#### PRACTICAL MANUAL

#### **PLANT VIROLOGY**

Course No. PPA 502 Credit Hrs. 3(2+1)

M.Sc. (Ag.) Plant Pathology



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2024

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Syllabus: Plant Virology
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**Practical:** This course provides a comprehensive understanding of plant viruses, focusing on their identification, transmission, and management. It includes hands-on experience in isolating and purifying viruses, utilizing bioassays, and applying modern diagnostic tools such as serological methods (ELISA) and nucleic acid techniques (PCR and LAMP).

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Objective: Study of s	symptoms caused	by plant viruses	•		
Activity: Collect differ	rent virus-infected s	amples from the f	ield and describe	their symptoms	
Materials required:					
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Procedure:					
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#### Objective: Bioassay of virus cultures on indicator plants and host differentials

Activity: Study the bioassay of virus cultures on indicator plants and other hosts for host range studies and the identification of a particular virus Materials required: .....

Objective: Study of different modes of plant virus transmission
Activity: Write about different modes of plant virus transmission



### Objective: Mechanical transmission of plant viruses through sap and study of disease development

Activity: Study the mechanical in Materials required:			
Procedure:			
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Observation and Result :	 		

Objective: Tra	ansmission o	of plant virus	ses by vec	tors and st	udy of dise	ase develo	pment
Activity: Determ	nine the relative	transmissior	n efficiencies	s of ToLCV by	y <i>B. tabaci</i> or	n healthy see	edlings
Materials require	ed:						
Procedure:							
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Objective: Transmiss				
<b>Activity:</b> Study the rela	tive transmission abili	ity of the moong	bean yellow mosa	aic virus through grafting
Materials required:				
Procedure:				
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Observation and Result	,			
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Activity: Purify plant virus  Activity: Purify plant virus
Materials required:
Procedure:
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# Objective: Electron microscopy of plant viruses Activity: Study the size and shape of different viruses through Electron microscopy Materials required: ..

# Objective: Monoclonal antibody production through Hybridoma technology Activity: To learn the monoclonal antibody production

Materials required:			
Procedure:			
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Objective: Detection and diagnosis of plant viruses with serological (ELISA) technique
Activity: To detect the antigen and antibody reactions.
Write the procedure of DAS ELISA

# Practical No. 11 Objective: Total DNA extraction from virus infected plants Activity: Extract the DNA from the virus infected plants Chemicals required: ...... Composition of CTAB buffer: Procedure: ..... ..... .....

#### **Objective:** To study about Polymerase Chain Reaction (PCR)

Activity: To	learn about p	olymerase cl	hain reaction				
	uired:			 			
Procedure:							
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# Objective: To study Gel electrophoresis Activity: To learn about gel electrophoresis Chemicals required: ..... .....

#### **Objective:** RNA extraction from virus infected plants

Chemicals required:
Procedure:

## Objective: Exposure to basic bio-informatic tools for viral genome analysis and their utilization in developing detection protocols and population studies

Activity: To know about NCBI, GenBank and	d BLAST:		
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## Objective: Perform multiple sequence alignment and construct a phylogenetic tree of sequences using ClustalW

Write about Multiple	sequence alignme	nt and ClustalW:			
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Procedure for phylogenetic tree construction

#### SYMPTOMS OF VIRUS DISEASES IN PLANTS

**Materials for collection of symptomatic plants**: Polythene bags, Hand lens, Knife, razor blade or scissors, Marker, Labels, Key to symptoms

#### Procedure:

- Visit a field of different crops and collect infected/suspected plants or uproot the plant keep them in polythene bag and bring to laboratory.
- The samples can be stored in a refrigerator at 4°C until processed.
- Compare and study the symptoms carefully and describe their identity in a tabular form along with their severity (very severe, severe, moderate and mild) and insect vectors associated, if any.

#### Types of Symptoms:

- Growth Reduction/Dwarfing: Growth reduction is a key symptom of virus infection, leading to decreased yield and
  quality of produce, often causing partial or total crop loss. It results in dwarfing when plants remain below their typical
  size, and stunting when plant growth and development are arrested. Example: Barley yellow dwarf virus, Peanut dwarf
  virus. etc.
- **Mosaic:** Dark and light coloration intermingled without a distinct boundary. Mosaic patterns may include green mosaic, pale yellow mosaic, yellow mosaic, or golden mosaic. *Example: Tobacco mosaic virus*.
- Vein Clearing: In some viral diseases, veins become light and translucent. Example: Citrus tristeza virus.
- **Vein Yellowing:** In some viral diseases, veins turn yellow, giving a net-like appearance of yellow vein symptoms. *Example: Bhendi yellow vein mosaic virus*.
- **Vein Banding:** Coloration develops around veins, causing them to thicken. *Examples:* Green vein banding in *Papaya ringspot virus* on watermelon, yellow vein banding in *Grapevine fan leaf virus* in grapes.
- Mottling: Light and dark coloration with distinct boundaries.
- Yellowing or Chlorosis: Chlorosis refers to a reduction in chlorophyll, resulting in a lighter leaf color. "Mild chlorosis" refers to light green leaves, while "severe chlorosis" or "yellowing" indicates a total depletion of chlorophyll, giving the leaves a yellow appearance.
- Rugose: Refers to rough leaves and includes etching (engraved), crinkling (wrinkled leaves), and leaf puckering (blister-like irregularities on the leaf). Examples: Etching of tobacco caused by Tobacco etch virus, leaf crinkling in urdbean, leaf puckering in cucumber caused by begomovirus.



- Leaf Roll and Leaf Curl: Leaf rolling describes the folding of leaves along their mid-axes, forming a tube-like structure. Example: Potato leafroll virus (PLRV). Leaf curling, on the other hand, involves more irregular folding, not resulting in a tube-like structure. Example: Tomato leaf curl virus (ToLCV).
- Leaf Distortion: Disturbed growth and development of leaves, ranging from minor deviations in shape to severe

deformations.

- **Epinasty:** Rapid growth of infected parts, such as the petiole and leaf blade, leading to downward bending or curling, as seen in peas.
- Leaf Enation: Abnormal outgrowths of vascular tissue on leaves, veins, or stems.
- Rosette: An abnormal condition where the leaves form a radial cluster on the stem. Example: Groundnut rosette
  umbravirus
- Necrosis: Rapid local cell death, often characterized by blackening or browning of tissues, such as local lesions on
  inoculated leaves. It can also result from degeneration of phloem in vascular bundles, leading to phloem necrosis.
  Necrosis may affect different parts, leading to vein necrosis, tip necrosis, or bud necrosis. If necrosis progresses, the
  entire plant may die rapidly. Examples: Tobacco rattle virus and Potato mop-top virus induce internal necrosis in potato
  tubers.
- **Stem Pitting:** Localized underdevelopment and shallow necrosis of certain tissues, resulting from cambium tissue failure. It appears as numerous scattered elongated pits visible when the bark is removed, as in *Citrus tristeza virus*.
- **Stem Grooving:** Occurs under similar conditions of phloem necrosis, resulting in deep, wide furrows or pits, as seen in *Apple stem grooving virus*.
- Ring Spotting: Localized spots on infected leaves, consisting of various forms of chlorosis and necrosis. Chlorotic ring spots appear as circular chlorotic areas, while necrotic ring spots show alternating necrosis with normal green areas. Example: Tobacco ringspot virus.

#### BIOASSAY OF VIRUS CULTURES ON INDICATOR PLANTS AND HOST DIFFERENTIALS

**Introduction:** Bioassays, also known as biological indexing, are one of the earliest techniques developed to detect plant viruses. The method relies on the ability of certain plants, called indicator plants, to develop distinct symptoms upon inoculation with viruses through mechanical means. Indicator plants are chosen based on their ability to exhibit clear disease symptoms when infected.

**Materials Required:** Indicator plants: *Chenopodium quinoa*, *C. amaranticolor*, *Gomphrena globosa*, *Vigna unguiculata*, *Nicotiana benthamiana*, *N. glutinosa*, etc. Infected plant material, 0.1 M phosphate buffer, Pestle and mortar, Carborundum, Celite, Mercaptoethanol, Cotton Water, Blotting paper

#### Procedure:

- 1. Collect infected plant samples showing symptoms from the field. Wash the infected leaves with tap water to remove dust, and dry them between folds of blotting paper.
- 2. Macerate the leaves in a chilled mortar and pestle using potassium phosphate buffer (pH 7.0, 0.05 M) at a ratio of 1 ml of buffer per gram of leaf tissue.
- 3. Squeeze the resulting pulp through absorbent cotton, and use the extract obtained as the standard inoculum.
- 4. To the standard inoculum, add celite (600 mesh) at a rate of 0.025 g/ml of extract and 0.02% mercaptoethanol.
- 5. Gently apply the inoculum to the upper surface of the indicator plant leaves using a small piece of absorbent cotton wool.
- 6. Wash the inoculated leaves 1–2 minutes after inoculation with distilled water using a fine jet from a squeeze bottle to remove excess inoculum. Place the plants in a glasshouse for observation.

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- 7. Observe local lesions on the indicator plants within 5-6 days and systemic symptoms after 15 days.
- 8. Repeat the procedure on different host plants to study the host range of the particular virus.

#### **MODES OF TRANSMISSION**

- **1. Mechanical Transmission:** Viruses are transmitted through physical contact between a healthy and infected plant. This can occur naturally through wind damage, insect feeding, or by human activities like pruning and grafting. Example: *Tobacco mosaic virus* (TMV) can spread through sap when leaves are touched.
- **2. Insect Vectors:** Many plant viruses are transmitted by insects that feed on plant sap. Insects can acquire the virus from infected plants and transmit it to healthy ones. Transmission can be of different types:
  - Non-persistent: Virus is carried on the insect's mouthparts for a short time (minutes to hours). Example: Cucumber mosaic virus (CMV) by aphids.
  - Semi-persistent: Virus stays in the insect's body for a longer time (hours to days), but does not multiply within the insect. Example: Lettuce infectious vellows virus by whiteflies.
  - Persistent (circulative): Virus circulates in the insect's body and can remain for life. Example: Barley yellow dwarf virus (BYDV) by aphids.
  - o Persistent (propagative): Virus multiplies in the insect. Example: Tomato spotted wilt virus (TSWV) by thrips.
- 3. Seed Transmission: Viruses can be transmitted from infected parent plants to their offspring through seeds. This is a

highly efficient form of transmission, as infected seeds can lead to a new generation of infected plants. Example: **Bean common mosaic virus (BCMV)** can be seed-borne in beans.

- **4. Pollen Transmission:** Viruses can spread through infected pollen grains during pollination, which can infect the developing seeds or the plant's reproductive tissues. **Example**: Cucumber mosaic virus (CMV) can be transmitted through pollen.
- **5. Vegetative Propagation:** Viruses can spread through vegetative parts such as cuttings, tubers, bulbs, or rhizomes. Plants propagated from infected tissues will carry the virus. **Example**: *Potato virus* Y (PVY) can be transmitted through potato tubers.
- **6. Soil Transmission:** Some viruses can be transmitted through soil, either through root contact with infected plant debris or soil-dwelling organisms like fungi, nematodes, or protozoa. **Example**: *Tobacco rattle virus* (TRV) can be transmitted by nematodes in the soil.
- **7. Grafting:** Infected scion or rootstock can transmit the virus to the healthy part of the grafted plant, spreading the virus systemically. **Example**: *Plum pox virus* (PPV) can be transmitted through grafting of infected plum trees.

#### MECHANICAL TRANSMISSION OF PLANT VIRUSES

**Materials Required:** Infected plant material, 0.1 M phosphate buffer, Pestle and mortar, Carborundum, Celite, Mercaptoethanol, Cotton, Water and Blotting paper

#### Procedure:

- 1. Preparation of 0.1 M Phosphate Buffer:
  - o For K<sub>2</sub>HPO<sub>4</sub> (Molecular Weight: 174.18), weigh 8.7 g.
  - o For KH<sub>2</sub>PO<sub>4</sub> (Molecular Weight: 136.09), weigh 6.8 g.
  - o Dissolve each separately in 200 ml of distilled water and make up the volume to 500 ml.
  - Store the buffer in the refrigerator at 4°C.

#### 2. Virus Inoculation:

- o Grind the infected leaves using 0.1 M phosphate buffer in a chilled pestle and mortar.
- Add a small quantity of celite to the crude sap and mix properly.
- Apply the crude sap to the leaves using a cotton swab.
- o Let it sit for 5 minutes.

#### 3. Post-Inoculation Care:

- Wash the inoculated leaves with water.
- Keep the plants in the shade for one day.
- o On the following day, transfer the plants to the bench for further observation.

#### TRANSMISSION OF PLANT VIRUSES BY VECTORS

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**Materials Required:** Whiteflies (*Bemisia tabaci*), Cotton plants, Insect-proof wooden cages, Muslin cloth, Healthy tomato plants, Plastic bottles, Plastic tubes, Aspirator

#### Procedure:

- 1. Maintenance of Whitefly Culture:
  - o Maintain a pure culture of indigenous whiteflies (Bemisia tabaci) on cotton plants.
  - Keep the cotton plants in insect-proof wooden cages.
  - Introduce healthy cotton plants grown in polythene bags into the cages to maintain the whitefly colony.

#### 2. Raising Healthy Tomato Seedlings:

- o Raise tomato seedlings in polyethylene bags filled with a soil and compost mixture in a 2:1 ratio.
- Keep the plants in insect-proof cages for use during the investigations.

#### 3. Preparation of Cages for Acquisition Access Feeding by B. tabaci:

- Use plastic or PVC bottles, tapering towards the narrow mouth.
- o Remove the bottom of the bottle with a soldering rod and cover it with muslin cloth.
- Cut the narrow mouth of the bottle a few centimeters above the screw cap and plug it with cotton to prevent flies from escaping.

#### 4. Preparation of Cages for Inoculation of Plants:

- Take plastic tubes, remove the bottom, and seal it with black muslin cloth to avoid moisture accumulation and ensure aeration.
- o Make a small hole (0.5 cm) in the middle of the tube to facilitate whitefly release.

o After inserting young leaflets into the tube, plug the open end with cotton.

#### 5. Collection of Whiteflies:

- Use an aspirator made of a glass tube (30 x 0.5 x 40 cm) and a 40 cm rubber tube to collect whiteflies.
- Suck whiteflies colonized on leaves into the glass tube and gently blow them into plastic tubes for use in transmission studies.

#### 6. Transmission of ToLCV using *B. tabaci*:

- o Insert a *ToLCV*-infected tomato twig inside the bottle containing whiteflies.
- o Allow the whiteflies to feed on the twig for 24 hours.
- o Release 10 whiteflies per seedling onto healthy tomato seedlings, which are covered with cylindrical plastic cages.
- o After a 48-hour inoculation access period (IAP), remove the whiteflies from the plants.
- Spray the plants with 0.03% Imidacloprid and keep them in insect-proof cages for symptom development.

#### TRANSMISSION OF PLANT VIRUSES (Mungbean yellow mosaic) BY GRAFTING

Introduction: Grafting is the union of cambial tissues between two hosts that are closely related, i.e., belonging to the same genus or family. If either the scion or rootstock is infected, the virus can pass to the healthy one through grafting. The common grafting methods are approach grafting, wedge (or top cleft) grafting, tongue grafting, and leaf grafting. The first two methods are easier and more convenient. Budding is also a common method for virus transmission. All virus and virus-like diseases of citrus are graft-transmissible. In approach grafting, slices of uniform size are made on both the scion and the stock and then united by matching the cambial tissues. In wedge or top cleft grafting, a wedge is made in the rootstock, and a scion of the same diameter or size is cut and inserted into it.

**Materials**: Infected mungbean plants, healthy cotton plants, healthy mungbean plants, sterilized knife or blade, distilled water, parafilm or polythene ribbons, plastic bags, beakers.

#### Procedure:

- Wash the diseased plants (scions) with sterilized/distilled water.
- On the potted test plants (stock), select branches of the same size and approximately the same age as the scion.
- Make a slanting cut of about 1 cm on the stock using a sterilized knife.
- Make a wedge-shaped cut on the scion, ensuring that the exposed area on the scion matches the cut area on the stock.
- Insert the wedge-shaped end of the scion into the cut on the stock.
- Immediately tie the union using parafilm or a polythene ribbon.
- Cover the plants completely with a plastic bag to maintain humidity.
- Maintain the plant at appropriate temperature (18-25°C) and humid conditions to enhance a successful union between the stock and scion.
- Include appropriate controls with healthy plants.

#### **PURIFICATION OF PLANT VIRUS**

**Materials Required**: Infected leaf samples, phosphate buffer, 1% beta-mercaptoethanol, muslin cloth, n-butanol, PEG (polyethylene glycol), NaCl.

#### Procedure:

- 1. Grind the leaf tissues with liquid nitrogen in a pestle and mortar to form a fine powder.
- 2. Homogenize the powdered tissues in a blender using 300 mL of 0.5 M phosphate buffer containing 1% betamercaptoethanol.
- 3. Strain the extract through two layers of muslin cloth.
- 4. Add n-butanol (8 mL per 100 mL of extract) and stir for 15 minutes in a cold room.
- 5. Centrifuge the mixture at 10,000 rpm for 30 minutes at 4°C and collect the supernatant.
- 6. Add PEG (molecular weight 600, 4 g per 100 mL) to the supernatant and stir for 2 hours at 4°C.
- 7. Centrifuge at 10,000 rpm for 15 minutes, collect the pellet, and resuspend it in 80 mL of 0.01 M phosphate buffer.
- 8. Add NaCl (0.2 M) and PEG (4 g per 100 mL) to the solution, and stir overnight at 4°C.
- 9. Collect the pellet by centrifugation at 10,000 rpm for 15 minutes, resuspend it in 2 mL of 0.01 M phosphate buffer, vortex well, and centrifuge again at 10,000 rpm for 2 minutes.
- 10. Collect the milky white supernatant containing the virus particles.
- 11. Load the supernatant onto a 10-40% sucrose gradient and centrifuge in a swing-out bucket rotor at 34,000 rpm for 1 hour at 4°C.
- 12. Collect the light-scattering fraction with a Pasteur pipette from the tubes and examine it under an electron microscope.

#### **ELECTRON MICROSCOPY OF PLANT VIRUSES**

Introduction: Electron microscopy (EM) provides virologists with a fundamental tool, much like the light microscope does for bacteriologists. EM reveals several important characteristics of virus particles, including their purity, substructures, and inclusion bodies in ultra-thin sections of infected tissue. The processes involved in EM, such as specimen preparation, grid preparation, film making, carbon coating, shadow casting, ultra-thin sectioning, and negative staining, are complex and beyond the scope of this manual. Handling EM equipment requires highly skilled personnel due to its specialized and costly nature. However, students should become familiar with some basic procedures, which are briefly described here.

#### Materials:

- Infected tissue (Tobacco mosaic virus TMV, Potato virus X PVX, Potato virus Y PVY) or virus preparations.
- Copper grids with formvar/collodion membrane supported by carbon coating.
- Phosphotungstic acid (PTA) or uranyl acetate (UAc).
- Filter paper

#### Procedure:

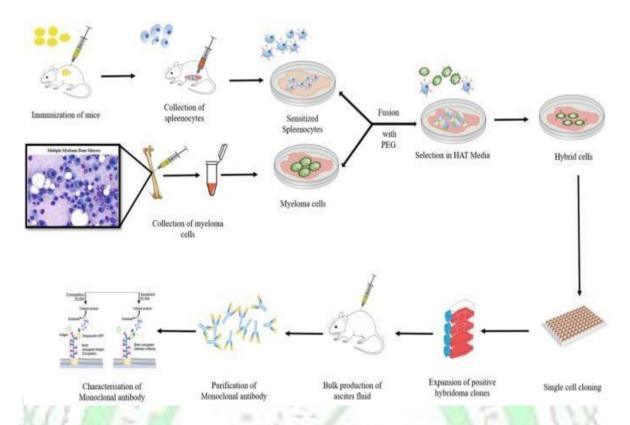
- 1. Place a drop of 2% PTA (pH 6.8) or UAc (pH 6.4) on the infected tissue and tease the tissue with a fine needle or the edge of a blade so that the cell exudate oozes out into the stain.
- 2. Alternatively, place a drop of stain on a copper grid, and gently touch the freshly cut edge of the virus-infected tissue to the grid. It is recommended to fix the tissue in 10% formaldehyde for 10-20 minutes to stabilize the virus particles.
- 3. For purified or partially purified virus preparations, mix the virus with the stain and place it on the grid. Drain the excess fluid with a piece of filter paper and allow the grid to dry. Observe the grid under an electron microscope and record the details of the virus particles.
- 4. Place the grid on a taped microscope slide, with the filmed side facing up.
- 5. Using a micropipette or syringe, place one drop of the specimen suspension on the grid. Allow the virus particles to settle on the grid for 2-3 minutes.
- 6. Blot the grid dry with a piece of filter paper, and immediately stain the grid with one drop of 2% PTA (pH 6.8). After 1-2 minutes, remove the excess fluid with filter paper, blot dry, and examine the grid under an electron microscope.

#### MONOCLONAL ANTIBODY PRODUCTION BY HYBRIDOMA TECHNOLOGY

**Materials Required:** Sp2 cells, immunized mice, serological pipettes (5 mL, 10 mL, 25 mL), 200  $\mu$ L pipette tips, micropipette (20  $\mu$ L-200  $\mu$ L), 1.5  $\mu$ L microtubes, 50 mL centrifuge tubes, dissecting board, 1L filter unit receiver, inverted microscope, washing solution, 1X PBS, plating medium, dissecting kits, 150 mm and 60 mm petri dishes, 5 mL syringes, autoclave bags, light duty tissue wipers, isoflurane.

#### Procedure:

- 1. **Immunization of Mice & Isolation of Splenocytes:** Mice are immunized with the antigen of interest, followed by blood screening for antibody production. Typically, two doses are administered: an initial "priming" dose and a second "booster" dose after 10 days. The antibody-producing splenocytes are then isolated for hybridoma production.
- 2. **Preparation of Myeloma Cells:** B cells are extracted from the spleen of the immunized mouse and cultured with myeloma cells (immortalized cancer cells). Once fused with spleen cells, the myeloma cells can result in hybridomas capable of unlimited growth. The myeloma cells are prepared for fusion.
- 3. **Fusion**: The myeloma cells and isolated B cells are fused in the presence of polyethylene glycol (PEG), which induces the fusion of cell membranes, forming hybridomas.
- 4. Selection of Hybridomas: The myeloma cells are HGPRT- (deficient in hypoxanthine-guanine phosphoribosyl transferase), while the B cells are HGPRT+. The hybridomas are grown in HAT (hypoxanthine-aminopterin-thymidine) medium, which supports only HGPRT+ cells. Myeloma cells that fuse with other myeloma cells or remain unfused die in HAT medium since they are HGPRT-. Similarly, B cells that fuse with other B cells or remain unfused die because they cannot divide indefinitely. Only hybridomas between B cells and myeloma cells survive, as they are both HGPRT+ and immortal.
- 5. Clone Screening and Selection: Each hybridoma must be screened to determine if it produces the desired antibody. Screening can be done using techniques such as SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and Western blotting, using the epitope of the desired antibody as a probe. Once a hybridoma producing the correct antibody is identified, it can be cultured indefinitely to produce monoclonal antibodies.
- 6. **Functional Characterization**: Confirm and validate the antibody production of each potentially high-producing hybridoma colony using methods like ELISA (enzyme-linked immunosorbent assay).
- 7. **Scale-Up and Weaning**: Scale up the production of clones that generate the desired antibodies and wean off the selection agents used in the culture medium.



Schematic diagram of Monoclonal antibody production by Hybridoma technology

#### DETECTION AND DIAGNOSIS OF PLANT VIRUSES USING THE SEROLOGICAL (ELISA) TECHNIQUE

**Principle**: Enzyme-Linked Immunosorbent Assay (ELISA) works by covalently linking an immunoglobulin molecule to an enzyme. This linkage retains the immunospecificity of the primary antibody and the catalytic properties of the enzyme. If the enzyme is directly linked to the immunoglobulin, it is called direct ELISA. If the enzyme is linked to a molecule that detects the immunoglobulin, it is classified as indirect ELISA.

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#### **Buffers and Reagents Used in ELISA:**

#### A) Coating Buffer (Carbonate Buffer/Antigen Buffer) (pH 9.6):

- Sodium carbonate (Na<sub>2</sub>CO<sub>3</sub>): 1.59 g
- Sodium hydrogen carbonate (NaHCO<sub>3</sub>): 2.93 g
- Sodium azide: 0.2 g
- Diethyl dithiocarbamate (sodium salt) at 0.001 M concentration (1.71 g/L)
- Distilled water: 1.0 L

#### B) Phosphate Buffer Saline (PBS) (pH 7.4):

- Disodium hydrogen phosphate (Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O): 2.9 g
- Potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>): 0.2 g
- Potassium chloride (KCI): 0.2 g
- Sodium chloride (NaCl): 8.0 g
- Distilled water: 1.0 L

#### C) Washing Buffer (PBST):

PBS: 1000 mL

#### D) Antibody Buffer:

- PBS-Tween: 100 mL
- Polyvinyl pyrrolidone (40000 MW): 2.0 g

#### E) Substrate Buffer (pH 9.8):

- Diethanolamine: 97 mL
- Distilled water: 800 mL

- Tween-20 : 0.5 mL
- Bovine serum albumin (BSA): 0.2 g
- Sodium azide: 0.2 g

#### **Procedure for DAC-ELISA:**

1. Coating: Prepare the antigen and dispense 200  $\mu L$  into each well of a microtiter plate using a micropipette. Incubate the

- plates at 37°C for 2-2.5 hours or at 4°C overnight.
- 2. **Washing**: Pour off the contents of the plate and rinse with PBS-Tween. Wash the plate by immersing it in wash buffer for 3 minutes. Repeat the washing process three times, ensuring the final wash drains off completely.
- 3. **Blocking**: Add 200 µL of blocking solution to each well and incubate at 37°C for 1 hour to block unoccupied sites in the wells. Wash the plates in PBS-Tween as described in step 2.
- 4. **Antibody Addition**: Dilute monospecific polyclonal antisera (1:1000 or 1:750 ratio, respectively) in antibody buffer, then add 200 μL per well. Incubate the plates at 37°C for 2-2.5 hours or at 4°C overnight. After incubation, wash the plates in PBS-Tween as described in step 2.
- 5. **Conjugate Addition**: Dilute anti-rabbit IgG-ALP labeled enzyme conjugate in enzyme buffer (1:10,000) and add 200 μL per well. Incubate the plates at 37°C for 2 hours, then wash the plates with PBS-Tween as described in step 2.
- 6. **Color Development**: Dissolve a 20 mg pNPP tablet in substrate buffer and add 200 µL per well. Incubate the plates at room temperature for 10-30 minutes to allow color development. Read the plates at 405 nm using an ELISA reader (e.g., EL310E from BIO-TEK Instruments, USA).

#### Important Critical Steps:

- 1. **Coating**: Proper preparation of samples is essential for optimal adhesion to microtiter plates.
- 2. Washing: Consistent washing of plates is crucial to reduce background colour development in negative control wells.
- 3. **Development**: The substrate may oxidize upon exposure to light, so it should be kept in the dark to enhance color development.

#### TOTAL DNA EXTRACTION FROM VIRUS-INFECTED PLANTS

#### Materials Required:

- CTAB extraction buffer
- Chloroform: alcohol (24:1)
- Isopropanol

- 70% ethanol
- 1X TE buffer
- Spectrophotometer

#### Procedure:

- 1. **Grind** 100-200 mg of infected leaves using a pestle and mortar to obtain a fine paste.
- 2. Add 1-2 mL of CTAB extraction buffer to the ground tissue and grind thoroughly.
- 3. Transfer 0.75 mL of the ground plant sap into a 1.5 mL microcentrifuge tube.
- 4. **Incubate** the tube at 60°C for 30 minutes in a water bath.
- 5. Add 750 µL of chloroform: alcohol (24:1) to the tube and mix well by inverting it to form an emulsion.
- 6. **Centrifuge** the mixture at 13,000 rpm for 10 minutes.
- 7. **Carefully collect** the supernatant and mix it with 300 µL of isopropanol. **Incubate** the mixture at -20°C for at least one hour to precipitate the DNA.
- 8. **Centrifuge** the tube at 13,000 rpm for 10 minutes and **discard** the supernatant, being careful not to lose the DNA pellet.
- 9. **Wash** the pellet with 500 µL of 70% ethanol and centrifuge at 14,000 rpm for 5 minutes.
- 10. **Remove** the ethanol carefully, and **dry** the pellet using a vacuum dryer for 5 minutes.
- 11. **Resuspend** the dried pellet in 100  $\mu$ L of 1X TE buffer and store it at -20 °C for future use.
- 12. **Quantify** the extracted DNA using a spectrophotometer.

#### **CTAB Extraction Buffer Composition:**

- 2% (w/v) CTAB1.4 M NaCl
- 20 mM EDTA

- 100 mM Tris-HCl (pH 8.0)
- 0.2% (v/v) β-mercaptoethanol

#### POLYMERASE CHAIN REACTION (PCR)

PARTICIPATION

Introduction: The polymerase chain reaction (PCR) enables researchers to produce millions of copies of a specific DNA sequence in approximately two hours. This automated process bypasses the need to use bacteria for amplifying DNA. PCR is a widely used technique in molecular biology, deriving its name from one of its key components: a DNA polymerase used to amplify a piece of DNA through in vitro enzymatic replication. As PCR progresses, the generated DNA serves as a template for further replication. This process amplifies a single or a few copies of a DNA fragment across several orders of magnitude, ultimately generating millions or more copies of that fragment. PCR can be extensively modified to perform a wide array of genetic manipulations. It is now a common and often indispensable technique used in medical and biological research labs for various applications. These include DNA cloning for sequencing, DNA-based phylogeny, functional analysis of genes, diagnosis of hereditary diseases, identification of genetic fingerprints, and detection and diagnosis of infectious diseases.

#### **Materials Required:**

- Tag polymerase: An enzyme that extends the growing DNA strand complementary to the DNA template.
- MgCl<sub>2</sub>: Provides ions necessary for the enzyme reaction.
- dNTPs: Nucleotides (adenine, cytosine, guanine, thymine) that serve as the building blocks for new DNA strands.
- Buffer: Maintains the optimal pH for the enzyme.
- Primers: Anneal to the single-stranded DNA template and provide an initiation site for the extension of new DNA.
  - o **Forward primer**: Anneals to the DNA antisense strand.
  - o **Reverse primer**: Anneals to the DNA sense strand.
- **DNA template**: The product of the 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.

- 1. **Denaturation step**: This is the first regular cycling event, consisting of heating the reaction mixture to 94-98°C for 20-30 seconds. This step causes the melting of the DNA template and primers by disrupting the hydrogen bonds between complementary bases of the two DNA strands, yielding single strands of DNA.
- 2. **Annealing step**: The reaction temperature is lowered to 50-65°C for 20-40 seconds, allowing the primers to anneal to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are formed only when the primer sequence closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.
- 3. Extension/elongation step: The temperature during this step depends on the DNA polymerase used; Taq polymerase has an optimum activity temperature of 75-80°C, with 72°C commonly set for elongation. During 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 the 5' to 3' direction. This process condenses 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 on both the DNA polymerase used and the length of the DNA fragment to be amplified. As a rule of thumb, at its optimum temperature, the DNA polymerase will polymerize approximately a thousand bases in one minute.

#### **GEL ELECTROPHORESIS**

**Principle of Electrophoresis:** The fundamental driving force of electrophoresis is the voltage applied to a mixture of charged molecules in solution. The separation speed of charged molecules is directly proportional to the surrounding voltage gradient.

#### **Materials Required**

- An electrophoresis chamber and power supply
- **Gel casting trays**, available in a variety of sizes and made of UV-transparent plastic. The open ends of the trays are closed with tape while the gel is being cast and are removed prior to electrophoresis.
- Sample combs, around which molten agarose is poured to form sample wells in the gel.
- **Electrophoresis buffer**, usually Tris-acetate-ethylene diamine tetraacetic acid (EDTA) (TAE) or Tris-borate-EDTA (TBE).
- Loading buffer, which contains a dense substance (e.g., glycerol) to allow the sample to "fall" into the sample wells, along with one or two tracking dyes that migrate in the gel, allowing visual monitoring of molecule movement.
- Ethidium bromide, a fluorescent dye used for staining nucleic acids.
- Transilluminator (an ultraviolet lightbox), used to visualize ethidium bromide-stained DNA in gels.

#### **Procedure**

- 1. **Preparation of Gel**: Agarose powder is mixed with the electrophoresis buffer to the desired concentration and then heated in a microwave oven until completely melted. Ethidium bromide is commonly added to the gel (final concentration of 0.5 µg/ml) to facilitate visualization of DNA after electrophoresis.
- 2. **Casting the Gel**: After cooling the solution to about 60°C, it is poured into a casting tray containing a sample comb and allowed to solidify at room temperature.
- 3. **Removing the Comb**: Once the gel has solidified, the comb is carefully removed without ripping the bottoms of the wells. The gel, still in its plastic tray, is inserted horizontally into the electrophoresis chamber and covered with buffer.
- 4. **Loading Samples**: Samples containing DNA mixed with loading dye are then loaded into the sample wells. The lid and power leads are placed on the apparatus, and a current is applied.
- 5. **Confirming Electricity Transmission**: The transmission of electricity is confirmed by observing bubbles coming off the electrodes. DNA fragments will migrate towards the positive electrode.
- 6. **Monitoring Migration**: The distance that the DNA fragments migrate through the gel can be judged by visually monitoring the migration of the tracking dyes. Bromophenol blue and xylene cyanol dyes migrate through agarose gels

- at roughly the same rate as double-stranded DNA fragments of 300 and 4000 bp, respectively.
- 7. **Visualizing DNA Fragments**: When adequate migration has occurred, DNA fragments are visualized on a ultraviolet transilluminator. It should be noted that DNA will diffuse within the gel over time. Documentation of DNA fragment migration should be accomplished shortly after the cessation of electrophoresis.

#### Note:

- Ethidium bromide is a known mutagen and should be handled carefully while wearing gloves.
- Always wear protective eyewear when observing DNA on a transilluminator to prevent damage to the eyes from UV light.

#### RNA EXTRACTION FROM INFECTED PLANTS

#### **Materials Required**

- Liquid nitrogen for grinding the sample.
- TRI reagent for homogenizing the leaf sample and RNA extraction (Sigma catalog no. T 9424).
- Chloroform for separating RNA, DNA, and protein phases.
- Isopropanol for precipitating the RNA.
- 75% Ethanol for washing the RNA pellet.
- Nuclease-free water or DEPC-treated water for resuspending the RNA pellet.

#### **Procedure**

- 1. **Sample Preparation**: Weigh 50-100 mg of the infected leaf tissue.
- 2. **Grinding the Sample**: Grind the sample immediately using liquid nitrogen in a pestle and mortar, then place it in a sterile microcentrifuge tube.
- 3. **Homogenization**: Homogenize the 50-100 mg leaf sample in 1 ml of TRI reagent.
- 4. **Centrifugation**: Centrifuge the homogenate at 9,000 rpm for 10 minutes at 2-8°C.
- 5. **Supernatant Transfer**: Transfer the supernatant into a fresh microcentrifuge tube and incubate the tube for 5 minutes at room temperature.
- 6. **Chloroform Addition**: Add 0.2 ml of chloroform to the microcentrifuge tube and shake vigorously for 15 seconds.
- 7. **Phase Separation**: Allow the tube to sit for 2-15 minutes at room temperature, then centrifuge at 9,000 rpm for 15 minutes at 2-8°C. Three separate phases will form: the colorless upper phase contains RNA, the interphase/mid phase contains DNA, and the bottom red organic phase contains protein.
- 8. **RNA Precipitation**: Transfer the colorless upper aqueous phase into a fresh microcentrifuge tube and add 0.5 ml of isopropanol. Mix well.
- 9. **Incubation and Centrifugation**: Incubate the tube for 5-10 minutes at room temperature, then centrifuge at 9,000 rpm for 10 minutes at 2-8°C.
- 10. Washing the RNA Pellet: Discard the supernatant and wash the RNA pellet with 75% ethanol while vortexing.
- 11. Centrifugation: Centrifuge the tube at 5,500 rpm for 5 minutes at 2-8°C.
- 12. **Ethanol Removal**: Carefully remove the ethanol without losing the pellet and dry the pellet for 5-10 minutes under a fan, keeping the lid of the microcentrifuge tube open. (Do not vacuum centrifuge the pellet.)
- 13. Resuspension of the Pellet: Finally, resuspend the dried pellet in 20-30 µl of nuclease-free water or DEPC-treated water
- 14. **Dissolving the Pellet**: Dissolve the pellet by repeated pipetting with a micropipette or by incubating at 55-60°C for 10-15 minutes. Store at -80°C.

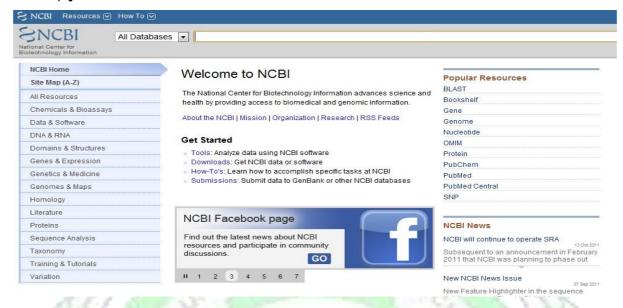
#### BASIC BIOINFORMATICS TOOLS FOR VIRAL GENOME ANALYSIS

Tools: BLASTn, Primer Designing Software, BioEdit, Clustal X/W, MEGA Software

NCBI (National Center for Biotechnology Information): NCBI is one of the leading online resources known for providing biological sequence information. It is maintained by two organizations in the U.S.: the National Library of Medicine (NLM) and the National Institutes of Health (NIH). As a national resource for molecular biology information, NCBI's mission is to develop new information technologies to aid in understanding the fundamental molecular and genetic processes that control health and disease. More specifically, NCBI has been tasked 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. User queries and sequence information are delivered through NCBI's search tool called "Entrez."

**Home Page:** NCBI features a simplified homepage from which users can navigate to different resources. The left pane of the homepage includes a site map followed by various categories that help narrow down the search for the right sequence. On the right side, there is a list of popular resources, which is very useful for first-time users.

#### The home page of NCBI can be seen as follows:

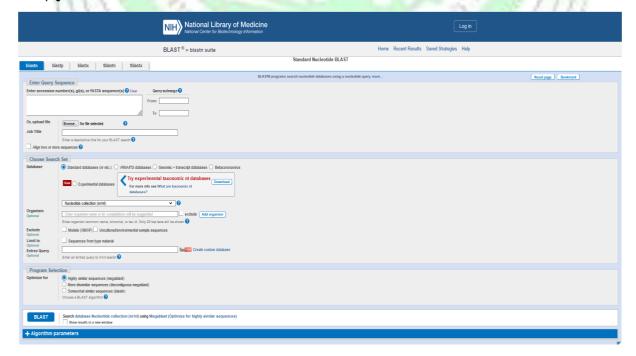


**GenBank:** The GenBank sequence database is an open-access, annotated collection of all publicly available nucleotide sequences and their protein translations. This database is produced and maintained by the National Center for Biotechnology Information (NCBI) as part of the International Nucleotide Sequence Database Collaboration (INSDC). GenBank and its collaborators receive sequences produced in laboratories worldwide from more than 100,000 distinct organisms. Since its establishment over 20 years ago, GenBank has become the most important and influential database for research in almost all biological fields, with its data 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 homepage of NCBI features a search box that directs users to Entrez. This tool is internally connected to various biological databases, increasing the likelihood of retrieving accurate information.

**BLAST:** BLAST stands for Basic Local Alignment Search Tool. It is a tool used to find sequences homologous to a particular query sequence. BLAST compares all the sequences in the database with the searched sequence and provides many hits, usually arranged in increasing order of the scores obtained. BLAST is available at the following URL: <a href="http://blast.ncbi.nlm.nih.gov/">http://blast.ncbi.nlm.nih.gov/</a>. It uses PAM and BLOSUM matrices for scoring the alignment.

#### Home page of BlastN



#### Multiple Sequence Alignment and Phylogenetic Tree of Sequences Using ClustalW

**Introduction:** 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 DNA and protein sequence databases. If similar sequences are found, they may match the complete length of the new sequence or only subregions of it. If more than one similar sequence is found, the next important step in the analysis is to align all of the sequences. Multiple alignments are a key starting point for predicting protein secondary structure, residue accessibility, function, and identifying 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. The optimal alignment of the two sequences usually requires the insertion of gaps in one or both sequences to achieve the best alignment. Aligning two residues implies that those residues are performing similar roles in the two different proteins. This allows information known about specific residues in one sequence to potentially be transferred to the residues aligned in the other. For example, if the active site residues of an enzyme have been characterized, aligning 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 legally obtained software equipped with a fast broadband Internet connection.

Web Resources Used: ClustalW

**Principle:** Phenetics is a novel phylogenetic method that considers as many variable characters as possible (OTUs = Operational Taxonomic Units), scoring these characters numerically and analyzing them using rigorous mathematical methods (numerical taxonomy).

**Phylogeny:** Phylogeny describes 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 referred to as cladistic methods. These trees are prepared by considering 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 structures or organism species) to aid in their classification.

Construction of Phylogenetic Tree: The preliminary step in constructing a phylogenetic tree is to compare nucleotide sequences through sequence alignment. Rigorous mathematical approaches have been devised to facilitate sequence alignment, which can be used to convert the data into a phylogenetic tree. The similarity approach aims to maximize the number of matched nucleotides (identical in the two compared sequences). Its complementary approach, the distance method, minimizes the number of mismatches. The final result of the two approaches is the same: the same alignment will be identified as the best, indicating a likelihood of identifying the same alignment if a relationship exists. Multiple alignments are often used for comparing more than two sequences, with Clustal W being a popular choice.

#### Procedure:

- 1. Go to <a href="http://www.ebi.ac.uk/clustalw/">http://www.ebi.ac.uk/clustalw/</a>.
- 2. Enter your email (if you want the results emailed to you; this step is not necessary) and choose an alignment title.
- 3. Paste the sequences into the box below using the FASTA format:

Copy code

>Name of Sequence #1

- >Name of Sequence #2
- 4. Every sequence MUST have a different name, or the alignment will not work. Alternatively, upload a file that includes all your sequences (such as a .doc file) in an acceptable format.
- 5. Press the run button to start the alignment.
- 6. When viewing your results, note the consensus symbols used by ClustalW:
  - o "\*" indicates that the residues or nucleotides in that column are identical across all sequences in the alignment.
  - o ":" indicates that conserved substitutions have been observed.
  - $\circ\,$  "." indicates that semi-conserved substitutions are observed.
- 7. If you would like to see your results in color, press the button that displays "Show Colors." Click "Hide Colors" to remove the color.
- 8. Click the button labeled "View Alignment File" to see the alignment on a larger scale (i.e., bigger font).