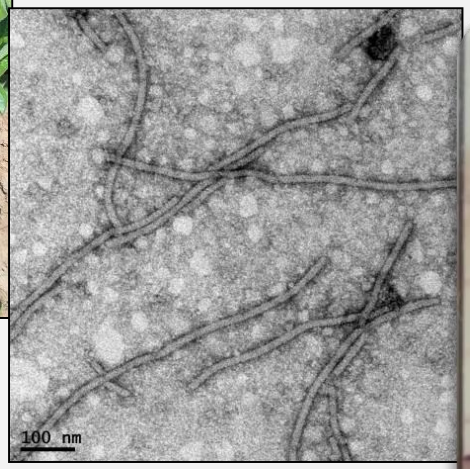
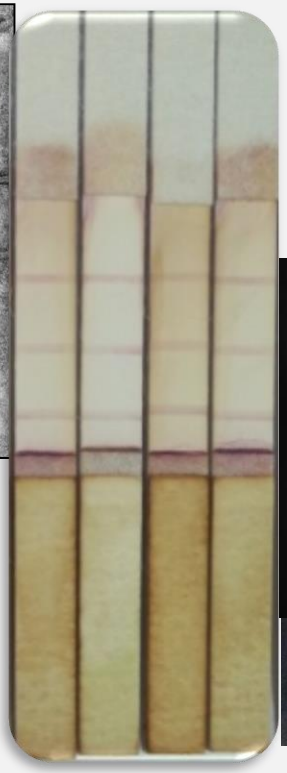
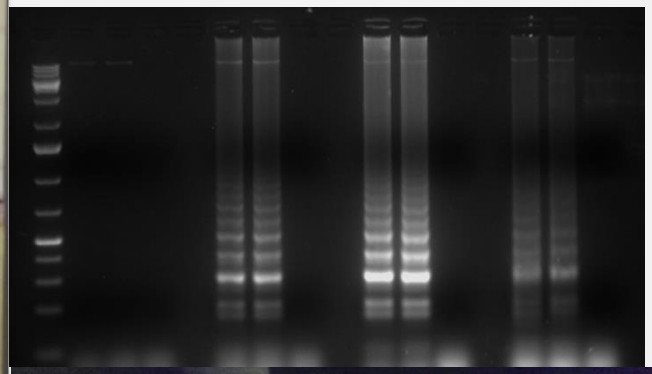
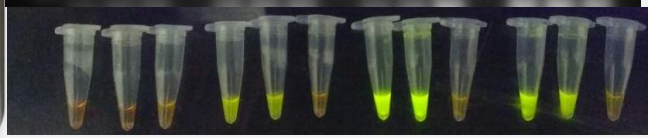
Virus detection techniques for quality seed production









Baswaraj Raigond
Scientist (Sr SI) raigond@gmail.com
Div. of Plant Protection, ICAR-CPRI, Shimla -171 001

INTRODUCTION

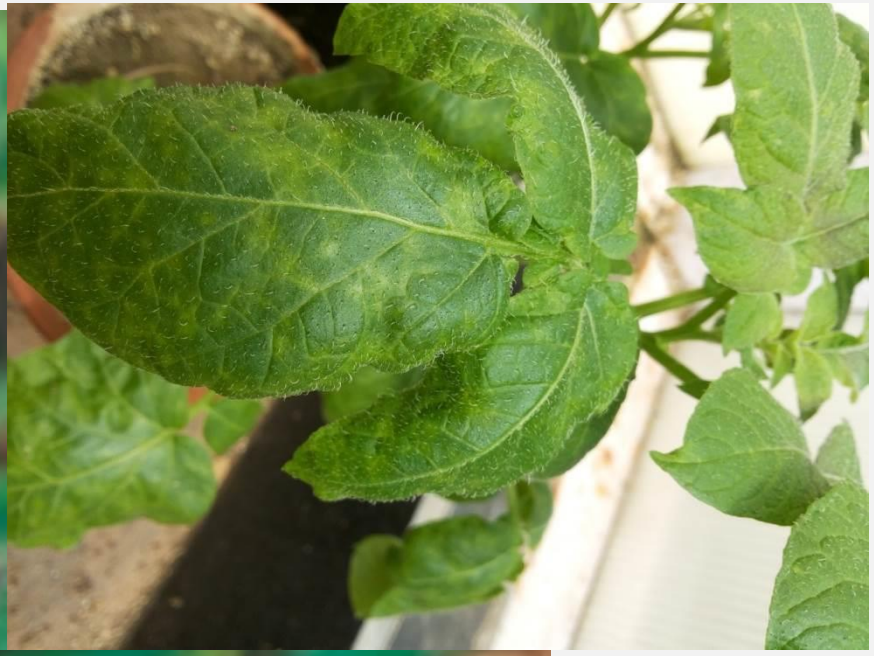
- **Viral infection affects seriously the quality and quantity of agricultural produce globally.**
- **So, the development and adaptation of efficient and rapid techniques for diagnosis and control of plant viruses constitute an imperative and relevant necessity.**
- **Therefore, any attempt to establish a control program for a viral disease must be, always, preceded by a correct and precise laboratory diagnosis.**

INTRODUCTION cont..

Several methods can be used for correct and definitive diagnosis of plant viruses as, it is very difficult to diagnose -symptoms

Symptoms vary:

- **Plant variety involved,**
- **Environmental conditions,**
- **Strain of the virus,**
- **Synergistic effect of infection caused by two different viruses**
- **Stage of the plant etc.,**

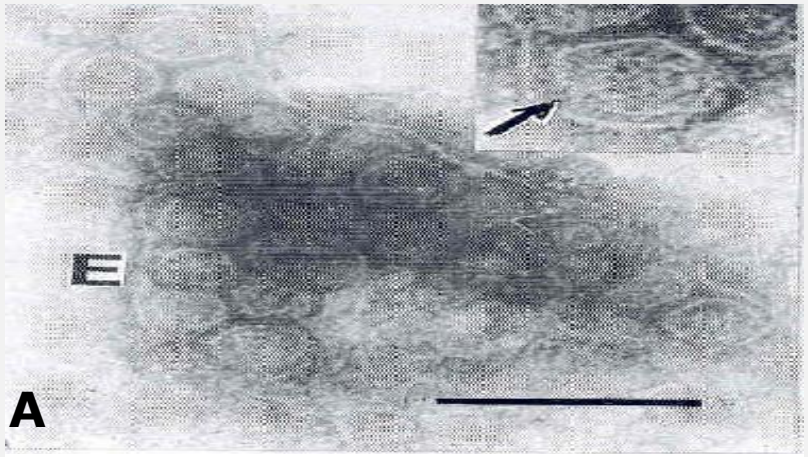


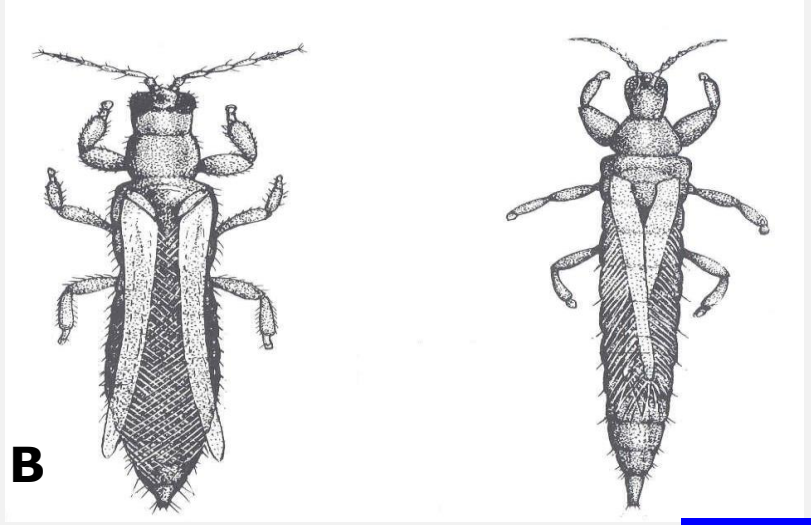

visuals:unlimited

Potato virus M

Potato Stem Necrosis Disease

Necrotic lesions on stem and petiole in tospovirus infected potato plant





Apical Leaf Curl Virus

Apical leaf curl and stunting in potato plant due to PALCV infection, White fly vector

Laboratory-based techniques

Over the last few decades techniques for accurate detection and diagnosis of diseases caused by plant viruses include

- TEM
- Serological and
- Molecular techniques.

Transmission Electron microscope

Most powerful scientific tools for carrying out detail structural studies of biological materials.

Purpose: to have magnified image of a specimen

Principle: In TEM, electron beam is used as a source of illumination where extremely small negatively charged electrons given off by a heated tungsten filament can be accelerated by high voltage to produce a coherent electron beam and can be focused by an electric field.

The image detected by the CCD may be displayed in real time on a computer monitor.

Both particles and ultrathin sections are held in the electron beam on carbon-coated grids..

Comparison of light microscope to TEM

Factors	LM	TEM
Illumination	Light rays	Electrons
Wave-length	400-800 nm	0.00037 nm (100 kV)
Medium	Air	Vacuum
Lenses	Glass	Electromagnetic
Image visibility	Direct	Fluorescent screen
Focusing	Mechanically	Electrically
Magnification	5-2000 X	Up to 5,00,000 or more
Resolving power	200 nm	0.2 nm

Leaf dip method

Grind an infected leaf - 2-3 mm diameter in phosphate buffer,

Place 10 µl of homogenate on parafilm or waxed slide in a wet Petriplate.

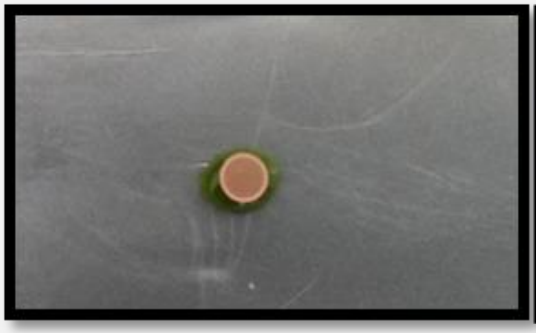
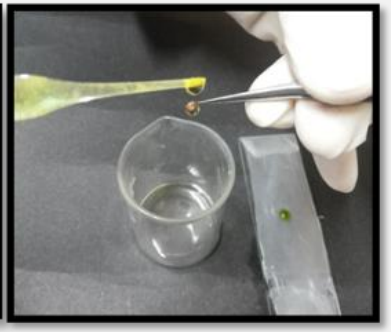
Place grids (film side downward) on the surface of the droplet, ensuring that the grid surface is wet and allow it for 2-5 minutes.

Pick up the grid by its edge with fine forceps and wash the grid with 10-15 drops of double distilled water (DDW) to remove the sap.

The grids are stained with 2% aqueous uranyl acetate (UA). The excess stain is immediately drained off by using Whatman filter paper.

Leaf dip method cont…



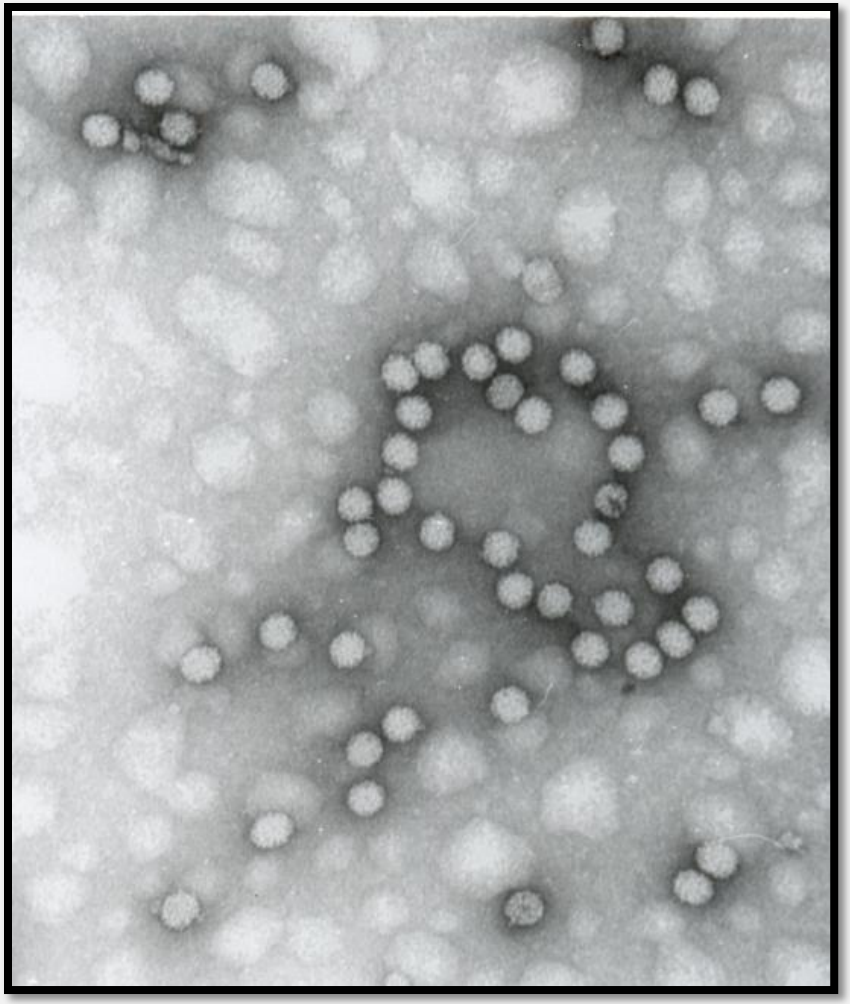




Immunosorbent electron microscopy (ISEM)

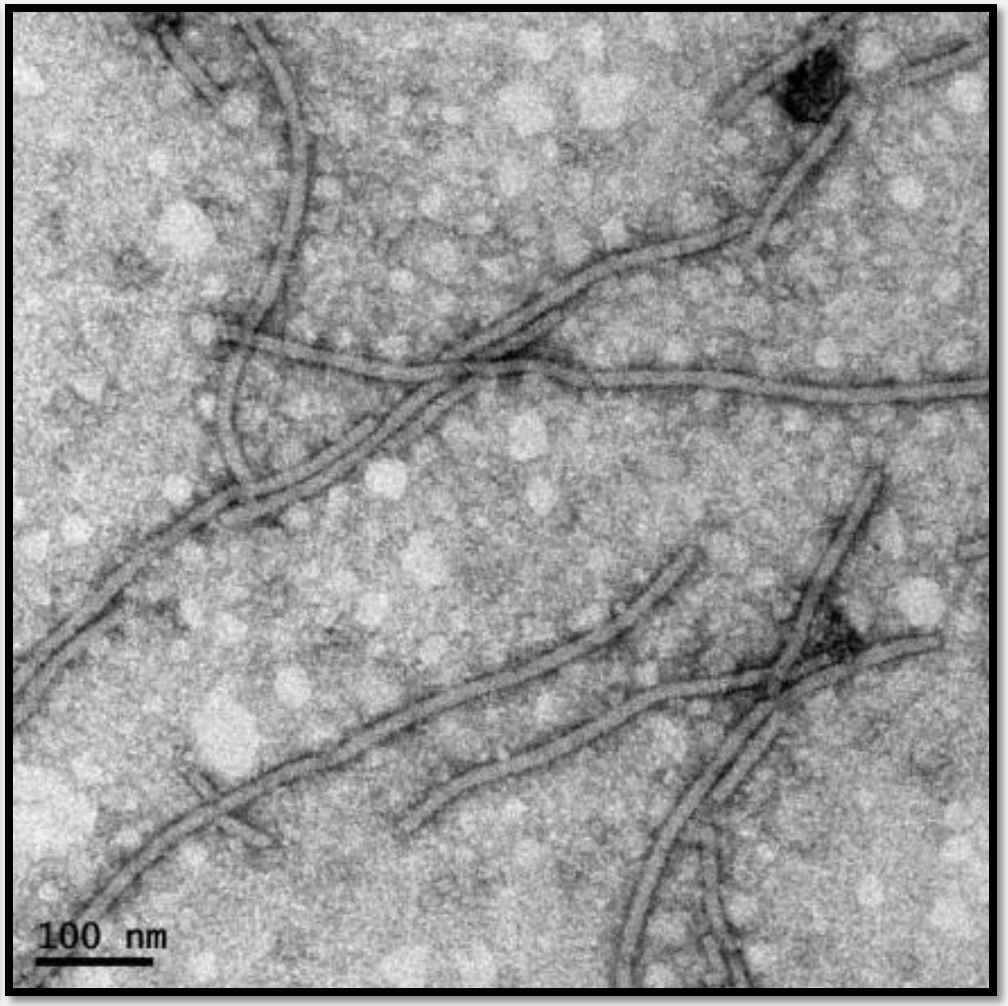
It is a procedure in which EM support film is first coated with a layer of antibody which serves to trap the virus preparation.

Trapping-

- Float the grid over a drop of diluted antiserum
- Wash the grid for 10-15 n phosphate buffer
- Drain briefly- place over a drop leaf extract and leave for 30 min. at room temp
- Remove the grid and wash with approximately 10-15 drops of DDW
- Stain with 2-4 drops of 2% freshly prepared uranyl acetate solution
- Dry the and examine the grid under the TEM



Potato leaf roll virus



Potato virus Y

Immuno-gold Electron microscopy

- This technique combines the specificity of serological properties with the morphology of the virus particles visualized in the electron microscope.
- Virus particles are selectively trapped on to antibody conjugated with gold nano particles.

Immuno-gold Electron microscopy cont…

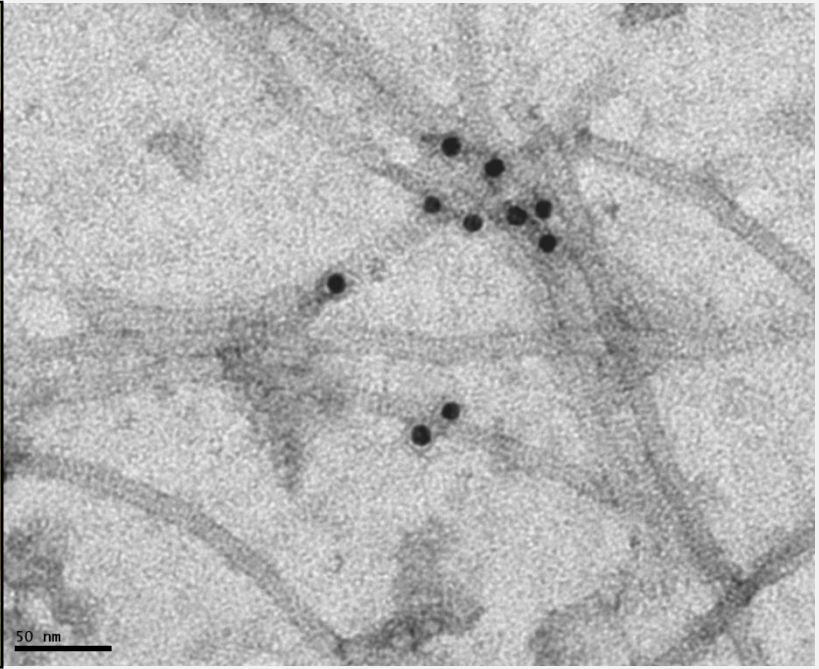
The antibody coated grids are washed with buffer and floated on drops of extracts from virus infected plant tissue at room temperature for 3 – 4 hrs.

After washing for three times, the grids are stained with 1.0% uranyl acetate.

Later dried and examined in the electron microscope.

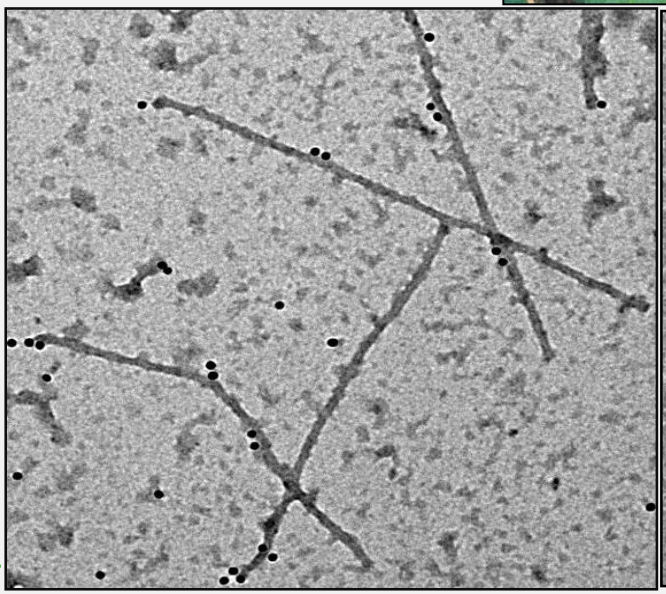
Great advantage: requires only small amounts of antigens and antiserum conjugated with gold nanoparticles.

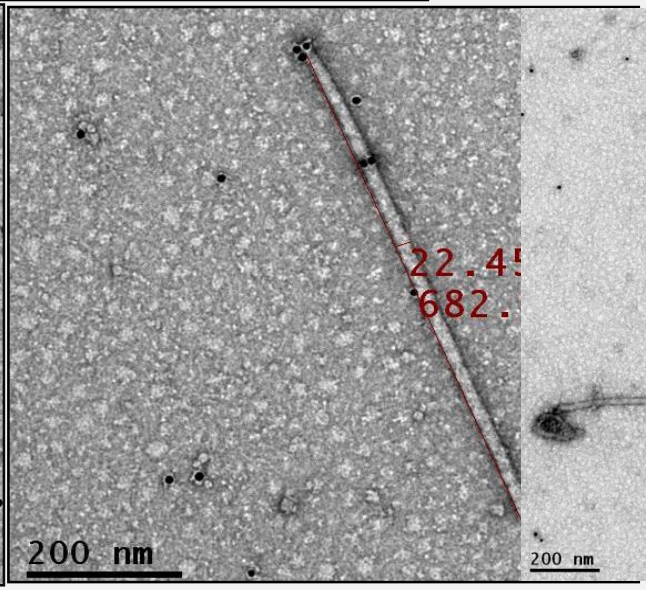
Potato virus A (PVA)

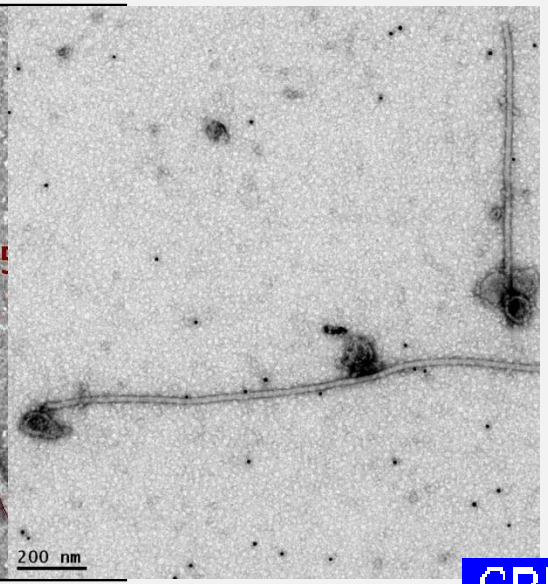


Potato virus M (PVM)

M

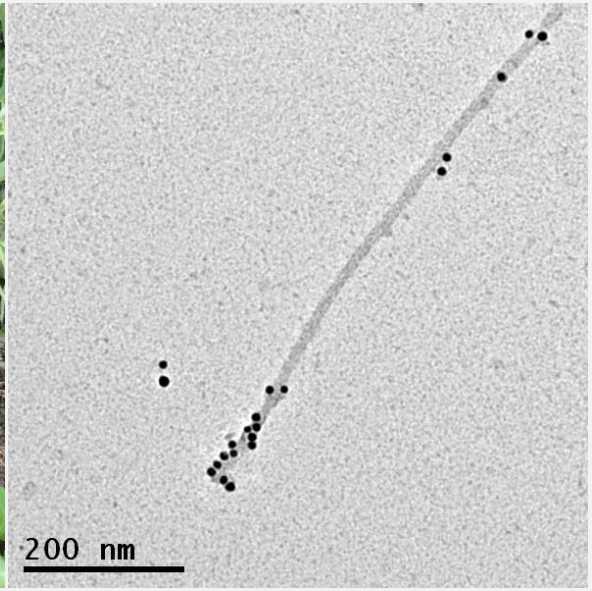


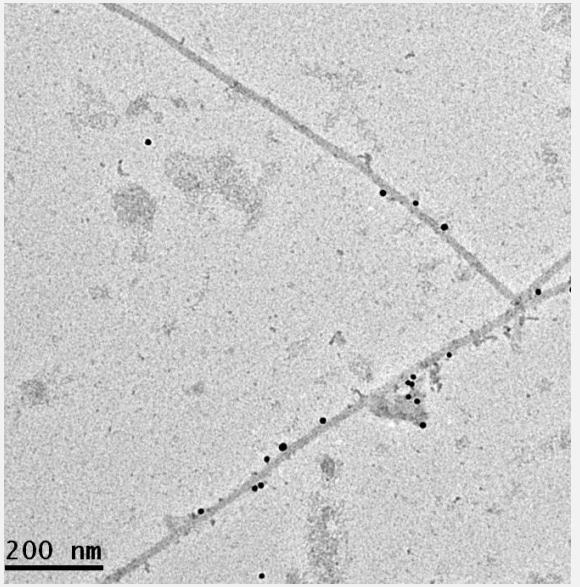




Potato virus M (PVM)

cont..





Serological techniques include

- Enzyme-linked immunosorbent assay (ELISA)
- Tissue blot immunoassays,
- Immunoelectron microscopy,
- Western blots,
- Dot blots,
- Lateral flow assays,
- Immunocapture PCR, and
- Double diffusion tests.

Among the above serological techniques:

Enzyme-linked immunosorbent assay (ELISA) is used extensively.

- ELISA is based on the specific reaction between the viral antigens and their specific antibodies.

- Antigen (virus protein/any other protein) when injected into a vertebrate animal (a mammal or a bird), can trigger an immune response in the animal resulting in production of specific antibodies.

- Antibodies are also proteins of the immunoglobulin group (IgG) produced against specific antigenic determinants and are present in the animal blood and most commonly involved in the serological tests for plant virus identification.

- Generally the methods that involve the antigen antibody reactions in vitro are simple and do not require sophisticated and expensive apparatus.

- Limitation in serology for plant virus identification and detection is the difficulty in producing a good virus specific antiserum.

- Most of the antisera used for plant virus identification and detection are usually prepared by immunizing mammals (Rabbits) or birds.

Enzyme-Linked Immunosorbent Assay (ELISA)

- Its very sensitive and specific technique introduced for identification of plant viruses in the 1970s (Clark & Adams).

- Because of its adaptability, high sensitivity, and economy in the use of reagents, it is used widely for indexing a large number of samples in a relatively short period of time.

Principle: consists of detecting the antigen-antibody interactions by enzyme induced colour reaction rather than by observing their precipitation.

- Its always recommended to include a homologous antigen for the specific virus antibody (positive control) and extracts from healthy plants to compare the absorption readings and to obtain a correct interpretation of the results.

- Different variations of this serological technique have been developed i.e., direct and the indirect ELISA.

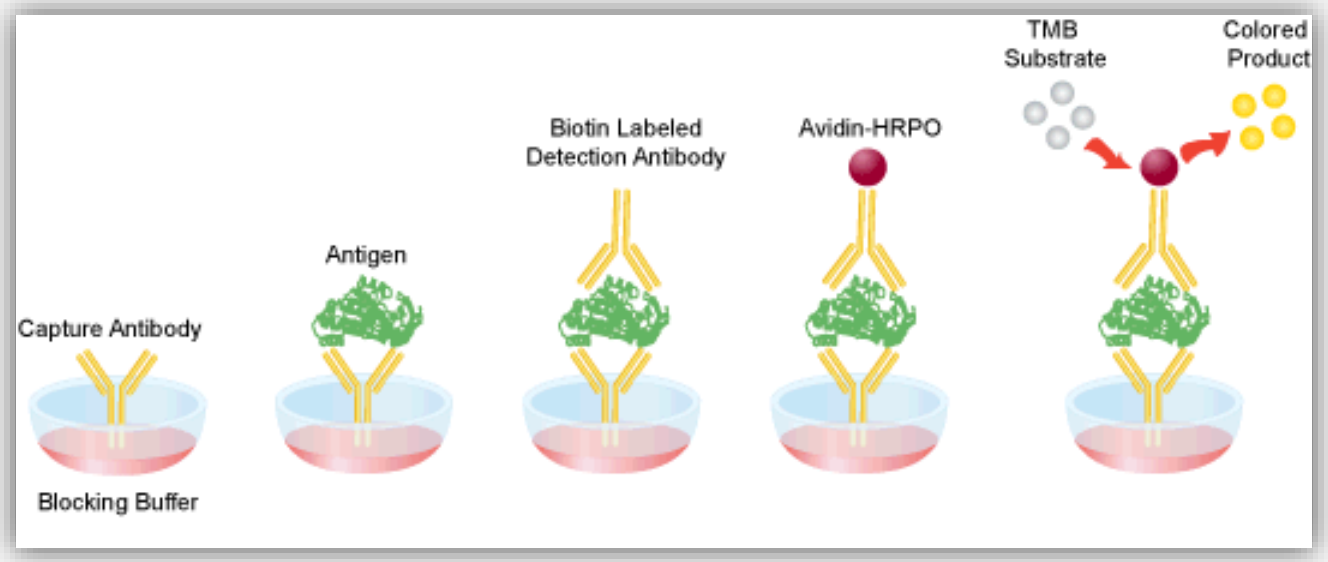
Direct ELISA

- Also called as double antibody sandwich (DAS-ELISA).
- The first step is adsorption of virus-specific antibodies to the wells of ELISA plates where unbound antibody is removed by washing, and the samples to be tested for virus antigen are added.
- Controls include extracts from known infected plants (positive control), and extracts from healthy plants (negative control) are to be used.
- After incubation and washing, the enzyme-antibody conjugate is added. If virus attached to the coating antibody is present, the enzyme-antibody conjugate will combine with the virus.

- Plates are washed, and the colourless substrate (p-nitrophenyl phosphate) is added.

- Positive wells will show a yellow reaction, due to the action of the conjugated enzyme (alkaline phosphatase) on the substrate and negative wells will remain colorless.

- The colorimetric changes are measured in an ELISA reader.



Indirect ELISA

- Indirect ELISA or plate-trapped antigen (PTA- ELISA) was developed to avoid the inconveniences and difficulties of conjugating the enzyme with the IgG specific for each virus species to be used in the second layer of antibodies in direct ELISA.

- For this reason, it requires antibodies produced in two different animal species and the virus particles are trapped in the wells of the ELISA plate.

- The indirect ELISA requires a universal IgG enzyme conjugate which can be used with the antibodies of all virus species.

This universal conjugate is composed of an IgG produced against the IgGs from the animal in which virus antibodies are raised .

For example:

If the virus antibodies are produced in rabbits an antirabbit IgGs are produced in a second animal such as goats or mice.

So, the detecting antibody conjugate binds specifically to the primary virus specific antibody.

- In this method, the wells of the ELISA plate are, initially, covered with extracts from infected plants and healthy plant samples.

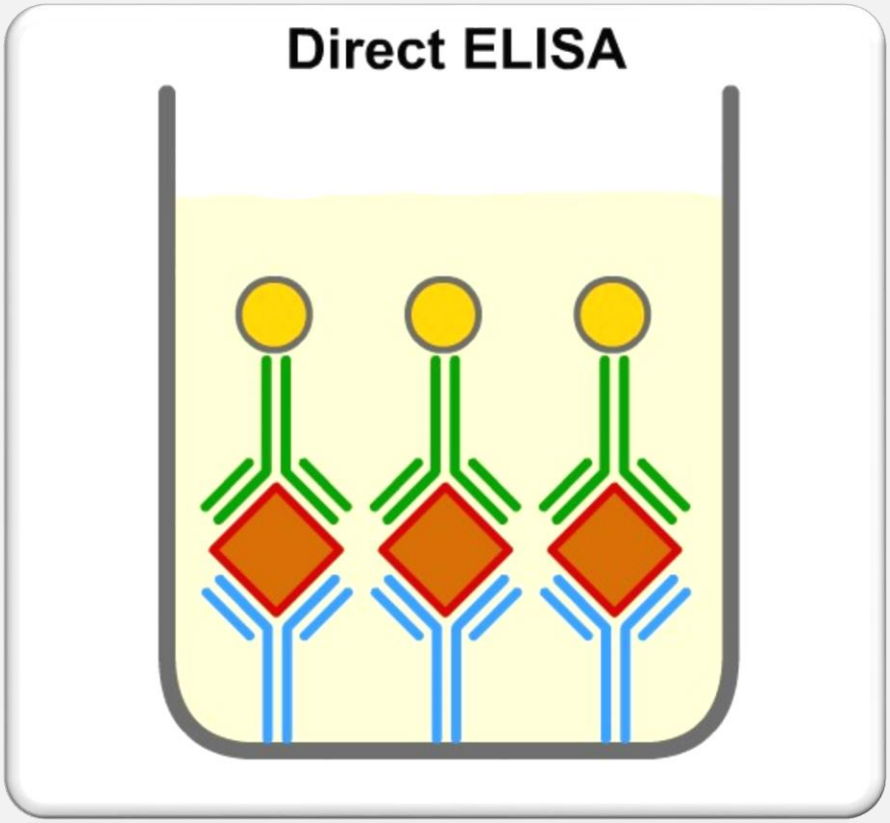
- Later the virus particles are covered with a layer of virus specific antibodies produced in a rabbit.

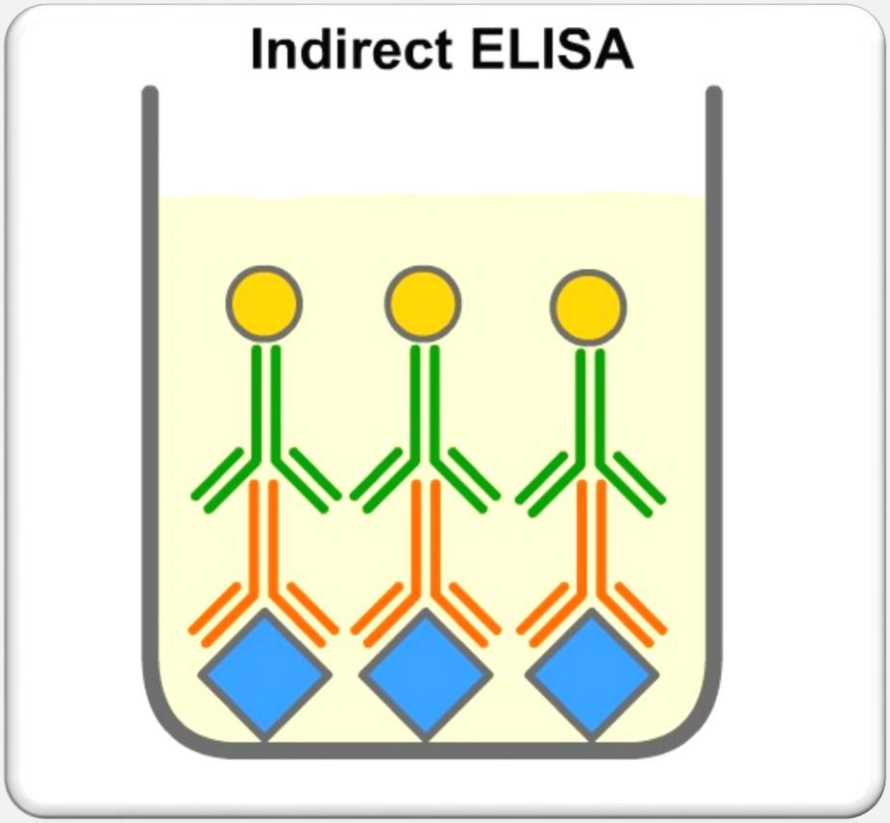
- The complex antigen-antibodies are covered with a universal conjugate i.e., anti-rabbit IgG produced in goats linked to the enzyme alkaline phosphate.

- The linked anti-IgG-enzyme reacts with the virus antibodies (IgG) which reacted with the virus particles adsorbed to the bottom of the ELISA plate wells will be detected by substrate added in the wells.

- Certain disadvantages such as competition between plant sap and virus particles for sites on the plate wells and, consequently, high background reactions.

Direct and Indirect ELISA





Triple Antibody Sandwich (TAS- ELISA)

- Another widely used ELISA variation is the triple antibody sandwich i.e., TAS- ELISA, similar to DAS- ELISA, except for an additional antibody produced in another animal is used.

- First, the plate is coated with virus antibody produced in a rabbit then virus antigen is added.

- Later second layer of virus specific antibody produced in mouse is added.

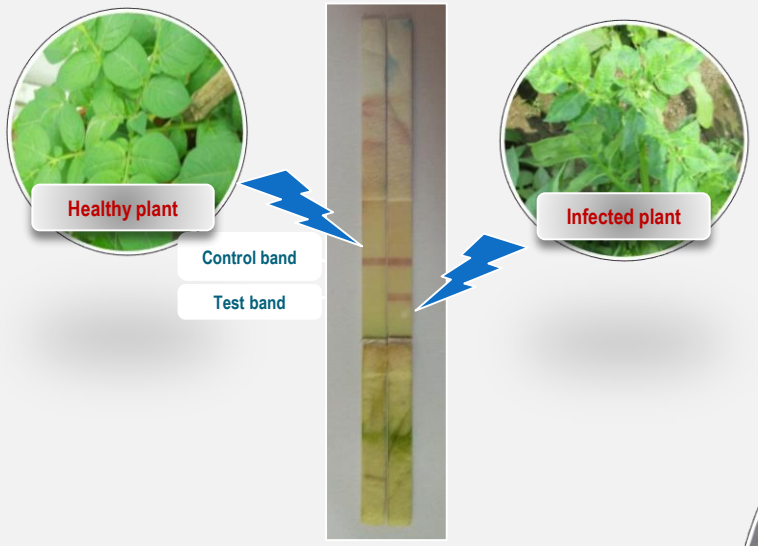
- Enzyme-conjugated specific antibody i.e., rabbit anti-mouse IgG, followed by colorimetric changes by adding substrate in the wells.

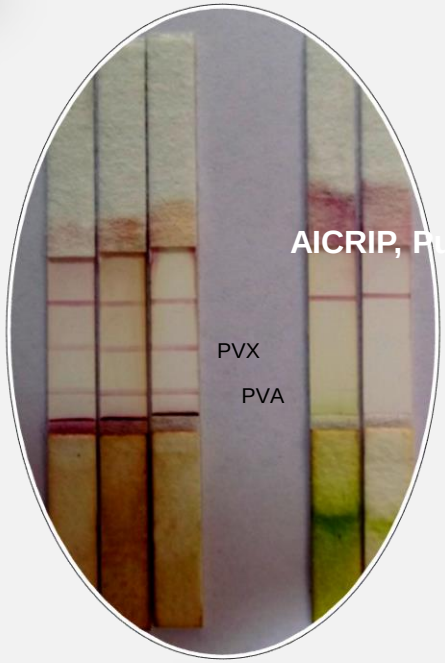
Dot Blot or Dot Immuno Binding Assay (DIBA)

This technique is a simple and easier method to prepare and apply the samples on nitrocellulose or nylon membranes.

- The samples containing the virus antigens are prepared by grinding tissues in Tris-buffered saline and the extracts are applied directly on the membrane.
- Later virus IgG produced in rabbit should be added.
- To this, the anti-rabbit IgG produced in mouse should be added.
- This protocols similar to indirect ELISA or PTA-ELISA, except that the positive reactions are recorded as coloured dots on the membrane.

Lateral flow immuno assay (LFIA)/ Dip stick





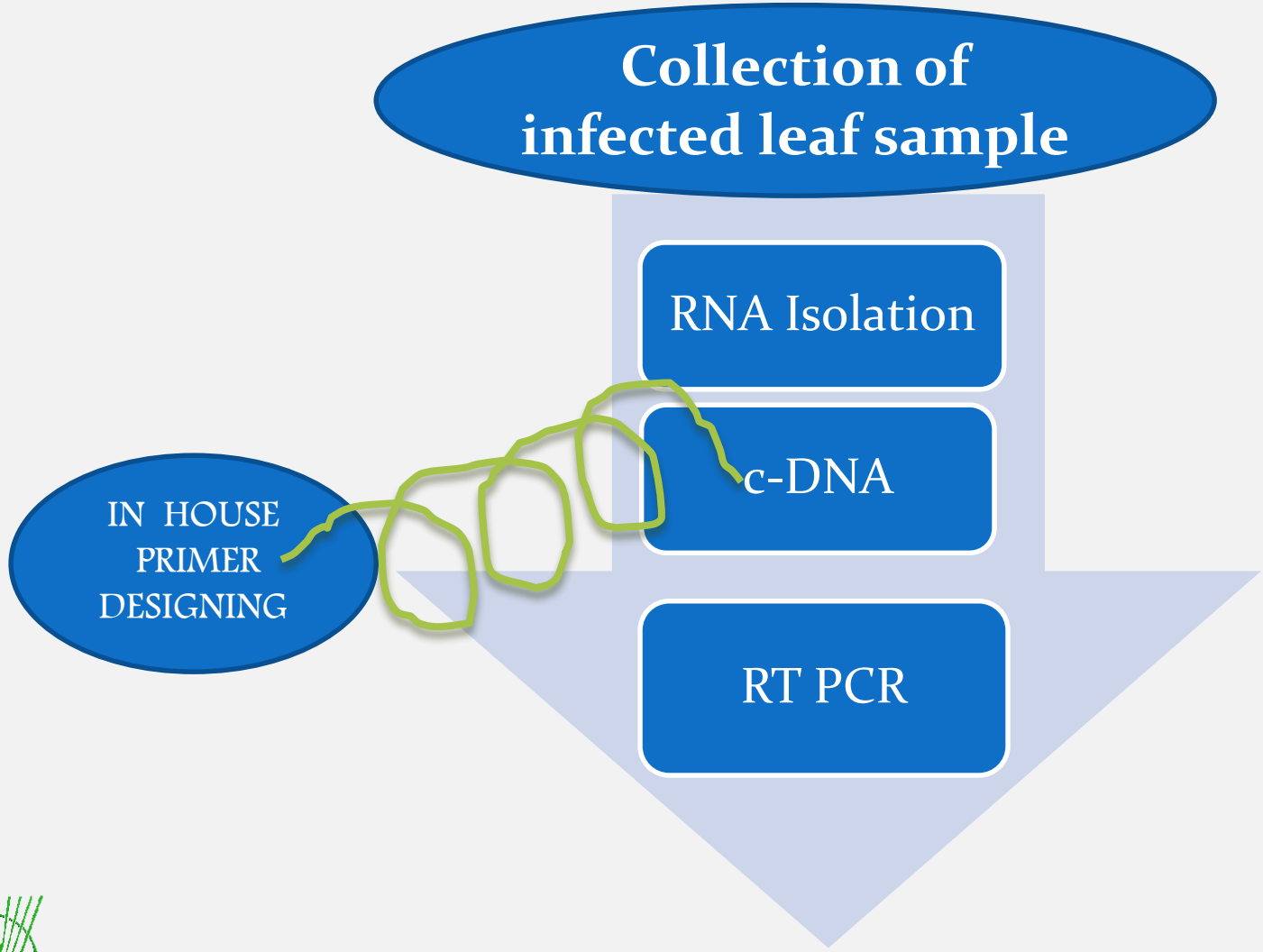
Nucleic acid based diagnostics

Polymerase Chain Reaction (PCR)

and

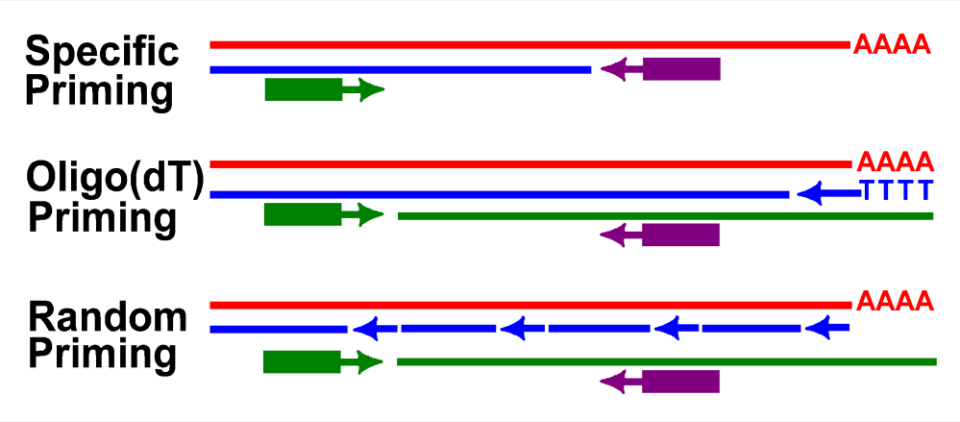
Reverse transcription-PCR (RT-PCR)

Methodology followed



c–DNA Synthesis

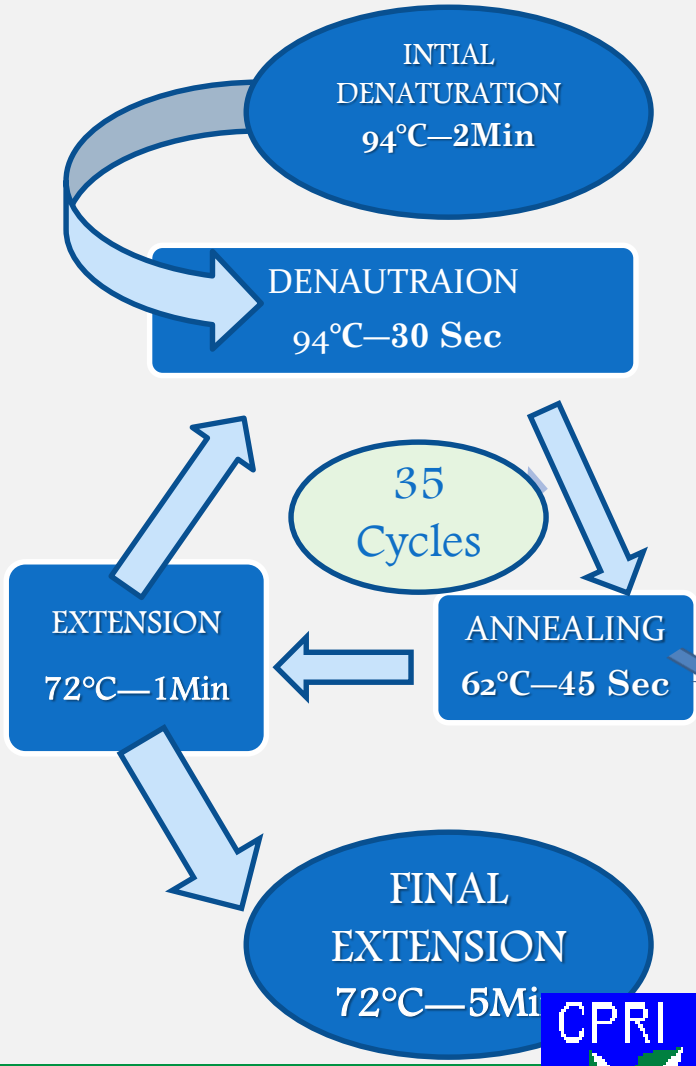
Components	Stock	Per RM – 20ul
BUFFER	5X	4.0 ul
dNTP	10 mM dNTP	2.0 ul
PRIMER	0.2µg/µl	1 .0 µl
RI (Rnase in hibitor)	20 U/ul	1.0 ul
Reverse transcriptase	200U/ul	1.0 ul
Template	-	6.0 ul
RNAse Water		5.0 ul



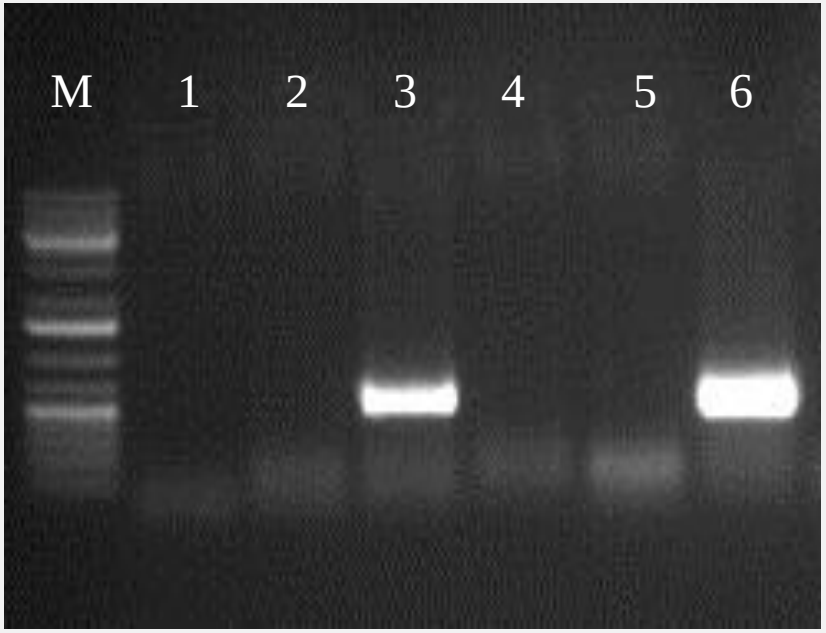
Incubated at 25°C for 05min, 42°C for 59min, 75°C for 10 min

Reverse Transcriptase PCR

COMPONENTS	STOCK	20 ul/RM
WATER (sterile nano pure water)	-	11.0 µl
BUFFER	10x Taq bufferA	2.5 µl
dNTP	2 mM dNTP mix	1.5 µl
FORWARD PRIMER	10 pM	1.0 µl
REVERSE PRIMER	10 pM	1.0 µl
TAQ POLYMERASE	1 U/µl	1.0 µl
TEMPLATE		2 µl cDNA



RT-PCR Results



M-1 Kb plus ladder, Lane-1,2,3 1F1R and
4,5,6- are 1F4R at 58, 60 and 62**°C** respectively

Immune Capture Polymerase Chain Reaction (IC-PCR)

This technique i.e., immune capture polymerase chain reaction (IC-PCR), combines the technical advantages of PCR with the practical advantages of serology.

It was developed for the detection of several plant viruses.

- In this test, microtiter tubes are coated with specific virus antibodies and incubated at 37°C for 2 hrs.
- Later virus particles are added than incubated and washed.
- The trapped virus particle will be disrupted followed by the release of viral nucleic acid (RNA/DNA).

- This virus nucleic acid is amplified by polymerase chain reaction (PCR) or reverse transcription PCR (RT- PCR),
- This entire procedure is carried out in a single microtiter tube.
- The IC-PCR has been shown to be a very useful alternative in virus detection from plant material and insect vectors (Mulholland, 2009).

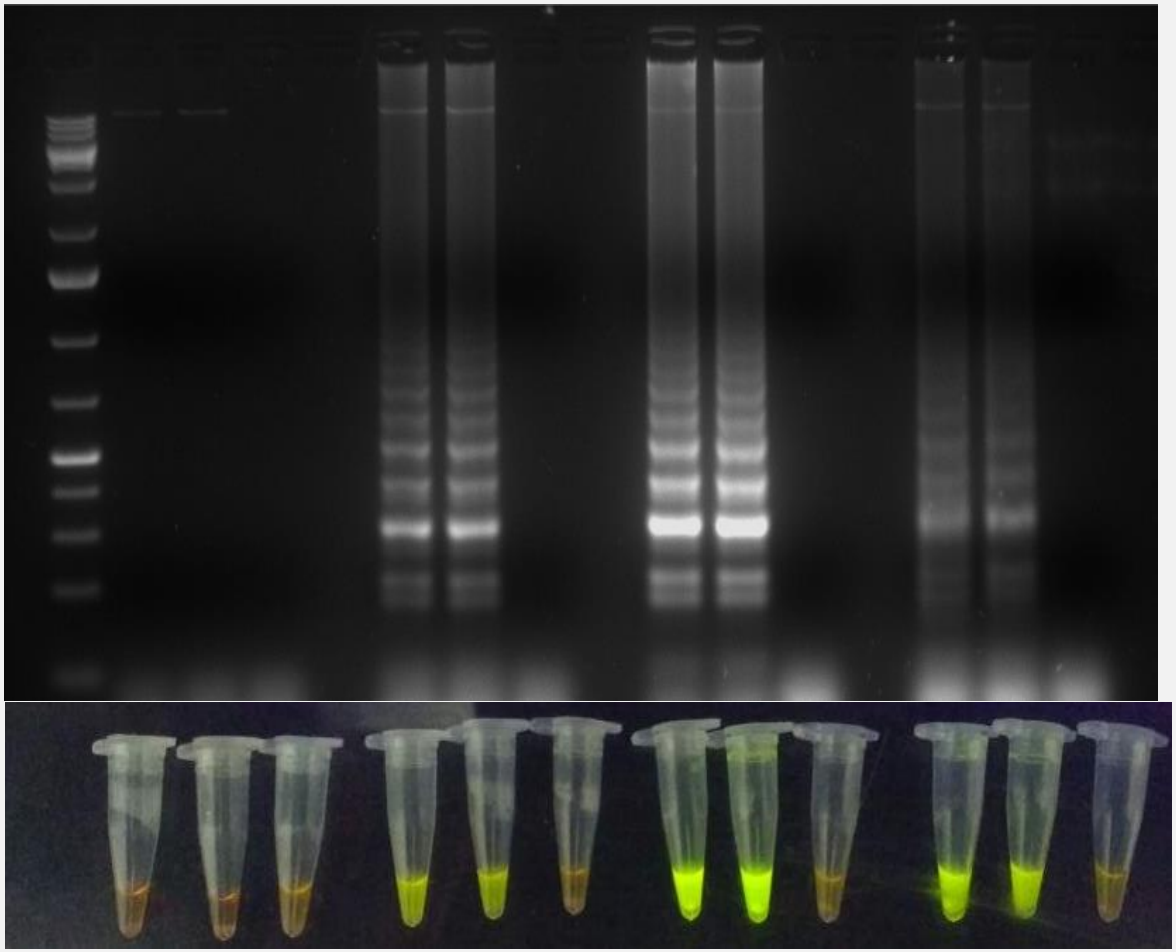
Print capture RT-PCR (PC-RT-PCR)

- This technique involves spotting of samples onto nitro cellulose membrane (NCM),
- eluting viral RNA from the NCM in sterile distilled water (20 µl) at 95°C for 10 min,
- cDNA synthesis followed by
- PCR amplification and analysis of the PCR product.

Loop-mediated isothermal amplification (LAMP)

- The LAMP test is carried out under isothermal conditions (60–65°C) and produces large amount of DNA (amplified 10^9–10^{10} times) in 15–60 min.
- Alternatively, gene amplification can be visualised by the naked eye either as turbidity or in the form of a colour change when SYBR Green, a fluorescent dsDNA intercalating dye, is employed.
- LAMP does not require a thermal cycler and can be performed simply with a heating block and/or water bath

LAMP assay for detection of potato virus



Thank you all