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DIRECTORATE OF DISTANCE EDUCATION

M.Sc., Botany

Paper – III

Microbiology and Plant Pathology

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Paper III: MICROBILOGY AND PLANT PATHOLOGY

Bacteriology: General Characteristics- Classification (Bergey`s), Ultra structure of bacterial cell- Gram +ve & gram –ve. Staining methods- Gram & Acid fast staining, Endospore staining, fission and Sporulation. Bacterial growth – Continuous & synchronous culture. Factors affecting growth, Determination Of bacterial growth- Direct method- haemocytometer, Viable plate count –Indirect method – turbidity.

Virology: General characters, classification, structure, multiplication. Bacteriophages- Classification Replication of DNA and RNA phages – lytic and Lysogenic Cycle, Viruses of Eukaryotes- Animal and Plant viruses- Viriods and prions.

Cultivation of microbes: Nutritional types of Microbes. Media, Steriliztion-Physical and chemical sterilizing agents- Principle, Mode of action and application. Culturing techniques, cultivation of bacteria, algae, fungi and viruses

General symptoms, pathogenesis Effect of environmental factors on disease development- temperature, moisture, wind, light, soil PH structure. Causative agent, transmission, symptom and control of plant diseases – Bacterial – Angular leaf spot of cotton. Fungal- blast of paddy. Viral- Bunchy top of banana. Plant protection: Exclusion (plant quarantine), eradication, chemical protection, biological control (organisms and mechanisms).Integrated plant protection.

Defense mechanism of plants against infection – preexisting structures (wax, cuticle, epidermis etc); Anatomical – cork layer, Tyloses, abscission layer; Biochemical – phenolics, phytolexins. Molecular biological aspects – PR proteins and avr genes. Effect of infection on the physiology of the host plant (permeability changes, photosynthesis, respiration, enzyme activity, nitrogen metabolism, phenol metabolism).

Text Books:

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M.Sc., Botany Paper-III

UNIT 1 BACTERIOLOGY

Structure

INTRODUCTION

Bacteria colonize the human environment: homes and businesses, the soil, even the human body. Tiny as they are, bacteria can pose enormous threats to public health if conditions allow them to thrive and multiply. Although all bacteria share certain structural, genetic, and metabolic characteristics, important biochemical differences exist among the many species of bacteria. These differences permit bacteria to live in many different, and sometimes extreme, environments. For example, some bacteria recycle nitrogen and carbon from decaying organic matter, and then release these gases into the atmosphere to be reused by other

living things. Other bacteria cause diseases in human and animals, help digest sewage in treatment plants, or produce the alcohol in wine, beer and liquors. Still others are used by humans to break down toxic waste chemicals in the environment, a process called bioremediation.

1.1 OBJECTIVES

After going through this unit, you will be able to

- describe the general characteristics of a basic bacterium
- recognize and name the general shapes and forms of bacteria
- explain the significance of the cell wall structures(such as the peptidoglycan) and the Gram stain
- describe the main factors that influence bacterial growth

1.3 Bacterial cell structure

Bacteria, despite their simplicity, contain a well-developed cell structure which is responsible for many of their unique biological properties. Many structural features are unique to bacteria and are not found among archaea or eukaryotes. Because of the simplicity of bacteria relative to larger organisms and the ease with which they can be manipulated experimentally, the cell structure of bacteria has been well studied, revealing many biochemical principles that have been subsequently applied to other organisms.

Cell morphology

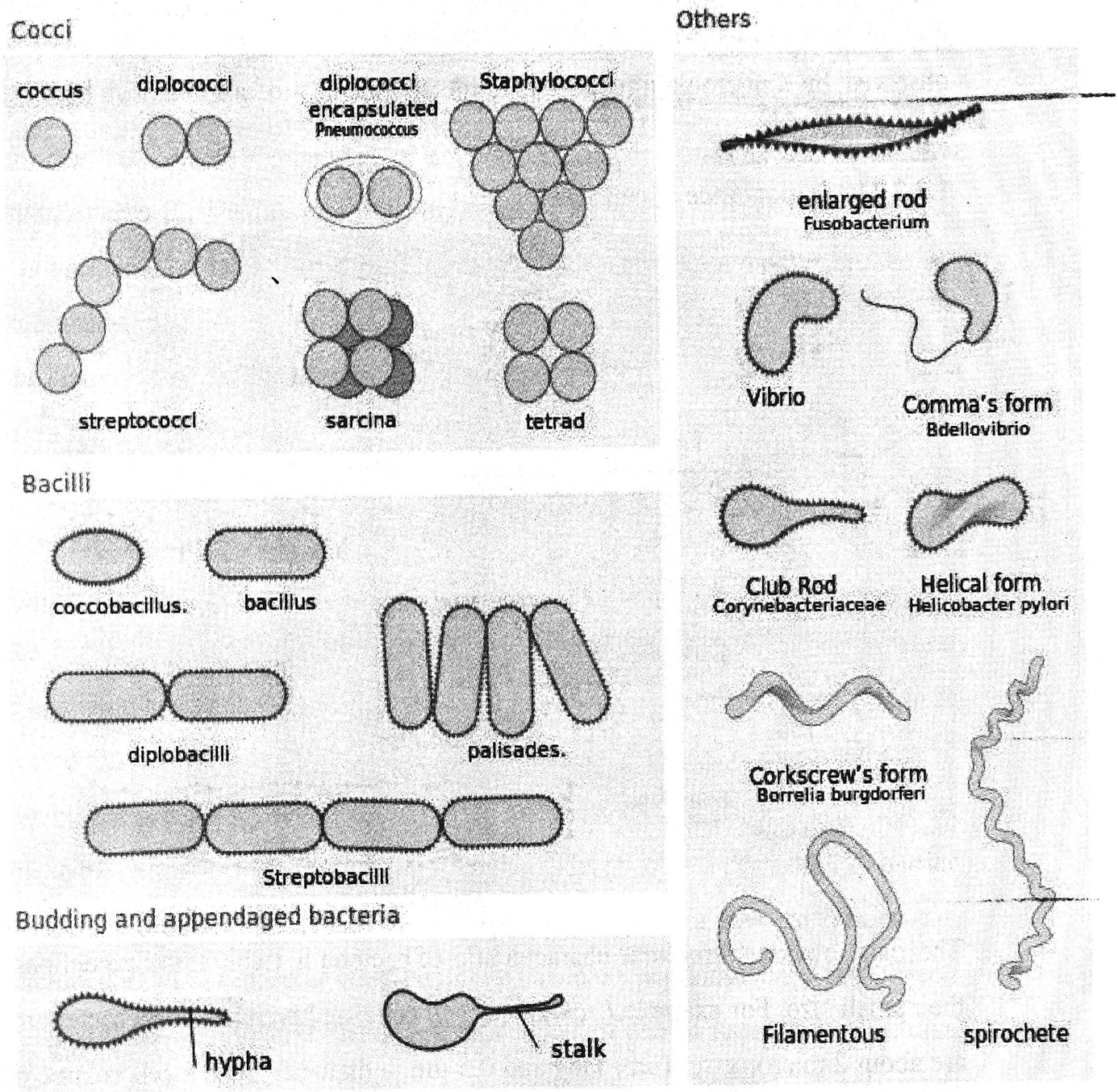


Figure-1

Bacteria come in a wide variety of shapes

Bacteria are classified into 5 groups according to their basic shapes: spherical (cocci), rod (bacilli), spiral (spirilla), comma (vibrios) or corkscrew (spirochaetes). They can exist as single cells, in pairs, chains or clusters.

Cell shape is generally characteristic of a given bacterial species, but can vary depending on growth conditions. Some bacteria have complex life cycles involving the production of stalks and appendages (e.g. *Caulobacter*) and some produce elaborate structures bearing reproductive spores

(e.g. *Myxococcus*, *Streptomyces*). Bacteria generally form distinctive cell morphologies when examined by light microscopy and distinct colony morphologies when grown on Petri plates. These are often the first characteristics observed by a microbiologist to determine the identity of an unknown bacterial culture.

1.3.2 The importance of cell size

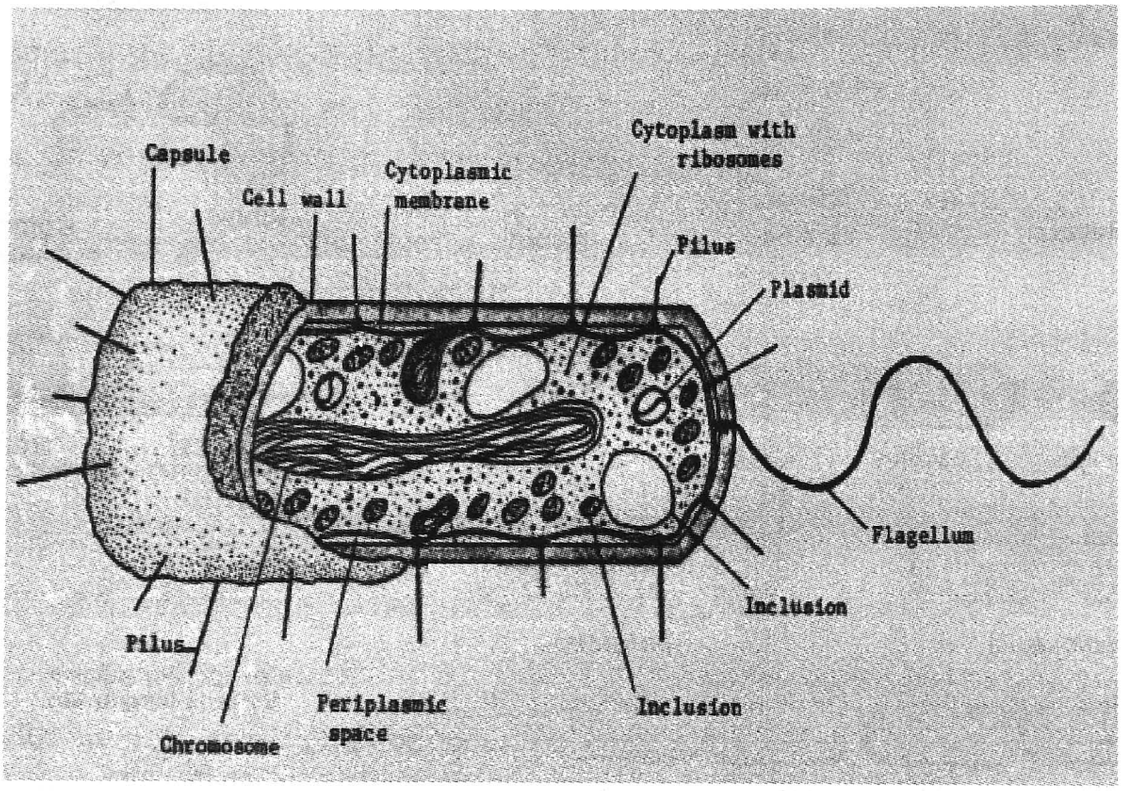


Figure-2

The most obvious structural characteristic of bacteria is (with some exceptions) their small size. For example,*Escherichia coli* cells, an "average" sized bacterium, are about 2 micrometres (μm) long and 0.5 μm in diameter, with a cell volume of 0.6 - 0.7 μm^3.[1] This corresponds to a wet mass of about 1 picogram (pg), assuming that the cell consists mostly of water. The dry mass of a single cell can be estimated as 20% of the wet mass, amounting to 0.2 pg. About half of the dry mass of a bacterial cell consists of carbon, and also about half of it can be attributed to proteins. Therefore, a typical fully grown 1-liter culture of *Escherichia coli* (at an optical density of 1.0, corresponding to ca. 10^9 cells/ml) yields about 1 g wet cell mass.

Small size is extremely important because it allows for a large surface area-to-volume ratio which allows for rapid uptake and intracellular distribution of nutrients and excretion of wastes. At low surface area-to-volume ratios the diffusion of nutrients and waste products across the bacterial cell membrane limits the rate at which microbial metabolism can occur, making the cell less evolutionarily fit. The reason for the existence of large cells is unknown, although it is speculated that the increased cell volume is used primarily for storage of excess nutrients.

The structure of peptidoglycan

As in other organisms, the bacterial cell wall provides structural integrity to the cell. In prokaryotes, the primary function of the cell wall is to protect the cell from internal turgor pressure caused by the much higher concentrations of proteins and other molecules inside the cell compared to its external environment. The bacterial cell wall differs from that of all other organisms by the presence of peptidoglycan (poly-*N*-acetylglucosamine and *N*-acetylmuramic acid), which is located immediately outside of the cytoplasmic membrane. Peptidoglycan is responsible for the rigidity of the bacterial cell wall and for the determination of cell shape. It is relatively porous and is not considered to be a permeability barrier for small substrates. While all bacterial cell walls (with a few exceptions e.g. extracellular parasites such as *Mycoplasma*) contain peptidoglycan, not all cell walls have the same overall structures. Since the cell wall is required for bacterial survival, but is absent in eukaryotes, several antibiotics (penicillins and cephalosporins) stop bacterial infections by interfering with cell wall synthesis, while having no effects on human cells, because human cells don't have cell walls, they only have cell membranes. There are two main types of bacterial cell walls, Gram positive and Gram negative, which are differentiated by their Gram staining characteristics. For both Gram-positive and Gram-negative bacteria, particles of approximately 2 nm can pass through the peptidoglycan.

The Gram positive cell wall

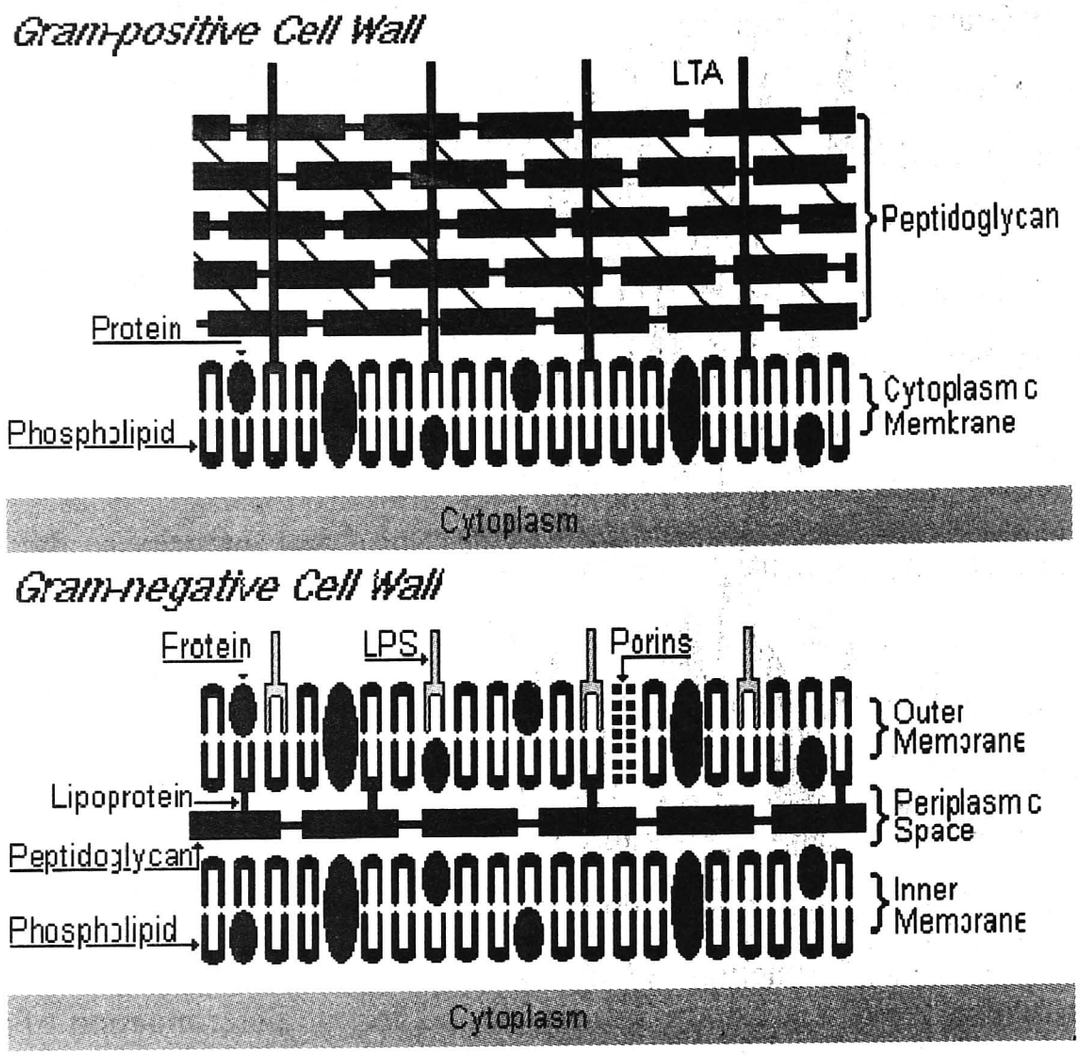


Figure-3

Peptidoglycans (mucopeptides, glycopeptides, mureins) are the structural elements of almost all bacterial cell walls. They constitute almost 95% of the cell wall in some Gram positive bacteria and as little as 5-10% of the cell wall in Gram negative bacteria. Peptidoglycans are made up of a polysaccharide backbone consisting of alternating N-acetylmuramic acid(NAM) and N-acetylglucosamine(NAG) residues in equal amounts. The cell wall of some Gram positive bacteria can be completely dissolved by lysozyme, as this enzyme attacks the bonds between GA and MA. In other Gram positive bacteria, e.g. *Staphylococcus aureus,* the walls are resistant to the action of lysozyme. They have O-acetyl groups on carbon-6 of some MA residues. The matrix substances in the walls of Gram positive bacteria may be polysaccharides or teichoic acids. The latter are very widespread, but have been found only in Gram positive bacteria. There are two main types of teichoic acid: ribitol teichoic acids and glycerol

teichoic acids. The latter one is more widespread. These acids are polymers of ribitol phosphate and glycerol phosphate, respectively, and only located on the surface of many Gram positive bacteria.

The Gram negative cell wall

Unlike the Gram positive cell wall, the Gram negative cell wall contains a thin peptidoglycan layer adjacent to the cytoplasmic membrane. This is responsible for the cell wall's inability to retain the crystal violet stain upon decolorization (differentiation) with ethanol-acetic acid during Gram staining. In addition to the peptidoglycan layer, the Gram negative cell all also contains an outer membrane composed by phospholipids and lipopolysaccharides, which face into the external environment. As the lipopolysaccharides are highly charged, the Gram negative cell wall has an overall negative charge. Lipopolysaccharides are often unique to specific bacterial strains (i.e. sub-species) and are responsible for many of the antigenic properties of these strains.

The bacterial cytoplasmic cell membrane

The bacterial cytoplasmic membrane is composed of a phospholipid bilayer and thus has all of the general functions of a cell membrane such as acting as a permeability barrier for most molecules and serving as the location for the transport of molecules into the cell. In addition to these functions, prokaryotic membranes also function in energy conservation as the location about which a proton motive force is generated. Unlike eukaryotes, bacterial membranes (with some exceptions e.g. *Mycoplasma* and methanotrophs) generally do not contain sterols. Unlike eukaryotes, bacteria can have a wide variety of fatty acids within their membranes. Along with typical saturated and unsaturated fatty acids, bacteria can contain fatty acids with additional methyl, hydroxy or even cyclic groups. The relative proportions of these fatty acids can be modulated by the bacterium to maintain the optimum fluidity of the membrane (e.g. following temperature change).

In the phospholipid bilayer, the lipid portion of the outer membrane is impermeable to charged molecules. However, channels called porins are present in the outer membrane that allow for passive transport of

many ions, sugars and amino acids across the outer membrane. These molecules are therefore present in the periplasm, the region between the cytoplasmic and outer membranes. The periplasm contains the peptidoglycan layer and many proteins responsible for substrate binding or hydrolysis and reception of extracellular signals. The periplasm exist as a gel-like state rather than a liquid due to the high concentration of proteins and peptidoglycan found within it. Due to its location between the cytoplasmic and outer membranes, signals received and substrates bound are available to be transported across the cytoplasmic membrane using transport and signalling proteins imbedded there.

Other bacterial surface structures

Fimbrae and pili

Fimbrae are protein tubes that extend out from the outer membrane in many members of the Proteobacteria. They are generally short in length and present in high numbers about the entire bacterial cell surface. Fimbrae usually function to facilitate the attachment of a bacterium to a surface (e.g. to form a biofilm) or to other cells (e.g. animal cells during pathogenesis)). A few organisms (e.g. *Myxococcus*) use fimbrae for motility to facilitate the assembly of multicellular structures such as fruiting bodies. Pili are similar in structure to fimbrae but are much longer and present on the bacterial cell in low numbers. Pili are involved in the process of bacterial conjugation. Non-sex pili also aid bacteria in gripping surfaces.

Capsules and slime layers

Many bacteria secrete extracellular polymers outside of their cell walls. These polymers are usually composed of polysaccharides and sometimes protein. Capsules are relatively impermeable structures that cannot be stained with dyes such as India ink. They are structures that help protect bacteria from phagocytosis and desiccation.

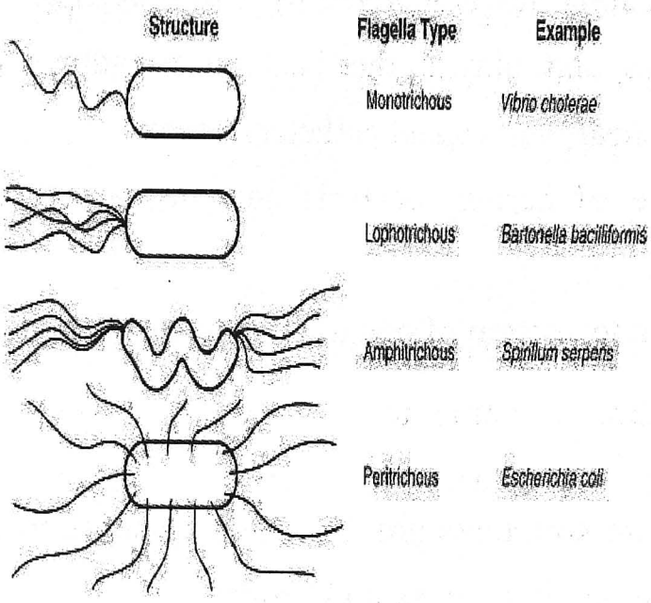


Figure-4

Slime layer is involved in attachment of bacteria to other cells or inanimate surfaces to form biofilms. Slime layers can also be used as a food reserve for the cell. Bacterial flagellum. The most recognizable extracellular bacterial cell structures are flagella. Flagella are whip-like structures protruding from the bacterial cell wall and are responsible for bacterial motility (i.e. movement).

The arrangement of flagella about the bacterial cell is unique to the species observed. Common forms include:

- Peritrichous - Multiple flagella found at several locations about the cell
- Monotrichous - Single flagellum found at one of the cell poles
- Lophotrichous - A tuft of flagella found at one cell pole
- Amphitrichous- A tuft of flagella found at two cell poles

The bacterial flagellum consists of three basic components: a whip-like filament, a motor complex, and a hook that connects them. The filament is approximately 20 nm in diameter and consists of several protofilaments, each made up of thousands of flagellin subunits. The bundle is held together by a cap and may or may not be encapsulated. The motor complex consists of a series of rings anchoring the flagellum in the inner and outer membranes, followed by a proton-driven motor that drives rotational movement in the filament.

GLYCOCALYX (Slime Layer):

- A polysaccharide coating that is secreted by many bacteria.
- Covers the surfaces and allows the bacteria to adhere firmly to various structures e.g. skin, heart valves,and catheters
- Mediates adherence of certain bacteria such as streptococcus mutants, to the surface of teeth.
- Important role in the formation of plaque, the precursor of dental caries.

SPORES:

- These highly resistant structures are formed in response to adverse conditions by two genera of medically important G+ rods:

– the genus *Bacillus* which includes the agent of anthrax, and

– the genus *Clostridium*, which include the agents of tetanus and botulism.

- Spore formation occur when nutrients such as sources of carbon and nitrogen are depleted.
- The spore forms inside the cell and contains bacterial DNA, a small amount of cytoplasm, cell membrane, peptidoglycan very little water and most important a thick keratin like coat that is responsible for the remarkable resistance of the spore to heat dehydration radiation and chemicals.
- The resistance may be mediated by dipicolinic acid, a calcium ion chelator found only in spores.

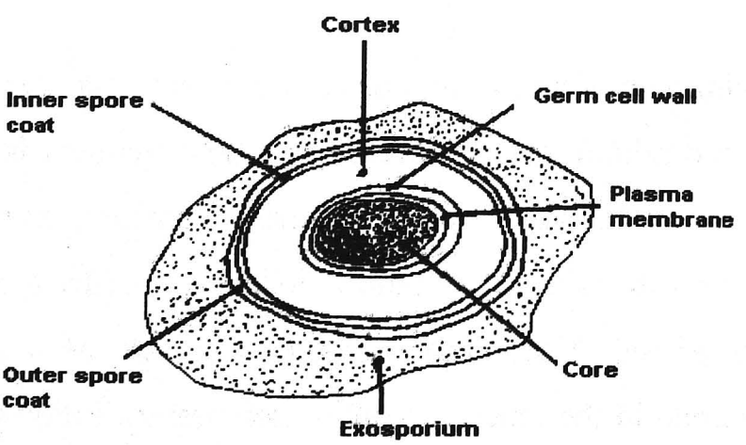


Figure-5

Cytoplasm:

The cytoplasm has two clear areas when seen in the electron-microscope.

1) A dense matrix that contains ribosomes,
2) Nutrient granules and plasmids.
3) An inner, nucleoid region composed of DNA.

The bacterial chromosome and plasmids

Unlike eukaryotes, the bacterial chromosome is not enclosed inside of a membrane-bound nucleus but instead resides inside the bacterial cytoplasm. This means that the transfer of cellular information through the processes of translation, transcription and DNA replication all occur within the same compartment and can interact with other cytoplasmic structures, most notably ribosomes. The bacterial chromosome is not packaged using histones to form chromatin as in eukaryotes but instead exists as a highly compact super coiled structure, the precise nature of which remains unclear. Most bacterial chromosomes are circular although some examples of linear chromosomes exist (e.g. *Borrelia burgdorferi*). Along with chromosomal DNA, most bacteria also contain small independent pieces of DNA called plasmids that often encode for traits that are advantageous but not essential to their bacterial host. Plasmids can be easily gained or lost by a bacterium and can be transferred between bacteria as a form of horizontal gene transfer.

Ribosomes and other multiprotein complexes

In most bacteria the most numerous intracellular structure is the ribosome, the site of protein synthesis in all living organisms. All prokaryotes have 70S (where S=Svedberg units) ribosomes while eukaryotes contain larger 80S ribosomes in their cytosol. The 70S ribosome is made up of 50S and 30S subunits. The 50S subunit contains the 23S and 5S rRNA while the 30S subunit contains the 16S rRNA. These rRNA molecules differ in size in eukaryotes and are complexed with a large number of ribosomal proteins, the number and type of which can vary slightly between organisms. While the ribosome is the most commonly observed intracellular multiprotein complex in bacteria other large complexes do occur and can sometimes be seen using microscopy.

Nutrient storage structures

Most bacteria do not live in environments that contain large amounts of nutrients at all times. To accommodate these transient levels of nutrients bacteria contain several different methods of nutrient storage in times of plenty for use in times of want. For example, many bacteria store excess carbon in the form of polyhydroxyalkanoates or glycogen. Some microbes store soluble nutrients such as nitrate in vacuoles. Sulfur is most often stored as elemental (S^0) granules which can be deposited either intra- or extracellularly. Sulfur granules are especially common in bacteria that use hydrogen sulfide as an electron source. Most of the above mentioned examples can be viewed using a microscope and are surrounded by a thin membrane to separate them from the cytoplasm.

Gas vesicles

Gas vesicles are spindle-shaped structures found in some planktonic bacteria that provides buoyancy to these cells by decreasing their overall cell density. Positive buoyancy is needed to keep the cells in the upper reaches of the water column, so that they can continue to perform photosynthesis. They are made up of a shell of protein that has a highly hydrophobic inner surface, making it impermeable to water (and stopping water vapour from condensing inside) but permeable to most gases. As the gas vesicle is a hollow cylinder, it is liable to collapse when the surrounding pressure becomes too great.

Carboxysomes

Carboxysomes are intracellular structures found in many autotrophic bacteria such as Cyanobacteria, Knallgasbacteria, Nitroso- and Nitrobacteria. They are proteinaceous structures resembling phage heads in their morphology and contain the enzymes of carbon dioxide fixation in these organisms (especially ribulose bisphosphate carboxylase/oxygenase, RuBisCO, and carbonic anhydrase). It is thought that the high local concentration of the enzymes along with the fast conversion of bicarbonate to carbon dioxide by carbonic anhydrase allows faster and more efficient carbon dioxide fixation than possible inside the cytoplasm.

Magnetosomes

Magnetosomes are intracellular organelles found in magnetotactic bacteria that allow them to sense and align themselves along a magnetic field (magnetotaxis). The ecological role of magnetotaxis is unknown but it is hypothesized to be involved in the determination of optimal oxygeconcentrations. Magnetosomes are composed of the mineral magnetite or greigite and are surrounded by a lipid bilayer membrane. The morphology of magnetosomes is species-specific.

1.4 Staining Methods

1.4.1 Gram Staining

Gram staining (or 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. A Gram positive results in a purple/blue color while a Gram negative results in a pink/red color.

The Gram stain is almost always the first step in the identification of a bacterial organism. While Gram staining is a valuable diagnostic tool in both clinical and research settings, not all bacteria can be definitively classified by this technique. This gives rise to Gram-variable and Gram-indeterminate groups as well.

Staining mechanism

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell envelope), which are stained purple by crystal violet, whereas Gram-negative bacteria have a thinner layer (10% of cell envelope), which are stained pink by the counter-stain. There are four basic steps of the Gram stain:

- Applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture. Heat fixing kills some bacteria but is mostly used to affix the bacteria to the slide so that they don't rinse out during the staining procedure.
- The addition of iodine, which binds to crystal violet and traps it in the cell,
- Rapid decolorization with alcohol or acetone, and

- *Counter staining* with safranin. Carbol fuchsin is sometimes substituted for safranin since it will more intensely stain anaerobic bacteria but it is much less commonly employed as a counterstain.

Crystal violet (CV) dissociates in aqueous solutions into CV^+ and chloride (Cl^-) ions. These ions penetrate through the cell wall and cell membrane of both Gram-positive and Gram-negative cells. The CV^+ ion interacts with negatively charged components of bacterial cells and stains the cells purple. Iodine(I^- or I^{-3}) interacts with CV^+ and forms large complexes of crystal violet and iodine (CV–I) within the inner and outer layers of the cell. Iodine is often referred to as a mordant, but is trapping agents that prevents the removal of the CV–I complex and, therefore, color the cell.

When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of the cell membrane. A Gram-negative cell will lose its outer lipopolysaccharide membrane, and the inner peptidoglycan layer is left exposed. The CV–I complexes are washed from the Gram-negative cell along with the outer membrane. In contrast, a Gram-positive cell becomes dehydrated from an ethanol treatment. The large CV–I complexes become trapped within the Gram-positive cell due to the multilayered nature of its peptidoglycan. The decolorization step is critical and must be timed correctly; the crystal violet stain will be removed from both Gram-positive and negative cells if the decolorizing agent is left on too long (a matter of seconds).

After decolorization, the Gram-positive cell remains purple and the Gram-negative cell loses its purple color. Counterstain, which is usually positively charged safranin or basic fuchsin, is applied last to give decolorized Gram-negative bacteria a pink or red color.

Some bacteria, after staining with the Gram stain, yield a Gram-variable pattern: a mix of pink and purple cells is seen.

The genera *Actinomyces*, *Arthobacter*, *Corynebacterium*, *Mycobacterium*, and *Propionibacterium* have cell walls particularly sensitive to breakage during cell division, resulting in Gram-negative staining of these Gram-positive cells. In cultures of *Bacillus*, *Butyrivibrio*, and *Clostridium*, a decrease in peptidoglycan

thickness during growth coincides with an increase in the number of cells that stain Gram-negative.[14] In addition, in all bacteria stained using the Gram stain, the age of the culture may influence the results of the stain.

Gram-positive bacteria

Gram-positive bacteria have generally a single membrane (*monoderm*) surrounded by a thick peptidoglycan. This rule is followed by two phyla - *Firmicutes* (except for the classes Mollicutes and Negativicutes) and the *Actinobacteria*. In contrast, members of the Chloroflexi (green non- sulfur bacteria) are monoderms but possess a thin or absent (class Dehalococcoidetes) peptidoglycan and can stain negative, positive or indeterminate. Members of the Deinococcus-Thermus group, stain positive but are diderms with a thick peptidoglycan.

The Gram-positive forms made up the phylum Firmicutes, a name now used for the large group. It includes many well-known genera such as *Bacillus*, *Listeria*, *Staphylococcus*,*Streptococcus*, *Enterococcus*,and *Clostridium*. It has also been expanded to include the Mollicutes, bacteria like *Mycoplasma* that lack cell walls and so cannot be stained by Gram, but are derived from such forms. Normally, if Gram's stain is done on acid-fast bacteria they will show up as if they are Gram-positive, mostly because of their thick cell wall.

Gram-negative bacteria

Gram-negative bacteria generally possess a thin layer of peptidoglycan between two membranes (*diderms*). Most bacterial phyla are Gram-negative, including the cyanobacteria, spirochaetes and greensulfur bacteria, and most Proteobacteria (exceptions being some members of the *Rickettsiales* and the insect-endosymbionts of the *Enterobacteriales*).

Gram-indeterminate bacteria

Gram-indeterminate bacteria do not respond to Gram staining and, therefore, cannot be determined as either Gram-positive or Gram-negative. Examples include Gram-variable and acid fast bacteria.

1.4.2 Acid fast

Acid-fastness is a physical property of certain bacteria (and, less commonly, protozoa), specifically their resistance to decolorization by acids during staining procedures.

Acid-fast organisms are difficult to characterize using standard microbiological techniques (e.g. Gram stain - if you gram stained an acid-fast bacillus (AFB) the result would be an abnormal gram positive organism, which would indicate further testing), though they can be stained using concentrated dyes, particularly when the staining process is combined with heat. Once stained, these organisms resist the dilute acid and/or ethanol-based de-colorization procedures common in many staining protocols—hence the name acid-fast.

The high mycolic acid content of certain bacterial cell walls, like those of *Mycobacteria*, is responsible for the staining pattern of poor absorption followed by high retention. The most common staining technique used to identify acid-fast bacteria is the Ziehl-Neelsen stain, in which the acid fast bacilli are stained bright red and stand out clearly against a blue background.

Another method is theKinyoun method, in which the bacteria are stained bright red and stand out clearly against a green background. Acid-fast bacteria can also be visualized by fluorescence microscopy using specific fluorescent dyes (auramine-rhodamine stain, for example).[3] Some bacteria may also be partially acid-fast. The eggs of the parasitic lung fluke *Paragonimus westermani* are actually destroyed by the stain, which can hinder diagnosis in patients who present with TB-like symptoms.

Ziehl–Neelsen stain

The Ziehl–Neelsen stain, also known as the acid-fast stain, was first described by two German doctors: the bacteriologist Franz Ziehl (1859–1926) and the pathologist Friedrich Neelsen (1854–1898).

It is a special bacteriological stain used to identify acid-fast organisms, mainly Mycobacteria.*Mycobacterium tuberculosis* is the most important of this group because it is responsible for tuberculosis (TB). Other important Mycobacterium species involved in human disease are *Mycobacterium*

leprae, *Mycobacterium kansasii*, *Mycobacterium marinum*, and members of the *Mycobacterium avium*complex. Acid fast organisms like Mycobacterium contain large amounts of lipid substances within their cell walls called mycolic acids. These acids resist staining by ordinary methods such as a Gram stain. It can also be used to stain a few other bacteria, such as *Nocardia*. The reagents used are Ziehl–Neelsen carbolfuchsin, acid alcohol, and methylene blue. Acid-fast bacilli will be bright red after staining.

A variation on this staining method is used in mycology to differentially stain acid-fast incrustations in the cuticular hyphae of certain species of fungi in the genus *Russula*. It is also useful in the identification of some protozoa, namely Cryptosporidium and Isospora. The Ziehl–Neelsen stain can also hinder diagnosis in the case of paragonimiasis because the eggs in an ovum and parasite sputum sample (OnP) can be dissolved by the stain, and is often used in this clinical setting because signs and symptoms of paragonimiasis closely resemble those of TB.

Procedure

1. Drop suspension onto slide
2. Air dry slide 10 minutes at 60 °C, heat-fix slide 10 minutes at 90 °C
3. Flood slide with Carbol Fuchsin
4. Hold a flame beneath the slide until steam appears but do not allow it to boil
5. Allow hot slide to sit for 3 to 5 minutes, rinse with tap water
6. Flood slide with 3% hydrochloric acid in isopropyl alcohol
7. Allow to sit 1 minute, rinse with tap water
8. Flood slide with Methylene Blue
9. Allow to sit 1 minute, rinse with tap water
10. Blot dry
11. View under oil immersion lens

Studies have shown that an AFB stain without a culture has a poor negative predictive value. An AFB Culture should be performed along with an AFB stain; this has a much higher negative predictive value.

1.4.2 Endospore staining

Endospore staining is a technique used in bacteriology to identify the presence of endospores in a bacterial sample, which can be useful for classifying bacteria. Within bacteria, endospores are quite protective structures used to survive extreme conditions, but this protective nature makes them difficult to stain using normal techniques. Special techniques for endospore staining include the Schaeffer–Fulton stain and the Moeller stain.

Schaeffer- Fultan stain: The Schaeffer–Fulton stain is a technique designed to isolate endospores by staining any present endospores green, and any other bacterial bodies red. The green stain is malachite green, and the counterstain is safranin, which dyes any other bacterial bodies red.

Procedure

Using an aseptic technique, bacteria are placed on a slide and heat fixed. The slide is then suspended over a water bath with some sort of porus paper over it, so that the slide is steamed. Malachite green is applied to the slide, which can penetrate the tough walls of the endospores, staining them green. After five minutes, the slide is removed from the steam, and the paper towel is removed. After cooling, the slide is rinsed with water for thirty seconds. The slide is then stained with diluted safranin for two minutes, which stains most other microorganic bodies red or pink. The slide is then rinsed again, and blotted dry with bibulous paper. After drying, the slide can then be viewed under a light microscope.

Moeller stain

Endospores are surrounded by a highly resistant spore coat. The spore coat is highly resistant to excessive heat, freezing and desiccation as well as chemical agents. More importantly, for identification, spores are resistant to commonly employed staining techniques; therefore alternative staining methods are required. Moeller staining involves the use of a steamed dye reagent in order to increase the stainability of the spores; Carbol fuchsin is the primary stain used in this method. Endospores are stained red, while the counterstain, Methylene blue stains the vegetative bacteria blue.

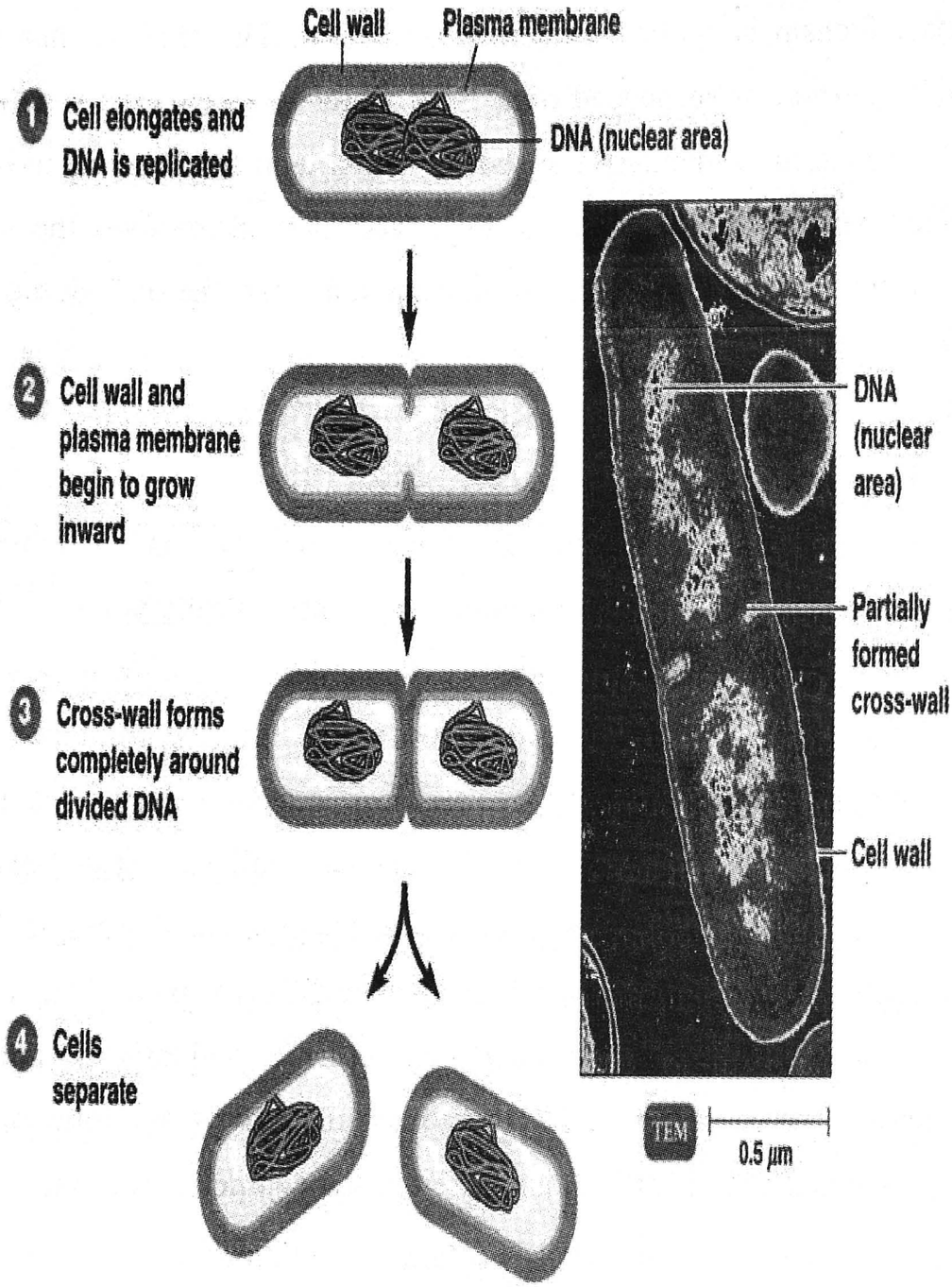
Method

Carbol fuchsin is applied to a heat-fixed slide. The slide is then heated over a bunsen burner, or suspended over a hot water bath, covered with a paper towel, and steamed for 3 minutes. The slide is rinsed with acidified ethanol, and counter-stained with Methylene blue. An improved method involves the addition of the surfactant Tergitol 7 to the carbol fuchsin stain, and the omission of the steaming step.

1.5 Fission

Binary fission of prokaryotes

Prokaryotic fission, which is binary fission, is a form of asexual reproduction and cell division used by all prokaryotes, (bacteria and archaebacteria), and some organelles within eukaryotic organisms (e.g.,mitochondria). This process results in the reproduction of a living prokaryotic cell (or organelle) by division into two parts that each have the potential to grow to the size of the original cell (or organelle). This type of division takes place without the formation of spindles. The single DNA molecule first replicates then attaches each copy to a different part of the cell membrane. When the cell begins to pull apart, the replicate and original chromosomes are separated. The consequence of this asexual method of reproduction is that all the cells are genetically identical, i.e. have the same genetic material.



(a) A diagram of the sequence of cell division.

(b) A thin section of a cell of *Bacillus licheniformis* starting to divide.

Figure-6

1.6 GROWTH CURVE

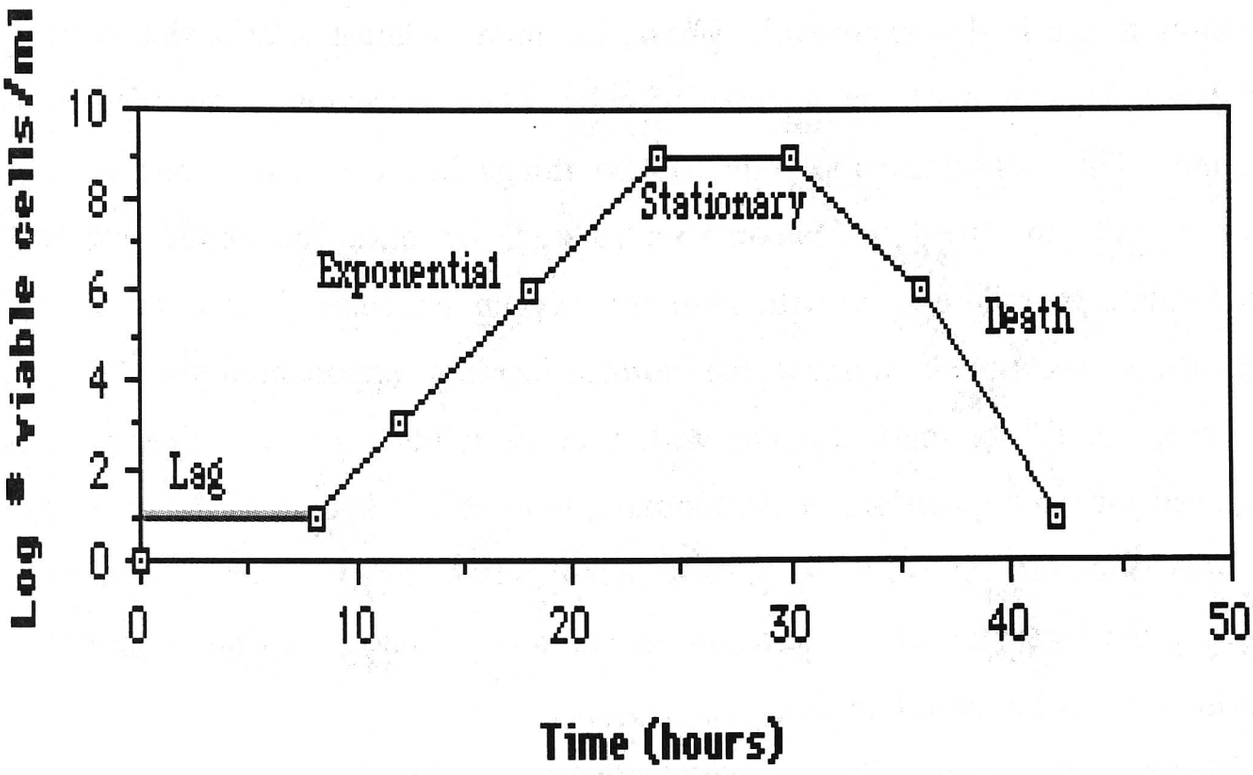


Figure-7

The growth curve indicates multiplication and death of bacteria. When a bacterium is inoculated in a medium, it passes through four growth phases which will be evident in a growth curve drawn by plotting the logarithm of the number of bacteria against time. Number of bacteria in the culture at different periods may be : (1) Total count. It includes both living and dead bacteria, and (2) Viable count. It includes only the living bacteria. Microbial concentration can be measured in terms of cell concentration, i.e. the number of viable cells per unit volume of culture, or of biomass concentration, i.e. dry weight of cells per unit volume of culture.

Growth Phases

1. **Lag Phase.** In this phase there is increase in cell size but not multiplication. Time is required for adaptation (synthesis of new enzymes) to new environment. During this phase vigorous metabolic activity occurs but cells do not divide. Enzymes and intermediates are formed and accumulate until they are present in concentration that permits growth to start. Antibiotics have little effect at this stage.

2. Exponential Phase or Logarithmic (Log) Phase. The cells multiply at the maximum rate in this exponential phase, i.e. there is linear relationship between time and logarithm of the number of cells. Mass increases in an exponential manner. This continues until one of two things happens: either one or more nutrients in the medium become exhausted, or toxic metabolic products, accumulate and inhibit growth. Nutrient oxygen becomes limited for aerobic organisms. In exponential phase, the biomass increases exponentially with respect to time, i.e. the biomass doubles with each doubling time. The average time required for the population, or the biomass, to double is known as the generation time or doubling time. Linear plots of exponential growth can be produced by plotting the logarithm of biomass concentration as a function of time. Importance: Antibiotics act better at this phase.

3. Maximal Stationary Phase. Due to exhaustion of nutrients or accumulation of toxic products death of bacteria starts and the growth cease completely. The count remains stationary due to balance between multiplication and death rate. Importance: Production of exotoxins, antibiotics, metachromatic granules, and spore formation takes place in this phase.

4. Decline phase or death phase. In this phase there is progressive death of cells. However, some living bacteria use the breakdown products of dead bacteria as nutrient and remain as persister.

1.6.1 Bacterial growth: Batch vs. Continuous

In batch cultivation, the bacteria are inoculated into the bioreactor (always stirred tank bioreactor). Then, under certain conditions (temperature, pH, aeration, etc.) the bacteria go through all the growth phases (lag, exponential, stationary). At last, the fermentation is stopped and the product is collected. Then, after cleaning and sterilization of the fermenter, The fermenter is ready for another batch.In continuous cultivation, the fresh medium flows into the fermentor continuously, and part of the medium in the reactor is withdrawn from the fermenter at the same flow rate of the inlet flow. The table below shows the advantages and disadvantages of different modes of operation of the stirred tank rector.

Mode of operation	Advantages	Disadvantages
Batch	Versatile: can be used for different reactions every day. Safe: can be properly sterilized. Little risk of infection or strain mutation Complete conversion of substrate is possible	High labor cost: skilled labor is required Sterilization, growth of inoculum, cleaning after the fermentation Safety problems: when filling, emptying, cleaning
Continuous	Works all the time: low labor cost, good utilization of reactor Often efficient: due to the autocatalytic nature of microbial reactions, the productivity can be high. Automation may be very appealing Constant product quality	Often disappointing: promised continuous production for months fails due to an. infection. ,spontaneous mutation of microorganisms to non producing strain Inflexible: can rarely be used for other productions without substantial retrofitting

From the above comparison, although continuous culture has some disadvantage, it can outperform batch culture by eliminating the inherent down time for cleaning and sterilization and the long lags before the organisms enter a brief period of high productivity. Continuous culture is superior to batch culture in several ways for research.

1.7 Factors affecting growth

A. Environmental Factors affecting Growth

1. Nutrients: Nutrients in growth media must contain all the elements necessary for the synthesis of new organisms. In general the following must be provided : (a) Hydrogen donors and acceptors, (b) Carbon source, (c) Nitrogen source, (d)

Minerals : sulphur and phosphorus, (e) Growth factors: amino acids, purines, pyrimidines; vitamins, (f) Trace elements: Mg,Fe,Mn.

Growth Factors:

A growth factor is an organic compound which a cell must contain in order to grow but which it is unable to synthesize. These substances are essential for the organism and are to be supplied as nutrients. Thiamine, nicotinic acid, folic acid and para-aminobenzoic acid are examples of growth factors.

Essential Metabolites:

These metabolites are essential for growth of bacterium. These must be synthesized by the bacterium, or be provided in the medium. Mg, Fe and Mn are essential trace elements.

Autotrophs live only on inorganic substances, i.e. do not require organic nutrients for growth. They are not of medical importance. Heterotrophs require organic materials for growth, e.g. proteins, carbohydrates, lipids as source of energy. All bacteria of medical importance belong to heterotrophs. Parasites may depend on the host for certain foods. Saprophytes grow, on dead organic matter.

2. pH of the medium: Most pathogenic bacteria grow best in pH 7.2-7.4. *Vibno cholerae* can grow in pH 8.2-9.0. 3.

3.GaseousRequirement

(a) Role of Oxygen. Bacteria may be classified into four groups on oxygen requirement:

(i) Aerobes. They cannot grow without oxygen, e.g. Mycobacterium tuberculosis.

(ii) Facultative anaerobes. These grow under both aerobic and anaerobic conditions. Most bacteria are facultative anaerobes,

e.g.Entero bacteriaceae.

(iii) Anaerobes. They only grow in absence of free oxygen,
e.g.Clostridium,Bacteroides.

(iv) Microaerophiles grow best in oxygen less than that present in the air,
e.g.Campylobacter.

Aerobes and facultative anaerobes have the metabolic systems:

(1) cytochrome systems for the metabolism of oxygen

(2) Superoxide dismutase,

(3)catalase.

Anaerobic bacteria do not grow in the presence of oxygen. They do not use oxygen for growth and metabolism but obtain their energy from fermentation reactions. Anaerobic bacteria are killed by oxygen or toxic oxygen radicals.

Multiple mechanisms play role for oxygen toxicity : (1) They do not have cytochrome systems for oxygen metabolism, (2) They may have low levels of superoxide dismutase, and (3) They may or may not have catalase.

4. Carbon dioxide: All bacteria require CO_2 for their growth. Most bacteria produce CO_2. *N. gonorrhoeae* and *N. meningitides* and *Br abortus* grow better in presence of 5 per cent CO_2.

5. Temperature. Most bacteria are mesophilic. Mesophilic bacteria grow best at 30-37°C. Optimum temperature for growth of common pathogenic bacteria is 37°C. Bacteria of a species will not grow but may remain alive at a maximum and a minimum temperature.

Organisms can be classified according to their optimum growth temperature:

- Psychrophiles- grow best between -5 and 20°C
- Mesophiles- grow best between 20°C and -45°C
- Thermophiles- grow best at temperatures above 45°C
- Thermoduric- organisms can survive high temperatures.
  Organisms which form endospores are thermoduric.

1.8 Determination of Bacterial growth

Microbial growth to determine 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.

1.8.1 Direct method

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).

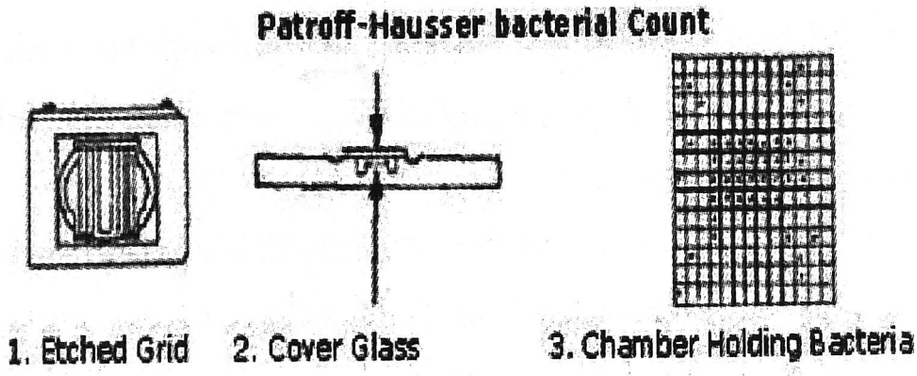


Figure-8

1. Etched Grid	2. Cover Glass	3. Chamber Holding Bacteria

The direct microscopic method is easy, inexpensive and relatively quick to count microbial cell number. However, using these method dead cells is not distinguished from living cells and also very small cells are usually missed.

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 f9rming 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). This results in a dilution of 1: 100 or $1 \times 10\text{-}^{2}$. 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 he dilution and of the volume used.

1.8.2 Indirect method

Measurement of Turbidity (Turbidometry)

Rapid cell mass determination is possible using turbidometry method. Turbidometry is based on the fact that microbial cells scatter light striking them. Since the microbial cells in a population are of roughly constant size, the amount of scattering is directly proportional to the biomass of cells present and indirectly related to cell number. One visible characteristic of growing bacterial culture is the increase in cloudiness of the medium (turbidity). When the concentration of bacteria reaches about 10 million cells (10^{7}) per ml, the medium appears slightly cloudy or turbid. Further increase in concentration results in greater turbidity. When a beam of light is passed through a turbid culture, the amount of light transmitted is measured, Greater the turbidity, lesser would be the transmission of light through medium. Thus, light will be transmitted in inverse proportion to the number of bacteria. Turbidity can be measured using instruments like spectrophotometer.

1.9 Points for Discussion

1. Are bacteria the only prokaryotic beings?
2. What are the main ecological roles of bacteria?

1.10 Check your progress

1. How does the cell's surface area/volume ratio compare with that of large organisms?
2. Compare the structure and chemistry of cell walls of gram positive and gram negative bacteria.
3. How are staining techniques classified?
4. What conditions promote bacterial growth?
5. What is the difference between batch and continuous culture?

UNIT 2 VIROLOGY

Structure

2.1 INTRODUCTION

A virus is a small infectious agent that can replicate only inside the living cells of organisms. Most viruses are too small to be seen directly with a light microscope. Viruses infect all types of organisms, from animals and plants to bacteria and archaea. Virus particles (known as virions) consist of two or three parts: the genetic material made from either DNA or RNA, long molecules that carry genetic information; a protein coat that protects these genes; and in some cases 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.

2.2 OBJECTIVES

After going through this unit, you will be able to

1. Identify the shape and know the structure of viruses
2. Understand the difference between an enveloped and unenveloped virus
3. Learn the multiplication and life cycle pattern of viruses

Definition:

Viruses are the most primitive cellular and non-cytoplasmic infectious agents. Russian botanist D.J. Iwanowski (1892) first discovered virus in an infected tobacco plant. However, M.W. Beijerink (1898) coined the term virus. Then American chemist W.M. Stanley (1935) isolated pure crystal of Tobacco Mosaic Viruses (TMV) and concluded that viruses are made of nucleoproteins.

2.3 General Characteristics of Viruses:

(i) Viruses are acellular, non-cytoplasmic infectious agents.

(ii) They are smaller than bacteria, and this can pass through bacteriological filter.

(iii) Viruses are transmissible from disease to healthy organisms.

(iv) All viruses are obligate parasites and can multiply only within the living host cells.

(v) Viruses contain only a single type of nucleic acid either DNA or RNA.

(vi) Viruses are host specific that they infect only a single species and definite cells of the host organisms.

(vii) Viruses are effective in very small doses. They are highly resistant to germicides and extremes of physical conditions.

2.4 Classification of Viruses

Viruses are not usually classified into conventional taxonomic groups but are usually grouped according to such properties as size, the type of nucleic acid they contain, the structure of the capsid and the number of protein subunits in it, host species, and immunological characteristics. It also means that when a new species of known virus family or genus is investigated it can be done in the context of the information that is available for other members of that group. Without a classification scheme each newly discovered virus would be like a black box, everything would have to be discovered and rediscovered. The development of a classification scheme is therefore an important and inevitable consequence. The

current classification scheme allows most newly described viruses to be labeled. In the best cases much can be assumed about the biology of the virus. Even in the worse case a framework for investigation would be suggested. Because there are so few virus discoveries now being made which do not fit into the existing classification scheme we can state with a degree of confidence that most of the major groupings of viruses infecting humans and domesticated animals have been identified.

How are viruses classified?

Two classification systems exist: The Hierarchical virus classification system and the Baltimore Classification System.

2.4.1 The Hierarchical virus classification system

In 1962 Lwoff, R. W. Horne, and P. Tournier advanced a comprehensive scheme for the classification of all viruses consisting of phylum - class - order - family - subfamily - genus - species - strain/type. The subsequently formed internat .commitee on the nomenclature of viruses accepted many principles of this system. The most important principle embodied in this system was that viruses should be grouped according to their shared properties rather than the protperties of the cells or organisms

http://www.nlv.ch/Virologytutorials/graphics/classificationtotal.jpgtheyinfect.

Four main characteristics are used:

Primary characteristics

1. Nature of the nucleic acid: RNA or DNA
2. Symmetry of the capsid
3. Presence or absence of an envelope
4. Dimensions of the virion and capsid

Secondary characteristics

1.Host range

2.Mode of transmission

3.Specific surface structures

At the moment classification is really only important from the level of families down. Members within a virus family are ordered with Genomics, the elucidation of evolutionary relationships be analyses of nucleic acid and protein sequence similarities. All Families have the suffix -viridaee.g. Caliciviridae, Picornaviridae, Reoviridae. Genera have the suffix -virus. Within the Picornaviridae there are 5 genera: enterovirus, cardiovirus, rhinovirus, apthovirus and hepatovirus The definition of `**species**' is the most important but difficult assignment to make with viruses.

2.4.2 The Baltimore Classification

The Baltimore system of virus classification provides a useful guide with regard to the various mechanisms of viral genome replication. The central theme here is that all viruses must generate positive strand mRNAs from their genomes, in order to produce proteins and replicate themselves. The precise mechanisms whereby this is achieved differ for each virus family. These various types of virus genomes can be broken down into seven fundamentally different groups, which obviously require different basic strategies for their replication. **David Baltimore**, who originated the scheme, has given his name to the so-called "Baltimore Classification" of virus genomes. By convention the top strand of coding DNA written in the 5' - 3' direction is + sense. mRNA sequence is also + sense. The replication strategy of the virus depends on the nature of its genome. Viruses can be classified into seven (arbitrary) groups:

I: Double-stranded DNA (Adenoviruses; Herpesviruses; Poxviruses, etc) Some replicate in the nucleus e.g. adenoviruses using cellular proteins. Poxviruses replicate in the cytoplasm and make their own enzymes for nucleic acid replication.

II. Single-stranded (+) sense DNA (Parvoviruses) Replication occurs in the nucleus, involving the formation of a (-) sense strand, which serves as a template for (+)strand RNA and DNA synthesis.

III: Double-stranded RNA (Reoviruses; Birnaviruses) These viruses have segmented genomes. Each genome segment is transcribed separately to produce monocistronic mRNAs.

IV: Single-stranded (+)sense RNA (Picornaviruses; Togaviruses, etc)

a) Polycistronic mRNA e.g. Picornaviruses; Hepatitis A. Genome RNA = mRNA. Means naked RNA is infectious, no virion particle associated polymerase. Translation results in the formation of a polyprotein product, which is subsequently cleaved to form the mature proteins.

b) Complex Transcription e.g. Togaviruses. Two or more rounds of translation are necessary to produce the genomic RNA.

V: Single-stranded (-)sense RNA (Orthomyxoviruses, Rhabdoviruses, etc) Must have a virion particle RNA directed RNA polymerase.

a) Segmented e.g. Orthomyxoviruses. First step in replication is transcription of the (-)sense RNA genome by the virion RNA-dependent RNA polymerase to produce monocistronic mRNAs, which also serve as the template for genome replication.

b) Non-segmented e.g. Rhabdoviruses. Replication occurs as above and monocistronic mRNAs are produced.

VI: Single-stranded (+)sense RNA with DNA intermediate in life-cycle(Retroviruses)

Genome is (+)sense but unique among viruses in that it is DIPLOID, and does not serve as mRNA, but as a template for reverse transcription.

VII: Double-stranded DNA with RNA intermediate (Hepadnaviruses) This group of viruses also relies on reverse transcription, but unlike the Retroviruses, this occurs inside the virus particle on maturation. On infection of a new cell, the first event to occur is repair of the gapped genome, followed by transcription.

2.5 STRUCTURE OF VIRUSES

(i) **Shape and size**:

The shape varies considerable. They may be spherical or golf ball-like, rod-shaped, tadpole-like, helical or polyhedral. Plant viruses are smaller than bacteria. Viruses have a very simple structure. The core of the viruses is made upon of nucleic acid, which is surrounded by a protein coat called capsid. The nucleic acid

always contains only a single kind of nucleic acid i.e. either DNA or RNA. The infectious property of a virus is due to its nucleic acid.

Capsid or the protein coats:

It is made up of many identical protein sub-units called capsomeres. The capsomeres are composed of either one or several type of proteins. Capsomeres are arranged in a very symmetrical manner and give a specific shape to a particular virus. The host specificity of virus is due to proteins of the capsid.

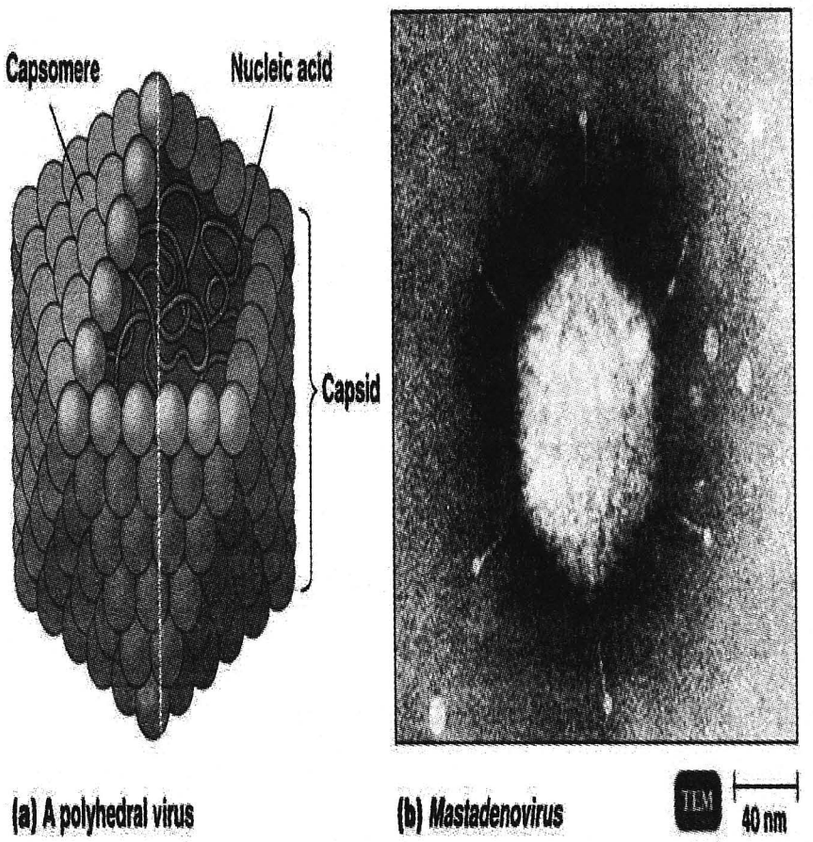


Figure-9

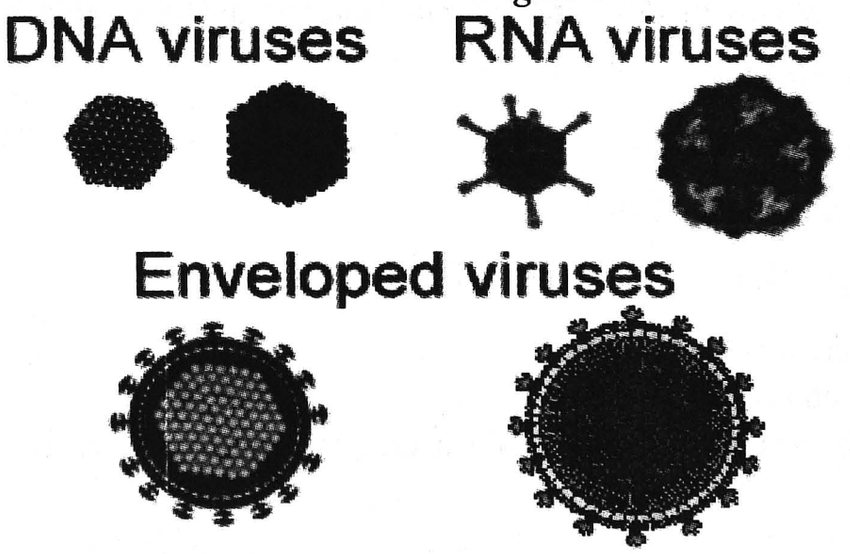


Figure-10

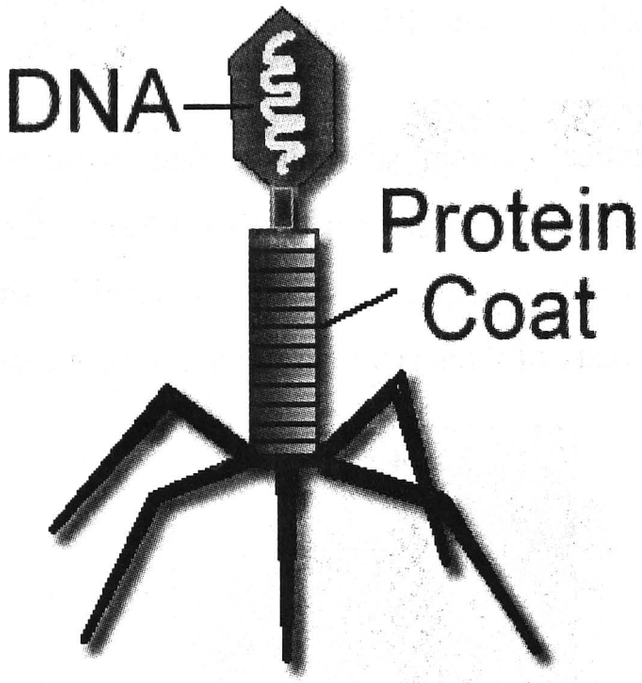


Figure-11

Biological position of viruses:

Viruses lack a cytoplasmic membrane and they do not have the basic component of a cell. They can only replicate inside the host cell. Outside the host cell, they are non-living. Thus, viruses show characters of both living and non-living.

(I) Non-living Characters of Viruses:

Following characters of viruses assign them as non-living:

(a) They can be crystallized.

(b) Outside the cell, they behave like inert chemicals.

(c) They do not show growth, development, nutrition, reproduction, etc.

(d) They can be precipitated.

(II) Living characters of viruses:

(a) They multiply within host cells.

(b) They possess genetic material, either DNA or RNA.

(c) There are definite races or strains.

(d) They exhibit mutations.

Because of the above reasons, viruses form unique bridge between living and non-living things.

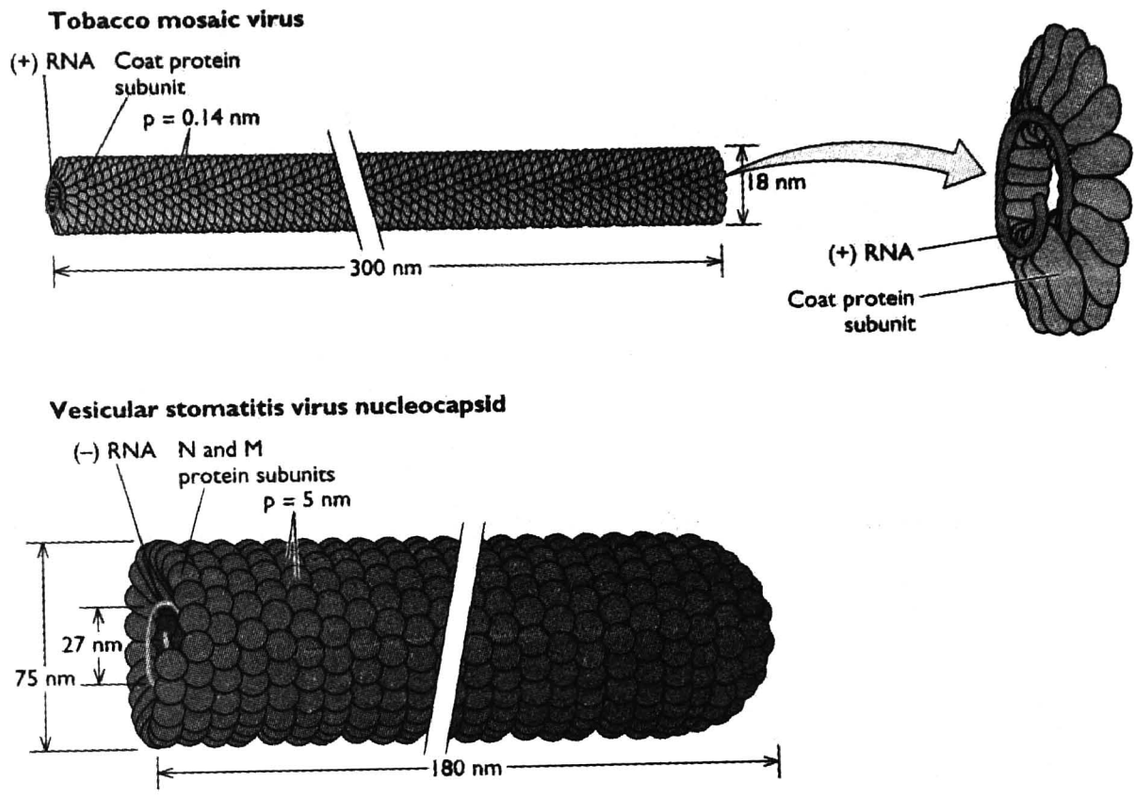


Figure-12

Morphology (Symmetry)

1. **Icosahedral** -The protomeres aggregate in groups of five or six to form the capsomere. In electron micrographs, capsomeres are recognized as regularly spaced rings with a central hole. The shape and dimensions of the icosahedron depends on characteristics of its protomeres. All icosahedral capsids have 12 corners each occupied by a penton capsomere and 20 triangular faces, each containing the same number of hexon capsomeres. Icosahedral symmetry is identical to cubic symmetry.

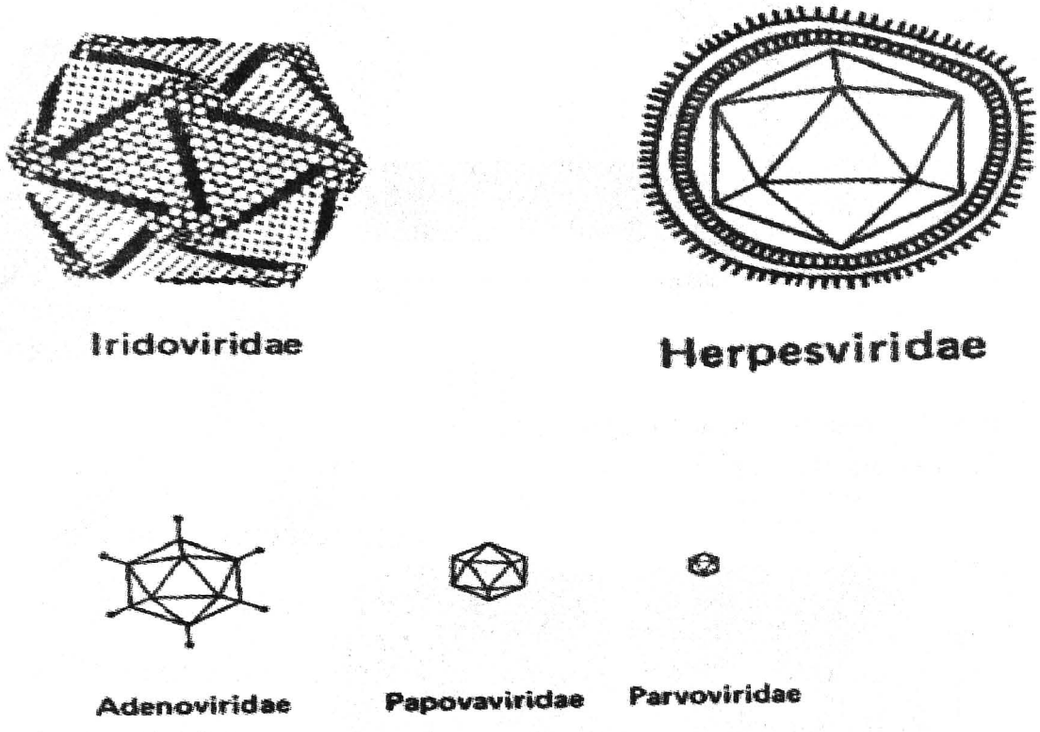


Figure-13

2. **Helical** -The protomeres are not grouped in capsomeres, but are bound to each other so as to form a ribbon-like structure. This structure folds into a helix because the protomeres are thicker at one end than at the other. The diameter of the helical capsid is determined by characteristics of its protomeres, while its length is determined by the length of the nucleic acid it encloses.

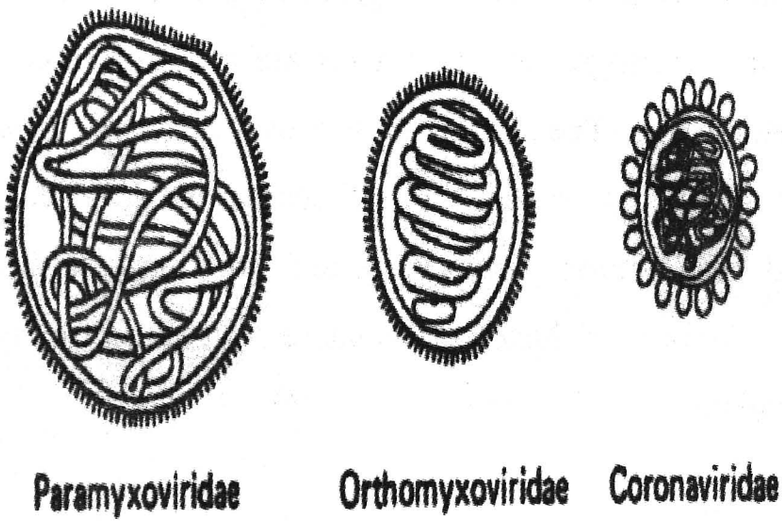


Figure-14

3. **Complex** -e.g., that exhibited by poxvirus and rhabdovirus. This group comprises all those viruses which do not fit into either of the above two groups.

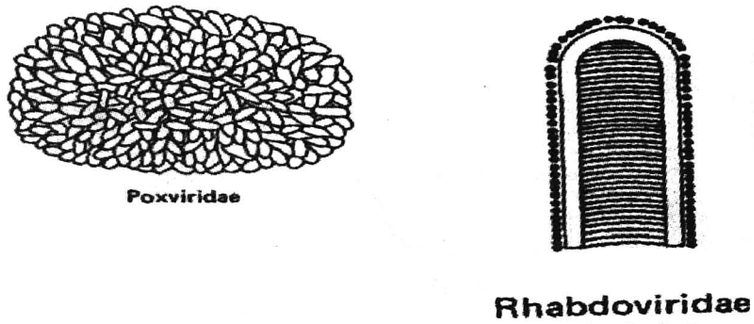


Figure-15

2.6 VIRAL MULTIPLICATION

Viruses do not contain enzymes for energy production or protein synthesis. For a virus to multiply, it must invade a host cell and direct the host's metabolic machinery to produce viral enzymes, viral proteins, and copies of its nucleic acid, using the host cell's ATP to power the reactions.

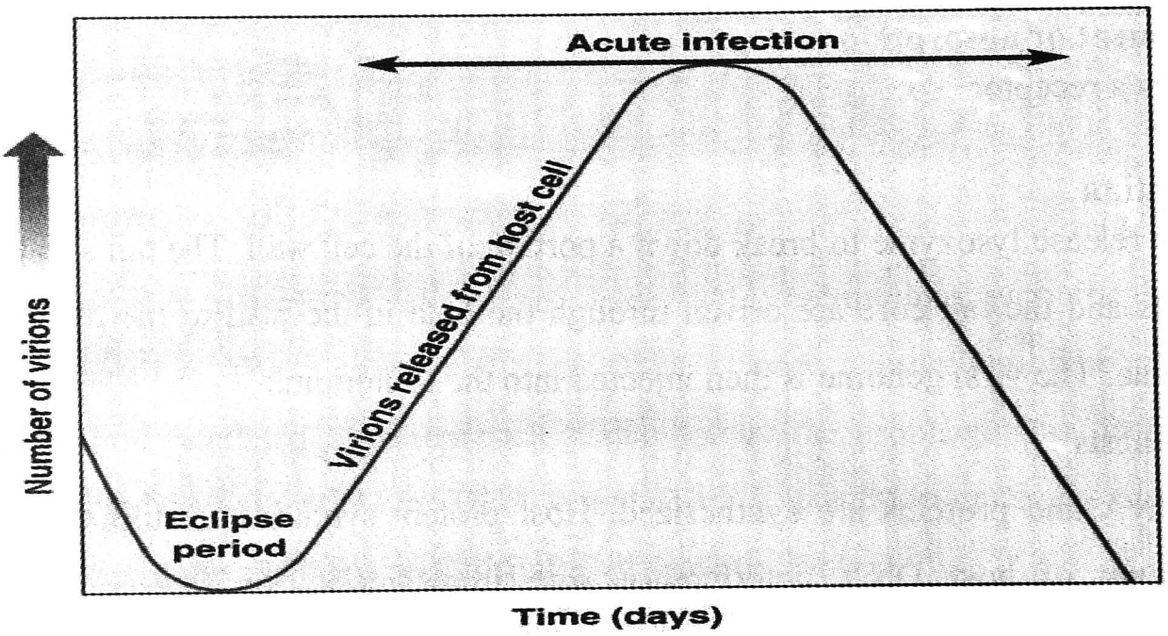


Figure-16

Viral particles disappear upon penetration, none are seen during biosynthesis and assembly, and eventually all cells die so no new virions can be produced.

The **eclipse period** is the period when all viral particles are present but before they are assembled.

Burst time is the time from phage adsorption to release.
Burst size is the number of newly synthesized phages produced from one infected cell.

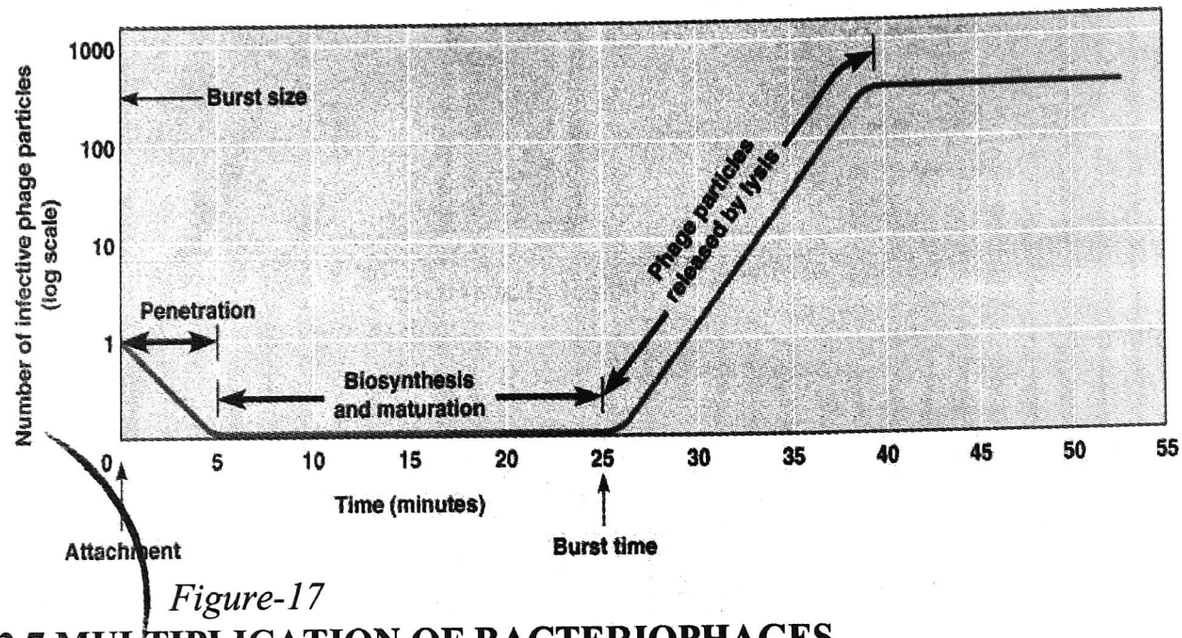


Figure-17

2.7 MULTIPLICATION OF BACTERIOPHAGES

The virus may cause lysis or lysogeny.

Events of the lytic cycle:

Attachment or adsorption

Requires a receptor

Penetration

T-evens release lysozyme to break down a portion of the cell wall. The tail sheath contracts and the tail core are driven through the hole in the wall to the plasma membrane. The viral genome is then injected into the bacterium.

Biosynthesis

Viral DNA and proteins are synthesized. Host protein synthesis is stopped by degradation of host DNA, interference with transcription, or repression of translation.

Maturation

During maturation or assembly phage DNA and capsids are assembled into complete viruses.

Release

Release occurs when phage lysozyme breaks down the cell wall and newly synthesized phage particles are released.

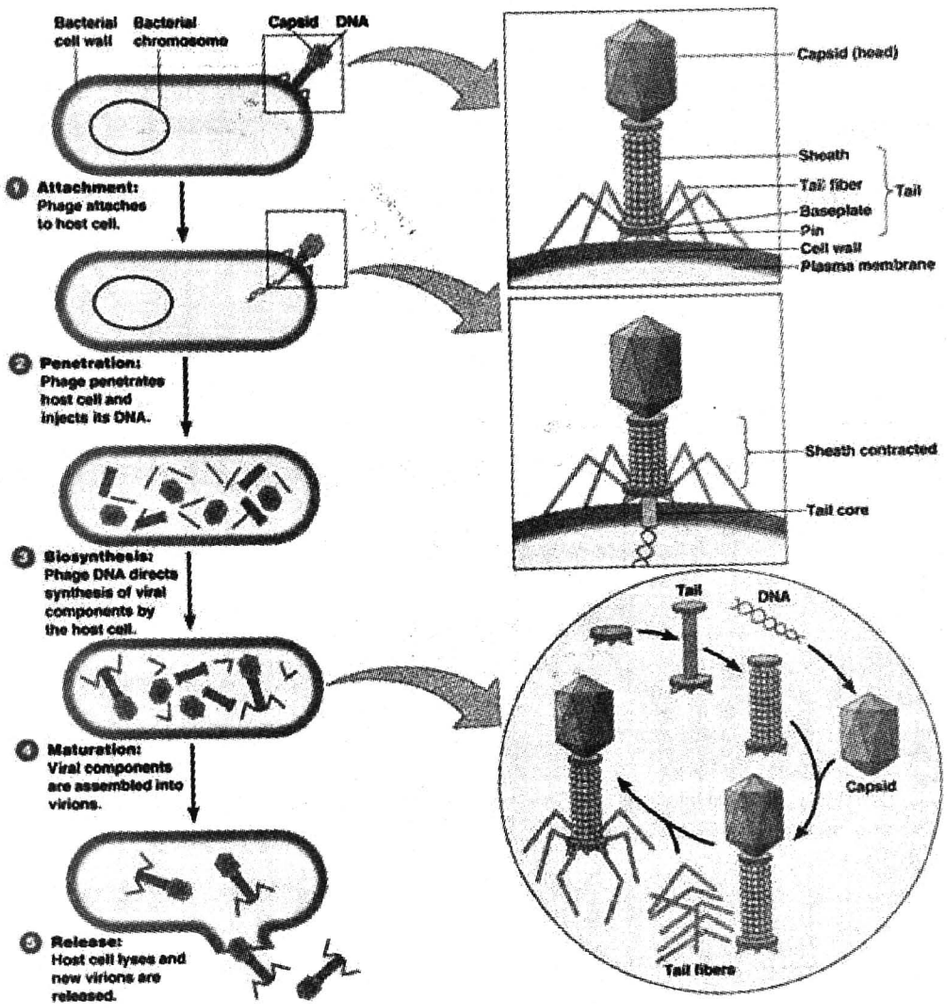


Figure-18

Lysogeny is a cycle in which the phage DNA recombines with the bacterial chromosome. The incorporated viral DNA is now a prophage. The prophage genes are regulated by a repressor coded for by the prophage, the prophage is replicated each time the host DNA is replicated. Exposure to mutagens can lead to excision of the prophage and initiation of the lytic cycle.

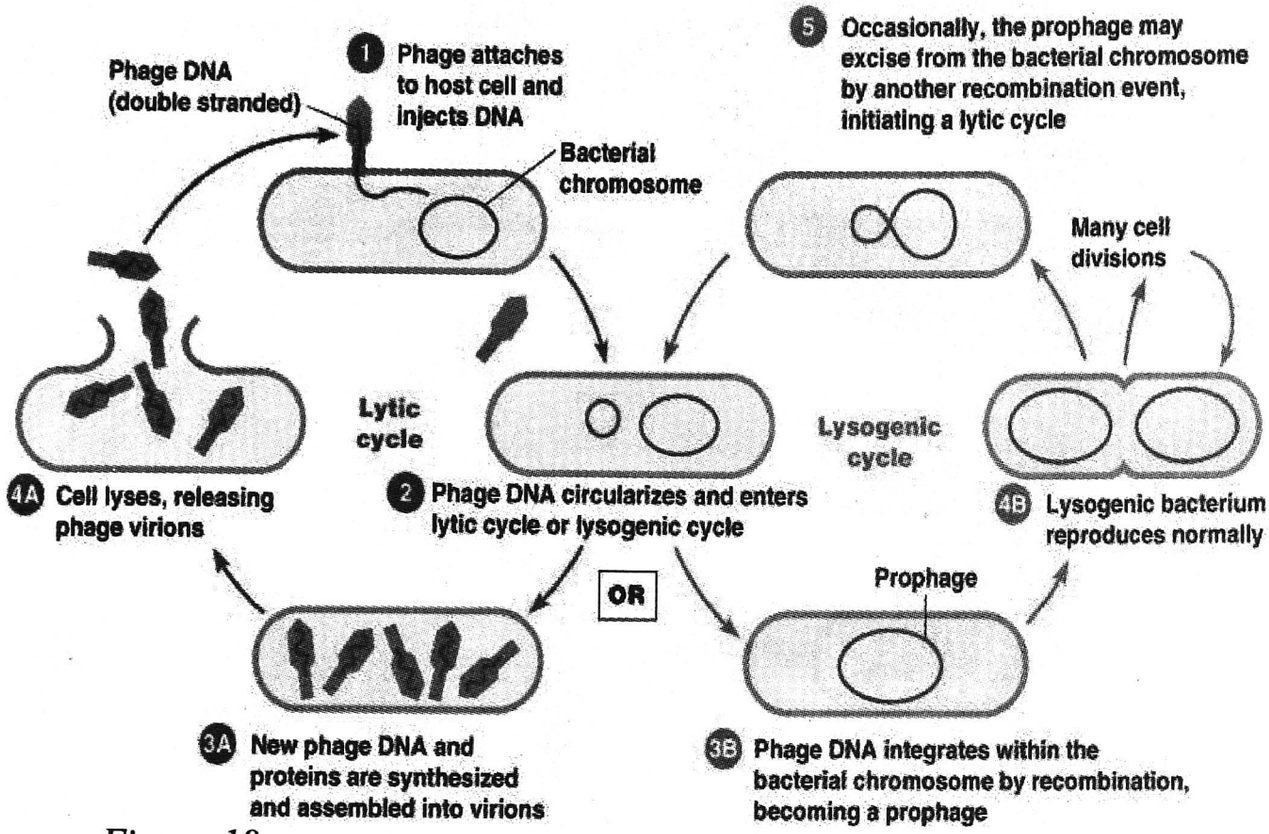
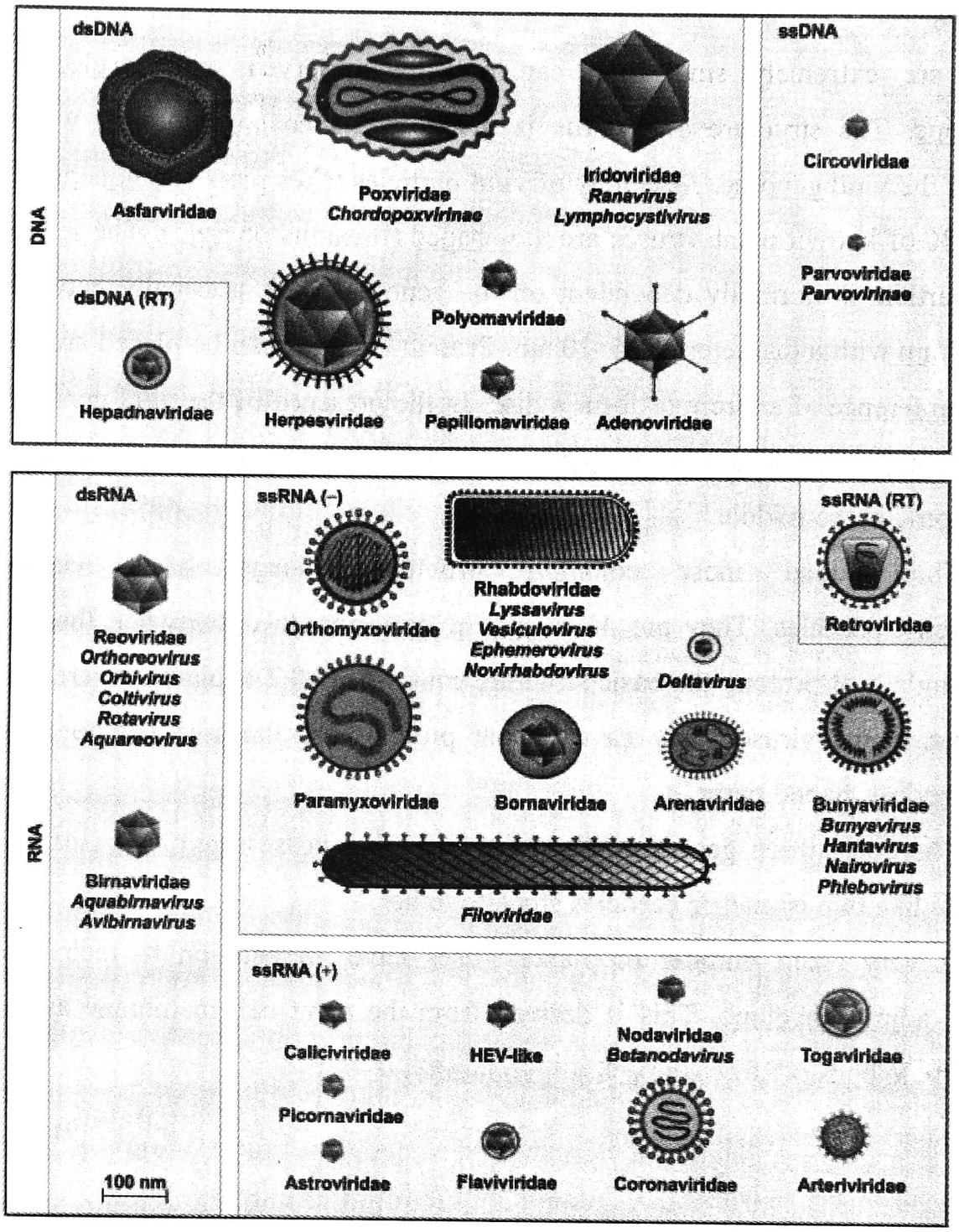


Figure-19

2.8 ANIMAL VIRUSES

At one time or another, we have all most likely been infected with a virus. The common cold and chicken pox are two common ailments caused by animal viruses. Animal viruses are intracellular obligate parasites, meaning that they rely on the host animal cell completely for reproduction. They use the host's cellular components to replicate, then leave the host cell to infect other cells. Viruses gain entry into host cells via several sites such as the skin, gastrointestinal tract, and respiratory tract. Once an infection has occurred, the virus may replicate in host cells at the site of infection or they may also spread to other locations. Animal viruses typically spread throughout the body mainly by way of the bloodstream, but can also be spread via the nervous system. Viruses have several methods to counter host immune system responses. Some viruses, like HIV, destroy immune system cells.



Viruses infecting vertebrates

Figure-20

2.9 PLANT VIRUSES

Plant viruses are viruses that affect plants. Like all other viruses, plant viruses are obligate intracellular parasites that do not have the molecular machinery to replicate without a host. Plant viruses are pathogenic to higher plants.

Structure

Viruses are extremely small and can only be observed with an electron microscope. The structure of a virus is given by its coat of proteins, which surround the viral genome. Assembly of viral particles takes place spontaneously. Over 50% of known plant viruses are rod-shaped (flexuous or rigid). The length of the particle is normally dependent on the genome but it is usually between 300–500 nm with a diameter of 15–20 nm. Protein subunits can be placed around the circumference of a circle to form a disc. In the presence of the viral genome, the discs are stacked, and then a tube is created with room for the nucleic acid genome in the middle.[

The second most common structure amongst plant viruses are isometric particles. They are 40–50 nm in diameter. In cases when there is only a single coat protein, the basic structure consists of 60 T subunits, where T is an integer. Some viruses may have 2 coat proteins are the associate to form an icosahedral shaped particle.

There are three genera of *Geminiviridae* that possess geminate particles which are like two isometric particles stuck together.

A very small number of plant viruses have, in addition to their coat proteins, a lipid envelope. This is derived from the plant cell membrane as the virus particle buds off from the cell.

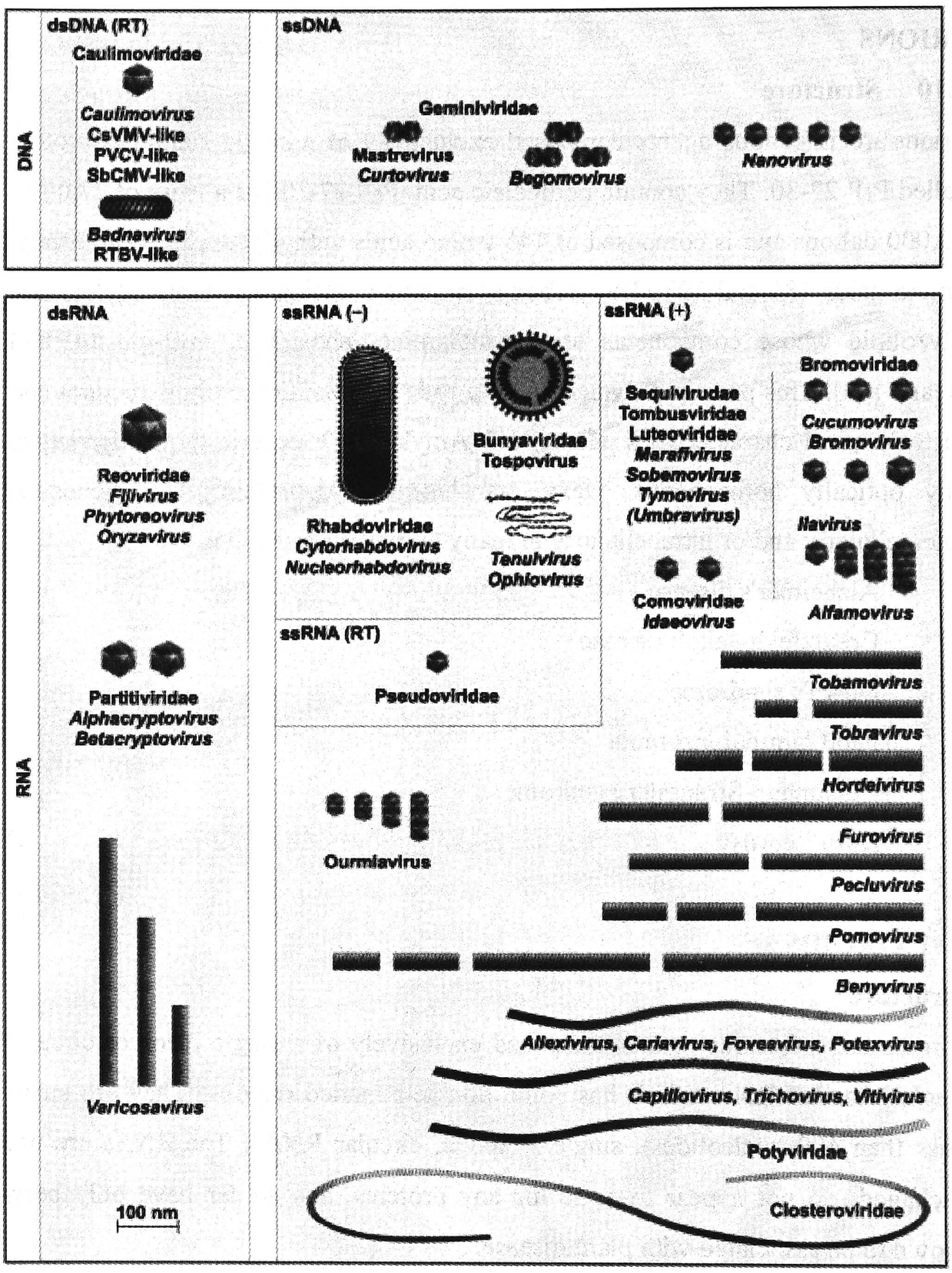


Figure-21 **Viruses infecting plants**

PRIONS

2.10 Structure

Prions are infectious agents composed exclusively of a single sialoglycoprotein called PrP 27-30. They contain no nucleic acid. PrP 27-30 has a mass of 27,000 - 30,000 daltons and is composed of 145 amino acids with glycosylation at or near amino acids 181 and 197. The carboxy terminus contains a phosphatidylinositol glycolipid whose components are ethanolamine, phosphate, myo-inositol and stearic acid. This protein polymerizes into rods possessing the ultrastructural and histochemical characteristics of amyloid. Amyloid is a generic term referring to any optically homogenous, waxy, translucent glycoprotein; it is deposited intercellularly and/or intracellularly in many human diseases such as:

- Alzheimer's disease
- Creutzfeldt-Jakob disease
- Down's syndrome
- Fatal familial insomnia
- Gerstmann-Straussler syndrome
- Kuru Leprosy

2.11 VIROIDS

Structure

Viroids are infectious agents composed exclusively of a single piece of circular single stranded RNA which has some double-stranded regions. They are small (less than 400 nucleotides), single stranded, circular RNAs. The RNAs are not packaged, do not appear to code for any proteins, and so far have only been shown to be associated with plant disease.

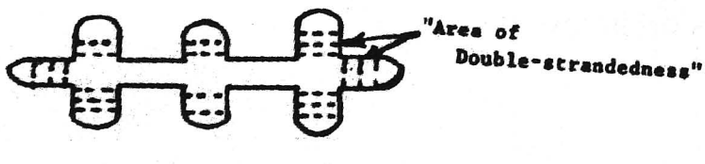


Figure-22

Because of their simplified structures both prions and viroids are sometimes called subviral particles. Viroids mainly cause plant diseases but have recently been reported to cause a human disease.

2.12 Points for Discussion

1. Are viruses' cellular beings?

2. Why is it a strong evolutionary hypothesis that although viruses are the structurally simplest beings they were not the first living beings?

2.13 Check your progress

1. What are viruses and how are they different from living organisms?

2. What is the basic structure of a virus?

3. How do viruses reproduce in an host cell?

4. What are bacteriophages?

5. How are viruses primarily classified?

UNIT 3 CULTIVATION OF MICROBES

Structure

3.1 INTRODUCTION

The survival of microorganisms in the laboratory, as well as in nature, depends on their ability to grow under certain chemical and physical conditions. An understanding of these conditions enables us to characterize isolates and differentiate between different types of bacteria. Such knowledge can also be applied to control the growth of microorganisms in practical situation

3.2 OBJECTIVES

After going through this unit, you will be able to

1. understand the different media and its components employed for isolation of microbes.
2. Identify proper methods and effects of sterilization of common laboratory materials.
3. study the effect of environmental factors on microbial growth.
4. apply basic techniques used for isolation and cultivation of microbes.

3.3 NUTRITIONAL TYPES OF MICROBES

Bacteria exhibit different modes of nutrition. On this basis, broadly two types of bacteria can be recognised autotrophic bacteria andheterotrophic bacteria.

3.3.1 Autotrophic Bacteria

These are bacteria which are able to synthesize their own organic food from inorganic substances. They use carbon dioxide for obtaining carbon and utilise hydrogen sulphide (H_2S) or ammonia (NH_3) or hydrogen (H_2) as the source of hydrogen to reduce carbon. These bacteria can be distinguished further into two types as follows:

3.3.2 Photoautotrophic Bacteria

The photoautotrophic bacteria possess photosynthetic pigments in membrane bound lamellae (or thylakoids) and utilise solar energy. The bacterial photosynthesis is different from that of green plants since here water is not used as a hydrogen donor. Hence oxygen is not released as a by product. For this reason, the process is described as anoxygenic photosynthesis.

$$CO_2 + H_2S \xrightarrow{\text{light energy}} \text{sugars} + \text{sulphur} + \text{water}$$

3.3.3 Chemosynthetic Bacteria

These are bacteria which manufacture organic compounds from inorganic raw materials utilising energy liberated from the oxidation of inorganic substances. Following are the common types of chemo autotrophic bacteria.

1. Nitrifying bacteria which derive energy by oxidizing ammonia into nitrates. E.g.: Nitrosomonas, Nitrobacter.

$NH_4^+ + 2O_2 \rightarrow NO_2 + 2H_2O + energy$

2. Sulphur bacteria which derive energy by oxidising hydrogen sulphide to sulphur. E.g.: Thiobacillus, Beggiatoa.

$2H_2S + O_2 \rightarrow 2S + 2H_2O + energy$

3. Iron Bacteria which derive energy by oxidising ferrous ions into ferric form. E.g.: Ferrobacillus, Gallionella.

$4Feco_3 + 6H_2O + o_2 \rightarrow 4Fe(OH)_3 + 4CO_2 + energy$

3.3.4 Heterotrophic Bacteria

These are bacteria which are unable to manufacture their own organic food and hence are dependent on external source. These bacteria can be distinguished into three groups as follows:

- Saprophytic Bacteria: These bacteria obtain their nutritional requirements from dead organic matter. They breakdown the complex organic matter into simple soluble form by secreting exogenous enzymes. Subsequently they absorb the simple nutrients and assimilate them, during which they release energy. These bacteria have a significant role in the ecosystem, functioning as decomposers.
- The aerobic breakdown of organic matter is called as decay or decomposition. It is usually complete and not accompanied by the release of foul gases. Anaerobic breakdown of organic matter is called fermentation. It is usually incomplete and is always accompanied by the release of foul gases. Anaerobic breakdown of proteins is called putrifaction.

The property of decomposition of organic compounds is employed in several industrial processes such as ripening of cheese, in the retting of fibres and in the curing of tobacco.

3.3.5 Symbiotic Bacteria

These are bacteria which live in a mutually beneficial association with other organisms. Such bacteria derive the essential nutrients from their host organisms and in that process help the host through some of their biological activities.

1. The most familiar example of symbiotic bacteria are the nitrogen fixing bacteria found in the root nodules of leguminous plants. Bacteria such as Rhizobium and Pseudomonas reside in the root nodules and reduce atmospheric nitrogen directly to ammonia. This becomes the source of nitrogen for the host plants. The plants in return provide bacteria with nutrients and protection.
2. The bacteria found in the human alimentary canal Escherichia coli are nonpathogenic. These bacteria check the growth of harmful putrefying bacteria. In addition, these bacteria release vitamins K and B12 which are necessary for blood components. The human host provides shelter and food for these bacteria.
3. A similar example is that of cellulose digesting bacteria which occur in the alimentary canal of ruminant mammals such as cows and goats.

3.3.6 Parasitic Bacteria

These are bacteria which occur in the body of animals and plants, obtaining their organic food from there. Most of these bacteria are pathogenic, causing serious diseases in the host organisms either by exploiting them or by releasing poisonous secretions called toxins.

3.4 MEDIA

1. Culture medium

a. Culture media are solutions containing all of the nutrients and necessary physical growth parameters necessary for microbial growth.
b. Note that not all microorganisms can grow in any given culture medium and, in fact, many can't grow in any known culture medium.
c. In addition to chemical and physical characteristics, media can be distinguished qualitatively as:

i. solid vs. broth
ii. non-synthetic vs. chemically defined
iii. reducing

iv. selective

v. differential

2. Solid medium [agar]

a. Solid medium is media containing agar or some other, mostly inert solidifying agent.

b. Solid medium has physical structure (broth lacks structure) and this allows bacteria to grow in physically informative or useful ways (e.g., as colonies or in streaks).

c. Solid medium is usually used as:

i. slants

ii. stabs

iii. petri dishes

3. Colony

a. A colony is a pile or mass of a sufficiently large number of cells, growing on or in solid medium, that they are visible to the naked eye.

4. Broth medium

a. Broth medium is media lacking a solidifying matrix.

5. Non-synthetic [chemically undefined] medium

a. Chemically undefined ingredient:

i. Non-synthetic medium contains at least one component that is neither purified nor completely characterized nor even completely consistent from batch to batch.

ii. Often these are partially digested proteins from various organismal sources.

iii. Nutrient broth, for example, is derived from cultures of yeasts.

b. May be broth or solid.

6. Simple non-synthetic medium

a. A simple non-synthetic medium, operationally defined, is capable of meeting the nutrient requirements of organisms requiring relatively few growth factors.

7.Complex non-synthetic medium

a.Undefined with more growth factors:

i. A more complex non-synthetic medium, on the other hand, supplies more (additional types of) growth factors.

ii. Complex non-synthetic medium are consequently able to support the growth of more fastidious microorganisms than can simple non-synthetic medium.

8.Chemically defined [synthetic] medium

a. A chemically defined medium is one prepared from purified ingredients and therefore whose exact composition is known.

b. Not trivial for fastideous:

i. To use a chemically defined medium you must know exactly what your microorganism's growth requirements are.

ii. If this is not known it must be discovered through trial and error and therefore is not a trivial process for fastideous microorganisms.

9. Preprepared medium

a. Media of many types can be obtained premixed, in an often dehydrated and powdered state. Such media are called preprepared media.

b. Ready to use:

i. It is even possible to obtain sterilized media that is ready to use.

ii. Such media can be especially desirable if one lacks an autoclave, filtration devices, or the desire to prepare even preprepared media from a can.

10. Enriched medium

a. Added growth factors:

i. Enriched media is preprepared (or equivalent) media to which additional growth factors have been added.

ii. These may be added individually or in complex mixtures.

iii. Enriched medium therefore may be chemically defined or chemically undefined, simple or complex.

b. An example of enriched medium employing the latter would be blood agar which is made from complex medium to which whole blood has been added.

11. Reducing medium

a. Oxygen scavenging media:

i. Reducing medium is employed for growing obligate anaerobes.

ii. Reducing medium particularly contains chemicals (reducing agents) that deplete molecular oxygen.

12. Selective medium

a. Differential growth suppression:

i. Selective medium is designed to suppress the growth of some microorganisms while allowing the growth of others (i.e., they select for certain microbes).

ii. Solid medium is employed with *selective medium* so that individual colonies may be isolated.

b. Examples of selective media include:

i. mannitol salts agar (selects against non-skin flora)

ii. MacConkey agar (selects against gram-positives)

iii. eosin-methylene blue agar (selects against gram-positives)

iv. phenylehyl alcohol agar (selects against gram-negatives)

13. Differential medium

a. Differential appearance:

i. Differential media allow the growth of more than one microorganism of interest but with morphologically distinguishable colonies.

ii. Note that almost any medium containing a specific substrate and well tailored indicator can be used as a differential medium.

b. Examples of differential media include:

i. mannitol salts agar (mannitol fermentation = yellow)

ii. blood agar (various kinds of hemolysis)

iii. MacConkey agar (lactose fermentation = yellow)

iv. eosin-methylene blue agar (various kinds of differentiation)

Sterilization

Sterilization (or sterilisation) is a term referring to any process that eliminates (removes) or kills all forms of microbial life, including transmissible agents (such as fungi, bacteria, viruses, spore forms, etc.) present on a surface, contained in a fluid, in medication, or in a compound such as biological culture media.[1][2] Sterilization can be achieved by applying the proper combinations of heat, chemicals, irradiation, high pressure, and filtration. The term has evolved to include the disabling or destruction of infectious proteins such as prions related to Transmissible Spongiform Encephalopathies

3.5 PHYSICAL AND CHEMICAL CONTROL OF MICROORGANISMS

In most medical settings, the control of microorganisms is of paramount concern. Decontamination refers to the destruction or removal of microorganisms from instruments, materials, body surfaces, etc.

Many agents and procedures have been developed to accomplish this end. It is imperative that you, as a medical professional, understand the modes of action, level of activity and other factors which influence the effectiveness of these procedures and agents.

A. Generally, decontamination involves physical and/or chemical agents. Physical agents include high temperature, radiation, filtration or cavitating sound waves. A myriad of chemical decontamination agents exists. For the most part, they are substances that react with and thus alter some important molecular component of the cell.

B. Microorganisms are not uniformly affected by physical and chemical decontamination. Susceptibility to the effects of physical and chemical agents depends upon the type of microorganism and at what stage in the microorganism's lifecycle they are exposed to the agent. When choosing and applying a method of decontaminating materials, it is important that you understand what type of organism is being targeted and the relative resistance of that organism.

1. The target with the highest resistance is the bacterial endospores. Endospores are ubiquitous in the environment. Many bacteria found in the soil are capable of forming these structures. Introduced into deep wounds or during surgical procedures, these spores can cause severe problems. Thus surgical equipment and other materials used in invasive procedures need to be decontaminated in such a way as to destroy these agents.

2. Targets with the moderate resistance include protist cysts, sexual fungal spores, nonenveloped viruses (many enteric viruses including those responsible for polio, Hepatitis A and Hepatitis E), Mycobacterium *tuberculosis*, *Staphylococcus aureus* and members of the genus *Pseudomonas*.

3. Targets with the least resistance include vegetative cells of most microbes, enveloped viruses (including those viruses responsible for AIDS and Hepatitis B), and asexual fungal spores.

C. There are several terms that have precise meanings. When these terms are used in a product description or as part of procedural instructions it is important that you are aware of these precise meanings.

1. Sterilization refers to any process that destroys or removes all infectious organisms including endospores and viruses.

2. Disinfection refers to any physical process or application of any chemical that will kill the growing (vegetative) microbial cells. These processes need not kill or inactivate endospores. A disinfectant is a chemical capable of killing microbial cells. It should be understood that if a chemical is referred to as a disinfectant, it is to be used on inanimate objects and not to be used on body surfaces.

3. Sanitize refers to any mechanical process (scrubbing, rinsing, etc.) that reduces the microbial load on a surface. Sanitizers are chemical agents that assist in this task. These are usually soaps or detergents.

4. Microbicidal agents are chemicals that will kill or destroy microorganisms. Among the microbicidal agents are those that target specific microorganisms including:

a. fungicidal agents which are designed to kill fungi;
b. bactericidal agents which are designed to kill bacteria;
c. sporicidal agents which are designed to destroy endospores;
d. viricidal agents which are designed to destroy viruses.

5. Microbiostasis refers to the inhibition of growth of microorganisms. This does not mean that the organisms are killed simply that they are unable to grow. Refrigeration and many antimicrobial drugs exert a microbistatic effect.
a. Bacteriostatic agents are chemicals that inhibit the growth of bacteria.
b. Fungistatic agents are chemicals that inhibit growth of fungi.

6. Disinfection refers to any physical process or application of any chemical that will kill the growing (vegetative) microbial cells. These processes need not kill or inactivate endospores. A disinfectant is a chemical capable of killing microbial cells. It should be understood that if a chemical is referred to as a disinfectant, it is to be used on inanimate objects and not to be used on body surfaces.

7. Antisepsis refers to those practices that keep microorganism from entering the sterile tissues. The application of these practices is referred to as aseptic technique. Antiseptics are those chemicals that can be applied to tissue surfaces to kill or inhibit the growth of microorganisms.

II. There are several factors that will influence the effectiveness of antimicrobial agents. When attempting to sterilize, disinfect or sanitize a surface and in the application of aseptic technique, these factors must be taken into consideration.
A. Time of exposure The amount of time that the microorganisms are exposed to any agent (physical or chemical) will greatly affect how many microorganism are destroyed. Short exposures often kill the most susceptible organism and thus select for the more robust organisms. This can be counter productive in that the robust organism will then come to dominate the population of microorganisms and will often rapidly replace the organisms killed by the brief exposure.

B. Microbial load The number of microorganisms must be also considered. Highly contaminated substances will require more protracted exposure to eliminate all living contaminates.

C. Type organism or organisms As mentioned earlier, different organisms display differing susceptibilities to antimicrobial agents. If elimination of vegetative cells is the aim, less stringent measures can be taken. If, on the other hand, endospores must be eliminated more rigorous measures will be required.

D. Temperature, pH and osmolarity Many antimicrobial agents lose their effectiveness under certain environmental conditions and become more effective under others. Generally speaking, higher temperatures lead to increased rates of antimicrobial affect. No such broad statement can be made for the relative effectiveness of agents under differing conditions of pH and osmolarity. For some agents, decreases in pH make them more effective while other agents become inactive as the pH drops. It becomes important that the affect which pH and osmolarity exert on the efficacy of an antimicrobial agent be understood and taken into account when using that agent.

E. Concentration or intensity of agent Usually for an agent to be effective it must be present at or above a certain concentration or intensity.

F. Milieu This term refers to other substances (proteins, solvents, etc.) that are present in the environment that you are trying to disinfect. These other substances may interfere with the action of the chemical or physical agent you intend to use to kill the bacteria. This is especially true of proteins. High levels of protein will interfere with the action of many chemical agents and will reduce the effectiveness of some physical agents.

III. Many chemical agents are available that are said to be effective at reducing of eliminating bacteria from the environment or from body surfaces. Disinfectants are chemical compounds that are designed to kill bacteria and are to be used only on inanimate objects. Antiseptics are compounds designed to kill or inhibit the growth of bacteria on external body surfaces or certain mucus membranes. In clinical settings these agents, when used properly, are an important part of aseptic technique. But overuse of these products, especially outside of clinical settings, carries several risks. First, the inappropriate reduction of nonpathogenic normal flora on external body surfaces and mucus membranes can lead to infection by pathogenic organisms. (i.e. Some yeast infections can be traced to the inappropriate use of antiseptic douche.) Secondly, genes for resistance to antimicrobial drugs have been shown to be found on the same plasmids as genes for resistance to certain antiseptics and disinfectants. Thus, inappropriate use of antiseptics and disinfectants selects for those organisms that carry these plasmids. As a consequence of this overuse of antiseptics and disinfectants, the level of drug resistance increases in those bacterial populations that are found in the environment and on the body. It is important that you are aware of the appropriate usage of antiseptics and disinfectants. It is equally important that you are not pulled in by the current media driven hyperbole regarding the need to kill every bacterium that is found on the body or in the environment.

IV. The actual manner in which a physical or chemical agent affects bacteria is referred to as its mode of action. Generally speaking, if the mode of action of a chemical or physical agent interferes with a process or destroys a structure that is common to both the target microorganisms and our cells, high levels of side effects can be expected. For many drugs, the mode of action entails interference with a process that is unique to the target microorganism, thus minimizing the impact the agent has on our cells.

A. The cell wall is a common target of antimicrobial action. Most bacteria and all fungi have cell walls while our cells lack them. Thus agents that interfere with the synthesis of or specifically destroy the cell wall can be used at high concentration with little chance of affecting our cells.

1. As we will see later, many antimicrobial drugs exert their effect by interfering with the processes that lead to the synthesis of the cell wall.

2. In the case of the gram-negative cell wall, destruction of the outer membrane by solvents and detergents can be easily accomplished.

3. Many bodily secretions contain the enzyme lysozyme. This enzyme digests the peptidoglycan of the gram-positive cell wall.

B. Many disinfectants damage the cell membrane. This can be accomplished by disrupting the phospholipid bilayer or altering the transmembrane proteins. Remember, the membrane is a structure shared by both our cells and the cells of microorganisms, most agents with this mode of action can not be used internally or on mucus membranes as they will harm our cells also.

1. Organic solvents and strong surfactants both act by dissolving the phospholipid bilayer. This destroys the barrier that usually limits movement of ions and other chemicals into or out of the cell.

2. Agents that alter transmembrane proteins destroy the ability of a cell to selectively import or export substances and, in the bacterial cell, can lead to the inactivation of cytochromes and ATP synthase. Inactivation of these proteins destroys the ability of the cell to generate ATP.

C. As previously discussed, microorganisms contain many different types of large biochemicals including proteins, DNA, RNA, and lipids. Agents that will damage

or inhibit the synthesis of these biological polymers will have an adverse effect on the microorganism.

1. Damage to a cell's DNA will inhibit that cell from properly reproducing and stop the use of the DNA as a guide to make RNA. This, in turn, will keep the cell from making proteins that were coded for by the damaged DNA. It appears that all living organisms have the ability to repair DNA. This repair mechanism involves enzymes that will remove the damaged DNA and replace it with functioning DNA. This process is very error prone and thus results in high levels of mutations in the DNA. It also takes time to carry out the repair process, so rapidly growing cells that divide before they have the time to fix the damaged DNA are more adversely effected than slow growing cells.

a. Bombardment of cells with radiation will lead to DNA damage.
b. Certain drugs bind to the enzymes needed to make DNA or RNA and interfere with the functioning of these enzymes.
c. Nucleotide analogs are chemicals that have considerable similarities to the nucleotides used in the synthesis of DNA. Often the enzymes that make DNA cannot distinguish between a real nucleotide and a nucleotide analog. When the analog is added to a growing DNA strand during replication, the synthesis of the DNA strand immediately stops. This keeps cells from copying their DNA completely. Cells that receive only partial copies of the DNA are usually not viable and immediately die.
2. It should be clear at this point the central role played by proteins in metabolic process. Without the action of those proteins known as enzymes, life could not continue. Thus by blocking the synthesis of proteins or inactivation of existing enzymes an organism can be killed. Many antimicrobials work solely or in part by altering the tertiary structure (shape) of a protein or by blocking the active site of an enzyme.
a. The osmolarity of a solution and hydrophilic attraction between amino acids in the protein and water help determine the tertiary structure of a protein. Under

normal conditions the proper shape is taken on. But altering the osmolarity or adding substances to the environment which alter hydrophilic bonds, will lead to the protein losing it proper shape.

b. Proteins assume their proper shape and are stable in that configuration only at a very limited temperature range. Outside of that range (either hotter or colder) the protein will take on a different shape and its functionality will be decreased or eliminated. Alteration of the shape of protein through chemical or physical means is referred to as denaturing.

c. Reactive chemicals will often covalently bind to proteins. This changes the shape of a protein in ways that leave it unable to function properly.

d. Many antimicrobial drugs bind to the ribosome or active site of RNA polymerase. By blocking the action of these two enzymes protein synthesis can be effectively shut down.

V. Chemical agents do not have equal levels of disinfection. Certain agents are very effective and will inactive even endospores. These agents are said to have a high level of activity. Agents with an intermediate level of activity will kill vegetative cells of the most resistant organism (TB, naked viruses), sexual fungal spores. Agents with a low level of activity kill vegetative cells of less resistant organisms and enveloped viruses.

A. Halogens react with proteins in such a way that secondary and tertiary structure is altered. Most halogens exert an intermediate level of activity. Examples of halogen based agents are bleach, chlorine and bromine gas (water purification for drinking and swimming pools), iodine and iodophores (Betadine, providone).

B. Phenolic agents disrupt membranes and alter secondary and tertiary structure of proteins. Most of these agents exhibit intermediate to low level of activity. Examples of common phenolic compounds include Hibiclens, creosote (a wood preservative) and amphyl.

C. Alcohols At 50-95% concentration are effective in disrupting membranes and alter protein tertiary structure. At 95-100% concentrations alcohols mainly serve to dehydrate cells. Alcohols exhibit an intermediate level of activity.

D. Hydrogen peroxide produces reactive hydroxyl radicals that oxidize proteins and other organic molecules. This chemical alteration leads to changes in the tertiary structure of proteins, which leads to reduced function by these proteins. Hydrogen peroxide exhibits a high level of activity.

E. Detergents mainly disrupt membranes but also will alter the tertiary structure of some proteins. Most detergents exhibit a low level of activity.

F. Ethylene oxide is the gas used to sterilize instruments that can not be autoclaved (referred to as gas sterilization). It chemically alters proteins, DNA and RNA. It exhibits a high level of activity

VI. Sterilization, sanitization or simply affecting microbiostasis of inanimate substances can be accomplished through several physical means.

A. Heat is widely used to sterilize and sanitize objects and solutions. The goal (whether you hope to render the substances sterile or simply reduce the bacterial load) and the possible target organisms must be considered. Most vegetative cells are easily destroyed by heat while endospores are much more resistant.

1. The thermal energy of heat has a greater effect in the form of moist heat. This involves exposing the solutions or items to be sterilized to boiling or steam. Boiling occurs at the 100° C and the steam produced by boiling is usually at that temperature. Though this temperature is effective against vegetative cells, it is not very effective against endospores. By allowing the boiling to occur in a pressurized chamber, the boiling point and the steam produced by this boiling is hotter. One of the most common types of medical sterilizers is the autoclave. The autoclave usually is pressurized so that the boiling point is pushed to 121^0 C by raising the pressure to 15 pounds per square inch.

2. Many solutions do not hold up well to the high heats and pressures of the autoclave. Gentler means of decontamination are needed. These means usually do not produce sterile solutions but reduce the bacterial count so that the solutions spoil more slowly. These methods utilize lower temperatures and target the vegetative cells.

a. Tradition pasteurization methods (known as batch pasteurization) involve heating the solution to 63^0 -65^0 C for 30min. These methods kill most of the

vegetative cells and decrease the rate of spoilage.

b. Flash pasteurization involves heating the solutions to 71.6^0 C for 15 seconds. These have similar effects as batch pasteurization.

c. Ultrahigh temperature (or ultra pasteurization) involves superheating the solution to 134^0 C for 1-2 seconds. This usually produces a sterile or nearly sterile solution.

3. To have the same decontaminating effect as moist heat, dry heat temperatures must be much higher. Commonly in laboratories, flaming of instruments (placing them in a flame and heating them to very high temperatures) is a common means for rapidly decontaminating an instrument. On a larger scale, hospitals incinerate (burn) contaminated wastes to kill any and all microorganisms contaminating these wastes.

B. Sanitizing surfaces often involves the use of surfactants. In this setting the surfactant is designed to loosen the bacteria from the surface by binding to the charged materials on the surface of the microorganism. These charged proteins and polysaccharides help the microorganism attach to surfaces. The surfactant binds to these charges and thus interferes with the ability of the microorganism to attach to the surface. The microorganism can then be simply wiped away.

C. In the home and in the medical setting, control of microbial growth is affected by keeping materials cold (freezing or refrigerating). It should be noted that this does not kill the microorganisms, cold simply stops or slows the rate of growth of the microbes. Desiccation involves removal of water from the material that you wish to preserve. Desiccation stops the activity of the enzymes of the microbes contaminating these materials which, in turn, stops growth. When the materials are rehydrated, the microbes often continue their growth.

D. Several types of radiation are commonly used to kill contaminating organisms.

1. Ionizing radiation penetrates organic matter very easily and when it strikes a molecule it will often cause the molecule to breakdown into highly reactive ions. If the radiation hits a DNA strand it will cause alteration of breakage of the strand. Other molecules in the vicinity of the DNA that are hit by the radiation can give rise to highly reactive ions. These ions then react with DNA, leading to breaks in

the "backbone" of the DNA strand. In either case the DNA is damaged. The most commonly used form of ionizing radiation is gamma radiation. It is used to sterilize drugs and medical supplies that are sensitive to heat. Increasingly, gamma radiation is being used to treat foods. Recently, poultry and beef producers received approval from the FDA to allow gamma irradiation of these meats to reduce the chances of transmission of several common pathogens.

2. Ultraviolet radiation (UV light) reacts with the pyrimidine bases of DNA (thymine and cytosine). When UV radiation hits DNA it imparts the pyrimidine bases with substantial amounts of energy. This energy allows the pyrimidines to form inappropriate covalent bonds with adjacent pyrimidine bases. This covalent linkage between the pyrimidine bases is known as a pyrimidine dimer. These dimers interfere with the ability of the effected pyrimidines to complementary base pair. This destroys the ability of the damaged DNA to carry out transcription or replication. Repair of this damage can occur but this process is error prone and thus introduces mutations into the repaired DNA.

E. For many solutions the most effective means of decontamination is to force the solution through a filter. Filtration is especially useful in sterilizing extremely sensitive drugs that would be adversely effected by any of the aforementioned means of decontamination. The size of the openings in the filter (pore size) will determine which pathogens are removed from the solution. Extremely small pores are necessary to remove viral pathogens.

Terms	Definitions
antisepsis	chemical agents are applied directly to expoosed body surfaces to destroy or inhibit vegetative pathogens
sanitization	cleansing technique that removes debris, microorganisms and toxins.
Oligodynamic action	Heavy metal germicides containing inorganic or organic metallic salt. Aqueous solution, tinctures, ointments, or soaps.
Fungicide	chemical that kills fungal spores, hypae, and yeast.
virucide	inactivates viruses, especially on living tissue.
sporicidal	destroy bacterial endospores. Sterilization agent.
Bacteriostatic	agents that prevent growth of bateria on tissues or objects in the environment.
fungistatic	chemicals inhibiting the growth and reproduction of fungi spores.
germicide/ microbicide	chemical agent that kills pathogenic organisms
disinfection	use of a physical process or chemical agent to destroy vegetative pathogens, not endospores though.
sepsis	growth of microorganisms in the blood and other tissues.
asepsis	prevents infection by preventing the entry of infectious agents into sterile tissues
sterilization	destroys or removes all viable microorganisms, including viruses.
bactericide	chemical that kills bacteria except endospores,
UV radiation	100nm-400nm=wavelength passes through air readily, liquid steadily, and only poorly through solids.
Cold Sterilization	Sterilizes in the absence of heat. Irradiation
Quaternary Ammonium Compounds QUATS	Positively charged cationic detergents.
Betapropiolactone BPL	Aerosol or liquid. Disenfects whole rooms, insstruments, sterilize bone and arterial grafts and & inactivate viruses in vaccines.
Ethylene Oxide	colorless substance that exists as a gas at room temp.
Formalin	Aqueous Solution of formaldehyde and water
Halogens	Fluorine, Iodine, Chlorine, Bromine. Nonmetallic elements with similar chemical proteins.
tinctures	antimicrobial chemicals that are dissoled in pure alcohol or water-alcohol solutions.
chemicals that sterilize	disenfectants, sterilants, and antiseptics
Aqueous Solutions	soultions contains pure water as the solvent

prymidine dimers	specific molecular damage occurs on the primidine bases (cytosine and thymine) and forms abnormal linkages between them.
degermation	scrubbing skin or immersing it in chemicals or both. -surgical scrub -alcohol wipes
microbial death	permanent loss of reproductive capability
mode of action	antimicrobial agents adverse effects on cells.
surfactants	microbial agents because they lower the surface tension of cell membranes.
moist heat	hot water, boiling water, or steam
dry heat	removes water from cell necessary for metabolic reactions. Denotes air with a low moisture content that has been heating coil.
Thermal Death Time	Shortest length of time required to kill all test microbes at a specific temp.
Thermal death point	lowest temperate required to kill all microbes in a sample in 10 mins.
Tyndallization	Selected substance that cannot withstand high temp of autoclave. Can be subjected to intermittent sterilization.
Pasteurization	heat is applied to liquids to kill potential agents of infection and soilage while retaining the liquids flavor and food value.
flash method	Pasteurization technique exposes the liquid to heat exchangers at 71 degrees C for 15 secs.
Thermoduric microbes	endospores
Incineration	flame or electric coil
desiccated	dehydrated
lyophilization	Freezing & drying. Preserves microorganisms in viable state for many years.
Ionizing Radiation	If radiation ejects orbital electrons from an atom, causes ions to form.
Nonionizing Radiation	UV radiation. Excites atoms by raising them to a higher energy state but doesn't ionize them.

3.6 CULTURING TECHNIQUES

A microbiological culture, or microbial culture, is a method of multiplying microbial organisms by letting them reproduce in predetermined culture media under controlled laboratory conditions. Microbial cultures are used to determine the type of organism, its abundance in the sample being tested, or both. It is one of the primary diagnostic methods of microbiology and used as a tool to determine the cause of infectious disease by letting the agent multiply in a predetermined medium. For example, a throat culture is taken by scraping the lining of tissue in the back of the throat and blotting the sample into a medium to be able to screen for harmful microorganisms, such as *Streptococcus pyogenes*, the causative agent of strep throat.[1]Furthermore, the term culture is more generally used informally to refer to "selectively growing" a specific kind of microorganism in the lab

Microbial cultures are foundational and basic diagnostic methods used extensively as a research tool in molecular biology. It is often essential to isolate a pure culture of microorganisms. A pure (or axenic) culture is a population of cells or multicellular organisms growing in the absence of other species or types. A pure culture may originate from a single cell or single organism, in which case the cells are genetic clones of one another.

For the purpose of gelling the microbial culture, the medium of agarose gel (agar) is used. Agar is a gelatinous substance derived from seaweed. A cheap substitute for agar is guar gum, which can be used for the isolation and maintenance of thermophiles

Bacterial Culture

Microbiological cultures can be grown in petri dishes of differing sizes that have a thin layer of agar-based growth medium. Once the growth medium in the petri dish is inoculated with the desired bacteria, the plates are incubated at the best temperature for the growing of the selected bacteria (for example, usually at 37 degrees Celsius for cultures from humans or animals, or lower for environmental cultures). Another method of bacterial culture is liquid culture, in which the desired bacteria are suspended in liquid broth, a nutrient medium. These are ideal

for preparation of an antimicrobial assay. The experimenter would inoculate liquid broth with bacteria and let it grow overnight (they may use a shaker for uniform growth). Then they would take aliquots of the sample to test for the antimicrobial activity of a specific drug or protein (antimicrobial peptides). As an alternative, the microbiologist may decide to use static liquid cultures. These cultures are not shaken and they provide the microbes with an oxygen gradient.

Culture collections

Microbial culture collections focus on the acquisition, authentication, production, preservation, catalogueing and distribution of viable cultures of standard reference microorganisms, cell lines and other materials for research in microbial systematics. Culture collection are also repositories of type strains.

Virus and Phage culture

Virus or phage cultures require host cells in which the virus or phage multiply. For bacteriophages, cultures are grown by infecting bacterial cells. The phage can then be isolated from the resulting plaques in a lawn of bacteria on a plate. Virus cultures are obtained from their appropriate eukaryotic host cells.

Isolation of pure cultures

For single-celled eukaryotes, such as yeast, the isolation of pure cultures uses the same techniques as for bacterial cultures. Pure cultures of multicellular organisms are often more easily isolated by simply picking out a single individual to initiate a culture. This is a useful technique for pure culture of fungi, multicellular algae, and small metazoa. Developing pure culture techniques is crucial to the observation of the specimen in question. The most common method to isolate individual cells and produce a pure culture is to prepare a streak plate. The streak plate method is a way to physically separate the microbial population, and is done by spreading theinoculate back and forth with an inoculating loop over the solid agar plate. Upon incubation, colonies will arise and single cells will have been isolated from the biomass.

3.6.1 Cultivation of bacteria

Instrument for Seeding Media

This is selected according to the nature of the medium and inoculum. Platinum or nichrome wires of different gauges are used. Nichrome is oxidising and hence in some cases nichrome wire is used.

Aerobic Incubation of Cultures

Incubation is usually done at 37°C. Some bacteria require special temperature such as Campylobacters grow better at 43°C and leptospires at 30°C. For prolonged incubations as are required for the growth of Mycobacterium tuberculosis screw capped bottles should be used instead of petri dishes or tubes to prevent the drying of medium. Extra carbon dioxide is needed for optimal growth of organisms such as *Brucellaabortus, Pneumococcus* and *Gonococcus*, etc. The concentration of additional carbon dioxide nedded is 5-10 per cent. The simplest method for having this environment is to put the plates in a container and generate $C0_2$ inside by lightings candle in it just before putting on the lid. Pure $C0_2$ can also be introduced in a container.

Methods of isolating pure cultures

When there is a mixture of bacteria, following methods can be utilised to isolate bacteria in pure cultures. Use of enrichment, selective and indicator media as described above can help in isolation of pure cultures. Pretreatment of clinical sample with substances which destroy unwanted bacteria e.g. culture of tubercle bacilli. The sputum is pretreated with alkali / acids. Plating on solid media to obtain isolated colonies, for selecting the desired ones. Obligate aerobes and anaerobes can be separated by incubating in appropriate conditions.

The vegetative forms and spore forms can be separated by heating at 80°C which will kill the vegetative forms. Motile and non-motile bacteria can be separated by inoculating the Craigie's tube which permits travel of motile bacteria to appear in the outside of the tube. Bacteria of different sizes can be separated by use of selective filters with different pore sizes. Pathogenic organisms can be separated from non pathogenic by animal inoculation tests.

Anaerobic cultures

For obligatory anaerobic bacteria, oxygen acts as a lethal poison and hence for the growth of these an environment which is free of oxygen has to be created. The MacIntosh and Fildes technique of 1916 is stillwidely practised with some modifications. In this the inoculated medium to be incubated anaerobically isplaced in the air tight jar and with the help of vacuum pump it is evacuated to -660 mm Hg. Hydrogen(90%) and carbon dioxide (10%) are added to this through built in valves. The oxygen is removed by itscombination with hydrogen in the presence of palladium catalyst which is present in the jar. Morehydrogen or gas mixture is then introduced to equilibrate the pressure and the jar is incubated at 37°C.Glove box or anaerobic chamber is used in modem laboratories with high quantum of work.

3.6.2 Cultivation of Algae

Like plants, algae use the sunlight for the process of photosynthesis. Photosynthesis is an important biochemical process in which plants, algae, and some bacteria convert the energy of sunlight to chemical energy. Algae capture light energy through photosynthesis and convert inorganic substances into simple sugars using the captured energy.

There are two main methods of cultivation

- Ponds
- Photobioreactors

Ponds

Since *algae* need sunlight, carbon-di-oxide and water for their growth, they can be cultivated in open ponds & lakes. More about open ponds are discussed here - Cultivation in open pond

Photobioreactors

A Photobioreactor is a controlled system that incorporates some type of light source. The term photobioreactor is more commonly used to define a closed system, as opposed to an open pond. A pond covered with a greenhouse could also be considered an unsophisticated form of photo*bioreactor*. Because these systems are closed, everything that the algae need to grow, (carbon dioxide,

water and light) need to be introduced into the system. More about photobioreactors are discussed here -Cultivation in photobioreactors

There are several factors to determine the growth rate of *algae*. The following are the important factors that determine the growth rate of algae

- Light - Light is needed for the photosynthesis process
- Temperature: There is an ideal temperature range that is required for algae to grow
- Medium/Nutrients - *Composition* of the water is an important consideration (including salinity)
- pH - *Algae* typically need a pH between 7 and 9 to have an optimum growth rate
- Algae Type - Different types of algae have different growth rates
- Aeration - The algae need to have contact with air, for its CO_2 requirements
- Mixing - Mixing prevents sedimentation of algae and makes sure all cells are equally exposed to light
- Photoperiod: Light & dark cycles

Algae *cultivation* can be done in a variety of environments. Algae cultivation in various environments are discussed in the following pages.

- Cultivation in open pond
- Cultivation in closed ponds
- Cultivation in photobioreactors
- Desert-based algae cultivation
- Cultivation in waste water
- Marine algae cultivation
- Cultivation next to power plants

Algae cultivation is an environmentally friendly process for the production of organic material by photosynthesis from carbon dioxide, light energy and water. The water used by algae can be of low quality, including industrial process water, effluent of biological water treatment or other waste water streams.The open systems, in order to increase their efficiency, are generally designed as a continuous culture in which a fixed supply of culture medium or influent ensures

constant dilution of the system. The organisms adapt their growth rate to this dilution regime, with the organism best adapted to the environment prevailing in the system winning the competition with the other organisms.

A drawback of the common open algae culture systems is the major risk of contamination by undesirable photosynthetic micro-organisms which can be introduced via air or rain. An alternative to the drawback of the open system could be to carry out algae cultivation in closed photobioreactors. In these, the process conditions can be accurately controlled, and no infection carrying alga species will occur. A major drawback of the closed photobioreactors resides in the high investment costs which lead to high production costs.

Thus, cultivating algae for fuel is an area where more experimentation and *research* are still required. As the viruses do not reproduce independent of living host cells, they cannot be cultured in the same as bacteria and eukaryotic microorganisms. However, the cultivation of viruses can be discussed under following heads: (i) cultivation of animal viruses, (ii) cultivation of plant viruses, and (iii) cultivation of bacteriophages.

3.6.3 Cultivation of Animal Viruses

(i) In Animal Cells

Suitable living mammals (such as sheep or calves or rabbits) are selected for cultivation of viruses. The selected animals should be healthy and free from any communicable diseases. The specific virus is introduced into the healthy animals. The site of administration varies according to the type of virus is allowed to grow in the living animal. At the end of incubation period, the animals are slaughtered and washed thoroughly and viruses are obtained from them.

(ii) In Chick-Embryo

The animal viruses can be successfully cultivated using chick-embryo technique. In this method fertile hen eggs are selected. Eggs must not be more then 12 days old. To prepare the egg for virus cultivation, the shell surface is first disinfected with iodine and penetrated with a small sterile drill. After inoculation, the drill hole is sealed with gelatin and the egg is then incubated. Viruses may be able to region. For convenience, the myxoma virus grows well on the chorioallantoic

membrane, whereas the mumps virus prefers the allantoic cavity. The infection may produce a local tissue lesion known as pock, whose appearance often is characteristic of the virus.

(iii) In Vitro Culture (Tissue Culture Technique)

More recently developed in vitro cultivation of animal viruses has eliminated the need to kill the animals. This technique has become possible by the development of growth media for animal cells and by the availability of antibiotics which prevent bacterial and fungal contaminations in cultures. Cultivating animal viruses using tissue culture technique involves following three main steps:

Cultured cells

- **Primary**

– Heterogeneous – many cell types

– Closest to animal

– Technical hassle

- **Diploid cell strain**

– Relatively homogeneous – fewer cell types

– Further from animal

– Technically less hassle

- **Continuous cell line**

– Immortal

– Most homogeneous

– Genetically weird – furthest from animal

– Hassle free

–Suspension or monolayer

Monolayer Preparation. Live tissues of vital organs (e.g., heart or kidney) are taken and the cells are separated from the tissue by digesting the intracellular cement substance with dispersing agents such as trypsins or collagenase or ethylenediaminetetraacetic acid (EDTA). The cell suspension is passed through screen filters so that the coarse particles are removed from the separated cells. The cells are washed free of dispersing agents. The cells are centrifuged if required and resuspended in nutrient medium contained in glass or plastic vessels. The

composition of medium and other conditions of incubation depends on the type of cells used. Upon incubation the cells quickly settle and attach firmly to the bottom of the flask. If undisturbed, these cells grow and spread to form monolayers.

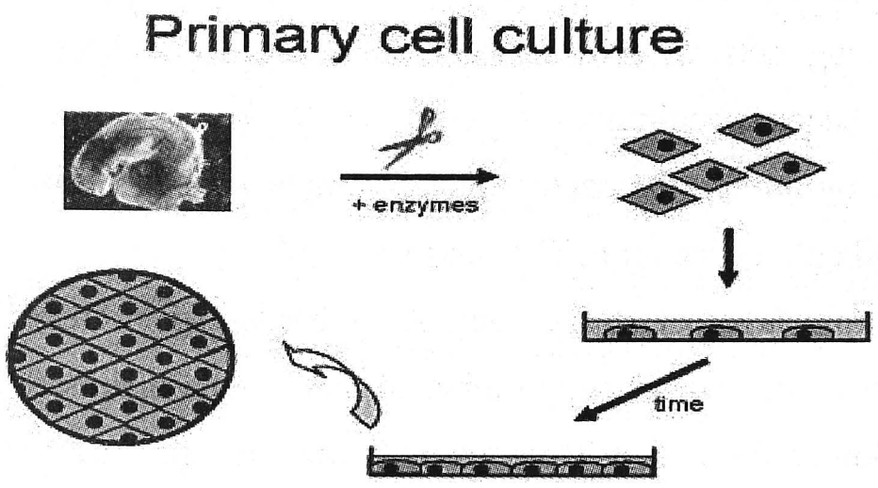


Figure-23

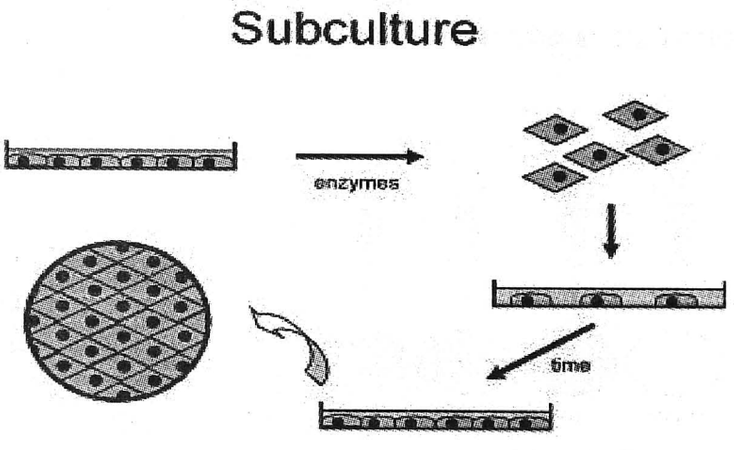


Figure-24

3.6.4 Cultivation of Plant Viruses

There are several methods of cultivation of viruses such as plant tissue cultures, cultures of separated cells, or cultures of protoplasts, etc. Viruses also can be grown in whole plants. Leaves are mechanically inoculated by rubbing with a mixture of viruses and an abrasive such as carborundum. When the cell wall is broken by the abrasive, the viruses directly contact the plasma membrane and infect the exposed host cells. (The role of the abrasive is frequently filled by insects that suck of crush plant leaves and thus transmit viruses.) A localized necrotic lesion often develops due to the rapid death of cells in the infected area. Even when lesions do not arise, the infected plant may show symptoms such as

change in pigmentation or leaf shape. Some plant viruses can be transmitted only if a diseased part is grafted onto a healthy plant.

Cultivation of Bacteriophages

Bacterial viruses of bacteriophages (phages for short) are cultivated in either broth or agar cultures of young, actively growing bacterial cells. Several host cells are destroyed that turbid bacterial cultures may clear rapidly because of cell lysis: Cultures are prepared by mixing the bacteriophage sample with cool, liquid agar and a suitable bacterial culture medium. The mixture is quickly poured into a petri dish containing a bottom layer of sterile agar. After hardening, bacteria in the layer of top agar grow and reproduce, forming a continuous, opaque layer or lawn. Wherever a virion comes to rest in the top agar, the virus infects an adjacent cell and reproduces. Finally, bacterial lysis generates a plaque or clearing in the lawn.

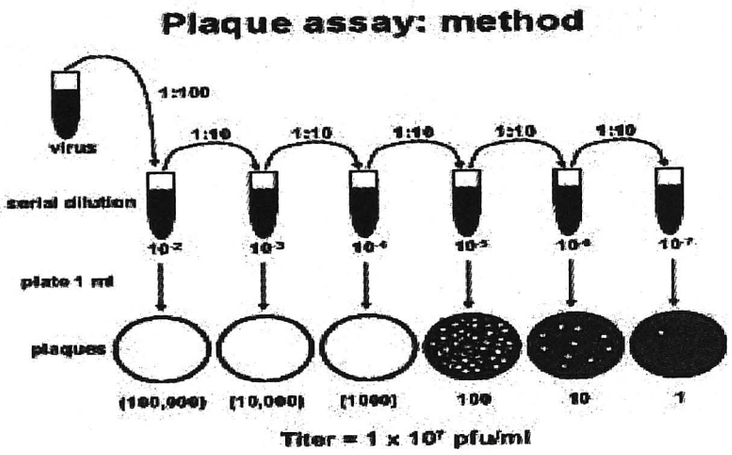


Figure-25

3.6.5 Culture Media for Fungi

Media generally contain a source of carbon, nitrogen and vitamins. Glucose (dextrose) is the most widely utilizable carbon source, and hence is the most commonly used in growth media. Fructose and mannose are the next most commonly utilized sugars by fungi and are found in media from natural sources. Sucrose (table sugar) may be used in some media. Nitrogen sources include peptone, yeast extract, malt extract, amino acids, ammonium and nitrate compounds. Casamino Acids, a Difco product, is acid-hydrolized casein, a mixture of amino acids. It is a good general source of nitrogen but is vitamin-free. Bacto-Peptone, another Difco product, contains nitrogen and a high peptone and amino acid content. Salts, including Fe, Zn and Mn, are often added to 'defined' media, but are usually not added to the common media used for routine culture.

Fungi have natural deficiencies for vitamins that are satisfied at M to nM concentrations. The most common naturally occurring vitamin deficiencies are thiamin and biotin. Deficiency of both is quite common among the Ascomycota. Other organic nutrients such as glucose are often contaminated with vitamins sufficient to supply the growth requirements of fungi. Ascomycota isolated from fruiting bodies forming on dung, wood or soil can be grown on Malt Agar, Potato Carrot Agar and Potato Dextrose Agar. The first step is to attempt to get the ascospores to germinate by streaking them out or getting forcible discharge onto a selective or isolation medium. Look for spore germination and transfer a small piece of agar with the germinating spore(s) to one of these richer media. Basidiomycota growing on wood can be grown on Malt Extract Agar. Cultures derived from mushrooms can be grown on Potato Dextrose Agar, Potato Sucrose Agar and Malt Extract Agar. Potato Dextrose Yeast Extract Agar (PDYA) also is a recommended medium for growing cultures of *Agaricus*, *Pleurotus*, *Lentinus*, *Stropharia*, *Flammulina*, and some of the *Psilocybe* species.

Medium recipes

Sabouraud agar can be purchased from a variety of commercial sources, either as the original recipe (Sabouraud agar, modified), or in a slightly altered version termed "Sabouraud agar, Emmons."

The neutral pH of the Emmons modification seems to enhance the growth of some pathogenic fungi, such as dermatophytes. Perliter of medium:

Peptone,10g

Glucose,40g

Agar,15g

1. Combine all ingredients in ~900 ml of deioinized water.
2. Adjust to pH 5.6 with hydrochloric acid and adjust final volume to 1 liter.
3. Autoclave 20 minutes at 121°C, 15 lb/in^2.
4. Cool to ~45 to 50°C and pour into petri dishes or tubes for slants.

Emmons modification of Sabouraud agar (1) Per liter of medium:

Neo-peptone,10g

Glucose,20g

Agar,20g

1. Follow steps 1 through 4, above, except adjust the pH to the range of 6.8 to 7.0 with hydrochloric acid before autoclaving, cooling, and pouring. Either Sabouraud agar or its Emmons version can be made more selective by adding antibiotics. Commonly used are gentamicin, which inhibits gram-negative bacteria, and/or chloramphenicol, which inhibits a wide range of gram-positives and gram-negatives, and cycloheximide, which inhibits primarily saprophytic fungi but not dermatophytes or yeasts

(3). Chloramphenicol and gentamicin are used at 50 mg/liter (dissolved in 10 ml of 95% ethanol before adding to molten media) and cycloheximide at 0.5 g/liter (dissolved in 2 ml of acetone first) (2).

Antibiotics should only be added after media has been autoclaved and then cooled to ~45 to 50°C. Keep all plates at 4°C until they are used, regardless of whether they contain antibiotics. Sabouraud agar plates can be inoculated by streaking (see the Streak Plate Protocol for an explanation of this method), as with standard bacteriological media, or by exposing the medium to ambient air. Typically, molds are incubated at room temperature (22 to 25°C) and yeasts are incubated at 28 to 30°C or 37°C if suspected of being dimorphic fungi. Incubation times will vary, from approximately 2 days for the growth of yeast colonies such as *Malasezzia*, to 2 to 4 weeks for growth of dermatophytes or dimorphic fungi such as *Histoplasma capsulatum*. Indeed, the incubation time required to acquire fungal growth is one diagnostic indicator used to identify or confirm fungal species.

Czapek Dox Broth

Czapek Dox Broth is a semisynthetic medium used for the general cultivation of fungi.

Ingredients Gms / Litre

Sucrose 30.000

Sodium nitrate 3.000

Dipotassium phosphate 1.000

Magnesium sulphate 0.500

Potassium chloride 0.500

Ferrous sulphate 0.010

Final pH (at 25°C) 7.3±0.2

**Formula adjusted, standardized to suit performance parameters

Directions

Suspend 35.01 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving

at 15 lbs pressure (121°C) for 15 minutes. Mix well and dispense as desired.

Principle and Interpretation

Fungi, including yeasts and filamentous species or moulds are ubiquitously distributed in nature. Czapek Dox Broth is a semisynthetic medium used for the cultivation of fungi, containing sodium nitrate as the sole source of nitrogen. Sucrose serves as the sole source of carbon while sodium nitrate serves as the sole source of nitrogen. Dipotassium phosphate buffers the medium. Magnesium sulphate, potassium chloride, ferrous sulphate serves as sources of essential ions.

Rose Bengal agar

Formula* in g/L

Peptone 5,00

Dextrose 10,00

Potassium phosphate 1,00

Magnesium sulfate 0,50

Rose bengal 0,05

Chloramphenicol 0,10

Agar 15,00

Final pH 7,2 ± 0,2 at 25°C

* Adjusted and /or supplemented as required to meet performance criteria

Directions

Suspend 32 g of powder in 1 L of distilled water and bring to the boil stirring constantly. Distribute in suitable containers and sterilize in the autoclave at 121°C for 15 minutes.

Description

Rose Bengal Agar is a selective medium used to detect and enumerate moulds and yeasts in food samples. In addition the nutritional requirements for moulds and yeasts, this medium also contains Rose Bengal, which apart from turning the yeast a pink colour, facilitates counting, by reducing the luxuriant growth of moulds

such as Rhizopus and Neurospora. This makes it is easier to detect other slower growing moulds.The chloramphenicol included in the formulation inhibits bacterial growth, but does not interfere with the growth of fungi.

Technique

After making a dilution bank, take 0,1 mL from each dilution and inoculate on Rose Bengal Agar plates with a Drigalsky Loop or glass spreader. Should the pour plate method be preferred, take 1 mL from each dilution and put it in an empty Petri dish. Pour the molten medium at 50°C and homogenize it by gently swirling the plate. Incubate at 22°C for 5 days enumerate the fungi. After making a dilution bank, take 0,1 mL from each dilution and inoculate with a Drigalsky Loop or glass spreader on Rose Bengal Agar plates. Should the massive seed method be preferred, take 1 mL from each dilution and put it in an empty plate. Pour the molten medium at 50°C and homogenize it by gently moving the plate in an eight (8) shape. Incubate at 22°C for 5 days and proceed to enumerate the fungi.

Limitations:

The low concentration of antibiotic that contains the culture medium can be expected that the growth of certain strains of bacteria is inhibited only partially. This medium is photo-sensible. Do not expose this medium to the light since photo-degradation of Rose Bengal produce compounds toxic to fungi. The prepared medium or ready-to-use plates haven a shelf-life of seven days at 4 ± 2°C in the dark.

3.6 Points for Discussion

1. Which type of organism would you most likely find to grow in food in your refrigerator? Mesophile? Psychrophile? Thermophile? Why?
2. Why is a solid medium used for purifying a microorganism?

3.7 Check your progress

1. What is isolation?
2. Why must you apply aseptic technique in isolation and cultivation of microbes?
3. Design an enrichment medium and a procedure for growing an endospore-forming bacterium that fixes nitrogen and uses cellulose for its carbon source.
4. Is nutrient broth a universal medium? Explain

UNIT IV DISEASES AND CROP PROTECTION

Structure

4.1 Introduction

Plant pathology (also **phytopathology**) is the scientific study of **plant diseases** caused by pathogens (infectious organisms) and environmental conditions (physiological factors). Organisms that cause infectious disease include fungi, oomycetes, bacteria, viruses, viroids, virus-like organisms, phytoplasmas, protozoa, nematodes and parasitic plants. Plant pathology also involves the study of pathogen identification, disease etiology, disease cycles, economic impact, plant disease epidemiology, plant disease resistance, pathosystem genetics, and management of plant diseases.

4.2 Objectives

After going through this unit, you will be able to

- Explain disease development, symptoms and the role of environmental factors in disease development
- Describe the various methods by which crop plants can be protected from pathogens

4.3 General symptoms

Diseases in plants cause change in appearance and functions. Infected plants manifest characteristic expressions of their pathological condition called **symptoms. Generalized symptoms** may be classified as local or systemic, primary or secondary, and microscopic or macroscopic. **Local symptoms** are physiological or structural changes within a limited area of host tissue, such as leaf spots, galls, and cankers. **Systemic symptoms** are those involving the reaction of a greater part or all of the plant, such as wilting, yellowing, and dwarfing. **Primary symptoms** are the direct result of pathogen activity on invaded tissues (*e.g.,* swollen "clubs" in clubroot of cabbage and "galls" formed by feeding of the root-knot nematode). **Secondary symptoms** result from the physiological effects of disease on distant tissues and uninvaded organs (*e.g.,* wilting and drooping of cabbage leaves in hot weather resulting from clubroot or root knot). The common symptoms caused by infection are given below.

Discoloration:

Appearance of yellow color is called **chlorosis.** The intensity of yellow color varies from light green to deep yellow. Partial chlorosis leads to **mosaic** like appearance which is common in viral diseases. **Vein clearing or yellow vein** is due to yellowing of veinal areas. **Interveinal chlorosis** is due to yellowing of lamina present in between the veins. in **marginal chlorosis**, leaf margin turns yellow. In **albication**, there is complete yellowing due to total absence of chlorophyll.

BLIGHTS: Symptoms include sudden withering and death of leaves and branches, or in the case of blossoms, wilting and discoloration. Conspicuous spots or irregular dead areas on leaves and twigs which cause foliage to distort and drop

prematurely could be a blight. Damage from blights can be minor, as in the case of some anthracnose blights, to serious, as in the case of fireblight.

CANKER: Symptoms usually form on woody stems and may be sunken areas, cracks, or raised areas of dead or abnormal tissue. Sometimes the cankers ooze conspicuously, or in the case of evergreens, drip sap onto the branches beneath. Cankers can sometimes be one of the symptoms manifested by another disease.

GALLS are swollen masses of abnormal tissue that range in size from small to quite large. Certain insects can also cause galls. Cut a gall open and search for signs of an insect inside- if you find none, the gall is probably caused by a disease.

LEAF BLISTERS are yellow bumps on the upper surfaces of the leaves with gray depressions on the lower surfaces. **LEAF CURL DISEASES** cause new leaves to be pale or reddish with the midrib deformed. The leaves pucker and curl as they expand. Certain insects cause similar symptoms, but on closer inspection, if insects are the cause the insects themselves or other signs of their presence can be seen.

MILDEWS are usually one of two types. Downy mildew is usually a white to purple, fuzzy growth, usually on the undersides of leaves and along stems. It turns black with age. Powdery mildew is a white to grayish powdery growth on the upper surfaces of leaves.

ROT DISEASES cause decay of roots, stems, wood, flowers, and fruit. They can be soft and squishy or hard and dry, and color can be either light or dark.

RUST DISEASES typically produce symptoms that include a powdery tan to rust-colored coating or soft tentacles.

WILT DISEASES cause permanent wilting (drooping of plant), often followed by death of part or all of the plant.

Necrosis: Localized or general death of cells or disintegration of tissues.

Shot hole: Dead spotting of leaves with diseased tissue dropping out, leaving small holes

Leaf spot: A definite, localized, round to regular lesion, often with a border of a different colour, characterized as to location (leaf spot, fruit spot) and colour

(brown spot, black spot); if numerous or if spots enlarge and merge, a large irregular blotch or blight may develop,

Fleck: A small, white to translucent spot or lesion visible through a leaf

Streak: Narrow, elongated, somewhat superficial necrotic lesions, with irregular margins, on stems or leaf veins

Blast: Sudden blighting or death of young buds, flowers, or young fruit; failure to produce fruit or seeds

Bleeding: Flow of sap, often discoloured, from a split crotch, branch stub, or other wound; usually accompanied by an odour of fermentation

Damping-off: Decay of seed in soil, rapid death of germinating seedlings before emergence, or emerged seedlings suddenly wilting, toppling over, and dying from rot at or near the soil line.

Dieback: Progressive browning and death of shoots, branches, and roots starting at the tips.

Hyperplastia and hypertrophy: an overdevelopment or overgrowth of plant cells, tissues, or organs; hyperplastic has come to mean an increase in number of cells (**hyperplastia**), and an increase in cell size(**hypertrophy**).

Curl: Distortion and crinkling of leaves or shoots resulting from unequal cell growth of opposite sides or in certain tissues

Mummification: Final stage in certain fruit rots, in which the dried, shriveled, and wrinkled fruit is called a "mummy".

Russeting: Usually a brownish, superficial roughening or corking of the epidermis of leaves, fruit, tubers, or other organs; often due to suberization (cork development) of cells following injury.

Fasciation, or witches'-broom: a distortion that results in a dense, bushy overgrowth of thin, flattened, and sometimes curved shoots, flowers, fruit, and roots at a common point; usually due to adventitious (abnormally located) development of organs. Pathogenecity is the ability of the pathogen to interfere with one or more of the essential functions of the host plant, thereby causing disease. A plant becomes diseased when it is attacked by a pathogen.

.4.1 Stages in the development of disease

The primary events in a disease cycle are inoculation, penetration, and establishment of infection, colonization, growth and reproduction of the pathogen.

4.4.1.1 Inoculation

Inoculation is the contact of a pathogen with a plant.

Types of Inoculum

An inoculum that survives dormant in the winter or summer and causes the original infections in the spring or in the autumn is called a primary inoculum, and the infections it causes are called primary infections. An inoculum produced from primary infections is called a secondary inoculum and it, in turn, causes secondary infections.

The inoculum is any part of the pathogen that can initiate infection. Thus, in fungi the inoculum may be spores, sclerotia (i.e., a compact mass of mycelium), or fragments

of mycelium. In bacteria, mollicutes, protozoa, viruses, and viroids, the inoculum is always whole individuals In nematodes, the inoculum may be adult nematodes, nematode juveniles, or eggs. In parasitic higher plants, the inoculum may be plant fragments or seeds. One unit of inoculum of any pathogen is called a propagule.

Sources of Inoculum

In some fungal and bacterial diseases of perennial plants, such as shrubs and trees, the inoculum is produced on the branches, trunks, or roots of the plants. The inoculum sometimes is present right in the plant debris or soil in the field where the crop is grown; other times it comes into the field with the seed, transplants, tubers, or other propagative organs or it may come from sources outside the field. Outside sources of inoculum may be nearby plants or fields or fields many miles away. In many plant diseases, especially those of annual crops, the inoculum survives in perennial weeds or alternate hosts, and every season it is carried from them to the annual and other plants. Inoculum that survives the off season periods (winter or summer) and causes the original infection in the growing season is called primary inoculums and the infection as primary infection. Inoculum produced from these primary infection that actually spreads the disease in the

field under favorable conditions, is called secondary inoculums that brings about secondary infections. Inoculum in the absence of its host from the field services in plant debris field, soil, seed, tubers, Transplants or other plant parts, perennial weeds, alternate hosts The inoculums is carried to host plants and this landing or arrival of inoculum is passive by wind, water, insects etc or in some cases also by active growth as in some root-infecting fungi like *Armillaria mella.*

4.4.1.2 Prepenetration: Attachment of Pathogen to Host

This phase includes all the events prior to actual entry of the pathogen. Such events include (i) germination of spores and seeds, (ii) hatching of eggs (nematodes), (iii) attachment of pathogen to host, and (iv) recognition between host and pathogen (early event-not still understood clearly). Lack of specific recognition factors in plant surface may not allow the attachment of pathogen to it. Such factors in plant include lectins (proteins of glycoproteins) and some oligo- and polysaccharides. In viral pathogen lack of recognition of its nucleic and by host ribosome's may lead to failure in disease. Pathogens such as mollicutes, fastidious bacteria, protozoa, and most viruses are placed directly into cells of plants by their vectors and, in most cases, they are probably immediately surrounded by cytoplasm, cytoplasmic membranes, and cell walls. However, almost all fungi, bacteria, and parasitic higher plants are first brought into contact with the external surface of plant organs. Before they can penetrate and colonize the host, they must first become attached to the host surface. Attachment takes place through the adhesion of spores, bacteria, and seeds through adhesive materials that vary significantly in composition and in the environmental factors they need to become adhesive. The propagules of these pathogens have on their surface or at their tips mucilaginous substances consisting of mixtures of water insoluble polysaccharides, glycoproteins, lipids, and fibrillar materials, which, when moistened, become sticky and help the pathogen adhere to the plant.

4.4.1.3 Spore Germination and Perception of the Host Surface

It is not clear what exactly triggers spore germination, but stimulation by the contact with the host surface, hydration and absorption of low molecular weight ionic material from the host surface, and availability of nutrients play a role.

Spores also have mechanisms that prevent their germination until they sense such stimulations or when there are too many spores in their vicinity. Once the stimulation for germination has been received by the spore, the latter mobilizes its stored food reserves, such as lipids, polyoles, and carbohydrates, and directs them toward the rapid synthesis of cell membrane and cell wall toward the germ tube formation and extension. The germ tube is a specialized structure distinct from the fungal mycelium, often growing for a very short distance before it differentiates into an appressorium. The germ tube is also the structure and site that perceives the host surface and, if it does not receive the appropriate external stimuli, the germ tube remains undifferentiated and, when the nutrients are exhausted, it stops growing. The perception of signals from plant surfaces by pathogenic fungi seems to be the result of signaling pathways mediated by cyclic adenosine monophosphate (cAMP) and mitogen -activated protein kinase

(MAPK). The size of the turgor pressure inside an appressorium is 40 times greater than the pressure of a typical car tire. The turgor pressure of an appressorium is due to the enormous accumulation of glycerol in the appressorium, which, due to its high osmotic pressure, draws water into the cell and generates hydrostatic pressure that pushes the thin hypha (appressorial penetration peg) outward through the host cuticle.

Germination of Spores and Seeds

Almost all pathogens in their vegetative state are capable of initiating infection immediately. Fungal spores and seeds of parasitic higher plants, however, must first germinate. Spores germinate by producing a typical mycelium that infects and grows into host plants or they produce a short germ tube that produces a specialized infectious structure, the haustorium. In order to germinate, spores require a favorable temperature and also moisture in the form of rain, dew, or a film of water on the plant surface or at least high relative humidity.

Hatching of Nematode Eggs

Nematode eggs also require conditions of favorable temperature and moisture to become activated and hatch. In most nematodes, the egg contains the first juvenile stage before or soon after the egg is laid. This juvenile immediately undergoes a

molt and gives rise to the second juvenile stage, which may remain dormant in the egg for various periods of time. Thus, when the egg finally hatches, it is the second -stage juvenile that emerges, and it either finds and penetrates a host plant or undergoes additional molts that produce further juvenile stages and adults.

4.4.1.4 Penetration

This is the actual entry of the pathogen into theirs host plants pathogens penetrate plant surfaces in different ways:

(i) Direct penetration through intact plant surfaces

(ii) Through natural openings, and

(iii) Through wounds

Some fungi penetrate tissues in only one of these ways, others in more than one. Bacteria enter plants mostly through wounds, less frequently through natural openings, and never directly through unbroken cell walls. Viruses, viroids, mollicutes, fastidious bacteria, and protozoa enter through wounds made by vectors, although some viruses and viroids may also enter through wounds. Parasitic higher plants enter their hosts by direct penetration. Nematodes enter plants by direct penetration and, sometimes, through natural openings. Penetration does not always lead to infection (penetration in plants that are not susceptible)

Direct Penetration through Intact Plant Surfaces

Direct penetration through intact plant surfaces is probably the most common type of penetration by fungi, oomycetes, and nematodes and the only type of penetration by parasitic higher plants. None of the other pathogens can enter plants by direct penetration.

Penetration through Natural Openings

Many fungi and bacteria enter plants through stomata, and some enter through hydathodes, nectarthodes, and Lenticels. Stomata are most numerous on the lower side of leaves. Bacteria present in a film of water over a stoma and, if water soaking occurs, can swim through the stoma easily and into the substomatal cavity where they can multiply and start infection. Fungal spores generally germinate on the plant surface, and the germ tube may then grow through the stoma. Hydathodes are more or less permanently open pores at the margins and

tips of leaves; they are connected to the veins and secrete droplets of liquid, called guttation drops, containing various nutrients. Some bacteria use these pores as a means of entry into leaves, but few fungi seem to enter plants through hydathodes. Some bacteria also enter blossoms through the nectarthodes or nectaries. Lenticels are openings on fruits, stems, and tubers that are filled with loosely connected cells that allow the passage of air. During the growing season, lenticels are open; but even so, relatively few fungi and bacteria penetrate tissues through them.

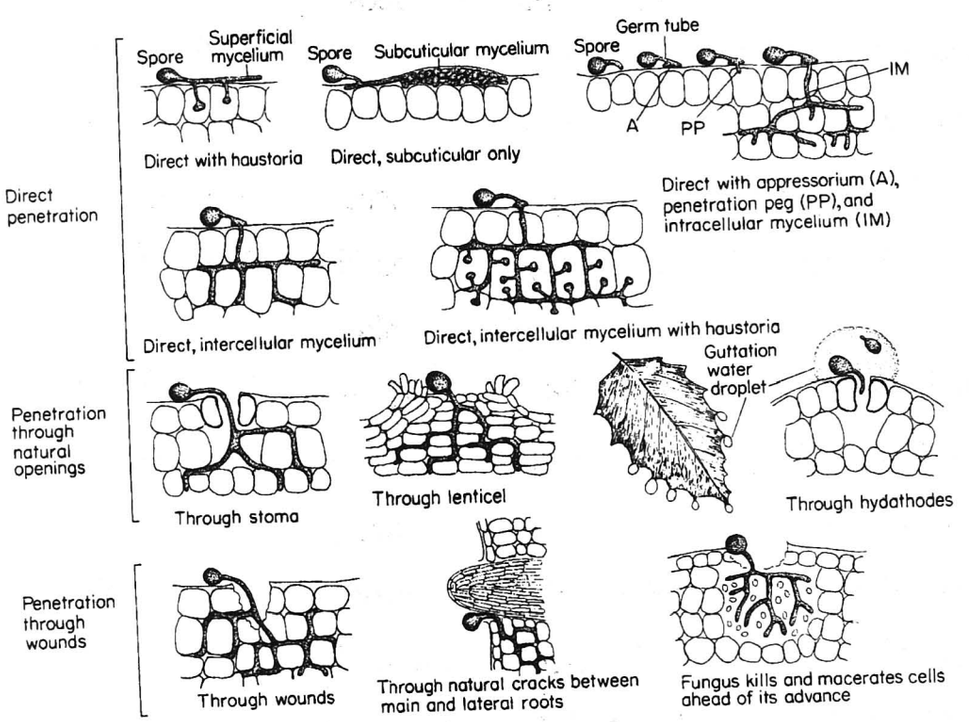


FIGURE 2-2 Methods of penetration and invasion by fungi.

Figure-26

4.4.1.5 Infection

This is the process by which a pathogen establishes contact with host cells or tissues and procures nutrients from them. This stage also includes invasion and to some extent growth and reproduction of the pathogen .During invasion, the pathogens colonise the host tissue in different ways and to different extent.

This time elapsing between penetration or more accurately spore germination and established infection is called period. Following infection, pathogens grow, multiply, or both within the plant tissues and invade and colonize the plant to a lesser or greater extent. Successful infections result in the appearance of symptoms. Some infections, however, remain latent, i.e., they do not produce

symptoms right away but at a later time when the environmental conditions or the stage of maturity of the plant become more favorable. Symptoms may appear as soon as 2 to 4 days after inoculation, as happens in some localized viral diseases of herbaceous plants, or as late as 2 to 3 years after inoculation, as in the case of some viral, mollicute, and other diseases of trees. In most plant diseases, however, symptoms appear from a few days to a few weeks after inoculation. The time interval between inoculation and the appearance of disease symptoms is called the incubation period. The length of the incubation period of various Diseases varies with the particular pathogen – host combination, with the stage of development of the host, and with the temperature in the environment of the infected plant.

Infection requirements:

1. The plant variety must be susceptible to the particular pathogen and at a susceptible stage.

2 -The pathogen must be in a pathogenic stage that can infect immediately without requiring a resting (dormancy) period first or infective juvenile stages or adults of nematodes.

3-The temperature and moisture conditions in the environment of the plant must favor the growth and multiplication of the pathogen.

Growth and reproduction of pathogen

Pathogens invade and infect tissue by growing and multiplying into them. In this way they colonize and infect more areas or paths of attacked plant. The period between infection, or more accurately spore germination and the appearance of visible symptoms is called incubation period. Thus incubation period includes the full life cycle of the pathogen. It may thus be seed that between spore germination and complete expression of the disease (symptoms), a series of events happens in the host. This chain of events between the time of infection, or more accurately spore germination and the complete expression of disease is called cycle or disease development. For spread of secondary inocula to perpetuate the disease in the field during growing season of plant dissemination is also sometimes included as a stage in disease development.

Seasonal carry-over of the inoculums to next season is also similarly included by some under disease development.

4.4.1.6 Invasion

Various pathogens invade hosts in different ways and to different extents. Some fungi, such as those causing apple scab and black spot of rose, produce mycelium that grows only in the area between the cuticle and the epidermis (subcuticular colonization); others, such as those causing powdery mildews, produce mycelium only on the surface of the plant but send haustoria into the epidermal cells. Most fungi spread into all the tissues of the plant organs (leaves, stems, and roots) they infect, either by growing directly through the cells as an intracellular mycelium or by growing between the cells as an intercellular mycelium. Fungi that cause vascular wilts invade the xylem vessels of plants. Bacteria invade tissues intercellulary, although when parts of the cell walls dissolve, bacteria also grow intracellularly. Bacteria causing vascular wilts, like the vascular wilt fungi, invade the xylem vessels. Most nematodes invade tissues intercellularly, but some can invade intracellularly as well. Many nematodes do not invade cells or tissues at all but feed by piercing epidermal cells with their stylets. Viruses, viroids, mollicutes, fastidious bacteria, and protozoa invade tissues by moving from cell to cell intracellularly. Viruses and viroids invade all types of living plant cells, mollicutes and protozoa invade phloem sieve tubes and perhaps a few adjacent phloem parenchyma cells, and most fastidious bacteria invade xylem vessels and a few invade only phloem sieve tubes. Many infections caused by fungi, bacteria, nematodes, viruses, and parasitic higher plants are local, i.e., they involve a single cell, a few cells, or a small area of the plant. These infections may remain localized throughout the growing season or they may enlarge slightly or very slowly. Other infections enlarge more or less rapidly and may involve an entire plant organ (flower, fruit, leaf), a large part of the plant (a branch), or the entire plant. Infections caused by fastidious xylem-or phloem inhabiting bacteria, mollicutes, and protozoa and natural infections caused by viruses and viroids are systemic, i.e., the pathogen, from one initial point in a plant, spreads and invades most or all susceptible cells and tissues throughout the plant.

4.4.1.7 Growth and Reproduction of the Pathogen (Colonization)

Individual fungi and parasitic higher plants generally invade and infect tissues by growing on or into them from one initial point of inoculation. Most of these pathogens, whether inducing a small lesion, a large infected area, or a general necrosis of the plant, continue to grow and branch out within the infected host indefinitely so that the same pathogen individual spreads into more and more plant tissues until the spread of the infection is stopped or the plant is dead. fungi causing vascular wilts often invade plants by producing and releasing spores within the vessels, and as the spores are carried in the sap stream they invade vessels far away from the mycelium, germinate there, and produce a mycelium, which invades more vessels. The bacteria, mollicutes, viruses, viroids, nematodes, and protozoa, do not increase much, if at all, in size with time, These pathogens invade and infect new tissues within the plant by reproducing at a rapid rate and increasing their numbers tremendously in the infected tissues. Plant pathogens reproduce in a variety of ways. Fungi reproduce by means of spores, which may be either asexual (mitospores , i.e., products of mitosis, roughly equivalent to the buds on a twig or the tubers of a potato plant), or sexual (meiospores , i.e. products of meiosis, roughly equivalent to the seeds of plants). Parasitic higher plants reproduce just like all plants, i.e., by seeds. Bacteria and mollicutes reproduce by fission in which one mature individual splits into two equal, smaller individuals. Viruses and viroids are replicated by the cell, just as a page placed on a photocopying machine. The rate of reproduction varies considerably among the various kinds of pathogens.

4.4.1.8 Dissemination of pathogen

After pathogen has grown and multiplied in or on the infected host, it spread to new, healthy plants. Dissemination is the transfer of inoculums from the site of its production to the susceptible host surface. Some pathogens disperse in active manner, whereas most passively with the help of an agent of dispersal. The chief agents of dissemination are (i) Air, (ii) water, (iii), vectors i.e. Insects, mites, nematodes etc. v)Man.

A few pathogens, such as nematodes, oomycetes, zoosporic fungi, and bacteria, can move short distances on their own power and thus can move from one host to another one very close to it. Fungal hyphae can grow between tissues in contact and sometimes through the soil toward nearby roots for a few to many centimeters. Both of these means of dissemination are quite limited, especially in the case of zoospores and bacteria. The spores of some fungi are expelled forcibly from the sporophore or sporocarp by a squirting or puffing action that results in the successive or simultaneous discharge of spores up to a centimeter or so above the sporophore. The seeds of some parasitic plants are also expelled forcibly and may arch over distances of several meters. Almost all dissemination of pathogens is carried out passively by such agents as air and insects. To a lesser extent, water, certain other animals, and humans may be involved. Dissemination by Air While airborne, some of the spores may touch wet surfaces and get trapped; when air movement stops or when it rains, the rest of the spores land or are "washed out" from the air and are brought down by the raindrops.

The spores of other fungi, particularly those of the cereal rusts, are very hardy and occur commonly at all levels and at high altitudes (several thousand meters) above infected fields. Spores of these fungi are often carried over distances of several kilometers, even hundreds of kilometers. Dissemination by Water, Insects, Mites, Nematodes, and Other Vectors Dissemination by Pollen, Seed, Transplants, Budwood, and Nursery Stock Dissemination by Humans some plant pathogens, e.g., the zoospores of some fungi and certain parasitic plants, can transmit viruses as they move from one plant to another (zoospores) or as they grow and form a bridge between two plants (dodder).

4. 5 Environmental effects on the development of plant diseases

Certain environmental conditions must exist for disease pathogens to cause infection. The specific conditions vary for different pathogens. High moisture and specific temperature ranges, for example, are necessary for many fungal diseases. These conditions must continue for a critical period of time while the pathogen is in contact with the host for infection to occur. Moisture, temperature, wind, sunlight, nutrition and soil quality affect plant growth. If one of these factors is

out of balance for the culture of a specific plant, that plant may have a greater tendency to become diseased. For example, lilacs growing in shade are more likely to be infected with powdery mildew than those growing in full sunlight. Environmental conditions also affect the growth and spread of disease pathogens. Very dry or wet weather will have an accompanying set of diseases that thrive under these conditions.

4.5.1Temperature

Each disease pathogen has a specific temperature range for growth and activity. There are warm-weather and cool-weather diseases. Many powdery mildew diseases are late summer, warmer temperature diseases. Temperature affects how rapidly pathogens multiply. Temperature also affects the incubation, or latent, period (the time between infection and the appearance of disease symptoms), the generation time (the time between infection and sporulation), and the infectious period (the time during which the pathogen keeps producing propagules). The disease cycle speeds up at higher temperatures, resulting in faster development of epidemics. The period of leaf wetness, combined with temperature information can be used to predict outbreaks of some diseases (infection periods) and be used to time preventative treatments, such as spraying. Soil temperature can also be critical for disease infection. Cool, wet soils promote fungal root diseases. Temperature extremes can cause stress in host plants, increasing susceptibility.

Several diseases (Fusarial and bacterial wilts, anthracnose) are favored by high temperature. In many canker diseases caused by fungi (*Nectria, Leucostoma, Phytophthora*), infection develop in early spring with high temperature. Pathogens like *Typhula, Fusarium* thrive only in cold season.

4.5. 2 Relative humidity

Relative humidity is very critical in fungal spore germination and the development of storage rots. *Rhizopus* soft rot of sweet potato (*Rhizopus stolonifer*) is an example of a storage disease that does not develop if relative humidity is maintained at 85 to 90 percent, even if the storage temperature is optimum for growth of the pathogen. Under these conditions, the sweet potato root produces suberized (corky) tissues that wall off the *Rhizopus* fungus.High

humidity favours development of the great majority of leaf and fruit diseases caused by fungi and bacteria. Moisture is generally needed for fungal spore germination, the multiplication and penetration of bacteria, and the initiation of infection. Germination of powdery mildew spores occurs best at 90 to 95 percent relative humidity. Diseases in greenhouse crops—such as leaf mold of tomato (*Cladosporium fulvum*) and decay of flowers, leaves, stems, and seedlings of flowering plants, caused by *Botrytis* species—are controlled by lowering air humidity or by avoiding spraying plants with water. Rain splash plays an important role in the dispersal of some fungi and nearly all bacteria, and a period of leaf wetness is necessary for the germination of most airborne spores. By using water for dispersal, propagules are dispersed at a time when they are likely to be able to germinate as well. Because the process of germination and infection takes time, the duration of leaf wetness also influences the success of the infection. The duration necessary for infection varies with temperature. Usually, a longer period of leaf wetness is needed to establish an infection in cooler temperatures, as germination and infection are generally accelerated in warmer conditions.

4.5.3 Wind and light

The combination of wind and sun affects how quickly plant surfaces dry. Faster drying generally reduces the opportunity for infection. Wind can spread pathogens from one area to another, even many miles. Wind and rain together can be a deadly combination. Windblown rain can spread spores from infected plant tissue, blowing these pathogens to new host plants. Sunlight is very important to plant health. Plants that do not receive the right amount of sunlight to meet their cultural requirements become stressed. This may make them more susceptible to infection. High soil temperatures early in the season may injure or kill plant tissues at the soil surface, resulting in a constricted stem; this is called heat canker. Bright sun, high temperatures, and strong dry winds may suddenly desiccate (dry) leaves of crops and garden plants, resulting in sunscald. When lightning strikes the ground it may kill plants in somewhat circular patches up to 50 feet in diameter. Reduced light intensity generally increases the susceptibility

of plants to viral diseases. Symptoms are severe when the plants are in normal light.

4.5.4 Soil moisture

High or low soil moisture may be a limiting factor in the development of certain root rot diseases. High soil-moisture levels favour development of destructive water mold fungi, such as species of *Aphanomyces*, *Pythium*, and *Phytophthora*. Excessive watering of houseplants is a common problem. Overwatering, by decreasing oxygen and raising carbon dioxide levels in the soil, makes roots more susceptible to root-rotting organisms. Diseases such as take-all of cereals (*Ophiobolus graminis*); charcoal rot of corn, sorghum, and soybean (*Macrophomina phaseoli*); common scab of potato (*Streptomyces scabies*); and onion white rot (*Sclerotium cepivorum*) are most severe under low soil-moisture levels.

4.5.5 Nutrition

Nutrition is a frequent cause of non-infectious disease. Either too much (excess) or too little (deficiency) can cause problems. For example, plants that are deficient in nitrogen develop a general yellowing, beginning with the lower leaves and progressing upward. Trace element deficiencies such as iron chlorosis, caused by iron deficiency, are common. Iron chlorosis occurs in many North Dakota trees and shrubs, especially silver maple, oak, and spirea. Iron chlorosis is recognized by progressively smaller leaves on the new growth; these leaves are yellow with green veins. When iron chlorosis is severe, leaves may turn brown and become brittle as well. Lime-induced chlorosis is common in our alkaline soils because the iron in the soil is not readily available to plants. Iron chlorosis also is common on certain soybean varieties. Zinc deficiency is common on dry beans and fairly common on flax, causing yellow leaves and stunted growth. Excess trace elements may also cause growth problems, but these are rare in North Dakota. Soil type can affect plant growth and also development of some pathogens. Light sandy soil low in organic matter favors growth of many types of nematodes. Damping-off disease increases in heavy, cold, water-logged soils. Soil pH affects pathogen development in some diseases. Clubroot of cabbage occurs in soils with

a low pH, for example. High soil pH is a factor in the development of scab on potatoes. Fertility affects a plant's growth rate and ability to defend against disease. Excessive nitrogen fertilization can increase susceptibility to pathogen attack. It causes formation of succulent tissue and delays maturity. This can contribute to certain patch diseases in lawns. Nitrogen deficiency results in limited growth and plant stress which may cause greater disease susceptibility.

4.5.6 Edaphic factors

The edaphic (soil) environment affects soil-borne diseases, largely by determining the amount of moisture available to pathogens for germination, survival and motility. Germination and infection success also rely on the temperature of the soil. The fertility and organic matter content of the soil can affect the development of disease. Plant defences are weakened by nutrient deficiency, although some pathogens, such as rusts and powdery mildews, thrive on well-nourished plants. Other diseases thrive in soils that are specifically low in organic matter. The pH of the soil is important in the occurrence and severity of plant diseases caused by certain soilborne pathogens. The club root of crucifers caused by *Plasmodiophora brassicae* is more prevalent and severe at pH 5.7, whereas the development drops sharply between pH 5.7 and 6.2 and it is completely checked at pH 7.8. Potato scab will be severe from pH 5.2 to 8. Cotton root rot fungus (Phymatotrichopsis) grows best at high pH (pH 7.2 -8).

4.6 Selected plant diseases

4.6.1 ANGULAR LEAF SPOT OF COTTON

Bacterial blight of cotton (also called angular leaf spot) is an important and potentially destructive bacterial disease. The disease is caused by a bacterium, *Xanthomonas campestris* pv. *malvacearum*.

Symptoms

Pathogen can attack all plant parts, infecting stems, leaves, bracts and bolls. On cotyledons small, green, water-soaked rounded (or irregular) spots form which turn brown. Cotyledons can be distorted if the infection is intense. Black and elongated lesions can girdle the hypocotyls and kill seedlings (**Seedling blight**). On the leaves, scattered small dark-green, water-soaked, areolate spots, form

measuring 1-2 mm on the lower surface, which appear translucent against transmitted light.. The spots increase in diameter to 5 mm, become angular, brown and later turn dark brown to blackish, becoming visible on the upper surface hence named **"angular leaf spot".** The angular appearance is due to restriction of the lesion by fine veins of the cotton leaf. Spots on infected leaves may spread along the major leaf veins. As disease progresses, leaf petioles and stems may become infected resulting in premature defoliation .Black cankers may girdle the stem or branches causing the portions to die above the canker (**'Black arm'**).rust containing the bacterium may form on old leaf spots or cankers. On bolls, small, rounded, raised, water-soaked lesions form, which later can expand to 1 cm in diameter, becoming irregular, brow to black and sunken, results in reduced boll size, poor production of lint and loss in viability in seed. Bolls may become infected causing boll rot which results in rotted seed and discolored lint. Infected bolls have round, rather than angular, lesions that initially may appear water-soaked. As infection proceeds, bolls lesions will be sunken and dark brown or black (**Boll rot**).

Transmission: Primary infection starts from the seeds, where the pathogen remains on the fuzz as a slimy mass. It enters the leaves through stomata. Secondary spread is favored by wind driven rain splash and dew.

Epidimiology: The extent to which bacterial blight will impact cotton yield in infested fields will depend on environmental conditions during the season. High rainfall, and humidity as well as warm temperatures favor disease development. High humidity and moderate temperature (28 °C) favours the development of the disease. Primary infection is favoured by 30 °C and secondary infection is better at 35 °C. Presence of moisture is very important for the first 48 hours. Dry and hot weather retards disease development.

Disease management:

1.Use of healthy seed from healthy plants.

2.Delinting seeds with concentrated sulphuric acid then floating the delinted seeds in water and removal of the floating seeds.

3.Disinfections of seeds with 1000 ppm streptomycin sulphate solution overnight.

4.Delinted seeds are treated with organomercurials like Agrosan or Ceresan(3g/kg of seed)

5.Secondary spread is controlled by spraying 1% Bordeaux mixtureor 0.25% copper oxychloride or 300ppm streptomycin sulphate .

6.Destruction of diseased plant debris, and

7.Killing of volunteer seedlings.

8.Crop rotation

9.Addition of potash to soil

4.6.2 BLAST DISEASE OF PADDY

Rice blast, caused by a fungus, causes lesions to form on leaves, stems, peduncles, panicles, seeds, and even roots. So great is the potential threat for crop failure from this disease that it has been ranked among the most important plant diseases of them all. It is caused by Pyricularia oryzae , a fungus. It was first recorded in China in 1637 In India it was first reported in Tanjore district of Tamil Nadu.

Systematic position of *P. oryzae*:
Division: Eumycota
Sub division: Deuteromycotina
Class: Hypomycetes

Order: Moniliales

Family: Moniliaceae

Its mycelium is branched filamentous, septate, hyaline or olive coloured and intercellular. Mature conidia of *P. oryzae* are usually three-celled or 2 septate (rarely 1 or 3), pyriform (pear-shaped), hyaline or colorless to pale olive, 19-27 x 8-10 µm in size and exhibit a basal appendage at the point of attachment to the conidiophore. Conidia usually germinate from the apical or basal cells. The conidiophores are pale brown, smooth and straight, or bending. The perfect stage is rarely found in the field.

Epidemiology: Day temperature (30°C), night temperature (20°C) and day light (14 hours) found to predispose the plants to infection. Relative humidity (92%) and free water required for conidial germination and infection. Spores do not germinate in direct sun light .Cloudy overcast weather, dew drops encourage blast spread . Conidia exhibit nocturnal pattern of diurnal periodicity with peak

concentration of spore dispersal occurring around 4AM favored by night temperature (25 - 27°C) and relative humidity (86 -98%). Conidia could remain viable under snow to over winter period and 4 - 6 months after harvest.

Symptoms :

Disease can infect paddy at all growth stages and all aerial parts of plant (Leaf, neck and node). Among the three leaf and neck infections are more severe. The symptoms of rice blast include lesions that can be found on all parts of the plant, including leaves, leaf collars, necks, panicles, pedicels, and seeds. Leaf spots are spindle shaped which are grey centered surrounded by brown margin. The symptoms on leaves may vary according to the environmental conditions, the age of the plant, and the levels of resistance of the host cultivars. On susceptible cultivars, lesions may initially appear gray-green and water-soaked with a darker green border and they expand rapidly to several centimeters in length. On susceptible cultivars, older lesions often become light tan in color with necrotic borders. On resistant cultivars, lesions often remain small in size (1-2 mm) and brown to dark brown in color. The collar of a rice plant refers to the junction of the leaf and the stem sheath. Symptoms of infection of the collars consist of a general area of necrosis at the union of the two tissues. Collar infections can kill the entire leaf and may extend a few millimeters into and around the sheath. The fungus may produce spores on these lesions.

The neck of the rice plant refers to that portion of the stem that rises above the leaves and supports the seed head or panicle. Necks are often infected at the node by the rice blast fungus and infection leads to a condition called rotten neck or neck blast. Infection of the necks can be very destructive, causing failure of the seeds to fill (a condition called blanking) or causing the entire panicle to fall over as if rotted. The rice blast fungus can also infect the panicles as the seeds form. Lesions can be found on the panicle branches, spikes, and spikelets. The lesions are often gray brown discolorations of the branches of the panicle, and, over time, the branches may break at the lesion. Seeds are not produced when pedicels become infected, a condition called blanking. Symptoms of rice blast on seeds themselves consist of brown spots and blotches.

Disease management:

- Use seed from a disease - free crop
- Destruction of wild collateral hosts
- Timely removal of weed hosts
- Destruction of infected plants
- Avoid excess N - fertilizer application
- Use of tolerant varieties (Penna, Pinakini, Tikkana, Sreeranga, Simphapuri, Palghuna, Swarnamukhi, Swathi, Prabhat, IR - 64, Jaya, IR - 36, MTU 9992, MTU 1005, MTU 7414)
- Burning of straw and stubbles after harvest

Chemical Control

- Seed treatment at 2.5 gm/kg seed with Capton or Carbendazim or Thiram or Triclyclazole.
- Spraying of Triclyclazole at 0.6 gm/liter of water or Edifenphos at 1 ml/lit of water or Carbendazim at 1.0 gm/lit.
- 3 to 4 sprays each at nursery, Tillering stage and panicle emergence stage may be required for complete control.

Nursery stage : Light Infestation - Spray carbendazim or Edifenphos @ 0.1 %.

Pre-Tillering to Mid-Tillering

- Light at 2 to 5 % disease severities - Apply Ediphenphos, Carbendazim or 1 BP 48 @ 0.1 %. Delay top dressing of N fertilizers when infection is seen.

Panicle initiation to booting :

- At 2 to 5% leaf area damage spray Edifenphos orCarbendazim or Pyroquilon or 1 BP 48 @0.1 %.

Flowering and after

At 5 % leaf area damage or 1 to 2 % neck infection Spray Edifenphos,Carbendazim@0.1 % or Triclyclazole @ 1 gm /lit of water

4.6.3 BUNCHY TOP OF BANANA

Banana Bunchy Top is a serious disease which threatens all banana varieties in commercial plantations and in backyards. *Banana bunchy top virus* (BBTV) is the pathogen which causes banana bunchy top disease of bananas. It is considered to

be the most economically destructive of the virus diseases affecting bananas worldwide.

Symptoms:

The first symptom is short dark dot-dash lines appearing along the veins of the youngest leaf starting from the mid-rib..Dark green stripes running along the mid-rib of the infected leaf may also be present. When the disease is more advanced, each new leaf becomes shorter, narrower and stands more upright, giving a 'bunched' leaf appearance – that's why it is called 'Bunchy Top'. On mature plants infected with BBTV, new leaves emerge with difficulty, are narrower than normal, are wavy rather than flat, and have yellow (chlorotic) leaf margins. They appear to be "bunched" at the top of the plant, the symptom for which this disease is named. Severely infected banana plants usually will not fruit, but if fruit is produced, the banana hands and fingers are likely to be distorted and twisted.

Transmission:

Bunchy Top can only be spread by aphids (*Pentalonia nigronervosa*) and infected planting materials. The aphid is a small black insect which looks similar to other aphids. The difference is that the banana aphid spreads Bunchy Top after it has fed for many hours on an infected plant. The aphid can retain the virus through its adult life, for a period of 15–20 days. Aphids can by carried many kilometres on the wind so they can progressively infect many banana plantations and backyard plants.

Control:

Bunchy top cannot be cured and affected plants must be destroyed. Control depends on prompt detection and destruction of infected stools by a trained inspector. There are strict quarantine restrictions to prevent movement of contaminated planting material. Control also depends on the use of uninfected planting material and intensive eradication schemes. Control of banana bunchy top is achieved by killing the banana aphids then destroying all infected material. The aphids should be controlled by spraying with an effective insecticide like metasystox 0.1 to 0.5 per cent or dimecron or parathion. Spraying of soap water also helps in checking of aphid population.

Injection of monocrotophos solution diluted with water at 1:4 ratio at 30-day interval twice or thrice at 2-3 months after planting is effective. Infected banana plants can be first sprayed with an insecticide like Sevin, or simply soapy water. Only the whorl and upper part of the trunk are sprayed to kill the aphids, which like to hide deep in the central whorl. Regular inspection and destruction of weeds and alternative hosts are important BBTV reducing factors. An isolation distance of 100-200 mts between two new banana orchards is very beneficial to control BBTV. Removal of diseased banana plants and replanting with virus- free banana plants leads to effective management strategy. Destruction of wild or unattended patches of bananas is must.

4.7 Plant protection – Exclusion

Exclusion is one of the methods adopted to prevent the spread of pathogens to areas where they do not already exist. It is effectively carried out through plant quarantine. **Plant quarantine** may be defined as the restriction imposed by duly constituted authorities on the production, movement and existence of plants or plant materials or products to prevent the introduction or spread of a pest or pathogen. When plant pathogens are introduced into an area in which they did not previously exist, they are likely to cause much more catastrophic epidemics than do the existing pathogens. Some of the worst plant disease epidemics that have occurred throughout the world, for example the downy mildew of grapes in Europe and the bacterial canker of citrus, the chestnut operation of the quarantine regulations. It is extremely difficult to predict accurately whether an exotic organism will become established, and once established, become economically important.

Examples of diseases introduced into other countries:

Disease	Pathogen	Introduced in	Introduced from
Late blight of potato	*Phytophthora infestans*	Europe	S. America
Citrus canker	*Xanthomonas citri*	USA	Asia
Downy mildew of grapes	*Plasmopara viticola*	France	USA
Paddy blast	*Piricularia oryzae*	India	South east Asia
Bunchy top of banana	Banana bunchy top virus	Srilanka	India

The materials which are imported or exported are thoroughly checked at the quarantine station and issued with a phytosanitory certificate/ phytopathological certificate if free of pest or pathogen. Plant materials on reaching the destination must be inspected at the customs ports and allowed their entry. There are 35 Plant quarantine Stations at different Airports, Seaports and Land frontiers implementing the Plant Quarantine regulations. The NPQS, New Delhi and RPQSs at Chennai, Kolkata, Amritsar and Mumbai have been strengthened with modern equipment for plant quarantine testing, etc., to facilitate speedy clearance of imports and exports.. The National Plant Quarantine Station at Rangpuri, New Delhi and four Regional Plant Quarantine Stations at Amritsar, Chennai, Kolkata and Mumbai are the major stations and are located at places having international airport/seaport/land frontier with neighboring countries. National Bureau of plant genetic resources, New Delhi which is the nodal institution for exchange of plant

genetic resources has been empowered under PQ order to handle quarantine processing of germplasm and transgenic planting material being imported for research purposes in the country. As per the recent amendments made under the PQ order, the Advanced Centre for Plant Virology at JARI, New Delhi, Indian Institute of Horticultural Research, Bangalore and Institute of Himalayan Bioresource Technology, Palampur have been identified for ensuring virus-free status in the imported *in vitro* material. Under the DIP Act, there is a provision of Domestic Quarantine to restrict the inter-state movement of nine invasive pests viz flute scale, sanjose scale, coffee berry borer, codling moth, banana bunchy top and mosaic viruses, potato cyst nematode, potato wart and apple scab.

Plant Quarantine Facilities in India:

Plant Quarantine regulatory measures are operative through the **"Destructive insects & pests Act, 1914 (Act 2 of 1914)"** in the country. The purpose and intent of this Act is to prevent the introduction of any insect, fungus or other pest, which is or may be destructive to crops. The import of agricultural commodities is presently regulated through the Plant Quarantine (Regulation of Import into India) Order, 2003 issued under DIP Act, 1914 incorporating the provisions of New Policy on Seed Development, 1988.

The primary objectives of the Scheme are to prevent the introduction and spread of exotic pests that are destructive to crops by regulating/restricting the import of plants/plant products and to facilitate safe global trade in agriculture by assisting the producers and exporters by providing a technically competent and reliable phytosanitary certificate system to meet the requirements of trading partners.

The major activities under the scheme include

- Inspection of imported agricultural commodities for preventing the introduction of exotic pests and diseases inimical to Indian Fauna and Flora
- Inspection of agricultural commodities meant for export as per the requirements of importing countries under International Plant Protection Convention (IPPC)
- Detection of exotic pests and diseases already introduced for containing/ controlling them by adopting domestic quarantine regulations.

- Undertaking Post Entry Quarantine Inspection in respect of identified planting materials.
- Conducting the Pest Risk Analysis (PRA) to finalise phytosanitary requirements for import of plant/plant material.

Plant diseases introduced in India before and after the enforcement of quarantine:

Disease	**Pathogen**	**Country introduced from**
Bunchy top of banana	Banana bunchy top virus	Srilanka
Downy mildew of cucurbits	*Erysiphe* sp.	Srilanka
Leaf rust of coffee	*Hemileia*	Srilanka
Late blight of potato	*Phytophthora infestans*	Europe
Onion smut	*Urocystis*	Europe
Paddy blast	*Piricularia oryzae*	South east Asia
Flag smut of wheat	*Urocystis tritici*	Australia
Wart disease of potato	*Synchytrium*	Netherland

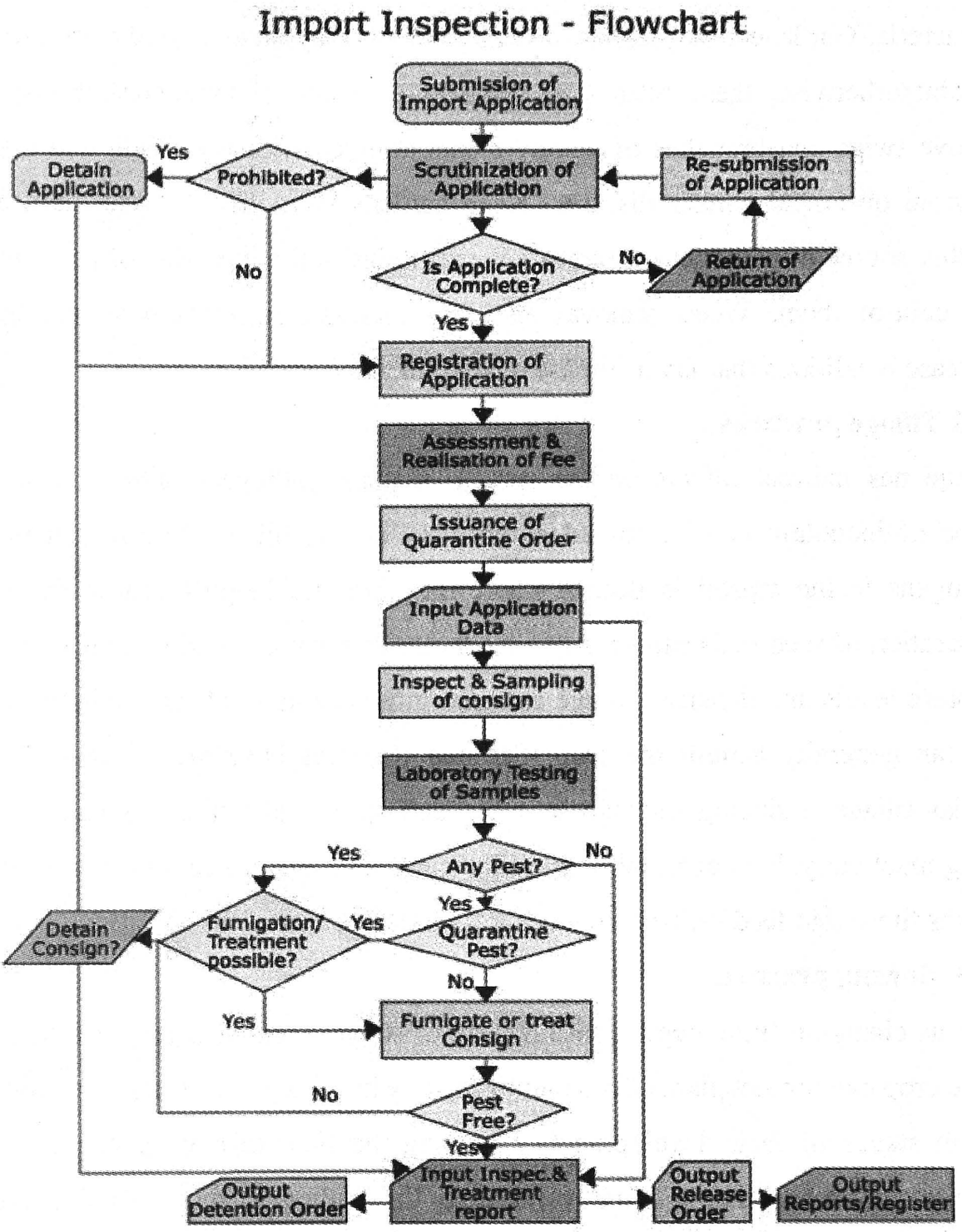


Figure-27

4.8 ERADICATION

Eradication is concerned with elimination of the disease agent after it has become established in the area of the growing host or has penetrated the host.

Methods of eradication

4.8.1 Sanitation

Plant pathogens are less likely to survive if organic matter is quickly decomposed. Remove plant debris or infected plant parts after each growing season. Turn the

soil after harvest to help break down small roots that may harbor nematodes, fungi or bacteria. Gardeners may compost dead plants if they have a good composting system; otherwise, these piles may serve as a source of pathogens. Prune or remove twigs and branches of woody plants affected with fire blight and other bacterial or fungal canker diseases. Keep gardens weed free. Weeds often are another source of pathogens. Eradicate weeds to break the life cycle of pathogens and control them. Weed removal also can increase air movement and thus decrease conditions that favor disease development.

4.8.2 Tillage practices

Tillage has indirect effects on the spread of plant pathogens, although, some forms of inoculum can be spread extensively during tillage. Tillage can bury pathogens in the topsoil in deeper where they are less likely to cause disease. Preparation of seed beds can greatly alter the soil's texture, aeration, temperature, moisture levels and density. Tillage can also influence nutrient release in the soil and can generally benefit the crop. Farming practices have moved away from regular tillage, reducing damage to roots and spread of pathogens caused by tilling machinery. However, minimum tillage can also encourage some pathogens, such as those that feed on crop residues left on the surface of the soil.

4.8.3 Sowing practices,

such as changing time, depth and direction of sowing, and changing the density of the crop can protect plants from pathogens to which they are susceptible only at certain stages of their development. Changing the time of sowing can exploit weather conditions that are unfavorable to the pathogen, thus reducing crop losses. This might require the use of a specific cultivar that is adapted to the selected growing period, but might also be susceptible to different pathogens. The depth of sowing can have a bearing on the chance of infection, as the seedling's pre-emergence stage, which is usually more susceptible to attack, is longer when seeds are planted deeper. However, deeper planting can stimulate germination.

4.8.4 Crop Rotation

Avoid planting the same crop in the same area of the garden year after year. Continuous culture of the same kind of crop provides an opportunity for pathogen buildup. For example, rotate leafy vegetables with grains or corn, or rotate annuals or biennials in seed and flower beds. It is best to grow the same or closely related plants in the same soil only once every three to five years. This practice starves out most pathogens that cause leaf, flower and stem diseases. Crop rotation is not as effective against soil-borne organisms, those fungi, bacteria and nematodes that persist in the soil for up to 10 years or more. Crop rotation is a frequently used strategy to reduce the quantity of a pathogen, usually soil-borne organisms, in a cropping area. Take-all of wheat (caused by *Gaeumannomyces graminis*) and soybean cyst nematode (*Heterodera glycines*) are two examples of soilborne diseases that are easily managed by short rotations of 1 and 2 years, respectively, out of susceptible crops, which may include susceptible weed hosts such as grasses in the case of take-all.

4.8.5 Eradication of alternate host

Barberry eradication and *Ribes* eradication can be done for reducing stem rust (caused by *Puccinia graminis*) of wheat and white pine blister rust respectively. The strategy is that removing these alternate hosts breaks the disease cycles and prevents infection of the economically more valuable host. However, stem rust can readily spread from wheat to wheat in many regions by the uredinial stage although elimination of the aecial host, barberry, may deter or diminish the development of pathogenic races of the rust. The white pine blister rust fungus is perennial in the pine host and eradication of the alternate host only protects noninfected trees but does not necessarily eliminate the pathogen from the area.

4.8.6 Eradication of over wintering hosts

Eradication may also be accomplished by destroying weeds that are reservoirs of various pathogens or their insect vectors. Elimination of potato cull piles is an effective method of eradicating over wintering inoculum of the late blight pathogen.

4.8.7 Removal of infected plant parts

Eradication can also be on a more modest scale such as the removal of apple or pear branches infected by the fire blight bacterium (*Erwinia amylovora*) or pruning to remove blister rust cankers (caused by *Cronartium ribicola*) on white pine branches. Or, it can be the sorting and removal of diseased flower bulbs, corms or rhizomes. Hot water seed-treatment of cereal seeds to kill smut mycelium in the seed and heat treatment to eliminate viruses from fruit tree budwood for grafting are other examples of pathogen eradication.

4.8.8 Cultural Management

Cultural disease management practices are the measures undertaken by humans to prevent and control disease by manipulating plants. Use good cultural management. Healthy plants are less likely to have disease problems than weak, undernourished ones. Grow plants under optimum conditions and there will be fewer disease problems Cultural management involves avoiding the onset of disease. To accomplish this without pesticides, create an environment unfavorable to pathogens.

- Fertilize plants properly based on soil nutrient analyses using either organic or commercially prepared (inorganic) fertilizers.
- **Crop density** and disease incidence are usually correlated, mainly due to the ease with which inoculum is transferred between plants when they are close together and their leaves and roots are able to touch. Also affecting plant susceptibility in densely planted crops is the microclimate created by their crowding. Temperatures are more uniform, humidity is increased and leaves stay wet for longer, all of which favour the development of disease. Crop density can be manipulated by sowing, pruning, thinning, trellising, fertilisation, water management, staking and harvesting some plants or plant parts.
- **Intercropping:** The practice of planting more than one crop in alternating rows, or intercropping, can reduce disease by increasing the distance between plants of the same species, and creating a physical barrier between plants of the same species. Intercropping is more labour intensive the more crops there are, but it is usually beneficial. How successful intercropping is depends partly on the

combination of crop plants chosen, since some combinations can actually make disease worse by providing an alternate host or a stimulus that encourages germination of inoculum on the neighboring species.

- **Strip farming** is a similar practice to intercropping, where areas of one crop are separated from each other with strips of another crop, the rationale being that the two different crops are unlikely to share the same pathogens, thus reducing the rate of spread. The main disadvantage of strip farming is the fact that it is relatively labour intensive.
- **Mulching and soil amendments:** Mulches are used to conserve moisture and organic matter and reduce erosion in the soil. They are usually some kind of organic matter, such as straw, sawdust, manure or aquatic plants. Some manufactured products, such as plastics, asphalt paper and paper are also used. If crop residues are used for mulch they can provide a food source and attractive environment for pathogens and increase the incidence of a disease. Mulch or crop residues can also influence disease incidence by altering the immediate environment. Water retention, nutrient enrichment of the soil, decrease in soil temperature, weed inhibition and seedling protection are all effects of mulching that can influence disease development. Spread of soil-borne diseases that rely on splash-dispersal can be reduced, but other diseases, for which the altered environment is favourable, can be enhanced. The addition of organic amendments to the soil can also reduce disease influence by increasing the activity of competing or predatory micro-organisms in the soil, although it can also increase the incidence or severity of diseases caused by pathogens that thrive on the amendment.
- Avoid excessive soil moisture. Overwatering enhances seed decay, damping-off and root rot diseases. Try not to plant in areas that have poor drainage or where water stands for several days following rains.
- **Flooding** can be used as a form of disease management, as in the example of growing rice in paddy fields. Its primary purpose is to reduce weeds, but it can also reduce the number of fungal propagules, insects and nematodes in the soil. While flooding can aid the destruction of crop debris carrying inoculum, it can

also carry propagules of some pathogens growing in the flood waters. Flooding has variable success in disease management, depending on the pathogens present.

- Most forms of **irrigation** play a detrimental, rather than beneficial role in plant disease management. For example, irrigation during dry seasons prevents the desiccation of propagules during drought, thereby increasing the level of inoculum. The irrigation water can also carry propagules and spread disease, unless it is treated before use. Similarly, overhead watering can prolong leaf wetness, increasing the likelihood of germination and infection by fungal spores. It can also facilitate splash-dispersal of inoculum. Alternate drying and wetting of soil encourages the activity of micro-organisms that destroy sclerotia, and brief overhead watering can wash spores away without causing long periods of leaf wetness. Trickle or drip irrigation is generally the least likely to encourage disease, since water is delivered directly to root zones and at a rate insufficient to disperse pathogens.

4.8.9 Soil Sanitation Treatments

Occasionally, disease-causing organisms that live in the soil may build up and prevent satisfactory growth of plants. Pathogen-free soil is desirable for houseplants, transplants and garden plots. Sterile potting mixes are available at many garden centers. There are several nonchemical methods available to eradicate or reduce pathogens in the soil. The use of dry, steam or solar heat are the most effective nonchemical means to sanitize soil. The time to treat soil is before seeding or transplanting. Soil to be treated must be easily crumbled and without clods or large pieces of plant debris. Add any amendments (manure, compost, peat moss, etc.) **before treatment**. Soil also must have proper moisture. To test for this, gently squeeze a handful of soil. When the hand is opened, the soil ball should break apart somewhat. If it doesn't and the ball cannot be broken apart by gently pushing down on top of the ball, the soil is too wet.

4.8.10 Soil fumigation

This has been a widely used eradication strategy. This technology involves introducing gas-forming chemicals such as carbon disulfide, methyl bromide, or chloropicrin into soil to kill target pathogens. However, undesirable side effects

such as killing beneficial organisms, contamination of groundwater, and toxicity of these chemicals have resulted in less reliance on this approach for disease management. Volatile fumigants like methyl bromide are injected into soil and sealed with a plastic film. Some water-soluble fumigants can be injected into the soil and the soil simply compacted to form a seal.

4,9 CHEMICAL PROTECTION

Chemical disease control employs the use of chemicals that are either generally toxic and used as disinfectants or fumigants or chemicals that target specific kinds of pathogens, as in the case of fungicides, bactericides (or antibiotics) and nematicides.

Fungicides are chemicals used in the control of fungal diseases. They are often classified as either protectant or systemic. Protectant fungicides are usually effective against a broad range of fungi and protect the plant against infection on the surfaces of the plant to which they are applied. Often, they require multiple applications during the growing season to maintain coverage as new growth emerges and weathering removes past coverage. Systemic fungicides can be absorbed by the plant without harming it, and transported to other tissues where they are toxic to fungi. These compounds can control and eradicate established infections, but they are also vulnerable to fungi developing resistance, as they generally only target one step in a biosynthetic pathway to kill the fungus.

I. SULPHER FUNGICIDES

a) Inorganic Sulpher fungicides - includes elemental Sulpher, wettable Sulpher & lime Sulpher. Sulpher fungicides effectively control powdery mildew of different crops such as chilli, okra, grape, rubber, mango, citrus, black gram & green gram. Sulpher controls tikka leaf spot of groundnut & Diplocapron black spot of rose. Sulpher dust is used as seed treatment also.

b) Organic Sulpher fungicides- Dithiocarba mates are the organic Sulpher fungicides.

They are divided into- (i) Dialkyldithiocarbamtes- Thiram, Ziram & ferban & Ziram is used as Protectant & sprayed before the outbreak of the disease. It controls early blight of potato & tomato and anthracnose disease of cucurbits &

beans. Thiram is commonly used for seed treatment. Thiram seed treatment controls seed-borne pathogens as well as soil born pathogens. It controls seed borne infection of *Colletotrichum capsici* of chilli, root rot of groundnut, sorghum grain smut & *Helminthosporium* leaf spot of rice. As seed treatment it controls soil-borne infection of *Pythium* spp. of tomato, tobacco & brinjal (Damping off) *Rhizoctonia solani* of cotton & Sheath blight of rice.

Ferban control diseases of apple. It controls leaf spot of banana, leaf mould of tomato & leaf spot of coffee.

(ii) Monoalkyldithio carbamates- maneb, Zineb, Mancozeb, vapam & nabam. Zineb controls anthracnose disease of bean, chilli, & cucurbits, rust disease of wheat, sorghum & bajra, downy mildew of grapevine, cucurbits, Onion & cabbage, *Cercospora* leaf spot of groundnut, cabbage, cauliflower, *Alternaria* leaf spot of potato, tomato singer. Mancozeb is widely used for the control of late blight of potato, *Cercospora* leaf spot of groundnut, cucurbits & sugar beet. *Helminthosporium* leaf spot of rice, ragi, maize & sorghum, downy mildew of grapevine & tobacco, rust disease of wheat groundnut, bajra & sorghum, *Alternaria* leaf spot of ginger, potato, tomato & wheat anthracnose of chilli, grapevine, sorghum, bean & cucurbits.

Vapam controls cotton wilt & damping off of papaya, tobacco & tomato. It controls nematodes also.

II. Copper Fungicides

(a) Copper Sulphate Preparations – It include Bordeaux mixture, Bordeaux Paste, Burgundy mixture, & chestnut compound. Bordeaux mixture is highly effective against late blight of potato, downy mildew of grapevine, coffee rust, betel vine wilt, pepper wilt, tomato early & late blights & coconut will & bud rot.

(b) Copper Carbonate Preparations- It controls many fungal diseases of apple, pear, peach, plums & apricot.

c) Copper Oxychloride Preparations – Some formulations available in the market are Fytolan, Blue copper, Blitox etc. They are generally effective against all diseases against which Bordeaux mixture has been found effective.

III. Mercury Fungicides-

Various mercury fungicides sold in the market are ceresan, aretan, agallol, wet ceresan, Dry ceresan etc. They are effective as seed treatment.

IV Heterocylic nitrogen compounds-

a) Captan- It is commercially marketed as Captan, orthocide, vancide etc. It is Protectant fungicide. It controls maize helminthosporiose, chilli fruit rot & apple scab. It is mostly used as seed treatment.

b) Folpet – It is commercially marketed as Phaltan. It controls rose black spot & apple scab.

c) Captofol – It is marketed as Difolotan, Foltaf etc. It effectively controls sheath rot of rice & mango anthracnose.

V. Quinone Fungicides-

a) Chloranil – It is commercially marketed as spergon. It is good seed dressing fungicide. It controls grain smut of sorghum & damping off of beans & cotton.

b) Dichlone- The commercial name of the fungicide is phygon. It controls peach leaf curl, apple scab & bean anthracnose.

Miscellaneous Fungicides-

a) Quintozene - Commercial names of the fungicide are Brassicol, Terraclor, PCNB & Tritisan. It is used to control soil borne pathogens. It is effective against Rhizoctonia Solani, Macrophomina Phaseolina & Sclerotiana Sclerotiorum.

b) Dinocap - It is marketed in the name of karthane, Arathane, Mildex etc. Dinocap is effective in controlling powdery mildews.

c) Fenaminsosulph- it is commercially known as Dexon. It is highly effective against phycomycetes like pythium phytophthora & Aphanomyces.

d) Dicloran_ Its trade name is Botran. It controls Botrytis infection in several crops.

e) Chlorothalonil- It is marketed as Daconil & Kavach. It controls both tikka leaf spot & rust disease of groundnut & betelvine wilt.

SYSTEMIC FUNGICIDES

Some of the systemic fungicides are-

a) Carbendazim – It is marketed as Bavistin, Derosal, B-Sten etc. It controls Powdery Mildews, smut diseases & bunts.

b) Benomyl- Benomyl is effective *against Fusarium , Rhizoctonia, Macrophomina, Cercospora, Colletotrichum, Puricularia, Verticillium, Phomopsis, Septoria, Erysiphe, Plasmodiophora, Botrytis, Ustilago, Urocystis & Tilletia* spp.

c) Thiabendazole – It is commercially available as Tecto & Mertect. It controls wheat bunt.

d) Carboxin- it is commercially available as vitavax. It is the most effective fungicide to control internally seed borne loose smuts of cereals. e) Oxycarboxin- it is commercially marketed as Plantvax. It is specifically effective against rust pathogens.

f) Pyracarbolid- it is commercially available as sicarol. It is effective against rust & smut & *Rhizoctonia* spp.

g) Metalaxyl- it is marketed as Ridomil & Apron. Metalaxyl is highly effective against phycomyces fungi like *Phytophthrora, Pythium, Scierospora, Pseudopernospora, Plasmopara, Sclerophthora & Albugo.*

h) Tride morph- its trade name is calixin. It is mainly used against powdery mildews as foliar sprays

i) Pyroquilon- it is commercially available as Fongorene. It effectively controls rice blast.

j) Kitazin- it is commercially marketed as Kitazin. It is highly effective against rice blast.

k) Tricyclazole – it is commercially marketed as Beam. It is highly specific for the control of rice blast.

l) Probenazole- its commercial name is oryzemate. It is also specific against rice blast.

m) Triadimefon- it is commercially market as Bayleton. It is highly effective against powdery mildews. It also controls rust diseases.

n) Biloxazole- it is marketed as Baycor. It is effective against cercospora diseases & rusts.

o) Triademenol- The commercial name of the fungicide is Bayton. As seed treatment it controls smut & powdery mildews.

MODE OF ACTION OF FUNGICIDES

Fungicides are metabolic inhibitors. Some fungicides target fungal intracellular membrane systems and their biological functions. A widely used fungicidal compound, acriflavine (3,6-diamino-10-methylacridin-10-ium chloride), increases mitochondrial permeability and releases cytochrome c in fungal cells, repressing plasma membrane receptor activation, disordering proton stream and collapsing the electrochemical proton gradient across mitochondrial membranes. As a consequence, ATP synthesis is decreased leading to cell death.

Inhibitors of electron transport chain: (Respiration in mitochondria)

- Sulfur - Disrupts electron transport along the cytochromes
- Strobilurins (azoxystrobin, kresoxim-methyl, pyraclostrobin, trifloxystrobin)
  - Inhibit mitochondrial respiration, blocking the cytochrome bc1 complex.

Inhibitors of enzymes:

- Copper
  - Nonspecific denaturation of proteins and enzymes.
- Dithiocarbamates (maneb, manzate, dithane, etc)
  - Inactivate –SH groups in amino acids, proteins and enzymes.
- Substituted aromatics (chlorothalonil, PCNB)
  - Inactivate amino acids, proteins and enzymes by combining with amino and thiol groups.
- Organophosphonate (fosetyl-Al)
  - Disrupts amino acid metabolism.

Inhibitors of nucleic acid metabolism and protein synthesis:

- Benzimidazoles (thiophanate-methyl)
  - Inhibit DNA synthesis (nuclear division).
- Phenylamides (mefenoxam)
  - Inhibits RNA synthesis.

- Dicarboximides (iprodione, vinclozolin)
- Inhibits DNA and RNA synthesis, cell division and cellular metabolism.

Inhibitors of sterol synthesis:

Ergosterol is the major sterol in most fungi. It is essential for membrane structure and function. Fungicides may inhibit demethylation of ergosterol.

Antibiotics:

Antibiotic is a compound produced by a microorganism that inhibits the growth of another microorganism. Antibiotics have been used since the 1950s to control certain bacterial diseases of high-value fruit, vegetable, and ornamental plants. They are essential for control of bacterial diseases of plants. Today, the antibiotics most commonly used on plants are ox tetracycline and streptomycin.

Streptomycin. In the U.S., streptomycin use is permitted on 12 plant species, but the primary uses are on apple, pear, and related ornamental trees for the control of fire blight caused by *Erwinia amylovora* . Minor uses include floriculture, seed treatment, and on seedlings of celery, pepper, potato, tomato, and tobacco in the greenhouse and/or field. At high concentrations, streptomycin can be phytotoxic to plants; thus it is applied to the surface of plants and not injected.

Oxytetracycline. This antibiotic is used primarily on peach, nectarine, and pear. Oxytetracycline also is used on an emergency basis on apple in specific regions where streptomycin-resistant strains of *E. amylovora* have been documented. Tetracycline derivatives are the only antibiotics that also can be used internally in plants. Tetracyclines are injected into the trunks of palm and elm trees to treat lethal yellows diseases caused by phytoplasmas. The injection of tetracycline into a tree is a labor-intensive and expensive treatment, which often must be repeated for abatement or delay of symptoms. Antibiotic injections are practical only for isolated high-value ornamental trees and not vast plantings of agricultural or forest trees.

4.10 BIOLOGICAL CONTROL OF PLANT PATHOGENS

Plant diseases are caused mainly by fungi, bacteria, viruses and nematodes. Biocontrol of plant disease involves the use of an organism or organisms to reduce disease.

Biological control agents (BCA)

4.10.1 Predators

Predators are mainly free-living species that directly consume a large number of prey during their whole lifetime. Ladybugs, and in particular their larvae which are active between May and July in the northern hemisphere, are voracious predators of aphids, and will also consume mites, scale insects and small caterpillars. The larvae of many hoverfly species principally feed upon greenfly, one larva devouring up to fifty a day, or 1000 in its lifetime. They also eat fruit tree spider mites and small caterpillars. Adults feed on nectar and pollen, which they require for egg production. Predatory Polistes wasps look for bollworms or other caterpillars on a cotton plant. Dragonflies are important predators of mosquitoes, both in the water, where the dragonfly naiads eat mosquito larvae, and in the air, where adult dragonflies capture and eat adult mosquitoes. Community-wide mosquito control programs that spray adult mosquitoes also kill dragonflies, thus reducing an important biocontrolagent. *Phasmarhabditis hermaphrodita* is a microscopic nematode that kills slugs, thereafter feeding and reproducing inside. The nematode is applied by watering onto moist soil, and gives protection for up to six weeks in optimum conditions. Other useful garden predators include lacewings, pirate bugs, rove and ground beetles, aphid midge, centipedes, spiders, predatory mites, as well as larger fauna such as frogs, toads, lizards, hedgehogs, slow-worms and birds. Cats and rat terriers kill field mice, rats, June bugs, and birds. Dachshunds are bred specifically to fit inside tunnels underground to kill badgers.

More examples:

- *Phytoseiulus persimilis* (against spider mites)
- *Amblyseius californicus* (against spider mites)
- *Amblyseius cucumeris* (against spider mites)
- *Typhlodromips swirskii* (against spider mites, thrips, and white flies)
- *Feltiella acarisuga* (against spider mites)
- *Stethorus punctillum* (against spider mites)
- *Macrolophus caluginosus* (against spider mites)

4.10.2 Parasitoid insects

Parasitoids are one of the most widely used biological control agents Parasitoids lay their eggs on or in the body of an insect host, which is then used as a food for developing larvae. The host is ultimately killed. Most insect parasitoids are wasps or flies, and usually have a very narrow host range.

Four of the most important groups are:

- Ichneumonid wasps: (5–10 mm). Prey mainly on caterpillars of butterflies and moths.
- Braconid wasps: Tiny wasps (up to 5 mm) attack caterpillars and a wide range of other insects including greenfly. A common parasite of the cabbage white caterpillar- seen as clusters of sulphur yellow cocoons bursting from collapsed caterpillar skin.
- Chalcid wasps: Among the smallest of insects (<3 mm). Parasitize eggs/larvae of greenfly, whitefly, cabbage caterpillars, scale insects and Strawberry Tortrix Moth (*Acleris comariana*).
- Tachinid flies: Parasitize a wide range of insects including caterpillars, adult and larval beetles, true bugs, and others.

Examples of parasitoids:

- *Encarsia formosa* A small predatory chalcid wasp which is a parasitoid of whitefly, a sap-feeding insect which can cause wilting and black sooty moulds. It is most effective when dealing with low level infestations, giving protection over a long period of time. The wasp lays its eggs in young whitefly 'scales', turning them black as the parasite larvae pupates.

  Diagram illustrating the life cycles of Greenhouse whitefly and its parasitoid wasp *Encarsia formosa*

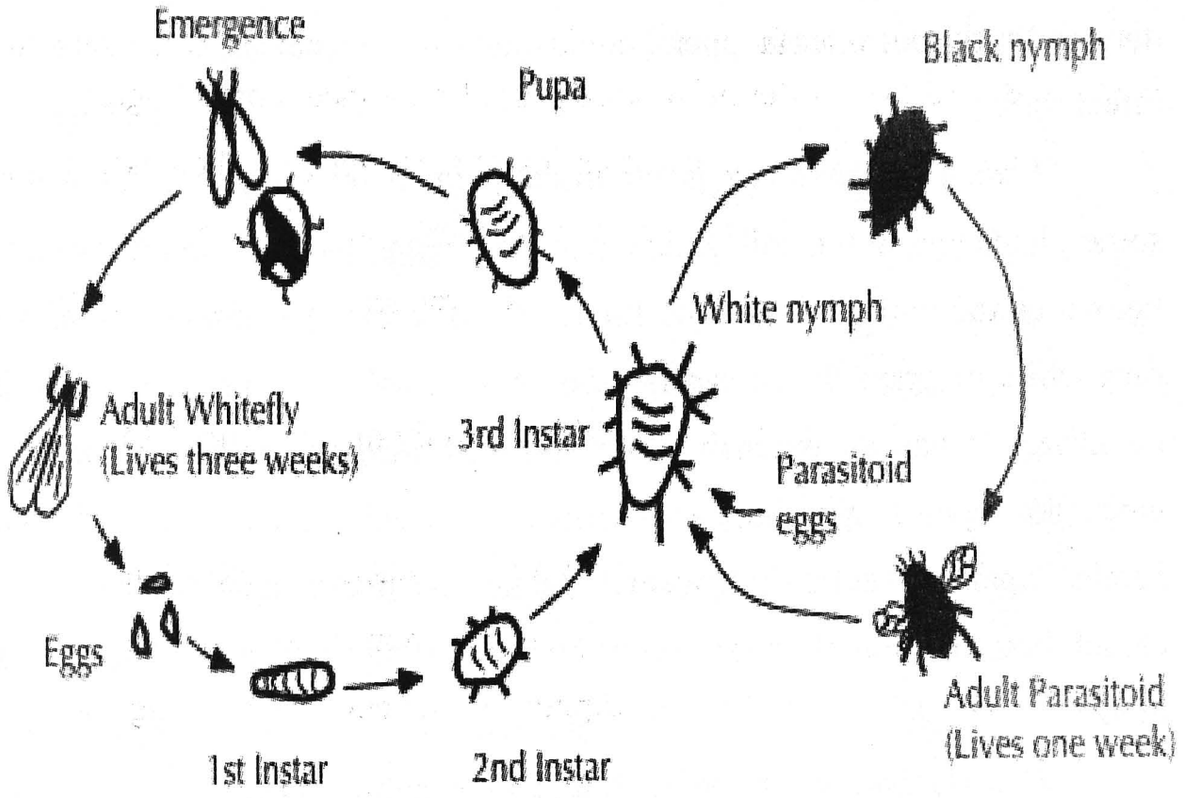


Figure-28

- *Eretmocerus* spp. (against white flies)
- *Aphidius colemani* (against aphids)
- *Gonatocerus ashmeadi* (Hymenoptera: Mymaridae) has been introduced to control the glassy-winged sharpshooter *Homalodisca vitripennis* (Hemipterae: Cicadellidae) in French Polynesia and has successfully controlled ~95% of the pest density. Commercially there are two types of rearing systems: short-term daily output with high production of parasitoids per day, and long-term low daily output with a range in production of 4-1000million female parasitoids per week.

4.10.3 Microorganisms

Insect-pathogenic Fungi

Entomophthora muscae infects flies. Susceptible pest species include the adults of the onion maggot, cabbage maggot, and seedcorn maggot. The fungus multiplies within the body of the adult fly which becomes enlarged; yellowish bands of fungal spores stripe the abdomen. Related species include *Pandora neoaphidis*, a common, naturally occurring pathogen of aphids that can be particularly effective during moist periods. *Zoophthora radicans* is another common fungus, with a broad host range. Strains have been isolated from several caterpillars, including

diamondback moth, leafhoppers, aphids, and some weevils. Other related species infect thrips.

Beauveria bassiana is an insect-pathogenic fungus found naturally on some plants and in the soil. Epizootics are favored by warm, humid weather. It is known as the white muscardine fungus because infected insect larvae eventually turn white or gray. *Beauveria* is used as a fungal microbial insecticide in some countries. It has an extensive host list that includes such important pests as whiteflies, aphids, grasshoppers, termites, Colorado potato beetle, Mexican bean beetle, Japanese beetle, boll weevil, cereal leaf beetle, bark beetles, lygus bugs, chinch bug, fire ants, European corn borer, codling moth, and Douglas fir tussock moth.

Metarhizium sp. is being tested as a natural enemy of corn rootworm, white grubs (scarabs), and some root weevils. It has a very broad host range and is extensively used in Brazil against spittle bugs in sugar cane and alfalfa. Other species of insect-pathogenic fungi have been tested as microbial insecticides for the control of pests.

Verticillium lecanii is used in Europe against greenhouse whitefly and thrips and aphids, especially in greenhouse crops.

Neozygites floridana has been successfully tested against spider mites, and Nomuraea rileyi has activity against green cloverworm, cabbage looper, imported cabbageworm, armyworms, corn earworm, and tobacco budworm. *Hirsutella thompsonii* infects mites and was previously registered for use in the United States.

Bacteria

Bacteria used for biological control infect insects via their digestive tracts, so insects with sucking mouth parts like aphids and scale insects are difficult to control with bacterial biological control. *Bacillus thuringiensis* is the most widely applied species of bacteria used for biological control, with at least four sub-species used to control Lepidopteran (moth, butterfly), Coleopteran (beetle) and Dipteran (true flies) insect pests. The bacteria are available in sachets of dried

spores which are mixed with water and sprayed onto vulnerable plants such as brassicas and fruit trees.

Fungi :

Fungi that cause disease in insects are known as entomopathogenic fungi, including at least fourteen species that attack aphids.[9] Beauveria bassiana is used to manage a wide variety of insect pests including: whiteflies, thrips, aphids and weevils. A remarkable additional feature of some fungi is their effect on plant fitness. Trichoderma species may enhance biomass production promoting root development, dissolving insoluble phosphate containing minerals.

Examples of entomopathogenic fungi:

- *Beauveria bassiana* (against white flies, thrips, aphids and weevils)
- *Paecilomyces fumosoroseus* (against white flies, thrips and aphids)
- *Metarhizium* spp. (against beetles, locusts and grasshoppers, Hemiptera, spider mites and other pests)
- *Lecanicillium* spp. (against white flies, thrips and aphids)
- *Cordyceps* species (includes teleomorphs of the above genera: that infect a wide spectrum of arthropods)
- *Trichoderma* species are used to manage certain plant pathogens. *Trichoderma viride* has been used against Dutch Elm disease, and to treat the spread of fungal and bacterial growth on tree wounds. It may also have potential as a means of combating silver leaf disease.

Biological control agent (type of organism)	**Trade Name**	**Target pathogens on one or more of the product labels**
Agrobacterium radiobacter (bacterium)	Galltrol A (strain 84)	*Agrobacterium tumefacians* (crown gall)
Ampelomyces quisqualis (fungus)	AG10	Powdery Mildew
Bacillus subtilis (bacterium)	Companion (GB03 strain)	*Pythium, Fusarium, Phytophthora,*
	Serenade	*Rhizoctonia, posdery mildew*

	Rhapsody (QST 713 strain)	*Colletotrichum, Erwinia, Pseudonomas,Xanthomonas, Diplocarpon, Cercospors*
	Cease	*Venturia*
Candida oleophila	Aspire	
Gliocladiumcatenulatum (fungus)	primastop	*Pythium, Rhizoctonia, Botrytis*
Steptomyces lydicus (actinomycete, fungus-like bacterium)	Actinovate Actino- Iron	*Pythium, Fusarium, Phytophthora, Rhizoctonia, Botrytis* ,powdery mildew, downy mildew, *Sclerotinia, Verticillium.*
Steptomyces griseoviridis	Mycostop	*Botrytis, Alternaria, Phomopsis, Pythium, Fusarium, Phytophthora*
Trichoderma harzianum (fungus)	PlantShield or RootShield	*Pythium, Fusarium, Phytophthora, Rhizoctonia, Botrytis ,powdery mildew, downy mildew, Sclerotinia*
Trichoderma virens (fungus)	SoilGard	*Pythium, Fusarium, Phytophthora, Rhizoctonia, Botrytis ,powdery mildew, downy mildew, Sclerotinia*

4.11 The Mechanism of Biocontrol

Biological control is principly achieved through antagonism (the inhibitory relationships between microorganisms including plants) which involves : *(i)* amensalism *i.e.* antibiosis and lysis, *(ii)* competition, and *(iii)* parasitism and predation.

4.11.1 Amensalism (Antibiosis and Lysis)

Amensalism is a phenomenon where one population adversely affects the growth of another population whilst itself being unaffected by the other population. Generally amensalism is accomplished by secretion of inhibitory substances. Antibiosis is a situation where the metabolites secreted by organism A inhibit

organism B, but organism A is not affected. It may be lethal also. Metabolites penetrate the cell wall and inhibit its activity by chemical toxicity. Generally antimicrobial metabolites are produced by underground parts of plants, soil microorganisms, plant residues, etc. Substances noxious to certain soil-borne plant pathogens are secreted by roots of maize, clover, lentil (glycine, phenylalanin) and other legumes, flax (hydrocyanic acid), pine(volatilemono-and sesquiterpenes) and by other plant roots. Other plant residues are the source of phenolic and non-volatile compounds. Similarly, antimicrobial substances (antibiotics) produced by microorganisms (soil bacteria, actinomycetes, fungi) are aldehydes, alcohols, acetone, organic acid, nonvolatile and volalite compounds which are toxic to microbes. Changes in microbial structures (cell wall, hyphae, conidia, etc.), may occur when microorganisms lack resistance against the attack by deleterious agents or unfavorable nutritional conditions. A chemical substance (*i.e.* melanin) is present in their cell walls to resist the lysis. Moreover, cell wall constituents, for example, xylan or xylose containing hetero polysaccharides, may also protect fungal cells from lysis.

4.11.2 Competition

exists between organisms that require the same resource for growth and survival. Use of the resource by one organism reduces its availability for the other organism. Competition for space or nutrients usually takes place between closely related species. Therefore, it can be effective to treat plants or seeds with a non-pathogenic strain of a related species that can out-compete the pathogenic organism. In some cases, the treating species need not be closely related to the pathogen, as long as it uses the same resources. For example, bacteria and yeasts can reduce fungal spore germination by competing with the spores for nutrients on the surface of leaves

4.11.3 Predation and Parasitism

Predation is an apparent mode of antagonism where a living microorganism is mechanically attacked by the other with the consequences of death of the farmer. It is often violent and destructive relationship. Parasitism is a phenomenon where

one organism consumes another organism, often in a subtle, non-debilitating relationship.

4.11.4 Mycoparasitism

When one fungus is parasitized by another one, the phenomenon is called as mycoparasitism. The parasitising fungus is called hyperparasite and the parasitized fungus as hypoparasite . Mycoparasitism commonly occurs in nature. As a result of inter-fungus interaction *i.e.* fungus-fungus interaction, several events take place which lead to predation viz., coiling, penetration, branching, sporulation, resting body production, barrier formation and lysis. In coiling (A) an antagonist, the hyperparasite *(a)* recognizes its host hyphae *i.e.* the hypoparasite *(h)* among the microbes and comes in contact and coils around the host hyphae. The phenomenon of recognition of a suitable host by the antagonists has been discovered in recent years. Cell wall surface of host and non-host contains D-galactose and N-acetyl D-galactosamine residues as lectin binding sites. With the help of lectins present on the cell wall, an antagonist recognizes the suitable sites (residues of lectins) and binds the host hypha. As a result of coiling, the host hypha loses the strength. If the antagonist has capability to secret cell wall, degrading enzymes, it can penetrate the cell wall of host hyphae and enter in lumen of the cells.

The event of entering in lumen of host cell is known as penetration.

Types of interspecies antagonisms leading to biological control of plant pathogens.

Type	**Mechanism**	**Examples**
Direct antagonism	Hyperparasitism/predation	Lytic/some nonlytic mycoviruses *Ampelomyces quisqualis* *Lysobacter enzymogenes* *Pasteuria penetrans* *Trichoderma virens*
Mixed-path antagonism	Antibiotics	2,4-diacetylphloroglucinol Phenazines

		Cyclic lipopeptides
	Lytic enzymes	Chitinases Glucanases Proteases
	Unregulated waste products	Ammonia Carbon dioxide Hydrogen cyanide
	Physical/chemical interference	Blockage of soil pores Germination signals consumption Molecular cross-talk confused
Indirect antagonism	Competition	Exudates/leachates consumption Siderophore scavenging Physical niche occupation
	Induction of host resistance	Contact with fungal cell walls Detection of pathogen-associated, molecular patterns Phytohormone-mediated induction

Mode of antagonism	*Plant pathogens*	*Antagonists (hosts)*	*Post-infection events*
Mycoparasitism:	*Rotrytis alii*	*Gliocladium roseum*	Penetration of hypae
	Cocchliobolus sativus	*Myrothecium verrucaria* and *Epicoccum purpurascens*	Antibiosis and penetration
	Rhizoctonia solani and *Fomes annosus*	*Trichoderma viride*	Coiling,c ytoplasm coagulation
	Sclerotium rolfsii	*T. harzianum*	Coiling, penetration and lysis

Source : Various mycological/microbiological research and review papers.

Sometimes host develops a resistant barrier to prevent the penetration inside the cell. Cytoplasm accumulates to form a spherical, irregular or elongated structure, so that the hypha of antagonist could not pass towards the adjacent cells of the hypha .Depending upon nutrition, the antagonist forms branches and sporulates (s) inside the host hypha . Until the host's nutrients deplete, the antagonist produces resting bodies, the survival structures, for example, chlamydospores *(c)* inside the host. Finally post-infection events lead to lysis of the host hypha due to loss of nutrients and vigor for survial.

4.11.5 Antibiotic-mediated suppression

Antibiotics are microbial toxins that can, at low concentrations, poison or kill other microorganisms. Most microbes produce and secrete one or more compounds with antibiotic activity. In some instances, antibiotics produced by microorganisms have been shown to be particularly effective at suppressing plant pathogens and the diseases they cause. Some examples of antibiotics reported to be involved in plant pathogen suppression are listed in the Table. In all cases, the antibiotics have been shown to be particularly effective at suppressing growth of the target pathogen *in vitro* and/or *in situ*.. The ability to produce multiple antibiotics probably helps to suppress diverse microbial competitors, some of which are likely to be plant pathogens. The ability to produce multiple classes of antibiotics, that differentially inhibit different pathogens, is likely to enhance biological control. More recently, *Pseudomonas putida* WCS358r strains genetically engineered to produce phenazine and DAPG displayed improved capacities to suppress plant diseases in field-grown wheat.

Some of antibiotics produced by BCAs

Antibiotic	Source	Target pathogen	Disease	Reference
2,4-diacetyl-phloroglucinol	*Pseudomonas fluorescens* F113	*Pythium* spp.	Damping off	Shanahanet al. (1992)
Agrocin 84	*Agrobacterium radiobacter*	*Agrobacterium tumefaciens*	Crown gall	Kerr (1980)
Bacillomycin D	*Bacillus subtilis* AU195	*Aspergillus flavus*	Aflatoxin contaminatio n	Moyneetal. (2001)
Bacillomycin, fengycin	*Bacillus amyloliquefacie ns* FZB42	*Fusarium oxysporum*	Wilt	Koumoutsi et al. (2004)
Xanthobaccin A	*Lysobacter* sp. strain SB-K88	*Aphanomyces cochlioides*	Damping off	Isla metal. (2005)
Gliotoxin	*Trichoderma virens*	*Rhizoctonia solani*	Root rots	Wilhiteetal. (2001)
Herbicolin	*Pantoea agglomerans* C9-1	*Erwinia amylovora*	Fire blight	Sandraetal. (2001)
Iturin A	*B. subtilis* QST713	*Botrytis cinerea* and *R. solani*	Damping off	Paulitz and Belanger (2001), Kloepper et al. (2004)
Mycosubtilin	*B. subtilis* BBG100	*Pythium aphanidermatum*	Damping off	Leclere et al. (2005)
Phenazines	*P. fluorescens* 2-79 and 30-84	*Gaeumannomyces graminis* var. *tritici*	Take-all	Thomashow et al. (1990)
Pyoluteorin,pyrro lnitrin	*P. fluorescens* Pf-5	*Pythium ultimum* and *R. solani*	Damping off	Howell and Stipanovic (1980)
Pyrrolnitrin, pseudane	*Burkholderia cepacia*	*R. solani* and *Pyricularia oryzae*	Damping off and rice blast	Homma et al. (1989)
Zwittermicin A	*Bacillus cereus* UW85	*Phytophthora medicaginis* and *P. aphanidermatum*	Damping off	Smith et al. (1993)

4.11.6 Lytic enzymes and other byproducts of microbial life

Diverse microorganisms secrete and excrete other metabolites that can interfere with pathogen growth and/or activities. Many microorganisms produce and release **lytic enzymes** that can hydrolyze a wide variety of polymeric compounds,

including chitin, proteins, cellulose, hemicellulose, and DNA. Expression and secretion of these enzymes by different microbes can sometimes result in the suppression of plant pathogen activities directly. While they may stress and/or lyse cell walls of living organisms, these enzymes generally act to decompose plant residues and nonliving organic matter. *Lysobacter* and *Myxobacteria* are known to produce copious amounts of lytic enzymes, and some isolates have been shown to be effective at suppressing fungal plant pathogens Furthermore, some products of lytic enzyme activity may contribute to indirect disease suppression. For example, oligosaccharides derived from fungal cell walls are known to be potent inducers of plant host defenses. Interestingly, *Lysobacter enzymogenes* strain C3 has been shown to induce plant host resistance to disease, though the precise activities leading to this induction are not entirely clear. The quantitative contribution of any and all of the above compounds to disease suppression is likely to be dependent on the composition and carbon to nitrogen ratio of the soil organic matter that serves as a food source for microbial populations in the soil and rhizosphere. However, such activities can be manipulated so as to result in greater disease suppression. For example, in post-harvest disease control, addition of chitosan can stimulate microbial degradation of pathogens similar to that of an applied hyperparasite. Chitosan is a non-toxic and biodegradable polymer of beta-1,4-glucosamine produced from chitin by alkaline deacylation. Amendment of plant growth substratum with chitosan suppressed the root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* in tomato.

Other microbial byproducts also may contribute to pathogen suppression. Hydrogen cyanide (HCN) effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. The production of HCN by certain fluorescent pseudomonads is believed to be involved in the suppression of root pathogens. *P. fluorescens* CHA0 produces antibiotics, siderophores and HCN, but suppression of black rot of tobacco caused by Thielaviopsis basicola appeared to be due primarily to HCN production. Volatile compounds such as ammonia produced by *Enterobacter cloacae* were involved in the suppression of *Pythium ultimum*-induced damping-off of cotton.

4.12 INTEGRATED PLANT PROTECTION

Integrated plant protection is the rational application of a combination of biological, biotechnical, chemical, cultural or plant-breeding measures whereby the use of chemical plant protection products is limited. The main goals of an integrated plant disease control program are to

- eliminate or reduce the initial inoculum
- reduce the effectiveness of initial inoculum
- increase the resistance of the host
- delay the onset of disease
- slow the secondary cycles

Plant diseases create challenging problems in commercial agriculture and pose real economic threats to both conventional and organic farming systems. Plant pathogens are difficult to manage for several reasons. Plant pathogens are constantly changing and mutating, resulting in new strains and new challenges to growers. Also, given the local, regional, and international movement of seed, plant material, and farming equipment, new and introduced pathogens periodically enter and cause new disease problems. Disease management is complicated by the presence of multiple types of pathogens. For any one crop the grower must deal with a variety of fungi, bacteria, viruses, and nematodes. This situation is even more complicated for organic vegetable growers because they usually produce a wide array of vegetable crops and are prohibited from applying conventional synthetic fungicides. The world market continues to be extremely competitive and continues to require that growers supply high-quality, disease-free produce with an acceptable shelf life. Disease management is therefore a critical consideration in organic vegetable production.

4.12.1 RESISTANT PLANTS AND CULTIVARS

One of the most important components in an integrated disease control program is the selection and planting of cultivars that are resistant to pathogens. The term resistance usually describes the plant host's ability to suppress or retard the activity and progress of a pathogenic agent, which results in the absence or reduction of symptoms. Tolerant plants can endure severe disease without

suffering significant losses in quality or yield; however, these tolerant plants do not significantly inhibit the pathogen's activity, and disease symptoms may be clearly evident. Resistant plants usually suppress the pathogen in some fashion. There are some distinct advantages to planting disease-resistant plant cultivars. Such selections are completely non-disruptive to the environment, and in fact their use enables growers to reduce and in some cases eliminate the application of chemicals used for pathogen control. The use of cultivars resistant to one disease is compatible with disease management steps taken to control other diseases. A final advantage is that of cultivars resistant to one disease is compatible with disease management steps taken to control other diseases. A final advantage is that for some host-pathogen systems the stability of that for some host-pathogen systems the stability of the resistance is long lasting and the cultivars can remain resistant for many years.

4.12.2 SITE SELECTION

Before planting crops, a grower should carefully plan out planting and crop rotation strategies to avoid insofar as possible any known problem areas. A grower can incur significant losses if he or she plants susceptible crops in a field known to be infested with persistent soilborne pathogens. Plant-pathogenic fungi such as Armillaria, *Fusarium* (the wilt-causing species), *Plasmodiophora, Sclerotium*, and *Verticillium* are true soil inhabitants and will persist in soil for many years, even in the absence of a plant host. Because not all fields are infested with these fungi, the grower is advised to select a planting site away from such fields. Soilborne fungi such as *Phytophthora*, *Pythium*, and *Rhizoctonia* often are much more widespread, so site selection might be less of an option in avoiding these organisms. There are also other planting situations that create risks that should be avoided. Pastures, foothills, river banks, grasslands, and other areas that support weeds and natural vegetation often are reservoirs of pathogens that cause virus and viruslike diseases. The vectors that carry such pathogens also can be found in these high-risk areas and often migrate into production fields.

Consider pertinent environmental factors when selecting a planting site. Crops planted very close to the seacoast tend to be more at risk from downy

mildew diseases as a result of the increased and persistent humidity. Just a few miles inland from the ocean, however, humidity can be significantly lower, decreasing the disease pressure for downy mildew. An understanding of soil factors is critical in avoiding some root and crown diseases. A site that has well-drained, sandy soil reduces the risk of damping-off and root rot for sensitive crops such as spinach. For any site selection decision, careful and detailed record keeping is essential keeping notes on previous soilborne disease problems associated with certain fields, the position of fields in relation to other key areas (weed reservoirs), the environmental characteristics of importance for each location, and the nature of soil, water, and other physical features of each site.

4.12.3 EXCLUSION

The practice of keeping out any materials or objects that are contaminated with pathogens or diseased plants and preventing them from entering the production system is known as exclusion. For some diseases, seed borne pathogens are a primary means of pathogen dissemination. Growers should purchase seed that has been tested and certified to be below a certain threshold infestation level or that has been treated to reduce pathogen infestation levels. Note that the designation "pathogen- free seed" really is not a valid term because it is not possible to know whether a seed lot is, in its entirety, absolutely free of all pathogens. Seed tests only examine representative samples, but in most cases the tests are accurate enough to give a true picture of the risk of diseases initiated by seed borne pathogens. If a grower produces or purchases transplants, they too should be as free as possible of pathogen contamination (where the pathogen is present on the plant but has not yet caused visible symptoms) and from disease (where symptoms are actually visible). Soil and water can harbor pathogens as well. Take care to see that no infested soil or water is introduced into uninfested areas. Tomato bushy stunt virus of lettuce, tomato, and other crops is found in river, flood, and runoff waters. Growers who have dredged up soil from ditches and dispersed it onto fields have found that their fields can become infested with the virus and subsequent plantings can be severely diseased. Water draining from fields can carry a number of pathogens, and growers should not recycle or reuse it

without carefully considering potential risks and then taking appropriate safety precautions. Soil adhering to tractor equipment and implements can spread soil borne pathogens from infested fields into clean fields. It is a good idea to reduce the off-site movement of these infested materials as much as possible.

4.12.4 APPLYING CONTROL MATERIALS

Once vegetable crops are in the field or greenhouse, it will sometimes be necessary to apply some sort or protectant or eradicant spray or dust material for disease control, if one is available. Unfortunately, the selection of effective, proven materials approved for organic use is limited. Inorganic disease control materials, primarily copper and sulfur-based fungicides, have been used for centuries. These inorganics are generally inexpensive and widely available, and they constitute minimal threats to the environment. However, their efficacy for disease control varies. While protectant copper fungicides have some activity against a wide range of fungal and bacterial pathogens, they are not extremely effective, and sole reliance upon them probably will not result in excellent disease control. Sulfurs also exhibit some activity against many pathogens, but they usually provide excellent control against only certain pathogens, such as powdery mildew fungi. Both coppers and sulfurs can burn sensitive vegetable crops under some environmental conditions. Oils, plant extracts, and other natural plant products are being investigated for use as disease-control sprays. Such products should be compatible with organic production practices, but reliable disease control has yet to be demonstrated. Bicarbonate-based fungicides have recently become available for control of plant diseases. Bicarbonates have demonstrated acceptable activity against powdery mildew and a few other diseases.

Disease control using microorganisms (biocontrol) or chemical by-products made by microorganisms is generating a good deal of interest. Very few effective, economically feasible biological control materials are commercially available. For the best results possible with any of these materials, appropriate application technique (proper equipment, spray volume, and plant coverage) and timing are essential. Most materials do not perform well if the disease is established, so applications should be made prior to extensive infection. Before

applying a product, a grower should confirm that the material is approved for use in organic production.

4.12.5 CULTURAL PRACTICES

There are a number of cultural practices that a grower should consider when designing an integrated disease control system. As a general approach, growers should take steps to grow vigorous, high-quality plants using the best farming practices possible. Listed below are some specific cultural practices that can help to manage diseases.

Crop rotation is an important consideration in disease management. Rotation using diverse crops, inclusion of cover crops, and appropriate use of fallow (host free) periods all can contribute to the reduction of inoculum levels for soil borne pathogens and the increase of diversity in soil microflora. In contrast, consecutive plantings of the same crop in the same field often lead to increases in soil borne pathogens. Too little crop rotation in a given area can also simulate a monoculture effect that might increase foliar diseases. When devising a crop rotation strategy, a grower should also be aware of which crops and cover crops might increase disease problems. A vetch cover crop, if planted into a field with a history of lettuce drop, can greatly increase the number of infective sclerotia of *Sclerotinia minor*. Vetch is a known host of root-knot nematode (Meloidogyne species) and also might increasesoil populations of *Pythium* and *Rhizoctonia* damping- off fungi. While oilseed radish could be a potential trap crop for cyst nematode (*Heterodera* species), as a cover crop it is a host of root-knot nematode and the club root fungus (Plasmodiophora *brassicae*).

There are many factors to consider in regard to **time of planting** a crop. If cauliflower is planted into *Verticillium*-infested fields in the spring or summer, it is likely to experience disease and possible crop loss. However, if cauliflower is planted into the same fields in the late fall or winter it will exhibit no *Verticillium* wilt symptoms, presumably because the soil temperatures are too cool to allow the fungus to develop and cause significant disease.

Disease can also be influenced by steps taken prior to and during the planting process. **Tillage** procedures should reduce plant residues left from

previous crops. Proper preparation of the field and the subsequent raised beds should reduce problems in areas that are subject to poor drainage, pooling of water, and other conditions that favor pathogens. Soil and bed preparation should result in good soil tilth so that seed or transplants are placed in a soil that favors plant development. Planting depth for seed or transplants should be tailored to enhance seed emergence or transplant establishment. Poor soil preparation can result in stressed and exposed plants and increased damping-off problems due to soil fungi.

Irrigation management is clearly an important factor when it comes to disease control. Regardless of the irrigation method a grower chooses (furrow, sprinkler, or drip), timing and duration of irrigations should satisfy crop water requirements without allowing for excess water. Over watering greatly favors most soil borne pathogenic fungi. For most foliar diseases, overhead sprinkler irrigation enhances pathogen survival and dispersal and disease development. Bacterial foliar diseases are particularly dependent upon rain and sprinkler irrigation. A grower should consider limiting or eliminating sprinkler irrigation if foliar diseases are problematic for a specific crop or field.

The **selection and application of fertilizers**, in a few documented situations, can significantly influence disease development. For example, the use of the nitrate form of nitrogenous fertilizers can increase the severity of lettuce corky root disease. The excessive use of nitrogen fertilizers can result in leaf growth that is overly succulent and more susceptible to some diseases. On the other hand, liming the soil to raise pH levels can reduce symptom expression for clubroot disease of crucifers. In general, however, fertilizer management is not directly related to disease control.

Field sanitation is the removal or destruction of diseased plant residues. In some field situations, sanitation is an appropriate step for managing diseases. Once lettuce has been harvested, for example, the remaining plants can act as a reservoir for lettuce mosaic virus. Sanitation in this case would include plowing down the old plants. Lettuce drop, caused by the fungus *Sclerotinia minor*, occurs when sclerotia develop on lettuce plant residues and remain in the top few inches

of soil. One form of sanitation involves deep plowing in which moldboard plows invert the soil and bury sclerotia. . Roguing is a special form of plant sanitation that involves the physical removal of diseased plants from the field.

The **management of other pests** is a cultural control that could greatly influence the development of plant diseases. In particular, virus disease management is more effective when weeds and insects are also controlled. Weeds are known reservoirs of a number of viral and bacterial pathogens. Soil solarization is the use of plastic tarps placed onthe soil surface to increase soil temperatures to a level that kills soil borne pathogens, weeds, and other crop pests. Soil solarization works best in areas with acceptably high summer temperatures. Soil solarization will not eradicate a pathogen from a field, but it may lower pathogen populations. Thus, in an integrated control program, several control methods are employed including regulatory inspections for healthy seed or nursery crop production, cultural practices(crop rotation, sanitation, pruning, etc.,) , biological control, physical control and chemical control.

4.13 Points for Discussion

1. How are symptoms useful in the diagnosis of plant diseases?
2. What are the various predisposing factors that favour plant diseases?
3. How are plant diseases controlled?

4.14 Check your progress

1. Define the common symptoms manifested by the infected plants.
2. What is the impact of temperature and humidity on disease development?
3. Elaborate on the various methods of eradication of plant pathogens.
4. Suggest ecofriendly methods for the control of plant pathogens and explain the mechanisms involved in them.
5. Explain Integrated system of disease management.

UNIT V DEFENSE MECHANISMS OF PLANTS AGAINST INFECTION

Structure

5.1 Introduction

Plants represent a rich source of nutrients for many organisms including bacteria, fungi, protists, insects, and vertebrates. Pathogens attack the host plants and cause severe damage as they interfere with the growth and metabolism. Although lacking an immune system comparable to animals, plants have developed a stunning array of structural, chemical, and protein-based defenses designed to detect invading organisms and stop them before they are able to cause extensive damage.

5.2 Objectives

After going through this unit, you will be able to

- Understand and appreciate the ways by which plants defend themselves against pathogens
- Explain the effect of infection on the physiology of the host plants

5.3 Defense mechanisms

Passive Defense: This type of defense response is due to the presence of some structural components or some type of metabolites present in the body of the plant. The outer covering of the plant surface may be a special type such as cuticle or wax, which cannot be attacked or digested by the infecting fungus or bacteria.

The presence of strong material such as lignin, tough bark, cuticle, etc. can effectively prevent the organisms from penetrating the plant surface. There are a large number of secondary metabolites such as alkaloids, tannins, phenols, resins, etc., which are toxic to pests and pathogens. Some of these compounds may have antimicrobial, antibacterial, or insecticidal properties. In addition to the secondary metabolites, there are certain proteins or peptides that have antimicrobial properties. For example, the antifungal peptides present in the seeds, which help in preventing the seeds from fungal infection; hydrolytic enzymes, which can lysing the bacteria and fungus; and proteins that inactivates the viral particle by digesting its coat protein and nucleic acids.

Active Defense: The defense response, which is produced newly and is not present previously in the cell or body, is called the active defense. The plant-cell wall is one of the sites where the change due to the defense response can be observed. All changes that happen in the cell wall due to an infection are collectively known as wall apposition. When a microorganism such as a fungus or bacteria starts infecting the plant body through the surface, immediately cell-wall thickness at that part is increased to make the penetration impossible. The change in thickness is due to the addition of new wall materials to the cell wall, specifically to the area of infection. Another interesting mechanism or response is called hypersensitive response (HR). In this response, the cells around the site of infection become necrotic. The metabolic activities of these cells also change. Their respiration becomes very slow or completely stopped. They begin to accumulate toxic compounds. Thus, an inhibitory effect or an unfavorable condition is created for the further growth and spread of the pathogen around the site of infection. The plant system or those cells (cells around the site of infection) also produce certain new chemicals in response to the infection known as phytoalexins. Phytoalexins are small molecular weight compounds produced when there is microbial attack or under conditions of stress, which are completely absent in healthy tissues.

5.3.1 Structural Defenses

All plant tissues contain pre-formed structural barriers that help limit pathogen attachment, invasion and infection. The cell wall is a major line of defense against fungal and bacterial pathogens. It provides an excellent structural barrier that also incorporates a wide variety of chemical defenses that can be rapidly activated when the cell detects the presence of potential pathogens. All plant cells have a primary cell wall, which provides structural support and is essential for turgor pressure, and many also form a secondary cell wall that develops inside of the primary cell wall after the cell stops growing. The primary cell wall consists mostly of cellulose, a complex polysaccharide consisting of thousands of glucose monomers linked together to form long polymer chains. These chains are bundled into fibers called microfibrils, which give strength and flexibility to the wall. The cell wall may also contain two groups of branched polysaccharides: cross-linking glycans and pectins. Cross-linking glycans include hemicellulose fibers that give the wall strength via cross-linkages with cellulose. Pectins form hydrated gels that help "cement" neighboring cells together and regulate the water content of the wall. Soft-rot pathogens often target pectins for digestion using specialized enzymes that cause cells to break apart: these organisms are extremely common, and anyone who has seen fruits or vegetables become brown and "mushy" have seen these pathogens in action.

Many cell walls also contain **lignin**, a heterogeneous polymer composed of phenolic compounds that gives the cell rigidity. Lignin is the primary component of wood, and cell walls that become "lignified" are highly impermeable to pathogens and difficult for small insects to chew. **Cutin, suberin**, and **waxes** are fatty substances that may be deposited in either primary or secondary cell walls (or both) and outer protective tissues of the plant body, including **bark**.

Cell walls contain proteins and enzymes that actively work to reshape the wall during cell growth yet thicken and strengthen the wall during induced defense. When a plant cell detects the presence of a potential pathogen, enzymes catalyze an **oxidative burst** that produces highly reactive oxygen molecules

capable of damaging the cells of invading organisms. Reactive oxygen molecules also help strengthen the cell wall by catalyzing cross-linkages between cell wall polymers, and they serve as a signal to neighboring cells that an attack is underway. Plant cells also respond to microbial attack by rapidly synthesizing and depositing **callose** between the cell wall and cell membrane adjacent to the invading pathogen. Callose deposits, called **papillae**, are polysaccharide polymers that impede cellular penetration at the site of infection, and these are often produced as part of the induced basal defense response.

Some plant cells are highly specialized for plant defense. **Idioblasts** ("crazy cells") help protect plants against herbivory because they contain toxic chemicals or sharp crystals that tear the mouthparts of insects and mammals as they feed. There are many classes of idioblasts including pigmented cells, sclereids, crystalliferous cells, and silica cells. **Pigmented cells** often contain bitter-tasting tannins that make plant parts undesirable as a food source. Young red wines often contain high levels of tannins that give wine a sharp, biting taste. **Sclereids** are irregularly-shaped cells with thick secondary walls that are difficult to chew: the rough texture of pear fruit (*Pyrus* spp.) is caused by thousands of sclereid **stone cells** that can abrasively wear down the teeth of feeding animals. Stinging nettles (*Urtica dioica*) produce **stinging cells** shaped like hypodermic needles that break off when disturbed and inject highly irritating toxins into herbivore tissues. Some stinging cells contain **prostaglandins**, hormones that amplify pain receptors in vertebrate animals and increase the sensation of pain. **Crystalliferous cells** contain crystals of calcium oxalate that may tear herbivore mouthparts when chewed and can be toxic if ingested. Members of the genera *Philodendron* and *Dieffenbachia* are very common tropical house plants that contain large amounts of these cells. Humans and pets who chew the leaves of these plants may experience a burning sensation in the mouth and throat that is often accompanied by swelling, choking, and an inability to speak. For these reasons, species of Dieffenbachia are commonly called **dumb cane**. Grasses and sedges contain rows of **silica cells** in their epidermal layers which give strength and rigidity to the growing leaf blades and deter feeding by chewing insects.

The epidermis constitutes the outermost protective tissue system of leaves, floral parts, fruits, seeds, stems, and roots of plants until they undergo considerable secondary growth. It is the first line of defense against invading pathogens and consists of both specialized and unspecialized cells. The epidermal cells of aerial plant parts are often covered in a waxy cuticle that not only prevents water loss from the plant, but also prevents microbial pathogens from coming into direct contact with epidermal cells and thereby limits infection. The cuticle can be relatively thin (aquatic plants) or extremely thick (cacti). The hydrophobic nature of the cuticle also prevents water from collecting on the leaf surface, an important defense against many fungal pathogens that require standing water on the leaf surface for spore germination. However, some fungal pathogens including *Fusarium solani* produce cutinases that degrade the cuticle and allow the fungi to penetrate the epidermis.

Trichomes ("leaf hairs") are specialized epidermal cells found on aerial plant parts that may provide both physical and chemical protection against insect pests. The velvety appearance of dusty miller (*Senecio cineraria*) is caused by thousands of tiny trichomes covering the plant's surface. Trichomes on the surface of soybeans (*Glycine max*) prevent insect eggs from reaching the epidermis and the larvae starve after hatching. The hook-shape of snap bean (*Phaseolis vulgaris*) trichomes impale caterpillars as they move across the leaf surface, and **glandular trichomes** in potato and tomato secrete oils that repel aphids. In woody plants, the **periderm** replaces the epidermis on stems and roots. Outer bark (**phellem**) is an excellent example of a preformed structural barrier that contains high amounts of water-resistant suberin and prevents many pathogens and i **Thorns** are modified branches that protect plants from grazing vertebrates, and include the honey locust tree (*Gleditsia triacanthos*). Many cacti produce thorn-like structures that are actually modified leaves or parts of leaves (e.g., stipules) called **spines** which serve similar purposes, such as in the barrel cactus (*Ferocactus* spp.). Botanically speaking, the "thorns" on the stem of rose plants (*Rosa* spp.) are neither true thorns nor spines: they are actually outgrowths of the epidermis called **prickles**.nsects from reaching the living cells underneath.

5.3.2 Anatomical structures formed after infection

5.3.2.1 Cork

Cork is an external, secondary tissue that is impermeable to water and gases, and is also called the phellem. The cork is produced by the Cork cambium which is a layer of meristematically active cells which serve as a lateral meristem for the periderm. The cork cambium, which is also called the phellogen, is normally only one cell layer thick and it divides periclinally to the outside producing cork. The phelloderm, which is not always present in all barks, is a layer of cells formed by and interior to the cork cambium. Together, the phellem (cork), phellogen (cork cambium) and phelloderm constitute the periderm. Cork cell walls contain suberin, a waxy substance which protects the stem against water loss, the invasion of insects into the stem, and prevents infections by bacteria and fungal spores. The cambium tissues, i.e., the cork cambium and the vascular cambium, are the only parts of a woody stem where cell division occurs; undifferentiated cells in the vascular cambium divide rapidly to produce secondary xylem to the inside and secondary phloem to the outside.

5.3.2.2 Tyloses

A common defense mechanism in xylem vessels against vascular wilt pathogens is the formation of tyloses. Tyloses are outgrowths of vessel-associated parenchyma cells which protrude into the xylem vessel through pits and block the spread of pathogens. They are formed during both compatible and incompatible interactions between the host and vascular wilt pathogens, although the time and extent of tylose formation significantly differs. Tyloses occur widely among plant species, and are induced by environmental stimuli such as wounding and pathogen infectionTyloses for much faster and more extensively in resistant plants when compared to susceptible plants.

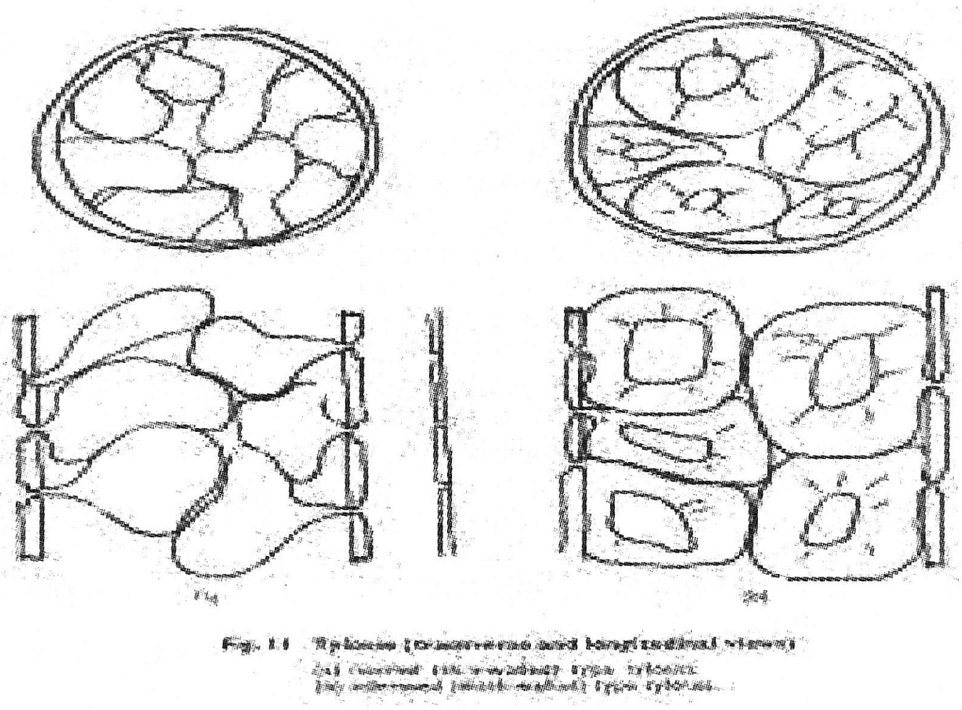


Figure-29

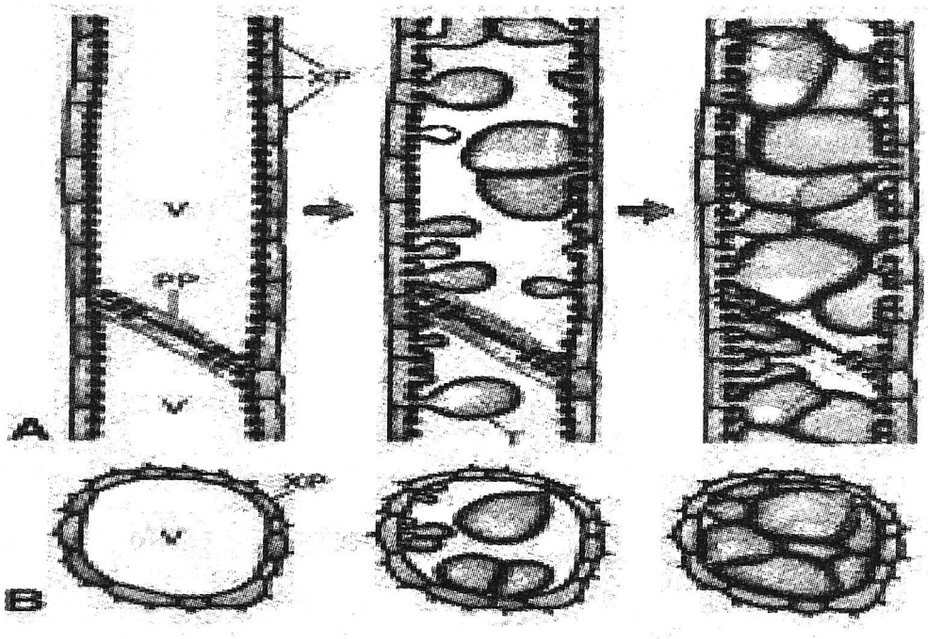


Figure-30

Tyloses impair xylem function by blocking vessels, but they are also a component in wound healing and may inhibit the intrusion and spread of pathogens. Several hypotheses have been advanced to explain tylose initiation. One persistent idea is that tyloses form in response to the formation of air embolisms in xylem vessels. Like wounding and pathogen infection, natural senescence, heartwood formation, frost, and flooding also stimulate tylose development.

5.3.2.3 Abscission layer

A plant will abscise a part either to discard a member that is no longer necessary, such as a leaf during autumn, or a flower following fertilization, or for the purposes of reproduction. Most deciduous plants drop their leaves by abscission before winter, while evergreen plants continuously abscise their leaves. Another form of abscission is fruit drop, when a plant abscises fruit while still immature; in order to conserve resources needed to bring the remaining fruit to maturity. If a leaf is damaged a plant may also abscise it to conserve water or photosynthetic efficiency, depending on the 'costs' to the plant as a whole. The abscission layer is a greenish grayish color.

Abscission can also occur in premature leaves as a means of plant defense. Premature leaf abscission has been shown to occur in response to infestation by gall aphids. By abscising leaves that have been made host to aphid galls, plants have been shown to massively diminish the pest population, as 98% of aphids in abscised galls died. The abscission is selective, and the chance of dropping leaves increases as the number of galls increase. A leaf with three or more galls was four times more likely to abscise than a leaf with one, and 20 times as likely to be dropped as a leaf without any galls.

The leaves of the infected plant fall down to remove the source of infection in order to protect the plant. This occurs by formation of an abscission layer at the base of the petiole. It takes place due to cytological and chemical changes in cells along which leaf will separate. This is called abscission zone. In this zone, two layers are formed abscission or separation layer and a protection layer. Here, structural changes facilitate the separation of the leaf. Beneath this, develops the protective layer which protects the exposed surface formed by falling of the leaf.

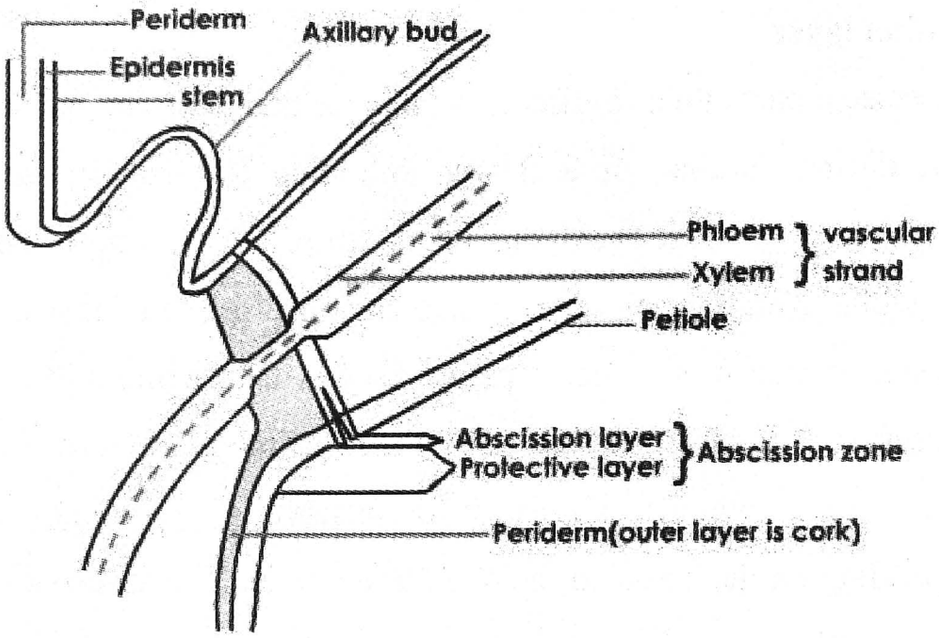


Figure-31

Abscission (separation) layer: Several cellular processes may be associated with abscission and it is not similar in all the species of plants. The actual separation may be preceded by cell division. The new cell walls may be degraded. Cell enlargement may also take place. The separation of leaf along the abscission layer may be caused by three methods. In the first case the middle lamella is dissolved, in the second case the primary walls are dissolved and in the third, the entire wall is dissolved. The dissolution of the middle lamella of the abscission layer occurs only in the tissues surrounding the vascular region. After the dissolution of the separation layer, the leaf is held by the vascular region only. The formation of tyloses in vessels and callose in sieve tissue stops supply of water and nutrition to the plant. A little disturbance in the environment like wind may cause the break in the vascular tissues and leaf fall.

Protective layer

As soon as the leaf falls, the protective layer develops on the site of abscission. It may be primary or secondary. It protects the exposed surface from dessication, injury and invasion of parasites. The primary protective layer develops by depositing lignin and suberin on the walls and intercellular spaces. The secondary protective layer is typically due to protective layer in woody plants. Auxin and ethylene are seen to be the best known agents in regulation of abscission. Auxin accelerates the formation of abscission zone when applied at the proximal side

and retards when applied to the distal end. Ethylene normally drives abscission to completion. The enzymes needed for cell wall degradation is peroxidase and phosphatase. Other chemical substances like gibberellic acid, cytokinin and abscisic add also influence abscission.

5.3.3 Biochemical Defense

Plant chemicals can be divided into two major categories: primary metabolites and secondary metabolites. **Primary metabolites** are substances produced by all plant cells that are directly involved in growth, development, or reproduction. Examples include sugars, proteins, amino acids, and nucleic acids. **Secondary metabolites** are not directly involved in growth or reproduction but they are often involved with plant defense. These compounds usually belong to one of three large chemical classes: terpenoids, phenolics, and alkaloids.

Terpenoids (terpenes) occur in all plants and represent the largest class of secondary metabolites with over 22,000 compounds described. The simplest terpenoid is the hydrocarbon **isoprene** (C_5H_8), a volatile gas emitted during photosynthesis in large quantities by leaves that may protect cell membranes from damage caused by high temperature or light. Terpenoids are classified by the number of isoprene units used to construct them. For example, **monoterpenoids** consist of two isoprene units, **sesquiterpenoids** (three units), **diterpenoids** (four units), and **triterpenoids.**

5.3.3.1 Phenolics

Phenolics are another large class of secondary metabolites produced by plants to defend themselves against pathogens. They are produced primarily via the shikimic acid and malonic acid pathways in plants, and include a wide variety of defense-related compounds including flavonoids, anthocyanins, phytoalexins, tannins, lignin, and furanocoumarins. Flavonoids are one of the largest classes of phenolics. Anthocyanins are colorful water-soluble flavonoids pigments produced by plants to protect foliage from the damaging effects of ultraviolet radiation. Anthocyanins are responsible for the showy colors of many plants and are present in high concentrations in flowers, fruits, and the leaves of deciduous plants in fall. Phytoalexins are isoflavonoids with antibiotic and antifungal properties that are

produced in response to pathogen attack. These toxic molecules disrupt pathogen metabolism or cellular structure but are often pathogen specific in their toxicity. Examples include medicarpin produced by alfalfa (*Medicago sativa*), rishitin produced by both tomatoes and potatoes (the *Solanaceae* family), and camalexin, produced by *Arabidopsis thaliana.*

Tannins are water-soluble flavonoid polymers produced by plants and stored in vacuoles. Tannins are toxic to insects because they bind to salivary proteins and digestive enzymes including trypsin and chymotrypsin resulting in protein inactivation. Insect herbivores that ingest high amounts of tannins fail to gain weight and may eventually die. The sharp taste of red wine is caused by grape tannins binding to salivary proteins in the mouth which results in protein coagulation.

Lignin is a highly branched heterogeneous polymer found principally in the secondary cell walls of plants, although primary walls can also become lignified. It consists of hundreds or thousands of phenolic monomers and is a primary component of wood. Because it is insoluble, rigid, and virtually indigestible, lignin provides an excellent physical barrier against pathogen attack.

Furanocoumarins are phenolic compounds produced by a wide variety of plants in response to pathogen or herbivore attack. They are activated by ultraviolet light and can be highly toxic to certain vertebrate and invertebrate herbivores due to their integration into DNA, which contributes to rapid cell death. In fact, grapefruit juice contains small quantities of furanocoumarins, which greatly increase the absorption of certain drugs into the bloodstream from the intestines. Some medicines carry warning labels cautioning people to avoid drinking grapefruit juice while taking the drugs in order to avoid an accidental overdose.

Role of natural phenols in the plant defense against fungal pathogens

Natural phenols play a role in the plant defense against fungal pathogens. In *Vitis vinifera* grape, *trans*-resveratrol is a phytoalexin produced against the growth of fungal pathogens such as *Botrytis cinerea* and delta-viniferin is another grapevine phytoalexin produced following fungal infection by *Plasmopara viticola*. Pinosylvin is a pre-infectious stilbenoid toxin (i.e. synthesized prior to infection),

contrary to phytoalexins which are synthesized during infection. It is present in the heartwood of *Pinaceae* It is a fungitoxin protecting the wood from fungal infection.

Sakuranetin is a flavanone, a type of flavonoid. It can be found in *Polymnia fruticosa* and rice, where it acts as a phytoalexin against spore germination of *Pyricularia oryzae*. In *Sorghum*, the *SbF3'H2* gene, encoding a flavonoid 3'-hydroxylase, seems to be expressed in pathogen-specific 3-deoxyanthocyanidin phytoalexins synthesis, for example in *Sorghum-Colletotrichum* interactions. 6-Methoxymellein is a dihydroisocoumarin and a phytoalexin induced in carrot slices by UV-C, which allows resistance to *Botrytis cinerea* and other microorganisms.

Danielone is a phytoalexin found in the papaya fruit. This compound showed high antifungal activity against *Colletotrichum gloesporioides*, a pathogenic fungus of papaya. Stilbenes are produced in *Eucalyptus sideroxylon* in case of pathogens attacks. Such compounds can be implied in the hypersensitive response of plants. High levels of polyphenols in some woods can explain their natural preservation against rot

5.3.3.2 Phytoalexins

Phytoalexins are antimicrobial and often antioxidative substances synthesized *de novo* by plants that accumulate rapidly at areas of pathogen infection. They are broad spectrum inhibitors and are chemically diverse with different type's characteristic of particular plant species. Phytoalexins tend to fall into several classes including terpenoids, glycosteroids and alkaloids; however, researchers often find it convenient to extend the definition to include all phytochemicals that are part of the plant's defensive arsenal.

Phytoalexins are low molecular weight antibiotics produced by many (but not all) plants in response to infection. There are many biotic elicitors of phytoalexin production, such as cell wall components, as well as abiotic elicitors, such as heavy metals and ultraviolet light. Phytoalexins inhibit the growth of bacteria and fungi *in vivo* and *in vitro*, and production of these antibiotics during an infection can induce resistance to subsequent infections by that pathogen. Over 350

phytoalexins are known in over 100 plant species. They include pterocarpans, sesquiterpenes, cryptophenols, isocoumarins, isoflavenoids, and others.

E.g.,

Phytoalexin	**Host**	**Pathogen**
Ipomeamarone	Sweet potato	*Ceratocystis* sp
Pisatin	Peas	Fungi
Phaseolin	Bean, Soybean	*Colletotrichum, Phytophthora*
Orchinol	Orchis	*Rhizoctonia*
Medicarpin	Alfalfa	Fungi
Cicerin	Cicer	*Aschochyta*
Rishitin	Potato	*Phytophthora*
gossypol	Cotton	*Verticillium, Rhizopus*
Capsidol	Pepper	Fungi
Isocaumarin	Carrot	*Ceratocystis*

Phytoalexins may be produced by any part of the plant, although different phytoalexins can accumulate in different organs. Generally, related plant species produce structurally-related phytoalexins, and many produce more than one, enabling the plant to present a toxic cocktail to invading pathogens. They are produced in cells surrounding an infection site and delivered to the infected cell packaged in lipid vesicles, creating a toxic micro-environment in the infected cell and, hopefully, preventing disease establishment. Phytoalexin accumulation is often associated with hypersensitive cell death, although only living cells can synthesise phytoalexins. Some plants can also sequester phytoalexins into vacuoles as stores of inactive sugar-conjugates, which can be cleaved and released quickly if initial defense responses are unsuccessful.

Function

Phytoalexins produced in plants act as toxins to the attacking organism. They may puncture the cell wall, delay maturation, disrupt metabolism or prevent reproduction of the pathogen in question. Their importance in plant defense is indicated by an increase in susceptibility of plant tissue to infection when phytoalexin biosynthesis is inhibited. Mutants incapable of phytoalexin

production exhibit more extensive pathogen colonization as compared to wild type. As such, host-specific pathogens capable of degrading phytoalexins are more virulent than those unable to do so. When a plant cell recognizes particles from damaged cells or particles from the pathogen, the plant launches a two-pronged resistance: a general short-term response and a delayed long-term specific response. As part of the induced resistance, the short-term response, the plant deploys reactive oxygen species such as superoxide and hydrogen peroxide to kill invading cells. In pathogen interactions, the common short-term response is the hypersensitive response, in which cells surrounding the site of infection are signaled to undergo apoptosis, or programmed cell death, in order to prevent the spread of the pathogen to the rest of the plant.

Long-term resistance, or systemic acquired resistance (SAR), involves communication of the damaged tissue with the rest of the plant using plant hormones such as jasmonic acid, ethylene, abscisic acid or salicylic acid. The reception of the signal leads to global changes within the plant, which induce genes that protect from further pathogen intrusion, including enzymes involved in the production of phytoalexins. Often, if jasmonates or ethylene (both gaseous hormones) is released from the wounded tissue, neighboring plants also manufacture phytoalexins in response. For herbivores, common vectors for disease, these and other wound response aromatics seem to act as a warning that the plant is no longer edible.

5.4 Molecular biological aspects

5.4.1 Pathogenesis-Related (PR) Proteins

Higher plants have a broad range of mechanisms to protect themselves against various threats including physical, chemical and biological stresses, such as wounding, exposures to salinity, drought, cold, heavy metals, air pollutants and ultraviolet rays and pathogen attacks, like fungi, bacteria and viruses. Plant reactions to these factors are very complex, and involve the activation of set of genes, encoding different proteins. These stresses can induce biochemical and physiological changes in plants, such as physical strengthening of the cell wall through lignification, suberization,and callose deposition; by producing phenolic

compounds, phytoalexins and pathogenesis-related (PR) proteins which subsequently prevent various pathogen invasion. Among these, production and accumulation of pathogenesis related proteins in plants in response to invading pathogen and/or stress situation is very important. Phytoalexins are mainly produced by healthy cells adjacent to localized damaged and necrotic cells, but PR proteins accumulate locally in the infected and surrounding tissues, and also in remote uninfected tissues. Production of PR proteins in the uninfected parts of plants can prevent the affected plants from further infection. PR protein in the plants was first discovered and reported in tobacco plants infected by tobacco mosaic virus. Later, these proteins were found in many plants. Most PR proteins in the plant species are acid-soluble, low molecular weight, and protease-resistant proteins. PR proteins depending on their isoelectric points may be acidic or basic proteins but they have similar functions. Most acidic PR proteins are located in the intercellular spaces, whereas, basic PR proteins are predominantly located in the vacuole. The PR proteins have been classically divided initially into 5 families based on molecular mass, isoelectric point, and localization and biological activity.

There is a range of novel proteins synthesised in response to infection, many of which have b-glucanase, chitinase or lysozyme activity. Some **pathogenesis**-related proteins disrupt pathogen nutrition. The presence of low levels of these proteins in healthy plants suggests that they might have other roles in plant growth and development aside from disease resistance. Chitinase and glucanase accumulate in the vacuoles, and glucanase is also sometimes secreted into the intercellular space. They dissolve the fungal cell wall, fragments of which then elicit hypersensitive cell death. The breakdown of the vacuole during decompartmentalisation of the cytoplasm results in a flood of hydrolytic enzymes, which have antiviral, antifungal and antibacterial activity. The accumulation of pathogenesis-related proteins peaks around 7-10 days after initial infection. The presence of these proteins *before* infection increases the plant's resistance to pathogens, as in the case of systemic acquired resistance.

Currently PR-proteins were categorized into 17 families according to their properties and functions (Table 2), including pea, bean, tomato, tobacco, maize, soybean, potato, and wheat.

Table 2: Classification of pathogenesis related proteins

Families	Type member	Properties
PR-1	Tobacco PR-1a	Antifungal
PR-2	Tobacco PR-2	β-1,3-glucanase
PR-3	Tobacco P,Q	Chitinase type I,II, IV,V,VI,VII
PR-4	Tobacco 'R'	Chitinase type I,II
PR-5	Tobacco S	Thaumatin- like PR
PR-6	Tomato Inhibitor I	Proteinase- inhibitor
PR-7	Tomato P69	Endoproteinase
PR-8	Cucumber chitinase	Chitinase type III
PR-9	Tobacco 'lignin forming peroxidase'	Peroxidase
PR-10	Parsley 'PR1'	Ribonuclease like
PR-11	Tobacco 'class V' chitinase	Chitinase type I
PR-12	Radish Rs- AFP3	Defensin
PR-13	Arabidopsis THI2.1	Thionin
PR-14	Barley LTP4	Lipid- transfer protein
PR-15	Barley OxOa (germin)	Oxalate oxidase
PR-16	Barley OxOLP	Oxalate oxidase-like
PR-17	Tobacco PRp27	Unknown

Functions

- An important common function of most PRs is their antifungal effects
- Some PRs also exhibit antibacterial, insecticidal or antiviral action.
- Function as signals that spread "news" of the infection to nearby cells.
- Infections also stimulate the cross-linking of molecules in the cell wall and the deposition of lignin, responses that set up a local barricade that slows spread of the pathogen to other parts of the plant

- Chitinase activity
- Peroxidase, ribonuclease and lysozyme activities
- Their hydrolytic, proteinase-inhibitory and membrane-permeabilizing ability.
- They inactivate the proteins secreted by the parasites in the invaded plant tissues

5.4.2 Avr genes:

Genetic analysis of plant–pathogen interactions has demonstrated that resistance to infection is often determined by the interaction of dominant plant resistance (R) genes and dominant pathogen-encoded avirulence (Avr) genes. It was postulated that R genes encode receptors for Avr determinants. A large number of R genes and their cognate Avr genes have now been analyzed at the molecular level. R gene loci are extremely polymorphic, particularly in sequences encoding amino acids of the leucine-rich repeat motif. A major challenge is to determine how Avr perception by R proteins triggers the plant defense response. Mutational analysis has identified several genes required for the function of specific R proteins.

The **gene-for-gene relationship** was discovered by Harold Henry Flor(1930) who was working with rust (*Melampsora lini*) of flax (*Linum usitatissimum*). Flor was the first scientist to study the genetics of both the host and parasite and to integrate them into one genetic system.Gene-for-gene relationships are a widespread and very important aspect of plant disease resistance. Flor showed that the inheritance of both resistance in the host and parasite ability to cause disease is controlled by pairs of matching genes. One is a plant gene called the resistance (*R*) gene. The other is a parasite gene called the avirulence (*Avr*) gene. Plants producing a specific R gene product are resistant towards a pathogen that produces the corresponding *Avr* gene product.

An avr gene is defined as a gene of a potentially virulent bacterium that renders the bacterium unable to cause disease in specific cultivars of the host plant. These Avr genes can easily be determined by the response of the host to a specific race of the pathogen after inoculation. Different races of a bacterium carry different avr genes that complement different resistance genes. The term "avirulence gene" remains useful as a broad term that indicates a gene that

encodes any determinant of the specificity of the interaction with the host. Thus, this term can encompass some conserved microbial signatures (also called pathogen or microbe associated molecular patterns (PAMPs or MAMPs)) and pathogen effectors (e.g.,bacterial type III effectors and oomycete effectors) as well as any genes that control variation in the activity of those molecules.

There is no common structure between avirulence gene products. Because there would be no evolutionary advantage to a pathogen keeping a protein that only serves to have it recognised by the plant, it is believed that the products of Avr genes play an important role in virulence in genetically susceptible hosts. Unlike the MAMP or PAMP class of avr genes that are recognized by the host PRRs, the targets of bacterial effector avr proteins appear to be proteins involved in plant innate immunity signaling, as homologues of Avr genes in animal pathogens have been shown to do this. For example, the AvrBs3 family of proteins possesses DNA binding domains, nuclear localisation signals and a acidic activation domains and are believed to function by altering host cell transcription.

Several avirulence genes and the proteins they code have been identified in and isolated from plant pathogenic fungi. These include the genes avr2, avr4, and avr9 of the fungus *Cladosporium fulvum* that are avirulent on tomato varieties carrying respectively the resistance lociCf-2, Cf-4 and Cf-9and the gene avrPi-ta of the rice blast fungus *Magnaporthe grisea,* which confers avirulence to rice varieties containing the resistant gene Pi-ta.

Role of avr genes in pathogenicity and virulence

Several avr genes such as the pthA gene from *Xanthomonas citri* and avrb6 from *X. campestris pv. Malvacearum* encode proteins that act as pathogenicity or virulence factors. In some cases, avr proteins seem to react with other proteins that play an intermediate role in transmitting the signals for plant defense. In a few cases as in the avr Pi-ta protein, they seem to interact directly with the R protein and to act off a cascade of defense reactions.

5.5 Effect of infection on the physiology of the host plant

Pathogens interfere with the various physiological functions of the host plant and lead to the development of symptoms depending on the plant organ and the tissues they infect. Thus, pathogen that infects and kills the flowers of a plant interferes with the ability of the plant to produce and seeds and multiply. A pathogen that infects and kills part or all of the roots of a plant reduces the ability of the plant to absorb water and nutrients and results in its wilting and death. Similarly, a pathogen that infects and kills parts of the leaves or destroys their chlorophyll leads to reduced photosynthesis, growth and yield of the plant.

5.5.1 Effect on permeability of cell membrane

Cell membranes consist of phospholipids bilayer in which many kinds of proteins are embedded. Membranes function as permeability barriers that allow passage into a cell only of substances the cell needs and inhibit passage out of the cell of substances needed by the cell. The lipid layer is permeable to most biological molecules. Small water soluble molecules such as ions, sugars and aminoacids flow through or are pumped through special membrane channels made of proteins. In plant cells, because of the cell wall, only small molecules reach the membrane. Disruption or disturbance of the cell membrane by chemical or physical factors alters (usually increases) the permeability of the membrane with a subsequent uncontrollable loss of useful substances, as well as the inability to inhibit the inflow of undesirable substances or excessive amounts of any substance.

Changes in cell membrane permeability are often the first detectable responses of the cells to infection by pathogens, to most hosts specific or several nonspecific toxins, to certain pathogen enzymes and to certain toxic chemicals such as air pollutants. The most commonly observed effect of changes in cell membrane permeability is the loss of electrolytes (small water soluble ions) and molecules from the cell. Electrolyte leakage occurs much sooner and at greater rate when the host- pathogen interaction is incompatible, and the host remains more resistant than when the host is susceptible and develops extensive symptoms.

5.5.2 Effect on photosynthesis

Plants and pathogens have developed dynamic interactions. Whereas plants tend to survive through different mechanisms following pathogen attack, the later looks for maximizing feed intake to insure its reproduction and dissemination. In this context, the photosynthetate – the energy source for both the plant and pathogens – synthesis and its availability is the focus of a struggle to death.

Process

In higher plants, the photosynthetic CO2 fixation occurs in the green leaves, considered as source organs, with the absorption of light by chlorophyll, much of which is located in the light-harvesting complexes (LHCs) of PSII and PSI within the thylakoid membrane of chloroplasts. The mesophyll cell of higher plants, due to its higher chloroplast content, is the most active photosynthetic tissue. In general photosynthesis proceeds through 2 major phases: a) a light phase that produces ATP and NADPH in the chloroplast thylakoids and released in the stroma and b) the CO2 reduction phase in presence of water in the stroma and that consumes the ATP and NADPH generated in phase a) to produce a triose phosphate through the Calvin-Benson cycle which comprises three stages. Briefly, the carboxylation of 3 molecules of ribulose 1,5 biphosphate fixes 3 molecules of CO2 and H2O under the ribulose 1,5 biphosphate carboxylase/oxygenase (rubisco) catalysis in the Calvin-Benson cycle and leads to 6 molecules of 3-phosphoglycerate. The 3-phosphoglycerates are then phosphorylated in presence of ATP produced during the light reaction by the catalytic action of 3-phosphoglycerate kinase into 1,3 bisphosphoglycerate which is further reduced by NADPH and NADP-glyceraldehyde 3-phosphate dehydrogenase into 6 molecules of glyceraldehyde 3-phosphate (6 triose phosphates). Of these six triose phosphates, one represents the net synthesis from CO2 fixation and, 9 ATP and 6 NADPH are utilized. The remaining five triose phosphates are used to regenerate the ribulose 1,5 biphosphate to insure continuous CO2 fixation . The outcome of CO2 fixation by higher photosynthetic plants is the production of carbohydrates. As a result of photosynthetic CO2 reduction during the day, starch granules accumulate in the chloroplast while an

excess of assimilates are continuously allocated, mostly in the form of sucrose, to sink tissues such as developing leaves, roots, meristems, fruits, and flowers, that are unable to produce sufficient amounts of assimilates by themselves and therefore require their net import via the phloem. Plant pathogens like viruses, fungi, oomycetes, and bacteria are known to interfere with the source-sink balance and in the case of a successful interaction; pathogens are believed to reprogram a plant's metabolism to their own benefit. This comprises the suppression of plant defence responses and the reallocation of photo assimilates to sufficiently supply the pathogen with nutrients. In accordance with this, the infected leaf is assumed to undergo a source to sink transition or retains its sink character. For example, infection of maize leaves with *Ustilago maydis* prevents establishment of C4 photosynthesis because *U. maydis*-induced leaf galls exhibited carbon dioxide response curves, CO2 compensation points and enzymatic activities that are characteristic of C3 photosynthesis. An indication for this is provided by a stimulation of cell wall-bound invertase (cw-Inv) that mobilizes hexoses at the infection site and a decreased rate of photosynthesis (Pathogen attacks result in the development of symptoms that include leaf and fruit wilt, stem and root rot , coverage of leaf surface with pustule, chlorosis and necrosis, a decreased rate of plant photosynthesis , and as a consequence plant death or yield loss ensues (Berger et al. Pathogen attacks result in a decreased rate of plant photosynthesis, and as a consequence yield loss. Pathogen infection often leads to plant death, the development of chlorotic and necrotic lesions and to a decrease in photosynthetic assimilate production. Foliar symptoms were associated with stomatal closure and alteration of the photosynthetic apparatus. A decrease in CO2 assimilation, transpiration, a significant increase in intercellular CO2 concentration, a strong drop in the maximum fluorescence yield and the effective Photosystem II quantum yields, and a reduction of total chlorophyll but a stable carotenoid content were reported.

5.5.3 Effect on respiration

The respiration rate of plants invariably increases following infection by fungi, bacteria or viruses. The higher rate of glucose catabolism causes a measurable increase in the temperature of infected leaves. An early step in the plant's response to infection is an oxidative burst, which is manifested as a rapid increase in oxygen consumption, and the release of reactive oxygen species, such as hydrogen peroxide (H2O2) and the super oxide anion (O2 -). The oxidative burst is involved in a range of disease resistance and wound repair mechanisms. In resistant plants, the increase in respiration and glucose catabolism is used to produce defence-related metabolite s via the pentose phosphate pathway. In susceptible plants, the extra energy produced is used by the growing pathogen.

5.5.4 Effect of pathogens on transcription and translation

Transcription of cellular DNA into messenger RNA and translation of messenger RNA to produce proteins are two of the most basic, general, and precisely controlled processes in the biology of any normal cell. They vary with the stage of development and the requirements of each cell. Nevertheless, disturbance of any one of these processes, by pathogens or environmental factors, may cause drastic, unfavorable changes in the structure and function of the affected cells by its effect on the expression of genes. Several pathogens, particularly viruses and fungal obligate parasites, such as rusts and powdery mildews, affect the transcription process in infected cells. In some cases, pathogens affect transcription by changing the composition, structure, or function of the chromatin associated with the cell DNA. In some diseases, especially those caused by viruses, the pathogen, through its own enzyme or by modifying the host enzyme (RNA polymerase) that makes RNA, utilizes the host cell nucleotides and machinery to make its own (rather than host) RNA.

5.5.5 Effect on phenol metabolism

Plants are capable of producing a wide array of secondary metabolites that serve a variety of functions, due to their bioactive, redox or structural properties. Subtle changes in the external or internal environment of the plant can cause significant changes in the array of secondary metabolites present in the tissue. Plant

phenolics are secondary metabolites that encompass several classes structurally diverse of natural products biogenetically arising from the shikimate-phenylpropanoids-flavonoids pathways. Plants need phenolic compounds for pigmentation, growth, reproduction, resistance to pathogens and for many other functions. The starting product of the biosynthesis of most phenolic compounds is shikimate. Plant phenolics are biosynthesized by several different routes and thus constitute a heterogeneous group from a metabolic point of view. Two basic pathways are involved: the shikimic acid pathway and the malonic acid pathway. The shikimic acid participates in the biosynthesis of most plant phenolics. The malonic acid pathway, although an important source of phenolic secondary products in fungi and bacteria, is of less significance in higher plants.

Phenols are acidic due to the dissociability of their -OH group. They are rather reactive compounds and as long as no steric inhibition due to additional side chains occurs, they form hydrogen bonds. Consequently, many flavonoids have intramolecular bonds. Another important feature is their ability to form chelate complexes with metals. Also, they are easily oxidized and, if so, form polymers (dark aggregates). The darkening of cut or dying plant parts is caused by this reaction. They have usually an inhibiting effect on plant growth. Among the phenylpropanol derivatives of lower molecular weight are a number of scents like the coumarins, cinnamic acid, sinapinic acid, the coniferyl alcohols and others. These substances and their derivatives are at the same time intermediates of the biosynthesis of lignin

The pathway of phenolic acid metabolism in plants requires the initial steps of general phenylpropanoid metabolism and provides the precursors for lignin biosynthesis. However, intermediates in the pathway and derivatives of these intermediates are ubiquitous in plants and accumulate to significant levels in tissues that do not synthesize lignin. The function of these intermediates, if any, is not yet clear, although several different roles have been proposed. Phenolic compounds, particularly hydroxycinnamates, are present at significant levels in plant cell walls, where they may act as molecular bridges. For example, in grasses, 4-coumaric acid and ferulic acid link lignin to polysaccharide polymers,

such as glucuronoarabinoxylan, through labile ester and/or ether bonds. Laccase and tyrosinase are the two most important phenol oxidases. Changes in the activity of phenol oxidases play important role in the regulation of metabolic pathways in diseased or injured tissues. Increased activity of phenolase in diseased plant tissues is generally accompanied by an increased concentration of phenolic substances.

5.6 Points for Discussion

1. Various stresses can induce biochemical and physiological changes in plants. Discuss

2.Why is the study of defense mechanism in plants important?

5.7 Check your progress

1 .What are defense mechanisms?

2. Classify defense mechanisms.

3. How PR proteins play an important role in defense mechanism?

4. A plant's first line of defense against infection is the physical barrier of the plant's "skin," the epidermis of the primary plant body and the periderm of the secondary plant body. Explain