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1. Microscopy of Fungi and Plant Pathogens

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1.1 Introduction:

Microscopes are specialized optical instruments designed to produce magnified visual or photographic (including digital) images of objects or specimens that are too small to be seen with the naked eye. The most familiar type of microscope is the optical, or light, microscope, in which glass lenses are used to form the image. Optical microscopes can be simple, consisting of a single lens, or compound, consisting of several optical components in line.

The hand magnifying glass can magnify about 3 to 20X. Single-lensed simple microscopes can magnify up to 300X and are capable of revealing fungi and bacteria, while compound microscopes can magnify up to 2,000X. A simple microscope can resolve below 1 micrometre (μm; one millionth of a metre); a compound microscope can resolve down to about 0.2 μm. Other types of microscopes use the wave nature of various physical processes. The most important is the electron microscope, which uses a beam of electrons in its image formation. The transmission electron microscope (TEM) magnify object more than 1,000,000X.

1.1.1 Magnification:

Magnification on a microscope refers to the amount or degree of visual enlargement of an observed object. Magnification is measured by multiples, such as 2X, 10X and 40X, indicating that the object is enlarged to 2 times as big, 10 times as big or 40 times as big, respectively. For a standard light-based microscope, the maximum magnification extends up to 1,500X;

beyond this, objects under view become excessively fuzzy because the wavelengths of light limit the clarity of images. Electrons, on the other hand, have much shorter wavelengths. According to Auburn University, electron microscopes produce useful images with magnifications up to about 200,000X. The standard eyepiece magnifies 10X. The most common objective lens magnifications for typical laboratory microscopes are 4x, 10x and 40x. Calculate total magnification by multiplying the eyepiece magnification by the objective lens magnification. So, typical laboratory microscopes magnify objects 40x, 100x and 400x.

1.1.2 Light Microscope:

The light microscope is an important tool in the study of microorganisms, particularly for identification purposes. The compound light microscope uses visible light to directly illuminate specimens in a two-lens system, resulting in the illuminated specimen appearing dark against a bright background.

The two lenses present in a compound microscope are the ocular lens in the eyepiece and the objective lens located in the revolving nosepiece. Compound light microscopes typically have the following components (as outlined below and set out in **(Figure 1.1)**.

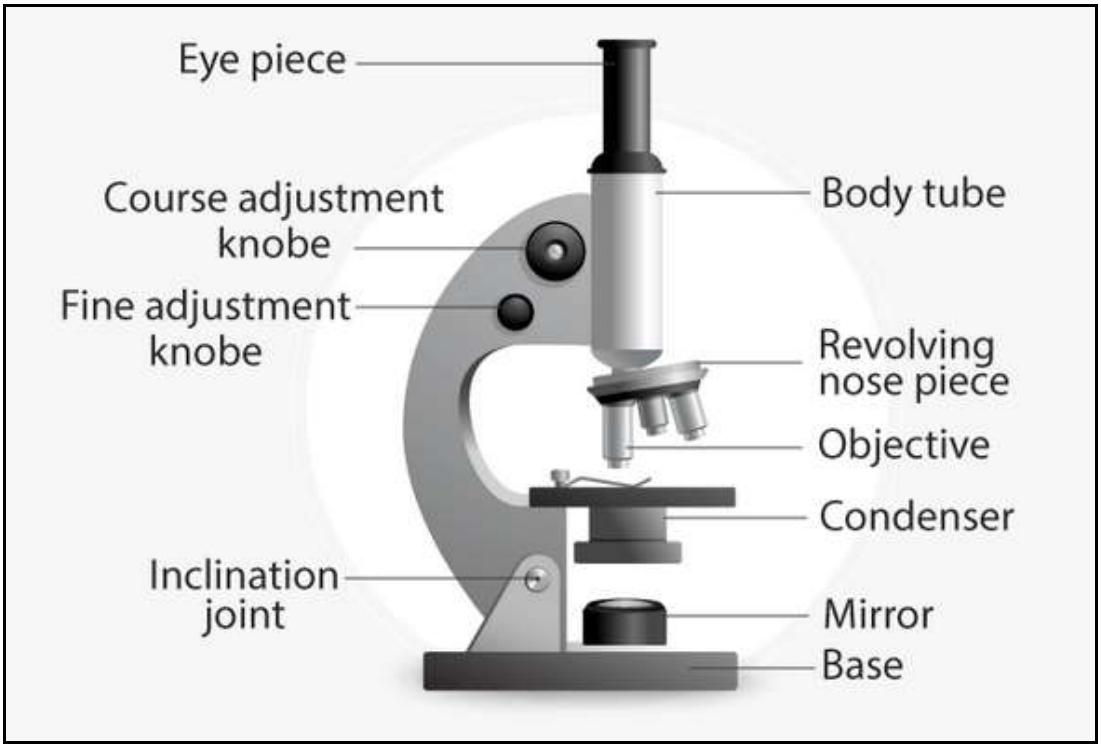

Figure 1.1: Different parts of Light Microscope

1.1.3 LPCB Staining:

Lactophenol Cotton Blue (LPCB) Staining is a simple histological staining method used for the microscopic examination and identification of fungi.

1.1.3.1 Principle of Lactophenol Cotton Blue (LPCB) Staining:

Lactophenol Cotton Blue (LPCB) staining method works on the principle of aiding the identification of the fungal cell walls. Fungi are eukaryotic organisms with both macroscopic and microscopic characteristics.

The fungal spore cell wall is made up of chitin of which the components of the Lactophenol Cotton Blue solution stain for identification. The lactophenol cotton blue solution acts as a mounting solution as well as a staining agent. The solution is clear and blue in colour and it is made up of a combination of three main reagents:

- **Phenol:** It acts as a disinfectant by killing any living organisms.
- **Lactic acid:** To preserve the fungal structures.
- **Cotton blue:** To stain or give colour to the chitin on the fungal cell wall and other fungal structures. The stain will give the fungi a blue-coloured appearance of the fungal spores and structures, such as hyphae.

1.1.3.2 Reagents of LPCB Staining:

A preparation of 50ml Lactophenol cotton Blue staining solution is made up of:

- Distilled water 50ml
- Cotton Blue (Aniline Blue) 0.125g
- Phenol Crystals ($C_6H_5O_4$) 50g
- Glycerol 100ml
- Lactic acid (CH3CHOH COOH) 50ml
- 70% ethanol

[Note: Lactophenol Cotton Blue solution is prepared at least 2 days before use.]

1.1.3.3 Preparation of LPCB solution:

Lactophenol Cotton Blue solution is prepared for over two days leaving the reagents undisturbed to allow dissolving and maturation.

A. Day 1: Dissolve the cotton blue in distilled water and leave to rest overnight. This eliminates insoluble dye.

B. Day2: Using protective gloves add phenol crystals to lactic acid in a glass beaker and stir using a magnetic stirrer until the crystals dissolve. Add glycerol. Filter the Cotton blue and the distilled water into the phenol + glycerol +lactic acid solution and mix. Store at room temperature.

1.1.3.4 Procedure of LPCB Staining:

On a clean microscopic glass slide, add a drop of 70% ethanol. Add the fungal specimen (from culture tube or diseased tissue) to the drop of alcohol using inoculation needle. Tease the fungal sample of the alcohol using a needle to ensure the sample mixes well with the alcohol.

Using a dropper or pipette, add one or two drops of Lactophenol Cotton Blue Solution before the ethanol dries off. Carefully cover the specimen with a clean coverslip without making air bubbles to the stain. Examine the specimen microscopically at 40X, to observe for fungal spores and other fungal structures.

1.2 *Rhizopus* sp.:

Systematic position
Kingdom: Fungi
Phylum: Zygomycota
Order: Mucorales
Family: Mucoraceae
Genus: *Rhizopus*

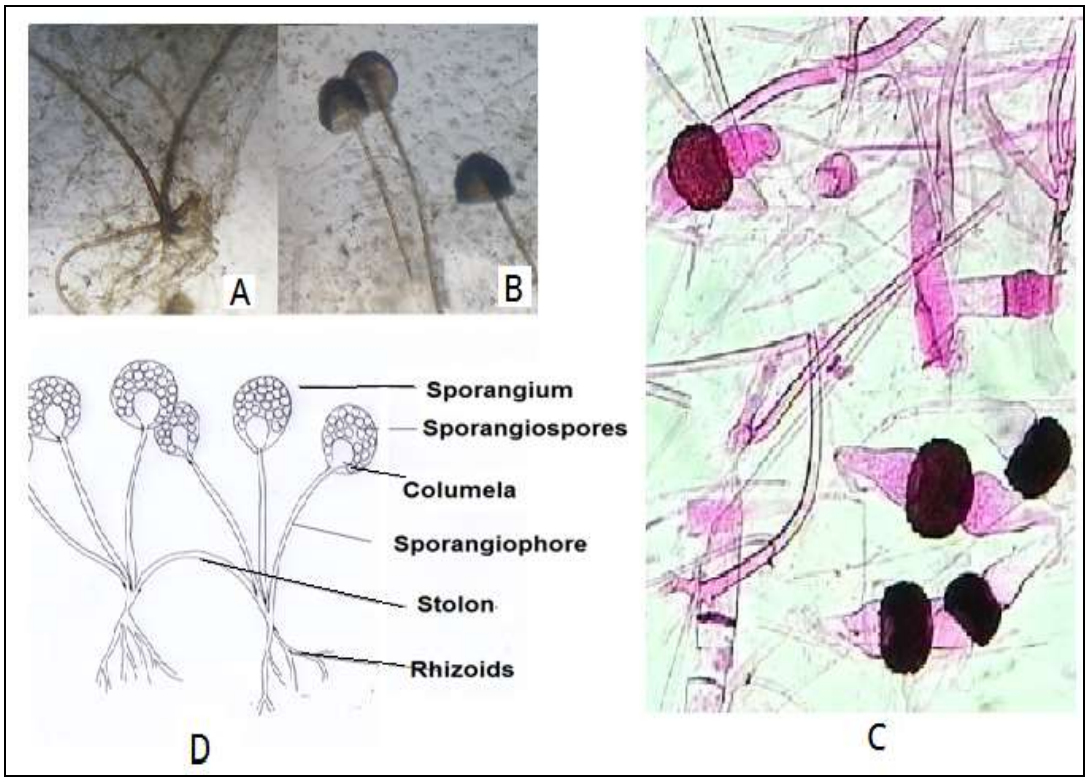

Figure 1.2: Specimen (*Rhizopus* sp). A- Nodal region with aerial hyphae and rhizoids, B- Sporangium, C- Zygospores, D- Free hand drawing of *Rhizopus* sp.

1.2.1 Description:

Thallus is white cottony, much branched mycelium. Mycelium is differentiated into nodes and internodes. The nodal region bears much branched **rhizoid** grows downward, inside the substratum for anchorage and absorption of nourishment. The internodal region is differentiate into sub aerial arching hyphae, known as **stolon** and aerial sporangium bearing hyphae, known as **sporangiophore**. The internodal region when touches the substratum forms node. The aerial hyphae enlarge and develop into a large round **sporangium**.

Sporangium consists of sporangiospores and vaculated columella. Cells are without septa i.e. coenocytic in nature. Septa only present to separate vegetative part where gametes form. It is whitish/grey colour. Many haploid sporagiospores are held together within the sporangium. It consists of diploid zygospores as sexual reproduction that is thicker-walled **(Figure 1.2).**

1.2.2 Diagnostic features of *Rhizopus sp.:*

- Saprophytic in nature, occurring in soil, fruits, other food and laboratory medium as contaminants.
- Plant body is thallophytic filamentous, without septa .i.e. coenocytic (multinucleated which consist of nodes and internodes. Two types of aerial branching hyphae (i.e. sub aerial and aerial) originated from node.
- The branched hyphae are of three types: sub aerial arch like internodes called as stolon, unbranched aerial spore bearing sporangiophores and underneath substratum the root like rhizoids.
- Asexual reproduction produces sporangiospores which are formed in a spherical structure known as a sporangium. The sporangia are produced in large numbers, which are dark, and are formed on a long stalk known as a sporangiophore.
- The sporangiospores are globose to ovoid, single-celled, hyaline to brown, and striate. The sporangiophores are aerial hyphae bearing sporangia at its tip.
- Sporangia consist of numerous sporangiospores and vaculated dome-shaped columella.
- Sexual reproduction produces dark non motile zygospores formed when two compatible mycelia fuse with each other.

----------therefore, the supplied specimen is *Rhizopus* sp.

1.3 Ascobolus sp.:

Systematic position
Kingdom: Fungi
Division: Ascomycota
Class: Pezizomycetes
Order: Pezizales
Family: Ascobolaceae
Genus: *Ascobolus*

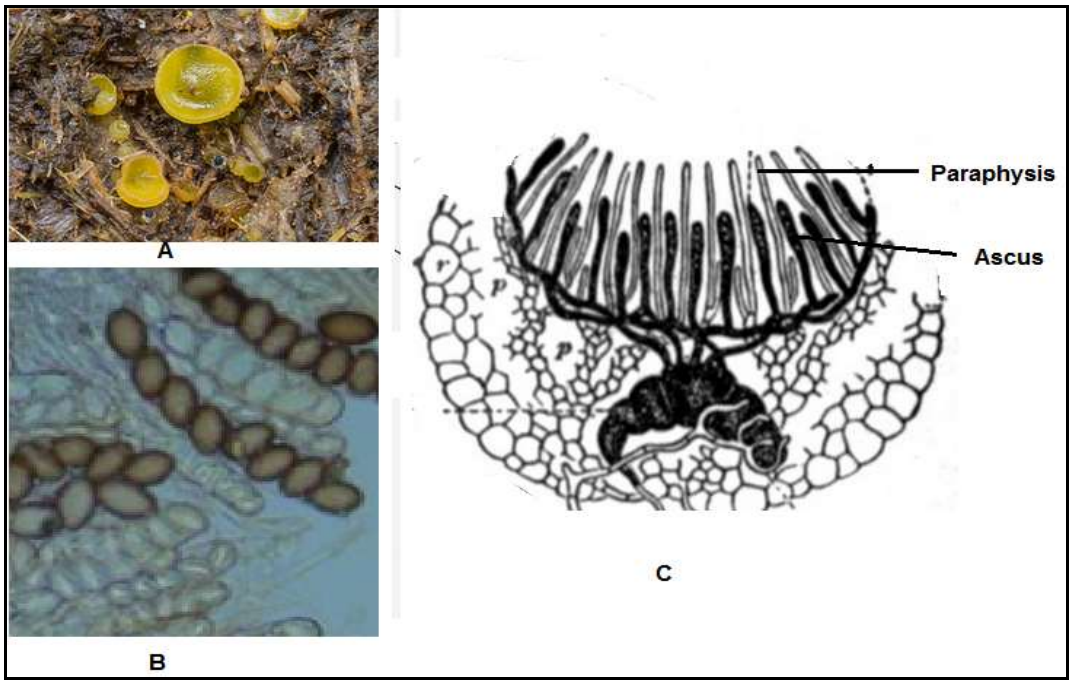

Figure 1.3: Specimen (*Ascobolus* sp). A- Ascocarp, B- Ascus with ascospores, C- Free hand drawing of fruiting body of *Ascobolus* sp.

1.3.1 Description:

These fungi usually live on dung or rotting plant remains and has a worldwide distribution. Thallus is represented by mycelium. It is well developed, perennial and consists of profusely branched hyphae. Hyphae are septate and the cells are multinucleate. The hyphae ramify within the substratum and form a complex system which helps in the absorption of the food material for the aerial fruiting bodies called apothecia. The apothecia are yellowish or greenish when young and purplish brown at maturity.

Ascobolus has small cup shaped yellow apothecia (fruiting bodies) with large asci protruding beyond the hymenium at maturity. Mature asci are long, consist of eight ascopores. The ascopores are one-celled, double-walled, ellipsoidal or spherical, purple or dark brown. Some vegetative hyphae grow up among the ascogenous hyphae and give rise to slender paraphysis **(Figure 1.3)**.

1.3.2 Diagnostic features of Ascobolus sp.:

- Plat body is thallus and represented by septate mycelium.
- The hyphae ramify within the substratum and form aerial cup shaped yellow fruiting bodies i.e. ascocarp called as apothecia.
- Asscocarp consist of asci which protruding beyond the hymenium at maturity.
- Mature asci are long, consist of eight ascopores. The ascopores are one-celled, double-walled, ellipsoidal or spherical, purple or dark brown.
- Some vegetative hyphae grow up and intermingled with ascci give rise to slender paraphysis.

----------therefore, the supplied specimen is *Ascobolus* sp.

1.4 *Peziza* sp.:

Systematic position
Kingdom: Fungi
Division: Ascomycota
Class: Pezizomycetes
Order: Pezizales
Family: Pezizaceae
Genus: *Peziza*

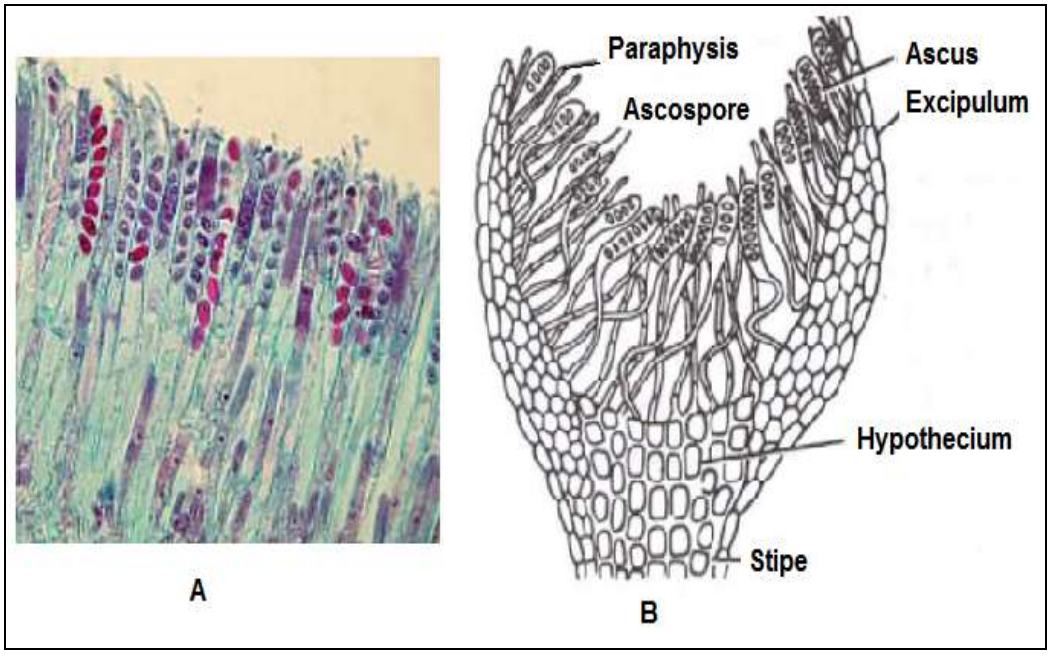

Figure 1.4: Specimen (*Peziza* sp). A- Ascus with ascospores, B- Free hand drawing of Apothecium of *Peziza* sp.

1.4.1 Description:

Peziza is a large genus of saprophytic cup fungi that grow on the ground, rotting wood, or dung. It is well developed, frequently perennial and consists of a dense network of hyphae. The hyphae are branched and septate.

The cells are uninucleate. A fruiting body is mostly cup or disc-shaped called an apothecium. The mature ascus is an elongated, cylindrical cell with eight ascospores.

Usually, the apothecium is a fleshy, sessile or sub-sessile cup or saucer-shaped body, regular in form, 2 to 40 cm in diameter, and without any hair, brightly coloured (but lacking carotenoids) to dark brown; smooth, velvety, hairy or bristly.

A vertical section through the apothecium shows that the upper surface constitutes the hymenium, a layer of elongated cells standing at right angle to the surface like a palisade.

It consists of asci intermingled with supporting and protective filamentous hyphae, the paraphyses. Immediate¬ly below the hymenium is a layer, thin or fairly thick, the hypothecium, consisting mainly of light-coloured hyphae and run¬ning parallel to the sur¬face of the hymenium.

The basal portion of the cup is known as the excipulum. The fully formed ascospores are oval, unicellular and hyaline.

When they become mature, each ascus opens ex¬plosively by a lid and the spores are shot out in a jet of liquid.

Each spore under favourable conditions germinates and forms a new mycelium **(Figure 1.4)**.

1.4.2 Diagnostic features of Peziza sp.:

- Plat body is thallus and represented by septate mycelium.
- The hyphae ramify within the substratum and form aerial cup shaped or disc-shaped fruiting bodies i.e. ascocarp called as apothecium.
- Apothecium is a fleshy, sessile or sub-sessile cup or saucer-shaped body, regular in form, 2 to 40 cm in diameter, and without any hair, brightly coloured (but lacking carotenoids) to dark brown; smooth, velvety, hairy or bristly.
- A vertical section through the apothecium shows upper hymenium layer that consists of asci intermingled with supporting and protective filamentous hyphae, the paraphyses.
- The fully formed ascospores are oval, unicellular and hyaline. When they become mature, each ascus opens ex¬plosively by a lid and the spores are shot out in a jet of liquid.

----------therefore, the supplied specimen is *Peziza sp*.

1.5 Aspergillus sp.:

Systematic position
Kingdom: Fungi
Division: Ascomycota
Class: Eurotiomycetes
Order: Eurotiales
Family: Trichocomaceae
Genus: *Aspergillus*

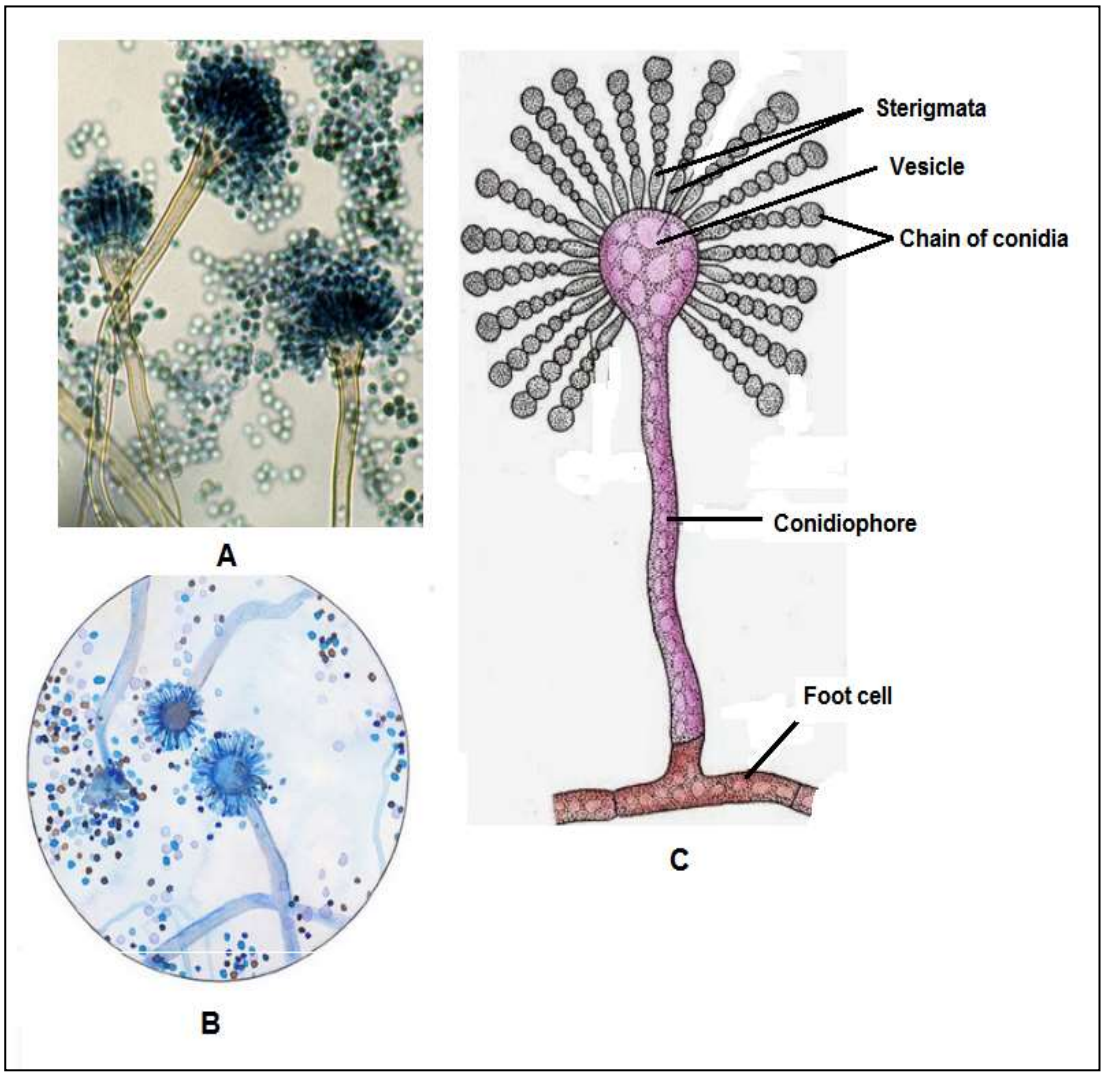

Figure 1.5: Specimen (*Aspergillus* sp). A- and B- conidia with conidiophores, C- Free hand drawing of conidia with conidiophores of *Aspergillus* sp.

1.5.1 Description:

Aspergillus Niger are filamentous fungi, which means that they tend to form filaments (hyphae) and thus resemble the structure of a plant. It is chiefly a saprophytic fungus which is widely distributed. It grows on decaying vegetables; on fatty media such as butter and ghee; on starchy media as bread and rice; on preserved fruits as jams and jellies.

Aspergillus appears in the form of greenish, smoky patches along with Mucor, Rhizopus and Penicilliutn on moist bread when kept under a bell jar for a couple of days. The other common colours are yellow, black and blue. When viewed under the microscope; A. Niger consists of a smooth and colourless conidiophores and conidia. Each conidium is globose. It has thick and unicellular.

The conidia are formed one below the other in a long chain at the top of somewhat bottle-shaped sterigma. The sterigmata arise side by side completely covering the entire surface of the globose, swollen head, the vesicle developed at the top of a special long, erect unbranched, unseptate, stout hypha called the conidiophore.

Each conidiophore arises as an outgrowth from a special thick-walled T-shaped cell of the mycelium. It is called the foot cell. Sometimes the sterigmata are produced in two layers. Those of the upper layer are called the secondary sterigmata and of the lower layer as primary. The former bear the conidia **(Figure 1.5)**.

1.5.2 Diagnostic Features of *Aspergillus* sp.:

- Plat body is thallus and represented by septate mycelium**.**
- The fungal morphology is defined by the hyphal conidia and conidiophores.
- **It is a spore-forming mold fungus reproduces asexually by producing spores in the form of conidia (*conidium: singular).* The conidia are densely found in the air.**
- Conidia are produced in column chains that are basipetal (facing downwards) from green phialides of 6-8 by 2-3um in size. The conidia are 2.5-3um in diameter, smooth surface or spiked (spinose). Each conidium is globose, thick and unicellular.
- Conidia are produced at the tip of a special long, erect unbranched, unseptate, stout hypha called the conidiophores that arises as an outgrowth from a special thick-walled T-shaped cell of the mycelium called as the foot cell.

---------- Therefore, the supplied specimen is *Aspergillus* sp.

1.6 *Penicillium* sp.:

Systematic position
Kingdom: Fungi
Division: Ascomycota
Class: Eurotiomycetes
Order: Eurotiales
Family: Trichocomaceae
Genus: *Penicillium*

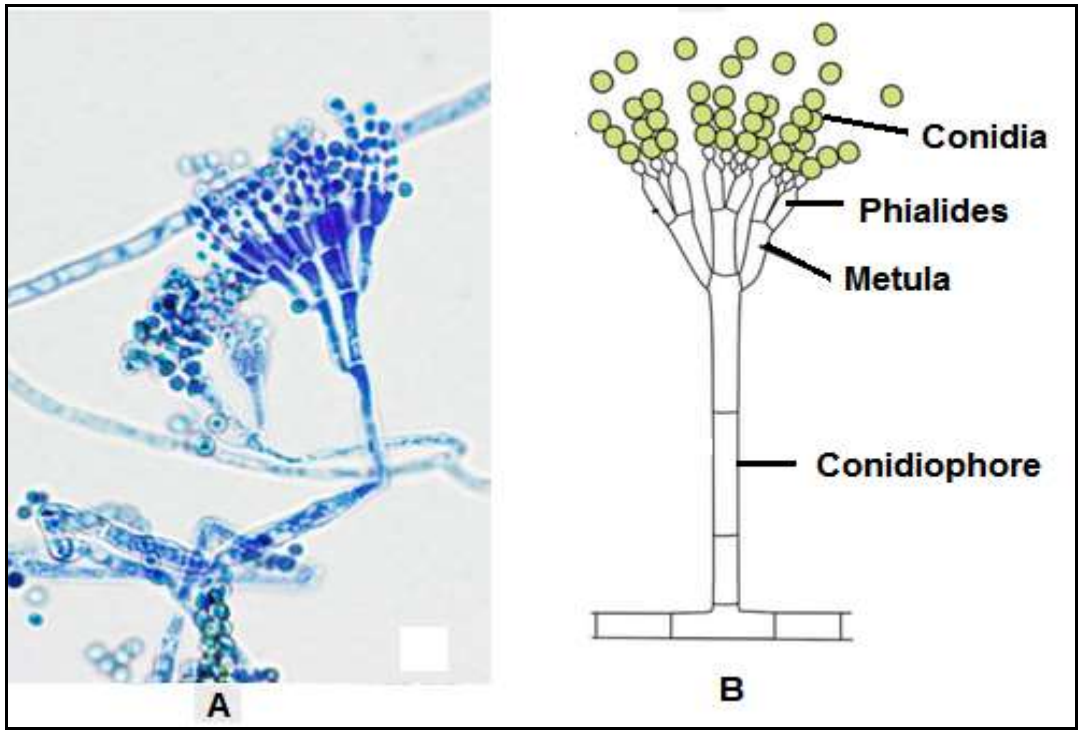

Figure 1.6: Specimen (*Penicillium* sp). A- And B- conidia with conidiophores, C- Free hand drawing of conidia with conidiophores of *Penicillium* sp.

1.6.1 Description:

Its vegetative body is known as mycelial and is to a great degree branched with septate hyphae, which is composed of thin-walled cells made up of one or more nuclei. Each septum possesses a central pore necessary for the maintenance of cytoplasmic continuity.

The organism is a saprophytic fungus mostly present in the soil, in the air, and in decaying organic matter and is most commonly referred to as the green or blue mold. *Penicillium* species are widely notable for their numerous and closely packed brush-like structures that produce spores which are called penicilli (sing.: penicillus). They possess simple or branching structures that are slightly elongated and end with clusters of flask-shapes structure known as phialides and conidiophores respectively.

The spores also are known as conidia. They are manufactured or produced in dry chains and they emanate from the tips of the phialides.

The oldest spores occupy the apex of the phialides while the youngest spores are found at the base of the phialides. One important feature in the identification of *Penicillium* species is its branching as can be seen in some species like P. glabrum which are unbranched and they just bear one cluster of phialides occupying the top of the stipe (**Figure 1.6**).

1.6.2 Diagnostic Features of *Penicillium* sp.

- Plat body is thallus and represented by septate mycelium**.**
- It produce numerous and closely packed brush-like structures that produce spores which are called penicilli (sing.: penicillus).
- They possess simple or branched slightly elongated hyphae that end in clusters of flask-shapes structure known as phialides and conidiophores respectively.
- The spores also are known as conidia. They are manufactured or produced in dry chains and they emanate from the tips of the phialides.
- The oldest spores occupy the apex of the phialides while the youngest spores are found at the base of the phialides.

----------therefore, the supplied specimen is *Penicillium* sp.

1.7 *Trichoderma* sp.:

Systematic position
Kingdom: Fungi
Phylum: Ascomycota
Class: Euascomycetes
Order: Hypocreales
Family: Hypocreaceae
Genus: *Trichoderma*

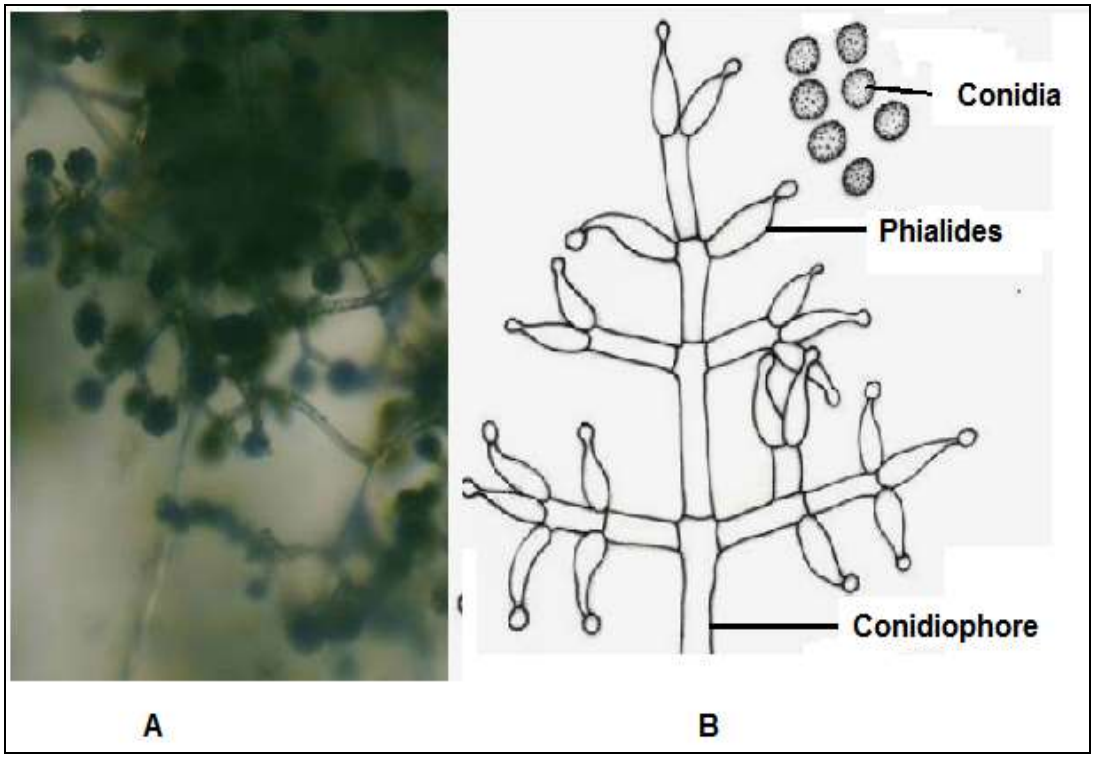

Figure 1.7: Specimen (*Trichoderma* sp). A- Conidia with conidiophores, B- Free hand drawing of conidia with conidiophores of *Trichoderma* sp.

1.7.1 Description:

The Genus *Trichoderma* is one of the most abundant fungi that have been shown to be present in all climatic zones. It is a filamentous fungus that is widely distributed in the soil, plant material, decaying vegetation, and wood. Although it is commonly considered as a contaminant, however, this fungus can also be found on various parts of plants including the leaves, seeds and grains.

Conidiophores are highly branched and thus difficult to define or measure, loosely or compactly tufted, often formed in distinct concentric rings or borne along the scant aerial hyphae.

Main branches of the conidiophores produce lateral side branches that may be paired or not, the longest branches distant from the tip and often phialides arising directly from the main axis near the tip. The branches may rebranch, with the secondary branches often paired and longest secondary branches being closest to the main axis.

All primary and secondary branches arise at or near 90° with respect to the main axis. The typical conidiophore with paired branches assumes a pyramidal aspect. The conidiophore terminates in one or a few phialides. Phialides are typically enlarged in the middle but may be cylindrical or nearly subglobose.

Phialides may be held in whorls, at an angle of 90° with respect to other members of the whorl, or they may be variously penicillate. Phialides may be densely clustered on wide main axis or they may be solitary.

Conidia typically appear dry but in some species they may be held in drops of clear green or yellow liquid. Conidia of most species are ellipsoidal, 3–5 x 2–4 µm; globose conidia are rare.

Conidia appear colourless to green, smooth to rough, and are in moist conidial masses, variable in shape and size. Conidiophores branch repeatedly, bearing clusters of phialides terminally in most cases (**Figure 1.7**).

1.7.2 Diagnostic Features of *Trichoderma sp.:*

- Plat body is thallus and represented by septate mycelium**.**
- Colonies are fast growing, at first white and downy, later developing yellowish-green to deep green compact tufts, often only in small areas or in concentric ring-like zones on the agar surface.
- Conidiophores are repeatedly branched, irregularly verticillate, bearing clusters of diergent, often irregularly bent, flask-shaped phialides.
- Conidia are mostly green, sometimes hyaline, with smooth or rough walls and are formed in slimy conidial heads (gloiospora) clustered at the tips of the phialides.

----------therefore, the supplied specimen is *Trichoderma* sp.

1.8 *Fusarium* sp.

Systematic position

Kingdom: Fungi
Phylum: Ascomycota
Order: Hypocreales
Family: Hypocreaceae
Genus: *Fusarium*

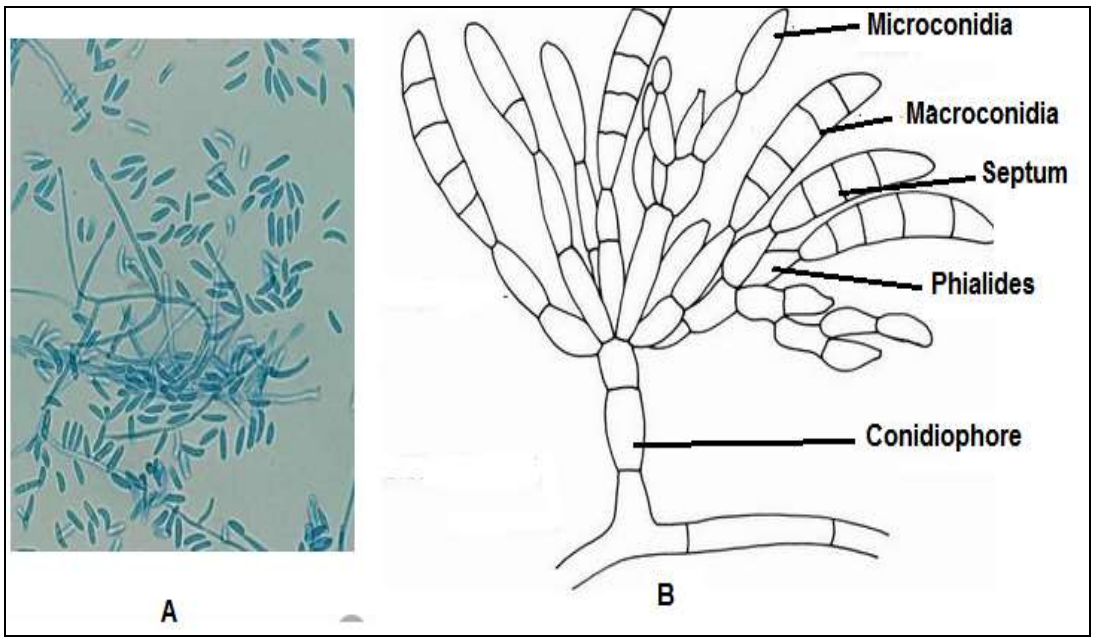

Figure 1.8: Specimen (*Fusarium* sp). A- Conidia with conidiophores, B- Free hand drawing of conidia with conidiophores of *Fusarium* sp.

1.8.1 Description:

Hyaline septate hyphae, conidiophores, phialides, macroconidia, and microconidia are observed microscopically. Phialides are cylindrical, with a small collarette, solitary or produced as a component of a complex branching system. Monophialides and polyphialides (in heads or in chains) may be observed.

Macroconidia (3-8 x 11-70 µm) are produced from phialides on unbranched or branched conidiophores. They are 2- or more celled, thick-walled, smooth, and cylindrical or sickle- (canoe-) shaped. Macroconidia have a distinct basal foot cell and pointed distal ends.

They tend to accumulate in balls or rafts. Microconidia (2-4 x 4-8 µm), on the other hand, are formed on long or short simple conidiophores. T

hey are 1-celled (occasionally 2- or 3-celled), smooth, hyaline, ovoid to cylindrical, and arranged in balls (occasionally occurring in chains). Chlamydospores, when present, are sparse, in pairs, clumps or chains.

They are thick-walled, hyaline, intercalary or terminal. Macroscopic and microscopic features, such as, color of the colony; length and shape of the macroconidia, the number, shape and arrangement of microconidia, and presence or absence of chlamydospores are key features for the differentiation of *Fusarium* species **(Figure 1.8)**.

1.8.2 Diagnostic Features of *Fusarium* sp.:

- **The Fungus** reproduces asexually and produces three kinds of spores known as macroconidia, microconidia, and chlamydospores.
- Macroconidia are produced in a sporodochium, which is an erumpent crowded cluster of conidiophores arising from stroma to form a cushion-like mass that supports the macroconidia.
- Macroconidia are also produced on mono-phialides (a conidiophore with a single opening through which an endoconidia is released) and poly-phialides (two or more openings or pores from which the endoconidia are forced out) on aerial mycelium.
- Microconidia are produced in the aerial mycelium. The microconidia can be produced on false heads or false chains on mono-phialides or poly-phialides. False Heads are a result of moisture drops on the conidiophore and they contain the endoconidia as they are produced.
- Microconidia have different shapes and sizes, the microconidia produced in chains have a truncate base.

----------therefore, the supplied specimen is *Fusarium* sp.

1.9 Agaricus sp.:

Systematic position
Kingdom: Fungi
Division: Basidiomycota
Class: Agaricomycetes
Order: Agaricales
Family: Agaricaceae
Genus: *Agaricus*

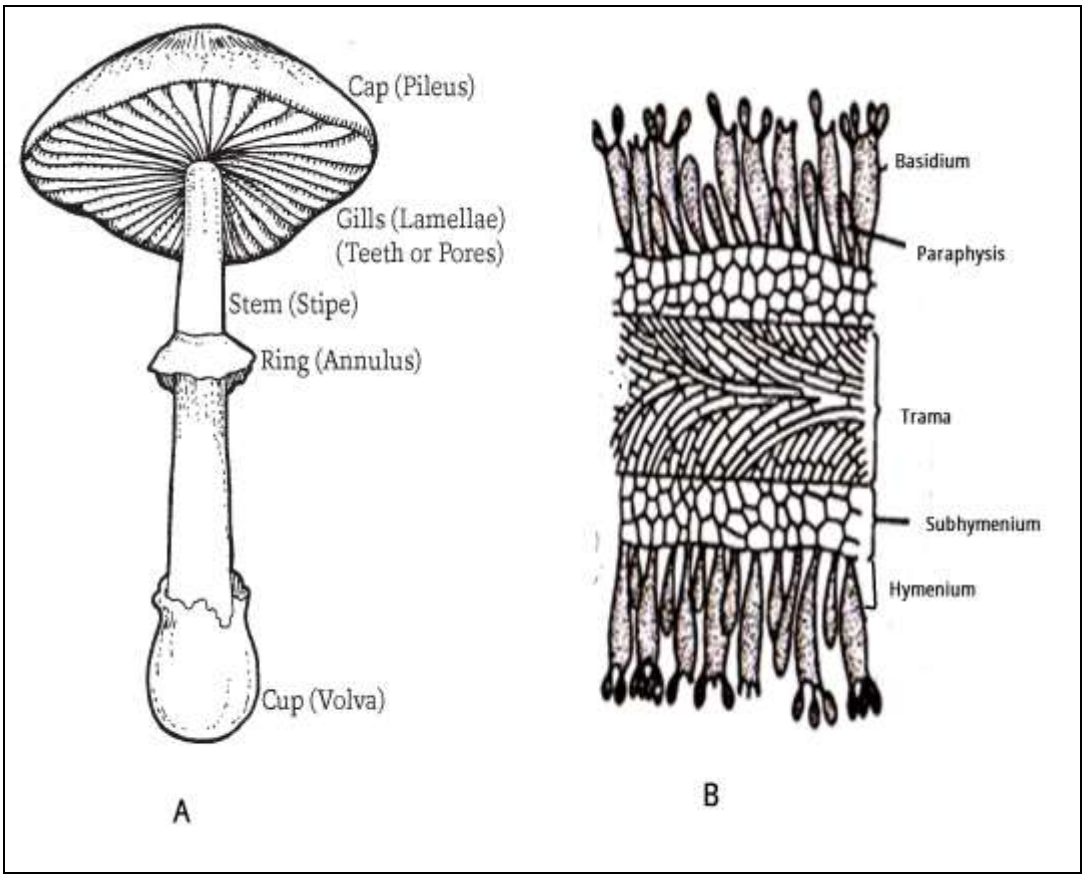

Figure 1.9: A- Basidiocarp of *Agaricus* sp.; B- T.S. of gill showing different parts.

1.9.1 Description:

It is a saprophytic fungus found growing on soil humus, decaying litter on forest floors, in the fields and lawns, wood logs and manure piles. It grows best in moist and shady places and is commonly seen during rainy season. It is cosmopolitan in distribution. The vegetative mycelium grows inside the soil.

Fruiting body is basidiocarp present above the soil and edible in young stage. The mature fruiting body can be differentiated into three parts i.e., stipe, pileus and annulus.

A transverse section of stipe shows that it is made up of two kinds of tissue, i.e. compactly arranged hypahe in the peripheral region known as cortex, loosely arranged hyphae in the central region known as medulla.

The stipe at its top supports a broad umbrella shaped cap called pileus. From the underside of the pileus hang approximately 300 to 600 plates of tissues known as gills or lamellae.

The gills are white or pinkish in young condition and turns brown or purplish black at maturity (**Figure 1.9**).

1.9.2 Diagnostic Features of *Agaricus* sp.*:*

- Plat body is thallus and represented by septate mycelium**.**
- The mature fruiting body is basidiocarp and differentiated into three parts i.e., stipe, pileus and annulus.
- A transverse section of stipe differentiate into two kinds of tissue, i.e. compactly arranged hypahe in the peripheral region known as cortex, loosely arranged hyphae in the central region known as medulla.
- The stipe at its top supports a broad umbrella shaped cap called pileus.
- From the underside of the pileus hang approximately 300 to 600 plates of tissues known as gills or lamellae.
- The gills are white or pinkish in young condition and turns brown or purplish black at maturity.

----------Hence, the specimen is *Agaricus* sp.

1.10 Polyporus sp.:

Systematic position
Kingdom: Fungi
Division: Basidiomycota
Class: Agaricomycetes
Order: Polyporales
Family: Polyporaceae
Genus: *Polyporus*

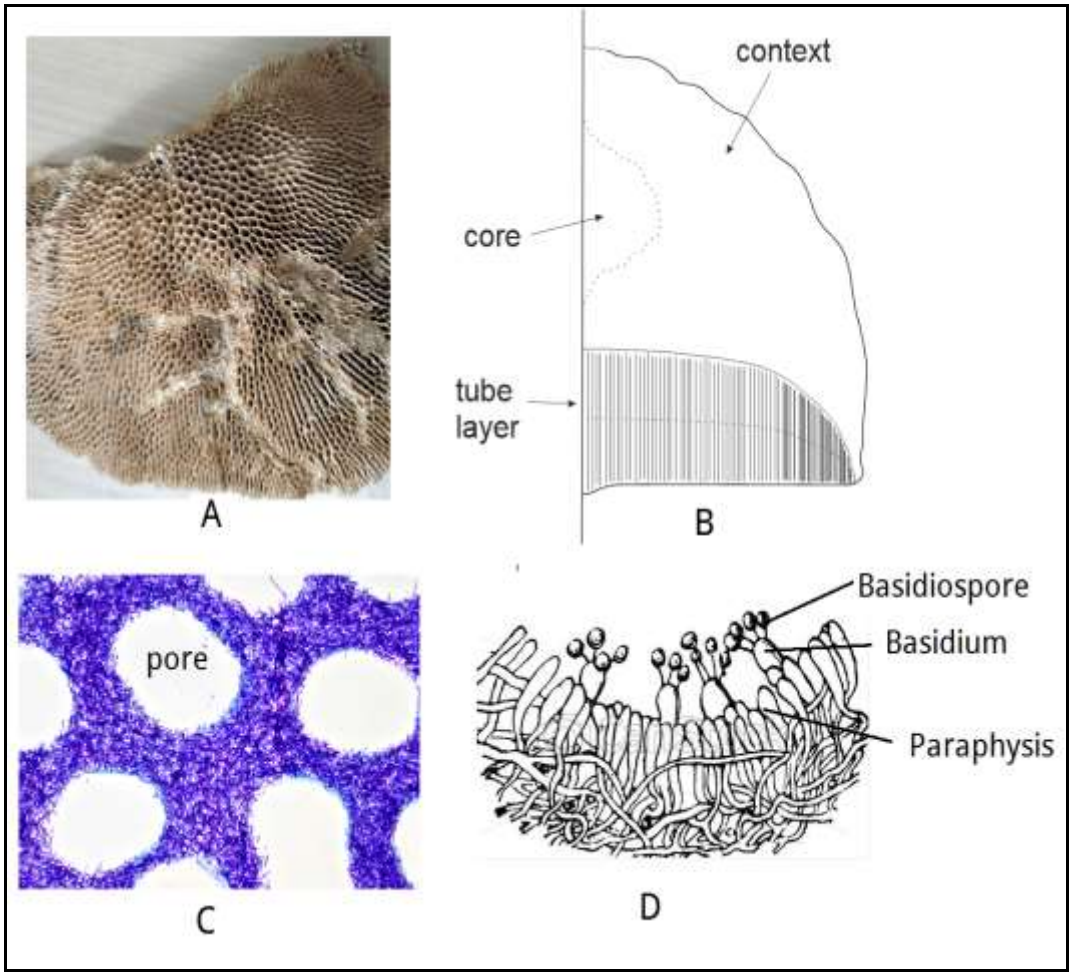

Figure 1.10: Polypore. A- a honeycomb-like basidiocarp, B- Tube layer and context, C- Pore layer of basidiocarp, D- pores consist of different parts

1.10.1 Description:

The fungal individual that develops the fruit bodies that are identified as polypores resides in soil or wood as mycelium. Polypores are often restricted to either deciduous (angiosperm) or conifer (gymnosperm) host trees. Most species of polypores develop new, short-lived fruit bodies annually or several times every year.

Abundant fruit takes place during the autumn or rainy season. Structure of the fruit bodies is simple, typically consist of two layers - a tube layer of vertically arranged tubes that open downwards, and supporting layer called subiculum that supports and attached the tubes to substrate.

In fruit bodies with a cap i.e. pileate fruit bodies in the upper surface and the pore layer is called context.

Some species of polypores also have a stalk i.e. stipe that attach to the cap either laterally or centrally depending on the species.

Polypore tubes are a honeycomb-like structure, where the individual tubes have fused together.

Their sides are covered with a spore-forming surface, the hymenium. The tubes offer shelter for developing spores and help to increase the area of the spore-producing surface.

Pore size and shape vary between species to species. A few polypores produce asexual spores (chlamydospores or conidia) in the upper surface of their or without the presence of a sexual fruit body (**Figure 1.10**).

1.10.2 Diagnostic Features of *Polyporus:*

- Plat body is thallus and represented by septate mycelium.
- Structure of the fruit bodies is simple, typically consist of two layers - a tube layer of vertically arranged tubes that open downwards, and supporting layer called subiculum that supports and attached the tubes to substrate.
- In fruit bodies with a cap i.e. pileate fruit bodies in the upper surface and the pore layer is called context.
- Polypore tubes are a honeycomb-like structure, where the individual tubes have fused together.
- Their sides are covered with a spore-forming surface, the hymenium. The tubes offer shelter for developing spores and help to increase the area of the spore-producing surface. Pore size and shape vary between species to species

----------Hence, the specimen is *Polyporus* sp.

1.11 *Cyathus* sp.:

Systematic position
Kingdom: Fungi
Division: Basidiomycota
Class: Agaricomycetes
Order: Agaricales
Family: Nidulariaceae
Genus: *Cyathus*

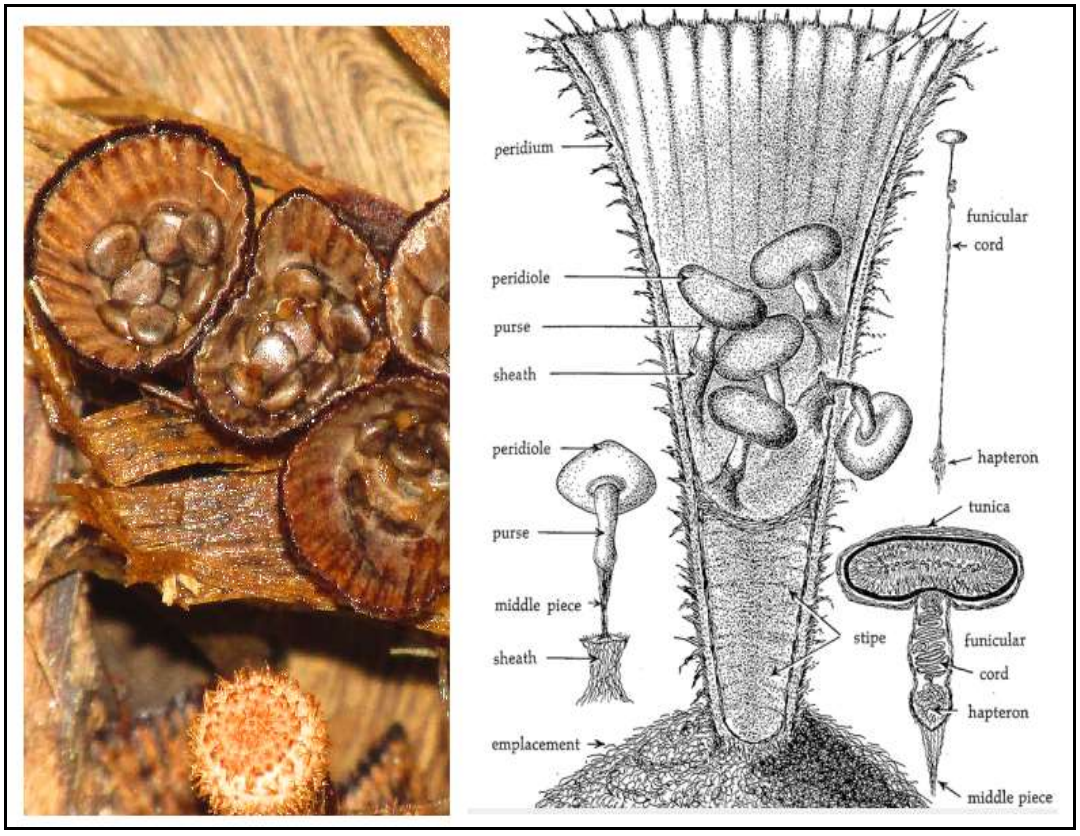

Figure 1.11: *Cyathus* sp.

1.11.1 Description:

The basidiocarp is deeper, inverted bell-shaped. Basidiocarps are formed. There are several species of *Cyathus* commonly known as the Bird's Nest Fungi. This fungus produces small, funnel-shaped cups, about 1 cm diameter.

The cups are initially covered by a thin membrane, but this ruptures as the fruit body revealing small clusters of disk-shaped, slate-coloured "eggs" called **peridioles**. These peridioles contain the basidiospores.

The funnel-like shape of the fruit bodies enables the peridioles to be splash-dispersed by rain drops that fall into the cup, causing the peridioles to be thrown up to a distance. The peridioles have a very complex structure.

They are attached to the wall of the splash-cup by a short stalk, within which there is a coiled **funicular cord** composed of hyphae. When the peridiole is splashed free from the fruitbody, this cord unwinds rapidly to a length of 5 cm or more, and it trails behind the peridiole.

At the base of the cord is a tangled mass of adhesive hyphae, which serve to attach the peridiole to any nearby substrate (**Figure 1.11**).

1.11.2 Diagnostic Features of *Cyathus:*

- The basidiocarp is deeper, inverted bell-shaped.
- The cups are initially covered by a thin membrane, but this ruptures as the fruit body revealing small clusters of disk-shaped, slate-coloured "eggs" called **peridioles**. These peridioles contain the basidiospores.
- The peridioles have a very complex structure which attached to the wall of the splash-cup by a short stalk, within which there is a coiled **funicular cord** composed of hyphae.

----------Hence, the specimen is *Cyathu*s sp.

1.12. *Alternaria* sp.:

Systematic position

Kingdom: Fungi
Phylum: Ascomycota
Class: Euascomycetes
Order: Pleosporales
Family: Pleosporaceae
Genus: *Alternaria*

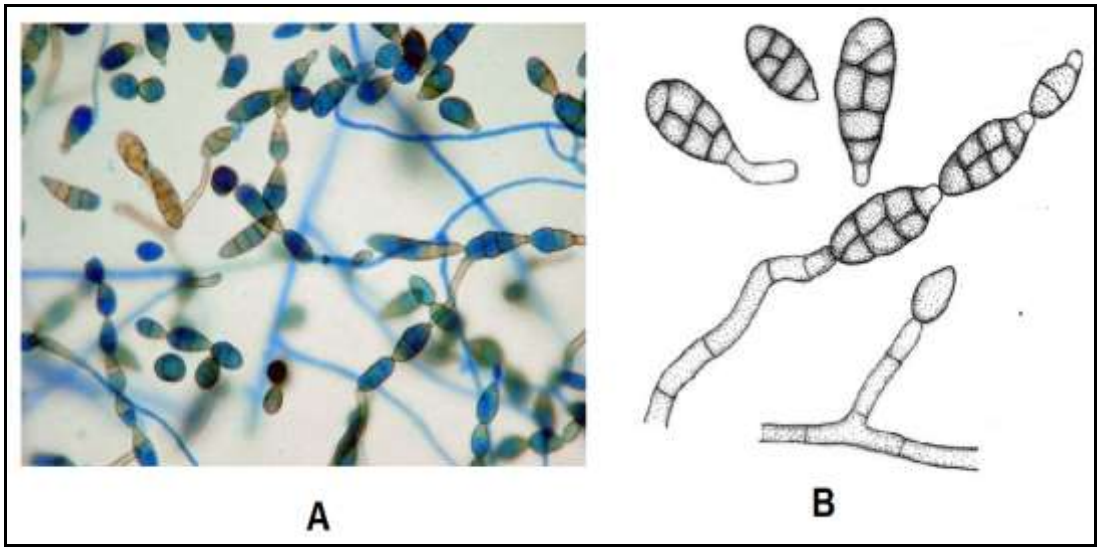

Figure 1.12: A- Spores of *Alternaria* sp. B- Free hand drawing of conidia, conidiophores and septa.

1.12.1 Description:

Alternaria species are saprophytic meaning that they thrive in decomposing materials and environments. They are also commonly found in organic materials and water or moisture areas.

Some are endophytic, therefore they live in various plant parts such as seeds, and fruits. It is also found on plants where it caused plant diseases and can be transferred to animals through plants causing human and animal infections. *Alternaria* sp. grows rapidly and the colony size reaches a diameter of 3 to 9 cm following incubation at 25°C for 7 days on potato glucose agar. The colony is flat, downy to woolly and is covered by grayish, short, aerial hyphae in time.

The surface is greyish white at the beginning which later darkens and becomes greenish black or olive brown with a light border. The reverse side is typically brown to black due to pigment production.

Alternaria sp. has septate, brown hyphae. Conidiophores are also septate and brown in color, occasionally producing a zigzag appearance.

They bear simple or branched large conidia (7-10 x 23-34 µm) which have both transverse and longitudinal septations.

These conidia may be observed singly or in acropetal chains and may produce germ tubes. They are ovoid to obclavate, darkly pigmented, muriform, and smooth or roughened.

The end of the conidium nearest the conidiophore is round while it tapers towards the apex. This gives the typical beak or club-like appearance of the conidia (Figure 12)

1.12.2 Diagnostic Features of *Alternaria* sp.:

- It grows as long chains with dark brown conidiophores.
- They thrive in environments with moisture and good nutrition, producing asexual spores known as conidiospores (conidia).
- They bear simple or branched large conidia (7-10 x 23-34 µm) which have both transverse and longitudinal septations.
- These conidia may be observed singly or in acropetal chains and may produce germ tubes.
- They are ovoid to obclavate, darkly pigmented, muriform, and smooth or roughened. The end of the conidium nearest the conidiophore is round while it tapers towards the apex. This gives the typical beak or club-like appearance of the conidia

……..Hence, it is *Alternaria* sp.

1.13 Curvularia sp.:

Systematic position

Kingdom: Fungi
Phylum: Ascomycota
Class: Euascomycetes
Order: Pleosporales
Family: Pleosporaceae
Genus: *Curvularia*

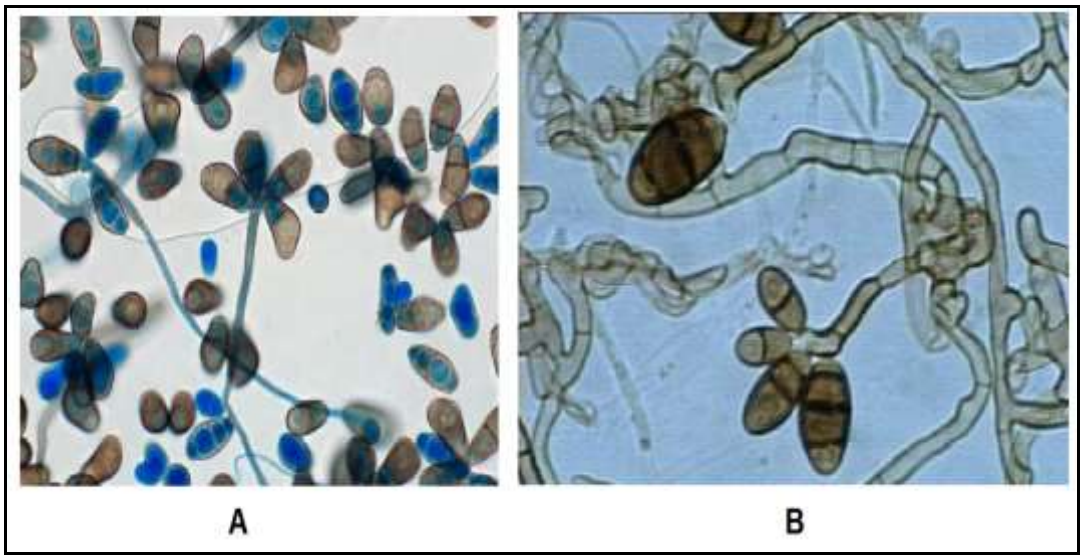

Figure 1.13: A and B- Conidiophore with Conidia of *Curvularia* sp.

1.13.1 Description:

Curvularia is a dematiaceous filamentous fungus. Most species of *Curvularia* are facultative pathogens of soil, plants, and cereals in tropical or subtropical areas, while the remaining few are found in temperate zones. As well as being a contaminant. *Curvularia* produces rapidly growing, woolly colonies on potato dextrose agar at 25°C. From the front, the color of the colony is white to pinkish gray initially and turns to olive brown or black as the colony matures. From the reverse, it is dark brown to black.

It is filamentous fungus, septate hyphae, generally brown in appearance due to brown hyphae, brown conidiophores and conidia. Conidiophores are simple or branched and are bent at the points where the conidia originate. This bending pattern is called sympodial geniculate growth. The conidia (8-14 x 21-35 µm), which are also called the poroconidia, are straight or pyriform, brown, multiseptate, and have dark basal protuberant hila. The septa are transverse and divide each conidium into multiple cells. The central cell is typically darker and enlarged compared to the end cells in the conidium. The central septum may also appear darker than the others. The swelling of the central cell usually gives the conidium a curved appearance. The number of the septa in the conidia, the shape of the conidia (straight or curved), the colour of the

conidia (dark vs pale brown), existence of dark median septum, and the prominence of geniculate growth pattern are the major microscopic features that help in differentiation of *Curvularia* spp. among each other. For instance, the conidia of *Curvularia lunata* have 3 septa and 4 cells, while those of *Curvularia geniculata* mostly have 4 septa and 5 cells (**Figure 1.13**).

1.13.2 Diagnostic Features of *Curvularia* sp.:

- It is filamentous fungus, septate hyphae, generally brown in appearance due to brown hyphae, brown conidiophores and conidia.
- Conidiophores are simple or branched and are bent at the points where the conidia originate. This bending pattern is called sympodial geniculate growth.
- The conidia (8-14 x 21-35 µm), which are also called the poroconidia, are straight or pyriform, brown, multiseptate, and have dark basal protuberant hila.
- The septa are transverse and divide each conidium into multiple cells. The central cell is typically darker and enlarged compared to the end cells in the conidium.
- The central septum may also appear darker than the others. The swelling of the central cell usually gives the conidium a curved appearance.

……..Hence, it is *Curvularia* sp.

1.14 Blast of Rice:

A. Causal Organism:

Rice blast disease is caused by *Magnaporthe oryzae* which belong to Ascomycota.

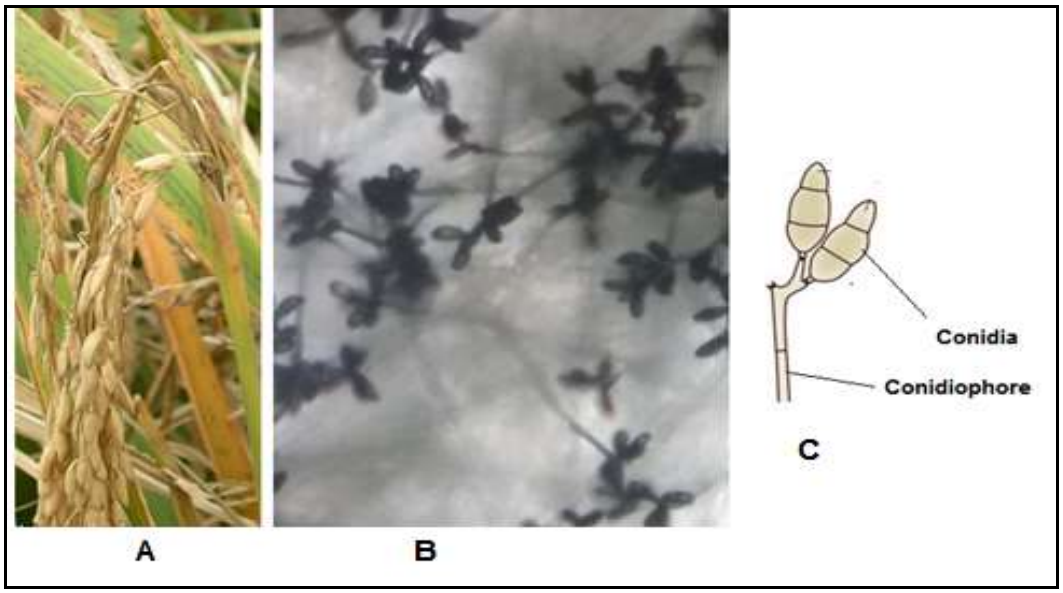

Figure 1.14: Blast of rice. A- Disease symptoms. B and C- Conidia with Conidiophore

B. Symptoms:

Rice blast disease occurs in about 80 countries on all continents where rice is grown.

The extent of damage depends on environmental factors, but worldwide it is one of the most devastating cereal diseases, resulting in losses of 10–30% of the global yield of rice.

In rice seedlings, small necrotic regions appear initially, which become larger and coalesce, and have chlorotic margins.

In older rice plants, disease symptoms can occur in leaves, collar junction of the leaf blade and leaf sheath, nodes, neck, and panicle. Neck rot and panicle blast are particularly devastating causing up to 80% yield losses in severe epidemics.

Triangular, purple-coloured lesions form on the neck node which elongate on both sides, seriously impairing grain development.

The panicles become white when young neck nodes are invaded; infection later in plant growth results in incomplete grain filling **(Figure 1.14)**.

1.15 False Smut of Rice:

A. Causal Organism:

The false smut disease of rice is caused by *Ustilaginoidea virens* (Cooke) Takahashi which belong to Ascomycotina.

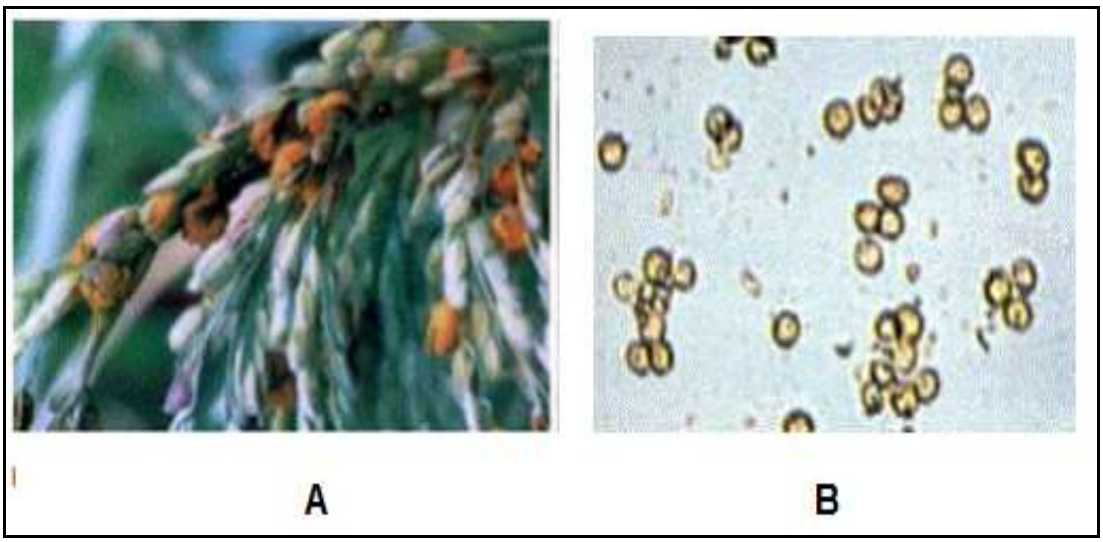

Figure 1.15: False smut of rice. A- Disease symptoms. B - Chlamydospores

B. Symptoms:

The false smut disease occurs in the field at hard dough to mature stage of the crop. The fungus transforms individual grains of the panicle into greenish spore balls that have velvety appearance.

The spore balls are small at first and visible in between glumes, grow gradually to reach 1 cm or more in diameter and enclose the floral parts. They are covered with a membrane that bursts as a result of further growth.

The colour of the ball becomes orange and later yellowish green or greenish black. At this stage, the surface of the ball cracks. The outermost layer of the ball is green and consists of mature spores together with the remaining fragments of mycelium.

Infection usually occurs during the reproductive and ripening stages, infecting a few grains in the panicle and leaving the rest healthy.

Chlamydospore is a thick- or double-walled asexual spore formed directly from a vegetative hyphal cell that functions as a resistant or overwintering stage formed on the spore balls Chlamydospores germinate in culture by germ tubes, which become septate and form conidiophores bearing conidia at the tapering apex. These conidia are ovoid and very minute (**Figure 1.15**).

1.16 Brown Spot of Rice:

A. Causal Organism:

Brown spot of rice is caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker which belong to subdivision Deuteromycotina (imperfect fungi), class Deuteromycetes, order Moniliales, and family Dematiaceae.

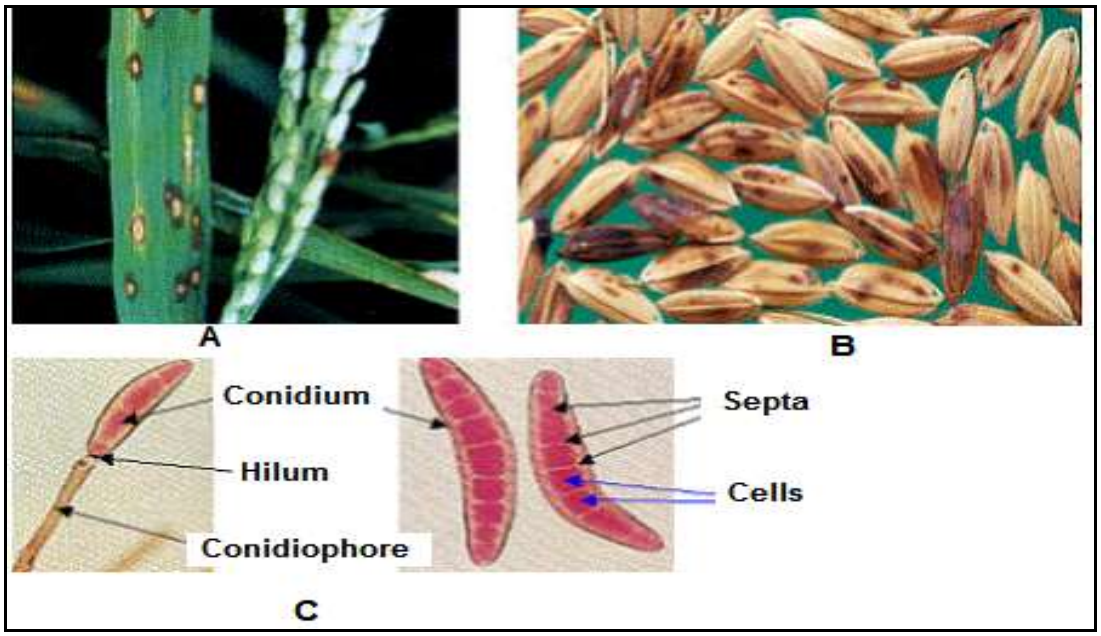

Figure 1.16: Brown Spot of rice. A and B- Disease symptoms, C – Conidia with conidiophore

B. Symptoms:

Brown spot may be manifested as seedling blight or as a foliar and glume disease of mature plants. On seedlings, the fungus produces small, circular, brown lesions, which may girdle the coleoptile and cause distortion of the primary and secondary leaves. In some cases, the fungus may also infect and cause a black discoloration of the roots. Infected seedlings are stunted or killed. On the leaves of older plants, the fungus produces circular to oval lesions that have a light brown to gray centre surrounded by a reddish brown margin.

On moderately susceptible cultivars, the fungus produces tiny, dark specks. When infection is severe, the lesions may coalesce, killing large areas of affected leaves. The fungus may also infect the glumes, causing dark brown to black oval spots, and may also infect the grain, causing a black discoloration.

The brown spot fungus produces multiseptate (three or more septae) conidiophore, singly or in bundles (generally 17). Conidia are generally curved, boat, or club-shaped, with 6 to 14 transverse septa or cross walls and often with a minute, slightly protruding hilum (dot at the point of attachment to a conidiophore) in (**Figure 1.16**).

1.17 Late blight of Potato:

A. Causal Organism

Late blight of potato is caused by *Phytophthora infestans* which belong to Oomycotina.

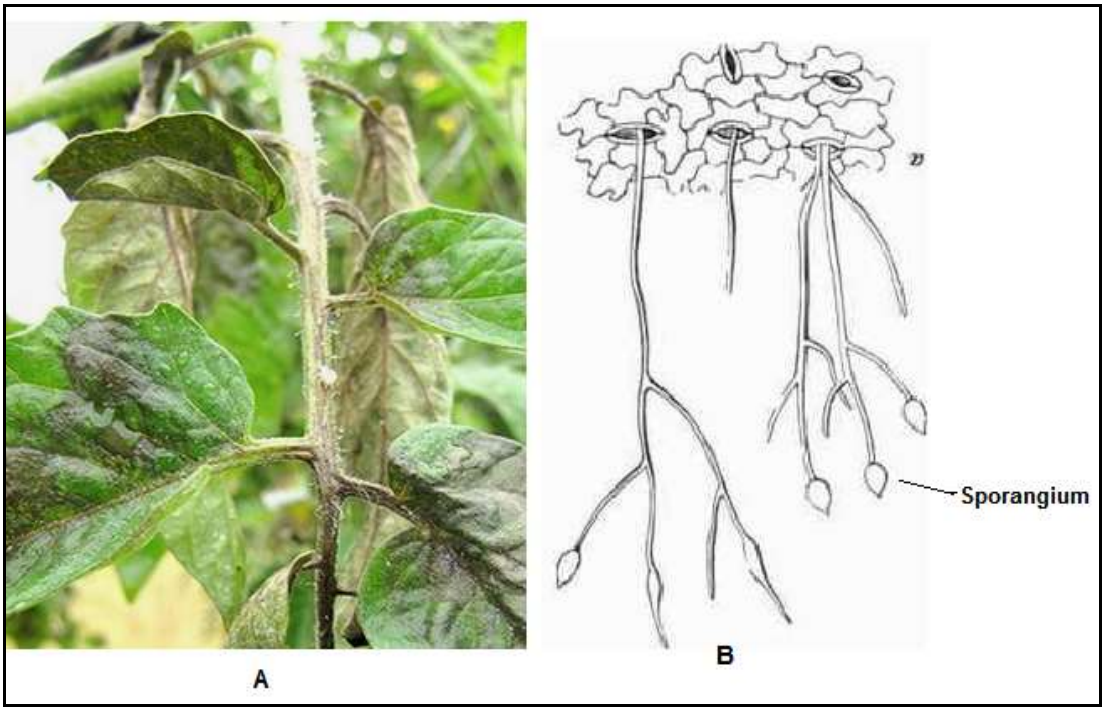

Figure 1.17: Late blight of potato. A- Disease symptoms, B– Sporangium

B. Symptoms:

The first symptoms of late blight in the field are small, dark, circular to irregularly shaped lesions, which appear 3 to 5 days after infection. These usually appear first on the lower leaves, where the microclimate is more humid. However, they may occur on upper leaves if weather conditions are favorable and the pathogen has been carried into the field by air currents.

Lesions often begin to develop on the compound leaf near the point of attachment to the petiole or at the leaf edges, where dew is retained longest.

During cool, moist weather, lesions expand rapidly into large, dark brown or black spots, often surrounded by a pale green to yellow border. As new infections occur and existing lesions coalesce, entire leaves may become blighted and killed within a few days.

On stems, lesions are often initiated at the point of attachment to the stem, and leaves become detached shortly after infection. The lesions continue to develop along the length of the stem and can remain active even in hot, dry weather (**Figure 1.17**).

1.18 Yellow Rust of Wheat:

A. Causal Organism:

Yellow (stripe) rust is caused by *Puccinia striiformis* f. sp. *tritici* (Pst), is a serious disease of wheat occurring in most wheat areas with cool and moist weather conditions during the growing season. The fungus belongs to basidiomycota which is an obligate biotrophic parasite that is difficult to culture on artificial media.

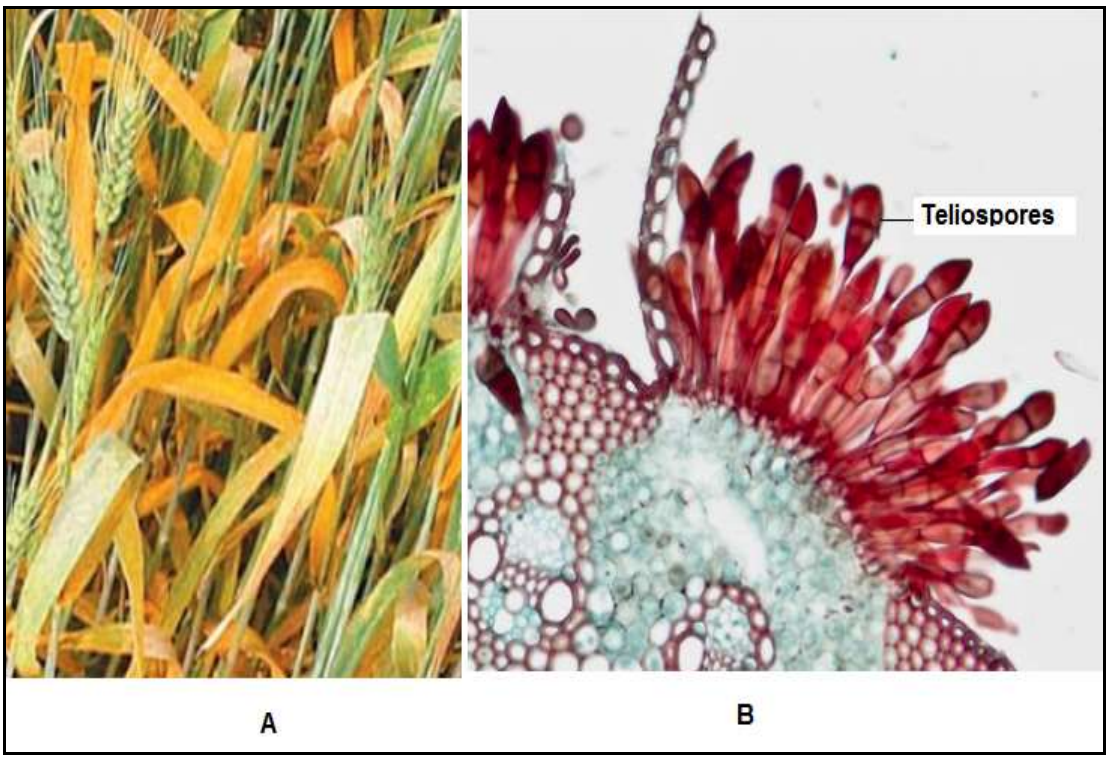

Figure 1.18: Yellow rust of wheat. A- Disease symptoms, B– Teliospore

B. Disease Symptoms:

Mainly occur on leaves than the leaf sheaths and stem. Bright yellow pustules (Uredia) appear on leaves at early stage of crop and pustules are arranged in linear rows as stripes.

The stripes are yellow to orange yellow. The teliospores are also arranged in long stripes and are dull black in colour.

The pustules of stripe rust, which, contain yellow to orange-yellow urediospores, usually form narrow stripes on the leaves. Pustules also can be found on leaf sheaths, necks, and glumes.

Teliospores consist of dikaryote cells which are often dark-coloured and thick-walled, especially in species where they overwinter (acting as chlamydospores) in (**Figure 1.18)**.

1.19 Wart Disease of Potato:

A. Causal Organism:

Wart disease of potato is caused by the soil-borne unicellular fungus *Synchytrium endobioticum* belong to **Chytridiomycotina**.

The fungus produce sporangia which are of two different types, the winter sporangia or resting sporangia (long live) and the summer sporangia (short live).

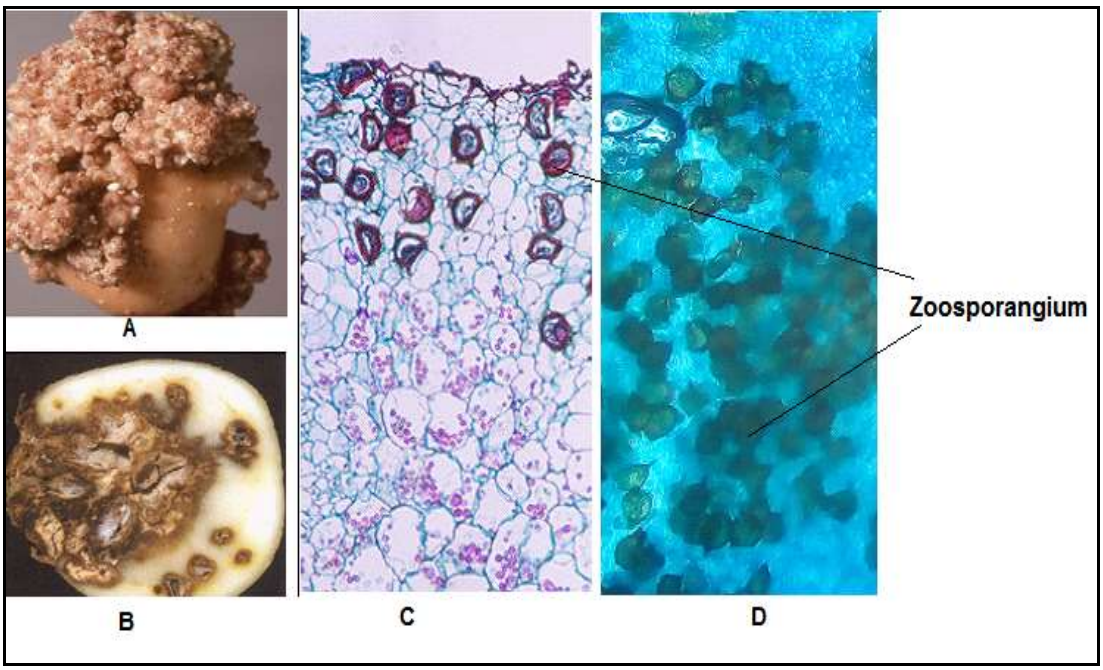

Figure 1.19: Wart disease of potato. A and B- Disease symptoms, C and D– Zoosporangium

B. Symptoms:

Only the below ground symptoms are apparent warty, cauliflower-like outgrowths from potato tuber eyes, stolon buds and the base of the stems.

Warts are initially white or green and darken and decay as they age. They vary in shape and size (1-8 cm diameter) and large masses may cover the entire tuber (**Figure 1.19**).

1.20 Fusarium Ear Rot:

A. Causal Organism:

Fusarium ear rot is caused primarily by *Fusarium verticillioides* fungus, which also causes stalk rot, root rot and seedling blight in maize.

Fusarium verticillioides is the accepted name of the species, which was also known as *Fusarium moniliforme*.

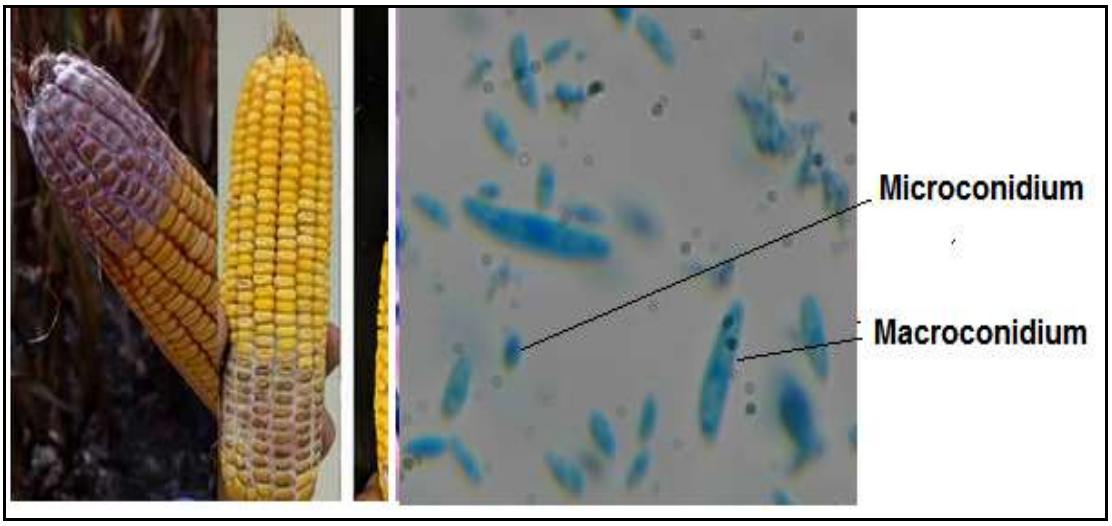

Figure 1.20: Fusarium ear rot disease. A- Disease symptoms, B– Conidia

B. Symptoms:

Symptoms include scattered individual kernels or groups of kernels with whitish-pink fungal growth.

These may also have a 'starburst' pattern of white streaks on the cap along the base of the kernel.

Infection is more frequent on damaged ear tips and on kernels with pericarp injuries due to insect feeding damage.

This type of ear rot can also infect kernels without showing visible symptoms.

Clean (first- grade) grain occasionally has an infection rate up to 90% without showing symptoms (**Figure 1.20**).

1.21 Loose Smut of Wheat:

A. Causal Organism:

Loose smut of wheat is caused by *Ustilago nuda* belong to Basidiomycotina.

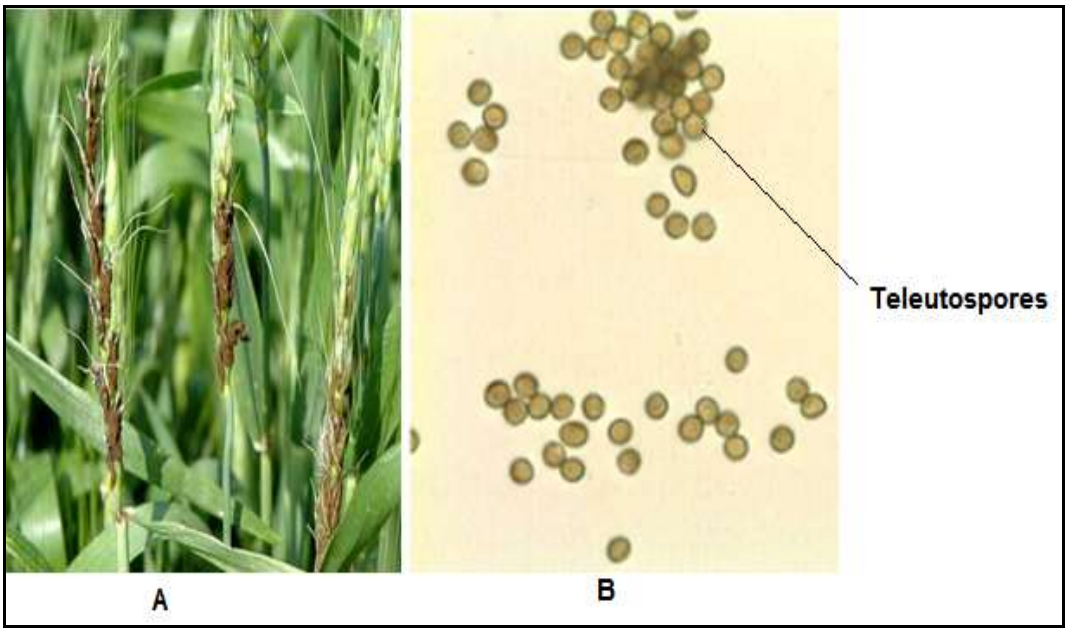

Figure 1.21: Loose smut of wheat. A- Disease symptoms, B– Teleutospores

B. Symptoms:

It is a seed borne disease; infection occurs during flowering stage through wind-borne spores. The seeds are infected during flowering and the infected seeds are the primary source of inoculum in the next crop.

The infection remains dormant inside the otherwise healthy looking seed but the plants grown from such seeds bear infected inflorescence. At this time, infected heads emerge earlier than normal heads. The entire inflorescence is commonly affected and appears as a mass of olive-black spores, initially covered by a thin gray membrane.

Once the membrane ruptures, the head appears powdery. The pathogen completely destroyed grains with black powdery smut consists of teleutospores. Teleutospores are distributed with the wind and spread the disease on large distances.

Teleutospores are formed from the cells of the mycelium. The pathogen survive in form of inactivate mycelium in the seed. Teleutospores observed under microscope have oval shape and smooth walls (**Figure 1.21**).

1.22 Scanning Electron Microscope:

The scanning electron microscope is a powerful microscopic tool that utilizes electrons to form a magnified image of specimen under study (**Figure 1.22**). It is a powerful magnification tool that produces high-resolution, three-dimensional images which provide information on the topography, morphology and composition of the sample/specimen. The information so obtained helps to assist a large number of science and industry applications. The SEM was developed by Dr. Charles Oatlev.

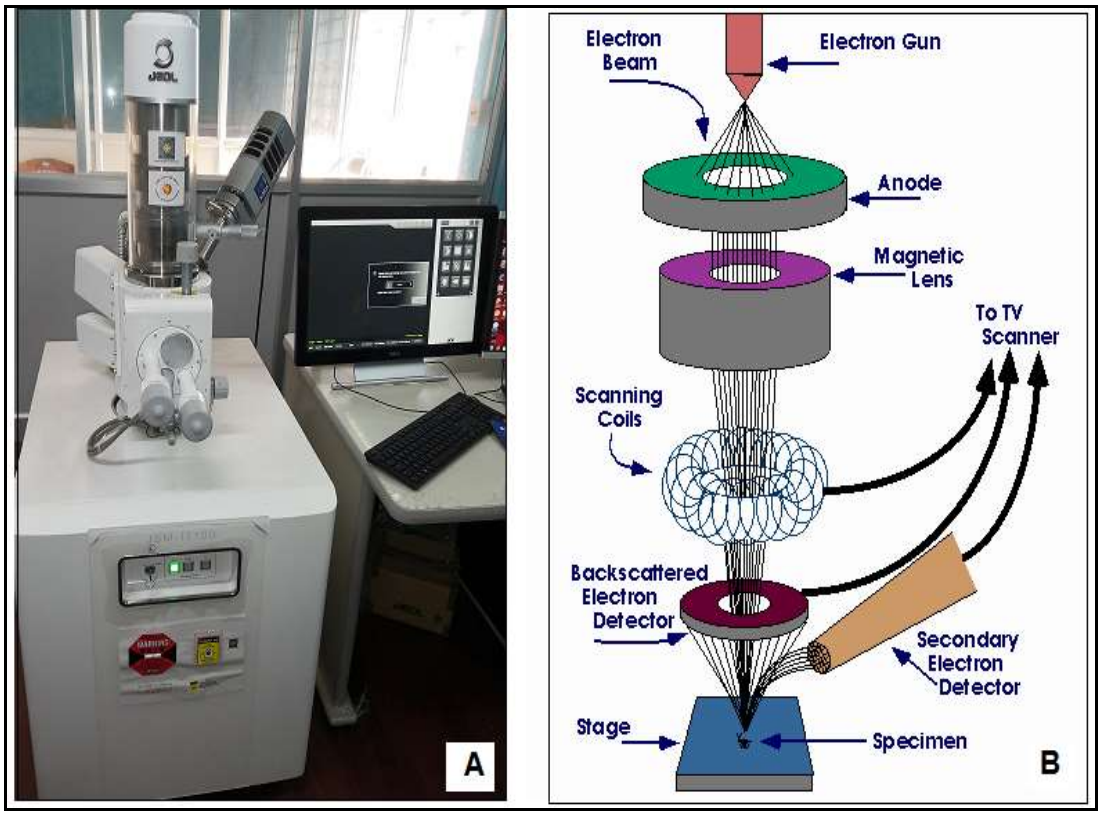

Figure 1.22: Scanning Electron Microscopy. A- Scanning Electron Microscope (SEM), B- Mechanism of SEM.

Principles of SEM:

The principle on which a SEM works is the same as that of the basic principles as light microscopes, but a focused beam of high-energy electrons is used instead of photons. The electrons carry significant kinetic energy. When the incident electrons strike the sample surface, the energy from these electrons is dissipated as a variety of signals. The signals are generated as a result of interactions that take place between electron and the sample. These signals include secondary electrons, backscattered electrons (BSE), and diffracted backscattered electrons (EBSD), X-Rays, visible light (cathodoluminescence) and heat. The production of SEM images utilize the secondary and back scattered, whereas the crystalline structures o orientation of minerals and micro-fabrics are determined by the diffracted backscattered electrons. The X-Rays are used for elemental analysis.

1.23 SEM Components:

The key components (**Figure 1.23**) which are essential for the operation of the scanning electron microscope are:-

a. Electron Source:

There are 3 common types of electrons sources:

I. Tungsten (W) Electron Filament:

This is basic type of electron source. It produces electrons when heated resistively.

II. Solid State Crystal (Lanthanum Hexaboride (LaB_6) or Cerium Hexaboride (CeB_6) :

This source is a thermionic emission gun. It is the most common high-brightness source and offers about 5-10 times the brightness as that of the tungsten filament. Also, the lifetime of the electron source is much longer lifetime than its tungsten filament counterpart.

III. Field Emission Gun (FEG):

As the name suggests, this source uses field electron emission for production of the electron beam. The small tip radius of the FEG provides for improved emission and focusing ability. The electrons generated by these sources are accelerated to a voltage range of 1-40 kV and then further focused into a narrow beam that can be used for the purpose of image formation and analysis of the same.

b. Lenses:

A series of condenser lenses are present which are used to focus the electron beam as it passes through the microscopic column. The narrowness of the beam determines what is going to be the size of the spot when it will be contacting the surface. These lenses are tubes, wrapped in coil and referred to as solenoids.

c. Scanning Oil:

The purpose of the scanning coils is to deflect the focused electron beam in the X and Y axes so that it scans in a raster fashion over the sample surface.

d. Sample Chamber:

Sample chamber consists of an evacuated chamber where the samples are mounted and placed.

It can also include additional devices to assist in sample imaging, such as translation stage, tilt and rotation devices, temperature stages, optical cameras etc.

e. SEM Detectors:

I. Secondary Electron Detector:

- Low energy electrons ejected from the k-orbitals of the sample atoms.
- Electrons are accelerated towards a scintillator, which in turn produces a current. The current is directed towards a photomultiplier and the amplified signal is read on the monitor.
- Everhart-Thornley detector is an example.

II. Backscattered Electron Detector:

- High energy electrons are produced as a result of elastic scattering interactions with specific atoms in the sample.
- The electrons are reflected backwards by sample atoms.
- These detectors can be scintillators or semiconductors.

III. Diffracted Backscatter Electrons:

- Determine crystallographic structures of sample.
- Determine orientation of minerals and micro-fabrics.

IV. X-Rays:

- Provide information on element and mineral.

V. Computer and display to view the images:

In addition, a stable power supply, vacuum and cooling system is required.

A vibration-free space that also isolates the instrument from ambient magnetic and electric fields is also needed**.**

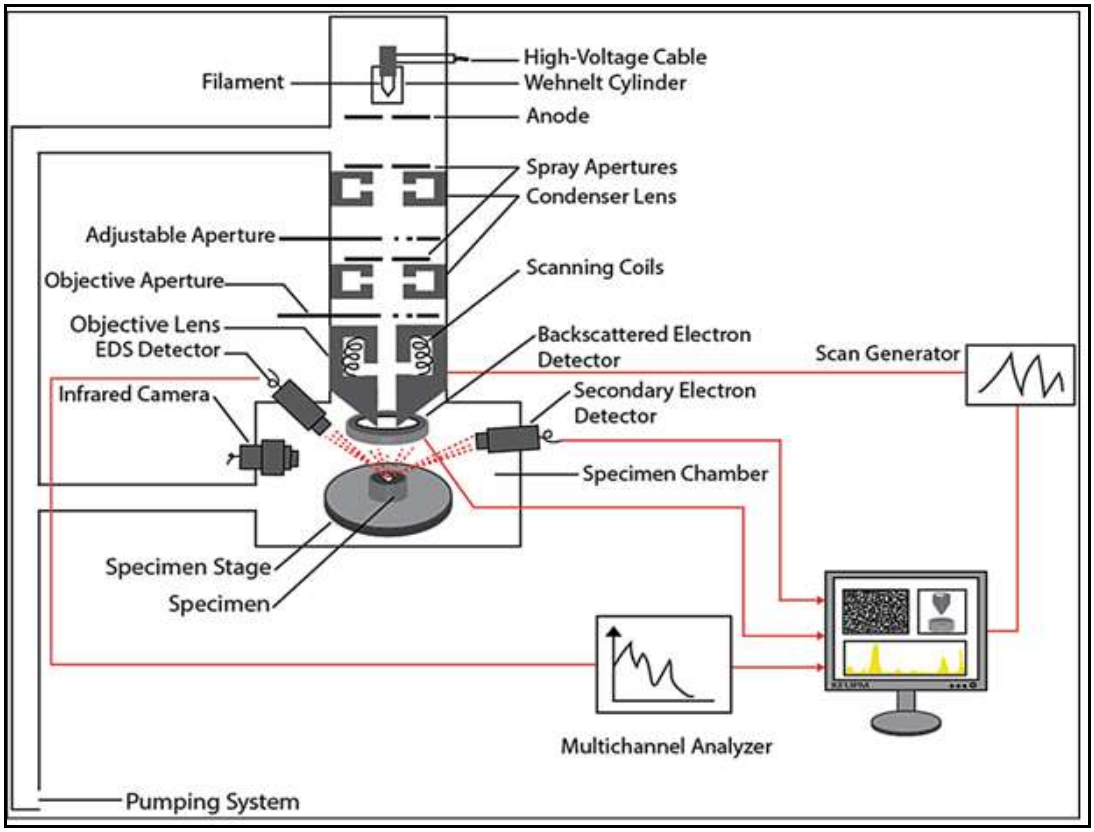

Figure 1.23: Components of SEM

1.23.1 Working of a SEM:

a. Electron guns placed at the top of the column produce high energy electrons. These are accelerated down and allowed to pass through a combination of electromagnetic lenses. The lenses help to produce a focused electron beam.
b. The electron beam moves across a vertical path through the microscope, in the presence of vacuum.
c. c) The sample chamber area is also evacuated by a combination of pumps. The sample is placed inside this chamber.
d. The scanning coils are adjusted to allow the electron beam to be focused on the sample surface. Beam scattering enables information for the sample to be collected on a defined area on which the beam has been focused.
e. The operator can adjust the beam through a computer to control magnification and surface area to be scanned.
f. Interaction between the incident electrons and the sample surface leads to the release of a number of energetic electrons from the sample surface
g. The interaction leads to specific scattering of electrons (e.g., backscattered electrons, secondary electrons etc.) which can provide information on size, shape, texture and composition of the sample.
h. The electrons are collected by detectors and converted into a signal. The signals are sent to a screen to produce a final black and white 3-dimensional image.

i. Areas ranging from approximately 1 cm to 5 microns in width can be imaged by SEM with a magnification ranging from 20X to approximately 30,000X (**Figure 1.23**).
j. The interaction between the incident electrons and the surface of the sample is determined by the acceleration rate of incident electrons.

1.23.2 SEM Sample Preparation:

Because the SEM utilizes vacuum conditions and uses electrons to form an image, special preparations must be done to the sample.

Two most commonly used methods for sample preparation in case of SEM are **sputter coating for non-conductive samples** and **dehydration of most biological specimens**.

In addition, it is required that all samples are able to handle the low pressure inside the vacuum chamber (Golding *et al.* 2016).

In case the sample to be observed under SEM **is a metal,** no preparation is required because metals are conducting in nature.

For biological specimens, dehydration is important.

The removal of water from sample is needed because the water would vaporize in the vacuum.

The samples are gradually treated in the presence of increasing concentrations of acetone (10%, 30%, 50%, 70% and 100%) to dehydrate the sample.

In case the sample to be observed under SEM **is a non-metal,** it has to be made conducting.

This is achieved by covering the sample with a thin layer of conductive material such as gold.

A device called a **"sputter coater"** which operates in the presence of electric field and argon gas is used to form this gold coating.

The argon gas and an electric field remove an electron from the argon atoms, making them positively charged.

The argon cations are then attracted to a negatively charged gold foil, where they knock down gold atoms from the surface of the foil.

The gold atoms therefore settle onto the sample surface to produce a gold coating.

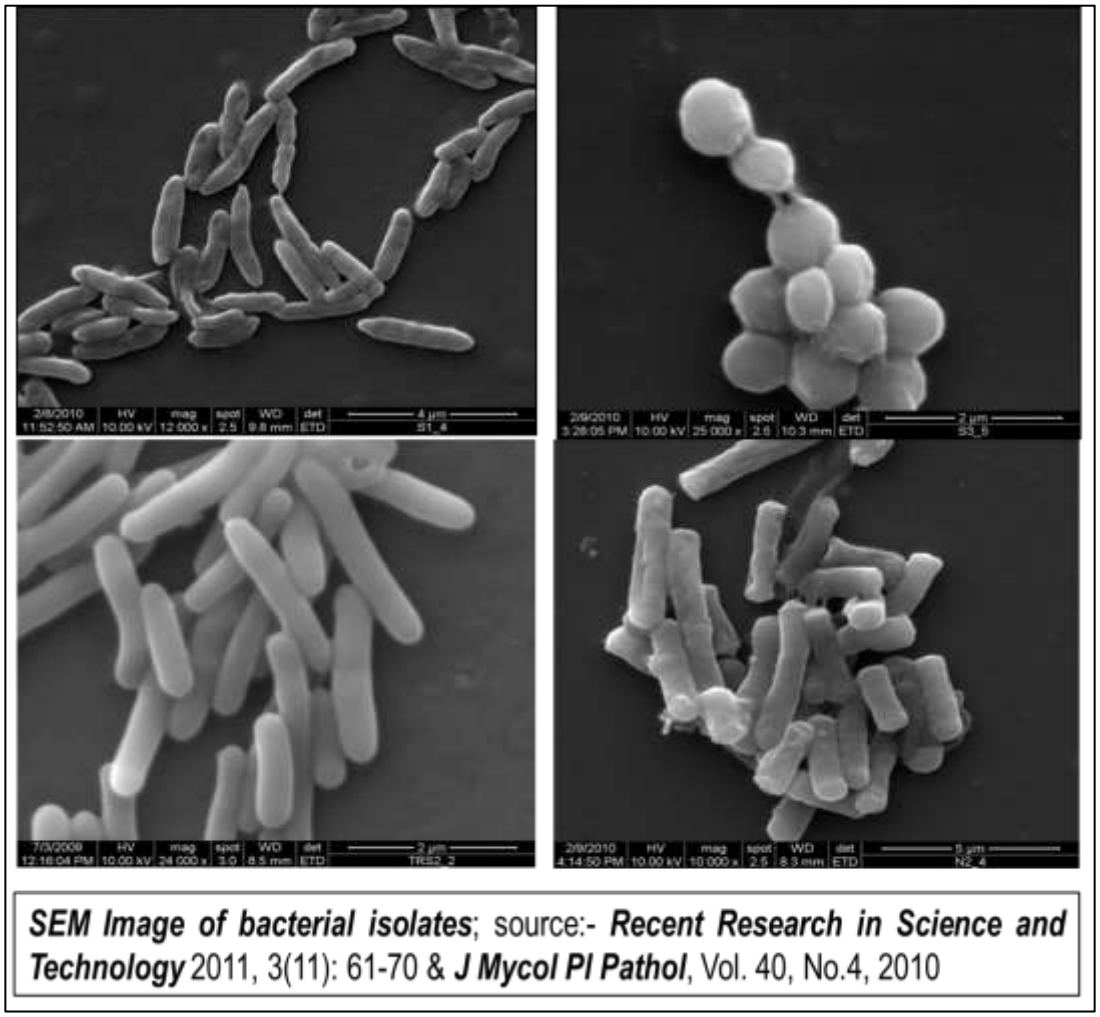

Figure 1.24: SEM image of bacterial isolates

Advantages of SEM:

- Three-dimensional imaging and topographical, morphological and compositional information obtained.
- User-friendly, fast and easy to operate.
- Detection, identification and analysis of surface fractures, microstructures, surface contaminations, crystalline structures etc.
- It provides higher resolution as well as larger area to be focused at one time as compared to traditional microscopes.

Applications of SEM:

- Essential research tool in life science, biology, medical and forensic science, metallurgy.
- Wide-array of applications, including industrial and technological applications.
- Deciphering spatial variations in chemical compositions.

- Measurement of very small features and objects down to 50 nm in size.
- Back scattered electron images (BSE) can be used for rapid discrimination of phases in multiphase samples.

1.23.3 Name of Institute Having Scanning Electron Microscopy (SEM) Facility:

a. University Science Instrumentation Centre (USIC), University of North Bengal (NBU) Website:- https://www.nbu.ac.in/cfs/usic.aspx
b. University Science Instrumentation Centre and Central Instrumentation Facility, University of Burdwan, Website:- http://www.buruniv.ac.in/usic
c. S. N. Bose Innovation Centre with Central Instrumentation Laboratory, University of Kalyani,Website:-http://162.144.89.90/index.php/services-and-facilities/instrumentation-facility/scanning-electron-microscopy
d. Central Instrument Facility/ Sophisticated Analytical Instrument Facility (SAIF), Bose Institute, Website: http://www.jcbose.ac.in/cif-instruments
e. FEI Quanta 200 Environmental Scanning Electron Microscope (eSEM) at ICMR-National Institute of Cholera and Enteric Diseases (NICED), Kolkata, Website:- http://www.niced.org.in/niced/NICED-Facilities.htm#ElectronMicroscopy
f. Field Emission Scanning Electron Microscope, Central Research Facility, IIT, Kharagpur , Website: http://www.crf.iitkgp.ac.in/CRF/Lab_Details/?id=19
g. The Quanta 200 FEG scanning electron microscope (SEM), Saha Institute of Nuclear Physics, Department of Atomic Energy, Govt. of India, Website: http://www.saha.ac.in/web/spd-facilities/spd-microscopy/spd-scanning-electron-microscope

1.24 Transmission Electron Microscopy:

Transmission electron microscopy (TEM) is a microscopy technique in which a beam of electrons is transmitted through a specimen to form an image (**Figure 1.25**).

The specimen is most often an ultrathin section less than 100 nm thick or a suspension on a grid.

An image is formed from the interaction of the electrons with the sample as the beam is transmitted through the specimen.

The image is then magnified and focused onto an imaging device, such as a fluorescent screen,

a layer of photographic film, or a sensor such as a scintillator attached to a charge-coupled device.

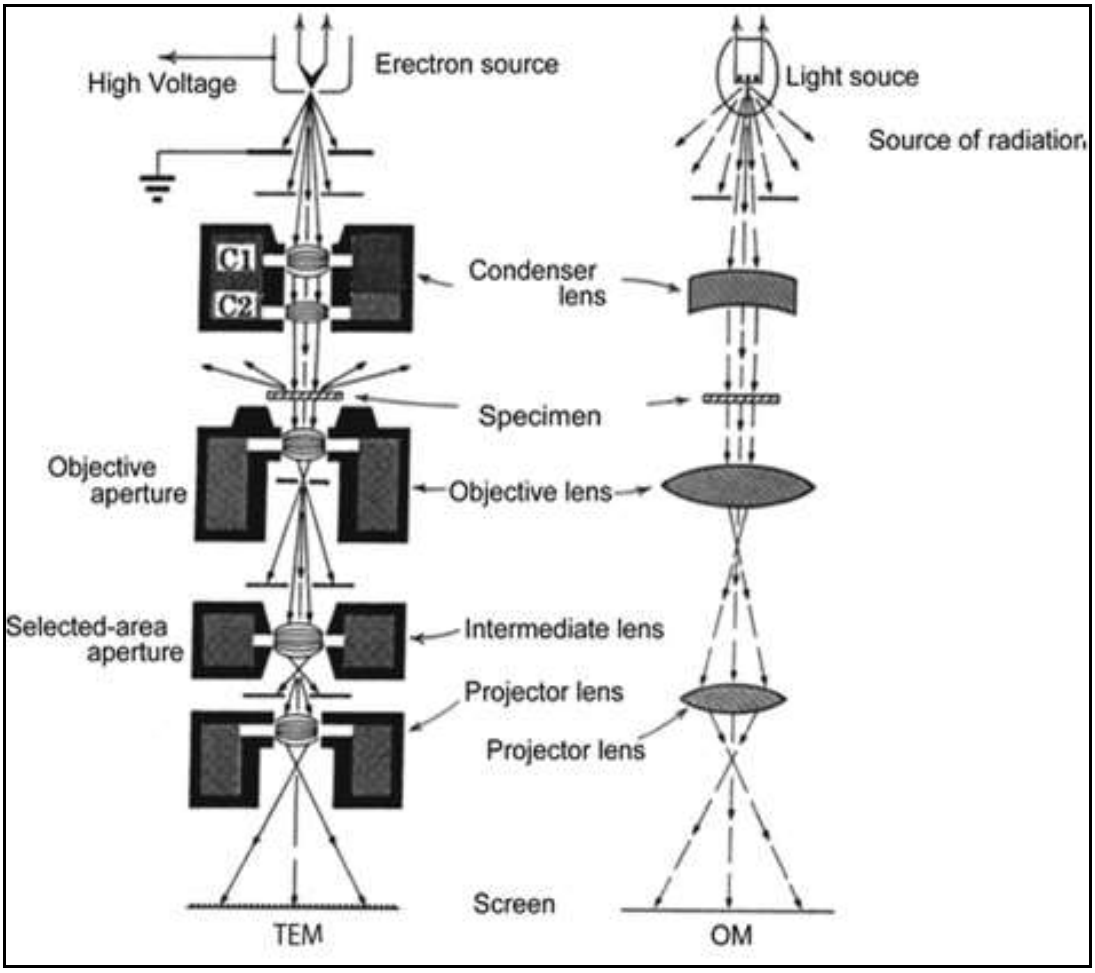

Figure 1.25: Transmission Electron Microscopy

1.24.1 Specimen Preparation:

a. Fixation:

Control and inoculated root/leaf samples (1-2 mm) were excised in 0.1M sodium phosphate buffer pH 7.4.

They were immediately transferred to 2.5% Glutaraldehyde in eppendorf tubes for 2-12 hours at room temperature.

b. Dehydration:

Dehydration was done in ascending grades of alcohol at intervals of 30 mins in 4°C (30%, 50%, 70%, 80%, 90%) and two changes in absolute alcohol at 1 hr interval each at 4°C in PLT-272(M) Fume Hood (Tanco).

c. Infiltration:

Infiltration was done twice in LR White resin (London Redin Co. Ltd) in absolute alcohol (1:1) for 1 hr each at 4° C.

d. Embedding:

The samples were dipped in LR White and kept overnight at 4° C. They were kept at room temperature for 3 hrs. A fresh change of LR white was done and kept at 56° C for 36 hrs.

1.24.2 Viewing Preparation:

a. Trimming:

Moulds containing the samples were roughly trimmed with a block trimmer (Reichert TM 60) fitted with a rotating milling cutter.

b. Sectioning:

A series of thick sections of the selected blocks were cut with Belgium glass strips in microtome (Leica EM UC7) to observe under an optical microscope. These semithin sections are stained with 1% aqueous toluidine blue solution. These sections can be viewed in light microscope. (Golding *et al.* 2016)

1.24.3 Immunogold labeling for Immuno-Transmission Electron Microscopy:

Ultrathin sections (60nm) were cut with fresh Belgium glass strips and picked up in nickel grids (100 mesh) for immunogold labeling.

a. Primary Antibod:

The grids containing ultrathin sections were floated in blocking solution containing 2% skimmed milk agar for 30 min. Primary antibody was diluted in 1% fish gelatin in the ratio 1:20. Grids were incubated the PAbs for 24 hrs at 4° C. Grids were washed on drops (100 µl) of fish gelatin pipetted on to parafilm 10X2 min.

b. Secondary antibody:

Grids were incubated with anti-rabbit IgG (Whole Molecule) gold antibody produced in goat affinity isolated antibody (Sigma-G7402) diluted in 1:5 in fish gelatin at room temp for 3 hrs.

c. Staining:

Sections were stained with 2% uranyl acetate for 15 min. The sections were washed in double distilled water. Post stain was done in 0.2% lead acetate for 5 min. Washed again in double distilled water.

d. Viewing:

Ultrastructural analysis of the section was performed with Morgagni 268D with iTEM Imaging System. Specificity of labeling was assessed by the control test by incubating sections with rabbit pre-immune serum instead of the primary antibody (**Figure 1.26**).

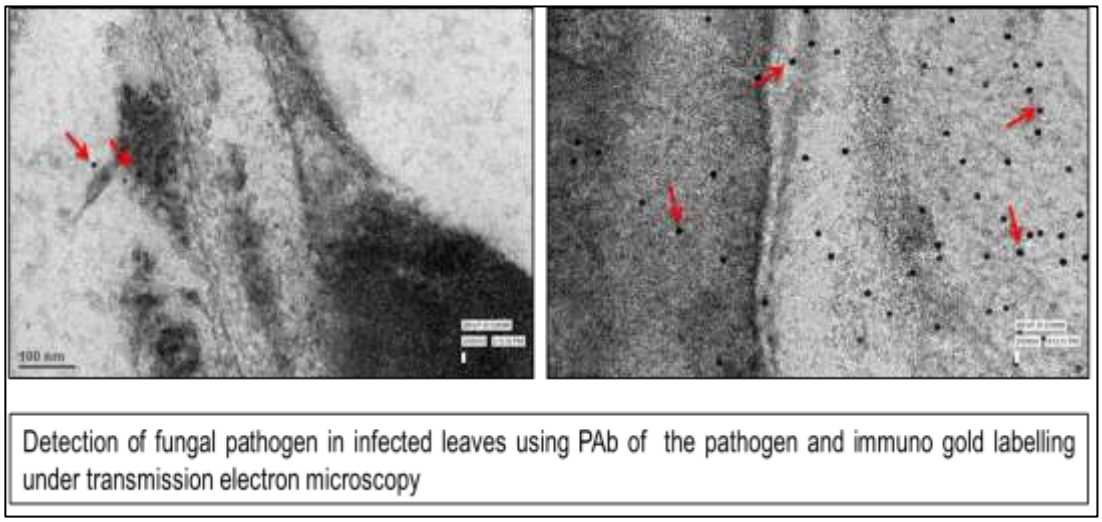

Figure 1.26: Detection of fungal pathogen by Transmission Electron Microscopy (TEM)

1.25 Reference:

1. Golding CG, Lamboo LL, Beniac DR, Booth TF. The scanning electron microscope in microbiology and diagnosis of infectious disease. Sci. Reports. 6: 26516, 2016.