm)	
area of paper standard (mm^2)	
weight of paper standard (g)	
weight of leaf tracing (g)	
area of one side of leaf (mm^2)	
total area of leaf (mm^2)	

NOTES

Table-2

Stomatal frequency data for the adaxial and abaxial sides of a Broad bean leaflet											
	Stomata $field^{-1}$										
	1	2	3	4	5	6	7	8	9	10	Mean
Abaxial											
Adaxial											

NOTES

Table-3

Microscope area of field used to calculate Broad bean stomatal frequency	
Magnification Used	
Diameter Of Field (Mm)	
Area Of Field (Mm^2)	

18.8 Viva-Voce

1. What are enzymes?
2. What do you mean by Rate of reaction?
3. What are the chemical properties of enzymes?
4. What is effect of substrate concentration on enzyme activity?
5. What is Km value?
6. Write down Michaelis-Menten equation.
7. What is Electrophoresis?
8. What is Rf value?
9. What do you mean by PAGE?
10. Define Chromatography.
11. What are buffers?
12. What is Water Potential?
13. Define Hypotonic, Hypertonic and Isotonic solutions.
14. Name different components of plant cell water potential.
15. What is semi-permeable membrane?
16. Which is the optimal temperature for cell to maintain its normal membrane function?
17. What is OD?
18. What is Titrable Acidity (TA)?
19. What is Total acidity?
20. What do you mean by Stomata frequency?
21. What is Stomatal Index?

18.9 References

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NOTES

Unit -19

Plant Physiology- II

NOTES

Structure of the Unit

19.0 Objectives

After going through this unit you will be able to understand the following physiological experiments;

- Extraction and separation of Chloroplast pigments and their separation, Estimation of Chlorophyll a and b ration in C3 and C4 plants,
- Determination of Absorption Spectra of Chlorophylls,
- Determination of rate of, respiration of germinating seeds, Estimation of nitrogen by Kjeldahl's method,
- Estimation of Indole Acetic Acid (IAA), Extraction of seed proteins and Determination of seed viability.

19.1 Extraction and Separation of Chloroplast Pigments

Introduction

Photosynthetic pigments are chemical compounds which reflect only certain wavelengths of visible light. This makes them appear "colourful". Flowers, corals, and even animal skin contain pigments which give them their colours. More important than their reflection of light is the ability of pigments to absorb certain wavelengths.

Chlorophylls are greenish pigments which contain a porphyrin ring. This is a stable ring-shaped molecule around which electrons are free to migrate. There are several kinds of chlorophyll, the most important being chlorophyll "a". This is the molecule which makes photosynthesis possible, by passing its energized electrons on to molecules which will manufacture sugars. All plants, algae, and cyanobacteria which photosynthesize contain chlorophyll "a". A second kind of chlorophyll is chlorophyll "b", which occurs only in "green algae" and in the plants. Carotenoids are usually red, orange, or yellow pigments, and include the familiar compound carotene, which gives carrots their colour. These compounds are composed of two small six-carbon rings connected by a "chain" of carbon atoms. As a result, they do not dissolve in water, and must be attached to membranes within the cell. Carotenoids cannot transfer sunlight energy directly to the photosynthetic pathway, but must pass their absorbed energy to chlorophyll. For this reason, they are called accessory pigments. One very visible accessory pigment is fucoxanthin the brown pigment which colours kelps and other brown algae as well as the diatoms. Similarly, Phycobilins are water-soluble pigments, and are therefore found in the cytoplasm, or in the stroma of the chloroplast. They occur only in Cyanobacteria and Rhodophyta.

NOTES

Principle

Chlorophyll a and chlorophyll b play a significant role in the plant growth process. Precise determination of its content could provide the scientific basis for the corps growth state, the plant pathology diagnosis and so on, and is the key point of implementing accurate agriculture. Observing the transmission spectrum using human eyes, the color of chlorophyll a and chlorophyll b was obtained. Thus, according to experiment curve of absorption spectrum of chlorophyll a and chlorophyll b, combining the three primary color principles of colorimetric, and choosing a standard light source with spectrum power distribution similar to D65 as the illumination.

It is determined their chromaticity coordinates in the 1931CIE-x, y chromaticity diagram: Chlorophyll a is (0.198 1, 0.334 1), which falls in the cyan color region; Chlorophyll b is (0. 270 4, 0. 566 3), which falls in the yellowish green region. The main point of above processing is: reducing the spectrum curves of chlorophyll a and the chlorophyll b to coordinates on the chromaticity diagram, and the result could offer the essential theoretical support for a new non-contact, long-distance and non-damage technology to determine the pigment content in single leaf or mass.

Requirements

Balance, Oven, Test tubes Beakers, Acetone, n-propanol and petroleum ether, Leaves

NOTES

Procedure

A. Extraction of Pigments

Extract photosynthetic pigments by grinding 2g of your leaves, torn into small pieces, in a mortar with a pinch of clean sand and a total of 10mL of 100% acetone. Initially, add only a small amount of acetone to begin the grinding process. It is much easier to grind the leaves if the extract is a pasty consistency. Add more solvent in small increments while continuing to grind the leaves. Pour the extract into a 15mL centrifuge tube and centrifuge in the bench top centrifuge for 3min. Remove the extract to a 10mL graduated cylinder using a Pasteur pipette. Record the volume of the extract in your lab notebook. Cover the extract with Para film to prevent evaporation. This extract will be used for chromatography.

B. Separation of Pigments by Paper Chromatography

1. Prepare 25mL of chromatography solvent by first adding 25mL of Petroleum ether into your chromatography jar followed by 250µL of n-propanol. Attach the lid tightly to allow saturation of gases in the jar. Label your jar with colored tape.
2. Obtain a piece of 3MM filter paper, being very careful not to touch the flat surface. Place the paper on the covered table, and draw two dots with a pencil about 2cm from the bottom (narrow edge), and 1cm from the left and right edges. Do not use a pen, because pen marks will develop with the pigments.
3. Using applicator or capillary, apply the pigment extract between the dots .Do not extend the line to the side edges-leave 1cm space on each side. Take more than one application of extract to obtain sufficient pigment in each band, and therefore, good absorption spectra. Apply the extract with the edge of the applicator, not the wide face. This will give a thinner line. The number of applications will depend on the type of plant used and the concentration of the extract.
4. Quickly, place the chromatogram in the chromatography jar in the hood, pigment line down and attach the lid tightly. Be sure the chromatography solvent (which contains petroleum ether with 1% n-propanol) in the jar will not stand higher than the line of applied pigments, that is, be sure the solution will migrate through the line and move the pigments up the paper rather than to diffuse them from the paper into the standing solvent.
5. Observe as the solvent front moves up the paper. Check after 5 minutes.
6. Remove the chromatogram and allow it to dry in the hood.

Observation and Results

Observes the colours, at the end, there should be 3–5 bands of pigment: 2 green bands and 1–3 yellow bands. Generally, there is a yellow pigment band (migrating with the solvent front), followed by 2 green bands, and finally 0–2 yellow bands. Also see their Rf values.

Green bands- Chlorophyll pigments

Yellow bands are of Xanthophylls

Rf = a / b

Where a = distance moved by substance from its original position;

b = distance moved by solvent from the same position.

NOTES

Precautions

Wash glassware with 70% ethanol as soon as possible.

Discard all ethanol in labeled bottle in hood.

Place glass pipettes tips down in pipette canister.

Discard Pasteur pipettes in red sharps container.

Rinse cuvettes with 95% ethanol and leave upright to dry.

19.2 Estimation of Chlorophyll a and b ration in C3 and C4 Plants

Introduction

Chlorophyll is in fact only one pigment in a group of closely related pigments commonly found in photosynthesising plants called photosynthetic pigments. This can be demonstrated by extracting the pigments from leaves with acetone and separating them by means of paper chromatography. With a bit of luck five pigments can be identified: chlorophyll a (blue-green), chlorophyll b (yellow-green), xanthophylls (yellow), carotene (orange) and phaeophytin (grey, it is a breakdown product of chlorophyll).

Principle

The most abundant plant pigments are chlorophyll a and b, the second group of plant pigment, the carotenoids can be divided into two different types; the carotenes and the xanthophylls. In Angiosperms (most land plants) there are typically two types of Chlorophyll (Chl) molecules, namely, chlorophyll a (Chl a) and chlorophyll b (Chl b). Both of these pigments absorb photons of light in the blue and red spectral regions, but the specific wavelengths of light they absorb are different. The absorbance of photons at 663 nm and 645 nm, specific for Chl a and Chl b, respectively, will be determined. The equation or this has been worked out and is known as Arnon's equation. Arnon's equation provide quantitative information about the Chl a and Chl b.

Requirements

Balance, Oven, Test tubes Beakers, mortar and pestle, Centrifuge, Spectrophotometer, Acetone, Calcium carbonate, Distilled water,, Leave of C3 plants (Tecoma), C4 plants (Maize)

Procedure

1. Cut the leaves into small pieces. Discard major veins and any tough, fibrous tissue.

NOTES

Weigh the pieces: you should keep about 0.10 g (100 mg) of material for grinding (Record total fresh weight of each sample).

2. Put the tissue into a mortar and add 10 ml of 80% acetone (acetone: water 80:20 v: v). Grind the tissue with a pestle. You want to pulverize the tissue completely, thus a few Grains of sand may help. This is your leaf homogenate.

3. Filter the leaf homogenate through the filter paper. The retentate is removed by the Filter paper (and discarded) and the extract (or filtrate) is collected in a test-tube.

Determination of Chlorophyll concentration

Obtain a clean cuvette for the spectrophotometer/colorimeter and fill two-thirds full with 80% acetone; this is the blank. Wipe the cuvette with a tissue and put it into the Spectrophotometer then set the wavelength to 663 nm. Cover the cuvette chamber and set the spectrophotometer to 0 absorbance with the blank in place. Remove the blank and save for the next measurement. Gently swirl your first extract in the test-tube and fill a second cuvette two-thirds full. Wipe it clean, insert into the spectrophotometer, and close the hatch. The readout should give the absorbance at 663 nm, the A663. Record this number, and repeat step 2 with the other extracts. Change the wavelength to 645 nm. Reinsert the blank cuvette, and re-zero the Spectrophotometer at the new wavelength. Remove the blank and insert a cuvette containing your first extract. Read and record A645. Repeat for the other extracts.

Observation and Results

Calculations: Use Arnon's equation (below) to convert absorbance measurements to mg Chl g-1 leaf tissue

Chl a (mg g-1) = [(12.7 × A663) - (2.69 × A645)] × ml acetone / mg leaf tissue

Chl b (mg g-1) = [(22.9 × A645) - (4.68 × A663)] × ml acetone / mg leaf tissue

Total Chl = Chl a + Chl b.

Carotenoids C x+c = 1000 A470 – 1.90Chla – 63.14 Chlb/214, (x = xanthophylls and carotenes).

19.3 Determination of Absorption Spectra of Chlorophylls

Introduction

Photosynthesis is a process used by plants and other organisms to convert light energy, normally from the Sun, into chemical energy that can be later released to fuel the organisms' activities. This chemical energy is stored in carbohydrate molecules, such as sugars, which are synthesized from carbon dioxide and water .An absorption spectrum is a measure of the light absorbing capacity of a substance across the entire range of

wavelengths (colours). In this experiment we measured the absortion spectrum of the pigment chlorophyll extracted from a sample.

Principle

NOTES

A spectrophotometer is a device that measures the relative amount of light absorbed by a sample dissolved in a liquid solvent. A spectrophotometer was used to measure the light absorbed by a sample of phycobillin extract in a special tube placed in its sample port The spectrophotometer had a Transmittance and Absorbance (T/A) Meter which had a scale that allowed us to read the amount of light that was transmitted by (passed Using a Spectrophotometer to Measure Light Absorption.

Requirements

Balance, Oven, Test tubes Beakers, mortar and pestle, Spectrophotometer, cuvette, Acetone, Distilled water, leaves

Procedure

Cut the chromatograms into horizontal strips. It is best to work with only one pigment at a time to avoid contamination of samples. If the band is light, use the lesser volume of acetone indicated in step. Pipette 1–2mL of 100% acetone into three 13mm test tubes. Taking each of the 3 types of pigment (top green, bottom green and one yellow) separately, cut the strip into small pieces that can be submerged in the solvent. Cover the opening of the tube with Para film to prevent acetone evaporation. Bring the test tubes and your matched glass cuvettes to the Beckman spectrophotometer. Once the baseline has been run from 700–400nm, using 100% acetone in the cuvette, runs an absorption spectrum for each pigment, rinsing the sample cuvette with acetone (from a squirt bottle in the hood!) between readings. The peaks and valleys will be adjusted automatically by the spectrophotometer, by changing the range of % absorbance on the y-axis. When all the spectra have been recorded, print the chlorophylls together either on one graph or separately. Print the carotenoids as a separate graph. Similarly, stock solution to prepare a series of 4 dilutions of a dietary supplement that contains chlorophyll among other compounds (stock chlorophyll derived compounds at 0.05mg/ml). Each dilution in the series will differ from the previous dilution by 1/2, so the dilution ratio for each tube is 1/2, 1/4, and 1/8. Calculate the concentrations of the chlorophyll derivative that the tubes (in micrograms/ml) will contain after the dilutions have been prepared, and record them in your lab notebook. Label four 13mm test tubes with the dilution ratios: 1/2, 1/4, 1/8, 1/16. With a 5.0mL serological pipette add 4.0mL distilled water to each tube. Using a clean pipette, add 4.0mL of stock solution to the tube labelled 1/2 and mix by inversion .Use square of Para film to cover test tube opening. Keeping thumb on Para film, invert test tube twice. Use a clean pipette to add 4.0mL of 1/2 dilution to the tube labelled 1/4; mix.

Use a clean pipette to add 4.0mL of 1/4 dilution to the tube labelled 1/8; mix. Use a clean pipette to add 4.0mL of 1/8 dilution to the tube labelled 1/16; mix.

Observation and Results

NOTES

Four dilutions (stock, 1/2, 1/4, 1/8, 1/16) demonstrate the relationship between absorbance and concentration. Set the spectrophotometer to the wavelength that gave maximal absorption for chlorophyll derivative in Part A and zeros the instrument using water as the blank. Read the absorbance of the four concentrations and record them in your lab notebook next to the appropriate concentrations.

19.4 Determination of rate of Respiration of Germinating Seeds

Introduction

Respiration is the transport of oxygen from the outside air to the cells within tissues, and the transport of carbon dioxide in the opposite direction. The physiological definition of respiration should not be confused with the biochemical definition of respiration, which refers to cellular respiration: the metabolic process by which an organism obtains energy by reacting oxygen with glucose to give water, carbon dioxide and ATP (energy). Although physiological respiration is necessary to sustain cellular respiration and thus life in animals, the processes are distinct: cellular respiration takes place in individual cells of the organism, while physiologic respiration concerns the bulk flow and transport of metabolites between the organism and the external environment.

$$C6H12O6\ (s) + 6\ O2\ (g) \rightarrow 6\ CO2\ (g) + 6\ H2O\ (l) + Energy$$

Principle

Germination and establishment are very important parts of a plant's life cycle through which numerous individuals do not survive. Germinating seeds are not yet able to acquire the energy they need through photosynthesis, and so they depend largely upon respiration for their early development. Therefore, measuring respiration rate of this stage under different environmental conditions will supply us with a greater understanding of these critical developmental stages.

Requirements

0.1 M Ba (OH) 2 solution -- Be careful. As a strong base, 1 M HCl, 0.1 % phenolphthalein

Cheese cloth, string, 1 % Tetrazolium

Procedure

1. The 250 ml Erlenmeyer flasks will be used as reaction vessels.

2. Collect 12 germinating seeds for each of the four treatments from the seeds germinated at room temperature

3. Determine their fresh mass.

4.Tie them up in moist cheesecloth.

5. Prepare a 250 ml solution of 10 mM Ba (OH) 2 from the 0.1 M (100 mM) stock solution provided. Add 25 ml to each of 8 flasks (four treatments plus four control flasks with no seeds).

NOTES

6. Hang the seeds by a string from the rubber stopper by passing the string along the side of the flask next to the stopper. The seeds must not touch the solution. The flask must be tightly sealed. Do not allow the Ba (OH) 2 solution to be exposed to the air any longer than necessary.

7. After all the flasks are ready, place them in either the freezer (-15 deg-C), the refrigerator (5 deg-C) at room temperature (25 deg-C) or in the oven (65 deg-C) and let them respire for 2 hours.

While waiting for the two hours, experiment with the Ba $(OH)_2$ solution and develop you titrating skills. For titrating, place about 10 ml of fresh 10 mM Ba $(OH)_2$ into an Erlenmeyer flask. Add about 4 drops of 1% phenolphthalein (pH indicator). Swirl to mix. The solution should turn deep pink. Using a transfer pipette, adds 0.1 M HCl drop wise until the colour just disappears. (You will need to make the 0.1 M HCl from the 1.0 M stock solution. Use a small beaker flask and a graduated cylinder.) When adding the HCl drop wise, swirl your flask between each drop added to the solution. Also, let 10 ml of Ba (OH)2 sit exposed to the air for an hour or so. Titrate this solution--it should take less HCl now because some of the base has been neutralized by carbonic acid from the CO_2 in the air. After the 2 hours are over, titrate the Ba $(OH)_2$ solution from each treatment. Analyse these results by comparing the HCl required for each temperature treatment. The actual value to compare is the difference between the control and the seed-containing treatments at the same temperature. That difference is then divided by the fresh mass of the seeds and divided by the time over which they respired. (Respiration rate = □ ml HCl / (fresh weight * time)) The units could be ml HCl g-1 min-1.

The respiration rate in the first experiment will be measured as CO_2 production. To estimate the amount of CO_2 produced in a 2 hour period, we will depend on the ability of a solution of barium hydroxide Ba $(OH)_2$] to absorb CO_2. In this process, barium carbonate is produced that is insoluble in water and will precipitate.

Ba $(OH)_2 + CO_2$ □ $BaCO_3$ + H2O

As CO2 is produced from respiration, it will precipitate as barium carbonate. So the trick is to be able to measure how much barium hydroxide has been "used up" in this process. Barium hydroxide is a strong base and will react in water by dissociation:

Ba $(OH)_2$ □ Ba++ + 2 OH-

NOTES

Observation and Results

We can measure how much barium hydroxide has reacted to form the barium carbonate precipitate by seeing how much acid it takes to neutralize the remaining base. This process takes place when we titrate the solution with acid in the presence of the indicator phenolphthalein. The more CO2 produced by respiration and then removed from the air, the less acid it will take to neutralize the barium solution.

Thus, greater respiration causes a greater decline in the pH of the barium hydroxide solution. When this happens, a lesser amount of HCl is necessary to titrate the solution to neutral. In other words, the control vials should require the most HCl and the most actively respiring vials should require the least HCl.

19.5 Nitrogen Determination by Kjeldahl Method

Introduction

Proteins, from the Greek proteios, meaning first, are a class of organic compounds which are present in and vital to every living cell. In the form of skin, hair, callus, cartilage, muscles, tendons and ligaments, proteins hold together, protect, and provide structure to the body of a multi-celled organism. In the form of enzymes, hormones, antibodies, and globulins, they catalyse, regulate, and protect the body chemistry. In the form of haemoglobin, myoglobin and various lipoproteins, they affect the transport of oxygen and other substances within an organism. Proteins are generally regarded as beneficial, and are a necessary part of the diet of all animals. Humans can become seriously ill if they do not eat enough suitable protein, the disease kwashiorkor being an extreme form of protein deficiency. Protein based antibiotics and vaccines help to fight disease, and we warm and protect our bodies with clothing and shoes that are often protein in nature .Micro- Kjeldahl method could be used for estimation for protein nitrogen and non-protein nitrogen.

Principle

The Kjeldahl method is the standard method of nitrogen determination. The method consists of three basic steps: 1) digestion of the sample in sulphuric acid with a catalyst, which results in conversion of nitrogen to ammonia; 2) distillation of the ammonia into a trapping solution; and 3) quantification of the ammonia by titration with a standard solution.

Requirements

Equipment

Kjeldahl flasks, 500 to 800 ml Kjeldahl digestion unit with fume removal manifold Kjeldahl distillation apparatus - Kjeldahl flask connected to distillation trap by rubber stopper. Distillation trap is connected to condenser with low-sulphur tubing. Outlet of

condenser should be less than 4 mm diameter. Erlenmeyer flask, 500 ml Analytical balance, sensitive to 0.1 mg.

Reagents

NOTES

Sulphuric acid, concentrated, 95-98%, reagent grade Sodium hydroxide, pellets, flakes, or 45% solution with specific gravity 1.36 (low N) dissolve 450 g in cool water and dilute to 1 L Potassium sulphate (K2SO4), alundum ,anhydrous Copper sulphate (CuSO4), anhydrous Titanium dioxide (TiO2) boiling stones, 8-14 mesh Pumice Methyl red indicator dissolve 1 g methyl red (sodium salt) in 100 ml methanol or ethanol Tributyl citrate (for antifoam) or paraffin or antifoam A or equivalent Lysine mono hydrochloride, reagent grade, dried at 110OC for four hr Hydrochloric acid standard solution, 0.5 N Prepare by diluting 430.1 ml 36.5 to 38% HCl to 10 L with distilled water and standardize by method 3.1.1 Sodium hydroxide standard solution Prepare 0.1 N sodium hydroxide (NaOH) solution and standardize by method 3.1.2. After standardizing hydrochloric acid and sodium hydroxide, check one against the other by titrating one with the other and calculating normality.

Procedure

Digestion

1. Weigh approximately 1 g ground sample into digestion flask, recording weight (W) to nearest 0.1 mg. Include reagent blank and high purity lysine HCl as check of correctness of digestion parameters. Weigh a second sub-sample for laboratory dry matter determination.
2. Add 15 g potassium sulphate, 0.04 g anhydrous copper sulphate, 0.5 to 1.0 g alundum granules, or add 16.7 g K2SO4, 0.01 g anhydrous copper sulphate, 0.6 g TiO2 and 0.3 g pumice. Then add 20 ml sulphuric acid. (Add additional 1.0 ml sulphuric acid for each 0.1 g fat or 0.2 g other organic matter if sample weight is greater than 1 g.)
3. Place flask on preheated burner (adjusted to bring 250 ml water at 25oC to rolling boil in 5 min).
4. Heat until white fumes clear bulb of flask, swirl gently, and continue heating for 90 min for copper catalyst or 40 min for CuSO4/TiO2 mixed catalyst.
5. Cool, cautiously add 250 ml distilled water and cool to room temperature (less than 25oC). Note: If bumping occurs during distillation, volume of water may be increased to ca. 275 ml.

NOTES

Distillation

1. Prepare titration flask by adding appropriate volume (V HCl) accurately measured acid standard solution to amount of water so that condenser tip is immersed (try 15 ml acid and 70 ml water if undecided). For reagent blank, pipet 1 ml of acid and add approximately 85 ml water. Add 3 to 4 drops methyl red indicator solution.
2. Add 2 to 3 drops of tributyl citrate or other antifoam agent to digestion flask to reduce foaming.
3. Add another 0.5 to 1.0 g alundum granule.
4. Slowly down side of flask, add sufficient 45% sodium hydroxide solution (approximately 80 ml) to make mixture strongly alkali. (Do not mix until

1. (Do not mix until after flask is connected to distillation apparatus or ammonia will be lost.)
2. Immediately connect flask to distillation apparatus and distill at about 7.5 boil rates (temperature set to bring 250 ml water at 25oC to boil in 7.5 min) until at least 150 ml distillate is collected in titrating flask.
3. Remove digestion flask and titrating flask from unit, rinsing the condenser tube with distilled water as the flask is being removed.

Titration

Titrate excess acid with standard sodium hydroxide solution to orange endpoint (colour change from red to orange to yellow) and record volume to nearest 0.01 ml (V NaOH). Titrate the reagent blank (B) similarly.

Observation and Results

Calculation:

Percent Nitrogen (N) %N (DM basis) = [(V HCl x N HCl) - (VBK x N NaOH) -(V NaOH x N NaOH)]/1.4007 X W X

- Where V NaOH = ml standard NaOH needed to titrate sample
- V HCl = ml standard HCl pipette into titrating flask for sample
- N NaOH = Normality of NaOH
- N HCl = Normality of HCl
- VBK = ml standard NaOH needed to titrate 1 ml standard HCl minus B
- B = ml standard NaOH needed to titrate reagent blank carried through method and distilled into 1 ml standard HCl
- 1.4007 = milliequivalent weight of nitrogen x 100
- W = sample weight in grams

Precautions

- Handle acid safely: use acid resistant fume hood. Always add acid to water unless otherwise directed in method. Wear face shield and heavy gloves to protect against splashes. If acids are spilled on skin, immediately wash with large amounts of water.
- Sulphuric acid and sodium hydroxide can burn skin, eyes and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concentrated acid or alkali.
- Keep baking soda and vinegar handy in case of chemical spills.

NOTES

19.6 Estimation of Indole Acetic Acid (IAA)

Introduction

Indole-3-acetic acid (IAA) is a phytohormone, a type of best characterized auxins, which is essential for the growth and development of plants. It is most common and at the same time physiologically most active. It is known to stimulate both rapid (e.g., increases in cell elongation) and long-term (e.g., cell division and differentiation) responses in plants .The capacity to synthesize IAA is widespread among soil- and plant associated bacteria. Tryptophan (Trp) is generally considered to be the precursor of IAA.

Principle

Indole-3-acetic acid (IAA) is commonly accepted as a naturally occurring plant growth hormone. It forms pink colour with Salkowisky s reagent.

Requirements

Colorimeter, Test tubes, pipettes, flasks, Standard IAA Solution, Ferric Chloride, Salkowisky s reagent, per chloric acid

Procedure

Extraction

Corn kernels were ground to 20 mesh and stored at -20°. A 15 g sample was extracted with 60 ml of 50 % aqueous acetone for 1 hr at room temperature with continuous agitation by a magnetic stirrer and then filtered. This procedure was repeated 4 more times. Each of the acetone extracts were then assayed for alkali labile IAA compounds. The extracts were made 1 N with respect to NaOH, then, after 15 min at room temperature, they were acidified to pH 2.5 with 5 N H_2SO_4 and extracted 3 times with an equal volume of peroxide free ether. The ether was evaporated to dryness, the residue redissolved in 1 ml of 95 % ethanol, 4 ml of Salkowski reagent added and IAA estimated calorimetrically. The corn meal residue was extracted with 100 ml of N NaOH for 15 min at room temperature. Take blank 0.0 and IAA solution (0.2-1.2).

Observation and Results

Colorimetric assays were corrected for interference by compounds which yield pink colour with Salkowski reagent. Optical density was measured at 530 nm. Calculate the amount of IAA in the given sample using standard graph.

NOTES

Calculation

0.5 ml of unknown IAA solution having concentration = X microgram (from standard IAA). Therefore 1 ml of unknown IAA solution having concentration = 1/0.5 x X microgram Concentration of IAA present in the given sample is expressed as micrograms of IAA per ml.

19.7 Extraction of Seed Proteins

Introduction

Seeds contain different components such as starch and complex carbohydrates that can reduce protein extraction. The proteins in cereal seeds are usually classified in four groups according to their solubility criteria: albumins, globulins, prolamins, and glutelins. They can be specifically extracted. Then several procedures mostly adapted to cereal seeds are reported for: (1) the whole storage proteins (mostly prolamins and glutelins); (2) the albumins-globulins extracted using salt buffer; (3) the amphiphilic proteins extracted using a phase partitioning process; and (4) the proteins strongly attached to or within the starch granules of the seed endosperm. These procedures have been used for 2-D electrophoresis and proteomic analyses

Principle

Seed proteins are extracted from Brassica sps. (~20 mg) as described by Shewry et al. (1995), with the exception that bromophenol blue was not added to the extraction buffer. The use of a Hamilton syringe to remove the supernatant prevents oil contamination.

Requirements

Seeds Brassica sps**.** Protein Extraction Buffer (+DTT/-Bromophenol Blue; BB), Protein Extraction Buffer (+DTT/+BB). Place 1 ml Protein Extraction Buffer (+DTT/-BB; above) into a microfuge tube and add/mix a small quantity of Bromophenol Blue (BB) powder on the tip of a wetted pipette tip. This Buffer is used as a sample buffer for running gels.

Procedure

A general procedure for extracting the proteins present in green seeds or immature cereal kernels is given-

1. Add 5 seed per sample into a 2 ml safe lock microfuge tube.
2. Weigh and record the weight of each sample. Add one 5 mm stainless steel bead to each tube.

3. Add 20mL Protein Extraction Buffer (+DTT/-BB) per mg sample weight to each tube. NOTE: the amount of buffer is dependent on the weight of the sample. Ensure the tubes are closed properly.
4. To grind seed, place tubes in Tissue Lyser (Qualiagen) and operate for 1 min at 30 Hz on either side (2 min total).
5. Centrifuge for 1 min at 13 000 rpm to collect cell debris. Protein can now be extracted.
6. Using a large magnetic flea, remove the bead from the tube by sliding the flea along the outside of the tube. Ensure that no cell debris remains on the bead before removing from tube. If cell debris remains, vortex briefly and pulse spin. Pulse spin sample to re-collect cell debris in tube.
7. Incubate for 1 h at room temperature. After 30 min of incubation, turn on heat block ready for step 15. Pierce lid of tube. Incubate at for 2 min at 100oC for 2 min.
8. Centrifuge sample for 30 min at 13 000 rpm.
9. Using a Hamilton syringe carefully remove supernatant, avoiding the oil layer. Rinse Hamilton syringe 5 xs in clean Milli Q water between samples.
10. Spin supernatant for 15 min at 13 000 rpm. Using a Hamilton syringe, carefully remove supernatant, avoiding the oil layer.
11. Aliquot 4x 10 µL supernatant into PCR tubes and freeze immediately at -80oC.

Observation and Results

Extracted seed protein can be used for protein quantification.

19.8 Determination of Seed Viability

Introduction

A viable seed is one which is capable of germination under suitable conditions. The definition includes dormant but viable seeds, in which case the dormancy must be broken before viability can be measured by germination. The Tetrazolium Chloride (TZ) test is often called the quick germination test. It's a chemical test used to determine seed viability, and results are usually available within 24 to 48 hours. This process is rapid evaluation of seed viability.

Principle

Germination test is the best indication of the potential of a seed lot to emerge under field conditions. However, it takes from days to weeks and in some cases even months to complete. Tetrazolium test, commonly known as the TZ test for seed viability have, therefore,. Developed to furnish quick estimates of seeds' germ inability. The test is very useful in processing, handling, storing and marketing. Large quantities of seed in a short

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time, testing dormant seed lots, vigour rating of the seed lots, supplementing germination test results and diagnosing the cause of seed deterioration.

Requirements

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Staining dished (watch glasses or Petri dishes),Beakers of 100 to 250 ml capacity for larger seeds, Single edge razor blade, Needles, Forceps, Magnifying devices, Medicine dropper, Dispensing bottle, Germinating blotters, filter paper , paper towel and Oven or incubator, TTC (Triphenyl Tetrazolium Chloride), seeds.

Procedure

1. Preparation

Seeds are soaked in water overnight. They may be pre-moistened, in which case the seeds are allowed to imbibe water between moistened germination paper blotters. Seeds are then dissected, either longitudinally or transversely, with a scalpel so that the embryo is exposed to the tetrazolium chloride solution. One half of this seed is used for the test and the other half is discarded.

2. Staining

A solution of 2, 3, 5 triphyenyl tetrazolium chloride (a salt) is added to water to form a colourless solution. The seeds are placed in a 1% solution (for legumes and grasses that are not bisected), or a dilute 0.1% solution for bisected grasses and cereals. Seed coats of legumes must usually be removed or peeled back before examination. Care must be taken to prevent breaking of radicles and other damage to the seeds.

Observation and Results

Observe the colour change in boiled and normal seed sample and discuss the results with reference to their reaction with TTC. Dehydrogenase enzymes present in living tissue reduce the tetrazolium chloride to formazan, a reddish, water-insoluble compound. This reaction occurs in or near living cells, which are releasing hydrogen in respiration processes. Depending on size, all seeds are examined under a microscope at 10-30 power. Larger seeds, such as peas, may be examined without a microscope.

% Viability =

No. of cotyledons showing pink colour / Total No. of cotyledons in the bath x 100

19.9 Viva-Voce

1. What are Photosynthetic pigments?
2. What is Paper Chromatography?
3. What do you mean by Rf value?
4. Define Photosynthesis.

5. Name one plant which has more chlorophyll content.
6. Can we extract chlorophyll with water?
7. What do you mean by OD?
8. How do you measure Absorption Spectra?
9. Define Photosynthesis.
10. How do you extract the pigments?
11. Can we extract chlorophyll with water?
12. What do you mean by OD?
13. Define Respiration.
14. Which is the standard method of nitrogen determination?
15. Write about the indicator mixture.
16. Write down the full form of IAA.
17. How do you prepare standard graph of IAA.
18. How do you calculate the amount of IAA in the given unknown sample?
19. How can you Extract seed proteins from the given sample?
20. Which extraction buffer is used in the physiology experiment?
21. How many types of protein present in the given seed sample.
22. What do you mean by seed viability?
23. Why are boiled cotyledons not coloured?
24. Which enzymes are involved in the reaction that imparts a pink colour to the cotyledon?

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19.10 References

1. Plant Physiology (Fundamentals and Applications) - A Kumar and S S Purohit
2. Plant Physiology- Salisbury and Ross
3. Plant Physiology- L. Taiz and E. Zeiger
4. Biochemistry- Lehninger
5. Biochemistry by Voet and Voet

Unit - 20

Biochemistry

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Structure of the Unit

20.0 Objectives

After going through this unit you will be able to understand the following

- Estimations like- Estimation of Amino Acid; Protein by Lowry's Method and
- Preparation of standard curve of BSA and Separation & identification of Amino Acid by using TLC method.
- Estimation of Fructose
- Reducing sugars, Determination of Amylase activity,
- Catalase Activity and Determination of Iodine number of edible oils.

20.1 Estimation of Amino Acid by Ninhydrin Method

Introduction

Amino acids are biologically important organic compounds composed of amine (-NH2) and carboxylic acid (-COOH) functional groups, along with a side-chain specific to each amino acid. The key elements of an amino acid are carbon, hydrogen, oxygen, and nitrogen, though other elements are found in the side-chains of certain amino acids. About 500 amino acids are known and can be classified in many ways. They can be classified

according to the core structural functional groups' locations as alpha, beta, gamma or delta amino acids; other categories relate to polarity, pH level, and side-chain group type (aliphatic, acyclic, aromatic, containing hydroxyl or sulfur, etc.). In the form of proteins, amino acids comprise the second-largest component (water is the largest) of human muscles, cells and other tissues. Outside proteins, amino acids perform critical roles in processes such as neurotransmitter transport and biosynthesis

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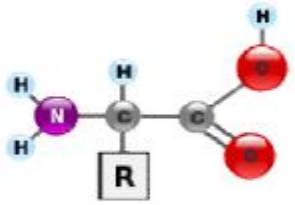

Amino-Acid

Principle

Amino acids are known as the building blocks of all proteins. There are 20 different amino acids commonly found in proteins. Amino acids are comprised of a carboxyl group and an amino group attached to the same carbon atom (the carbon). They vary in size, structure, electric charge and solubility in water because of the variation in their side chains (R groups). Detection, quantification and identification of amino acids in any sample constitute important steps in the study of proteins. Alpha amino acids react with Ninhydrin involved in the development of colour which is explained by the following five steps.

1. **alpha-amino acid + Ninhydrin ---> reduced ninhydrin +Alpha amino acid +H_2O**

This is an oxidative deamination reaction that elicit two hydrogen from the alpha amino acid to produce an alpha imino acid. Also the ninhydrin reduced and loses an oxygen atom with the formation of water molecule.

2. **alpha-amino acid + H_2O ---> alpha-keto acid +NH_3**

The rapid hydrolysis of NH group in the alpha – amino acid will cause the formation of an alpha- keto acid with an ammonia molecule. This alpha-keto acid further involved in the decarboxylation reaction of step.

3. **alpha-keto acid + NH_3 ---> aldehyde + CO_2**

Under a heated condition to form an aldehyde that has one less carbon atom than the original amino acid. A carbon dioxide molecule is produced along with aldehyde. These first three steps produce the reduced ninhydrin and ammonia that are required for the production of colour .The overall reaction for the above reactions is simply explained in Reaction (4) as follows:

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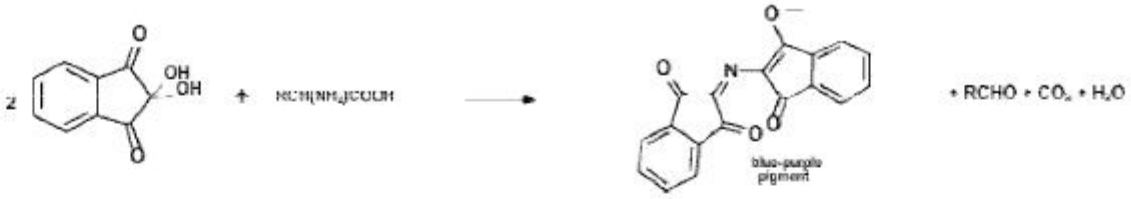
4.alpha-amino acid + 2 ninhydrin---> CO_2 + aldehyde + final complex(BLUE) + $3H_2O$

Ninhydrin is originally yellow, reacts with amino acid and turns deep purple. It is this purple colour that is detected in this method. Ninhydrin will react with a free alpha-amino group, NH_2-C-COOH. This group is present in all amino acids, proteins or peptides. Whereas, the decarboxylation reaction will proceed for a free amino acid, it will not happen for peptides and proteins. Theoretically only amino acids produce colour with ninhydrin reagent.

The ninhydrin reaction, one of the most important methods of detecting amino acids, both technically and historically, has been conventionally used to detect their microgram amounts. When amino acids with free alpha amino groups are treated with an excess of ninhydrin, they yield a purple coloured product. Under appropriate conditions, the colour intensity produced is proportional to the amino acid concentration. The primary amino groups react with ninhydrin to form the purple colour dye now called Riemann's purple (RP) was discovered by Siegfried Ruhemann in 1910. Amino acids like proline, the guanidine group of arginine, the amide groups of asparagine, the indole ring of tryptophan, the sulfhydryl group of cysteine, amino groups of cytosine and guanine, and cyanide ions also react with ninhydrin to form various chromophores that can be analysed. The overall reaction can be written as follows:



Requirements

1. Ninhydrin reagent= dissolve 500 mg ninhydrin in 100 ml acetone.
2. 0.2 M Citrate buffer (Ph 5.0)
3. Standard amino Acid solution

Methods

Plant material was macerated in 80% ethanol. It was further centrifuged and supernatant was used for the estimation of free amino acids whereas, residue was utilized for the estimation of protein linked amino acids.

2.0 ml of ninhydrin reagent added to 1.0 mi of ethanoic plant extract then heated on water bath at 100 C, cool, diluted, a violet blue colour developed. OD was taken at 570 nm. Standard curve was prepared with glycine.

Observations and Results

Record OD values of samples of different concentrations, blank, 0.1ml to 0.6 ml. Draw a standard graph. Finally calculate amino acid concentration in the test solution and express it as mg/100ml.

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20.2 Estimation of Protein by Lowry's Method and Preparation of Standard Curve of BSA

Introduction

Quantitation of total protein content is a measurement common to many applications in basic science research and routine clinical laboratory practice. Most biochemical studies that involve the measurement of a biological activity require the normalization of that activity to the protein content. The specific activity of a particular enzymatic activity is of particular importance when proteins are being purified or different samples are being compared. The most utilized methods to assay total protein rely on the reduction of copper in the presence of a chromogenic reagent.

Principle

Protein was estimated by protocol of Lowry et al. (1951). The method combines the reactions of copper ions with the peptide bonds under alkaline conditions with the oxidation of aromatic protein residues. The Lowry method is best used with protein concentrations of 0.01–1.0 mg/mL and is based on the reaction of Cu+, produced by the oxidation of peptide bonds, with Folin–Ciocalteu reagent (a mixture of phosphotungstic acid and phosphomolybdic acid in the Folin–Ciocalteu reaction). The reaction mechanism is not well understood, but involves reduction of the Folin–Ciocalteu reagent and oxidation of aromatic residues (mainly tryptophan, also tyrosine). Experiments have shown that cysteine is also reactive to the reagent. Therefore, cysteine residues in protein probably also contribute to the absorbance seen in the Lowry Assay. The concentration of the reduced Folin reagent is measured by absorbance at 750 nm.

Materials

1. Complex-forming reagent: Prepare immediately before use by mixing the following stock solutions in the proportion 100:1:1 (by vol), respectively:

Solution A: 2% (w/v) Na_2CO_3 in distilled water.

Solution B: 1% (w/v) $CuSO_4 \cdot 5H_2O$ in distilled water.

Solution C: 2% (w/v) sodium potassium tartrate in distilled water.

2. 2 N NaOH.

3. Folin reagent (commercially available): Use at 1 N concentration.

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4. Standards: Use a stock solution of standard protein (e.g., bovine serum albumin fraction V) containing 2 mg/mL protein in distilled water, stored frozen at –20C. Prepare standards by diluting the stock solution with distilled water as follows:

Stock solution (mL) 0 2.5 5 12.5 25 50 125 250 500

Water (mL) 500 498 495 488 475 450 375 250 0

Protein conc. (mg/mL) 0 10 20 50 100 200 500 1000 2000

Method

1. To 0.1 mL of sample or standard adds 0.1 mL of 2 N NaOH. Hydrolyse at 100 C for 10 min in a heating block or boiling water bath.

2. Cool the hydrolysate to room temperature and add 1 mL of freshly mixed complex-forming reagent. Let the solution stand at room temperature for 10 min

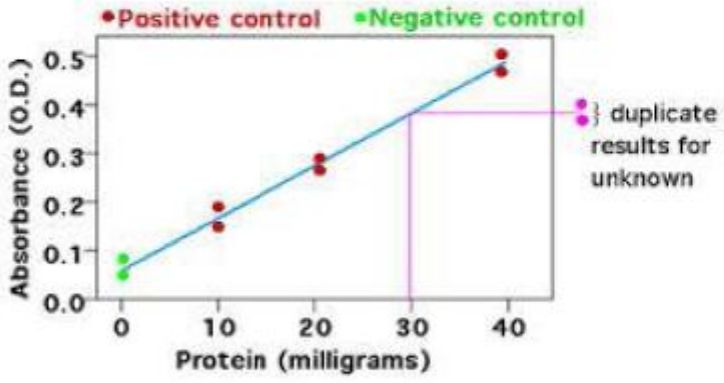
3. Add 0.1 mL of Folin reagent, using a vortex mixer, and let the mixture stand at room Temperature for 30–60 min (do not exceed 60 min)

4. Read the absorbance at 750 nm if the protein concentration was below 500 mg/mL or at 550 nm if the protein concentration was between 100 and 2000 mg/mL.

5. Plot a standard curve of absorbance as a function of initial protein concentration and use it to determine the unknown protein concentrations

Observations and Results

A standard curve was prepared as follows. Bovine serum albumin (BSA) powder was dissolved in distilled water and diluted to a concentration of 1 µg/ml. A series of dilutions (0, 1, 2.5, 5, 10, and 20 µg/well) were made in replicates of 4 with a final volume of 100 µl. Samples were diluted such that they would fall within the BSA standard range (0-25 µg / 100 µl) and 100 µl placed in each well. After standards and samples were diluted and transferred to the micro plate, 200 ul of biuret reagent was added to each well and mixed thoroughly with repeated pipeting. Biuret reagent was prepared by mixing 0.5 ml of 1% cupric sulphate with 0.5 ml of 2% sodium potassium tartrate, followed by the addition of 50 ml of 2% sodium carbonate in 0.1 N NaOH.



The mixture was then allowed to incubate at room temperature for 10-15 minutes prior to the addition of 20 µl per well of 1.0 N Folin & Ciocalteu's reagent. Samples were mixed immediately with repeated pipeting with each addition. Colour was allowed to develop for 30 minutes at room temperature and the absorbance measured at 650 nm and blanked on the water only control. Although in these experiments the plates were read immediately, the reaction was found to be stable for up to an hour.

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20.3 Separation & Identification of Amino Acid by using TLC Method

Introduction

Thin layer chromatographic (TLC) technique readily provides qualitative information and with careful attention to details, it is possible to obtain quantitative data. Thin layer chromatography is a technique used to separate and identify compounds of interest. A TLC plate is made up of a thin layer of silica adhered to glass or aluminium for support. The silica gel acts as the stationary phase and the solvent mixture acts as the mobile phase. In the ideal solvent system the compounds of interest are soluble to different degrees. Separation results from the partition equilibrium of the components in the mixture. In the simplest form of the technique, a narrow zone or spot of the sample mixture to be separated is applied near one end of the TLC plate and allowed to dry. The strip or plate is then placed with this end dipping in to the solvent mixture, taking care that the sample spot/zone is not immersed in the solvent. As the solvent moves towards the other end of the strip, the test mixture separates into various components. This is called as the development of TLC plates. The separation depends on several factors; solubility: the more soluble a compound is in a solvent, the faster it will move up the plate. (b) attractions between the compound and the silica, the more the compound interacts with silica, the lesser it moves, (c) size of the compound, the larger the compound the slower it moves up the plate. The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value. The plate is removed after an optimal development time and dried and the spots/zones are detected using a suitable location reagent. An important characteristic used in thin layer chromatography is Rf value.

Principle

Chromatography is the most useful techniques available for the separation of closely related compounds in a mixture. Here the separation is effected by differences in the equilibrium distribution of the components between two immiscible phases, viz., the stationary and the mobile phases. These differences in the equilibrium distribution are a

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result of nature and degree of interaction of the components with these two phases. The stationary phase is a porous medium like silica or alumina, through which the sample mixture percolates under the influence of a moving solvent (the mobile phase). There are a number of interactions between the sample and the stationary phase and these have been well exploited to effect the separation of compounds.

$$Rf = \frac{distance\ moved\ by\ the\ substance\ from\ origin}{distance\ moved\ by\ solvent\ from\ origin}$$

Requirements

Silica gel,Alanine,Tryptophan,Acetic acid, Ninhydrin reagent,Water,Iodine,silica gel plate• mobile phase: 1-butanol, glacial acetic acid and water (4:1:1), known solutions of amino acids, unknown solutions of amino acids, micropipette, developing tank, 2% ninhydrin solution, heat gun

Methods

1. Carefully hold the silica plate by the sides to prevent disturbing the silica gel layer. Draw a pencil line about 1.5 cm from the bottom plate.

2. Mark one point on the line for each one of your known and unknown solutions. (If you have four known solutions and 2 unknowns, mark six points.) Leave margins of at least 1.5 cm on both sides. Number each point.

3. At point number 1 apply a very small drop of one of your known 1 2 3 4 5 6 7 solutions. The silica beyond a diameter of 2-3 mm. locating the centre of large spots will be difficult later when the spot has moved along the paper.

4. After the liquid has evaporated (only a few seconds), add a second drop to the same spot. Record the name of the amino acid and the number of the spot.

5. Repeat this procedure for the remaining solution. Remember to record the name of the amino acid or unknown number and the number of the spot.

6. Allow all the spots to dry completely.

7. Place your TLC plate in the developing tank with the mobile phase with the spots toward the bottom.

8. Allow the solvent to ascend the silica gel to at least ¾ of its height, which will to require 1 hour or less. (The further the solvent ascends, the greater the separation. Immediately remove the plate, if the solvent reaches the top.)

9. Remove the plate and quickly mark the farthest advance of solvent front with a pencil, unless it reached the top of the paper.

10. Dry the plate with a heat gun. Be careful to move the heat gun around and not heat one point continuously. Do this procedure in the hood.

11. Spray the plate with the 2% ninhydrin solution in the hood.

12. Do not allow the ninhydrin solution to stream down the plate, because this may move some of the compounds.

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13. Dry the plate again with the heat gun. Do not over heat the plate. Long heating times may cause browning of the plate over the entire surface.

14. Circle each coloured spot with a pencil. The ninhydrin spots fade gradually, so circle at once.

15. Measure the distance from the origin to the centre of each coloured spot and calculate the Rf values for all spots.

16. Record the Rf values and the colour of each ninhydrin spot.

17. Identify the unknown amino acids.

Observations and Results

$$Rf = \frac{distance\ moved\ by\ the\ substance\ from\ origin}{distance\ moved\ by\ solvent\ from\ origin}$$

Since amino acids are colourless compounds, ninhydrin is used for detecting them. To identify this, after development, the TLC plate is sprayed with ninhydrin reagent and dried in an oven, at 105°C for about 5 minutes. Ninhydrin reacts with amino acids that results in purple coloured spots [due to the formation of the complex - Rheuman's purple] Rf values can be calculated and compared with the reference values to identify the amino acids. The Rf value for each known compound should remain the same provided the development of plate is done with the same solvent, type of TLC plates, method of spotting and in exactly the same conditions.

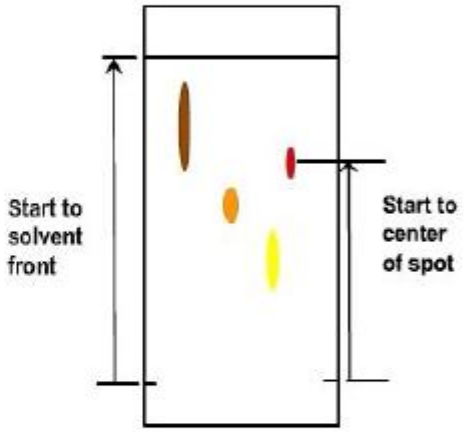

Fig. 20.1 Separation of Amino Acid by using TLC

Precautions

Saturate the chamber with mobile phase at least half an hour.

Spotting should be done at 1.0 cm from the bottom.

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20.4 Estimation of Fructose by Resorcinol Method

Introduction

Carbohydrates are large biological molecules, or macromolecules, consisting of carbon (C), hydrogen (H), and oxygen (O) atoms, usually with a hydrogen: oxygen atom ratio of 2:1 . Its empirical formula is Cm (H2O) n .Carbohydrates are technically hydrates of carbon; structurally it is more accurate to view them as polyhydroxy aldehydes and ketones. Quantitative measurement of fructose was carried out by the Resorcinol method. Seliwanoff's test reagent dehydrates ketohexoses to form 5-hydroxymethylfurfural.5-hydroxymethylfurfural further reacts with the resorcinol present in the test reagent to produce a red product within two minutes.

Principle

Carbohydrates are widely prevalent in the plant kingdom, comprising the mono-, di-, oligo-, and polysaccharides. The common monosaccharides are glucose, fructose, galactose, ribose etc. The disaccharides, i.e., the combination of two monosaccharides include sucrose, lactose and maltose. Starch and cellulose are polysaccharides consisting of many monosaccharide residues. Cellulose is the most abundant organic compound on this planet since it forms part of the cell wall in plants. Aldehydes (–CHO) and ketones (= CO) are active groups in carbohydrates. Carbohydrates contain many hydroxyl groups as well. The number of hydroxyl groups varies with the number of carbon atoms. Monosaccharides contain the free aldehyde or ketone group. Some disaccharides have the free aldehyde group (maltose) and some do not have the free ones (sucrose). The polysaccharides, starch and cellulose, are polymers of monosaccharides linked through the active groups.

Requirements

Test tubes, water bath, colorimeter, test tube stand.

Reagents-

1. Resorcinol-thiourea reagent. Dissolve 0.1 gm. of resorcinol and 0.25 gm. of thiourea in 100 cc. of glacial acetic acid. This reagent should be kept in a brown bottle.

2. 30% hydrochloric acid. To 1 part of distilled water add 5 parts of concentrated HCI, sp. gr. 1.19.

3. Standard Fructose solution solution. Prepare a stock fructose solution containing 1 mg. per cc. Dilute this 1:50 to obtain a working inulin standard containing 0.02 mg. per cc.

Procedure

In this experiment the quantity of HCl was reduced to 2ml instead 2,5ml. To 0, 5 ml of Selliwanoff's reagent (0,1g resorcinol and 0,1g of urea dissolved in 100ml of ethanol) in glass tubes, 25µl of fructose standard (45, 60, 90, 150, 270, 300,450, 600 mg/dl), and 2 ml HCl 37%. The content were mixed and placed in a boiling water bath (100oC) for 5 minutes, removed and cooled. The absorbance of the standard was read against reagent blank at 546nm.

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Observations and Results

The preliminary result indicated that is possible to reduce the amount of HCl, making this method less expensive and less toxic. The other modifications will be held. Identify the concentration of fructose in the sample of test solution using its OD values and standard curve and calculate fructose content in the given samples.

20.5 Estimation of Reducing Sugars

Introduction

Carbohydrates are the most abundant class of organic compounds found in living organisms. They originate as products of photosynthesis, an endothermic reductive condensation of carbon dioxide requiring light energy and the pigment chlorophyll.

$$n\,CO2 + n\,H2O + \text{energy} \longrightarrow CnH2nOn + n\,O2$$

The carbohydrates are a major source of metabolic energy, both for plants and for animals that depend on plants for food. Aside from the sugars and starches that meet this vital nutritional role, carbohydrates also serve as a structural material (cellulose), a component of the energy transport compound ATP, recognition sites on cell surfaces, and one of three essential components of DNA and RNA. Carbohydrates are also called as saccharides or sugars.

Principle

Benedict's quantitative reagent is a modification of qualitative aspects. It contains copper sulphate-sodium acetate and sodium carbonate. It also contains potassium thiocyanate and small amount of potassium ferrocyanide. The inclusion of acetate prevents the precipitation of copper car boning by chelating Cu3+ ions. The thiocyanate causes with the precipitation of white cuprous thiocyanate rather than red cupric oxide. On the reduction of Cu3+ ions, which inhibits the end point of the titration digest the transition from blue to white to be readily observed. Methylene blue will be used as an additional indicator. The small amount of potassium ferrocyanide prevents the pre oxidation of copper .The non-stichometric reaction is on which not follow a defined pathway and

cannot be described by an equation either quantitatively or qualitatively. The reduction of Cu3+ ions by sugar is a non-stichometric equation and is only constant over a small range of sugar concentration.

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Requirements

(i) Standard glucose solution

200mg of glucose was weighed accurately and made up to 100ml with distance water. (Conc.2mg/ml).

(ii) Benedict's quantitative reagent

100mg of sodium citrate and 62.5gm potassium thiocyanate were dissolved in 300ml of distilled water by warming gently and filtered.18% of copper sulphate is dissolved in 50ml of water, added with continuous stirring. 2.5ml of 5% potassium ferrocyanide is added and volume is made up to 500ml with water.

(iii) Anhydrous sodium carbonate

(iv) Lead acetate

Procedure

Take 5ml of Benedict's reagent into a clean conical flask. About 600mg. of anhydrous sodium carbonate was added to provide the required alkalinity with a few porcelain bits and heated to boiling over a moderate flame. Standard glucose solution is taken in a burette. When the Benedict's solution boils continuously, glucose solution is added drop by drop (1 drop/sec) till last trace of blue colour disappears. The volume of glucose rundown is noted and the titrations are repeated to concordant values.

The given unknown sugar solution was made up to 100ml in a standard flask with distilled water. Then the burette was filled with unknown sugar solution and the Benedict's reagent was titrated as before. The volume of sugar solution rundown was noted and titrations are repeated for concordant values.

Observations and Results

The amount of glucose present in 100ml of given unknown solution is mg. Firstly glucose is standardized against Benedict solution.

For e.g. 5 ml of Benedict solution is reduced by X ml of glucose solution.

1 ml of standard glucose solution contains 2 mg glucose.

X ml of standard glucose solution contains 2 mg glucose =?

X x 2 = Y

5 ml of Benedict solution is reduced by Y mg of glucose.

Similarly, titration with fruit extract;

5 ml of Benedict solution has reduced by Z ml of fruit extract.

Z ml of fruit extract contains Y mg of glucose

250 ml fruit extract contains Y mg of glucose?

250 x Y / Z= (A) mg

20 g of fruit extract contains (A) mg

100 gm of fruit extract contains?

100 x (A)/ 20 + (B) g

Amount of reducing sugar in the given sample is = (B) g.

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20.6 Determination of Amylase activity

Introduction

Enzymes are macromolecular biological catalysts. They are responsible for thousands of metabolic processes that sustain life. Enzymes are highly selective catalysts, greatly accelerating both the rate and specificity of metabolic chemical reactions, from the digestion of food to the synthesis of DNA. Most enzymes are proteins, although some catalytic RNA molecules have been identified. Enzymes adopt a specific three-dimensional structure, and may employ organic (e.g. biotin) and inorganic (e.g. magnesium ion) cofactors to assist in catalysis.

Principle

The decrease with time of the intensity of colour obtained with the diluted iodine solution is used as an index of starch degradation. Iodine gives blue colour on reaction with starch.

Requirements

Centrifuge, Pestle and mortar, tubes, muslin cloths

Buffer soln. of Ph =6, Soluble starch, KI Solution and gram seeds.

Procedure

Take 20 g seeds without seed coat, macerated, and add 5-10 ml chilled buffer solution. Filter the homogenate through muslin cloth and centrifuge it at 3000 rpm for 5 min. Collect supernatant and make it 100 ml with buffer. This extraction contains amylase enzyme.

Observations and Results

Plot a graph with varying concentrations on X and rate of reaction (time) on the Y axis.

20.7 Determination of Catalase Activity

Introduction

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen (such as vegetables, fruit or animals). It catalyses the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative

damage by reactive oxygen species (ROS). Likewise, catalase has one of the highest turnover numbers of all enzymes; one catalase molecule can convert approximately 5 million molecules of hydrogen peroxide to water and oxygen each second.

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Catalase is a tetramer of four polypeptide chains, each over 500 amino acids long. It contains four porphyrin heme (iron) groups that allow the enzyme to react with the hydrogen peroxide. The optimum pH for human catalase is approximately and has a fairly broad maximum (the rate of reaction does not change appreciably at pHs between 6.8 and 7.5). The pH optimum for other catalases varies between 4 and 11 depending on the species. The optimum temperature also varies by species.

Principle

The reaction of catalase in the decomposition of hydrogen peroxide in living tissue:

$$2\ H2O2 \longrightarrow 2\ H2O + O2$$

The presence of catalase in a microbial or tissue sample can be tested by adding a volume of hydrogen peroxide and observing the reaction. The formation of bubbles, oxygen, indicates a positive result. This easy assay, which can be seen with the naked eye, without the aid of instruments, is possible because catalase has a very high specific activity, which produces a detectable response.

Requirements

K MnO4, Hydrogen peroxide, Buffer, Sulphuric acid.

Reagents for extraction buffer

1 Mm EDTA

0.05% TritonX100

2% PVP

- 1Mm Ascorbate in 50Mm phosphate buffer of P^H 750mM phosphate buffer P^H7
- Hydrogen peroxide (3.125mM) – 350 µl of H_2O_2 make up the volume up to 100ml

Phosphate buffer

0.2M Na_2HPO_4

0.2M NaH_2PO_4

Procedure

For the determination of enzymes activity, 1 gm material was homogenized in 5 ml of extraction buffer by grinding with a pre-cooled mortar pestle. The homogenate was centrifuged at 15000 rpm for 25 minutes at 4°c. The pellet containing cell debris was discarded and supernatant containing the soluble enzymes was stored on ice till the assay was carried out.

The activity of catalase was assayed using this method.

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PHOSPHATE BUFFER 50Mm pH7

(2.8ml)

↓

0.1ml H_2O_2 (3.125Mm)

↓

TAKE OD AT 240nm

↓

ADD 0.1ml ENZYME EXTRACT

↓

TAKE OD AT 240 nm

Observations and Results

Record the titration values of sample and blank. Express the catalase activity by

Mg H2O2 destroyed by 1 g plant tissues = 25/2 x V/W x 0.85

25 is total volume of enzyme extract

2 is enzyme extract

V is difference of blank and sample titration value

W is weight of sample.

20.8 Determination of Iodine Number of Edible Oils

Introduction

Lipids are a group of naturally occurring molecules that include fats, waxes, sterols, fat-soluble vitamins (such as vitamins A, D, E, and K), monoglycerides, diglycerides, triglycerides, phospholipids, and others. The main biological functions of lipids include storing energy, signalling, and acting as structural components of cell membranes. Lipids have applications in the cosmetic and food industries as well as in nanotechnology.

Principle

The determination of the iodine value is based on the addition of iodine to the double bonds of unsaturated fatty acids. The iodine value (or "iodine adsorption value" or "iodine number" or "iodine index") in chemistry is the mass of iodine in grams that is consumed by 100 grams of a chemical substance. Iodine numbers are often used to determine the

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amount of unsaturation in fatty acids. This unsaturation is in the form of double bonds, which react with iodine compounds. The higher the iodine number, the more C=C bonds are present in the fat.[1] It can be seen from the table that coconut oil is very saturated, which means it is good for making soap. On the other hand, linseed oil is highly unsaturated, which makes it a drying oil, well suited for making oil paints.

Requirements

- Sample changer with Swing Head and DIS-Cover
- Titrator with DET mode
- 2x Burette 20 mL (Glacial acetic acid, Mg (CH3COO)2)
- 4x Burette 50 mL (H2SO4, ICl, KI, Na2S2O3)
- Propeller Stirrer

Electrodes

Iodine, KI Solution, Starch solution, chloroform, coconut oil

Procedure

For the manual determination of the iodine value the beakers have to be placed in the dark after adding the reaction solution, magnesium acetate solution and glacial acetic acid. Before the titration the potassium iodide solution has to be added, all these steps are laborious and time consuming. The automated determination is done with brown glass beakers and the Robotic DIS-Cover system. This method leads to good and reproducible results.

Observations and Results

The result is given as g I2 consumed by 100 g sample and is a measure for the unsaturation of oil.

Calculate the difference in titrate value of blank and sample.

Blank – Sample= X

1ml of Na2S2O2 consumes 6.325 mg of iodine

X ml of Na2S2O2 consumes 6.325 mg of iodine?

X ml x 6.325 = Y

Iodine number = Y

20.9 Viva-Voce

1. Which colour is formed by amino acid and ninhydrin reaction?
2. What is the wave length at which Amino acid is measured?

3. What is the wave length at which Protein is measured?
4. Why we use ninhydrin in Amino-acids reaction?
5. What do you mean by Rf value?
6. Which colour is formed by fructose and Resorcinol reagent?
7. Fructose belongs to which carbohydrate group.
8. What are carbohydrates?
9. What are reducing sugars
10. What are Enzymes?
11. Which type of seeds is used in this experiment?
12. What are Catalase Enzymes?
13. What is role of KMnO4 in this experiment?
14. What is the role of H2SO4 in this experiment?
15. What do you mean by Iodine number?

NOTES

20.10 References

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