Techniques for Detection and Diagnosis of Plant Diseases

PRACTICAL MANUAL

Course Code: PPA-506 2(0+2)

For Post Graduate Students

2024

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Name of Student.....

Roll No.....

Batch.....

Session.....

Semester.....

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Credit.....

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CERTIFICATE

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Date: Course Teacher

SYLLABUS

Practical: Detection of plant pathogens 1. Based on visual symptoms, 2. Biochemical test 3. Using microscopic techniques, 4. Cultural studies; (use of selective media to isolate pathogens). 5. Biological assays (indicator hosts, differential hosts) 6. Serological assays 7. Nucleic acid based techniques (Non-PCR– LAMP, Later flow microarray & PCR based- multiplex, nested, qPCR, immune capture PCR, *etc.*). Phenotypic and genotypic tests for identification of plant pathogens. Molecular identification (16S rDNA and 16s-23S rDNA intergenic spacer region sequences-prokaryotic organisms; and eukaryotic organisms by ITS region) and whole genome sequencing. Volatile compounds profiling by using GC-MS and LC-MS. FAME analysis, Fluorescence in-situ Hybridization (FISH), Flow Cytometry, Phage display technique, and biosensors for detection of plant pathogens. Genotypic tools such as genome/specific gene sequence homology comparison by BLAST (NCBI and EMBL) and electron microscopy techniques of plant virus detection and diagnosis.

Content

Practical No. 1

Objective: To get familiar with general plant pathological laboratory equipment

The students will visit laboratory of Plant Pathology to acquaint with different appliances, tools, glass-wares, and other miscellaneous items, which they will be using in various exercises and experiments to be conducted.

1. Identify the laboratory equipment available in the Plant Pathology lab:

(a) Laboratory appliances/tools:

(i)		(ii)	
(iii)		(iv)	
(v)		(vi)	
(vii)		(viii)	
(ix)		(x)	
(xi)		(xii)	
(xiii)		(xiv)	
(xv)		(xvi)	
(xvii)		(xviii)	
(xix)		(xx)	

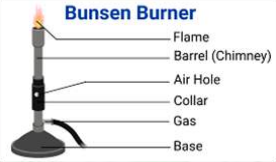
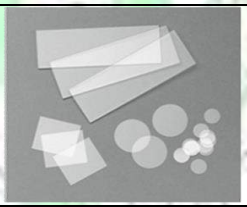
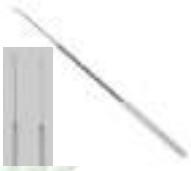
(b) Glass-wares:

(i)		(ii)	
(iii)		(iv)	
(v)		(vi)	
(vii)		(viii)	
(ix)		(x)	

2. Label the following laboratory instrument/equipment and state its principles and functions.

Auto Clave:	
Laminar Air Flow:	

BOD Incubator:	
Hot Air Oven:	
Thermocycler:	
Spectrophotometer:	
Gel Electriphoresis unit:	
Electronic balances:	

p^H meter:	
Centrifuge:	
Heater:	
Bunsen Burner:	
Dissecting Needle:	
Slide and Cover slip:	
Inoculating needle and loop:	
Pestle and Mortar:	
Haemocytometer/ Hemocytometer:	

Practical No. 2

Objective: Detection of plant pathogens based on visual Symptoms

Practical No. 3

Objective: Collection and preservation of disease specimen

Material required:-.....

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1) Dry preservation: .....

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2) Wet preservation: .....

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Note: Prepare of herbarium file.

Practical No. 4

Objective: Detection of plant pathogens based on Biochemical test

Biochemical tests are used for microbial identification based on different lye in their biochemical activities exhibited by different types of bacteria. Different biochemical tests are listed below that are used to identify gram positive and negative bacteria.

S. No.	Biochemical tests	S. No.	Biochemical tests
i.		ii.	
iii.		iv.	
v.		vi.	
vii.		viii.	
ix.		x.	
xi.		xii.	
xiii.		xiv.	

Catalase test.....

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Oxidase test:- .....

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Indole test:- .....

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Sulfur test:- .....

Urease test:-

Triple sugar iron test:-

Nitrate test:-

Starch hydrolysis test:-

Carbohydrate fermentation test: .....

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Methyl red test: .....

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Voges-Proskaur test:- .....

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Citric Acid Utilization (CAU) test:- .....

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Analytical Profile Index (API) Test:- .....

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Practical No. 5

Objective: Detection of plant pathogens based on using Microscopic techniques

Draw the illustrated diagram of Compound Microscope

Practical No. 6

Objective: Histopathological studies for detection of Bacteria

Gram's staining

Material Required: .....

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Principle: .....

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Procedure: .....

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Observation: .....

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Practical No. 7

Objective: Histopathological studies for detection of Fungi

1) Prepare the temporary slide from a given diseased sample, observe under the microscope and identify the causal organism.

Material Required: .....

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Principle: .....

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Procedure: .....

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Observation: .....

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Practical No. 8

Prepare the temporary slide from a given diseased sample, observe under the microscope and identify the causal organism.

Material Required: .....

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Principle: .....

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Procedure: .....

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Observation: .....

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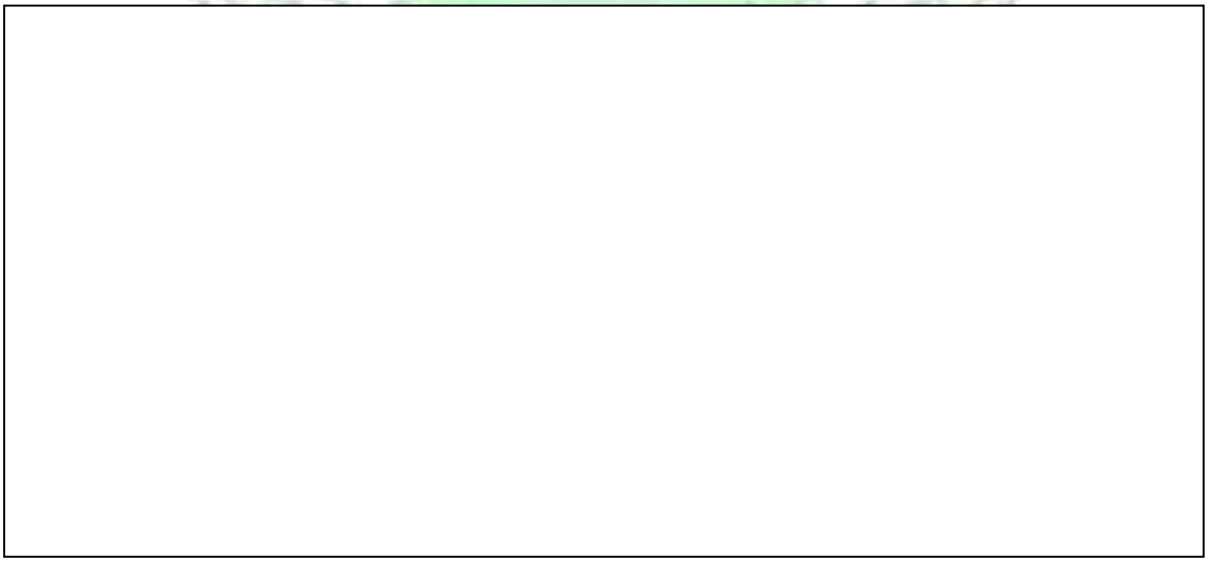

Practical No. 9

Prepare the temporary slide from a given diseased sample, observe under the microscope and identify the causal organism.

Material Required: .....

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Principle: .....

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Procedure: .....

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Observation: .....

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Practical No. 10

Prepare the temporary slide from a given diseased sample, observe under the microscope and identify the causal organism.

Material Required: .....

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Principle: .....

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Procedure: .....

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Observation: .....

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Practical No. 11

Prepare the permanent slide from a given diseased sample, observe under the microscope and identify the causal organism.

Material Required: .....

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Principle: .....

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Procedure: .....

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Observation: .....

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Practical No. 12

Objective: Detection of plant pathogens based on Cultural studies

1) Prepare Potato Dextrose Agar (PDA) medium

Material Required: .....

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Procedure: .....

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2) Prepare Nutrient Agar (NA) medium

Material Required: .....

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Procedure: .....

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Practical No. 13

Objective: Isolation and purification of Plant Pathogens from infected plant tissues

Isolation of plant pathogenic of fungi

Material Required: .....

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Procedure: .....

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Observation: .....

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Practical No. 14

Isolation of plant pathogenic bacteria

Material Required: .....

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Procedure: .....

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Observation: .....

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Practical No. 15

Objective: Detection of plant pathogens based on biological assays (Indicator hosts and Differential hosts)

Materials required: .....

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Procedure: .....

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Activities: .....

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Observation: .....

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Practical No. 16

Objective: Detection of plant pathogens based on Serological Assays (ELISA)

Principle: .....

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Materials required: .....

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A) Coating buffer (Carbonate buffer/antigen buffer) (p^H 9.6): .....

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B) Phosphate buffer saline (PBS) p^H 7.4: .....

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C) Washing buffer (PBST): .....

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D) Antibody buffer: .....

E) Substrate buffer (p^H 9.8):

Procedure:

Observation:

Practical No. 17

Objective: Isolation and purification of genomic DNA using CTAB

Materials required: .....

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Procedure: .....

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Observation: .....

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Practical No. 18

Objective: Assessing the quantity and quality of isolated Nucleic Acids (DNA or RNA)

A) Spectrophotometric method:-

Materials required: .....

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Procedure: .....

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Observation:

Sample	A260 (nm)	A280 (nm)	Ratio (260/280 nm)	Concentration	Remarks
Sample 1					
Sample 2					
Sample 3					
Sample 4					
Sample 5					

Comments: .....

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B) Agarose Gel Electrophoresis:-

Materials required: .....

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Procudure: .....

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Observation.....

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Comments.....

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Practical No. 19

Objective: Polymerase Chain Reaction (PCR) amplification

Materials required: .....

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Procedure: .....

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Observation: .....

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Practical No. 20

Objective: Agarose gel Electrophoresis

Material Required: .....

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Preparation

i) 10X TBE Buffer: .....

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ii) Gel loading buffer (10 ml): .....

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Procedure: .....

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Observation and comment: .....

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Practical No. 21

Objective: Isolation and purification of total genomic RNA

Materials required:

Procedure:

Observation and comment:

Practical No. 22

Objective: cDNA preparation of isolated RNA

Materials required: .....

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Procedure: .....

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Observation and comment: .....

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Practical No. 23

Objective: To get exposure on different bioinformatics tools

Practical No. 24

Objective: Multiple sequence alignment (MSA) and Phylogenetic tree construction

Practical No. 25

Objective: Synthesis of Primers for PCR

Properties of Primers:

Practical No. 26

Objective: Electron microscopy techniques for plant virus detection and diagnosis

Material: .....

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Procedure: .....

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Observations and results: .....

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Practical No. 27

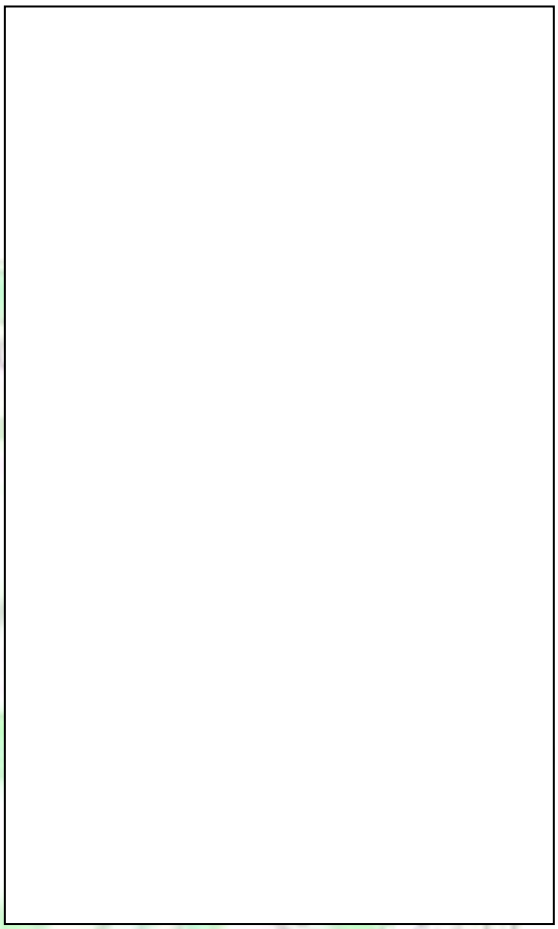
Objective: To study and Identification of parasites in higher plants (Phanerogams)

Practical No. 28

Objective: To study about different morphological features of plant parasitic nematodes.

Draw the illustrated diagram of a Plant parasitic nematodes

Nematode Morphology: .....



Body parts of Nematodes: .....

Practical No. 29

Objective: Mounting of plant parasitic nematodes and their characterization using a microscope.

Material required: .....

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Procedure:.....

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Observation: .....

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Practical No. 30

Prepare the temporary slide from the given sample, observe under the microscope and identify the plant pathogenic nematodes.

Material required: ....

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Procedure:..

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Observation: ....

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Practical No. 31

Objective: Molecular identification of prokaryotic organisms using 16S rRNA and 16s-23S rRNA (intergenic spacer region sequences)

Practical No. 32

Objective: Molecular identification of eukaryotic organism using ITS region

Practical No. 33

Objective: Volatile compounds profiling for Plant pathogen detection.

Material Required:.....

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Procedure: .....

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Observation: .....

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Practical No. 34

Objective: To prepare Percent, Molar and Normal solutions

I) Percent solutions:

II) Molar solutions:

III) Normal solutions:

Annexures

General Plant Pathological Laboratory Equipment

1.	Autoclave	**6.**	Spectrophotometer	**11.**	Bunsen Burner/Sprit Lamp	**16.**	Hot Plate	21.	Freeze
2.	Laminar Air Flow	**7.**	Gel Electrophoresis	**12.**	Cork-borer	**17.**	Forceps	22.	Scalpel
3.	BOD Incubator	**8.**	Electronic balances	**13.**	Dissecting Needle	**18.**	Shaker	23.	Scissor
4.	Hot-air oven	**9.**	p^H meter	**14.**	Inoculating needle and loop	**19.**	Blade	24.	Gel Doc
5.	Thermocycler	**10.**	Centrifuge	**15.**	Colony counter	**20.**	Centrifuge	25.	Micropipette

(b) Glass-wares:

1.	Conical flask (different sizes)	**5.**	Beaker (different sizes)	**9.**	Slides
2.	Measuring cylinder (different capacity)	**6.**	Pippet (different volume)	**10.**	Watch glass
3.	Petri dishes	**7.**	Culture tubes	**11.**	Dropping bottle
4.	Cover-slip	**8.**	Nematode counting dish	**12.**	Bearman funnel

(c) Miscellaneous items:

1.	Cotton	**5.**	Blotting paper	**9.**	Washing brush
2.	Aluminum foil	**6.**	Wash bottle	**10.**	Washing powder
3.	Trays	**7.**	Thread	**11.**	Wire basket
4.	Sieve of different sizes	**8.**	Rubber bands	**12.**	Mortar and pestle

Different Methods for Detection and Diagnosis of Plant Pathogens

Detection: - Identification of the causal organism or microorganism.
Diagnosis: - To conclude symptoms that a particular pathogen is associated.
Different Methods for Detection and Diagnosis of Plant Disease

1) Based on visual symptoms
2) Biochemical test
3) Using microscopic techniques
4) Cultural studies; (selective media to isolate pathogen).
5) Biological assays (indicator hosts, differential hosts)
6) Serological assays.

Detection of plant pathogens based on visual Symptoms

Effect of a pathogen on the plants: - During pathogenesis, normal activities of the infected host plant undergo malfunction. Consequently, morphological and physiological changes occur.

1) **Morphological or structural changes: -** Physiological malfunctioning of the host cells causes disturbances in chemical reaction which ultimately lead to some structural changes viz., overgrowth, phyllody, sterile flowers, hairy roots, witches' broom, bunchy top, crown gall, root knot, leaf curling, rolling, puckering etc.

2) Physiological changes: -

i. Disintegration of the tissues by the enzymes of the pathogen.
ii. The effect on the growth of the host plant is due to growth regulators produced by the pathogen or by the host under the influence of the pathogen.
iii. Effect on uptake and translocation of water and nutrients.
iv. Abnormality in respiration of the host tissues due to disturbed permeability of cell membrane and enzyme system associated with respiration.
v. Impairing photosynthesis due to loss of chlorophyll and destruction of leaf tissue.
vi. Effect on the process of translation and transcription.
vii. Overall reproduction system of the host.

Sign & Symptom: -

Sign: - A **sign** of plant disease is physical evidence of the pathogen. For example, fungal fruiting bodies, and spores are a sign of disease.

Symptom: - The external and internal reactions or alterations of a plant as a result of a disease. A visible or detectable abnormality expressed on the plant as a result of disease or disorder is called a symptom. The totality of symptoms is collectively called a **syndrome** while the pathogen or its parts or products seen on the affected parts of a host plant is called a **sign**.

Different types of disease symptoms are cited below:

Anthracnose: - A disease that appears as black, sunken, leaf, stem, or fruit lesions, caused by fungi that produce their asexual spores in an acervulus.

Atrophy: - It is known as hypoplasia or dwarfing which results from the inhibition of growth due to a reduction in cell division or cell size.

Blight: - A disease characterized by general and rapid killing of leaves, flowers, and stems.

Blotch: - A disease characterized by large, irregularly shaped, spots or blots on leaves, shoots, and stems or A large area of discolouration of a leaf, fruit etc. giving a blotchy appearance.

Colour change: - It denotes conversion of green pigment of leaves into other colours mostly to yellow colour, in patches or covering the entire leaves-

i. **Albino: -** Lack of any pigment and turned into white or bleached
ii. **Chlorosis: -** ¬Yellowing of normally green tissue due to chlorophyll destruction or failure of chlorophyll formation. It occurs due to infection of viruses, bacteria, fungi, low temperature lack of iron etc.
iii. **Chromosis: -** Red, purple or orange pigmentation due to physiological orders etc.
iv. **Etiolation: -** Yellowing due to lack of light.
v. **Virescent: -** A normally white or coloured tissue that develops chloroplasts and becomes green.

Die-back: - Drying of plant organs such as stem or branches which starts from the tip and progresses gradually towards the main stem or trunk is called die-back or wither tip or Progressive death of shoots, branches, and roots, generally starting at the tip.

Enation: - Tissue malformation or overgrowth, induced by certain virus infections.

Exudation: - Such symptom is commonly found in bacterial diseases when masses of bacterial cells ooze out to the surface of affected plant parts and form some drops or smear, it is called exudation. This exudation forms a crust on the host surface after drying.

Gummosis: - Production of gum by or in a plant tissue.

Leaf spot: - A self-limiting lesion on a leaf.

Mildew: - White, grey or brown coloured superficial growth of the pathogen on the host surface is called mildew.

Mosaic: - Symptom of certain viral diseases of plants characterized by intermingled patches of normal and light green or yellowish colour.

Mummy: - A dried, shrivelled fruit.

Necrosis: - Dead and discoloured. It indicates the death of cells, tissues and organs resulting from infection by a pathogen. Necrotic symptoms include spots, blights, burns, cankers, streaks, stripes, damping-off, rot etc.

Overgrowth: - Excessive growth of the plant parts due to infection by pathogens. Overgrowth takes place by two processes-

i) **Hyperplasia: -** abnormal increase in size due to excessively more cell division.
ii) **Hypertrophy: -** abnormal increase in size or shape due to excessive enlargement of the size of a cell of a particular tissue.

Phyllody: - Excessive production of leaves in place of shoots and blossoms.

Pustule: - Small blister-like elevation of epidermis created as spores form underneath and push outward.

Ring spot: - A circular area of chlorosis with a green centre; a symptom of many virus diseases.

Rosette: - Short, bunchy habit of plant growth.

Rot: - The softening, discolouration, and often disintegration of succulent plant tissue as a result of fungal or bacterial infection.

Rusts: - Numerous small pustules grow out through host epidermis which gives rusty (rust formation on iron) appearance of the affected parts.

Scab: - A roughened, crust-like diseased area on the surface of a plant organ; a disease in which such areas form.

Sclerotia: - These are dark and hard structures of various shapes composed of dormant mycelia of some fungi. Sometimes, sclerotia are developed on the affected parts of the plant.

Scorch: - Burning of leaf margins as a result of infection or unfavourable environmental conditions.

Shock symptoms: - The severe, often necrotic symptoms produced on the first new growth following infection with some viruses; also called acute symptoms.

Shot hole: - A symptom in which small diseased fragments of leaves fall off and leave small holes in their place.

Smuts: - Charcoal-like and black or purplish-black dust-like masses developed on the affected plant parts, mostly on floral organs and inflorescence are called smut.

Wilt: - Loss of rigidity, drooping and withering of plant parts, starting from some leaves to growing tip occurs suddenly or gradually. It is generally caused by insufficient water in the plant and also takes place due to blockage in the translocation system caused by the pathogen.

Witches' broom: - Broom-like growth or massed proliferation caused by the dense clustering of branches of woody plants.

Different types of signs and symptoms are produced on host plants due to plant pathogen infection

Anthracnose of chilli

Early light of Patato

Purple blotch of Onion

Plant Albinism

Chlorosis

Chromosis

Citrus die back

Citrus Gummosis

Leaf spot

Powdery mildew

Downey mildew

Mummified fruit

Mosaice (YVMV)

Sesamum Phyllody

Rust pustules

Stem rot

Apple scab

Leaf scorch

Shot hole symptoms

Smut of wheat

White blister

Sclerotia

Bacterial wilt

Fusarium wilt

Witches' broom

Stem gall of coriander

Leaf curl

Citrus canker

COLLECTION AND PRESERVATION OF DISEASE SPECIMEN

1) Dry Preservation:

a) Collection and drying: The sample should have distinctively visible symptoms. Dry the specimen in between the multiple layers of blotting sheets under shade/sunlight or in a hot air oven for a few days. Frequently change the blotter paper.

b) Labelling and packaging: The material should be kept in herbarium packets. This is attached to a chart paper sheet. Label each specimen pocket as per the information sheet and paste it into the herbarium sheet. The name of a pathogen, host, locality, date, and name of a scientist who identified the specimen, should be mentioned on the label.

c) Disinfection and storage: The specimen folders are fumigated with methyl bromide in fumigation chamber for 24-48 hours before storage.

2) Wet Preservation: Wash fresh diseased specimens then put in a boiling mixture of 1 part of glacial acetic acid saturated with normal copper acetate crystals and 4 parts of water till the green colour reappears and then kept preserved in 5 per cent formalin in the transparent glass/plastic jar.

All mounted or preserved specimens must be labelled with as much of the following information as far as possible:

1. **Name of the Host plant:**
2. **Name of the causal organism:**
3. **Place where collected (location):**
4. **Date of collection:**
5. **Name of the collector:**

When a new organism or diseased specimen is reported for the first time, it must be deposited in any of the following herbaria:

a) **Herb. IMI. Kew, Surrey, England.**
b) **Herb. Mycological Division, USDA, Beltsville.**
c) **Herb. Crypt. Indias, Orientalis, IARI, New Delhi.**

Herbarium Cryptogamae Indiae Orientalis (HCIO): The foundation of **HCIO**, a national fungal herbarium facility was laid down by Sir Edwin John Butler at Pusa, Bihar in 1905. After a disastrous earthquake at Pusa, Bihar in 1934, the HCIO was shifted to the Division of Mycology and Plant Pathology, Indian Agricultural Research Institute, New Delhi. Ever since its inception in 1905, the HCIO continued with the identification, classification and conservation of fungal specimens.

DETECTION OF PLANT PATHOGENS BASED ON BIOCHEMICAL TEST

Biochemical tests are used for microbial identification based on different lye in their biochemical activities exhibited by different types of bacteria. Different biochemical tests are listed below that are used for identification of gram positive and negative bacteria.

S. No.	Biochemical tests	S. No.	Biochemical tests
xv.	**Catalase Test**	xvi.	**Oxidase Test**
xvii.	**Indole Test**	xviii.	**Sulfur Test: -**
xix.	**Urease Test**	xx.	**Triple sugar iron test**
xxi.	**Nitrate Test**	xxii.	**Starch Hydrolysis Test**
xxiii.	**Methyl Red Test**	xxiv.	**Voges-Proskaur Test**
xxv.	**Citric Acid Utilization Test**	xxvi.	**Analytical Profile Index (API) Test:-**

i. **Catalase Test:** Test is used to check microorganisms that produce the catalase enzyme. Catalase enzyme-producing bacteria will neutralize the hydrogen peroxide and bubbles will be produced that are indicative of a positive test. Mostly, catalase enzyme is produced by obligate aerobes and facultative anaerobic bacteria. The test is performed by tube or slide method by mixing the colony of bacteria with a few drops of 3% H_2O_2 on a slide and looking for bubble formation within 10 seconds.

ii. **Oxidase Test:** It is helpful in the identification of microorganisms having ability to produce cytochrome oxidase enzyme. The test helps to differentiate oxidase positive Pseudomonas and negative Enterobacteriacea families. Cytochrome oxidase will oxidize the electron donor and the color will change to dark purple. This test is performed by impregnation of 1 per cent tetra-methyl-p-phenylenediamine dihydrochloride acting as an artificial electron donor into a filter paper and dried. The bacterial colonies are smeared on a paper strip and checked for colour change within 10 sec.

iii. **Indole Test:** This test is helpful in the identification of bacteria having the ability to produce tryptophanase enzyme. This enzyme will convert tryptophane amino acid into indole gas. Thus, gas can be checked by adding different reagents such as Ehrlich's reagent or Kovac's reagent. Indole gas reacts with the reagent and the red colour rosindole dye will form which indicates a positive test.

iv. **Sulfur Test:** The **s**ulfur test is helpful in the identification of microorganisms having the ability to produce cysteine desulfurase enzyme. This enzyme catabolizes the cysteine (amino acid) or reduces it to thiosulphate which will reduce the sulfur into hydrogen peroxide. Due to the production of hydrogen sulfide the color of medium changes to black color.

v. **Urease Test:** The urease test is used to identify bacteria that can produce the urease enzyme. Urease is an enzyme that breaks down urea into NH_3 and CO_2. The creation of ammonia raises the pH of the medium to alkaline, and the colour changes to pink at p^H 8.1, signifying positive findings.

vi. **Triple sugar iron test:** This test is useful for identifying members of the Enterobacteriaceae family. The medium contains 3 sugars: 0.1% glucose, and 1% each of lactose and sucrose. Phenol red and ferrous sulfate are used as indicators. The medium is prepared as a butt and slant. If the inoculated bacteria can utilize glucose under both aerobic and anaerobic conditions, the slant and butt will turn yellow due to acid production within 6-8 hours. If the bacteria can utilize sucrose and lactose, the acid production will continue, and the medium will remain yellow. If it cannot utilize sucrose or lactose, the bacteria will start using amino acids, making the medium alkaline and turning it red due to the phenol red indicator.

vii. **Nitrate Test:** The nitrate test is useful for identifying microorganisms that can convert nitrate to nitrite by releasing the nitratase enzyme. This test can be used to distinguish between Gram (+)ve and Gram (–)ve bacterial species. After adding the bacterial colonies, the test tubes containing the nitrate broth were incubated and examined for the presence of gas.

viii. **Starch Hydrolysis Test:** This test helps identify microorganisms that produce the starch-hydrolyzing enzymes alpha-amylase and oligo-1,6-glucosidase. It is commonly used to distinguish Clostridium and Bacillus species. Starch is too large to pass through the bacterial cell wall and be used directly as a carbon source. Enzymes are released to hydrolyze the starch into glucose, which can then be metabolized. When iodine is added to the agar medium, it turns a dark brown color, indicating that the starch has been hydrolyzed, which suggests a positive result.

ix. **Methyl Red Test:** This is an extension of the Methyl red test, which detects microbes that can produce butylene as a byproduct. Acetoin, the reaction's intermediate product, can be identified using alpha-naphthol and 40 per cent KOH. In the presence of KOH, acetoin is oxidized to diacetyl, which then reacts with the guanidine component of peptone in the presence of alpha-naphthol, resulting in a red color that indicates a positive test.

x. **Voges-Proskaur Test:** This is an extension of the Methyl red test, which detects microbes that can produce butylene as a byproduct. Acetoin, the reaction's intermediate product, can be identified using alpha-naphthol and 40 percent KOH. In the presence of KOH, acetoin is oxidized to diacetyl, which then reacts with the guanidine component of peptone in the presence of alpha-naphthol, resulting in the formation of a red color that indicates a positive test. This reaction is used to detect the presence of such microbes, rather than through the use of magnetic resonance imaging.

xi. **Citric Acid Utilization (CAU) Test:** The CAU test is used to identify microbes that can use citrate as an energy source. It is performed on citrate agar, which contains citrate and ammonium as carbon and nitrogen sources, respectively. The test identifies microbes that produce the enzyme citrate permease, which converts citrate to pyruvate and fuels the organism's metabolism. As microbes consume citrate, the medium's pH rises, causing the bromothymol blue indicator to change from green to blue.

xii. **Analytical Profile Index (API) Test:** The Analytical Profile Index is a method for rapidly classifying bacteria through the use of biochemical tests. However, this approach can only identify known microbes, as it relies on comparing test results to a database of previously characterized organisms. The API 20E/NE system is a specific application of this technique, designed for the identification of a limited set of Gram-negative Enterobacteriaceae and non-Enterobacteriaceae bacteria. In contrast, the API system is also used for Gram-positive microbes such as Staphylococcus and Micrococcus species, as well as other related bacteria. The API 20E/NE kit consists of 20 small wells containing dehydrated substrates, which are used to assess the enzymatic activity of the test organism.

DETECTION OF PLANT PATHOGENS BASED ON USING MICROSCOPIC TECHNIQUES

Microscope: - It is an optical instrument, which magnifies small objects and helps to see clearly and distinctly.

Simple Microscope: A convex lens of short focal length used to get a magnified virtual image is called simple. It cannot produce sufficient magnification to observe very small objects like bacteria, cells etc. Therefore, we use compound microscope.

Compound microscope: It differs from a simple one in the sense that, it consists of two sets of lenses fitted co-axially in a tube. One of the lenses is of a smaller focal length and the other is of a larger focal length. The lens of a smaller focal length is directed towards the object, it is known that the objective. The other lens of larger focal length is held close to the eye and it is called the eyepiece. The diameter of the eyepiece is greater than that of the objective, this helps to collect more light and gives a brighter image. The distance between the optic centre (centre of the lens) and principal focus (the point at which the rays after refraction converge) is **focal length**.

Principles of Microscope: The light rays reflected from mirror pass through the diaphragm and then to the condenser where the rays are condensed and pass through the specimen and reach the objective. The real image is formed within body tube which again serves for further magnification by ocular lens system which forms a virtual image.

Different parts of a microscope

1) **The body with its draw tube:** It is a metallic part and draw tube is provided with a millimetre scale that shows the total length of the working tube.

2) **Stage:** It is a platform that accommodates the microscopic glass slide, on which the object to be examined is placed. It has an aperture in the centre to permit light to pass through. The stage may be equipped with two metal clips, which are of mechanical type where the slides can be moved by rack and pin adjustment.
3) **Coarse adjustment:** It is a metallic part of the microscope which is a big circular screw and it moves the nosepiece up and down rapidly for focusing.
4) **Fine adjustment:** It is the metallic part of microscope which is a small screw, and it moves the nose piece very slowly for definite sharp focusing.
5) **Nose-piece:** It bears different objective lenses and can be rotated to change from one objective to another according to the requirement.
6) **Arm:** It resembles English letter 'C' which supports the upper portion of the microscope.
7) **Base:** This is horseshoe shaped metallic part and supports the entire microscope.
8) **Irish diaphragm:** This is made of metal and controls the amount of light striking the object. It can be opened and closed with the lever.

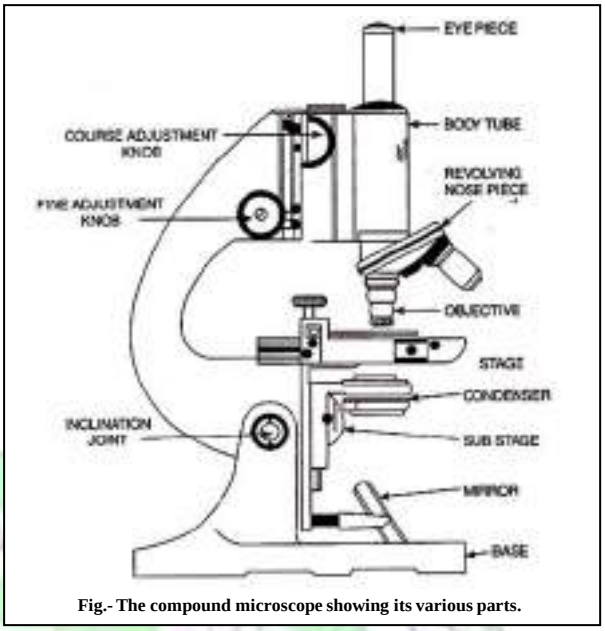


Fig.- The compound microscope showing its various parts.

9) **Eyepiece:** It is the optical part of the microscope and is also called as ocular. It has two lenses - (i) **Field lens- the upper small lens** (ii) **Eye lens- lower larger lens**.
10) **Objectives:** This is an optical part of the microscope, which is nearest to the stage. Ordinary microscopes have three objectives, which are fixed in a revolving nosepiece.
    i. **Low Power Objective (l0x):** It is the objective where the working distance is more. If the ocular is l0x, then the magnification is 100 times.
    ii. **High Power Objective (40 x or 45 x):** Here the working distance is less as compared to low power. If the ocular is l0x, then the magnification is 400 or 450 times.
    iii. **Oil Immersion Objective (l00x):** This requires the use of a drop of either cedarwood oil or liquid paraffin between the lens and the object. Oil serves to prevent the loss of light rays due to refraction. It has a refractive index of 1.51. Here the working distance is very small. If the ocular is l0x then the magnification is 1000 times.
11) **Condenser:** It is an optical part, which is made of lenses. It condenses the light rays thereby preventing the escape of light rays. It also controls light intensity as well.
12) **Mirror:** It has two reflecting surfaces (i) plane and (ii) concave. The plane mirror reflects the light rays parallel to one another while the concave mirror concentrates the light.

Resolving power: The resolving power of a lens is its ability to show two closely adjacent points as distinct and separate.

$$\textbf{Resolving power} = \frac{\textbf{Wave length}}{\textbf{2 x Numerical aperture}}$$

Numerical aperture: It is the function of the effective diameter of the objectives concerning its focal length and refractive index of the medium between the specimen and the objective.

Magnification: It is the ratio between size of the image and size of the object.

$$\mathbf{M = M_0 \times M_E}$$

Where, M = Total magnification

M_0 = Magnification of the objective

M_e = Magnification of the eyepiece

Working distance: It is the distance between the specimen and the objectives.

Working with microscope

Materials required: - A slide with a smear lens, cleaning paper, cedarwood oil or liquid paraffin and microscope.

Procedure:

1. Take a grease free slide and take a small drop of mount to fix the specimen.
2. Now keep the specimen on the mount with the help of a clean needle and carefully put a cover slip on it.
3. Keep the prepared slide accurately over the hole in the centre of the stage.
4. Adjust the light source until it passes maximum amount of light through the specimen with low power objective.
5. Look through the eye-piece and adjust with a coarse adjustment knob until the specimen is in approximate focus and then bring the specimen to sharp focus with the help of fine adjustment knob.

6. After examining with low-power objectives change to the high power by rotating the nose-piece without changing the position of the slide.
7. Look through eye-piece and bring the image into final accurate focus by using fine adjustment.
8. For focusing the specimen by the oil immersion/100x objective put a small drop of cedar wood oil on the object. Now raise the condenser, fully open the iris diaphragm and turn the mirror to get maximum amount of light then use a fine adjustment knob to get a clear sharp image of the specimen.
9. Each time after using the oil immersion objectives clean the oil from the objective lens with a lens cleaning paper or cloth.

HISTOPATHOLOGICAL STUDIES FOR DETECTION OF BACTERIA

Histology- The study of tissues and cells under a microscope. Also known as microscopic anatomy or microanatomy.

Histopathology- The study of diseased tissue and cells using a microscope

Microbial Staining- giving colour to microbes or process in which microbes are stained because they are colourless and highly transparent structures.

Stains - Stain is a dye used to color the living or dead organelles. Stains are organic compounds that carries either positive charges or negative charges or both.

Importance of Staining:

- ✓ It highlights the structures of microorganisms allowing them to be seen under a microscope.
- ✓ Used for the identification of microorganisms.
- ✓ It is also used to differentiate different microorganisms.

Fixing: - The fixing of a sample refers to the process of attaching cells to a slide.

Bacteria

Types of Stain/dyes: -

A) Based on the charges: e.g. Methylene blue, crystal violet, etc.

i) Basic stain/dyes- stain with (+) ve charge.

- To stain (–) ve charged molecules of bacteria
- Mostly used because cell surface is –ve charge.

ii) Acidic stain/dyes- stain with (–) ve charge. e.g. Acid fuchsine, malachite green, nigrosin, Indian ink, etc.

- To stain (+) ve charge molecules of bacteria.
- Used to stain the bacterial capsules.

iii) Neutral stain/dyes- stain with both charges. e.g. Geimsa's stain etc.

B) Based on function of stain:

1	**Simple staining**	**Positive Staining**
2	**Differential staining**	
3	**Special staining**	**Negative Staining**

Positive Staining: - where the actual cells are themselves coloured and appear in a clear background.

Negative Staining: where the cells remain clear (uncoloured) and the background is coloured to create a contrast to aid in the better visualization of the image.

1. Simple staining- only one dye is used differentiation among bacteria is impossible**.** Simple to perform because only one basic stain is used (Crystal violet, Methylene blue, Basic fuschin, Malachite green, etc.)

Principle:

- All bacteria in smear take stain and appear in colour of stain.
- Basic stains have more affinity towards bacterial surfaces & stain the bacteria.

Uses:- To study morphology and arrangement of bacteria.

2. Differential staining- more than one dye is used- Differentiation among bacteria is possible (e. g. Gram staining and acid-fast staining).

Gram Staining: -Danish Bacteriologist Hans Christian Gram (1880)

- Based on this reaction, bacteria are classified into Gram positive and Gram negative bacteria.
- The cell wall composition differences make a difference.

Requirements: -

	Staining Reagents	Role		Staining Reagents	Role
1	Crystal violet	**Primary stain**	**3**	Ethanol (95%)	**decoloriser**
2	Gram's iodine	**mordant/fixative**	**4**	Safranine	**counterstain**

Principle: -

i. **Crystal violet-** all bacteria take crystal violet- so all appear violet.
ii. **Iodine-** Crystal Violet-iodine (CV-I) complex is formed.
iii. **Ethanol** (95%)**-** bacteria with high lipid content lose CV-I complex (appear colourless) but bacteria with less lipid content retain CV-I complex (appear violet).
iv. **Safranine-** only colourless bacteria takes (appear pink).

Procedure: -

- Crystal violet- 1 min - wash.
- Iodine- 1 min - wash.
- Acetone add drop by drop and watch out if colour comes out - wash immediately.
- Safarnine- 1 min - wash.
- Allow to dry - examine under a microscope.

Note: Results should be confirmed only with 100x.

3. Special staining- more than one dye used. Used to stain special structures of bacteria like; capsules, spores, flagella, and metachromatic granules.

i) Capsule Stain: - Negative stain- Nigrosin ink + indian ink. Look for unstained structures against a stained background.

ii) Spore Stain: - *Clostridium* and *Bacillus* species.

- **Malachite green**- 2 min- heat stain till steam rises -2 min - wash.
- Counterstain with **safranin** -1 min- wash.
- Dry the slide and examine.

iii) Flagellar Stain- **Silver Stain:** This stain increases the thickness of flagella thus easy to see under a light microscope.

HISTOPATHOLOGICAL STUDIES FOR DETECTION OF FUNGI

Lactophenol cotton blue: The lactophenol cotton blue (LPCB) wet mount is the most widely used method of staining and observing fungi under a microscope.

Preparation: - The preparation has three components: **Phenol, Lactic acid** and **Cotton blue**.

Sl. No.	Ingredients	Quantity	Role
1	Cotton blue (Aniline blue)	0.05 g	which stains the chitin in the fungal cell walls
2	Phenol crystals	20 g	which will kill any live organisms
3	Glycerol	40 ml	prevent drying
4	Lactic acid	20 ml	which preserves fungal structures, and
5	Distilled water	20 ml	to dissolve Cotton blue crystal
Lactophenol cotton blue		**100 ml**	

Procedure: -

- On the first day, dissolve the Cotton blue in the distilled water. Leave it overnight to eliminate insoluble dye.
- On the second day, wearing gloves add the phenol crystals to the lactic acid in a glass beaker and place on a magnetic stirrer until the phenol is dissolved.
- Add the glycerol.
- Filter the Cotton blue and distilled water solution and add it into the phenol/glycerol/lactic acid solution.
- Mix and store at room temperature in an amber colour bottle.

Procedure for preparing microscopic slide of a fungal specimen using Lactophenol and Lactophenol cotton blue:

- Place a drop of 70% alcohol on a microscope slide.
- Immerse the specimen/material in the drop of alcohol.
- Add one, or at most two drops of the lactophenol/cotton blue mountant/stain before the alcohol dries out.
- Holding the coverslip between forefinger and thumb, touch one edge of the drop of mountant with the coverslip edge, and lower gently, avoiding air bubbles. The preparation is now ready for examination.

Observation: Stain will give the fungi a blue-coloured appearance of the fungal spores and structures, such as hyphae.

Some other stains: -

✓ **Fluorescent Staining of Microorganisms: -**

- **Ethidium bromide (0.1% solution)-** Staining of fungal nuclei.
- **DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride)-** Nuclear Staining of fungi, detection of Phytoplasmal and mycoplasmal infections in cell cultures and plant tissues and granules in cyanobacterial.

✓ **Vital staining: -** live-dead staining
- **Fluorescein diacetate (FDA)-** FDA is a fluorescence dye taken up by viable cells.

DETECTION OF PLANT PATHOGENS BASED ON CULTURAL STUDIES

Medium (pl. media) is the substance that provides nutrients for the growth of microorganisms. The nutrients on which microorganisms are cultivated are known as a culture medium (pl. culture media). Based on chemical composition, media can be classified into three groups (Natural, Semi-synthetic and Synthetic).

1) Natural medium- Culture media of which, the exact chemical composition is not known is called natural or empirical culture media. **Examples** are vegetable juices, meat extracts, beef and tomato juice etc.

2) Semi-synthetic- Culture media, the chemical components of which are partially known and partially obscure are termed as semisynthetic culture media. **Examples**- Potato dextrose agar (PDA), Czapek-Dox agar (CDA), oatmeal agar (OMA), corn meal agar (CMA), Nutrient agar (NA), etc.

3) Synthetic medium- Such media are composed of substances that are chemically known. These media are very useful in studying the physiology, metabolic nature and nutritional requirements of microbes. Both autotrophs and heterotrophs can be grown in these media. **Examples**- Mineral glucose medium, Richard's solution, etc.

Different types of culture mediums and their composition-

1) Potato Dextrose Agar (PDA):

S. No.	**Composition**	
	Ingredients	**Quantity**
1	Peeled potatoes	200.0 g
2	Dextrose	20.0 g
3	Agar-agar powder	20.0 g
4	Distilled water	800-1000 ml make up the volume 1000 ml.
5	Final pH	5.6 ± 0.2.

Preparation: -

- Take 200 g unpeeled potatoes and boil in 500 ml of distilled water for 30 min.
- Filter the liquid (potato infusion) through clean cheesecloth in a clean glass jar and discard potato slices.
- Mix in all other ingredients (Dextrose and agar powder) and boil to dissolve.
- Stir the media regularly with a clean glass rod.
- Fill the media in a conical flask and put on the cotton plug.
- Autoclave for 20-25 min at 121°C.

2) Nutrient Agar (NA):

S. No.	**Composition**	
	Ingredients	**Quantity**
1	Beef extract	200.0 g
2	Peptone	5.0 g
3	Agar-agar powder	20.0 g
4	Distilled water	make up the volume 1000 ml.
5	PH of the medium	7.2

Preparation: -

- Take 800 ml of distilled water in a clean beaker and heat it.
- Mix in all other ingredients and boil to dissolve.
- Stir the media regularly with a clean glass rod.
- Fill the media in a conical flask and put on the cotton plug and Volume make up to 1000 ml.
- Autoclave for 20-25 min at 121°C.

Some other media

3) Richards's solution			**4) Czapek's Medium**		
S. No.	**Ingredients**	**Quantity**	**S. No.**	**Ingredients**	**Quantity**
1	Potassium nitrate	10.00 g	**1**	$NaNO_3$	2.0 g
2	Potassium dihydrogen phosphate	5.00 g	**2**	K_2HPO_4	1.0 g
3	Magnesium sulphate	2.50 g	**3**	$MgSO_4.7H_2O$	0.5 g
4	Ferric chloride	0.02 g	**4**	KCl	0.5 g

5	Sucrose	50.00 g		**5**	$FeSO_4.7H_2O$	0.01 g
6	Distilled Water	1000 ml		**6**	Sucrose	30 g
				7	Agar-Agar	20 g
				8	Distilled water	make up the volume 1000 ml

5) Corn meal medium			**6) Malt extract medium**			**7) Oat meal medium**		
S. No.	**Ingredients**	**Quantity**	**S. No.**	**Ingredients**	**Quantity**	**S. No.**	**Ingredients**	**Quantity**
1	Corn meal	25.00 g	**1**	Malt extract	20.00 g	**1**	Oat meal	40.00 g
2	Peptone	20.00 g	**2**	Agar Agar	20.00 g	**2**	Agar Agar	20.00 g
3	Glucose	20.00 g	**3**	Distilled Water	1000 ml	**3**	Distilled Water	1000 ml
4	Agar Agar	20.00 g						
5	Distilled Water	1000 ml						

ISOLATION AND PURIFICATION OF PLANT PATHOGENS FROM INFECTED PLANT TISSUES

Plant pathogenic organisms seldom occur alone in their natural habitat without other microorganisms. Hence, to study them they have to be separated from other contaminating microorganisms.

Collection of the diseased samples: The sample showing clear symptoms of the disease should be collected from the naturally infected host and brought back to laboratory for further studies. Always use a clean paper bag or zip poly packet for collection, storage and transportation of diseased specimens, to avoid saprophytic growth. A dried sample is always more suitable for this purpose than wet samples. Separate diseased samples should be collected and stored separately.

Isolation of the pathogen: The sample showing clear symptoms of the disease should be selected for isolation of the pathogen. Isolation of plant pathogens from the infected/ diseased tissues is achieved by following techniques-

Material required: Infected plant material, Petri dishes, Alcohol, Mercuric chloride, Potato Dextrose Agar (for fungi), Nutrient Agar (for bacteria), Sterilized distilled water, Inoculation needle, Inoculation loop, Sprit lamp, slants etc.,

1) Procedure for isolation of plant pathogenic Fungi

i. Collect the naturally infected plant tissues (leaves, twigs, fruits, flowers, stem etc.).
ii. Wash the infected tissues with running tap water and keep them on a clean plastic tray for drying.
iii. Cut the diseased tissue into small pieces of 1-2 mm having both healthy and infected tissues.
iv. Surfaces sterilize with 0.1% Mercuric Chloride for 1 minute.
v. Wash them in sterile distilled water successively 3 times and then keep them on sterilised blotter paper for drying.
vi. Transfer these pieces into Potato Dextrose Agar (PDA) slants or a Petri dish aseptically with the help of flame sterilized Inoculation needle.
vii. Incubate them at 27 ±2 °C and observe the inoculated plates regularly.

2) Procedure for isolation of plant pathogenic Bacteria

i. Collect the naturally infected plant tissues (leaves, twigs, fruits, flowers, stem etc.).
ii. Wash the infected tissues with running tap water and keep them on a clean plastic tray for drying.
iii. Cut the diseased tissue into small pieces of 1-2 mm.
iv. Surface sterilizes them with 0.1 % Mercuric Chloride minutes.
v. Wash the pieces 3 times in sterile water.
vi. Transfer these pieces into a test tube containing 2 ml of sterile distilled water and crush with sterile glass rod to liberate (ooze) the bacterial cells.
vii. Streak a loopful of this suspension on a slant or aseptically.
viii. Incubate them at 28 ±1°C for 24 hrs.

DETECTION OF PLANT PATHOGENS BASED ON BIOLOGICAL ASSAYS- INDICATOR HOSTS AND DIFFERENTIAL HOSTS

Bioassays or biological indexing is one of the earliest active virus tests developed to detect plant viruses. The procedure is based on the ability of certain plants, called indicator plants, to produce symptoms when inoculated with viruses by mechanical inoculation. Indicator plants are chosen for their ability to display relatively distinct disease symptoms when infected.

Materials required:

Indicator plants: *Chenopodium quinoa*, *C. amaranticolor*, *Gomphrena globosa*, *Vigna unguiculata*, *Nicotiana benthamiana*, *N. glutinosa etc*. infected planting materials, 0.1 M phosphate buffer, Pestle and mortar, Carborandum, Celite, Mercaptoethanol, Cotton, Water, Blotting Paper.

Procedure:

- Infected plants showing symptoms is collected from field, wash the infected leaves in tap water to remove the dust particles adhering to them and dried between the folds of blotting paper.
- The leaves is then macerated in chilled mortar and pestle using potassium phosphate buffer (pH 7.0, 0.05M) at the rate of 1ml/gm of leaf tissue.
- The resultant pulp is squeezed through absorbent cotton and the extract thus obtained is used as standard inoculum.
- To the standard inoculum add celite (600 mesh) at the rate of 0.025 g/ml of the extract and 0.02% mercaptoethanol.
- Apply the inoculum gently on the upper surface of the leaves of any indicator plants with a small piece of absorbent cotton wool.
- Wash the inoculated leaves 1-2 minutes after inoculation to remove the excess inoculum with a fine jet of distilled water from a squeeze bottle and keep the plants under observation in the glass house.
- Observe the local lesion on indicator plants within 5-6 days and system symptoms within 15 days.
- Repeat the practical on different hosts to study the host range of a particular virus.

Activities:

1. **Inoculate the virus on indicator plants and different host species**
2. **Study the nature of infection of virus and list out the local lesion and systemic host.**

DETECTION OF PLANT PATHOGENS BASED ON SEROLOGICAL ASSAYS (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a method of target antigen (or antibody) capture in samples using a specific antibody (or antigen) and of target molecule detection/quantitation using an enzyme reaction with its substrate.

Principle: In ELISA, various antigen-antibody combinations are used, always including an enzyme-labelled antigen or antibody, and enzyme activity is measured colourimetrically. The enzyme activity is measured using a substrate that changes colour when modified by the enzyme. Light absorption of the product formed after substrate addition is measured and converted to numeric values. Depending on the antigen-antibody combination, the assay is called a direct ELISA, indirect ELISA, sandwich ELISA, competitive ELISA etc.

Material Required:

A) Coating buffer (Carbonate buffer/antigen buffer) (p^H 9.6):

S. No.	Chemical/Reagents	Quantity
1.	Sodium carbonate (Na2CO3	1.59 g
2.	Sodium hydrogen Carbonate (NaHCO3)	2.93 g
3.	Sodium azide	0.2 g
4.	Distilled water	1.0 L
5.	The diethyl dithiocarbamate (sodium salt) was added at 0.001 M conc.	1.71 g/L

B) Phosphate buffer saline (PBS) p^H 7.4:

S. No.	Chemical/Reagents	Quantity
1.	Disodium hydrogen phosphate (Na2HPO4, 2 H2O)	2.9 g
2.	Potassium di hydrogen phosphate (KH2PO4)	0.2 g
3.	Potassium chloride (KCl)	0.2 g
4.	Sodium chloride (NaCl)	8.0 g
5.	Distilled water	1.0 L

C) Washing buffer (PBST):

S. No.	Chemical/Reagents	Quantity
1.	PBS	1000 ml
2.	Tween-20	0.5 ml

D) Antibody buffer:

S. No.	Chemical/Reagents	Quantity
1.	PBS-Tween	100 ml
2.	Polyvinyl Pyrrolidone (40000 MW)	2.0 g
3.	Bovine Serum Albumin (BSA)	0.2 g

E) Substrate buffer (p^H 9.8):

S. No.	Chemical/Reagents	Quantity
1.	Diethanolamine l	97 m
2.	Distilled water	800 ml
3.	Sodium azide	0.2 g

Procedure for DAC- ELISA

i. Prepare the antigen and dispense it into each well of microtitre plates at the rate of 200µl per well using a micropipette and incubate the plates at 37°C for 2-2.5 hr or at 4°C overnight.
ii. The contents of the plate are poured off and rinsed in PBS-Tween. Washing is carried out by immersing the plate in a wash buffer for 3 min. repeat the washing into three times and at the final wash plate is taken to drain off buffer completely.
iii. 200µl blocking solution is added to each well and incubated at 37°C for one hour to block the unoccupied sites in wells of microtitre plates and wash the plates in PBS-Tween as in step 2.
iv. Monospecific Polyclonal antisera is dilute (1:1000 ratio and 1:750 ratio) respectively (v/v) in antibody buffer and add 200µl per well. Then incubate the plates at 37°C for 2-2.5 hr or at 4°C overnight. After the incubation wash the plates in PBS-Tween as in step 2.
v. In the next step antirabbit IgG-ALP labelled enzyme conjugate diluted in enzyme buffer (1: 10,000) is added 200 per well. Then the plates are incubated at 37°C for 2 hr and wash the plates with PBS-Tween as in step 2.
vi. To develop color, 20 mg tablet of pNPP is dissolved in Substrate buffer and add 200µl per well. The plates are incubated at room temperature for 10-30 min for colour development. Read the plates at 405 nm by using an EL310E ELISA reader (BIO-TEK instruments, USA).

Important critical steps

a. **Coating:** proper preparation of the samples should be done to improve adhesion to microtiter plates.
b. **Washing:** Plates should be washed carefully and consistently a number of times to reduce background color development in negative control wells.
c. **Development:** Substrate gets oxidized when it gets exposed to light. Hence keep the substrate at dark for better color development.

Observation and results: Look for the development of yellow colour on wells at the end of the Practical. Read the absorbance at 405 nm and record the readings.

ISOLATION AND PURIFICATION OF GENOMIC DNA OF PLANT PATHOGEN USING CTAB

Material Required: CTAB buffer, Microfuge tubes, Mortar and pestle, Isopropanol (Ice cold), 70 % Ethanol (Ice cold), Ammonium Acetate (7.5 M), Water bath (55 ºC), Phenol:Chloroform:Iso Amyl Alcohol (25:24:1), Nuclease free water (Sterile), Agarose.

Procedure:

i. Grind 100-200 mg of fungal mycelium (100-200 mg) or bacterial culture (50-100 mg) or infected leaves (virus) using a pestle and mortar.
ii. Add 1-2 ml CTAB extraction buffer and grind thoroughly.
iii. Transfer 0.75 ml of grinded sap into a 1.5 ml micro centrifuge tube and incubate at 60º C for 30 min in a water bath.
iv. After incubation, add 750 µl of phenol: chloroform: isoamyl alcohol (25:24:1) and mix well to form an emulsion by inverting the tube.
v. Centrifuge the mixture at 13,000 rpm for 10 min.
vi. Collect the supernatant and mix thoroughly with 300 µl of isopropanol and incubate at -20º C for at least one hour.
vii. Centrifuge the contents centrifuged at 13,000 rpm for 10 min and discard the supernatant without losing pellet.
viii. Wash the pellet with 500 µl of 70 per cent ethanol and centrifuge at 14,000 rpm for 5 min.
ix. Remove the ethanol and dry the pellet in a vacuum drier for 5 min.
x. Finally the dried pellet is resuspended in 100 µl of 1X TE buffer and stored at -20º C.
xi. Quantify the DNA by using a Spectrophotometer.

<u>Assessing the quantity and quality of isolated Nucleic Acids (DNA or RNA)</u>.

A) Spectrophotometric method: Spectrophotometry is a fast technique that relies on the properties of light interacting with samples for precise quantification. Samples are loaded into an instrument (the spectrophotometer) that passes light at varying wavelengths through them, which is then picked up by a detector. The detector provides information regarding sample quantity and quality that is then translated via computer software. Absorbance measurements are taken at **260 nm** and **280 nm**, and the ratio of the two indicates the purity of the sample. For pure DNA and RNA, the **260/280 ratio** should be

between **1.8** and **2.0**. A ratio less than 1.8 and more than 2.0 indicates the probable presence of contaminants.

Procedure:

- To measure the concentration of DNA/RNA it is diluted approximately with TE buffer (either 1:50 or 1:100).
- The spectrophotometric absorbance of the sample is recorded at 260/280 nm, using TE as blank.
- The DNA concentration of the sample is calculated as follows:

Conc. of DNA/RNA (µg/ml) = OD_{260} × 50 × dilution factor

B) Agarose Gel Electrophoresis: Agarose gel electrophoresis separates nucleic acids based on their molecular weight by pulling them through a solidified gel matrix in the presence of an electric current. Extracted nucleic acids are compared to a molecular weight standard run in parallel, containing fragments of known sizes and quantities.

Procedure:

i. Prepare a 1% solution of agarose by melting 1 g of agarose in 100 ml. of TBE buffer in a microwave for approximately 2 min. Allow to cool for a couple of minutes then add 2.5 µl of ethidium bromide, stir to mix.
ii. Cast a gel using a supplied tray and comb. Allow the gel to set for a minimum of 20 min at room temperature on a flat surface.
iii. Load the following into separate wells
    - 10 µL 1kb ladder
    - 5 µL sample + 5 µl. water + 2 µL 6x Loading Buffer
iv. Run the gel for 30 min at 100 V
v. Expose the gel to UV light and take a photograph of the gel using a Gel Doc system.

Confirm DNA quality, presence of a highly resolved high molecular weight band indicates good quality DNA, presence of a smeared band indicates DNA degradation.

POLYMERASE CHAIN REACTION (PCR) AMPLIFICATION

Polymerase chain reaction is a widely used molecular biology technique that enables researchers to produce millions of copies of a specific DNA sequence. It derives its name from one of its key components; a DNA polymerase which is used to amplify target DNA (template DNA) by *in vitro* enzymatic replication. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints; and the detection and diagnosis of infectious diseases.

Materials required:

- **Taq polymerase:** Enzyme that extends growing DNA strand complementary to DNA template
- **MgCl2:** Provides ions needed for enzyme reaction.
- **dNTPs:** Nucleotides (Adenine, Cytosine, Guanine, Thymine) building blocks for new DNA strands.
- **Buffer:** Maintains optimal pH for an enzyme.
- **Primers:** Anneal to single-stranded DNA template, provide initiation site for extension of new DNA. Forward primer: Anneals to DNA anti-sense strand; Reverse primer: Anneals to DNA sense strand.
- **DNA template:** the product of our DNA extraction.

Procedure: The PCR usually consists of a series of 30 to 35 cycles. Most commonly, PCR is carried out in three steps, often preceded by one temperature hold at the start and followed by one hold at the end.

Stage I:-

Initialization step: This step consists of heating the reaction mixture to a temperature of 94-96°C (or 98°C if extremely thermo stable polymerases are used), which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

Stage II: -

Denaturation step: This step is the first regular cycling event and consists of heating the reaction mixture to 94-98°C for 20-30 seconds. It causes melting of DNA templates and primers by disrupting the hydrogen bonds between complementary bases of the two DNA strands, yielding single strands of DNA.

Annealing step: The reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are formed only formed when the primer sequence very closely match the template sequence. The polymerase binds to the primer template hybrid and begins DNA synthesis.

Extension/elongation step: The temperature at this step depends on the DNA polymerase used; Taq polymerase has its optimum activity temperature at 75-80°C, and commonly a temperature of 72°C is set for elongation. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl

group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. As a rule of thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases in one minute.

Stage III: -

Final Extension: This single step is occasionally performed at a temperature of 70- 74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single stranded DNA is fully extended.
Final hold: This step at 4°C for an indefinite time may be employed for short term storage of the reaction.

AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis is a technique used to separate DNA fragments based on their size. This process involves applying an electric current that causes the negatively charged DNA molecules to migrate through an agarose gel matrix. The smaller DNA fragments can navigate the gel pores more easily than the larger fragments, resulting in their separation by size. The DNA bands are visualized by staining with ethidium bromide, a dye that intercalates between the DNA bases and fluoresces under UV light. Unknown DNA samples are typically run alongside a "ladder" of known fragment sizes, allowing the approximate size of the unknown DNA bands to be determined. Additionally, the sensitivity of the technique allows for the detection of approximately 10 nanograms of DNA in a single band on a horizontal agarose gel.

Materials: Agarose, TBE buffer, Gel casting tray, Comb, Power pack; Sample DNA; Loading dye; Sterile microtips; EtBr staining solution; UV transilluminator or Gel Doc. System

Solutions preparation:

i) 10X TBE Buffer

Tris base	108 g
Boric acid	55 g
0.5 M EDTA (p^H 8.0)	20 ml

Dissolve in dd H_2O and volume make up to 1 L. Autoclave before use.

ii) Gel loading buffer (10 ml)

Glycerol	5 ml
40X TBE	250 µl
Bromophenol blue saturated	1 ml
Xylene cyanol, 10% suspension	1 ml
H_2O	2.75 ml

Mix well, divide into 1 ml aliquots and store at -20 °C

Procedure:

i. Weigh 0.8 g agarose and put it in a 250 ml. conical flask. Add 100 ml of 1x TBE buffer and gently boil the solution in a microwave oven with occasional mixing until all agarose particles are completely dissolved, allowing it to cool to 50 °C. Prepare the gel mould and keep the comb in position. Pour the cooled gel solution into the gel mould and allow the gel to set for 20 minutes. Before powring add Ethidium bromide (EtBr).
ii. Fill the horizontal electrophoresis chamber with 1x TBE. Remove the comb from the gel and place gel with the tray in the electrophoresis chamber.
iii. Load the digested DNA sample carefully into the wells. In one well load a standard marker (lambda DNA restricted with Hind III).
iv. Run the gel at 20 mA overnight.
v. Stain the gel in 100 ml sterile distilled water containing 1 µg/ml ethidium bromide for 10 minutes. Briefly destain with sterile water.
vi. Visualize the DNA bands on a UV Transilluminator and place a ruler next to the gel to be able to determine the fragment sizes later on. Take a picture using a Polaroid camera with a red filter.

ISOLATION AND PURIFICATION OF GENOMIC RNA OF PLANT PATHOGENS

TRI reagent method is followed to extract the total RNA from infected plant tissue.

Material Required: TRI reagent, Microfuge tubes, Refrigerated Centrifuge, Mortar and pestle, Liquid Nitrogen, Isopropanol (Ice cold), 70 % Ethanol (Ice cold), Water bath (55 ºC), Chloroform, Nuclease free water (Sterile).

Procedure:

i. Weigh 50-100 mg of the infected leaf tissue.
ii. Grind the sample immediately using liquid nitrogen in a pestle and mortar and place it in a sterile micro centrifuge tube.

iii. Homogenize the 50-100 mg leaf sample in 1 ml of TRI reagent.
iv. Centrifuge the homogenate at 9,000 rpm for 10 min at 2-8º C.
v. Transfer the supernatant into a fresh microcentrifuge tube and incubate the tube for 5 min at room temperature.
vi. Add 0.2 ml of Chloroform to the microcentrifuge tube and shake vigorously for 15 sec.
vii. Keep the tube for 2-15 min at room temperature and centrifuge at 9,000 rpm for 15 min at 2-8º C. Three separate phases will form. The colourless upper phase consists of RNA, interphase consists of DNA and bottom red organic phase consists of protein.
viii. The colourless upper aqueous phase is transferred into a fresh microcentrifuge tube and adds 0.5 ml of Isopropanol and mixed properly.
ix. Keep the tube for 5-10 min at room temperature and centrifuge at 9,000 rpm for 10 min at 2-8º C.
x. Discard the supernatant and RNA pellet is washed with 75 per cent ethanol with vertexing.
xi. The tube is centrifuged at 5,500 rpm for 5 min at 2-8º C.
xii. Remove the Ethanol without losing the pellet and dry the pellet for 5-10 min under a fan by keeping the lid of the microcentrifuge tube open (The pellet should not be vacuum centrifuged).
xiii. Finally the dried pellet is resuspended in 20-30 µl of nuclease free water (sterile).
xiv. The pellet is dissolved by repeated pipetting with a micro-pipette or by keeping it at 55-60º C for 10-15min and stored at -80º C.

COMPLEMENTARY DNA (cDNA) PREPARATION AND PCR AMPLIFICATION

cDNA (short for copy DNA; also called **complementary DNA**) is synthetic DNA that has been transcribed from a specific mRNA through a reaction using the enzyme reverse transcriptase. While DNA is composed of both coding and non-coding sequences, cDNA contains only coding sequences. The isolated RNA from the plant samples is used for RT-PCR. First, cDNA is synthesised from viral RNA in a 20 µl reaction using a **cDNA preparation Kit**. Reverse transcription is carried out in thermocycler. RT reaction is set up for CMV by adopting the manufacturer's instructions which are as follows:

i. 5 µl of template RNA + 2 µl of reverse primer + 4 µl of nuclease free water is added into a 0.2 m polypropylene tube.
ii. The mixture is incubated at 70º C for 5 min.
iii. The mixture is immediately chilled on ice for 2 min.
iv. All the reaction components including 4 µl 5 X BIOSCRIPT reaction buffer (BIOLINE), 2 µl 10mM dNTPs, and 2 µl nuclease free water are then added into the tube and the tube is incubated at 37º C for 1 min.
v. 200 units of RT enzyme (200 U/1 µl) were added to the tube and incubated at 42º C for 60 min.
vi. The reaction is stopped by heating the mixture at 70º C for 10 min and stored at -20º C. The c-DNA is used for performing PCR.

Reverse transcription polymerase chain reaction (RT PCR): The cDNA synthesized by reverse transcription is amplified by RT-PCR. The PCR reaction mixture of 25 µl is prepared as follows:

Procedure: 25 µl of reaction mixture is prepared by adding the following ingredients into the Eppendorf tube.

BIOINFORMATICS TOOLS

Tools: BLASTn tool, Primer designing software, Bioedit tool, Clustal X/W, MEGA Software.

NCBI (National Centre for Biotechnological Information): NCBI is one of the leading online resources known for providing Biological sequence information. NCBI is maintained by two organizations in US, National Library of Medicine (NLM) and National Institute of Science (NIH). As a national resource for molecular biology information, NCBI's mission is to develop new information technologies to aid in the understanding of fundamental molecular and genetic processes that control health and disease. More specifically, the NCBI has been charged with creating automated systems for storing and analyzing knowledge about molecular biology, biochemistry, and genetics. NCBI is connected to various other sequence databases to be more efficient in answering sequence queries. The user queries and sequence information are delivered through NCBI's search tool called

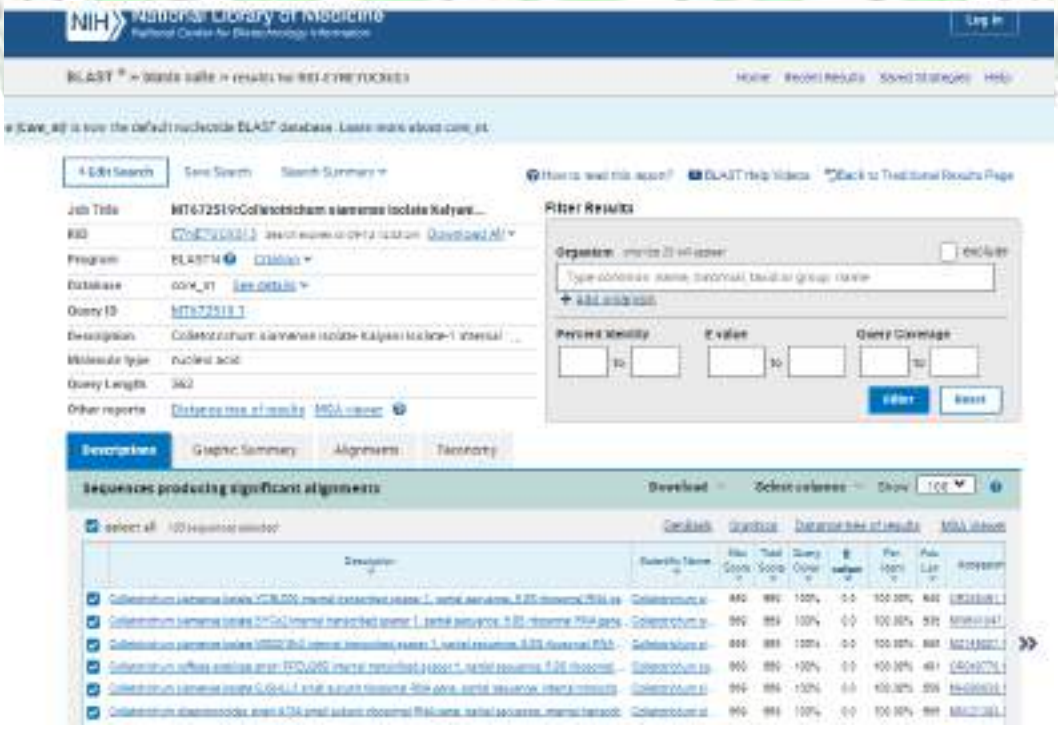
the “entrez”.

GenBank: The GenBank sequence database is an open-access, annotated collection of all publicly available nucleotide sequences and their protein translations. This database is produced and maintained by the National Center for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database Collaboration (INSDC). The National Center for Biotechnology Information is a part of the National Institutes of Health in the United States. GenBank and its collaborators receive sequences produced in laboratories throughout the world from more than 100,000 distinct organisms. In more than 20 years since its establishment, GenBank has become the most important and influential database for research in almost all biological fields, whose data were accessed and cited by millions of researchers around the world. GenBank continues to grow at an exponential rate, doubling every 18 months.

Entrez: The NCBI database accepts queries and delivers data via a custom made search engine called Entrez. The Home page of NCBI has a search box that directs the user to Entrez. Entrez is internally connected to various biological databases which increases the probability of getting the correct information.

BLAST: BLAST stands for Basic Local Alignment Search Tool. BLAST is a tool that is used to find the sequences homologous to a particular sequence. BLAST compares all the sequences in the database with the one that is searched for and provides many hits which are usually arranged in the increasing order of the score obtained. BLAST uses **PAM** and **BLOSUM** matrices for scoring the alignment. BLAST is available at the URL **http://blast.ncbi.nlm.nih.gov/**

Output: The file format of the particular virus after BlastN is shown as follows:



MULTIPLE SEQUENCE ALIGNMENT (MSA) AND PHYLOGENETIC TREE CONSTRUCTION

Multiple sequence alignment: When a protein sequence is newly determined, an important goal is to assign possible functions to the protein. The first computational step is to search for similarities with sequences that have previously been deposited in the DNA and protein sequence databases. If similar sequences are found, they may match the complete length of the new sequence or only to sub-regions of the sequence. If more than one similar sequence is found, then the next important step in the analysis is to multiply and align all of the sequences. Multiple alignments are a key starting point for the prediction of protein secondary structure, residue accessibility, function, and the identification of residues important for specificity.

A protein sequence is represented by a string of letters coding for the 20 different types of amino acid residues. A protein sequence alignment is created when the residues in one sequence are lined up with those in at least one other sequence. Optimal alignment of the two sequences will usually require the insertion of gaps in one or both sequences to find the best alignment. The alignment of two residues implies that those residues are performing similar roles in the two different proteins. This allows for information known about specific residues in one sequence to be potentially transferred to the residues aligned in the other. For example, if the active site residues of an enzyme have been characterized, alignment of these residues with similar residues in another sequence may suggest that the second sequence possesses similar catalytic activity to the first.

CLUSTAL W combines a good hierarchical method for multiple sequence alignment with an easy-to-use interface. The software is free, although a contribution to development costs is required when purchasing the program. CLUSTAL W runs on most computer platforms and incorporates many of the techniques described in the previous section. The program uses a series of different pair-score matrices, biases the location of gaps, and allows you to realign a set of aligned sequences to refine the alignment.

Requirement: Computer system with (legal software) equipped with Internet Connection preferably fast Broadband.

Web resources used: ClustalW: www.ebi.ac.uk/tools/clustalW2/index.html

Principle:

Phenetics is a novel phylogenetic method that takes into account as many variable characters as possible (OTUs = Operational Taxonomic Units), these characters being scored numerically and analyzed by rigorous mathematical methods (numerical taxonomy).

Phylogeny: Phylogeny is the description of biological relationships, especially from an evolutionary point of view and is usually expressed as a "tree". There are different ways to represent these trees:

Cladogram: Character-based methods are called cladistic methods. The trees are prepared by considering the various possible pathways of evolution and are based on parsimony or likelihood methods. The resulting tree is called a cladogram.

Dendrogram: A phylogenetic tree generated by taking into account the similarity (coefficient) or dissimilarity seen in the characters of different entities e.g., protein structure or organism species, genera etc., to be useful in their classification.

Construction of Phylogenetic Tree: In constructing a phylogenetic tree the preliminary step is to compare the nucleotide sequences. This is done by Sequence alignment. Rigorous mathematical approaches have been devised to sequence alignment to be used to convert the data into a phylogenetic tree. The similarity approach aims to maximize the number of matched nucleotides (identical in two sequences compared). Its complementary approach is the Distance method that allows the minimizing of the number of mismatches. The final result of the two approaches is the same i.e., the same alignment will be identified as the best i.e., there is a likelihood of identifying the same alignment if there is a relationship. Multiple alignments is often used for the comparison of more than two sequences with the help of clustal W as the popular choice.

Procedure:

1) Go to http://www.ebi.ac.uk/clustalw/
2) Put in your e-mail (if you want the results e-mailed to you not necessary) and an alignment title of your choice.
3) Paste sequences in the box below using the FASTA format:
   >Name of Sequence #1
   >Name of Sequence #2
4) Every sequence MUST have a different name given to it or the alignment will not work. Or upload a file that includes all your sequences (such as a .doc file) in an acceptable format.
5) Press run button to start alignment reading.
6) When viewing your results, these are the consensus symbols used by ClustalW:
   a. "*" means that the residues or nucleotides in that column are identical in all sequences in
   b. ":" means that conserved substitutions have been observed.
   c. "." means that semi-conserved substitutions are observed.
7) If you would like to see your results in color, push the button that displays Show Colors. Click Hide Colors to get rid of color.
8) Click on the button named View Alignment File to see the alignment on a larger scale (ie bigger font).

SYNTHESIS OF PRIMERS FOR PCR

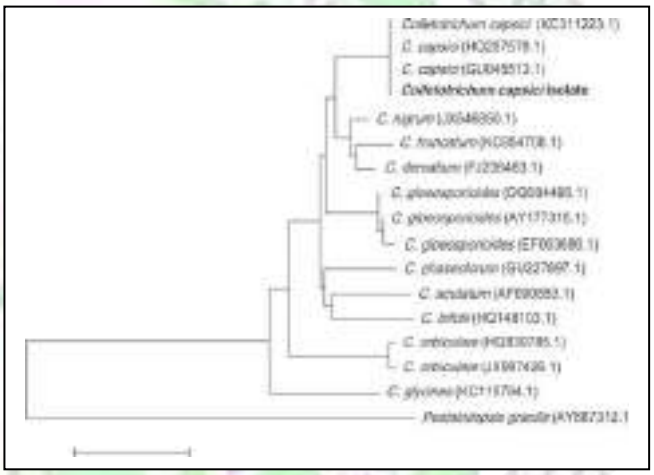
The use of software in biological applications has given a new dimension to the field of bioinformatics. Many different programs for the design of primers are now available. Freeware software is available on the internet and many universities have established servers where a user can log on and perform free analyses of proteins and nucleic acid sequences. There are a number of simple stand-alone programs as well as complex integrated networked versions of the commercial software available. These software packages may be for complete DNA and protein analysis, secondary structure predictions, primer design, molecular modeling, development of cloning strategies, plasmid drawing or restriction enzyme analyses. Companies engaged in biosoftware development include, Alkami Biosystems, Molecular Biology Insights, PREMIER Biosoft International, IntelliGenetics Inc., Hitachi Inc., DNA Star, Advanced American Biotechnology and Imaging.

Properties of Primers:

- Length of 18-24 bases
- 40-60% G/C content
- Start and end with 1-2 G/C pairs
- Melting temperature (Tm) of 50-60°C
- Primer pairs should have a Tm within 5°C of each other
- Primer pairs should not have complementary regions

Primer length: For broad-spectrum studies, primers of typically 18-30 nucleotides in length are the best. Primers should be at least 18 nucleotides in length to minimize the chances of encountering problems with a secondary hybridization site on the vector or insert. Primers with long runs of a single base should generally be avoided. It is especially important to avoid 4 or more G's or C's in a row.

Melting Temperature (Tm): The optimal melting temperatures for primers in the range 52-58°C, generally produce better results than primers with lower melting temperatures. Primers with melting temperatures above 65°C should also be avoided because of potential for secondary annealing. It is then advisable to do the sequencing reaction with annealing and extension at 60°C. A good working approximation of this value (generally valid for oligos in the 18–30 base range) can be calculated using the formula of Wallace et al. (1979), Tm = 2(A+T) + 4(G+C).



GC Content (Tm and Ta are interrelated): GC% is an important characteristic of DNA and provides information about the strength of annealing. Primers should have GC content between 45 and 60 per cent. For primers with a G/C content of less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 50°C. GC content, melting temperature and annealing temperature are strictly dependent on one another.

3'-End Sequence: It is well established that the 3' terminal position in PCR primers is essential for the control of mispriming. Primers should be "stickier" on their 5' ends than on their 3' ends. A "sticky" 3' end as indicated by a high G/C content could potentially anneal at multiple sites on the template DNA. A "G" or "C" is desirable at the 3' end but the first part of this rule should apply. This GC clamp reduces spurious secondary bands.

Dimers and false priming cause misleading results: Primers should not contain complementary (palindromes) within themselves; that is, they should not form hairpins. If this state exists, a primer will fold back on itself and result in an unproductive priming event that decreases the overall signal obtained. Hairpins that form below 50°C generally are not such a problem. Primers should not contain sequences of nucleotides that would allow one primer molecule to anneal to itself or the other primer used in PCR reactions (primer dimer formation).

Specificity: As mentioned above, primer specificity is at least partly dependent on primer length. There are many more unique 24 base oligos than there are 15 base pair oligos. However, primers must be chosen so that they have a unique sequence within the template DNA that is to be amplified. A primer designed with a highly repetitive sequence will result in a smear when amplifying genomic DNA. However, the same primer may give a single band if a single clone from a genomic library is amplified.

Degenerate Primers: Degeneracy in primer sequence should also be taken into consideration. Degenerate primers based on the amino acid sequence of conserved regions were also used to search for members of a gene family.

Complementary primer sequences: Primers need to be designed with absolutely no intra-primer homology beyond 3 base pairs. If a primer has such a region of self-homology, “snap back” can occur. Another related danger is inter-primer homology: partial homology in the middle regions of two primers can interfere with hybridization. If the homology should occur at the 3' end of either primer, primer dimer formation will occur.

Activities:

1. Design the primers for predominant viruses
2. Identify the virus from obtained sequence (from your sequences) through BlastN analysis
3. Do multiple sequence analysis and construct Phylogeny by using ClustalW tool

PHYLOGENETIC TREE CONSTRUCTED WITH THE ITS rDNA SEQUENCE OF COLLETOTRICHUM ISOLATES

Electron microscopy techniques for plant virus detection and diagnosis: Electron microscopy provides virologists with the basic tool a light microscope does for the bacteriologist. An electron microscope reveals several characteristics and features of virus particles, purity of virus preparations, substructures and inclusion bodies in ultra-thin sections of infected tissue. A series of processes are involved in electron microscopy such as specimen preparation; preparation of grids, filmmaking, carbon coating, shadow casting, ultra-thin sectioning and negative staining, which are beyond the scope of this manual. Handling of the specialized and costly equipment as EM requires highly skilled manpower. However, students must be well acquainted with some basic procedures, which are described briefly.

Material:

- Infected tissue (TMV, PVX, PVY) or virus preparations
- Copper grids with formavar/collodion membrane supported by carbon coating.
- Phosphotungstic acid (PTA) or Uranyl acetate (UAc).
- Filter paper.

Procedure:

- Place a drop of 2% PTA, pH 6.8 or UAc, pH 6.4 on an infected tissue and tease the tissue with a fine needle or edge of blade so that the cell exudate oozes out in the stain.
- Alternatively, place a drop of stain on a copper grid and freshly cut edge of virus infected tissue is touched. It is advisable to fix the tissue for 10-20 minutes in 10% formaldehyde to stabilize virus particles.
- Purified or partially purified virus preparation can be mixed with stain and taken on the gird. Drain fluid with a piece of filter paper and allow the gird to dry. Observe the gird in an EM and record details of virus particles.
- Place the grid on a taped microscopic slide, filmed side up.
- Put one drop of specimen suspension on the grid with a micropipette or syringe. Allow virus particles to settle on the grid for 2-3 minutes.
- Blot dry with a piece of filter paper, and immediately stain the grid with one drop of 2% PTA stain, pH 6.8. Remove the excess fluid after 1-2 minutes with a piece of filter paper, and blot dry and examine under an EM.

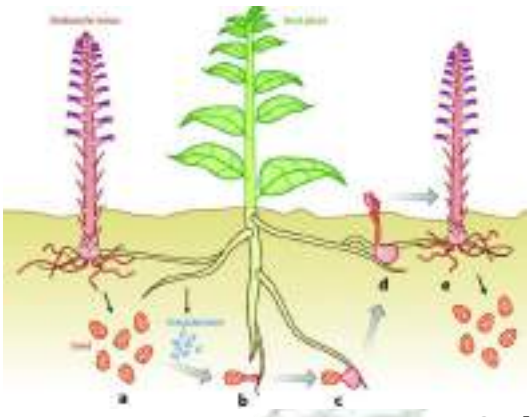
IDENTIFICATION OF PARASITES HIGHER PLANT (PHANAEROGAMS)

Some flower and seed bearing higher plants (phanerogams) live parasitically on other living plants and can cause important diseases on agricultural crops and also in forest trees. The phaneroganic parasitic plants are divided into two:

1. Stem parasites		**2. Root parasites**	
Total parasite- Cuscuta (Dodders)	**Semi parasite-** Loranthus	**Total parasite-** Orabanche (Broomrapes)	**Semiparasite-** Striga (Witchweeds)

Cuscuta sp.

Loranthus sp.



Orabanche sp.



Striga sp.

Dodder (*Cuscuta* sp.):- This is a non-chlorophyllous, leafless, parasitic seed plant, which attaches its yellow, orange or pink, thread-like stem to host plants (cultivated or wild plants). Leaves are represented by minute scales. It sends minute root like organs (haustoria) to the host cortex, which serve as an anchor as well as organs of food absorption. It bears tiny, white, pink or yellow flowers in cluster. Clover, berseem, flax and many oilseed crops are commonly attacked by this stem parasite.

Loranthus (*Dendrophthiae* sp.):- It is a common parasite of fruit trees. The parasite attacks aerial parts of host trees. It is devoid of a true root system of its own and hence, is dependent on host for water and mineral. Leaves are leathery and evergreen and possess chlorophyll. The stem is thick, erect or flattened at the nodes and appears to arise in cluster at the point of infection. Flowers are borne in clusters. They are long and tubular in shape and greenish-white or red in colour. The infected area of host becomes swollen and forms attachment disc.

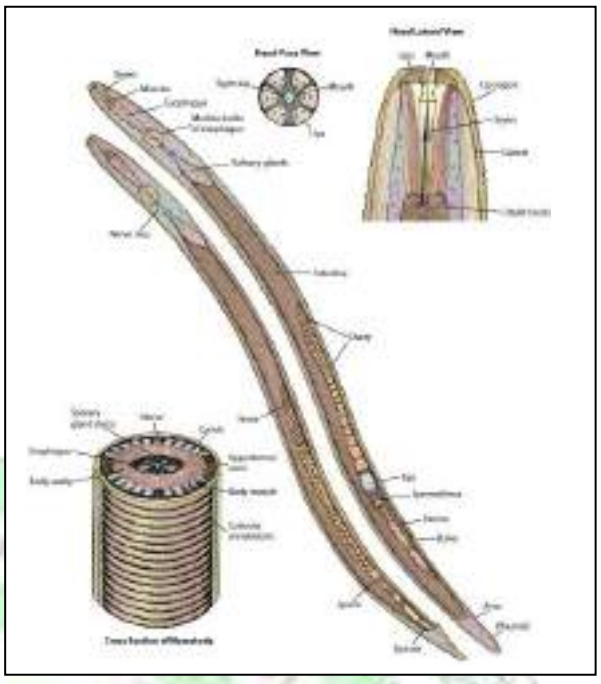
Broomrape (*Orohanche* sp.*)*:- It is a total root parasite affecting tobacco, brinjal, tomato, cabbage, cauliflower, turnip and many other Solanaceous and Cruciferous plants. The parasite consists of stout, fleshy stem, 15-20 cm tall. Stem is pale yellow or brownish-red in colour and covered by small, thin and brown scaly leaves. Flowers appear in axil of scales and are white and tubular. A large number of parasitic stems may be seen.

Witch's weed (*Striga* sp.):- Witchweed is a well-known parasite of sugarcane, cereals, maize and millets in India. The parasite is a small plant, 15-30 cm tall with bright green, slightly hairy stem and leaves. Leaves are narrow, long and in opposite pairs. The flowers are small and usually brick red or scarlet, although some may be yellowish-red, yellowish or almost white. The seeds are borne in a capsule and are very minute to see with naked eye. Infected roots bear a large number of witch's weed haustoria, which are attached to root to feed on it.

MORPHOLOGICAL FEATURES OF PLANT PARASITIC NEMATODES

Morphology: Pant parasitic adult nematodes are elongated worms ranging in length from about 0.30mm to over 5.0mm. The anterior end tapers to a rounded or truncated lip region, the body proper is more or less cylindrical, and the posterior and tapers to a terminus which may be pointed or hemispherical Proportions of the elongated body vary greatly. Females have greatly expanded bodies, sometimes nearly spherical, but always with a distinct neck. The adult males are always slender worms. Plant parasitic nematodes have no appendages. The mouth of a nematode is at the ANTERIOR end, and the terminus is at the POSTERIOR end. The excretory pore, vulva, and anus are on the VENTRAL side; and the opposite side is called DORSAL. The right and left sides are called LATERAL. The cuticle is attached to several other layers of tissue, which are separated laterally, dorsally and ventrally by chords. These contain nerves, excretory organs, etc., and separate

four bands of muscles, which move the body.



A. **Alimentary canal –** The alimentary canal starts at the mouth and ends at the anus. It includes the oesophagus, intestine, intestine, and rectum.
B. **Stylet –** In plant parasitic nematodes of the "Tylenchida" group, the mouth contains a stylet or mouth spear, a hardened, hollow, culticular structure similar to a hypodermic needle. Muscles are attached to three knobs at the posterior end of stylet and extend forward. They are used to pull the stylet forward so that it projects from the mouth opening and can be used to pierce plant cells. The food of the nematode is taken through the stylet.
C. **Oesophagus –** A slender tube is attached to the posterior end of the stylet. This is the oesophageal tube leading to the median bulb, which in turn is attached by means of another slender tube to the intestine. Posterior to the median bulb, the oesophagus contains three glands, one dorsal and two subventral, each with a nucleus. Three glands may form a terminal bulb to which the intestine is attached, or may form a lobe lying alongside the intestine. In either case, the dorsal gland has a duct leading anteriorly through the median bulb and connecting with the oesophageal tube. The connection is called the dorsal gland orifice.
D. **Dorsal Gland Orifice –** This in most species of plant parasitic nematodes is located behind the stylet at a distance seldom exceeding the stylet length and generally much closer. At this point there is an opening into the oesophageal tube and often an abrupt bend in it.
E. **Median bulb –** The median bulb contains a "valve" to which muscle fibres are attached. In cross-section, this structure is tri-radiate. When activated by muscles, it functions as a pump, sucking food through the stylet and forcing it into intestine.
F. **Intestine –** It is a simple tube with walls one cell thick. It functions as a storage organ and is usually filled with globules of fatty substances. Posteriorly it narrows to a rectum, which terminates at the anus.
G. **Excretory system –** Nematodes have an excretory system, but in the plant parasites, the only part usually seen is a section of the excretory tube leading to the excretory pore.

MOUNTING OF PLANT PARASITIC NEMATODES

Step 1. Killing and fixing nematodes: Collect live nematode specimens in distilled or deionized water in a small beaker or watch glass. Concentrate the nematodes in a minimal volume of water and add an equal volume of hot (90C) fixative solution and buffered formalin (Humason, 1972) to it. Nematodes may be killed with heat before adding fixative, though adding hot fixative directly is also effective. Buffered formalin provides a very good fixation. Leave the specimens in the fixative for 1-2 days. Nematodes may be stored in buffered formalin indefinitely; it does not clear characters. Buffered formalin solution is prepared as follows:

Formalin (40% formaldehyde)- 10.0 ml; Water-90 ml; Sodium acid phosphate-0.4 g; Anhydrous disodium phosphate-0.65 g

Step 2. Processing Specimens to glycerin

1. Prepare the following two solutions and keep them at room temperature

Seinhorst I solution: 20 parts 95% ethanol; 1 parts glycerin; 79 parts water

Seinhorst II solution: 95 parts 95% ethanol; 5 parts glycerin

Place fixed nematodes in a BPI dish. Draw-off excessive fixative and concentrate the nematodes in a small volume. Add 6-8 ml of Seinhorst solution I to the nematode suspension. (A very small quantity of rose Bengal, acid fuchsin, or aqueous picric acid may be added to the solution to stain the nematodes. This is optional. Place the open BPI dish in a larger closed glass container with 95% ethanol at the bottom, and place it in oven at 35-40C for at least 12 hours. This removes most of the water in the BPI dish. (Do not close or allow ethanol from the glass container to over-flow into the BPI dish.). Remove the dishes from oven and draw-off the excess Seinhost solution1 from the BPI dish using a pipette under a dissecting microscope to avoid loss of specimens. Add Seinhorst solution 2 to the BPI dish, place it in a partially covered Petri-dish and keep it in an oven at 40C. Several hours (at least 3 hours) later, draw-off excess solution from the BPI dish and repeat step 5. Keep the dishes in oven until all the alcohol has evaporated (at least 3 hours) and nematodes are in pure glycerin.

Step 3. Mounting nematodes

Temporary Mounts

1. Place a small drop of the fixative in the center of a clean glass slide.

2. Using a nematode pick under a dissecting microscope, pick up the desired specimens and place them in the fixative on the center of the slide.
3. Place the slide under dissecting microscope, and arrange the nematodes in the centre of the slide and bottom of the drop.
4. Place glass wool (about 5mm in length) or glass microbeads in a triangular position near the edge of the drop.
5. Place a cover glass (18mm wide) gently over the drop using a forcep or supporting it with a needle. Draw off excess fixatives carefully using filter paper.

Permanent Mounts

1. Fix a clean cover glass (25mm wide) in the center of a Cobb aluminium slide by supporting with appropriate size white cardboard pieces.
2. Place a small drop of anhydrous glycerin in the centre of the cover glass on the aluminium slide.
3. Pick up nematodes from the fixative, as in step 2 of (A), and place them in the glycerin drop.
4. Arrange the nematodes in the center of the slide and place glass wool as in steps 3-4 of (A).
5. Carefully place a cover glass (18mm wide) over the drop, and seal the edges of the cover glass as in steps of 5-6 of (A).
6. After the sealant has dried, a second coat of sealant may be added. Allow to dry, label the slides on the white cardboard, and examine them under a compound microscope. Excess of glycerin on the slide is difficult to remove and can cause smudges, which interferes with the sealing process.
7. Store the slides in a flat position to avoid settling of nematodes towards the edge of the cover glass.

MOLECULAR IDENTIFICATION OF PROKARYOTIC ORGANISMS USING 16S rRNA AND 16S-23S rRNA

rRNA: rRNA or ribosomal RNA are molecules found in the cells involved in the protein synthesis of organelles, referred to as ribosomes that spread out to the cytoplasm. This is to aid in translating the information contained in the mRNA (messenger RNA) into the proteins. Three important RNAs are occurring in the cells- rRNA, mRNA, and tRNA (transfer RNA). The rRNAs found in Archaea and Bacteria differ. This is essential to know, as the archaeal and bacterial lines seem to have separated from the common precursor slightly before the development of the eukaryotic cells.

What is 16S rRNA? 16S ribosomal RNA (or 16S rRNA) is the component of the 30S small subunit of a prokaryotic ribosome, roughly 1500 base pairs. Fig 1 (A) shows how 16S rRNA is involved in a prokaryotic ribosome. The bacterial 16S rRNA gene contains nine hypervariable regions (V1-V9) ranging from 30-100 base pairs, flanked by conserved regions (Fig. 1C). You can find 16S rRNA sequences in databases such as Ribosomal database project (RDP), Greengenes database, Silva, and Human microbiome project (HMP).

16S rRNA is a sequence of DNA encoding the RNA of the small subunit of the ribosome of bacteria. This 16S rRNA gene can be seen in all bacteria, with an associated form occurring in all the cells even in eukaryotes. Research on 16S rRNA sequences from several entities suggests that some part of the molecule experiences speedy genetic alterations, hence differentiating between various species in the same genus.

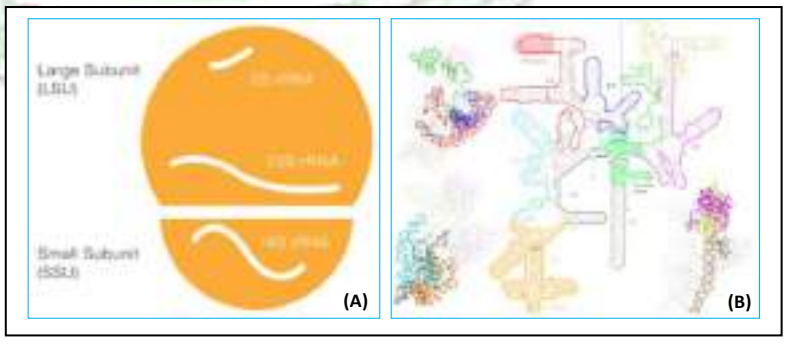
Features of 16S rRNA: -

Number of copies: - Bacteria comprise approximately 5 to 10 copies of the 16S rRNA, making the detection extremely sensitive.

Size: - The size of the 16S rRNA coding gene is close to 1500bp containing 50 functional domains.

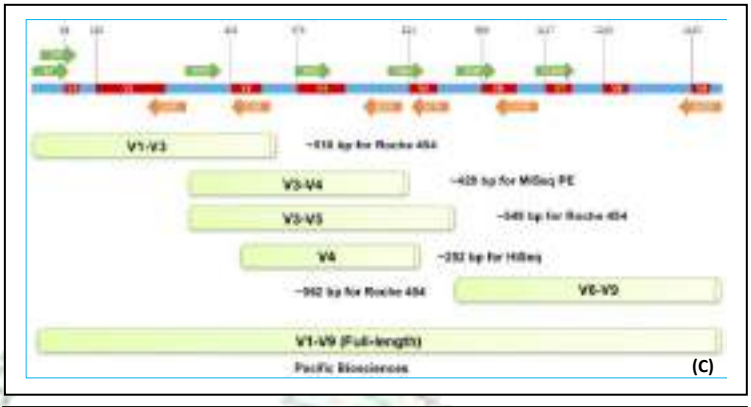
Information: - The internal structure of 16S rRNA gene comprises conserved and variable regions. The universal primers of different bacteria could be framed as per the conservative region, and particular primers of particular bacteria could be framed as per the variable region. The interspecific variation of information in the different areas of the 16S rRNA makes the recognition specific.

16S rRNA as a molecular marker: 16S rRNA gene is characterized by ubiquity and evolutionary properties, which allow it to become an important molecular marker in microbial ecology. Carl Woese and George E. Fox pioneered the use of 16S rRNA in phylogenetics. According to Yang *et al*. (Fig. 2), red regions (V2 and V8) have a poor phylogenetic resolution at the phylum level. Green regions (V4, V5, and V6) are associated with the shortest geodesic distance, which implies that they may be the optimal choice for phylogeny-related analyses, including phylogenetic analysis of novel bacteria phyla.



16S rRNA gene. (A) The involvement of 16S rRNA gene in a prokaryotic ribosome. (B) The 2D and 3D structure of the 16S

rRNA gene. Individual regions are shown in the same colour (Yang et al. 2016). (C) The regions and primers of 16S rRNA.



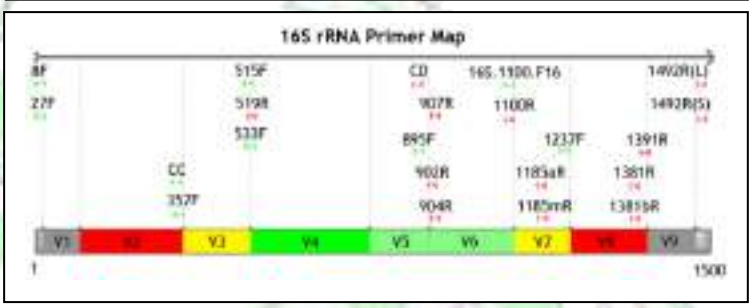


Illustration of different variable regions (Yang *et al*. 2016).

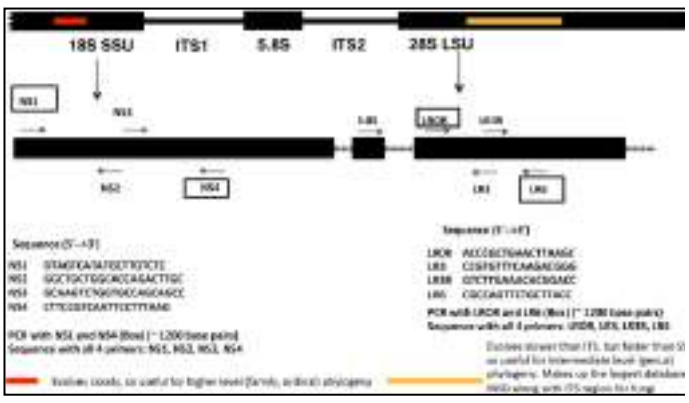
16S rRNA sequencing: - Since 16S rRNA gene is conserved in bacteria, and contains hypervariable regions that can provide species-specific signature sequences, 16S rRNA sequencing is widely used in identification of bacteria and phylogenetic studies. 16S rRNA sequencing is featured by fast speed, cost-efficiency, and high precision. It has been widely applied in basic research, as well as medical, forensic, agricultural, and industrial microbiology. 16S rRNA sequencing generally uses the next/third generation sequencing technology to read the PCR products which are amplified with suitable universal primers of one or several regions of 16S rRNA. The common sequencing platforms for 16S rRNA sequencing include Illumina MiSeq, Roche 454, Illumina MiSeq/HiSeq, and Pacific Bioscience. The former three platforms only cover 100 to 600 base pairs per single read, while the Pacific Bioscience can produce full-length 16S rRNA.

Advantages and applications of 16S rRNA sequencing

- Identification and taxonomic classification of bacterial species
- Discovery of novel pathogens
- Phylogenetic classification
- Metagenomic survey of bacterial populations
- Clinical microbiology

MOLECULAR IDENTIFICATION OF EUKARYOTIC ORGANISM (FUNGI) USING ITS REGION

Fungi are morphologically, ecologically, metabolically, and phylogenetically diverse. They are known to produce numerous bioactive molecules, which makes them very useful for natural products researchers in their pursuit of discovering new chemical diversity with agricultural, industrial, and pharmaceutical applications. Traditionally, mycologists have relied on fungal morphology, such as spore-producing structures, to identify species. However, this approach may not always be reliable for lower-level classifications, as morphological characters can be contentious or problematic, particularly in highly diverse fungal lineages. Morphology can be misleading due to factors like hybridization, cryptic speciation, and convergent evolution, limiting its effectiveness for accurate species-level identification within an evolutionary framework.

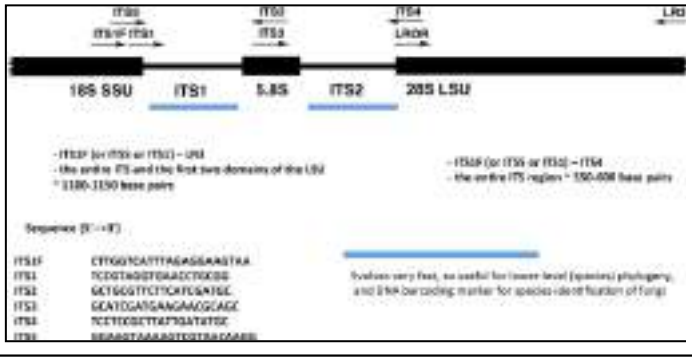


Primers for amplification of small-subunit (SSU) nrRNA and large-subunit (LSU) nrRNA

In the following sections, we discuss two methods used in mycology for sequence-based identification of fungi, namely, DNA barcoding using the ITS region and DNA taxonomy using one or multiple genes in sequence alignments and employing tree-building tools to estimate phylogenetic relationships. In DNA barcoding, the user compares an unknown sequence against a sequence database, such as either International Sequence Database (INSD: GenBank at the National Center for Biotechnology Information, GenBank, NCBI; the European Nucleotide Sequence Archive of the European Molecular Biology Laboratory, EMBL; and the DNA Data Bank of Japan, DDBJ) or UNITE (User-friendly Nordic ITS Ectomycorrhiza Database), and identifies species based on sequence similarity.

The use of molecular data for fungal identification began over 20 years ago with a seminal paper on ribosomal operon primers. These primers have enabled the generation of fungal DNA sequences for the large subunit, small subunit, and internal transcribed spacer region, ushering in a new era of molecular phylogenetic identification. The different regions exhibit varying levels of genetic variation, with the small subunit evolving the slowest and the internal transcribed spacer

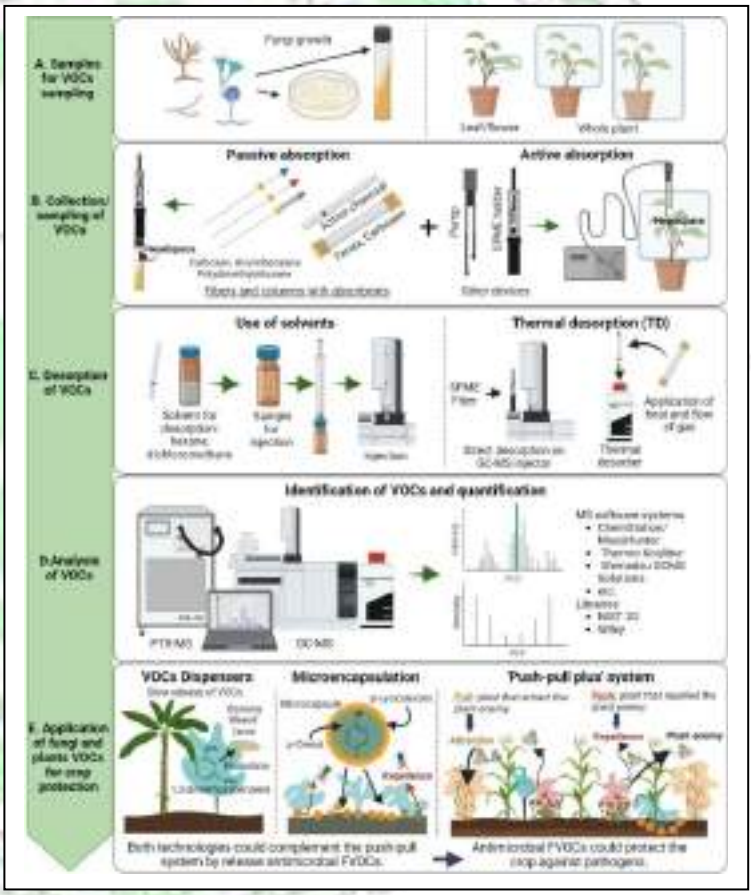
region evolving the fastest. Researchers can use these regions to identify fungi at different taxonomic levels, with the small subunit for higher-level placement, the large subunit for intermediate-level identification, and the internal transcribed spacer for species-level classification. The internal transcribed spacer was chosen as the official fungal barcode due to its ease of amplification, widespread use, and appropriately large barcode gap. For natural products research, the first two domains of the large subunit gene and the entire internal transcribed spacer region should be sequenced due to their prevalence in fungal taxonomy and systematics.



Primers for amplification of the ITS region

VOLATILE COMPOUNDS PROFILING FOR PLANT PATHOGEN DETECTION

As part of their metabolism, plants and fungi release diverse volatile organic compounds (VOCs) into the air. At the global level, plants alone account for 67% of the VOC emissions present in the atmosphere. Plant VOCs are emitted in response to biotic and abiotic stresses and play an important role in defence signalling and communication between plants. They can serve as priming agents to enhance resistance to both herbivores and plant pathogens and can serve as important stimuli in the stress memory of plants. Plant VOCs can affect hormone levels and are implicated in regulating the senescence processes of competitive plants, such as weeds. About 250 fungal-specific VOCs have been shown to have characteristic odours. The rate of emission of VOCs from plants and fungi varies depending on the physicochemical and biological factors and the nature of the interaction between the pathogen and the plant. However, many VOCs emitted from plants in response to biotic stress factors are nonspecific, as different pathogens can cause similar tissue damage and elicit the same chemical response. VOC emissions can also be used in pest and disease management strategies.



PREPARATION OF PERCENT, MOLAR AND NORMAL SOLUTIONS

I) Percent solutions: The per cent concentration of a solution can be determined in two ways:

i. Ratio of the mass of the solute divided by the mass of the solution (Mass per cent)
ii. Ratio of the volume of the solute divided by the volume of the solution (Volume per cent)

1) Mass Percent: When the solute in a solution is a solid, a convenient way to express the concentration is by mass per cent (mass/mass)**,** which is the gram of solute per 100g of solution.

Formula: Percent by mass = mass of solute/mass of solution × 100%

Suppose that a solution was prepared by dissolving 25.0g of sugar into 100 g of water. The per cent by mass would be calculated by:

Percent by mass = 25g sugar / 125g solution × 100% =20% sugar

Sometimes you may want to make up a particular mass of solution of a given percent by mass and need to calculate what mass of the solvent to use.

For example, you need to make 3000g of a 5% solution of sodium chloride. You can rearrange and solve for the mass of solute:

Mass of solute = percent by mass / 100% × mass of solution = 5% / 100% × 3000g = 150g NaCl

You need to weigh out 150g of NaCl and add it to 2850g of water. Notice that it was necessary to subtract the mass of the NaCl (150g) from the mass of solution (3000g) to calculate the mass of the water that would need to be added.

2) Volume Percent: The percentage of solute in a solution can more easily be determined by volume when the solute and solvent are both liquids. The volume of the solute divided by the volume of the solution expressed as a per cent, yields the per cent by volume (volume/volume) of the solution.

Formula: Per cent by volume = volume of solute/volume of solution × 100%

If a solution is made by adding 40 ml of ethanol to 200ml of water, the per cent by volume is:

= 40mL ethanol / l240 ml solution × 100% = 16.7%ethanol

II) Molar solutions: The most common unit of solution concentration is molarity (M). The molarity of a solution is defined as the number of moles of solute per one litre of solution. Note that the unit of volume for molarity is litres, not millilitres or some other unit. Also, note that one litre of solution contains both the solute and the solvent. Molarity, therefore, is a ratio between moles of solute and litres of solution. To prepare laboratory solutions, usually a given volume and molarity are required. To determine molarity, the formula weight or molar mass of the solute is needed. The following examples illustrate the calculations for preparing solutions.

Moles = Weight of solute in grams / Gram Molecular Weight

It's essential to remember that, the term mole represents the amount of substance (in grams), irrespective of the volume where the substance is dissolved.

Example:

No. of moles of sodium hydroxide (NaOH) present in 40 gm is = 40 / 40= 1

Molecular weight of NaOH is **Na: 22.99**; **O: 16**; **H: 1** i.e., 39.99 = 40

Let us understand this with a simple exercise of How to prepare 1 L of 1 M solution of NaOH.

Since molecular weight of NaOH is 40, dissolve 40 grams of NaOH in 1 liter of water to make a 1M NaOH solution per 1 L.

Similarly, one mole of sodium chloride (NaCl) is 58.45 (MW 58.45).

III) Normal solutions: Normality (N) is also another way of expressing the concentration of solute in the solution. This is partially similar to Molarity but uses gram equivalent weight (eq.w t) rather than gram molecular weight (GMW) of solute per litre.

Thus, it may explained as "1N solution contains 1 gram-equivalent weight of solute per liter of a solution" it is also defined as "number of gram equivalents of solute per litre of the solution". To obtain gram equivalent weight one should know the number of hydrogen atoms that can be added or removed from the given substance. When you divide the GMW of the substance with nunber of replaceable hydrogens you'll get EW.

Formula:

Eq. Wt (EW) = gram molecular weight / number of replaceble hydrogen atoms

For example: Molecular weight of NaOH is Na: 22.99; O: 16; H: 1 i.e., 39.99 = 40;

NaOH possess 1 hydrogen atom that can be replaced, hence

Eq. Wt of NaOH is: = 40 / 1 = 40

Let us understand this with another example of How to prepare 500 mL of 0.5 N solution of NaOH.

(Atomic weight of NaOH is Na: 22.99; O: 16; H:1 i.e., 39.99 = 40)

Weight in grams = desired normality × volume required in litres × GMW / Valence

Weight in grams = 0.5 N × 500 mL ×40 GMW / 1 EW = 10 g of NaOH

Hence, 10 grams of NaOH is needed to prepare 500 mL of 0.5N solution of NaOH.